AUTISM SPECTRUM DISORDERS: DEVELOPMENTAL TRAJECTORIES, NEUROBIOLOGICAL BASIS, TREATMENT UPDATE, VOLUME 2

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AUTISM SPECTRUM DISORDERS: DEVELOPMENTAL TRAJECTORIES, NEUROBIOLOGICAL BASIS, TREATMENT UPDATE, VOLUME 2

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This book is dedicated to Francesco Saverio
—Roberto Canitano

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EDITORIAL
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Editorial: Autism Spectrum Disorders: Developmental Trajectories, Neurobiological Basis, Treatment Update, Volume 2

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Editorial on the Research Topic

Autism Spectrum Disorders: Developmental Trajectories, Neurobiological Basis, Treatment Update, Volume 2

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In this collection of articles on Autism Spectrum Disorder (ASD), various themes have been covered with the aim to widen the perspective of updating the readers on the recent advancements in this research field. Continuing expanding areas of investigation have attracted the interest of authors and the resulting Research Topic contains different contributions in different fields of ASD research, including classification, endophenotypes, gender differences, comorbidities, and biological underpinnings.

The publication of DSM-5 and ICD-11 has changed ASD classification and diagnostic criteria (1), introducing ASD as a behaviorally defined neurodevelopmental disorder and eliminating previous diagnoses of Autistic Disorder, Asperger's Disorder, and Pervasive Developmental Disorder—not otherwise specified, present in the previous version of DSM. Oberman and Kaufmann argue in their article that the term Autism Spectrum Disorder, and its criteria, is preferred over the plural form Autism Spectrum Disorders that is still prevalent in the basic science and genetic literature. In addition, they find that the diagnosis of ASD is inaccurate in many individuals with intellectual disability, and advocate to assess and use other diagnostic entities, such as Social Communication Disorder or Stereotypic Movement Disorder, in order to avoid over-diagnosing ASD in those individuals.

Investigating ASD behavioral, functional, and neuroanatomical endophenotypes represents a hot topic in ASD research (2, 3). A number of cognitive models focused on executive function (EF) have been proposed to explain the symptom clusters observed in ASD, as detailed by Demetriou et al. Empirical studies pointed out a broad impairment in EF. The observed heterogeneity of EF performance is considered a limiting factor in establishing EF as a cognitive endophenotype in ASD. Further understanding of the neurobiological basis that underpins EF performance, such as the excitation/inhibition hypothesis, will likely be important to shed light on these components. Importantly, the authors state that application of the research domain criteria (RDoC) framework could improve our understanding of EF impairment in ASD and facilitate targeted interventions. To investigate alterations in neural processing of social visual information in children with ASD, Jan et al. used high density electroencephalography and high-resolution eye-tracking. The study highlighted differences in the neural processing of dynamic cartoons containing human-like social interactions. ASD children, as compared with controls, showed decreased prefrontal and

cingulate activation, impaired activation of the premotor cortex, and increased activation of parietal, temporal, occipital, and cerebellar regions. Thus, impairments in brain regions involved in processing social cues are present from an early age in ASD children and deserve further investigation. Spera et al. used machine learning to evaluate altered functional connectivity in resting-state fMRI datasets of individuals with ASD and matched controls. Theirs results indicate that both under- and over-functional connectivity occurred in a selected cohort of ASD children as compared with controls, and that these functional alterations are spread in different brain networks including the precuneus, the inferior frontal gyrus, and the hippocampus. Repetitive transcranial magnetic stimulation (rTMS) is a novel treatment that has been used in a limited number of studies in children with ASD. Yang et al. evaluate the use of high-frequency rTMS over the left inferior parietal lobule in 11 low-functioning children with ASD. This preliminary study provides positive evidence for efficacy and that larger and controlled studies are warranted. In the study by Malatesta et al., the absence of left-cradling shown in mothers of typically developing children was not observed in mothers of ASD children, who exhibited a significant left-cradling bias in the 6–12 months age group. It remains to be further investigated whether this pattern is related to the overstimulation in which ASD mothers try to engage the infants in response to their lack of social interaction. Ruta et al. instead validated the Quantitative Checklist for Autism in Toddlers (Q-CHAT) in an Italian cohort of young children with ASD and developmental disorders, showing that Q-CHAT has good psychometric properties and external validity to distinguish ASD children from both typically-developing children and children with developmental delay.

The significant gender bias in ASD incidence (4:1 male to female ratio) has been postulated to have neurodevelopmental origins (4, 5). However, existing studies indicate minimal sex differences in core ASD symptoms (6). Mahendiran et al. investigated sex differences in social and communication skills in ASD, ADHD, and typically developing children. The authors found that females with ASD had worse performances than males at older ages, in spite of better communication skills in earlier age. This suggests that a developmental approach to find out sex differences over time might have multiple implications. In another study (Mahendiran et al.), the same authors performed a metanalysis of 11 original studies on sex differences in children with ASD and ADHD, and did not detect sex differences in social and communication function. However, the authors found a remarkable heterogeneity between the analyzed studies with respect to psychometric measurements and population differences. In particular, several of the studies included a low number of females, thus likely being underpowered to detect sex differences. Future larger studies, controlling for measure and with adequate numbers of female participants are required to further understand sex differences in social and communication domains.

ASD presents a wide range of comorbidities (7). Scandurra et al. instead evaluated adaptive skills in children with ASD, ADHD, or ASD+ADHD. A worse general adaptive profile was ascertained in the ASD and ASD+ADHD groups, as compared with the ADHD-only group, indicating the load of autism ASD symptoms on overall adaptive profile. The externalizing history of a cohort of young violent offenders with ASD, compared with offenders without ASD, is described in the article by Hofvander et al. A very high prevalence of externalizing and antisocial behaviors in the history of these offenders were detected and there were few differences between the groups. Placements in foster homes were overrepresented in ASD-offenders, which were also overrepresented in sex crimes with a child victim. This portion of ASD individuals causes significant challenges to the criminal justice system and additional knowledge is needed to prevent these individuals from committing crimes and also to receive a fair judicial treatment. Feeding problems are prevalent in children with ASD. In order to examine this further, Catino et al. studied interactions between parents and infants diagnosed with ASD, during feeding, using the Scale for the Assessment of Feeding Interactions (SVIA), a new assessment tool. This study supports the psychometric robustness of the SVIA, highlights the importance of direct observation of the parent-child dyad during feeding, and supports a high rate of feeding problems in children with ASD.

Finally, a group of four papers addressed the biological underpinnings of ASD (8). Oxidative stress and polymorphisms in genes encoding antioxidant enzymes (such as glutathione transferases, GSTs) might be involved in the development of ASD. Mandic–Maravic et al. found specific perinatal complications as significant risk factors for ASD. GSTM1 polymorphism might serve as a moderator of the effect of some prenatal factors on the risk of ASD such as using medication during pregnancy. In their review article, Balasco et al. addressed the neurobiological bases of sensory processing in ASD, with a specific focus of tactile sensitivity. Sensory abnormalities affect 90% of ASD individuals, and are recognized as diagnostic criteria for ASD. The article summarizes the most recent findings in this domain, focusing on both clinical studies and preclinical research on ASD mouse models. Modi et al. described a loss of inhibition that resulted in increased excitation/inhibition balance in the CA2 hippocampal circuit of Neuroligin 3 knockout mouse, a non-syndromic ASD mouse model. These defects were associated to social cognition deficits and confirmed the emerging role of the CA2 hippocampal region in controlling social behaviors. Finally, Sanfeliu et al. used RNA sequencing in *Mecp2* mutant mice and age-matched controls to identify differentially regulated genes and pathways. The authors found that some genes and pathways were differentially expressed in the brain and blood of *Mecp2* mutant mice at a symptomatic, but not pre-symptomatic, stage. Genes controlling circadian rhythms and immune response were specifically enriched in *Mecp2* mutant brain and blood, respectively.

AUTHOR CONTRIBUTIONS

All the authors equally contributed to the writing of the manuscript.

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ORIGINAL RESEARCH
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Transcriptomic Analysis of *Mecp2* Mutant Mice Reveals Differentially Expressed Genes and Altered Mechanisms in Both Blood and Brain

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Rett syndrome is a rare neuropsychiatric disorder with a wide symptomatology including impaired communication and movement, cardio-respiratory abnormalities, and seizures. The clinical presentation is typically associated to mutations in the gene coding for the methyl-CpG-binding protein 2 (*MECP2*), which is a transcription factor. The gene is ubiquitously present in all the cells of the organism with a peak of expression in neurons. For this reason, most of the studies in Rett models have been performed in brain. However, some of the symptoms of Rett are linked to the peripheral expression of *MECP2*, suggesting that the effects of the mutations affect gene expression levels in tissues other than the brain. We used RNA sequencing in *Mecp2* mutant mice and matched controls, to identify common genes and pathways differentially regulated across different tissues. We performed our study in brain and peripheral blood, and we identified differentially expressed genes (DEGs) and pathways in each tissue. Then, we compared the genes and mechanisms identified in each preparation. We found that some genes and molecular pathways that are differentially expressed in brain are also differentially expressed in blood of *Mecp2* mutant mice at a symptomatic—but not presymptomatic—stage. This is the case for the gene *Ube2v1*, linked to ubiquitination system, and *Serpin1*, involved in complement and coagulation cascades. Analysis of biological functions in the brain shows the enrichment of mechanisms correlated to circadian rhythms, while in the blood are enriched the mechanisms of response to stimulus—including immune response. Some mechanisms are enriched in both preparations, such as lipid metabolism and response to stress. These results suggest that analysis of peripheral blood can reveal ubiquitous altered molecular mechanisms of Rett and have applications in diagnosis and treatments' assessments.

Keywords: Rett syndrome, methyl-CpG-binding protein 2, gene expression, transcriptomics, cerebellum, blood

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INTRODUCTION

Rett syndrome (RTT) is a rare neurological disease, affecting approximately 1 in every 10,000 live female births. Approximately 95% of RTT cases present with mutations in the *MECP2* gene, which is located in the long arm of the X chromosome (1). Its genomic location explains why the majority of patients are females. Females can compensate for loss of *MECP2* function with an extra intact

copy on the homologous X chromosome, but this is not the case for males. Consequently, males have a severe phenotype and represent less than 1% of RTT patients.

The symptoms manifest after a period of apparent normality, corresponding to the first 6–18 months of life. After this stage, patients present neurological features (microcephaly, seizure), motor disability (ataxia, loss of purposeful hand use, stereotyped hand movements, loss of the ability to walk, hypotonia), social impairment (loss of speech, unresponsiveness to social cues, lack of emotional expression), and autonomic complications (respiratory anomalies, cardiac dysfunction, constipation) (2). The symptoms and their severity can be variable from one patient to another. One of the reasons for this variability is thought to be skewed X-inactivation, as patients with an X-inactivation biased to the nonmutated copy of *MECP2* have shown little to no symptoms (3).

The strong association between *MECP2* mutations and the disease has prompted the generation of mutant mice, which present specific mutations in *Mecp2* or a lack of its expression (4–10). These mice show signs that resemble the symptoms in patients; hence, they are considered valuable models for shedding light on the molecular mechanisms underlying RTT (4, 5).

MECP2 encodes for methyl-CpG-binding protein 2 (MeCP2), a chromatin binding protein (11) that is expressed ubiquitously in the body with major expression in the central nervous system (CNS). As MeCP2 was first postulated as a transcriptional repressor, several groups used the mouse models to study gene expression changes (12–14). These studies have revealed that MeCP2 can both upregulate and downregulate gene expression, and that gene expression changes are specific to different brain areas and cell types (12–14).

Although *MECP2* is highly expressed in the brain, it is also present in several other tissues/organs, and a recent mouse model showed that a small portion of symptoms are still present when *Mecp2* is exclusively expressed in the CNS but not in the rest of the body (15), supporting the possibility that molecular signatures of dysfunctions in RTT may be present in peripheral tissues, and they are possibly linked to changes in the brain.

In our study, we used RNA sequencing to compare the differential gene expression in brain and in blood in a mouse model of RTT. This analysis reveals associations between genes expressed in the two tissues and has important applications in the detection of peripheral biomarkers for Rett syndrome.

RESULTS

MeCP2 Protein Expression Levels Are High in Mouse Cerebellum at 7 Weeks of Age

In the brain, the expression of *Mecp2* is dynamically modulated during development (16, 17). Additionally, *Mecp2* expression can differ between brain areas (15), as well as the genes that *Mecp2* regulates (13). For these reasons, we understand that to perform a transcriptomic analysis, it is necessary to use a specific brain area, and that the area should ideally have high levels of MeCP2 expression at the developmental stage in which the study is conducted.

To identify the ideal brain region for the developmental stage of our study (i.e., 7 weeks, when symptoms are advanced in the *Mecp2*-null male), we measured the temporal expression of MeCP2 protein in wild-type (WT) C57Bl/6 male mice in different brain areas (cortex, cerebellum, hippocampus, and hypothalamus) and compared them to the full brain expression (**Figure 1**). *P*-values were calculated using a Kruskal–Wallis test followed by a Dunn's multiple comparisons test.

At 7 weeks, the only area that showed significantly higher levels of MeCP2 compared to full brain was the cerebellum (4.29-fold, p-value = 0.0002). We also screened for the expression of MeCP2 protein at 4 and 12 weeks of age, at which stage there were no significant differences between the full brain and the specific areas.

The results show that MeCP2 expression changes during development and at 7 weeks, the MeCP2 protein is highly expressed in the cerebellum.

RNA Sequencing Reveals Differentially Expressed Genes in Cerebellum and Blood of *Mecp2-Null* Mice

RNA sequencing (RNAseq) was performed on male *Mecp2*-null mice at 7 weeks of age and compared to wild-type (WT)

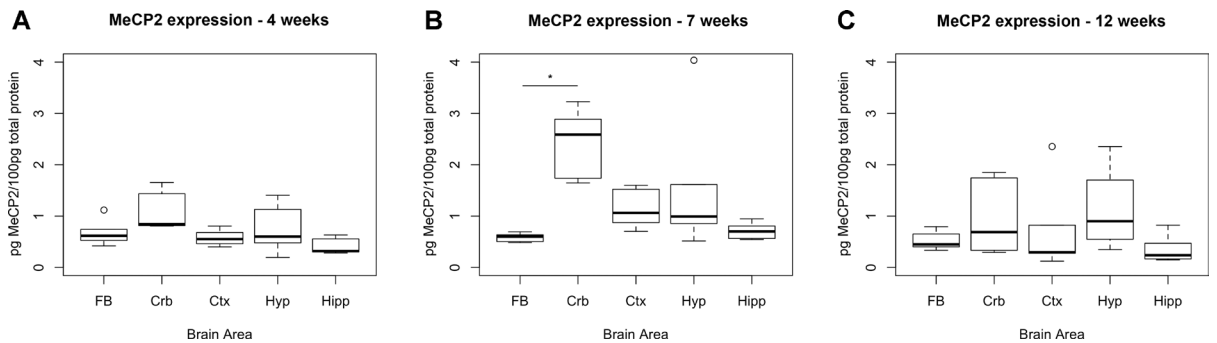

FIGURE 1 | Expression levels of MeCP2 peak in cerebellum at 7 weeks. Expression of MeCP2 protein in full brain (FB), cerebellum (Crb), cortex (Ctx), hypothalamus (Hyp), and hippocampus (Hipp) of WT C57Bl/6 male mice, at the ages of 4 **(A)**, 7 **(B)**, and 12 weeks **(C)**. Six mice per group were examined. At 7 weeks, the cerebellum is the only area where MeCP2 protein expression is significantly different than the full brain. The data was analyzed with a Kruskal–Wallis test, considering significant p-values < 0.05. No significant differences between areas were found at 4 or 12 weeks of age.

matched controls. The experiment was performed independently in cerebellum and blood. Tissue-dependent EdgeR analysis revealed 81 differentially expressed (DE) genes (DEGs) in cerebellum, of which 44 were upregulated and 37 downregulated (**Figure 2A**). In blood, 205 DEGs were found to be significantly different between WT and mutants: 105 upregulated and 100 downregulated. We found similar levels of gene expression in both tissues (R^2 Spearman WT = 0.95, R^2 Spearman MUT = 0.93, **Figure 2B** and **C**). DEGs show different profiles in the heatmaps of expression (**Figure 2D** and **E**). **Table 1** contains the first 10 genes of each group ordered by *p*-value. The full list is available in **Supplementary File 1**. As an intrinsic control, we checked the expression of *Mecp2*, and we confirmed a strong difference between WT and mutants: a log2 fold change (log2FC) of −3.88 in the cerebellum of *Mecp2*-null mice relative to the controls, with an FDR-corrected *p*-value [false discovery rate (FDR)] of 5.38E-27. In blood, *Mecp2* showed a log2FC of −2.50, and an FDR of 4.07E-06. *Bdnf*, which is known to be downregulated in RTT, showed a log2FC of −1.25 and an FDR of 1.05E-04 in cerebellum, while in blood, it was not detectable.

Altogether, the comparison between blood and brain revealed high correlation of genes' expression in both tissues.

Identification of Overlapping Genes Between Cerebellum and Blood

We then proceeded to identify DEGs present in both cerebellum and blood. We found two genes with an FDR-corrected *p*-value < 0.05 in both tissues: *Mecp2* and *Ube2v1*. *Mecp2* showed a log2FC of −3.88 and an FDR-corrected *p*-value of 5.38E-27 in cerebellum, and a log2FC of −2.50 and an FDR-corrected *p*-value of 4.07E-06 in blood. *Ube2v1* showed a log2FC of −2.91 and an FDR-corrected *p*-value of 0.02 in cerebellum, and a logFC of 2.58 and an FDR-corrected *p*-value of 4.52E-10 in blood. These genes were selected

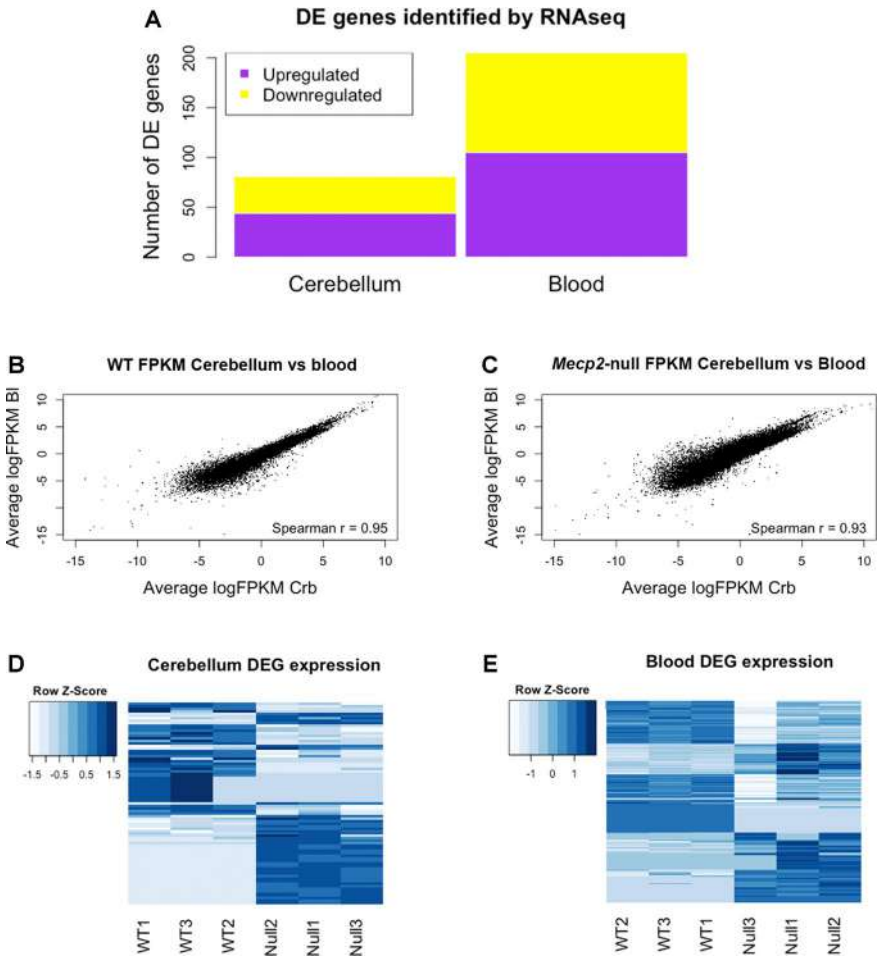

FIGURE 2 | RNAseq analysis reveals differentially expressed genes (DEGs) in cerebellum and blood and identifies gene expression correlation between them. Summary of the *Mecp2*-null vs WT RNAseq analysis. **(A)** Number of DEGs identified in blood and cerebellum between *Mecp2*-null (N = 3) and WT mice (N = 3). 205 DEGs were identified in blood (105 upregulated and 100 downregulated), and 81 were identified in cerebellum (44 upregulated and 37 downregulated). For statistical comparison, we used EdgeR package with FDR correction and a significance level of p < 0.05. **(B, C)** Scatterplots of average gene expression in cerebellum versus average gene expression in blood, in logFPKM in WT **(B)** and *Mecp2*-null **(C)**. **(D, E)** Heat maps of expression Z-scores (normalized FPKM values) of the DEGs identified in cerebellum and blood.

TABLE 1 | Top 10 differentially expressed genes (DEGs) ordered by *p*-value in cerebellum and blood. In both tissues, we detected a strong downregulation of Mecp2, which acts as a positive control of the model and the methodology. This is also the case for Bdnf in cerebellum. Log2CPM represents the log2-average counts per million across all samples. Log2FC represents the log2-ratio Mecp2-null/wild type (WT); hence, positive values mean upregulation in Mecp2-null and vice versa.

Gene symbol	Log2CPM	Log2FC	FDR-corrected p-value
Cerebellum			
Mecp2	6.49	–3.88	5.38E-27
Gm27640	0.05	6.92	1.94E-23
eLsm12	4.5	1.91	5.39E-19
Gpr21	0.06	5.22	5.09E-16
Gm10408	–1.41	9.60	8.96E-13
Zdhhc24	3.96	1.31	1.17E-06
Tenm2	5.53	0.99	6.32E-06
Bdnf	4.2	–1.25	5.29E-05
Paip2	7.07	1.00	1.05E-04
Gm3298	–3.48	7.30	2.22E-04
Blood			
Ube2v1	5.71	2.58	4.52E-10
Bpifa1	4.78	15.53	2.70E-09
Tmem164	3.59	3.28	4.46E-09
Tnnc2	0.24	10.97	6.73E-09
Mmrn1	5.01	–3.25	4.73E-08
Camp	7.57	7.69	8.33E-08
Scgb3a1	3.88	14.63	1.75E-06
Mpo	4.54	10.18	1.75E-06
Bace2	–0.34	10.39	3.01E-06
Mecp2	3.85	–2.50	4.07E-06

for quantitative polymerase chain reaction (qPCR) validation with additional samples (n = 12/group). As expected, *Mecp2* differential expression was confirmed, with no expression in the mutant samples and average delta Ct values (dCt) of 5.31 in cerebellum and 6.26 in blood. *Ube2v1* showed significant upregulation in blood of P50 *Mecp2*-null mice (ddCt = 0.46, FC = 1.42, p-value = 0.012), and it was downregulated in cerebellum of age-matched *Mecp2*-null mice (ddCt = −0.27, FC = 0.84, p-value = 0.032), (**Figure 3**). An analogous comparison in presymptomatic mice showed no significant difference between mutant mice and controls (cerebellum: ddCt = 0.058, p-value = 0.354, blood: ddCt = −0.354, p-value = 0.314). Additionally, RNA quantification performed in presymptomatic females did not reveal any differences of *Ube2v1* expression between heterozygous *Mecp2*-null and WT (data not shown). All this suggests that the differential expression of *Ube2v1* is linked to the appearance of the symptoms.

Our independent validation confirmed the dysregulation of *Ube2v1* in both brain and blood, identifying a particular form of ubiquitination as a mechanism broadly altered in *Mecp2* mutants.

Gene Pathways and Network Analysis Reveals Mechanisms Dysregulated in Both Brain and Blood

To identify the cellular mechanisms differing between WT and mutant mice, we used the genes identified in the single gene analysis to perform pathway analysis, protein interaction network, and biological function analysis [gene ontology (GO)].

Potentially dysregulated pathways were identified with the iPathway software, which takes as an input differential expression data between two conditions and computes the overrepresentation and possible perturbation of biological pathways according to the differences in gene expression. The analysis revealed five significant (p-value < 0.05) pathways in cerebellum and 33 in blood. After FDR correction, the only significant pathway in cerebellum was complement and coagulation cascades and the only significant pathway in blood was platelet activation. The behavior of the complement and coagulation cascades pathway was driven by the dysregulation of the following genes: *Fga*, *Serpina1c*, and *Serpina1e*, while the affectation of the platelet activation pathway was driven by the genes: *Ptgs1*, *Mylk*, *P2rx1*, *Hpr2*, *Gp5*, *Vwf*, and *Actg1*. Interestingly, although there is no overlap between pathways in brain and blood, *Serpina1c* was identified by RNAseq to be DE both in brain and blood, so it was selected for validation with PCR (see paragraph below). The full list of genes with an uncorrected p-value < 0.05 can be found in **Supplementary File 2**.

The analysis of protein interaction network was performed with the STRINGapp in Cytoscape, which predicts interactions between the members of a protein input list, and related interactors. As input, we used the DE genes from cerebellum and blood separately. The software can identify which are the higher connected nodes in the network ("hubs") and rank them according to the number of connections (degree).

We set up the analysis to add a maximum of 30 additional interactors per group. We then ranked the nodes by degree, in order to find proteins with the highest connectivity. In the cerebellum, the top scoring protein was Alb (Albumin), while in the blood it was Spna2 (Spectrin alpha 2). We then identified the network proteins present in both tissues, in order to find possible common mechanisms: Alb, App, Hsp90aa1, Hsp901b1, Ilt6, Kng1, Kng2, Nos1, and Spna2. **Figure 4** depicts the network obtained from cerebellum, with the proteins also present in the blood analysis highlighted. The full list of proteins and their interactors, ranked by degree, can be found in **Supplementary File 3**.

The common interactors are involved in the nitric oxide biosynthesis pathway, which in STRING results statistically significant (p-value = 0.016 after FDR).

We then performed a functional enrichment analysis on the output of the STRING analysis—including both DE genes and their interactors, in order to identify overrepresented gene ontology (GO) biological process categories. The analysis of functional enrichment revealed 462 and 477 significant GO categories in cerebellum and blood, respectively. The most significantly enriched process in the cerebellum is "circadian behavior," while in the blood it is "response to stress." The top 10 biological processes significantly enriched in cerebellum and blood are depicted in **Figure 5A** and **B**. We found 152 overlapping significant GO biological processes between cerebellum and blood, the most significant being "response to stress." The top 10 most significant overlapping biological processes are depicted in **Figure 5C**. The full list of GO categories enriched in cerebellum, blood, and their overlap can be found in **Supplementary File 4**.

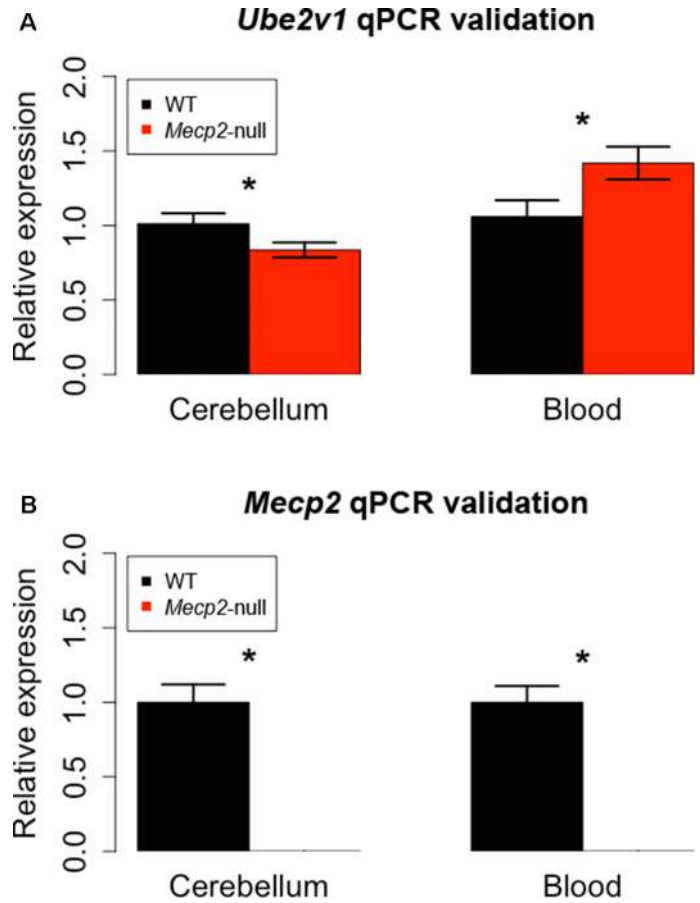

FIGURE 3 | qPCR on different biological samples validate *Ube2v1* and *Mecp2* dysregulation in brain and blood. Validation by qPCR of *Ube2v1* **(A)** and *Mecp2* **(B)** differential expression between *Mecp2*-null ($N = 6$) and WT mice ($N = 6$). Expression is represented as relative expression, calculated as 2^{ddCt}. Regarding *Ube2v1*, the ddCt between *Mecp2*-null and WT is 0.46 in blood (p-value = 0.012, FC = 1.42) and –0.27 in cerebellum (p-value = 0.032, FC = 0.84). In *Mecp2*-null mice, no *Mecp2* expression was detected.

The gene-sets analysis reveals common mechanisms activated in blood and brain with an enrichment of mechanisms involved in system's homeostasis, metabolic processes such as nitric oxide synthesis, and coagulation-related processes.

qPCR Validation of Additional Genes

In addition to the identified overlapping genes in differential expression analysis, we considered for validation with qPCR additional genes that were selected according to different criteria. First, we selected genes that were significant in brain and also in blood and vice versa (**Table 2**). Of these, we tested with qPCR genes that were linked to the multiple gene analysis and/or were lined to mechanisms known to be associated to RTT: *Dnah14*, *Serpina1c*, *Lsm12*, *Mup10*, *Ankrd63*, *Hal*, *Ankrd63*, *Slc6a4*, *Crispld2*, *Tnnc2*, *Mpo*, *Gpx3*, *Paip2*, and *Fkbp5*. We also tested, in blood, genes that showed a high fold change or were dysregulated in cellular pathways in blood: *S100a9*, *Ms4a3*, *Snx31*, and *Prg3*, *Gstm2*, *Gsta3*, and *Itgb3*. Two of the selected genes (*Fkbp5* and *S100a9*) had already been identified by other screenings (18). The additional tested genes are reported in **Table 2**.

We considered significantly different in the qPCR analysis those genes with a p-value < 0.05, and we considered "trending" the genes with a p-value between 0.1 and 0.05. The genes that confirmed to be dysregulated after qPCR are: *Serpina1c*, *Ankrd63*, *Crispld2*, *Mpo*, and *Gsta3*. The validation results are reported in **Supplementary File 5**. In the case of *Serpina1c*, we performed an additional PCR in liver extracts as this gene is mostly expressed by hepatocytes (19). We confirmed the downregulation of *Serpina1c* in mutants also for this preparation (ddCt = −1.006, p-value = 0.008). We also tested the differential expression of *Serpina1c* between mutant and WT in cerebellum and blood of presymptomatic mice, obtaining negative results (cerebellum: ddCt = 1.804, p-value = 0.18, blood: ddCt = 3.563, p-value = 0.363). RNA quantification performed in presymptomatic females did not reveal any differences of *Serpina1c* expression between heterozygous *Mecp2*-null and WT (data not shown). Like in the case of *Ube2v1*, this suggests that the dysregulation of *Serpina1c* is linked to the appearance of symptoms.

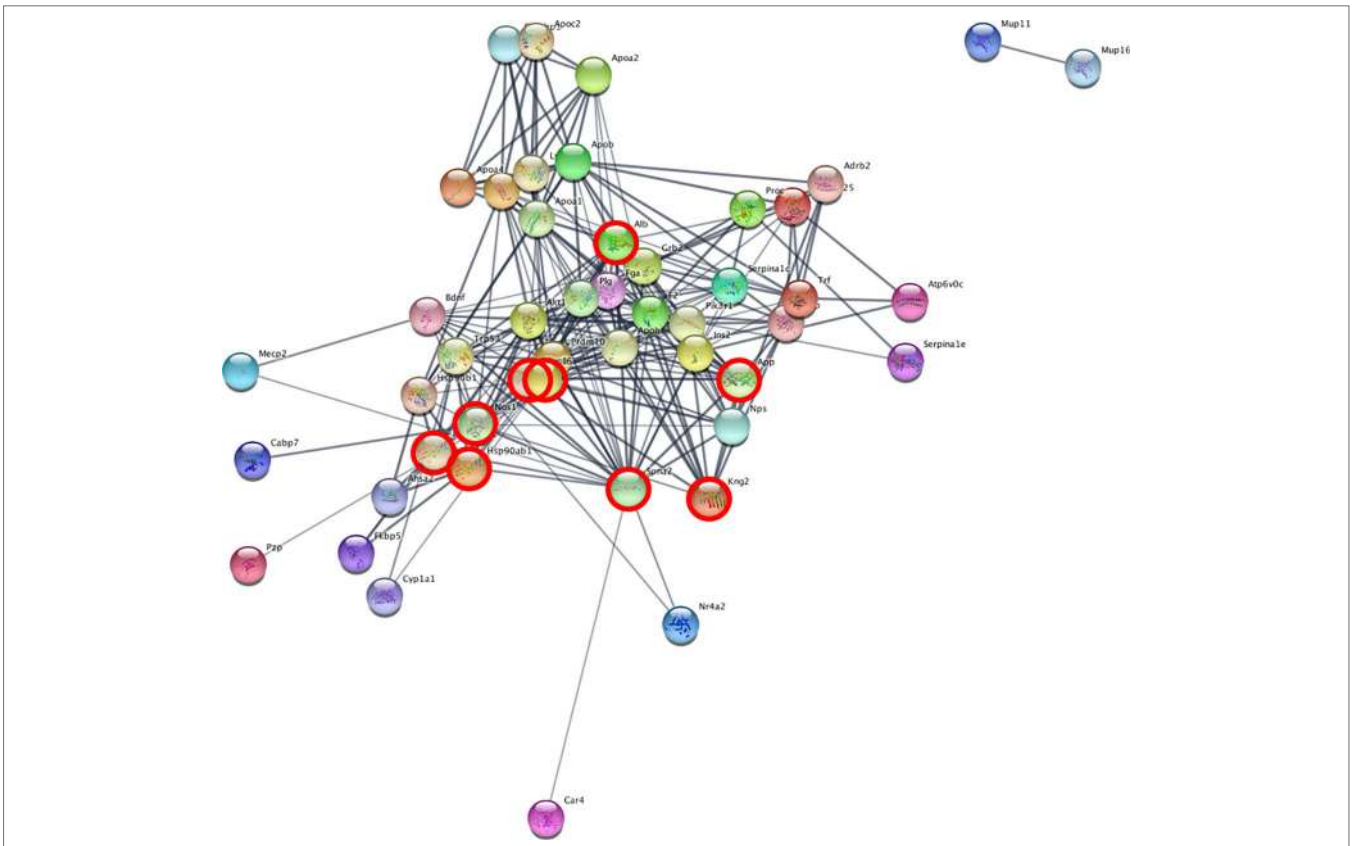

FIGURE 4 | Network analysis reveals interacting genes across cerebellum and blood. STRING analysis network of cerebellum DE genes. The highlighted nodes correspond to proteins overlapping between blood and cerebellum.

Altogether, the multiple RNA measurements on different samples confirmed reveal that there are genes and mechanisms DE in both blood and brain.

DISCUSSION

The quest for understanding and treating neurodevelopmental disorders is hampered by several factors, one of these being access to the brain. Considering the limited molecular etiology, and the impact of *MECP2* mutations in multiple tissues to the clinical presentation (15), RTT represents an ideal model to study the mechanisms of disease present in both CNSs and peripheral systems, with the aim to identify markers in peripheral blood that would be accessible for diagnostic, prognostic, and treatment purposes.

In this study, we compare the expression of genes and molecular pathways in blood and brain of *Mecp2* mutant mice to identify common mechanisms dysregulated across different tissues. Since gene expression is strongly dependent on brain area and developmental stages, we first investigated which brain regions highly express the MeCP2 protein in P50 male mice—an age when symptoms are advanced. We find that in P50 mice, the cerebellum is the region with higher expression of MeCP2 compared to hippocampus, cortex, and hypothalamus (**Figure 1**), but protein expression in the cerebellum decreases in adult mice, confirming the results of Ross and colleagues (15). We used *Mecp2*-null male mutants and matched controls at P50, and we compared the DEGs in cerebellum to the DEGs in the blood. There are several studies that look at the gene expression profile in Rett patients (20) and mouse models (12, 14, 18, 21–39). However, to our knowledge this is the first study that compares directly differential gene expression in brain and blood of *Mecp2*-null mice versus matched controls.

The analysis of the expression levels shows a reasonable correlation between the genes expressed in the two preparations (R^2_WT = 0.95, R^2_MUT = 0.93, **Figure 2A**), although there is a higher variability in the blood compared to the cerebellum (**Supplementary Figure 6**). This variability could generate results less consistent in blood, reinforcing the necessity of validating the results with another method (PCR) on independent biological samples. Both the differential expression analysis (EdgeR) and the gene pathways and network analysis identify overlapping associations across the preparations.

The most significant gene DE in the two tissues—other than *Mecp2* itself—is *Ube2v1* (also known as *Uev1a*), which is downregulated in cerebellum and upregulated in the blood of the mutant mice. Its differential expression was confirmed by qPCR both in the blood and in the cerebellum (**Figure 3**). *Ube2v1* encodes for Ubiquitin Conjugating Enzyme E2 V1, which is a ubiquitin-conjugating E2 enzyme variant (UEV) protein. UEVs are similar in sequence to ubiquitin-conjugating E2 enzymes but lack their enzymatic activity (40). This type of ubiquitination is

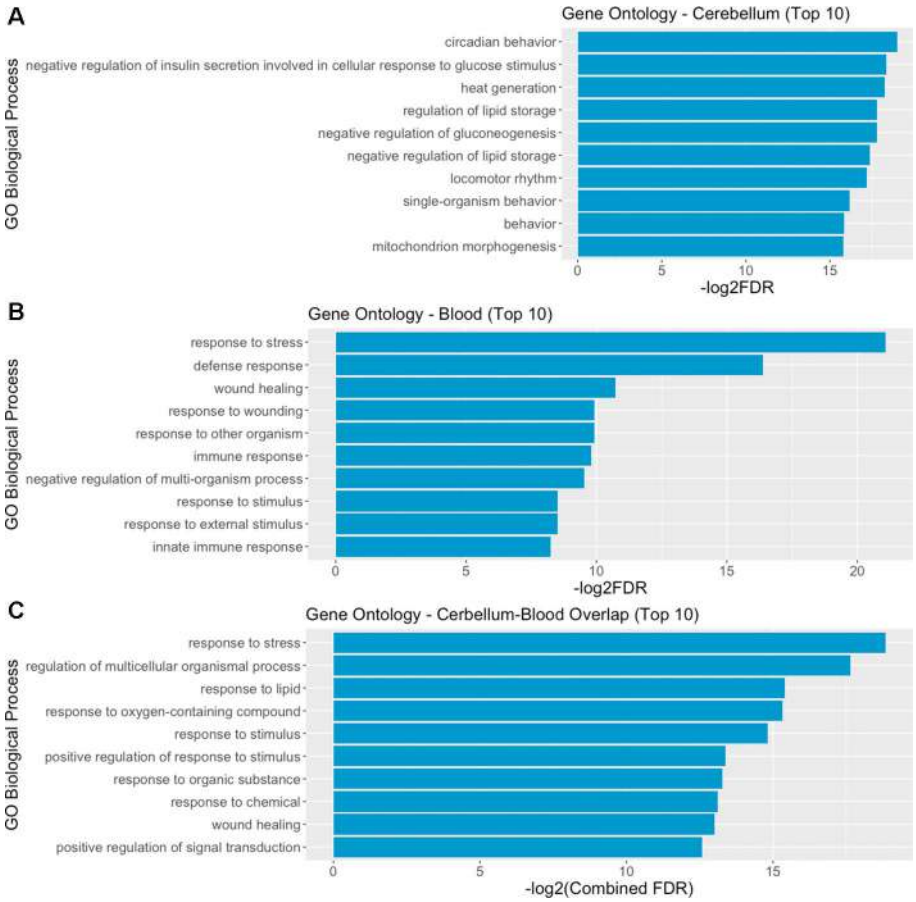

FIGURE 5 | Gene ontology (GO) analysis reveals overlapping biological mechanisms in cerebellum and blood. **(A, B)** Top 10 gene ontology biological process categories enriched in cerebellum and blood DE genes and their interactors predicted by STRING. **(C)** Top 10 gene ontology biological processes overlapping between blood and brain, according to their combined FDR. Combined FDR was computed as follows: FDRCerebellum + FDRBlood – FDRCerebellum * FDRBlood.

not linked to proteolysis, but it acts as a system of nonproteolytic cell signaling instead (41) and has been associated to elements involved in synaptic plasticity and function. In the brain, the function of Ube2v1 is to modulate the protein organization at synaptic level and the ability of the neurons to respond to changes in activity (**Figure 5A**). **Figure 6** represents speculations regarding possible roles of Ube2v1 in RTT.

In a mouse study, postsynaptic density-95 (PSD95) and Ube2v1 were copurified using tandem affinity purification (42) and Ube2v1 mediates Lys63-linked polyubiquitination (L63-polyUb) of PSD95 in an activity-dependent and nonproteolytic manner. Such modification of PSD95 regulates two main properties associated to synaptic function: first, it affects PSD95's scaffolding properties, promoting synaptic formation, maturation, and strength (43). Second, the ubiquitination of PSD95 is known to mediate N-methyl-D-aspartate-mediated α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) endocytosis (44). Interestingly, the *Ube2v1* homologous *uev-1* regulates AMPAR trafficking in *Caenorhabditis elegans*, possibly by modulating a clathrin-independent AMPAR recycling pathway (45). Trafficking of AMPARs has been shown to be altered in the hippocampus of *Mecp2*-null mice (46). There is further evidence suggesting an important function of *Ube2v1* in the brain; knocking out its associate *Ubc13* in mouse results in impaired cerebellar synapse formation (47). This hypothesis, although only theoretical, suggests that *Mecp2*, through the activity-dependent regulation of *Ube2v1*, influences L63-polyUb of PSD95. PSD95 modulates synaptic maturation and function, which indeed are impaired in RTT (48).

Outside the brain, *Ube2v1* exerts control over cell cycle and differentiation (40), response to DNA damage through p53 (49), and regulates pathways responsible for immune inflammatory response, mainly through the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway (50, 51) (**Figure 5B**). For instance, the UBE2V1–UBC13–TRAF6 complex activates nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IkB) kinase (IKK) in response to proinflammatory cytokines (52, 53). This mechanism is consistent with the increased levels of NF-kB observed in MeCP2-deficient human peripheral blood mononuclear cells (PBMCs) and in the human monocyte line THP1 (54). The same authors found that the upregulation of NF-kB caused by MeCP2 deficiency enhances the expression of tumor necrosis factor alpha (TNFa), interleukin 6 (IL-6) and interleukin 3 (IL-3)

TABLE 2 | Summary of additional genes tested by qPCR. The list includes differentially expressed (DE) genes overlapping between cerebellum and blood, some genes selected for their high fold change in blood and some genes selected from predicted dysregulated pathways. Log2FC refers to the ratio Mecp2-null/WT; hence, positive values mean upregulation in Mecp2-null and vice versa.

Additional genes tested by qPCR				
Genes identified in cerebellum that are also significant in blood				
Gene	**log2FC cerebellum**	**FDR cerebellum**	**log2FC blood**	**Uncorrected *p*-value blood**
Dnah14	2.04	2.51E-02	8.54	3.88E-03
Fkbp5	1.33	3.70E-02	1.40	4.90E-03
Serpina1c	–10.52	2.76E-02	9.87	5.50E-03
Gm28374	6.52	1.34E-02	–6.55	1.84E-02
Lsm12	1.91	5.39E-19	–1.30	3.24E-02
Paip2	1.00	1.06E-04	–1.06	3.49E-02
Ahsa2	–0.85	4.13E-02	–0.83	4.15E-02
Mup10	–10.75	3.30E-02	7.12	4.28E-02
Genes identified in blood that are also significant in cerebellum				
Gene	**log2FC blood**	**FDR blood**	**log2FC cerebellum**	**Uncorrected *p*-value cerebellum**
Atp6v0d2	10.74	4.30E-02	5.91	3.44E-04
Hal	9.73	2.87E-02	–5.88	3.65E-04
Ankrd63	8.90	3.79E-02	2.07	5.56E-04
Hist1h2be	–3.32	1.08E-02	–0.86	4.59E-03
Slc6a4	–1.99	2.27E-02	2.75	1.13E-02
Crispld2	2.54	4.87E-02	0.66	1.22E-02
RP23-253I14.4	11.74	1.05E-02	3.12	1.28E-02
C430002N11Rik	–8.55	5.89E-03	4.84	1.32E-02
Tnnc2	10.97	6.73E-09	3.44	1.37E-02
Scgb3a2	14.25	2.26E-03	4.57	1.91E-02
Mpo	10.18	1.75E-06	1.18	2.65E-02
Gm5741	–8.52	9.00E-03	2.67	3.33E-02
Gpx3	3.03	1.64E-02	0.73	3.37E-02
High fold change in blood				
Gene	**log2FC RNAseq**			**FDR RNAseq**
S100a9	2.87			2.01E-02
Snx31	–8.80			3.09E-03
Ms4a3	13.05			5.27E-03
Prg3	12.26			5.27E-03
Genes from predicted dysregulated pathways (blood)				
Gene	**log2FC RNAseq**			**FDR RNAseq**
Gsta3	8.98			4.06E-03
Gstm2	7.56			2.34E-04
Itgb3	–3.15			1.00E-03

(55), which can contribute to the subclinical immune dysregulation observed in RTT patients (56), including increased levels of TNFa and IL-6 in the blood, among other cytokines (57). Alteration of the NF-kB pathway was also suggested after transcriptomic analysis on the blood of RTT patients (58). NF-kB is also dysregulated in cortical neurons of *Mecp2*-null mice, with direct effects on dendritic complexity that can be rescued by reducing NF-kB signaling (59).

The presence of *Ube2v1* across different processes and cell types, and especially in the immune system, makes it an interesting candidate to examine the function of *MECP2* in the periphery and to reflect brain function in peripheral blood. A transcriptomic analysis performed in human PBMCs revealed enrichment of gene ontology categories related to regulation of protein ubiquitination (60). This could support the idea of *Ube2v1* also playing a role in the human condition.

Serpina1c

Serpina1c resulted to be a DEG in both blood and brain. Its downregulation in the mutants was confirmed in cerebellum and showed a strong trend in blood. In blood, the qPCR expression analysis contradicts the one originally obtained by RNAseq, which indicated a strong upregulation in the blood of mutants. The results in the sequencing analysis though, are driven by one single sample, while the results in PCR have been replicated

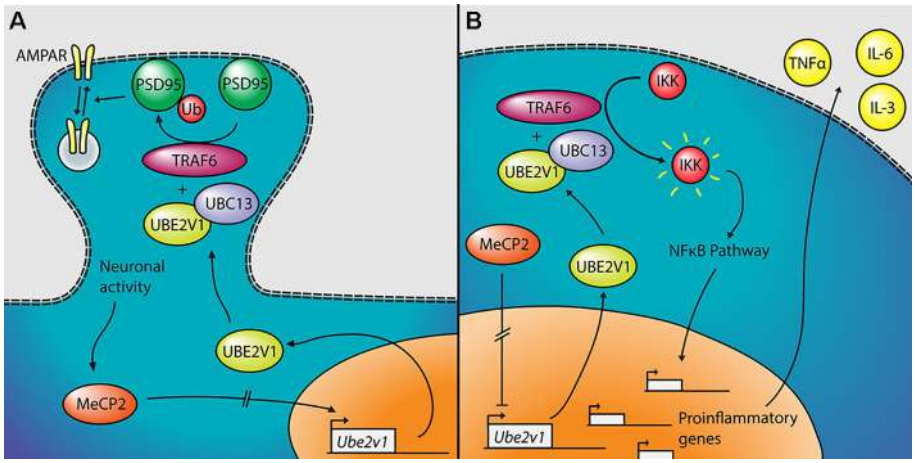

FIGURE 6 | Proposed mechanism linking *Mecp2*, *Ube2v1*, and Rett syndrome (RTT). **(A)** In neurons, MeCP2 would upregulate the transcription of *Ube2v1*, directly or indirectly. UBE2V1, together with UBC13 and TRAF6, would promote Lys63-linked ubiquitination of PSD95, which would in turn regulate AMPA receptor (AMPAR) trafficking. **(B)** In peripheral blood mononuclear cells (PBMCs), MeCP2 would repress the transcription of *Ube2v1* (directly or not). UBE2V1, together with UBC13 and TRAF6, would activate IκB kinase (IKK), which would activate the NFκB pathway. This would promote the transcription of proinflammatory genes and the upregulation of cytokines such as TNFα, IL-6, and IL-3.

across 12 independent biological samples; hence, for this gene, we trust the decrease measured with PCR. The nature of *Serpina1c* gives reasons to pay attention to its possible involvement in RTT.

Serpina1c encodes for α-1-antitrypsin (α1AT) 1–3, which is part of the serine protease inhibitor (Serpin) family. While in mouse, *Serpina1* has five different variants (Serpina1a-e) with distinct specificity (61), in human, there is only one *SERPINA1* gene.

In mouse, the protein network for *Serpina1c* shows interactions with the elements of the Akt pathways, which has been shown to be dysregulated in *Mecp2* mutant mice (62).

In humans, mutations that result in a deficit of α1AT are mostly known to be a cause of pulmonary emphysema and liver disease (63). The liver is the main secretor of α1AT in both human and mouse, and it also expresses MeCP2. We confirmed the downregulation of *Serpina1c* RNA in the liver of mutant mice by qPCR. The MeCP2 and α1AT coexpression suggests a role of *Serpina1c/SERPINA1* in RTT.

In normal conditions, circulating α1AT protects the lungs by inhibiting neutrophil elastase, which degrades the connective tissue. Patients with low plasma levels of α1AT have an elevated risk of pulmonary emphysema, due to excessive degradation of the connective tissue. To our knowledge, respiratory deficiencies present in RTT have not been yet linked to this kind of pathology. However, emphysema-like features have been observed in the lungs of *Mecp2*-null mice (64).

The role of α1AT in liver pathogenesis would be less relevant in RTT, as its main disease-causing mechanism is the aggregation and accumulation of abnormal forms of α1AT in the hepatocytes (65, 66). The most intriguing result of the *Serpina1c* analysis is that diagnostic grade blood tests are already available to quantify the levels of circulating α1AT (67) and the same methods could be used in RTT patients for prognostic purposes.

Regarding its brain function, α1AT has been proposed to be involved in Alzheimer's disease (AD) (68), Parkinson's disease (PD) (69), schizophrenia (70), and amyotrophic lateral sclerosis (ALS) (71), but no specific mechanisms have been described. α1AT has been shown to drastically reduce excitotoxicity *in vitro* through the inhibition of calpain (72), but there is no evidence of this phenomenon being physiologically significant *in vivo*. α1AT is also therapeutic against stroke in rats (73).

It is also possible that Serpina1c in the brain acts through the interaction with other proteins. α1AT is an inhibitor of activated protein C (APC) (74). APC, in turn, neutralizes plasminogen activator inhibitor 1 (PAI) (75), which inhibits tissue plasminogen activator (tPA) (76). tPA is a protease that catalyzes the conversion of plasminogen to plasmin. Aside from its important anticoagulant role, plasmin is responsible for the cleavage of the inactive precursor brain derived neurotrophic factor (proBDNF) into active mature BDNF (mBDNF) (77). BDNF is considered relevant in RTT: it has been shown to be dysregulated in both RTT patients (78, 79) and in animal models (80, 81), and its overexpression in mice ameliorates the symptoms of *Mecp2*-null mice (80). MeCP2 seems to directly regulate the expression of BDNF in an activity-dependent manner (82), suggesting that the involvement of BDNF in RTT is transcription dependent. However, it is possible that the decreased expression of BDNF in RTT is dependent both by an MeCP2-dependent transcription, and by an abnormal posttranslational cleavage of BDNF. BDNF has been studied as possible peripheral biomarkers of mood disorders (83), and the tPA–BDNF pathway in serum is a target for the treatment of depression (84). Moreover, the fact that tPA's main known role is related to hemostasis would be in accordance with the dysregulation of the platelet activation mechanisms, identified with the pathways analysis.

Interestingly, RNA expression analysis of both *Ube2v1* and *Serpina1c* in presymptomatic mice shows no difference

between WT and mutant mice, suggesting that their altered expression may be linked to presentation of symptoms and severity of the condition.

Platelet Activation Pathway

Pathway analysis predicted a potential disruption of the platelet activation pathway in the blood of *Mecp2*-null mice. Interestingly, an altered coagulation pathway was identified in the brain of the same mutant mice, driven by the already discussed *Serpina1c*. If, according to the previously described hypothesis, the levels of plasmin were altered in RTT, an abnormal hemostatic state could be expected, although it has never been reported in patients.

Other mechanisms could potentially link platelet activation and RTT. In a metabolomic screening of *Mecp2*-null mice, an alteration of the platelet-activating factor (PAF) cycle was predicted (85). PAF is a multifunctional phospholipid, which acts through its G-coupled receptor PAFR (86) and is involved in activation of platelets and leucocytes and in synaptic function (87, 88).

Regarding its role in the synapse, PAF has been described as a retrograde messenger in hippocampal long-term potentiation (LTP) (89). PAF also mediates synaptic facilitation in striatal slices (90) and LTP in cortical slices (91). PAF can enhance presynaptic vesicle exocytosis through calcium signaling (92, 93). Animal models lacking PAFR have shown differing results, with a study claiming LTP attenuation (94) and another claiming a normal synaptic function (95). It has also been observed that PAF needs to be properly regulated: elevated levels of PAF can cause excitotoxicity (96), and it seems to be involved in various CNS diseases, such as AD, PD, epilepsy, stroke, or multiple sclerosis (97). LTP defects have been repeatedly observed in mouse models of RTT (46, 98–101), and PAF could be a contributing factor.

We speculate that the alteration of the platelet activation pathway could be influenced by abnormal levels of nitric oxide (described below), which has a limiting effect on platelet activation (102).

Nitric Oxide

Our protein interaction analysis revealed that some genes overlapping between blood and cerebellum could be related to the nitric oxide (NO) synthesis pathway. NO is a signaling molecule present in several biological processes. In the brain, it can act as an anterograde and retrograde neurotransmitter, and it can induce dendritic and presynaptic growth (103). As previously mentioned, synaptic function and morphology are abnormal in RTT. NO could play a role in anxiety (104)—which is characteristic of RTT (105)—and it has also been linked to other pathologies of the CNS such as schizophrenia, bipolar disorder, depression, autism, and fragile X syndrome (106, 107). Abnormal upregulation of neuronal NO synthase has been observed in enteric neurons of *Mecp2*-null mice (108). Conversely, a reduced NO availability has been observed to contribute to vascular dysfunction in *Mecp2*-null mice (109). Our data is not enough to describe if the NO levels are most likely to be up- or downregulated in our model, but it remains a hypothesis to explore further. NO would also be a potential target for a treatment. L-lysine, an inhibitor of NO synthesis, has been used in a trial as an adjuvant of risperidone in schizophrenia (110). Bumetanide, a molecule that has been shown to be effective in the treatment of ASD (111), has been suggested to achieve its effect by increasing the levels of NO (112), but no experiments have been performed in that regard.

The analysis of enriched biological functions with GO shows consistency with other function dysregulated in RTT. The enrichment of circadian behavior in the brain preparation is consistent with sleep disorders in patients with RTT (113), and with disruption found in *Mecp2* mutant mice. *Mecp2* mutants have altered nocturnal activity and present structural abnormalities of hypothalamic centers controlling circadian rhythms (114). In addition, both MeCP2 expression and the MeCP2-binding to promoters of regulated genes are correlated to circadian rhythms (115). The altered nocturnal activity correlates with anxiety behaviors and increased plasma concentration of corticosterone—the stress hormone—linking the enrichment of circadian behavior function to the increased response to stress function, present both in brain and in blood. Other functions that appear enriched in the GO analysis of blood and brain systems include metabolism, and response to stimuli such as immune response, which have been reported to be indeed altered in patients with RTT (116, 117).

Taken together, our findings point at several genes overlapping between brain and blood and connecting the multiple aspects of RTT. These results have implications not only for the understanding of the biological mechanisms of RTT and the broad action of MeCP2 across different tissues. In fact, the presence of peripheral markers associated to brain dysfunction and linked to the symptoms of RTT, would facilitate the monitoring of the disease and the evaluation of the functional effects of candidate treatments.

METHODS

Mice

For screening the MeCP2 expression in control conditions, we used WT C57/BL6 male mice. For the sequencing experiment, we used *Mecp2*$^{tm1.1Bird}$ male mice available from Jackson (Stock no.: 003890) with a deletion of exons 3 and 4. WT C57/BL6 male mice were used as controls, as they match the background of the mutants. For qPCR, we used additional mice from the colony. Mice were genotyped using a standard PCR on DNA extracted from ear punches, using the protocol described in the Jackson website (www.jax.org). For the selection of presymptomatic and symptomatic mice, we used the criteria defined by Stearns et al. (118), where the authors run a battery to behavioral tests to define the onset of symptoms of RTT in male mice (after 28 days after birth—P28). For female mice, the onset of symptoms is after 3 months (P90). Mice were housed in the animal facility at 12 L/D cycle. All procedures on animals were authorized by the National Authority in Animal Welfare [Health Products Regulation Authority (HPRA)] Department of Comparative Medicine in Trinity College Dublin (TCD) (Authorization number: AE1936/I108, AE1936/P067).

Protein Extraction and Enzyme-Linked Immunosorbent Assay (ELISA)

Tissue was harvested at postnatal ages of 4, 8, and 12 weeks. Cortex, hippocampus, hypothalamus, cerebellum, and full brain were dissected and stored at −80°C until protein extraction. Tissue was homogenized in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl,

1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH = 8) containing cOmplete Protease Inhibitor Cocktail Tablets (Sigma), using pestles. The homogenate was incubated for 30 min in ice and then sonicated. The homogenate was centrifuged for 30 min at 20,000xG at 4°C, and the supernatant was stored at −80°C until further use. MeCP2 concentration was measured using a precoated sandwich ELISA assay (ELISAGenie). The MeCP2 concentration of each sample was normalized by its total protein concentration, measured using a Pierce™ BCA Protein Assay Kit (ThermoFisher). Statistical analysis was performed using a Kruskal–Wallis nonparametric test, and results were considered significant with a p-value < 0.05.

RNA Extraction

Mice were sacrificed using CO_2 at 8 weeks of age. Tissue harvesting was operated between 12 and 3 PM, and littermates were used as matching controls in the majority of cases (unless matched controls were not available). All the experiments comparing mutants and WT were run simultaneously. Cerebellums were dissected and stored at −80°C until RNA extraction. Blood was extracted immediately after euthanasia by suction from the heart and stored in RNAprotect Animal Blood Tubes (Qiagen). RNA was purified from brain and blood using the miRNeasy Mini Kit (Qiagen) and the RNeasy Protect Animal Blood Kit (Qiagen), respectively. RNA purity and concentration were assessed by the A260/230 and A260/280 obtained with a NanoDrop.

RNAseq

Three *Mecp2*-null and 3 WT mice were used for RNAseq. RNA from blood was depleted from globin mRNA using a mouse/rat GLOBINclear™ kit (Ambion). RNA integrity and concentration were measured using a bioanalyzer. For the subsequent steps, only samples with an RIN (RNA Integrity Number) > 7 were used. Sequencing cDNA libraries were prepared using a NEBNext Ultra RNA Library Prep Kit for Illumina, along with a NEBNext Poly(A) mRNA Magnetic Isolation Module and the NEBNext Multiplex Oligos for Illumina (New England Biolabs). RNA (200 ng) were used as a starting material for each sample. Library concentration and mean fragment length were measured using a bioanalyzer. Libraries were pooled, and a preliminary sequencing run was performed in a MiSeq (Illumina). Then, they were sent for sequencing to Edinburgh Genomics, where a HiSeq 2000 system (Illumina) was used. Sixty million, 2x75bp paired-end reads were obtained for each sample. The quality of the reads was determined using FastQC. All reads had qualities above Q28 among their whole extent (a representative sample of the FastQC analysis is depicted in **Supplementary Figure 7**). Reads were aligned to the Ensembl GRCm38 mouse genome construct, using Hisat2 (119), with default parameters. Abundance tables were generated using Stringtie (119), using the –e and –B options (simplified protocol) and the rest of parameters on default. To obtain count tables, the output of Stringtie was processed with the prepDE.py script provided by the developers. To obtain fragments per kilobase of transcript per million mapped reads (FPKM) tables, the output of Stringtie was processed with the R package Ballgown. The gene expression tables were filtered by removing counts corresponding to miRNA and genes presenting 0 counts in all samples. Differential expression analysis was performed using EdgeR (120), a statistical package specifically designed to analyze transcriptomic data, and we selected a likelihood ratio test. Results were corrected for multiple testing. Genes were considered as DE with an FDR-corrected p-value < 0.05.

Protein Interaction and Functional Enrichment Analysis

Protein interaction and functional enrichment analysis were performed in the STRINGapp plugin for Cytoscape (v.1.8.0), which uses information from the STRING database (string-db.org). DE genes obtained by RNAseq were used as an input. A maximum number of 30 maximum additional interactors was selected. The confidence score cutoff was set at 0.6. Functional enrichment analysis was also performed on Cytoscape, using the STRING Enrichment plugin.

Pathway Analysis

Pathway analysis of the results obtained by RNAseq was performed by using the iPathway software (Advaita). The software generates the p-value associated to each result considering the correction for multiple testing.

Identification of Common Differentially Expressed Genes in Blood and Cerebellum

Identification of the significant DEG in each tissue was performed selecting the significant genes identified by EdgeR after multiple testing correction (P value ≤ 0.05). For the selection of DEG in both blood and brain, we used the list of DEG in one tissue (i.e., brain) and we tested the hypothesis that they were also significant in blood with the appropriate correction. We repeated the procedure for the DEG in blood also significant in brain.

qPCR

Six *Mecp2*-null and 6 WT mice were used for qPCR. RNA was reverse transcribed using the Quantinova RT Kit (Qiagen). Reactions (20 µl) were set up, using 2x Gene Expression Master Mix (Applied Biosciences) and PrimeTime® qPCR Probe Assays (Integrated DNA Technologies). We used the following primer–probe sets:

- *S100a9*: F: GGAATTCAGACAAATGGTGGAAG R: CATCA GCATCATACACTCCTCA; probe:/56-FAM/TGACATCAT/ ZEN/GGAGGACCTGGACACA/3IABkFQ/
- *Ms4a3*: F: TCAATACCCAGGCTTTCAAGG R: GAGAAT CAGCATTAAAGACACCAG; probe:/56-FAM/TGCAGACAT/ ZEN/CAGGTGACGGTGAAG/3IABkFQ/
- *Serpina1c*: F: GGAATCACAGAGGAGAATGCT R: GAATAA GGAACGGCTAGTAAGACT; probe:/56-FAM/TGTGCATAA/ ZEN/GGCTGTGCTGACCA/3IABkFQ/
- *Lsm12*: F: CCTAGCTTCACTCAATGTTAGTAAG R: ATGG TCTTGTGAATGGTCTGG; probe:/56-FAM/TCAGCTTCT/ ZEN/CCTCCTTCTCCGTCC/3IABkFQ/

- *Ankrd63*: F: CCAGCTTGATTTCCTTGTCCT R: CCTGAG CCATCCACCTTTC; probe:/56-FAM/AGAAGCAGC/ZEN/CG TTGTTCACACCT/3IABkFQ/
- *Crispdl2*: F: TCTGAGTGTCCATCCAGCTA R: TTCCA CCTCGTTCATCATATCC; probe:/56-FAM/AGAAGCAGC/ ZEN/CGTTGTTCACACCT/3IABkFQ/
- *Scgb3a2*: F: CTGGTATCTATCTTTCTGCTGGTG R: GTC GTCCAAAGGTACAGGTAA; probe:/56-FAM/TGGTTATTC/ ZEN/TGCCACTGCCCTTCT/3IABkFQ/
- *Prg3*: F: CTATGTGCTGGTGAGGACTC R: AACTATA ACTGTGGACGGAAGC; probe:/56-FAM/ATCTCCTGC/ZEN/ AGACTCTCTGAGCCT/3IABkFQ/
- *Fam69c*: F: TGAGCCATTTCGACAGTGAC R: CCATGTCTAC GTCAATAGCTACC; probe:/56-FAM/TGATGTCAA/ZEN/ACCT GAGAACTTCGCCA/3IABkFQ/
- *Has2*: F: AGTCATGTACACAGCCTTCAG R: GACCTTCA CCATCTCCACAG; probe:/56-FAM/CATAATCCA/ZEN/CGCT TCGCCCCAGT/3IABkFQ/
- *Vmn2r85*: F: CCACAGAGTCAACAACTTCA R: GTACAT GTCACACTGCACATTG; probe:/56-FAM/ATGGGCCAC/ZEN/ AGGAGGAACATCAG/3IABkFQ/
- *Acc2os*: F: CATCCCTCCTGTTGTTATTATTCATC R: TCTGC TCCACTGAGTTTACTG; probe:/56-FAM/AGCTAAGCC/ZEN/ TGGTTCCTTTGTTCCTG/3IABkFQ/
- *Gapdh*: F: AATGGTGAAGGTCGGTGTG R: GTGGAGT CATACTGGAACATGTAG; probe:/5Cy5/TGCAAATGGCAGCC CTGGTG/3IAbRQSp/
- *Slc14a2*: F: CAACCGCATCTACTTCCTGAC R: GCTC TCTTCTGCCTTCCAC; probe:/56-FAM/ACTGCTCTC/ZEN/ CACTGCCACCATT/3IABkFQ/
- *Snx31*: F: CCAGATGAGCAGAGTGAAGTG R: CTAGGTT CTGGTTGAGAGTTCG; probe:/56-FAM/AGCAGAGTT/ZEN/ CCAAGGAAAGTGACCTG/3IABkFQ/
- *Bpifa1*: F: CCTCTCCTGAACAACATCCTC R: AGACTTCC AACTACGGGCATA; probe:/56-FAM/CCATCGTCT/ZEN/CTAT GTCACCATCCCTCT/3IABkFQ/
- *Tfap2d*: F: TGAGCCAGGATAGATCACCA R: GCTTAGA GCTGCACATATTGC; probe:/56-FAM/CCAGACCCA/ZEN/ CTCCCATTCTAGACCT/3IABkFQ/
- *Ube2v1*: F: CACTTACAAGATGGACAGGCA R: GGTACTTA GGCCCACACTCTA; probe:/56-FAM/ACCTCCACG/ZEN/AAC AATCTATGAAAACCGAA/3IABkFQ/
- *Dnah14*: F: TCAGTATAGAAGTCCTCTCAGTCA R: TGCACA CGACATATTGATCCG; probe:/56-FAM/CCAGTACGA/ZEN/ ACCTGACAGAATAGCTGC/3IABkFQ/
- *Mup1*: F: TGAGAAGCATGGAATCCTTAGAG R: ATGAAC ACCAACCCACTCC; probe:/56-FAM/TATCCAATG/ZEN/CCA ATCGCTGCCTCC/3IABkFQ/
- *Atp6v0d2*: F: AGTCTTACCTTGAGGCATTCTAC R: GCC AAATGAGTTCAGAGTGATG; probe:/56-FAM/TCCCATTCT/ ZEN/TGAGTTTGAGGCCGAC/3IABkFQ/
- *Hal*: F: CCATCAGAAATCGCAGAAAGC R: AGTTCT GTAGTGATGATGTCCTTC; probe:/56-FAM/CGTACACCT/ ZEN/TACGCTGCTGTCCAC/3IABkFQ/
- *Hist1h2be*: F: CGCAAACGCTACTGAAAGGA R: TTCTTGCC GTCCTTCTTCTG; probe:/56-FAM/TCTGAAGAT/ZEN/GCCT GAGCCAGCC/3IABkFQ/
- *Slc6a4*: F: CATCGTCTGTCATCTGCATCC R: CGTTGG TGTTTCAGGAGTGAT; probe:/56-FAM/TCCTTAAGT/ZEN/ GTCCCTGGAGTGCTGA/3IABkFQ/
- *Tnnc2*: F: GAGTGCGGAGGAGACAAC R: CCATCAGCAT CGAACATGTCA; probe:/56-FAM/AACCATGAC/ZEN/GGACC AACAGGCT/3IABkFQ/
- *Mpo*: F: CCCGCATTCCTTGTTTTCTG R: GCTTCTCCCC ATTCCATCG; probe:/56-FAM/CTCACCTCC/ZEN/ATGCACA CCCTCTTT/3IABkFQ/
- *Gpx3*: F: GCAGTATGCAGGCAAATATATCC R: CCCAGAAT GACCAAGCCAA; probe:/56-FAM/TCTGTCAGA/ZEN/CCTC AGTAGCTGGCT/3IABkFQ/
- *Paip2*: F: GACAGGATTCGTTGGCTACC R: GACTTGGAT CTTTCATGGTTGG; probe:/56-FAM/TCGTTGTCG/ZEN/TTT TTAACCCAGTGCAC/3IABkFQ/

qPCR was performed in a Quantstudio 5 Real Time PCR System (Applied Biosciences), using the following cycle: 2 min at 50°C, 10 min at 90°C, and 40x (15 s at 95°C and 1 min at 60°C). All samples were analyzed in triplicate, and *Gapdh* was used as a loading control. For target genes, we used the reporter fluorophore fluorescein amidite (FAM), and for *Gapdh*, we used Cy5. For each sample, the average *Gapdh* Ct value was subtracted from the average target Ct value, obtaining a dCt value. The average dCt of the WT group was used to calculate the ddCt value for each sample. Statistical significance was assessed using a *t*-test on the ddCt values, in Microsoft Excel. Significance was considered with a p-value < 0.05.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Gene Expression Omnibus, under accession number [GSE129387].

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of National Animal Welfare Authority, Ireland. The protocol was approved by the Animal Ethical Committee Trinity College Dublin and HPRA.

AUTHOR CONTRIBUTIONS

AS performed the experiments and wrote the paper; KH provided assistance in the design and analysis of the RNAseq experiment; DT contributed to sample extraction and establishment of the colony; and DT and MG designed and supervised all the parts of the research and the writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2019.00278/full#supplementary-material

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High-Frequency Repetitive Transcranial Magnetic Stimulation Applied to the Parietal Cortex for Low-Functioning Children With Autism Spectrum Disorder: A Case Series

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Background: Repetitive transcranial magnetic stimulation (rTMS) is a safe and efficacious technique to stimulate specific areas of cortical dysfunction in several neuropsychiatric diseases; however, it is not known whether high-frequency rTMS (HF-rTMS) over the left inferior parietal lobule, in low functioning children with autism spectrum disorder (ASD), improves core symptoms.

Method: Eleven low-functioning children with ASD completed two separate HF-rTMS treatment courses, 6 weeks apart. Each treatment course involved five 5-s trains at 20 Hz, with 10-min inter-train intervals, on left inferior parietal lobule each consecutive weekday for a 3-week period (15 treatments per course). Subjects were assessed at five time points: immediately before and after the first HF-rTMS course, immediately before and after the second HF-rTMS course, and 6 weeks after the second rTMS treatment course. Treatment effectiveness was evaluated using the Verbal Behavior Assessment Scale (VerBAS) and Autism Treatment Evaluation Checklist (ATEC). The latter test consists of four subtest scales: Language, Sociability, Sensory, and Behavior. In addition, daily treatment logbooks completed by parents were considered as one of the outcome measures.

Results: Participants showed a significant reduction in language- and social-related symptoms measured by ATEC from pretreatment to the 6-week follow-up after the second treatment course. Moreover, some possible improvements in imitation and cognition were reported by caregivers.

Conclusions: Our findings suggest that HF-rTMS over the left parietal cortex might improve core deficits in low-functioning children with ASD.

Keywords: repetitive transcranial magnetic stimulation, autism spectrum disorder, inferior parietal lobule, social relating, language

INTRODUCTION

Autism spectrum disorder (ASD) is characterized by deficits in social communication and stereotyped behaviors (1). Despite the spectrum's extreme heterogeneity, deficits in social cognition, including reduced social responsiveness, difficulty interacting with others, and recognizing others' intentions and emotions, are core features of ASD (1).

Dysfunction of the mirror neuron system (MNS) has been postulated in the pathophysiology of ASD (2). Mirror neurons are visuomotor cells that discharge not only when an individual performs a particular action but also when a similar action is observed (2, 3). The mirror neuron system (MNS) enables individuals to interpret motor acts of others and promotes the development of social cognition, such as emotion and empathy (3). Besides, MNS facilitates motor coordination and participates in memory, speech, and action planning (3–5).

MNS predominantly comprises the inferior frontal gyrus, inferior parietal lobule (IPL), and posterior superior temporal sulcus (6). Recent studies suggest that a dysfunction of the MNS might generate social and cognitive impairments related to ASD (7). It has been found that motor neurons of the IPL can code motor goals (8) and process the congruence between the executed and the observed motor act (8, 9). It has also been demonstrated that any damage to the parietal cortex affects the imitation or understanding of an observed action (10). Therefore, IPL is a likely neurobiological target for the treatment of ASD.

Repetitive transcranial magnetic stimulation (rTMS) offers a noninvasive approach for modifying cortical excitability. It potentially evokes a short-term functional reorganization in the brain (11). Effects of rTMS are not limited to the primarily stimulated cortex, because of anatomical and functional connections of cortical regions within a distributed network (11–13). Studies have suggested that low-frequency rTMS (<1 Hz) decreases cortical excitability, whereas HF-rTMS (>5 Hz) increases it (14, 15). Neuroenhancement of MNS in typically developing individuals has been reported using high-frequency (20 Hz) rTMS (HF-rTMS) (16).

A limited number of research studies have evaluated the therapeutic effects of rTMS in ASD. For example, it has been reported that applying low-frequency rTMS to the dorsolateral prefrontal cortex causes a reduction in stereotypical behaviors (17). Stimulation of IPL, however, has not been undertaken in ASD. Moreover, few studies investigated the effects of rTMS in children with ASD and intellectual disability. In the present study, we examined the effects of HF-rTMS on IPL in autism associated with severe intellectual disability. We hypothesized that HF-rTMS application to IPL would result in improvements in social functioning.

METHODS

Participants

Thirteen participants with ASD (age range 3–12 years) were recruited from Xuanwu Hospital, Capital Medical University, Beijing, China. Diagnosis was made by an experienced physician according to the *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition* (DSM-V) (1), and further confirmed with the Autism Diagnostic Interview–Revised (ADI-R) (18) and Autism Behavior Checklist (ABC) (19), administered by physicians trained to clinical reliability. Cases with a personal and family history of seizure, the presence of metal implants, were excluded. No subjects were on psychotropic medications. All 13 participants had IQ <70 measured by the Wechsler Intelligence Scale for Children, Fourth Edition (WISC-IV) (20). Two patients withdrew from the study during the first course of the treatment due to family reasons. The data of these two individuals were excluded in the final sample. Participant information is summarized in **Table 1**.

This study had the approval of the ethics committee of Xuanwu Hospital, and all participants' parents provided written informed consent before the study.

Procedures

A Magstim Super Rapid stimulator (The Magstim Company Ltd., Whitland, UK) connected to a 70-mm figure-of-eight coil was used to perform rTMS. The stimulation was applied on the left IPL [electrode P3 on the electroencephalography (EEG) cap] (21). Participants completed two separate courses that were 6 weeks apart. Each treatment course consisted of five 5-s trains at 20 Hz, with 10-min intertrain intervals, each consecutive weekday for a 3-week period (15 treatments per course). Because most participants could not participate in motor threshold assessments, we referred to the resting motor thresholds (RMT) measured in children (7–13 years old) with Tourette syndrome and children (8–13 years old) with attention deficit hyperactivity disorder in our laboratory. RMT of these children mostly ranged from 40% to 50%. Thus, the stimulation intensity was set uniformly at 50% of stimulator output.

Subjects were evaluated at five time points: immediately before the first HF-rTMS course ("pre-1"), immediately after the first HF-rTMS course ("post-1"), immediately before the second HF-rTMS course ("pre-2"), immediately after the second HF-rTMS course ("post-2"), and 6 weeks after the second rTMS treatment course (6 weeks later, "6wl"). Treatment effectiveness was assessed using the Verbal Behavior Assessment Scale (VerBAS) (22) and Autism Treatment Evaluation Checklist (ATEC) (23). ATEC consists of four subtest scales: Scale I (Speech/Language/Communication), Scale II (Sociability),

TABLE 1 | Participant demographics.

Participant number	Gender	Age (years)	ABC (scores)
1	Male	7	73
2	Male	7	93
3	Male	6	63
4	Female	5	70
5	Female	5	71
6	Male	11	64
7	Female	9	82
8	Female	9	86
9	Male	4	81
10	Male	3	85
11	Male	12	107

ABC, Autism Behavior Checklist.

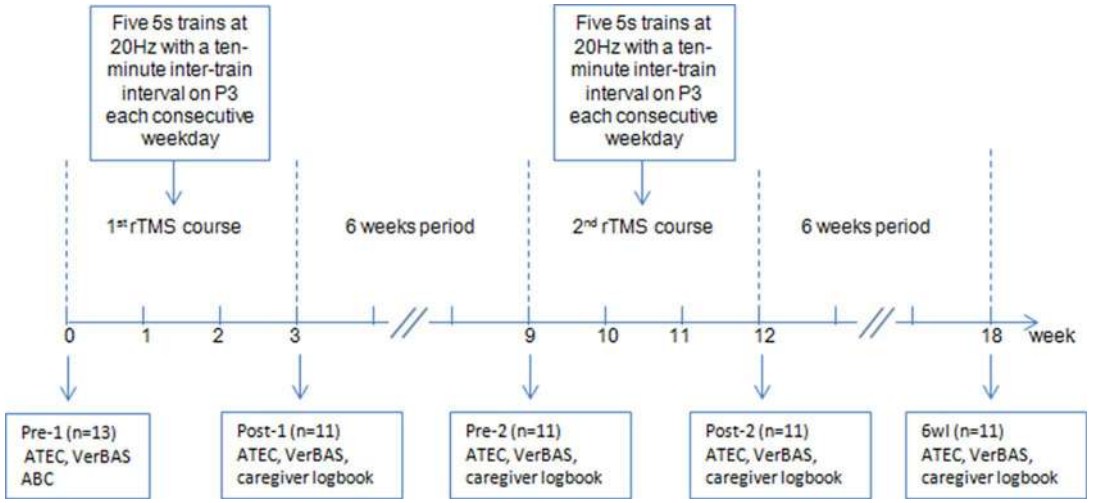

FIGURE 1 | Flow diagram of the experiment. Note: ATEC, Autism Treatment Evaluation Checklist; VerBAS, Verbal Behavior Assessment Scale; ABC, Autism Behavior Checklist; P3, left parietal electrode.

Scale III (Sensory/Cognitive awareness), and Scale IV (Health/Physical/Behavior) (23). In addition, daily logbooks completed by caregivers were considered as one of the outcome measures.

Moreover, we monitored any side effects during the stimulation courses and instructed caregivers to report any side effects they noted during and after treatment. The protocol flow diagram is shown in **Figure 1**.

Data Analysis

Statistical analysis was completed using Statistical Product and Service Solutions (SPSS) software (version 19.0, SPSS Inc., IL, USA). A P value <0.05 was considered significant for all analyses. One-way ANOVA with repeated measures was used to examine differences in the effects of HF-rTMS on the four ATEC scale scores, as well as VerBAS scores among different time points (pre-1 vs. post-1 vs. pre-2 vs. post-2 vs. 6wl). Bonferroni correction was used to adjust P values in *post hoc* analyses.

RESULTS

Eleven individuals (7 boys, 4 girls) with a mean age of 7.09 ± 2.88 years completed the two treatment courses and follow-up assessments. There were no reports of serious adverse events. Transient irritability during or after HF-rTMS was reported in three cases by caregivers (**Table 2**). One participant became irritable during the first 3 days in each treatment course. Another was more emotional after the second treatment course and recovered in 5 days. A third was hyperactive and irritable during the first 5 days of the first course.

The repeated-measures ANOVA revealed significant changes over time in the ATEC language scale [$F(4,50) = 2.685$, $P = 0.042$] and ATEC social scale [$F(4,50) = 2.636$, $P = 0.045$]. The least significant difference (LSD) method was used in *post hoc* analysis. The ATEC language scale significantly decreased from "pre-1" to "post-2" ($P = 0.048$) and from "pre-1" to "6wl" ($P = 0.003$). There was also a significant reduction in ATEC social scale from "pre-1" to "post-2" ($P = 0.021$) and from "pre-1" to "6wl" ($P = 0.005$).

TABLE 2 | Side effects reported by caregivers during and after high-frequency repetitive transcranial magnetic stimulation (HF-rTMS) treatment courses.

Participant no.	Side effects
1	Irritable during the first 3 days of each treatment course. For example, crying for a longer period of time if a need was not immediately met.
2	More emotional and restless after the second course, which recovered in 5 days; occasionally hitting the head against the wall in episodes of anger.
9	Hyperactive, irritable during the first 5 days of the first course.

However, after P value correction by Bonferroni method, the difference between "pre-1" and "post-2" did not achieve statistical significance in the ATEC language and social scales. The ATEC language scale significantly decreased from "pre-1" to "6wl" ($P = 0.025$) (lower ATEC scores reflect reduced impairments). The ATEC social scale significantly decreased from "pre-1" to "6wl" ($P = 0.048$).

No statistically significant changes over time were found in ATEC sensory and cognitive awareness scale [$F(4,50) = 0.234$, $P = 0.918$], ATEC health and behavioral scale [$F(4,50) = 0.398$, $P = 0.809$], or VerBAS [$F(4,50) = 1.086$, $P = 0.374$]. Summary data for clinical measures were presented in **Table 3**.

According to the clinical observations and caregiver reports, HF-rTMS might be more effective in male children than in female children. Most caregivers reported their children displayed possible improvements in imitation and cognition (e.g., language imitation and behavior imitation) after the HF-rTMS treatments. We summarized some improvements from caregiver logbooks in 11 participants in **Table 4**.

TABLE 3 | Autism Treatment Evaluation Checklist (ATEC) scale scores and Verbal Behavior Assessment Scale (VerBAS) scores at each assessment time point (mean ± SD).

	Pre-1	Post-1	Pre-2	Post-2	6wl
ATEC language scale	16.1 ± 5.3	13.6 ± 5.0	12.9 ± 4.2	12.1 ± 4.4	9.8 ± 4.1*
ATEC social scale	19.8 ± 7.5	17.0 ± 7.0	15.6 ± 5.6	13.6 ± 5.1	12.2 ± 4.7*
ATEC sensory and cognitive awareness scale	21.3 ± 5.0	20.1 ± 6.0	19.8 ± 6.0	19.4 ± 5.4	19.2 ± 5.8
ATEC health and behavioral problems scale	21.2 ± 8.2	19.5 ± 7.8	17.4 ± 7.3	18.2 ± 7.1	18.6 ± 7.7
VerBAS scale	30.6 ± 8.8	34.4 ± 10.2	34.9 ± 9.7	36.9 ± 9.4	38.6 ± 9.7

**Significantly different from "pre-1" ($P < 0.05$).*
For ATEC, lower scores reflect reduced impairments.
For VerBAS, higher scores reflect reduced impairments.
6wl, 6 weeks later.

TABLE 4 | Improvements in the quality of life of 11 participants, following HF-rTMS treatments. Records from caregiver's logbooks.

No.	Posttreatment assessment			
	Language	**Social skills**	**Imitation, cognition, learning, fine motor skills**	**Behaviors and emotions**
1	Increased active language, e.g., initiatively saying "go home" after treatment	More eye contact. Showed greater affection toward family members. Helped parents do housework. Willing to play games with other children	Enhanced learning and imitation ability. Accepted new knowledge faster than before. Improved comprehension and execution	A slight decrease in repetitive behavior
2	Decreased self-talk	Willing to play games with other children. Taking the bus quietly instead of shouting, especially when there were no seats available. Showed greater affection toward family members. Aware of location of parents when taking the bus	Improved attention and comprehension. Could understand the explanation of game rules. Improved imitation. Showed more patience with writing and painting. Improved fine motor skills.	A slight decrease in repetitive behavior
3	Louder voice and clearer speech. Expanded vocabulary. Could say "no" to express unwillingness. Increased active language, e.g., naming objects he recognized on TV	N/A	Improved attention, comprehension, and imitation. Improved discernment of color and shape. Improved fine motor skills	More physically active
4	Speaking loudly and clearly	Closer to parents. More eye contact	Faster reaction time. Could understand some instructions	Laughed more than before
5	Louder speech. Increased active language, e.g., actively calling "Dad," "Mom" (first time occurrence since birth). Often says "Ah," with pitch variation	N/A	Faster reaction time	Improvement in bad temper. More smiles than before
6	N/A	Willing to play games with other children. More understanding of surrounding environment, e.g., looking around when crossing the road. Quietly sitting for 2–3 h during a conference and applauding with others	Improved concentration and comprehension. Could understand and carry out two simultaneous instructions	N/A
7	Increased active speech, e.g. actively calling "Dad," "Mom"	Closer to parents and sister. Willing to play games with other children	Could understand and carry out some instructions	Obvious decrease in frequency of crying
8	N/A	Closer to parents and sister; willing to play games with other children	Could understand and carry out some instructions	N/A
9	During the third week of the first treatment course, passive language imitation gradually increased. At the beginning of the second course, spontaneously imitated what parents and teachers said. Could answer some simple questions, such as his name, age, and parents' names	More eye contact; willing to be together with family members	Improved comprehension, memory, and imitation	A slight decrease in repetitive behavior (not obvious)
10	Increased active language. Clearer speech. Could say five-to-six-word vs. two-to-three-word sentences before treatment. Could answer some simple questions, e.g., age, name, and what he liked to eat	Paid attention to other children when playing. Likes to be close to family. If parents go out, he would catch up or become unhappy	Improved comprehension, memory, and execution. Became interested in reading.	N/A
11	Reduced repetitive language. More accurate oral expression. Initiatively expressed his opinions, e.g., "I want to sit down," when tired	Could wait in line and quietly ride public transportation	Improved learning and imitation. Could sometimes understand parents' words	Greatly reduced impulsive and violent behaviors

N/A, not applicable.

DISCUSSION

This study provides preliminary evidence for the effectiveness and safety of HF-rTMS over the left IPL as a treatment option to improve core symptoms in low-functioning autism. Specially, HF-rTMS significantly reduced social and speech deficits as measured by ATEC and parents' report. At the same time, children's imitation and cognition might be improved following treatment.

The specific mechanisms underlying these effects may reflect specific neuroplastic effects associated with high-frequency stimulation and will require further investigation. Physiological experiments in humans indicate that HF-rTMS evokes long-term potentiation (LTP) of synaptic transmission (24, 25). These changes are not only restricted to the site of stimulation but also observed in a widespread cortical and subcortical network (26, 27). The use of HF-rTMS (20 Hz) to adaptively modulate properties of the MNS in humans has been reported in typically developing individuals (16).

From a neurophysiological perspective, we hypothesize that these clinical effects resulted from stimulation of IPL and associated MNS, which have been linked to ASD (7). Stimulation of the IPL may induce long-lasting changes in the excitability of regions within the MNS network (28). Such alterations may improve one's understanding of social environment and may reinforce the capacity for imitation. Thus, an enhanced interpretation of social context may lead to improvements in language and social skills, as shown in the current trial (28).

Growing evidence suggests that dopaminergic dysfunction is implicated in the pathogenesis of ASD (29, 30). Human studies show that HF-rTMS of the frontal cortex induces the release of dopamine in the cortical, limbic, and striatal brain regions (31). In this study, HF-rTMS on parietal cortex might alter dopamine activity in specific brain regions, which is related to social cognition in ASD (30).

To the best of our knowledge, this study is the first attempt at HF-rTMS over the IPL in intellectually disabled individuals with ASD. According to clinical observations and caregiver reports, HF-rTMS in this trial might be more effective in boys than in girls. The underlying reasons for the significant gender disparities in treatment outcome are not clear. However, female individuals with ASD seem to exhibit lower IQ (32), more severe phenotypes (33), overall autistic symptoms (34), and psychopathological problems (35). Moreover, it is important to note that the four girls in our study were two sets of twins; thus, genetic factors may play a critical role in their pathogenesis.

It may be confusing that the clinical effects measured by scales did not turn up at the time point of "immediately after the treatment course." We thought there may be two reasons to account for this. Firstly, the number of participants in our study was relatively small, which may have a great impact on statistical analysis. Despite the limited validity of parental reports as outcome measures, the improvements in social cognition and speech were indeed observed during and after the treatment course. However, the improvements were not reflected by statistical analysis, as we expected. Secondly, repeated sessions of HF-rTMS could produce remodeling with an increase in active synapses (36), which may be responsible for cumulative rTMS effects. This may explain why the difference achieved statistical significance only at the time point of "six weeks after the second treatment course."

Our study had several limitations that should be mentioned, including the fact that the study does not contain a control group (e.g., sham HF-rTMS), the conclusions are limited by the small sample size, limited validity of parental reports as outcome measures, and lack of neuroimaging and/or neurophysiological assessments. In future follow-up studies, large case–control clinical trials are necessary to explore the use of HF-rTMS as a unique treatment for improving core symptoms in ASD.

CONCLUSION

Our original findings suggest that HF-rTMS on IPL has the potential to become a distinct therapeutic method aimed at treating core symptoms of ASD.

ETHICS STATEMENT

This study was carried out in accordance with the ethics committee of Xuanwu Hospital with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the ethics committee of Xuanwu Hospital, Capital Medical University (LYS2017063).

AUTHOR CONTRIBUTIONS

YY participated in the design of the study, conducted the analyses, and wrote the manuscript; HW was involved in the study design, supervised the data analysis, and revised the manuscript; QX and ZH helped collected participants and coordinated the study; YW conceived and coordinated the design of the study and revised the manuscript. All authors read and approved the final manuscript.

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Validation of the Quantitative Checklist for Autism in Toddlers in an Italian Clinical Sample of Young Children With Autism and Other Developmental Disorders

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Background: The Quantitative Checklist for Autism in Toddlers (Q-CHAT) is parent-report screening questionnaire for detecting threshold and sub-threshold autistic features in toddlers. The Q-CHAT is a dimensional measure normally distributed in the general population sample and is able to differentiate between a group of children with a diagnosis of autism and unselected toddlers.

Objectives: We aim to investigate the psychometric properties, score distribution, and external validity of the Q-CHAT in an Italian clinical sample of young children with autism versus children with developmental delay and typically developing children.

Method: N = 126 typically developing children (TD), n = 139 children with autism, and n = 50 children presenting developmental delay (DD) were administered the Q-CHAT. Standardized measures of cognitive functions, language, and behaviors were also obtained.

Results: The Q-CHAT scores were normally distributed and demonstrated adequate internal consistency and good item to total score correlations. The mean Q-CHAT score in the autism group was significantly higher than those found in the DD sample and TD children. No difference on the mean Q-CHAT score between DD and TD children was found. The accuracy of the Q-CHAT to discriminate between autism and TD was very good. Two different cut-points (27 and 31, respectively) maximized sensitivity and specificity for autism versus TD and DD, respectively. Finally, higher Q-CHAT scores were correlated with lower language and social communication skills.

Conclusions: In clinical settings, the Q-CHAT demonstrated good psychometric properties and external validity to discriminate autism children not just from children with typical development but also from children with developmental delay.

Keywords: autism, early screening, toddlers, Quantitative Checklist for Autism in Toddlers, Autism Spectrum Disorders (ASD)

INTRODUCTION

Autism spectrum conditions (autism) are a neurodevelopmental condition that significantly impairs social communication and includes unusually narrow interests and difficulties adjusting to unexpected change (1). Autism begins very early in life and is lifelong. There is consolidated evidence that early intervention has a significant impact on reducing the severity of symptoms and improving social communicative and adaptive skills with consequent better functioning and greater independence later in life (2, 3). However, early intervention is possible only if children at risk can be detected accurately through autism-specific screenings by the age of 18–24 months and immediately referred for diagnostic assessment. For these reasons, the American Academy of Pediatrics (4) and the Centers for Disease Control and Prevention's National Center on Birth Defects and Developmental Disabilities (NCBDDD) (5) have recommended the use of routine screeners within developmental surveillance to help pediatricians develop a strategy for early identification of children with autism. Different screening instruments for autism, with different scoring approaches (categorical versus continuous), have been developed since the late 1990s and used as first-level screeners in community samples and/or as level 2 screeners in clinical settings (6–8). Among them, the Checklist for Autism in Toddlers (CHAT) (9), the Modified Checklist for Autism in Toddlers (M-CHAT) (10), and the M-CHAT-Revised with Follow-up (11) (M-CHAT/RF) have been tested in the general population. Results indicated that the CHAT at 18 months had a high specificity and positive predictive value but low sensitivity, dropping too many affected children. The M-CHAT and M-CHAT/RF, which replaced the CHAT, have been validated across multiple studies, cultures, and populations, mostly in mixed samples of high- and low-risk children and have demonstrated moderate psychometric properties (12, 13). In high-risk samples of children referred for developmental concerns, as expected considering the higher prevalence of autism, the M-CHAT demonstrated higher positive predictive values (PPVs) of 0.74 (14) and 0.79 (15), respectively, in two independent samples. Similar PPVs were also reported for other screeners such as the Social Communication Questionnaire (SCQ) (PPV of 65%) (16) and the ESAT (PPV of 79%) (17). Other studies, conducted in clinical settings, compared the score distribution and accuracy of different screeners in young children with a diagnosis of autism, children with other developmental conditions, and typically developing children. Stone et al. (18) reported that scores on the Screening Tool for Autism in Two-Year-Olds (STAT) in children with autism were significantly higher than those reported in children with developmental delay and/or language impairment. Similarly, Matson et al. (19) tested the validity of the Baby and Infant Screen for Children with aUtIsm Traits (BISCUIT) to identify autism in a cohort of children presenting either developmental delay and/or medical conditions likely to result in a developmental delay. BISCUIT-Part 1 total scores in the autism group were significantly higher than those reported in the control group with developmental conditions. In another preliminary study on the Quantitative Checklist for Autism in Toddlers (Q-CHAT), Allison et al. (20) examined the clinical validity of the Q-CHAT as a dimensional measure of threshold and sub-threshold autistic features and found that the Q-CHAT was normally distributed in the general population sample and was able to differentiate between a group of children with a diagnosis of autism and unselected toddlers. In a subsequent study (21), a short version of the Q-CHAT (QCHAT-10), including the 10 items that best differentiated between children with and without autism, was tested and the screening cut-point of 3 demonstrated sensitivity and specificity estimates as high as 91% and 89%, respectively. Although the Q-CHAT results were promising, the full range of psychometric characteristics was not reported and the accuracy of the instrument with regard to other developmental conditions was not explored.

The current study aims to further investigate the Q-CHAT validity and score distribution in an independent clinical sample of young children with a diagnosis of autism, children with a diagnosis of developmental delay, and typically developing children. We also analyzed the accuracy of the Q-CHAT total scores in predicting diagnostic status in children with both autism and developmental delay. Finally, we explored the predictive validity of screening scores on the Q-CHAT with regard to measures of cognitive functioning, language, behaviors, and autism symptom severity.

METHODS

Participants

A group of n = 315 young children [M/F = 206:109 (65%:35%), mean age (SD) = 31.6 (8.8) months] from three Italian regions (Piedmont, Tuscany, and Sicily) took part in the study. N = 126 were typically developing children (TD) [mean age (SD) = 33.2 (9.3) months], n = 139 children had a diagnosis of autism [mean age (SD) = 31.6 (8.0) months], and n = 50 children were presenting Developmental Delay (DD) [mean age (SD) = 27.6 (8.3) months]. TD children were recruited in mainstream nursery schools. Parents were given the QCHAT through the teachers, and the completed questionnaires were collected back by a member of the research team at school. Autism and DD children were diagnosed and tested at the clinical facilities within the Autism Centre (C.A.S.A.) of the NHS Unit CN1 in the province of Cuneo (Piedmont), the Scientific Foundation "Stella Maris"

in Pisa (Tuscany), and the University Hospital "G. Martino" in Messina (Sicily). Parents were given the QCHAT by a member of the research team and filled out the questionnaire during the child's assessment. All parents were explicitly asked to fill out the questionnaire together.

Procedure

The study was conducted as part of a large population-based screening program funded by the Ministry of Health and Tuscany Region (GR-2010-2319668). The study was approved by the local Ethic Committees in each region, and all the participants signed a written consent form to be enrolled in the study. All the participants, including TD children, were given the Griffith's Mental Development Scale (22) to assess their language and performance developmental quotient (LDQ and PDQ). TD children presenting either language or global developmental delay (n = 2) as well as autistic traits (n = 1) were excluded from the study and offered a separate dedicated diagnostic assessment. The Autism Diagnostic Observation Schedule, Second Edition (ADOS-2) (23) was used as part of the diagnostic assessment in the autism group. DD and autism diagnoses were made by multidisciplinary teams comprising psychologists and child neuropsychiatrists according to DSM 5 criteria of ASD and global developmental delay. Furthermore, parents of autism and TD children completed the Child Behavior Checklist 1.5-5 (24).

Validation of the Italian Q-CHAT

The Q-CHAT is a 25-item caregiver-report screening measure for autistic traits in toddlers. Items are rated on a five-point Likert scale (0–4), with higher ratings indicating more autistic traits and a Q-CHAT total score ranging from 0 to 100. Thirteen items are reverse scored. The scoring procedure used in the study was exactly the same as that used in the original Q-CHAT study by Allison et al. (20). To maintain the functional and conceptual equivalence of words and sentences between English and Italian, a back-translation was conducted and points of divergence were discussed with the authors who developed the instrument (CA and SBC) to ensure that the items were accurately reflecting the same meaning as that in the original language.

Statistical Analyses

All statistical analyses were conducted using the Data Analysis and Statistical Software STATA Release 8.1 (25). As per Allison et al. (20), incomplete or ambiguously answered Q-CHAT items were conservatively scored "0." If seven or more Q-CHAT items were missing, then the checklist was excluded from analysis [n (%) = 3 (0.9%)]. Accordingly, for the CBCL, missing items were conservatively scored as "0," whilst questionnaires with more than eight missing items were excluded [n (%) = 3 (1.1%)] (24). Descriptive analysis was conducted on personal history as well as socio-demographic status, accounting for group, gender, and region. In particular, categorical variables were analyzed using the chi-squared test, while quantitative variables were analyzed using either the Student t test or the analysis of variance (ANOVA). Cramer's V and eta-squared were computed as measures of effect size for categorical and quantitative variables, respectively. Multiple comparisons were performed by applying Holm–Bonferroni's correction to Fisher's exact probability test and for categorical variables, and the Tukey test for quantitative variables. The Shapiro–Wilk test was used to assess normality in the Q-CHAT score distribution. Q-CHAT item distribution and item–total correlations were also examined using Spearman's rho non-parametric correlation coefficient in each group separately. Cronbach's alphas were calculated to examine the Q-CHAT total score internal consistency in each group and the overall sample. A between-group analysis of covariance, accounting for the effect of age and PDQ, was conducted to assess group differences in the Q-CHAT total scores. In addition, a multiple linear regression model was applied to assess the effect of group, gender, age, Performance Developmental Quotient (PDQ), and parental education on QCHAT total scores. A receiver operating characteristic (ROC) curve of the Q-CHAT total score was produced to plot sensitivity and 1-specificity in relation to both an autism and DD diagnosis. The area under the curve (AUC) is a measure of the overall predictive validity, where an AUC = 0.50 indicates random prediction of the independent variable and an AUC > 0.90 indicates excellent validity. Potential cutoff scores on the Q-CHAT for differentiating between children with autism, DD, and TD were also evaluated using ROC analysis to determine the cut-point corresponding to the best combination of sensitivity and specificity. The relationship between the Q-CHAT scores LDQ and PDQ as well as the ADOS-2 scores in the ASD group was examined using a multiple linear regression model that accounted for the effects of age, gender, and parental education. Finally, convergent validity between the Q-CHAT total score and the CBCL 1.5-5 domains in autism and TD children separately was assessed using Spearman's rho non-parametric correlation coefficient.

RESULTS

Demographic and Clinical Characteristics of the Sample

Table 1 reports the demographic and clinical characteristics of the sample.

Within each group, no regional differences were found for the main demographic and clinical characteristics of the sample (all $p > 0.05$ after Bonferroni–Holm correction). Furthermore, neither a main effect of region nor a region by group interaction was found for the Q-CHAT scores; hence, all the relevant analyses were conducted on the whole sample. As expected, a significant group difference in gender distribution was found (Chi squared = 40.61, df = 2, $p < 0.001$). The autism group had more males than females compared to the DD and TD groups ($p < 0.001$ for both comparisons), while no difference in gender distribution was found between DD and TD children. A significant difference between groups was also found for age [$F(2,303) = 7.39$, $p < 0.001$]. DD children in the sample were significantly younger than autism and TD children ($p < 0.01$ for both comparisons), while age between autism and TD children did not significantly differ. Furthermore, Performance Developmental Quotient (PDQ) scores were significantly different between the three groups [$F(2,288) = 84.59$, $p < 0.001$], with TD children having a

TABLE 1 | Descriptive and clinical characteristics of children with autism, developmental delay (DD), and typically developing children (TD).

	Autism	DD	TD	Group	Region	Group*region
N	139	50	126	/	/	/
Age in months (mean, SD)	31.6 (8)	27.6 (8.3)	33.2 (9.3)	$F(2,297) = 4.8$, $p = 0.009$	$F(2,297) = 1.3$, $p = .3$	$F(4,297) = 4.4$, $p = 0.002$§
Gender M:F (N, %)	116:23 (83:17)	29:21 (58:42)	59:67 (47:53)	$X^2(2) = 40.6$, $p < 0.001$	$X^2(2) = 2.3$, $p = .3$	$X^2(8) = 48$, $p < 0.001$§
PDQ	88 (34.1)	61.8 (9.6)	119.6 (21.5)	$F(2,282) = 67.7$, $p < 0.001$	$F(2,282) = 0.6$, $p = .6$	$F(4,282) = 0.98$, $p = .4$
Q-CHAT total score*	39.4 (13.1)	27.1 (6.3)	21.1 (6.7)	$F(2,272) = 72.6$, $p < 0.001$	$F(2,272) = 1.4$, $p = .2$	$F(4,272) = 1.2$, $p = .3$
Personal history						
Term pregnancy (N, %)	116 (86)	35 (78)	104 (88)	$X^2(2) = 2.9$, $p = .2$	$X^2(2) = 1.6$, $p = .4$	$X^2(8) = 10.9$, $p = .2$
Pregnancy complications (N, %)	22 (18)	4 (10)	10 (9.5)	$X^2(2) = 3.7$, $p = .2$	$X^2(2) = 9.1$, $p = 0.01$§	$X^2(8) = 15.9$, $p = 0.04$§
Birth weight in gr. (Mean, SD)	3,280 (549.6)	2,876.9 (768.1)	3,170.1 (537.8)	$F(2,287) = 1.7$, $p = .2$	$F(2,287) = 3.9$, $p = 0.02$§	$F(4,287) = 3$, $p = 0.02$§
APGAR score (Mean, SD)	9 (0.7)	9.1 (1.1)	9.3 (0.7)	$F(2,153) = 2.6$, $p = 0.08$	$F(2,153) = 1.5$, $p = .2$	$F(4,153) = 1$, $p = .4$
Perinatal problems (N, %)	19 (15)	8 (19)	14 (12)	$X^2(2) = 1.4$, $p = .5$	$X^2(2) = 11.6$, $p = 0.003$§	$X^2(8) = 14.2$, $p = 0.07$
Gait in months (Mean, SD)	14.1 (2.7)	18.2 (1.8)	12.7 (2.2)	$F(2,256) = 42.2$, $p < 0.001$	$F(2,256) = 4.5$, $p = 0.01$§	$F(4,256) = 1.8$, $p = .1$
First words in month (Mean, SD)	17.5 (8.7)	17 (2.3)	10.8 (3.6)	$F(2,220) = 25.5$, $p < 0.001$	$F(2,220) = 0.2$, $p = .8$	$F(4,220) = 0.5$, $p = .7$
Nursery school (N, %)	89 (66)	25 (50)	119 (95)	$X^2(2) = 50$, $p < 0.001$	$X^2(2) = 4.5$, $p = .1$	$X^2(8) = 66.8$, $p < 0.001$§
SES						
Education mother (N, %)				$X^2(6) = 19.6$, $p = 0.01$	$X^2(6) = 18.1$, $p = 0.02$§	$X^2(16) = 38.9$, $p = 0.001$§
Pre-primary, primary	24 (17)	12 (26)	7 (6)			
Secondary	57 (42)	18 (39)	53 (42)			
Bachelor, Master Degree, PhD	56 (41)	16 (35)	65 (52)			
Occupation mother (N, %)				$X^2(6) = 35.5$, $p < 0.001$	$X^2(6) = 12.5$, $p = 0.05$	$X^2(24) = 47.7$, $p = 0.003$§
Not working	56 (41.5)	24 (52)	25 (20.5)			
Manual, technical	10 (7.5)	2 (4)	5 (4)			
Clerical, sales	37 (27)	16 (35)	34 (28)			
Administrative, professional, management	32 (24)	4 (9)	58 (47.5)			
Ethnicity mother				$X^2(6) = 11.5$, $p = 0.07$	$X^2(6) = 10$, $p = .1$	$X^2(24) = 40.5$, $p = 0.02$§
Caucasian	133 (96.5)	43 (95.5)	123 (98.5)			
Asiatic	0	2 (4.5)	2 (1.5)			
African	2 (1.5)	0	0			
Other	3 (2)	0	0			
Education father				$X^2(6) = 8.3$, $p = .4$	$X^2(6) = 15.5$, $p = 0.05$	$X^2(16) = 30.9$, $p = 0.01$§
Pre-primary, primary	34 (25)	15 (32.5)	23 (20)			
Secondary	59 (43)	29 (43.5)	51 (44)			
Bachelor, Master Degree, PhD	43 (32)	11 (24)	42 (36)			
Occupation father				$X^2(6) = 19.4$, $p = 0.004$	$X^2(6) = 15$, $p = 0.02$§	$X^2(24) = 41.8$, $p = 0.01$§
Not working	4 (3)	7 (15.5)	5 (4)			
Manual, technical	47 (35)	15 (33.5)	25 (21)			
Clerical, sales	35 (26)	9 (20)	29 (25)			
Administrative, professional, management	49 (36)	14 (31)	59 (50)			
Ethnicity father				$X^2(6) = 5.3$, $p = .3$	$X^2(6) = 11.2$, $p = 0.02$§	$X^2(24) = 32.7$, $p = 0.01$§
Caucasian	136 (98)	43 (96)	119 (99)			
Asiatic	0	1 (2)	1 (1)			
African	1 (2)	1 (2)	0			
Other	0	0	0			

**Controlled for age and PDQ; §p > 0.05 after Bonferroni-Holm correction.*

significantly higher PDQ than ASD and DD children and autism children having in turn higher PDQ scores than DD children ($p < 0.01$ for each of the three pairwise comparisons).

Q-CHAT Internal Consistency, Item Score Distribution, and Item–Total Correlations

The QCHAT scores were normally distributed in the ASD, DD, and TD groups ($W = 0.98$, $p = 0.07$, $W = 0.97$, $p = 0.32$, and $W = 0.996$, $p = 0.97$). Internal consistency was good in the overall sample as well as the autism group (Cronbach's alpha = 0.87 and 0.84, respectively), and adequate in the DD and TD groups (Cronbach's alpha = 0.70 for both). The item–score distribution of the Q-CHAT in the autism, DD, and TD groups is shown in **Table 2**.

Most of the items were significantly correlated with the Q-CHAT total score in the overall group of children, with large effect sizes ($0.50 \leq rho \leq 0.65$) for items 1, 2, 4, 6, 8, 9, 10, 12, 15, 17, 19, and 25, moderate effect sizes ($0.40 \leq rho < 0.50$) for items 16 and 20, and small effect sizes ($0.20 \leq rho < 0.40$) for items 5, 7, 11, 13, 21, 23, and 24. Low effect sizes ($rho < 0.20$) were found for items 3, 14, 18, and 22.

Group Differences in the Q-CHAT Scores

The mean Q-CHAT scores (SD) were 39.4 (13.1) in the autism group, 27.1 (6.3) in the DD sample, and 21.1 (6.7) for TD children.

Figure 1 shows the Q-CHAT total score distribution in the three groups.

Since age and PDQ were significantly different between the three groups, an ANCOVA was performed to control for the effect of these variables. Adjusting for age and PDQ, a main effect of group on the QCHAT total scores was found [$F(2,278) = 87.4$, $p < 0.001$, eta squared = 0.46]. Pairwise comparisons indicated that Q-CHAT scores in the autism group were significantly higher than in the DD and TD groups (both p-values < 0.001). No difference in the Q-CHAT scores between DD and TD children was found ($p = 0.56$). When the effect of gender (controlled for age and PDQ) was explored, no main effect of gender [$F(1,274) = 0.17$, $p = 0.68$] nor gender by group interaction [$F(2,274) = 0.24$, $p = 0.79$] on Q-CHAT total score was found. Adjusted mean (SD) Q-CHAT scores by gender were as follows: autism males = 39.2 (10.4); autism females = 40.0 (10.0); DD males = 24.3 (10.6); DD females = 22.9 (10.7); TD males = 23.3 (10.7); TD females = 22.1 (10.8). In agreement with the ANCOVA, the multiple linear regression model including group, gender, age, PDQ, and parents' education showed no significant effect on the Q-CHAT total score of gender (Beta = −0.27, $p = 0.85$), age (Beta = −0.10, $p = 0.17$), and the father's education (medium- vs low-level: Beta = −1.90, $p = 0.24$; high- vs low-level: Beta = −3.01, $p = 0.12$). The mother's medium-level and high-level education and PDQ were associated with lower QCHAT scores (education: Beta = −3.92 and −5.72, $p = 0.05$ and 0.01; PDQ: Beta = −0.06, $p = 0.007$). Finally, the QCHAT total score was markedly affected by the autism condition (Beta = 16.2, $p < 0.001$), but not by the DD condition (Beta = −0.2, $p = 0.94$) as compared to the TD condition.

TABLE 2 | Item–score distribution in children with autism, developmental delay (DD), and typically developing children (TD).

	Autism					DD					TD				
	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
1. Look when call name	15.8	28.1	33.1	20.1	2.9	64.0	30.0	6.0	0.0	0.0	66.7	31.0	2.4	0.0	0.0
2. Eye contact	10.8	49.6	33.1	5.8	0.7	54.0	46.0	0.0	0.0	0.0	71.4	28.6	0.0	0.0	0.0
3. Lineup objects[a]	33.1	23.0	25.2	12.9	5.8	16.0	10.0	34.0	22.0	18.0	6.3	21.4	37.3	23.8	11.1
4. Understand child's speech	5.0	10.8	21.6	13.7	48.9	32.0	26.0	24.0	6.0	12.0	33.3	41.3	19.0	6.3	0.0
5. Protoimperative pointing	41.7	24.5	5.0	5.0	23.7	66.0	18.0	8.0	2.0	6.0	53.2	27.0	11.9	4.8	3.2
6. Protodeclarative pointing	24.5	25.9	11.5	6.5	31.7	74.0	10.0	4.0	6.0	6.0	69.0	15.9	5.6	6.3	3.2
7. Interest in spinning object[a]	44.6	36.0	14.4	4.3	0.7	48.0	34.0	12.0	4.0	2.0	49.2	44.4	4.8	1.6	0.0
8. Number of words[a]	11.5	7.9	21.6	29.5	29.5	26.0	18.0	28.0	26.0	2.0	56.0	19.2	16.8	7.2	0.8
9. Pretend play	23.0	25.9	15.1	10.1	25.9	50.0	26.0	16.0	2.0	6.0	71.2	15.2	12.0	0.8	0.8
10. Follow a look	19.4	41.7	12.9	9.4	16.5	52.0	30.0	14.0	2.0	2.0	61.9	26.2	7.9	1.6	2.4
11. Sniff/lick unusual objects[a]	13.7	41.7	20.1	12.9	11.5	4.0	42.0	14.0	22.0	18.0	17.5	48.4	13.5	12.7	7.9
12. Use of hand as tool[a]	15.1	11.5	18.0	30.2	25.2	26.0	8.0	12.0	36.0	18.0	48.4	21.4	7.1	16.7	6.3
13. Walk on tiptoes[a]	27.3	30.9	24.5	12.9	4.3	40.0	24.0	30.0	6.0	0.0	50.8	23.0	19.8	6.3	0.0
14. Adapt to change in routine	42.4	42.4	9.4	3.6	2.2	38.0	56.0	6.0	0.0	0.0	40.5	50.8	7.1	1.6	0.0
15. Offer comfort	12.9	15.8	23.0	20.9	27.3	24.0	40.0	26.0	8.0	2.0	35.7	36.5	23.8	2.4	1.6
16. Does same thing over and over again[a]	19.4	17.3	22.3	25.9	15.1	20.0	10.0	14.0	24.0	32.0	27.8	24.6	19.8	17.5	10.3
17. Typicality of first words	34.8	24.6	9.4	1.4	29.7	62.0	24.0	6.0	2.0	6.0	73.8	23.8	1.6	0.8	0.0
18. Echolalia[a]	29.7	5.8	8.7	26.8	29.0	12.0	2.0	6.0	34.0	46.0	7.1	5.6	10.3	27.8	49.2
19. Gestures	31.7	28.8	8.6	11.5	19.4	68.0	28.0	4.0	0.0	0.0	70.4	24.8	4.0	0.8	0.0
20. Unusual finger movements[a]	68.3	9.4	4.3	12.2	5.8	76.0	6.0	10.0	6.0	2.0	88.9	4.0	4.8	0.8	1.6
21. Check reaction	17.3	28.8	29.5	15.8	8.6	34.0	38.0	24.0	4.0	0.0	32.8	41.6	20.8	4.8	0.0
22. Maintenance of interest[a]	49.3	34.1	9.4	5.8	1.4	48.0	32.0	18.0	2.0	0.0	52.0	29.6	16.0	2.4	0.0
23. Twiddle objects repetitively[a]	72.7	8.6	8.6	7.2	2.9	70.0	12.0	10.0	4.0	4.0	73.0	11.9	10.3	4.8	0.0
24. Oversensitive to noise[a]	43.2	20.1	23.7	12.2	0.7	50.0	30.0	14.0	6.0	0.0	45.2	32.5	18.3	2.4	1.6
25. Stare at nothing with no purpose[a]	56.1	18.7	8.6	12.9	3.6	86.0	6.0	4.0	4.0	0.0	90.5	7.9	1.6	0.0	0.0

[a]*Reverse-scored items.*

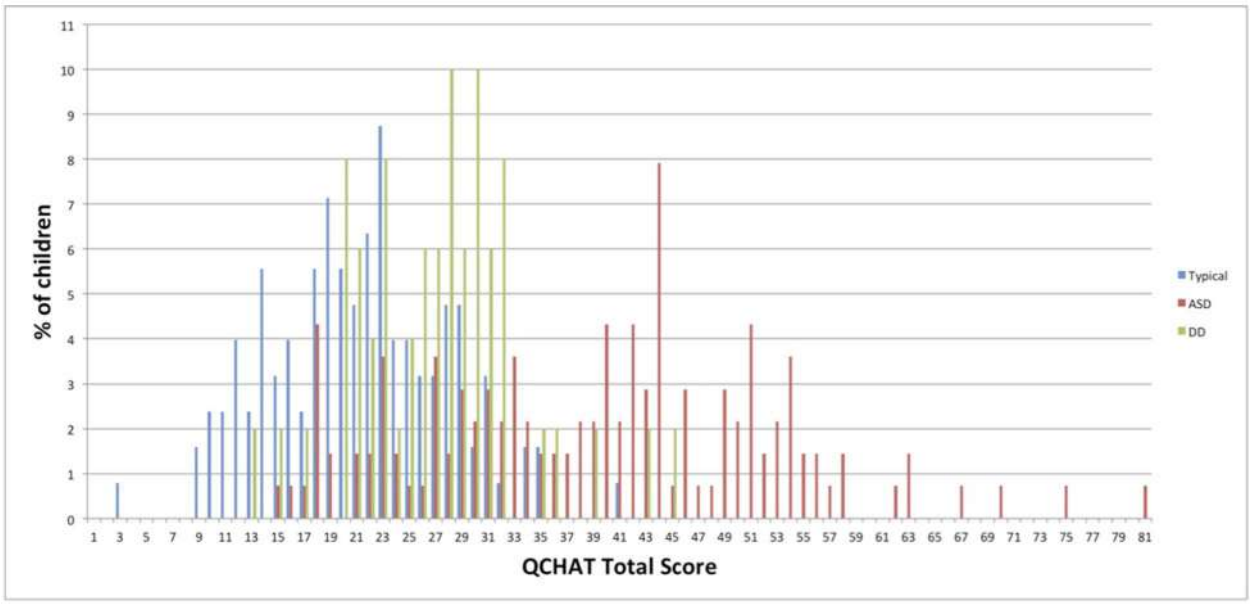


FIGURE 1 | Q-CHAT score distribution in children with autism, developmental delay (DD), and typically developing children (TD).

Accuracy of the Q-CHAT in Predicting ASD and Q-CHAT Cut-Points

Figure 2 shows the area under the curve (AUC) for the Q-CHAT total score in the ASD versus TD, ASD versus DD, and DD versus TD groups.

Sensitivity and specificity associated with different cutoff scores for autism and DD children are presented in **Table 3**.

Based on ROC analysis, the Q-CHAT total score that better differentiated between autism and TD children maximizing sensitivity (i.e., correctly identifying all children at risk for autism) while maintaining adequate specificity (i.e., correctly identifying all children not at risk for autism) was 27 (Sens. = 83%, Spec. = 78%). When autism children were compared to DD children, a higher cut-point of 31 or above indicative of an autism condition was found (Sens. = 73%, Spec. = 76%).

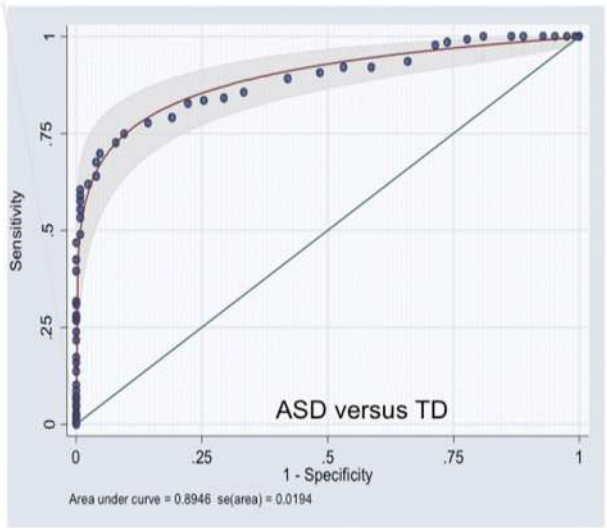
Convergent Validity of the Q-CHAT With the Griffiths Development Quotient, the ADOS 2, and the CBCL.

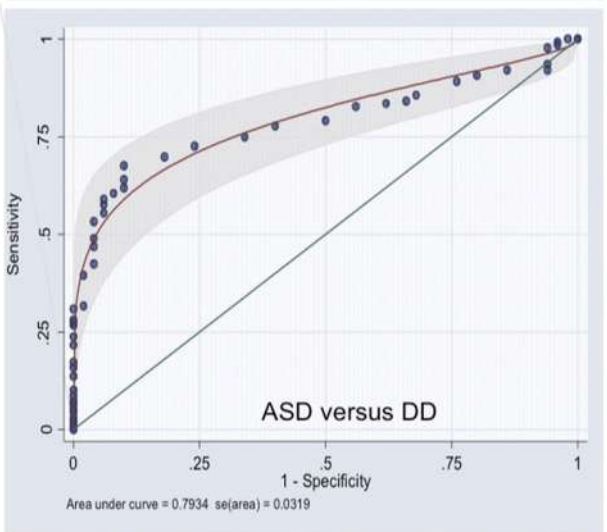
In the autism sample, the QCHAT total scores were positively correlated with the ADOS 2 social affect (Beta = 0.94, $p < 0.001$) and negatively correlated with the Griffiths LDQ (Beta = −0.1, $p = 0.02$). No main effect of PDQ and ADOS 2 restricted and repetitive behaviors was found (Beta = 0.01, $p = 0.72$ and Beta = 0.13, $p = 0.82$). Furthermore, in both the autism and TD groups, the QCHAT total score was positively correlated with most of the CBCL domains with medium to large effect sizes in both groups (Spearman rho from 0.29 to 0.44 in autism and from 0.46 to 0.57 in TD children). The correlations between all the CBCL domains and the QCHAT scores in the autism and TD groups are reported separately in **Table 4**.

DISCUSSION

This study aimed to investigate the psychometric properties of the Q-CHAT among children with a diagnosis of autism and children presenting other neurodevelopmental conditions such as developmental delay versus typically developing children. Furthermore, the external validity of the Q-CHAT towards measures of cognitive functioning, language, behavior, and autism symptom severity were analyzed.

Similarly to previous studies using the Q-CHAT in both clinical and population-based settings (26–28), we found a normal distribution of the Q-CHAT scores. This result confirms the unique potential of this instrument as a dimensional measure of autistic traits along a continuum in the population and makes the Q-CHAT a particularly suitable tool to be used in genetic and biomarker stratification approaches at a very early developmental stage. As expected and consistent with the findings reported by Allison et al. (20), children with a diagnosis of autism scored significantly higher than those with typical development. Furthermore, in our study, we explored the Q-CHAT score distribution in children with developmental delay (DD) and found that Q-CHAT scores in autism children were significantly higher than those reported in DD children. Conversely, scores on the QCHAT in the DD group, after controlling for PDQ and age, were slightly higher but not significantly different from TD children. Furthermore, while an autism condition strongly predicted the Q-CHAT score, a DD condition did not. These results are worthy of attention, in that the Q-CHAT has been specifically designed as a quantitative measure for autism rather than a broadband tool for neurodevelopmental conditions (including autism) in general. Consequently, it may be expected that the Q-CHAT would be less accurate in identifying children





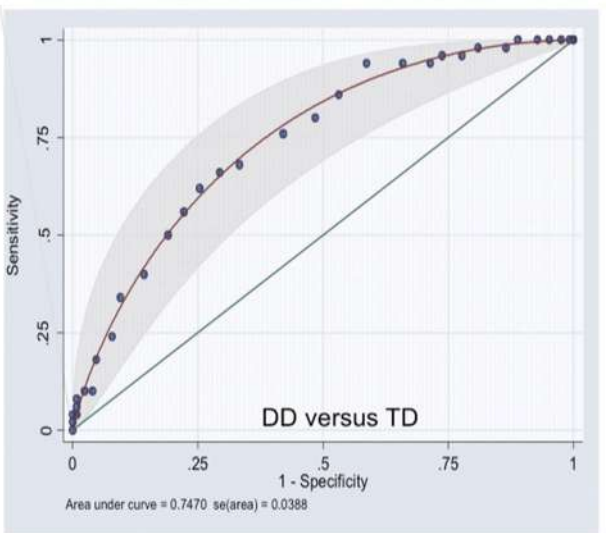


FIGURE 2 | Area under the curve for the Q-CHAT.

with a DD diagnosis than those with an autism diagnosis. This was in fact the case. Children with DD were not classified consistently by the Q-CHAT (AUC = 75% indicating a modest accuracy), while the discriminant validity of the Q-CHAT for autism was very good (AUC = 89%) and in line with that reported by Allison et al. (21) (AUC = 92%). Unlike the previous findings where an effect of gender (with boys scoring higher in the unselected sample) and age (a small negative correlation in the autism group) on the Q-CHAT scores were reported (20), we did not replicate these results. Also, the mean Q-CHAT scores in boys and girls in Allison et al.'s study were somewhat higher [mean score of 27.5 (7.8) for boys and of 25.8 (7.7) for girls] than our sample [TD males = 21.6 (7.6); TD females = 20.8 (5.7)]. However, it should be considered that in Allison et al.'s study, the Q-CHAT questionnaires were sent by post and no direct assessment was possible to exclude potential children with atypical development and/or mild neurodevelopmental conditions. In our study, all the TD children were tested for language and performance development using the Griffiths test as well as for behavior using the CBCL 1.5-5, and indeed, three children (2.3%) were excluded from the study because of language/developmental delay or autism traits. The same scoring pattern has been found in the autism group with Allison et al.'s sample reporting rather higher Q-CHAT scores [mean score of 51.3 (SD = 14.1) for boys and of 54.6 (SD = 14.9) for girls] than our sample. Again, it is likely that the sample characteristics in the two studies are different in that the autism children in our study have been referred and diagnosed within clinical facilities, whilst in Allison et al.'s study the autism sample was mainly recruited through the Autism Research Centre website and parents who volunteered might have had more impaired children and/or over-reported symptoms. Also in Allison et al.'s study, neither independent verification of an autism diagnosis nor IQ assessment was possible. As for age, the unselected group in Allison et al.'s study was young [mean age (SD) = 21.2 (2.1) months], whereas children in the autism group were significantly older [mean age of 44.5 (10.2) months]. In our study, the autism and TD samples were more consistently matched [mean age (SD) = 31.6 (8) months and mean age (SD) = 33.2 (9.3) months in the autism and TD group, respectively] and an effort has been made to recruit autism children as young as possible, before the age of 3 years, to comply with the purpose of the instrument as an early screener for autism. When the Q-CHAT total score that better differentiated between autism and TD children was explored, we found that a cut-off of 27 maximized sensitivity (83%) without compromising specificity too much (78%). In a previous study, using a short version of the Q-CHAT (Q-CHAT-10), Allison et al. (21) reported a higher sensitivity and specificity (91% and 89%, respectively) at the screening cut-point. However, it should be considered that the Q-CHAT-10 included selectively only the 10 most discriminating items, and therefore, higher sensitivity and specificity may be expected. In another study, in a community clinical sample, Charman et al. (29) explored the accuracy of two other commonly used screeners, the MCHAT and the SCQ, in predicting autism versus non-autism status. While the M-CHAT demonstrated adequate sensitivity (84%) but poor specificity (50%), the SCQ conversely

TABLE 3 | Sensitivity and specificity of different Q-CHAT cut-points in predicting an autism and a DD status.

Cut-off	ASD versus TD		ASD versus DD		Cut-off	ASD versus TD		ASD versus DD	
	Sensitivity	Specificity	Sensitivity	Specificity		Sensitivity	Specificity	Sensitivity	Specificity
> = 9	100.00%	0.79%			> = 37	58.99%	99.21%	58.99%	94.00%
> = 10	100.00%	2.38%			> = 38	57.55%	99.21%	57.55%	94.00%
> = 11	100.00%	4.76%			> = 39	55.40%	99.21%	55.40%	94.00%
> = 12	100.00%	7.14%			> = 41	48.92%	99.21%	48.92%	96.00%
> = 13	100.00%	11.11%	100.00%	0.00%	> = 42	46.76%	100.00%	46.76%	96.00%
> = 14	100.00%	13.49%			> = 43	42.45%	100.00%	42.45%	96.00%
> = 15	100.00%	19.05%	100.00%	2.00%	> = 44	39.57%	100.00%	39.57%	98.00%
> = 16	99.28%	22.22%	99.28%	4.00%	> = 45	31.65%	100.00%	31.65%	98.00%
> = 17	98.56%	26.19%	98.56%	4.00%	> = 46	30.94%	100.00%	30.94%	100.00%
> = 18	97.84%	28.57%	97.84%	6.00%	> = 47	28.06%	100.00%	28.06%	100.00%
> = 19	93.53%	34.13%	93.53%	6.00%	> = 48	27.34%	100.00%	27.34%	100.00%
> = 20	92.09%	41.27%	92.09%	6.00%	> = 49	26.62%	100.00%	26.62%	100.00%
> = 21	92.09%	46.83%	92.09%	14.00%	> = 50	23.74%	100.00%	23.74%	100.00%
> = 22	90.65%	51.59%	90.65%	20.00%	> = 51	21.58%	100.00%	21.58%	100.00%
> = 23	89.21%	57.94%	89.21%	24.00%	> = 52	17.27%	100.00%	17.27%	100.00%
> = 24	85.61%	66.67%	85.61%	32.00%	> = 53	15.83%	100.00%	15.83%	100.00%
> = 25	84.17%	70.63%	84.17%	34.00%	> = 54	13.67%	100.00%	13.67%	100.00%
> = 26	83.45%	74.60%	83.45%	38.00%	> = 55	10.07%	100.00%	10.07%	100.00%
> = 27	82.73%	77.78%	82.73%	44.00%	> = 56	8.63%	100.00%	8.63%	100.00%
> = 28	79.14%	80.95%	79.14%	50.00%	> = 57	7.19%	100.00%	7.19%	100.00%
> = 29	77.70%	85.71%	77.70%	60.00%	> = 58	6.47%	100.00%	6.47%	100.00%
> = 30	74.82%	90.48%	74.82%	66.00%	> = 62	5.04%	100.00%	5.04%	100.00%
> = 31	72.66%	92.06%	72.66%	76.00%	> = 63	4.32%	100.00%	4.32%	100.00%
> = 32	69.78%	95.24%	69.78%	82.00%	> = 67	2.88%	100.00%	2.88%	100.00%
> = 33	67.63%	96.03%	67.63%	90.00%	> = 70	2.16%	100.00%	2.16%	100.00%
> = 34	64.03%	96.03%	64.03%	90.00%	> = 75	1.44%	100.00%	1.44%	100.00%
> = 35	61.87%	97.62%	61.87%	90.00%	> = 81	0.72%	100.00%	0.72%	100.00%
> = 36	60.43%	99.21%	60.43%	92.00%	> 81	0.00%	100.00%	0.00%	100.00%

In light gray is reported the best cut-point in predicting a DD status, in dark gray is reported the best cut-point in predicting an autism status.

TABLE 4 | Correlation of the QCHAT total score with the CBCL scores.

	Spearman rho		p-value	
	ASD	TD	ADS	TD
EMOTIONALLY REACTIVE	0.27	0.52	0.02	<0.001
ANXIOUS DEPRESSED	0.19	0.40	0.08	0.003
SOMATIC COMPLAINTS	0.003	0.45	0.98	0.001
WITHDRAWN	0.52	0.41	<0.001	0.003
SLEEP PROBLEMS	0.10	0.13	0.37	0.35
ATTENTION PROBLEMS	0.34	0.28	0.002	0.05
AGGRESSIVE BEHAVIOR	0.19	0.43	0.09	0.001
INTERNALIZING	0.38	0.57	<0.001	<0.001
EXTERNALIZING	0.29	0.46	0.01	0.001
PDD	0.44	0.49	<0.001	<0.001
ADHD	0.24	0.22	0.03	0.11
AFFECTIVE	0.23	0.27	0.04	0.05
ANXIETY	0.07	0.41	0.55	0.002
OPPOSITIONAL DEFIANT	0.26	0.34	0.02	0.01
TOTAL SCORE	0.34	0.55	0.002	<0.001

demonstrated low sensitivity (64%) and moderate specificity (75%). Overall, the Q-CHAT in our sample replicated the good sensitivity of the M-CHAT whilst maintaining a sub-optimal but still higher specificity than the SCQ. When an autism versus a DD status was contrasted, a higher cut-point of 31 was the most appropriate in our sample to better discriminate between the two conditions, still ensuring adequate sensitivity (73%) and specificity (76%). The latter cutoff, although not reaching the recommended sensitivity and specificity of at least 80% (30), nevertheless is still acceptable, especially considering that when there is a greater overlapping of scores, such as in the case of autism and DD, sensitivity and specificity are consequently lower. In the light of these results, two different cut-points (27 and 31, respectively) may be proposed, depending on whether the Q-CHAT is intended to be used as a broader first-level screener or more specifically used to discriminate between autism and other developmental conditions. Finally, we explored the external validity of the QCHAT with regard to measures of cognitive functioning, language, autism symptom severity, and behaviors. In the autism group, we found that Q-CHAT scores were positively correlated with the severity of symptoms in the *Social Affect* domain of the ADOS-2 and negatively correlated with the language abilities on the Griffiths test. These findings indicated that the lower the language and social communication skills, the higher the Q-CHAT scores were. Furthermore, both in autism and TD children, the Q-CHAT scores were positively correlated, with medium to large effect sizes in both groups, with the CBCL PDD subscale, as well as with the internalizing subscale (in particular emotional reactivity and withdrawn) and the externalizing subscale (attention and oppositional-defiant problems in particular). These findings are consistent with those reported by Magiati et al. (27) in a large population-based sample using the Q-CHAT and by Constantino et al. (31) and

Duku et al. (32) in two independent samples of children with a diagnosis of autism using the Social Responsiveness Scale.

There are limitations to this study that must be acknowledged. First of all, there are unequal proportions of children in the three groups, with the DD group having half the sample size of the autism and TD groups.

Furthermore, DD children in our sample were significantly younger than children in the other two groups. Although age did not predict Q-CHAT scores and we controlled statistically for age, a replication in a larger and better age-matched sample of children with DD is recommended. In addition, the PDQ in TD children was high and maybe not be a representative of the general population. Nevertheless, the effect of PDQ was controlled for in all the analyses, and the results were confirmed.

While these factors have been controlled for statistically, in the application of the QCHAT in clinical and community settings, we should consider their possible effects with respect to the cut-off while deciding "caseness."

CONCLUSIONS

In conclusion, we demonstrated that in a clinical setting of children already diagnosed with an ASD or developmental delays as compared to typically developing children, the Q-CHAT is a quantitative, normally distributed measure with satisfying psychometric properties and external validity, able to discriminate autism children not only from children with typical development but also from children with other developmental conditions such as developmental delay. Future research should aim to replicate the findings in clinical samples from a larger community as well as in population samples with follow-up prospective designs before recommending the Q-CHAT as a clinical instrument for early autism screening.

ETHICS STATEMENT

The protocol was approved by the Scientific Foundation "Stella Maris' Ethic Committee" (Prot. n. 11/2012) and a written informed consent in accordance with the Declaration of Helsinki was obtained from all subjects.

AUTHOR CONTRIBUTIONS

LR conceived of the study, participated in its design and coordination, and drafted the manuscript. FM, SB, and CA participated in the design and interpretation of the data. GA, FA, and AG participated in the design and coordination of the study. EL, NT, CC, RM, NC, and VC performed the measurement. FC participated in the design of the study and performed the statistical analysis. GP and GT participated in the coordination of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

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Possible Implication of the CA2 Hippocampal Circuit in Social Cognition Deficits Observed in the Neuroligin 3 Knock-Out Mouse, a Non-Syndromic Animal Model of Autism

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Autism spectrum disorders (ASDs) comprise a heterogeneous group of neuro-developmental abnormalities with a strong genetic component, characterized by deficits in verbal and non-verbal communication, impaired social interactions, and stereotyped behaviors. In a small percentage of cases, ASDs are associated with alterations of genes involved in synaptic function. Among these, relatively frequent are mutations/deletions of genes encoding for neuroligins (NLGs). NLGs are postsynaptic adhesion molecules that, interacting with their presynaptic partners neurexins, ensure the cross talk between pre- and postsynaptic specializations and synaptic stabilization, a condition needed for maintaining a proper excitatory/inhibitory balance within local neuronal circuits. We have focused on mice lacking NLG3 (NLG3 knock-out mice), animal models of a non-syndromic form of autism, which exhibit deficits in social behavior reminiscent of those found in ASDs. Among different brain areas involved in social cognition, the CA2 region of the hippocampus has recently emerged as a central structure for social memory processing. Here, *in vivo* recordings from anesthetized animals and *ex vivo* recordings from hippocampal slices have been used to assess the dynamics of neuronal signaling in the CA2 hippocampal area. *In vivo* experiments from NLG3-deficient mice revealed a selective impairment of spike-related slow wave activity in the CA2 area and a significant reduction in oscillatory activity in the theta and gamma frequencies range in both CA2 and CA3 regions of the hippocampus. These network effects were associated with an increased neuronal excitability in the CA2 hippocampal area. *Ex vivo* recordings from CA2 principal cells in slices obtained from NLG3 knock-out animals unveiled a strong excitatory/inhibitory imbalance in this region accompanied by a strong reduction of perisomatic inhibition mediated by CCK-containing GABAergic interneurons. These data clearly suggest that the selective alterations in network dynamics and GABAergic

signaling observed in the CA2 hippocampal region of NLG3 knock-out mice may account for deficits in social memory reminiscent of those observed in autistic patients.

Keywords: NLG3 KO mice, CA2 hippocampal region, sociability and social memory, excitatory/inhibitory dysfunction, CCK-positive neuron

INTRODUCTION

Autism spectrum disorders (ASDs) comprise a heterogeneous group of neurodevelopmental disorders with a strong genetic component, characterized by deficits in verbal and non-verbal communication, impaired social interactions, restricted interests, and stereotyped behaviors (1). The high incidence of these disorders in recent years (1/100 children with males affected four to six times more than females) can be attributed to the improvement of the diagnostic criteria in general, to the increased attention of the medical community, and to environmental and epigenetic factors acting upon a genetically vulnerable background (2). Most of the genes implicated in ASDs encode proteins relevant for synapse formation, transcriptional regulation, and chromatin remodeling (3). Although mutations or deletions in genes encoding for synaptic proteins are relatively rare (4–8), nevertheless they are important since they point to synapses as possible sites of origin of ASDs, which can be considered synaptopathies (9). One class of non-syndromic forms of ASDs has been found to be associated with mutations or deletions in neuroligin genes (Nlgn1-4) (10–12). Neuroligins (NLGs) are postsynaptic adhesion molecules which bind to their presynaptic partners neurexins to functionally couple the postsynaptic densities with the transmitter release machinery, thus contributing to synapses formation and stabilization (9). To study the mechanisms by which these mutations/deletions contribute to ASD, animal models have been generated that recapitulate important aspects of the human disorder.

Previous studies from NLG3^{R451C} knock-in and NLG3-knock-out mice have revealed the presence of circuit-specific and cell-specific synaptic dysfunctions (13–16). Interestingly, both NLG3^{R451C} knock-in and NLG3-knock-out mice exhibit deficits in social interaction/memory reminiscent of those found in autistic patients (13, 17).

Among brain areas comprising the "social brain" the CA2 region of the hippocampus, characterized by peculiar molecular, morphological, and physiological properties (18, 19), has recently emerged as a central structure for social memory processing (20, 21). This region is responsible not only for encoding social memory, namely, the capacity of an animal to recognize a conspecific (20, 21), but also for social aggression (22). Previous data from mice lacking the Nlgn3 gene have focused on the cerebellum (14) and on the CA1 hippocampal synaptic connectivity (23). However, the CA2 hippocampal circuit, selectively related to social cognition, whose impairment constitutes one of the core symptoms of ASDs, has never been explored.

Here, behavioral, *in vivo* and *ex vivo* electrophysiological recordings from NLG3 knock-out mice have allowed identifying alterations of the CA2 hippocampal circuit probably related to deficits in social memory.

METHODS

Animals

All experiments were performed in accordance with the Italian Animal Welfare legislation (D.L. 26/2014) that implemented the European Committee Council Directive (2010/63 EEC) and were approved by local veterinary authorities, the EBRI ethical committee, and the Italian Ministry of Health (1084/PR15). All efforts were made to minimize animal suffering and to reduce the number of animals used. NLG3 KO mice were obtained from Prof. P. Scheiffele (Biozentrum, Basel). Experiments were performed on offspring male derived from heterozygous mating after 10 backcrossing with C57BL6/J. The experiments were performed and the results were analyzed blindly before genotyping. Control experiments were performed on wild-type littermates (controls). Genotyping was carried out on tail biopsy DNA by PCR using a standard protocol. At least five male mice for each genotype were used in a given experiment. Western blot analysis from hippocampal lysates confirmed the lack of NLG3 protein in KO mice (**Figure S1**).

Western Blot

Hippocampi from controls and NLG3 KO mice were homogenized using a lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% CHAPS, 1 mM Na_3VO_4, 50 mM NaF, and protease inhibitor mixture (Sigma). Equal amounts of total protein extracts were run on a 10% Sodium Dodecyl Sulfate Polyacrylamide Gel (SDS-PAGE). Western blot analysis was performed with the following primary antibodies: an anti-NLG3 polyclonal antibody (1:1,000, Synaptic System, #129 311) and an anti-actin-HRP (1:5,000, Santa Cruz Biotechnology). Anti-NLG3 Primary antibody was revealed by HRP-conjugated anti-rabbit secondary antibody (Sigma) followed by ECL (Amersham Biosciences).

Slice Preparation

Transverse hippocampal slices (320 µm thick) were obtained from postnatal (P) day P30–P40 old animals, using a standard protocol (24). Briefly, after being anesthetized with an intraperitoneal injection of a mixture of tiletamine/zolazepam (80 mg/kg) and xilazine (10 mg/kg), mice were decapitated. The brain was quickly removed from the skull, placed in artificial cerebrospinal

fluid (ACSF) containing the following (in mM): 75 sucrose, 87 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 7 $MgCl_2$, 0.5 $CaCl_2$, 25 $NaHCO_3$, and 25 glucose. After recovery, an individual slice was transferred to a submerged recording chamber and continuously perfused at 33–34°C with oxygenated ACSF at a rate of 3 ml/min. ACSF saturated with 95% O_2 and 5% CO_2 containing the following (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$, 25 $NaHCO_3$, and 25 glucose. The osmolarity was 310–320 mOsm.

Electrophysiological Recordings in Slices

Cells were visualized with a 60× water immersed objective mounted on an upright microscope (Nikon, eclipse FN1) equipped with a CCD camera (Scientifica, UK). Whole-cell patch clamp recordings in voltage-clamp mode were performed with a MultiClamp 700B amplifier (Axon Instruments, Sunnyvale, CA, USA). Patch electrodes were pulled from borosilicate glass capillaries (WPI, Florida, USA); they had a resistance of 3–4 MΩ when filled with an intracellular solution containing the following (in mM): 70 $CsMeSO_3$, 70 CsCl, 10 Hepes, 0.2 EGTA, 2 $MgCl_2$, 4 MgATP, 0.3 MgGTP, and 5 Na-phosphocreatine; the pH was adjusted to 7.2 with CsOH; the osmolarity was 290–300 mOsm. The holding membrane potential value was not corrected for the liquid junction potential of 10 mV (calculated with the Clampex software; Molecular Devices, Sunnyvale, CA, USA). The stability of the patch was checked by repetitively monitoring the input and series resistance during the experiments. Series resistance (10–20 MΩ) was not compensated. Cells exhibiting 15% changes were excluded from the analysis.

Spontaneous $GABA_A$-mediated inhibitory postsynaptic currents (sIPSCs) and AMPA-mediated postsynaptic currents (sEPSCs) were recorded in the CA2 region of the hippocampus from a holding potential of −70 mV in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) and picrotoxin (100 μM), respectively. GABAergic currents (IPSCs) were evoked in CA2 principal cells by stimulation of GABAergic inputs with a glass pipette (filled with ACSF) positioned in stratum pyramidale (at 100–200 μM from the patched cell). Cells were recorded from a holding potential of −50 mV, in the presence of CNQX (10 μM) and DL-2-amino-5-phosphonopentanoic acid (DL-APV, 100 μM), to block α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors, respectively. The intensity of stimulation (delivered at 0.1 Hz) was set at around 60% of the maximum response. Synaptic currents mediated by cholecystokinin-positive interneurons were obtained by subtracting from the total current that sensitive to ω-conotoxin GVIA (ω-CTx-GVIA, 1 μM), known to block N type of voltage-dependent calcium channels, responsible for triggering the release of GABA from CCK+ interneurons (25). Synaptic currents mediated by parvalbumin-positive interneurons were obtained by subtracting from the total current that sensitive to ω-Agatoxin (ω-Agtx-IVA, 300 nM), known to block the P/Q types of voltage-dependent calcium channel responsible for triggering the release of GABA from PV+ interneurons (26). Drugs were applied in the bath, and the ratio of flow rate to bath volume ensured complete exchange within 2–3 min.

In Vivo Electrophysiological Recordings From Anesthetized Animals

Mice (P50–70) were anesthetized with i.p. injection of a mixture of tiletamine/zolazepam (Zoletil; 80 mg/kg) and xilazine (10 mg/kg) to induce anesthesia before surgery and during recordings. A craniotomy for recording sites was drilled between −1.6 and −1.7 mm posterior from bregma, and lateral coordinates were adjusted after extracellular mapping to locate the CA2 and CA3 pyramidal cell layers. Temperature was maintained between 36°C and 37°C using a feedback-controlled heating pad (FHC). Recordings were obtained with a Multiclamp 700B amplifier connected to the Digidata 1550 system. Data were acquired with pClamp 10 (Molecular Devices) and analyzed off-line with Clampfit 10.4 (Molecular Devices).

Extracellular recordings of field potentials were obtained with glass electrodes (Hingelberg, Malsfeld, Germany) prepared with a vertical puller PP-830 (Narishige), and the tip was broken to obtain a resistance of 1–2 MΩ. Electrodes were filled with a standard Ringer's solution containing the following (in mM): 135 NaCl, 5.4 KCl, 5 HEPES, 1.8 $CaCl_2$, and 1 $MgCl_2$.

Juxtacellular recordings of spontaneous neuronal firing were obtained using glass electrodes (7–10 MΩ) filled with Ringer solution. Bursts behavior (a burst was defined as a sequence of two or more action potentials occurring at frequency ≥20 Hz) (27) was assessed using the features of spike firing pattern illustrated by plots of interspike interval (ISI) distributions. Bursting behavior showed a distribution of ISI skewed on the left. A clampfit algorithm was used to detect bursts from the total events found setting a threshold. The interval for burst detections ranged from 20 to 50 ms.

Data Analysis

Data were transferred to a computer hard disk after digitization with an A/D converter (Digidata 1550, Molecular Devices). Data acquisition (digitized at 10 kHz and filtered at 3 kHz) was performed with pClamp 10.4 software (Molecular Devices, Sunnyvale, CA, USA). Input resistance and cells capacitance were measured online with the membrane test feature of the pClamp software. Spontaneous EPSCs and IPSCs were analyzed with pClamp 10.4 (Molecular Devices, Sunnyvale, CA, USA). This program uses a detection algorithm based on a sliding template. The template did not induce any bias in the sampling of events because it was moved along the data trace by one point at a time and was optimally scaled to fit the data at each position.

The rise time of the evoked IPSC was estimated as the time needed for 20–80% increase of the peak current response. The decay time was fitted with first order exponential function in the following form:

$$y(t) = A^{*} e(-t/\tau)$$

where τ and A are the time constants and relative fractions of the respective components.

LFP analysis

Unsupervised Decomposition of Local Field Potentials (LFPs)

Raw LFPs data were sampled at 10 kHz and decomposed into their elementary signals or intrinsic mode functions (IMFs) using the "Complete Empirical Ensemble Mode Decomposition with Adaptive Noise "CEEMDAN" algorithm (28). One of the main advantages of this algorithm is that it deals well with non-stationary signals, thus diminishing filtering artifacts (such as harmonics and side band-related distortions) caused by convolution filters for cross-frequency coupling analysis (29–31). We extracted theta (5–14 Hz), low (25–55 Hz), and high gamma (56–120 Hz) signals by combining IMFs with mean instantaneous frequencies [see Ref. (32)].

Power Spectral Density (PSD) Analysis

Welch's PSD was computed using MATLAB *pwelch* function. The Hanning (2048) window was chosen in order to achieve frequency resolution of 5 Hz with overlap of 50%.

Average Relative Power of Individual Bands

Average relative amplitude of LFP bands was calculated as *z*-score of individual bands, with standard deviation of raw LFP. This gives us the relative amplitude of each band. Average relative power was obtained by taking the square of relative amplitude and dividing it by the length of the signal.

Band Occurrence Frequency

To obtain band occurrence frequency, we used *z*-scored amplitude of individual LFP bands. This was obtained using MATLAB function *z*-score. We then counted the number of cycles (*cycle count*) where amplitude exceeded the *threshold* of 2 standard deviations. Dividing the *cycle count* by total duration gives us the band occurrence frequency for a given LFP band in a given recording file. The choice *threshold = 2 standard deviations* was motivated by the observation that amplitudes in a given LFP band is distributed normally and we wanted to include only those cycles in *cycle count* which takes the amplitude beyond the 95th percentile.

Spike Time Extraction

To extract spikes from LFPs we used "wave clus," a MATLAB tool developed by Chaure et al. (33) that filters raw signals using band-pass filter in the range 300 to 4,500 Hz. Spike detection threshold was set at 5 standard deviations.

Power During Spikes

We studied variation in power in LFPs preceding and following 1 s of spike time. This time (1 s) was divided into 10 bins of 100 ms each. We then computed the average relative power as described above for each bin. This allows evaluating how LFP power varies before and after a spike.

Spike-Triggered Average LFP Analysis

Analysis of the temporal relationship between multi-unit-activity and ongoing activity was analyzed using custom written scripts in Matlab (The MathWorks, Inc., Natick, Massachusetts, United States). Briefly, spikes were extracted using a threshold on the high-passed version of the recorded membrane potential (cut frequency was set at 500 Hz). The threshold was set at 5 times the standard deviation of this signal. To compute the spike-triggered average, we cut 1 s time segment around the occurrence of each spike using a band-pass-filtered LFP signal (cut frequencies were set at 0.2 and 50 Hz). Experiments where multi-unit-activity were either absent or very sparse (below 1 Hz) were excluded from the analysis.

The power spectrum of **Figure 2C** was computed using running windows of 0.5 s and tapers on the high-pass-filtered membrane signal (cut frequency was set at 3 Hz) using Matlab and the toolbox chronux (http://chronux.org/).

Drugs

Drugs were applied in the bath by gravity by changing the superfusion solution to one differing only in its content of drug(s). Drugs used were the following: CNQX, DL-APV, ω-CTx-GVIA, and picrotoxin purchased from Tocris (UK) and ω-Agtx-IVA from SIGMA (Italy). Stock solutions were made in distilled water and then aliquoted and frozen at −20°C. Picrotoxin and CNQX were dissolved in DMSO. The final concentration of DMSO in the bathing solution was 0.1%. At this concentration, DMSO alone did not modify the membrane potential, input resistance, or the firing properties of CA2 neurons.

Behavioral Experiments

Three Chamber

In order to evaluate sociability and interest in social novelty, we tested animals by using the three-chamber test, adapted from Moy et al. (34). The social testing area was a homemade rectangular, three-chambered box (each chamber was 20 × 40 × 21 cm in size). Dividing walls were made from clear Plexiglas, with rectangular openings (6 × 8.5 cm) allowing access to each chamber. The light intensity was distributed equally in different parts of the apparatus (6 lux). Between trials, the chambers of the arena were cleaned with 70% ethanol to eliminate any lingering smells.

Mice were habituated to handling 5 min a day for 5 days before the test. The day before the test, mice were placed in the empty chamber and allowed to explore for 30 min. On the test day, a 10 min habituation phase in the empty apparatus took place prior to the test phase. The test phase consisted of three consecutive trials. In the first two trials, sociability was assessed. An unfamiliar C57BL7/6J male mouse (stranger 1) was placed in a wire cup (ø 10.5 cm × 10.5 cm h) in one of the side chambers. In the other side chamber, an identical wire cup was placed to ensure that the test mouse had a choice between a novel object and a novel social context. The position of "stranger 1" was alternated between the first and second trials, to prevent side preference. The test mouse was placed in the middle compartment and allowed to explore the entire social test arena for 10 min. The interaction time with the stranger mouse was recorded by the video-tracking system (ANY-maze, Stoelting

Co, IL, US). The score for sociability was calculated as the difference between the investigation time for the compartment containing the novel mouse and that for the compartment with the novel object. After a 1 h inter-session interval a second unfamiliar C57BL7/6J male mouse (stranger 2 or novel) was placed into the previously empty wire cage, while "stranger 1" (familiar) remained inside its cup. The subject mouse was given 10 min to explore all three chambers. "Strangers mice" were 4–5 weeks old (juvenile) to exclude any effect due to mutual aggression. The score for social novelty was calculated as the difference between the investigation time for the compartment containing the novel mouse and that for the compartment with the familiar mouse.

Open Field

Mice were also tested in the open field for general locomotor activity, anxiety, and willingness to explore. The experimental apparatus consisted of a black rectangular open field (60 cm × 60 cm × 30 cm; Panlab, US). Mice were allowed to acclimate in their home cage to the procedure room for 30 min before the test. The arena was cleaned with 70% ethanol between trials to eliminate any lingering smells. During the test, each animal was placed in the center of the arena and allowed to freely move for 10 min while being recorded with an overhead camera. Mouse activity was then analyzed by an automated tracking system (ANY-maze, Stoelting Co, IL, US) for the following parameters: total ambulatory distance and velocity. A series of 12 × 12 cm zones were identified and used to evaluate the time spent in the center (inner zone) or peripheral zones (outer zone). The outer zone consisted of 12 blocks close to walls, while the inner zone consisted of 9 blocks in the center. Greater time spent in the outer zones of the maze was indicative of amplified anxiety-related behavior (35).

Tissue Preparation for Immunohistochemistry

Mice (P60–90) were anesthetized with i.p. injection of a mixture of tiletamine/zolazepam (Zoletil; 80 mg/kg) and xilazine (10 mg/kg) and perfused transcardially with ice-cold oxygenated ACSF (pH 7.4) for 2 min, as previously described (36). Brains were rapidly dissected and fixed overnight in 4% paraformaldehyde (PFA) phosphate buffered saline solution (PBS). To identify the tracks of microelectrodes used to record local field potentials *in vivo*, brains were removed after the completion of electrophysiology experiments and were fixed in 4% PFA overnight. After rinsing in PBS, brains were incubated with 30% (wt/vol) sucrose in PBS at 4°C overnight, frosted with dry ice-cold isopentane, and stored at −80°C. Brains were embedded in the OCT compound (Leica, Germany) and sectioned by cryostat (Leica CM1850 UV, Germany; 60-µm-thick coronal sections).

Immunohistochemistry

Free-floating sections were rinsed in PBS and incubated overnight at 4°C in primary antibody solution (50 mM Tris, 150 mM NaCl, 0.2% Triton X-100, 2% normal goat and donkey serum, pH 7.4) with antibodies anti-PCP4 (rabbit, 1:200;# SIGMA), anti-CCK8 (mouse, 1:500; #ab37274 Abcam) and anti-PV (guinea pig, 1:1,000; #24428, ImmunoStar). Sections were washed 3 times for 15 min in PBS and incubated in secondary antibody solution (50 mM Tris, 150 mM NaCl, 0.05% Triton X-100, 2% normal goat and donkey serum, pH 7.4) for 1 h at room temperature with secondary antibodies raised in goat and donkey (rabbit AlexaFluor-647, 1:400;#A21245, ThermoFisher, US; mouse AlexaFluor-555, 1:500; #A31570; guinea pig AlexaFluor-488, 1:500; #A11073). Sections were washed 3 times for 15 min in PBS, incubated for nuclear staining DAPI (1:2,000; 3 min), and washed in PBS. Slices were mounted on superfrost slides (Thermo Scientific, US) and cover slipped with Fluoromount Aqueous Mounting Medium (SIGMA).

Image Acquisition

z-Stack images (four optical sections, 0.5 µm step size) were recorded of all specimens using a spinning disk (X-Light V2, Crest Optics) microscope Olympus IX73 equipped with a LED light source (Spectra X light Engine, Lumencore, US) and an Optimos camera (QImaging, Canada). Images were acquired using a 60× or 40× objective with numerical aperture of 1.35 and 1.30, which had a pixel size of 108.3 × 108.3 nm^2 and 106.2 × 106.2 nm^2, respectively. The excitation wavelengths used were at 408, 470 and 647 nm. The *z*-stacks were done with a motorized stage (HLD117, Prior Scientific, UK) controlled by MetaMorph software (Molecular Devices). All imaging parameters were kept constant among genotypes. Puncta detection and analysis was performed in the CA2 area identified by PCP4 marker on maximum intensity projections created from *z*-stacks using Image J. A minimum of three mice per group was used, and statistical tests were performed using pooled data points from all mice per group.

Statistical Analysis

Values are given as the mean ± SEM of n experiments. Significance of differences was assessed by Student's paired or unpaired *t* test, as indicated. A Mann–Whitney test and Wilcoxon rank sum were used for comparison of two groups. Statistical differences were considered significant at $p < 0.05$. Statistical analysis was performed with GraphPad Prism 8.0 software.

RESULTS

NLG3 KO Mice Exhibit Deficits in Social Behavior

One of the core symptoms of autism is the impairment in social cognition. Previous studies from NLG3 KO mice have revealed altered social memory, probably related to olfactory deficiency with no alterations in time spent in social interaction (17). However, when tested in the three-chamber apparatus, while control mice exhibited normal levels of sociability, spending more time in exploring the mouse cage with respect to the empty one (object), NLG3 KO mice did not show any preference for the mouse cage (**Figures 1A**, **B**). The exploration time of mouse cage was significantly lower in NLG3 KO mice as compared to controls (111 ± 10.2 s *vs* 84 ± 6.6 s, $p = 0.08$ Mann–Whitney test and 155 ± 8.4 s *vs* 85.4 ± 5.3 s, $p < 0.0001$, Mann–Whitney test,

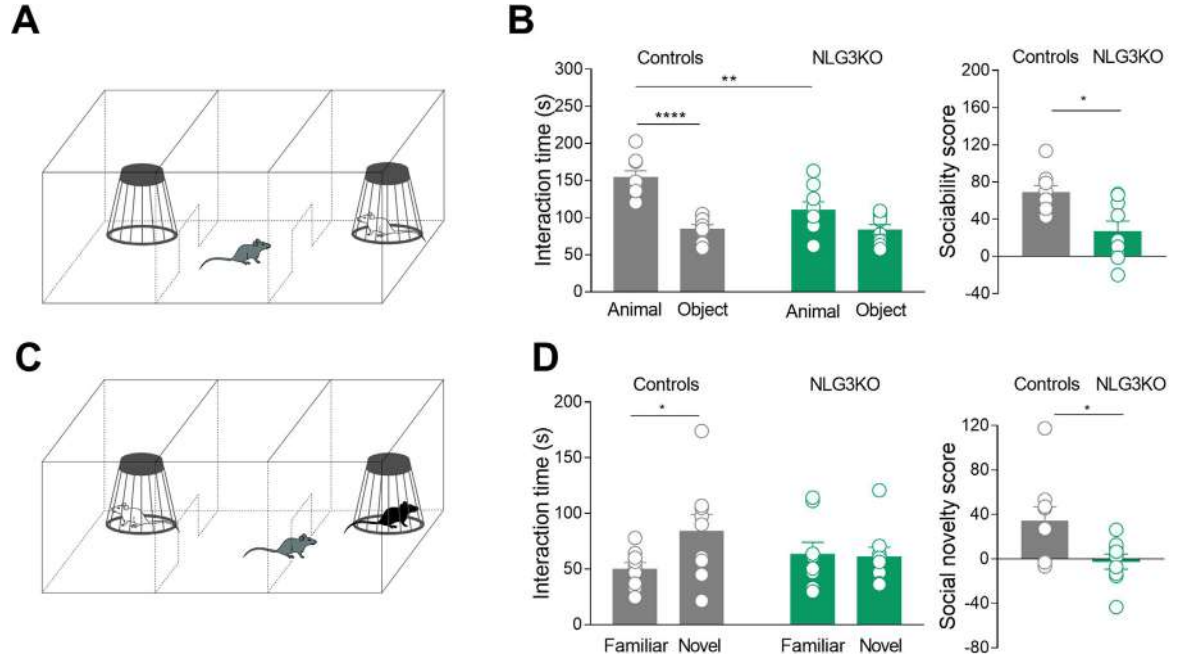

FIGURE 1 | NLG3 KO mice exhibit deficits in social interaction and social memory. **(A)** Schematic representation of three-chamber apparatus to evaluate social interaction. **(B)** Left: scatter plots showing interaction times spent to explore the animal and the object during sociability task in control (gray, $n = 9$) and in NLG3 KO mice (green, $n = 9$); right: scatter plots showing the sociability score in control (gray, $n = 9$) and in NLG3 KO mice (green, $n = 9$). **(C)** Schematic representation of three-chamber apparatus to evaluate social memory (performed 1 h after the sociability test). **(D)** Left: scatter plot showing interaction times spent to explore the novel and the familiar animal in the social novelty task in control (gray, $n = 9$) and in NLG3 KO mice (green, $n = 9$). Right: scatter plot showing the social novelty score in control (gray) and in NLG3 KO mice (green). Open circles are values from single animals, and bars are mean ± SEM.*$p < 0.02$; **$p < 0.001$; ****$p < 0.0001$; Mann–Whitney test.

in NLG3 KO and control mice, respectively) suggesting a reduced interest in investigating a conspecific.

When tested for social novelty, an hour after the sociability test, while control mice spent more time exploring the "novel" than the "familiar" mouse, NLG3 KO mice showed, as in Radyushkin et al., (17), no preference for the novel animal (**Figures 1C**, **D**). The interaction time was 84.2 ± 15 s *vs* 50.2 ± 5.6 s ($p = 0.02$, Mann–Whitney test) and 61.2 ± 8.4 s *vs* 63.7 ± 10 s ($p = 0.1$, Mann–Whitney test) in control and KO mice, respectively. To understand if the social impairment is associated to locomotor alterations or changes in anxiety levels, we tested both NLG3 KO mice and control littermates in the open field test. According to previous work (37, 38) NLG3 KO mice showed a strong tendency for an increased total ambulatory distance (**Figures S2A, B**; controls: 39.4 ± 3.2 m, $n = 10$; NLG3 KO: 51.7 ± 5.2 m, $n = 9$; $p = 0.057$, unpaired t test) and velocity (**Figure S2C**; controls: 65.8 ± 5.3 mm/s; NLG3 KO: 86.2 ± 8.7 mm/s, $n = 9$; unpaired t test, $p = 0.057$) as compared to controls. No differences in the time spent in the center (**Figure S2D**; controls: 58.7 ± 5.8 s; NLG3 KO: 61.8 ± 7.8 s; $p = 0.75$, unpaired t test) or peripheral (**Figure S2D**; controls: 541 ± 5.8 s; NLG3 KO: 538 ± 7.8 s; $p = 0.75$, unpaired t test) zones were detected in both genotypes indicating that there were no changes in anxiety levels. Taken together, these data show a critical role for NLG3 in social behavior including sociability and social memory, reminiscent of those observed in autistic children.

Impairment of Spike-Related Slow Wave Activity in the CA2 Region of the Hippocampus of NLG3 KO Mice

In order to understand whether defects in social behavior observed in NLG3 KO mice depend on alterations in network dynamics, local field potentials (LFPs) were recorded in *in vivo* anesthetized animals from the CA2 (**Figure 2A**) and CA3 hippocampal regions. Firstly, we analyzed the relation between LFPs and multi-unit activity (MUA) recorded in stratum pyramidale. In the presence of anesthetics, LFPs were characterized by slow waves, occurring at the frequency of about 1 Hz (**Figures 2B**, **C**). By filtering the LFP signals for frequencies above 500 Hz, we could clearly see the signatures of MUA (**Figure 2B**, bottom). We then computed the spike-triggered average (STA) LFP by averaging the LFP waveform in the time window (−1, +1 s) around each MUA (see Methods, **Figures 2D**, **E**). When calculating the average value of the LFP in the first 250 ms following a MUA, we observed a strong reduction for NLG3KO as compared to control mice for data recorded in the CA2 region (**Figure 2D**, right; −0.034 ± 0.001 mV and −0.006 ± 0.006 mV in control and NLG3 KO, respectively; $p = 0.02$, two-sided Wilcoxon rank sum test). In CA3 recordings, we observed a tendency to weaker oscillations (although not significant) around the MUA, (**Figure 2E**; −0.014 ± 0.013 mV and 0.02 ± 0.007 mV in control and NLG3 KO mice, respectively; $p = 0.4$, two-sided Wilcoxon rank sum test). These results clearly demonstrate that the lack of NLG3 selectively

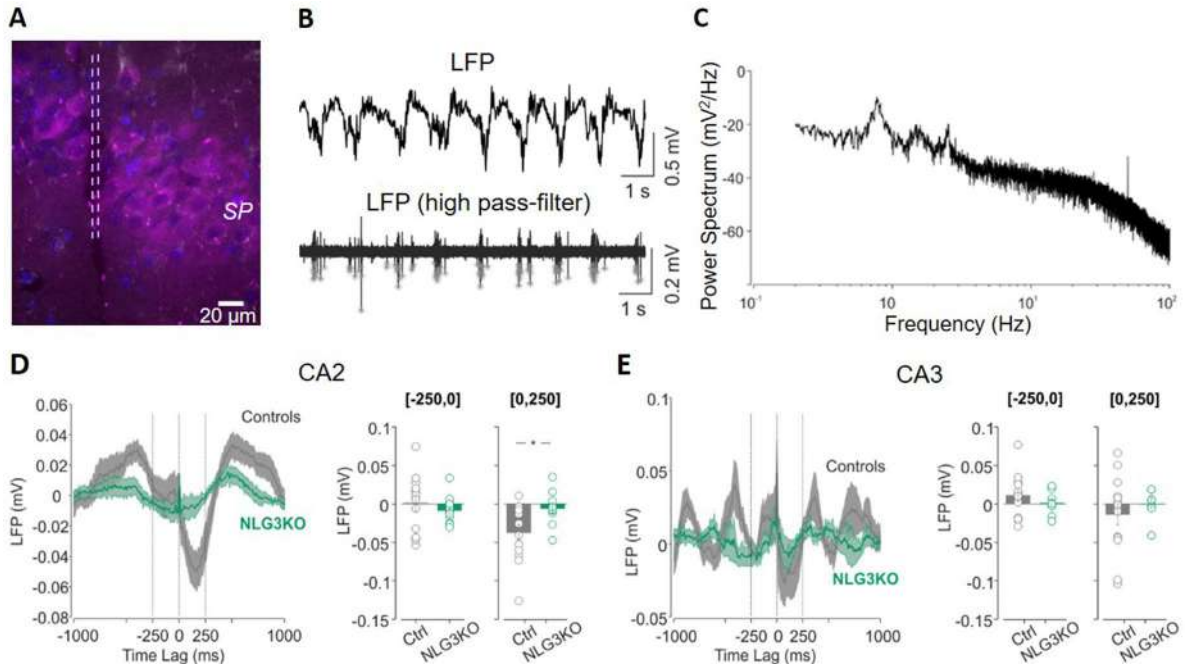

FIGURE 2 | Decreased spike-triggered local field potential (LFP) in the CA2 hippocampal region of NLG3 KO mice. **(A)** Confocal image showing the electrode track (lesion highlighted by dashed lines) in the CA2 pyramidal layer (SP) marked with anti-PCP4 antibody (magenta). **(B)** Samples traces of LFPs recorded in the CA2 region from an anesthetized animal, filtered at 0.2–50 Hz (top) and at 500 Hz (bottom). Gray stars show single action potentials obtained after high-pass filtering. **(C)** Representative power spectrum showing strong oscillations around 1 Hz, typical of anesthesia conditions. **(D)** Left: spike-triggered average of LFPs recorded in area CA2 from control (gray, $n = 12$) and NLG3 KO mice (green, $n = 12$). Average LFP value in the interval preceding (250–0 s, middle) and following (0–250 s, right) the occurrence of the spike. **(E)** Same as **(D)**, but for the CA3 region (control, $n = 14$; NLG3KO, $n = 8$). Bars are mean ± SEM.*$p < 0.05$, two-sided Wilcoxon rank sum test.

alters synchronization between action potentials and LFP in the CA2 area of the hippocampus.

Reduced Power of Theta and Gamma Oscillations in Hippocampal Subfields of NLG3 KO Mice

To dissect out possible alterations in neural oscillations, observed in several neurodevelopmental disorders (39, 40), raw LFP data were used to extract various bands occurring in the theta (5–14 Hz), low gamma (25–55 Hz), and high gamma (56–120 Hz) frequencies with Ensemble Empirical Mode Decomposition algorithm. While high-gamma oscillations are thought to contribute to memory encoding (41), low-gamma ones are believed to promote memory retrieval (42). To assess the average network activity, we measured power using the Welch Power Spectrum method and analyzed average relative power in individual LFP bands. In order to normalize for variations in raw LFP across animals and genotypes, we considered the relative power in each LFP band with respect to its corresponding raw LFP. This allowed us to compare normalized power across genotypes. We found a significant reduction in the average relative power of both low gamma and high gamma bands in the CA2 region of NLG3 KO mice as compared to controls (**Figures 3A**, **B**; theta: 0.045 ± 0.002 mV^2 and 0.033 ± 0.001 mV^2, $p = 0.1$; low gamma: 0.08 ± 0.003 mV^2 and 0.06 ± 0.001 mV^2, $p < 0.0001$; high gamma: 0.005 ± 0.0003 mV^2 and 0.002 ± 0.0002 mV^2, $p < 0.0001$ in controls and in NLG3 KO, respectively; Mann–Whitney test). Interestingly, also the CA3 region of NLG3 KO mice showed a reduced power in gamma as well as in theta bands (**Figures 3C**, **D**; theta: 0.057 ± 0.002 mV^2 and 0.038 ± 0.002 mV^2, $p < 0.0001$; low gamma: 0.11 ± 0.005 mV^2 and 0.068 ± 0.003 mV^2, $p < 0.0001$; high gamma: 0.008 ± 0.0006 mV^2 and 0.003 ± 0.0002 mV^2, $p < 0.0001$ in controls and in NLG3 KO, respectively; Mann–Whitney test). To assess whether the reduction in power relied on alterations in band occurrence frequency, the latter was quantified for each LFP bands in both CA2 and CA3 hippocampal regions. We found a significant increase in band occurrence frequency of high gamma band in both CA2 and CA3 regions of NLG3 KO mice as compared to controls. No significant alterations in band occurrence frequency of theta and low gamma rhythms of either regions were observed (**Figures S3A and B**; theta: 2.5 ± 0.16 Hz and 0.3 ± 0.12 Hz, $p = 0.17$; low gamma: 11.01 ± 0.17 Hz and 10.5 ± 0.2 Hz, $p = 0.06$; high gamma: 22.0 ± 1.1 Hz and 25.4 ± 0.7 Hz, $p = 0.01$ in controls and in NLG3 KO mice, respectively; Mann–Whitney test). These data suggest that the occurrence frequency of theta and low gamma are not responsible for reduction in power in theta and low gamma bands observed in NLG3 KO mice. To understand how theta and gamma power vary in CA3 and CA2 regions before and after a spike, we extracted spikes from raw LFP using the WaveClus, an unsupervised spike-sorting algorithm. Spike

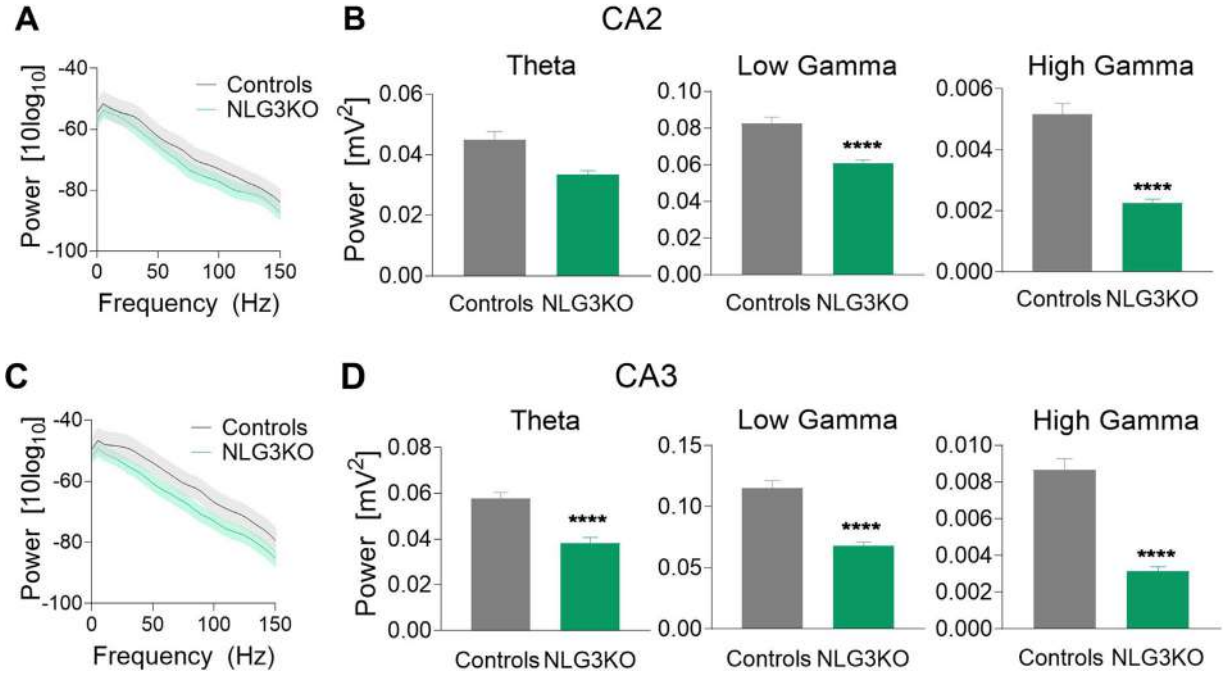

FIGURE 3 | Reduced power in the theta and gamma frequency bands in NLG3 KO mice. **(A)** Welch's Power spectrum (1–150 Hz) of LFP recorded from the CA2 region of anesthetized control (gray) and NLG3KO (green) mice (shaded areas: SEM). **(B)** Summary plots of relative LFP power in the theta (5–14 Hz, left), the low gamma (25–55 Hz, middle), and high gamma (56–120 Hz, right) bands in controls (gray, n = 17) and in NLG3 KO mice (green, n = 14). **(C, D)** Same as A, B but for the CA3 area (control, n = 15; NLG3KO, n = 11). Bars are mean ± SEM.****$p < 0.0001$, Mann–Whitney test.

times were extracted, and LFP power, in and between theta and gamma bands, were analyzed 1 s before and after a spike (**Figure 4**). We found a significant reduction in power before spikes in theta and fast gamma bands in the CA2 region of NLG3 KO mice as compared to controls (**Figure 4A**; theta: 0.04 ± 0.002 mV^2 and 0.03 ± 0.0002 mV^2, $p < 0.0001$; low gamma: 0.1 ± 0.01 mV^2 and 0.07 ± 0.005 mV^2, $p = 0.019$; high gamma: 0.004 ± 0.0006 mV^2 and 0.002 ± 0.0004 mV^2, $p < 0.0001$ in controls and in NLG3 KO, respectively; Mann–Whitney test). In agreement with the reduction in power in both theta and gamma bands of NLG3 KO, we also found a significant decrease in power before spikes in all bands of CA3 region in NLG3 KO mice (**Figure 4B**; theta: 0.05 ± 0.0004 mV^2 and 0.02 ± 0.0001 mV^2, $p < 0.0001$; low gamma: 0.1 ± 0.0006 mV^2 and 0.05 ± 0.0001 mV^2, $p < 0.0001$; high gamma: 0.006 ± 0.0001 mV^2 and 0.002 ± 8.6e^{-5} mV^2, $p < 0.0001$ in controls and in NLG3 KO respectively; Mann–Whitney test) suggesting altered dynamics in both CA2 and CA3 network activities.

Increased Bursting Activity in *In Vivo* Recordings From CA2 Principal Cells of NLG3 KO Mice

To understand whether alterations of network activity observed in the CA2 and CA3 hippocampal regions of NLG3 KO mice were associated to changes in their output, single neurons firing was recorded in *in vivo* anesthetized animals. Based on spontaneous firing frequency, firing mode and spike waveform, two populations of neurons could be clearly recognized in juxtacellular recordings from single CA2 neurons, corresponding to putative interneurons and pyramidal cells, respectively (43). While interneurons preferentially fire narrow action potentials at high rate, principal cells fire single action potentials intermixed with short duration bursts. Since interneurons are highly heterogeneous, we restricted the comparison between NLG3 KO and control mice to bursting neurons. The spontaneous firing [monitored for 5–10 min (**Figure 5A**)] and spike properties (i.e., half width, firing rate, bursting behavior) were evaluated in both controls and NLG3 KO mice. Similar half width were observed in CA2 neurons recorded from NLG3 KO and control mice suggesting that we targeted the same population of neurons (**Figure 5B**; NLG3 KO: 0.33 ± 0.3 ms and 0.3 ± 0.02 ms in controls and NLG3 KO mice, respectively; $p = 0.59$, Mann–Whitney test). A significant increase in the frequencies of total spikes (within and out of burst, **Figure 5C**, left; 0.97 ± 0.3 Hz and 3.6 ± 0.9 Hz in controls and NLG3 KO mice, respectively; $p = 0.01$, Mann–Whitney test), single spikes (**Figure 5C**, middle; 0.4 ± 0.1 Hz and 1.9 ± 0.6 Hz, in controls and NLG3 KO mice, respectively; $p = 0.007$, Mann–Whitney test), and bursts (**Figure 5C**, right; 0.2 ± 0.06 Hz and 0.98 ± 0.2 Hz in controls and NLG3 KO mice, respectively; $p = 0.007$, Mann–Whitney test) were observed in NLG3 KO mice as compared to controls. No differences in spike frequency within single bursts were observed (controls: 92.4 ± 8.8 Hz; NLG3 KO: 74.3 ± 12 Hz; $p = 0.38$, Mann–Whitney test). Similar half widths were observed in CA3 neurons recorded from NLG3 KO and control mice suggesting that we targeted the same population of neurons (**Figures 5D**, **E**; controls: 0.33 ± 0.3 ms;

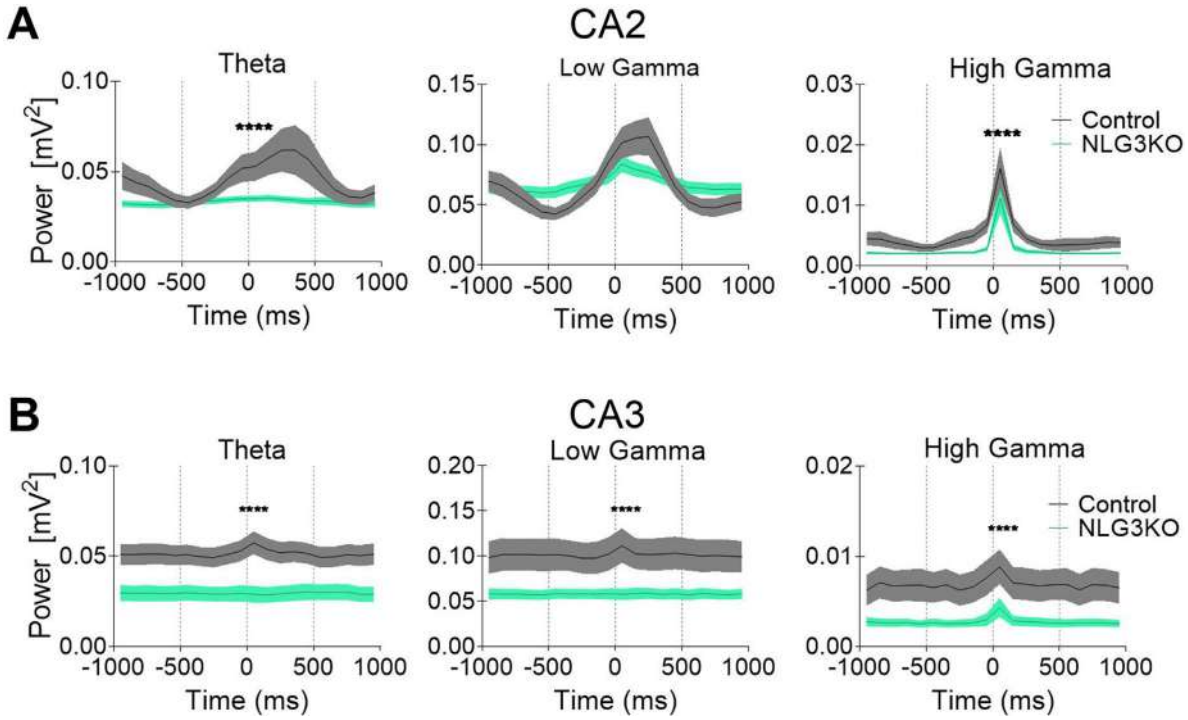

FIGURE 4 | Reduced power in the theta and gamma frequency bands during spikes in NLG3 KO mice. **(A)** Summary plots showing relative power at theta (left), low gamma (middle), and high gamma (right) bands in the interval preceding (–1 s) and following (1 s) the occurrence of a spike in control (gray, n = 15535) and in NLG3 KO mice (green, n = 26092) in the CA2 region. **(B)** Same as A but for the CA3 region (control, n = 34804) and in NLG3 KO, n = 10875). N = spikes extracted from LFP, shaded areas: SEM.****$p < 0.0001$, Mann–Whitney test.

NLG3 KO: 0.3 ± 0.02 ms; p = 0.59, Mann–Whitney test). Interestingly, no changes in the firing frequency of CA3 bursting neurons were observed (**Figures 5D**, **F**) indicating that the lack of NLG3 causes a selective enhancement of neuronal excitability in the CA2 hippocampal area, known to process information relevant for social cognition.

An Excitatory/Inhibitory Imbalance May Account for Altered Network Activity in the CA2 Region of the Hippocampus of NLG3 KO Mice

To understand whether the enhanced firing rate could be related to an excitatory/inhibitory imbalance within the CA2 hippocampal region, spontaneous excitatory and inhibitory postsynaptic currents mediated by AMPA and $GABA_A$ receptors, respectively, were recorded in *ex vivo* slices obtained from controls and NLG3 KO mice.

A significant increase in frequency (**Figures 6A**, **B**; controls: 0.17 ± 0.05 Hz and NLG3 KO: 0.6 ± 0.13 Hz; p = 0.017, Mann–Whitney test) but not in amplitude (**Figures 6A**, **C**; controls: 33 ± 3.8 pA, NLG3 KO 30.1 ± 7 pA; p = 0.25, Mann–Whitney test) of sEPSCs, recorded from CA2 principal cells in the presence of picrotoxin, was found in NLG3 KO as compared to controls. This effect was associated to a significant decrease in frequency (but not in amplitude) of sIPSCs recorded in the presence of CNQX and DL-APV (**Figures 6D**, **E**; controls: 10 ± 1.8 Hz, NLG3 KO: 5.4 ± 0.4 Hz; p = 0.03, Mann–Whitney test) but not in amplitude (**Figures 6D–F**; controls: 53.6 ± 13.6 pA, NLG3 KO: 32.4 ± 4.3 pA; p = 0.13, Mann–Whitney test).

These results strongly suggest that an excitatory/inhibitory imbalance within the CA2 hippocampal region may be instrumental for the *in vivo* dynamic changes observed in NLG3 KO mice.

Impairment of Perisomatic Inhibition Mediated by GABAergic Interneurons Expressing CCK in NLG3 KO Mice

To determine whether alterations in network oscillations observed in *in vivo* and in *ex vivo* recordings from NLG3 KO mice are due to impairments of perisomatic inhibition, which efficiently suppresses repetitive sodium-dependent action potentials (44) and entrains network oscillations (45), inhibitory synaptic currents evoked in CA2 pyramidal cells by stimulation of GABAergic inputs were analyzed. Stimulation of GABAergic inputs, with an intensity equal to 60% of that necessary to evoke a maximal response, induced monosynaptic IPSCs. IPSCs showed similar rise (controls: 4.89 ± 2 ms, NLG3 KO: 3.3 ± 1.6 ms; p = 0.6, Mann–Whitney test) and decay times (controls: 31.1 ± 2.4 ms, NLG3 KO: 26.2 ± 3.7 ms; p = 0.2, Mann–Whitney test) in both genotypes.

GABAergic terminals present in the pyramidal layer belong mainly to CCK+ and PV+ GABAergic interneurons

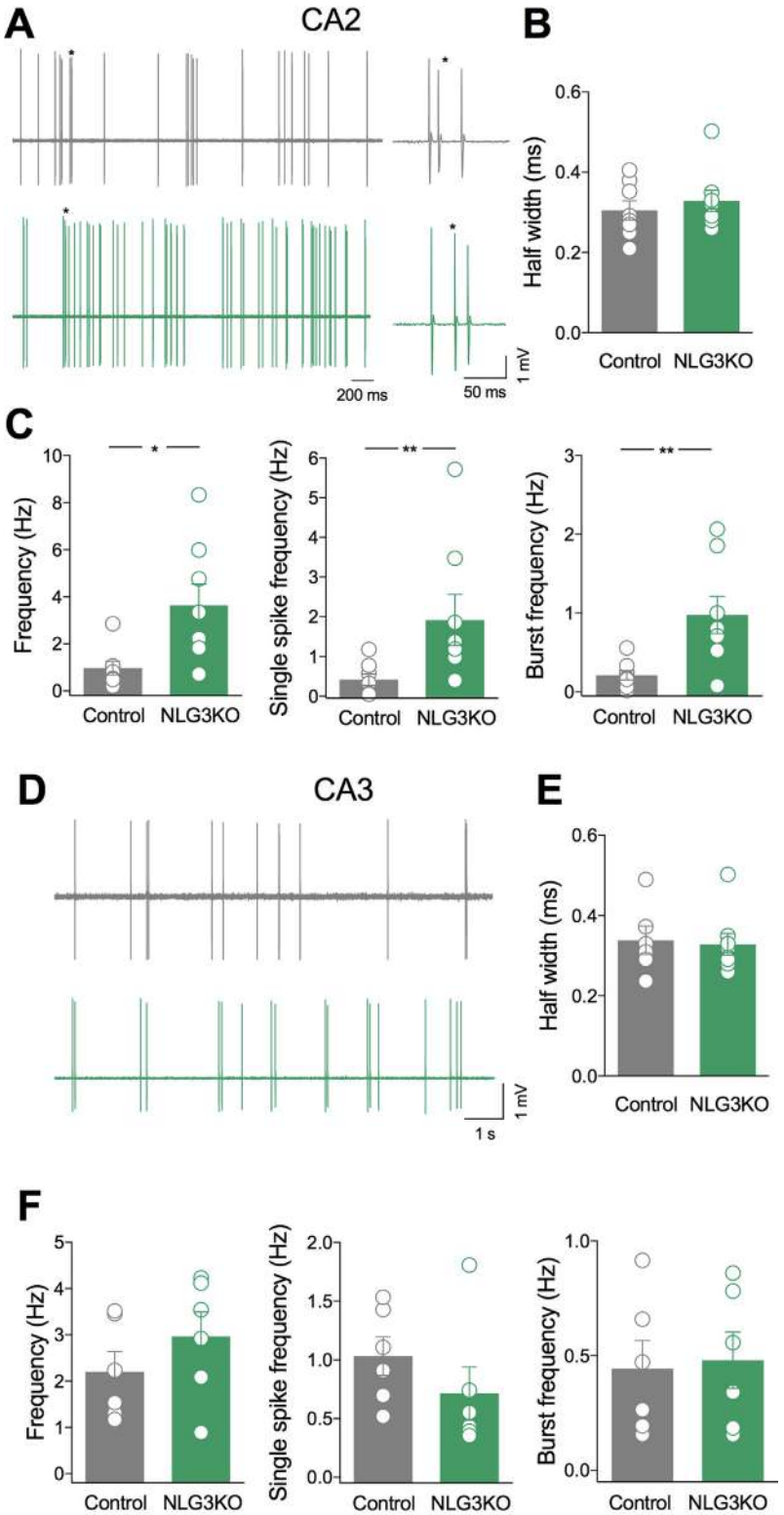

FIGURE 5 | Selective enhancement of CA2 principal cells firing in NLG3 KO mice. **(A)** Sample traces of bursting neurons from a control (gray) and a NLG3 KO mouse (green) in the CA2 region. On the right individual bursts showed on an expanded time window (asterisks) **(B–C)** Scatter plots showing half spike width **(B)**, total, single spike frequency and burst frequency **(C)** of CA2 principal cells in control (gray, $n = 8$) and in NLG3 KO mice (green, $n = 8$). $^{*}p < 0.02$; $^{**}p < 0.001$, Mann–Whitney test). **(D)** Sample traces of bursting neurons from a control (gray) and a NLG3 KO mouse (green) in the CA3 region. **(E–F)** Scatter plots showing half spike width **(E)**, total single spike frequency and burst frequency **(F)** of CA3 principal cells in control (gray, $n = 6$) and in NLG3 KO mice (green, $n = 6$). Open circles represent values from single animals, and bars are mean ± SEM.

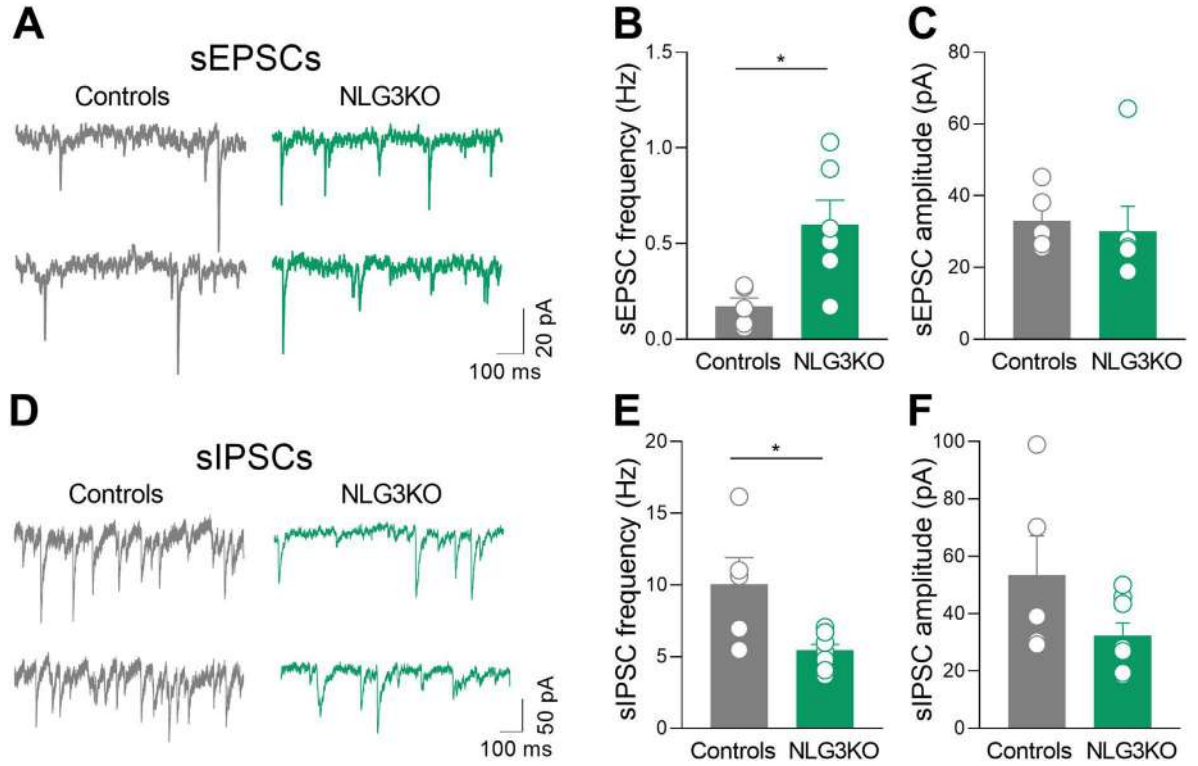

FIGURE 6 | Enhanced excitatory and reduced inhibitory postsynaptic transmission in the CA2 region of NLG3 KO mice. **(A)** Sample traces showing sEPSCs from CA2 pyramidal neurons in hippocampal slices obtained from a control (gray) and a NLG3 KO mouse (green). **(B, C)** Scatter plots showing the mean frequency **(B)** and amplitude **(C)** of sEPSC recorded from CA2 pyramidal neurons in control (gray, $n = 6$) and in NLG3 KO mice (green, $n = 5$). Bars represent SEM; open circles represent values from single cells. **(D)** Sample traces showing sIPSCs from CA2 pyramidal neurons in hippocampal slices obtained from a control (gray) and a NLG3 KO mouse (green). **(E, F)** Scatter plots showing the mean frequency **(E)** and amplitude **(F)** of sIPSC recorded from CA2 pyramidal neurons in control (gray, $n = 6$) and in NLG3 KO mice (green, $n = 5$). Bars represent SEM. Open circles represent values from single cells. *$p < 0.05$, Mann–Whitney test.

(46). Interestingly, GABA release from these two interneuron subtypes is triggered by calcium entry *via* different voltage-gated calcium channels, N types, and P/Q, respectively, sensitive to different toxins. While the release of GABA from CCK+ interneurons is selectively blocked by ω-Ctx-GVIA, that from PV+ ones is selectively blocked by ω-Agtx-IVA (25, 26, 47). Taking advantage of these two toxins, we found that while the CCK component of the evoked IPSC (sensitive to ω-Ctx-GVIA, 1 μM) was strongly reduced in NLG3 KO mice as compared to controls (**Figure 7A**, **B**, controls: 58.2 ± 5.5%, NLG3 KO: 21 ± 4.8%; $p = 0.0003$, Mann–Whitney test), the PV component (sensitive to ω-Agtx-IVA, 300 nM) was unaltered in both genotypes (**Figures 7C**, **D**; controls: 39.8 ± 21%, NLG3 KO: 34 ± 11%; $p = 0.9$, Mann–Whitney test).

To study whether the lack of NLG3 alters inhibitory short-term plasticity, GABAergic interneurons were stimulated with brief train of five stimuli at 20 Hz. As previously shown for somatic targeting GABAergic synapses (48) this stimulation paradigm led to a similar short-term depression in both NLG3 KO and control mice (**Figure S4**, ratio last/first response, controls: 0.48 ± 0.08, NLG3 KO: 0.28 ± 0.04; $p = 0.1$, Mann–Whitney test), indicating that the lack of NLG3 does not affect GABAergic short-term plasticity. As expected, application of ω-Ctx-GVIA strongly reduced the amplitude of IPSCs, without altering in both genotypes the degree of depression of the remaining components (**Figure S4**, ratio last/first response; controls: 0.58 ± 0.2, NLG3 KO: 0.3 ± 0.1; $p = 0.65$, Mann–Whitney test). These data clearly indicate that deficits in basal GABAergic signaling *via* CCK+ interneurons contribute to enhance cell excitability and to alter network dynamics in the CA2 region of the hippocampus.

Loss of NLG3 Does Not Significantly Affect the CCK- and PV-Mediated Innervation of the CA2 Hippocampal Region

To assess whether a reduction in CCK innervation could play a role in the decreased CCK-mediated current observed in NLG3 KO mice, we performed immunohistochemical analysis of CCK terminals in the CA2 pyramidal layer using a CCK marker (49). Although a tendency toward a reduction of CCK positive puncta in NLG3 KO mice was observed as compared to controls (**Figures 8A–C**; controls: 61.6 ± 7.3, NLG3KO: 49.8 ± 8.8; $p = 0.07$, Mann–Whitney test), this did not reach statistical significance. This result suggests that a reduced probability of GABA release from CCK terminals is mainly responsible for the reduced amplitude of CCK-mediated current in mice lacking NLG3. In agreement with electrophysiological data, no differences in the number of PV

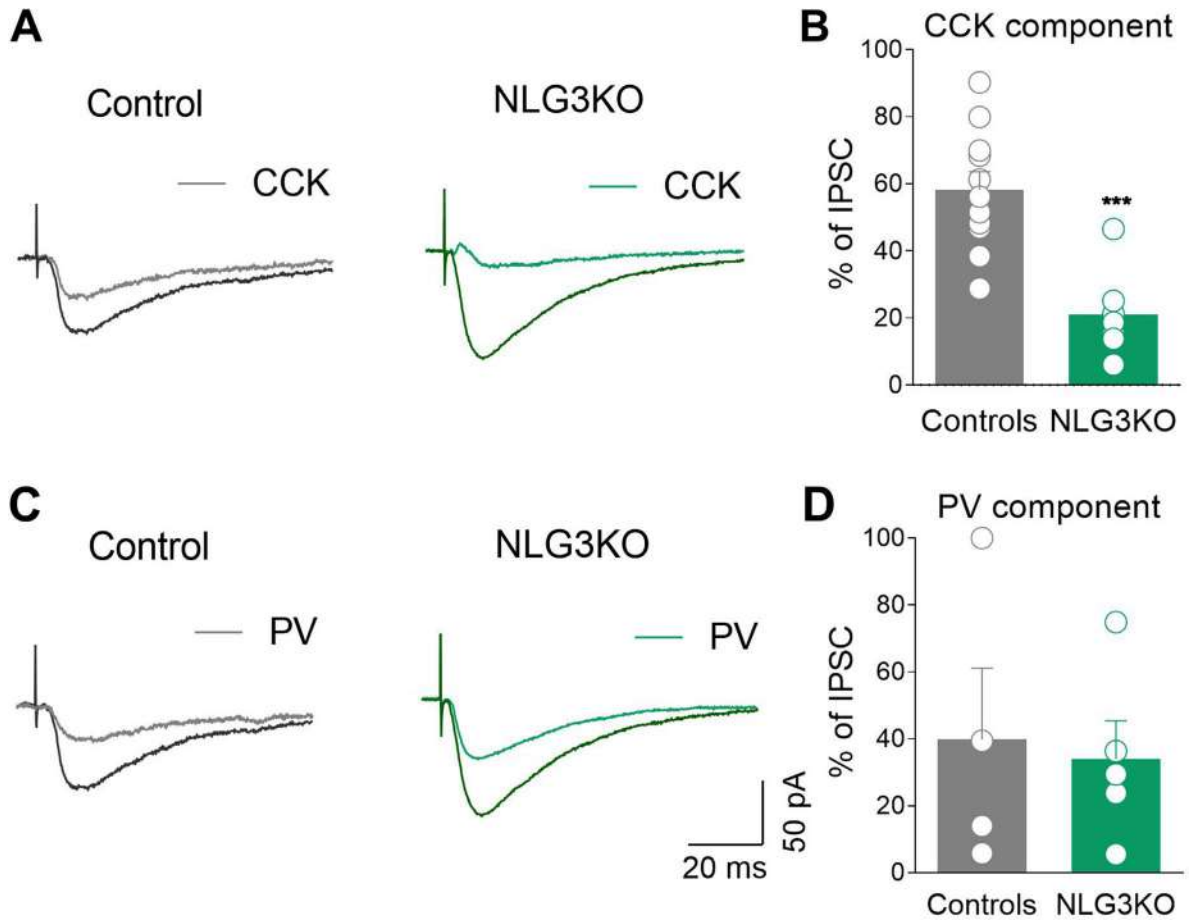

FIGURE 7 | Reduced CCK-mediated inhibition in CA2 region of NLG3 KO mice. **(A)** Sample traces of IPSCs evoked in CA2 pyramidal neurons by stimulation of GABAergic inputs in the pyramidal layer in control and in NLG3 KO mice before (black and dark green, respectively) and after (gray and light green, respectively) application of ω-Ctx-GVIA 1 μM. **(B)** Summary plot showing the percentage amplitude of CCK-mediated responses (ω-Ctx-sensitive currents) in control (gray, $n = 11$) and in NLG3 KO mice (green, $n = 7$). **(C)** Sample traces of IPSCs evoked in CA2 pyramidal neurons by stimulation of GABAergic inputs in the pyramidal layer in control and in NLG3 KO mouse before (black and dark green, respectively) and after (gray and light green, respectively) application ω-Agtx-IVA 300 nM. **(D)** Summary plot showing the percentage amplitude of PV-mediated responses (ω-Agtx-IVA-sensitive currents) in control (gray, $n = 4$) and in NLG3 KO mice (green, $n = 5$). Open circles represent values from single cells, and bars are mean ± SEM. ***$p = 0.0003$; Mann–Whitney test.

positive puncta measured in the principal layer of CA2 region were observed (**Figures 8D–F**; controls: 63.5 ± 12, NLG3KO: 64.7 ± 11; $p = 0.6$, Mann–Whitney test).

DISCUSSION

Here we provide evidence that the NLG3 KO mouse, an animal model of non-syndromic form of autism, exhibits clear alterations in network dynamics in the CA2 region of the hippocampus, probably at the origin of social memory defects reminiscent of those found in autistic patients. In agreement with previous studies (17, 38), NLG3-deficient mice did not show any preference for the novel mouse in the social novelty test, suggesting an impairment in the capacity to remember a conspecific, an effect associated to an enhanced exploratory behavior in the open field. However, in contrast with Radyushkin et al., (17), NLG3 KO mice exhibited clear sociability deficits as assessed by the reduced time in exploring the animal as compared to their control littermates and by the lack of preference for the animal *versus* the object. This discrepancy may be due to the fact that in contrast with Radyushkin et al., (17), who have used as controls age-matched C57Bl/6 male, we have employed littermates reared, as NLG3 KO, in the same cage, a condition known to critically affect, through peers interactions, the acquisition of social behavior (50).

To identify the circuitry responsible for social deficits in ASDs represents a key step for understanding the pathophysiology of these disorders. Several brain areas encode social behavior, including the medial prefrontal cortex (51), the basolateral amygdala (52), and the CA2 region of the hippocampus (20, 53). Our *in vivo* experiments from NLG3 KO mice clearly demonstrate selective changes in the amplitude of slow waves preceding or following the occurrence of spikes (spike-triggered average or STA). These modifications reflect alterations in electrophysiological signals produced by large neuronal ensembles in that specific region of

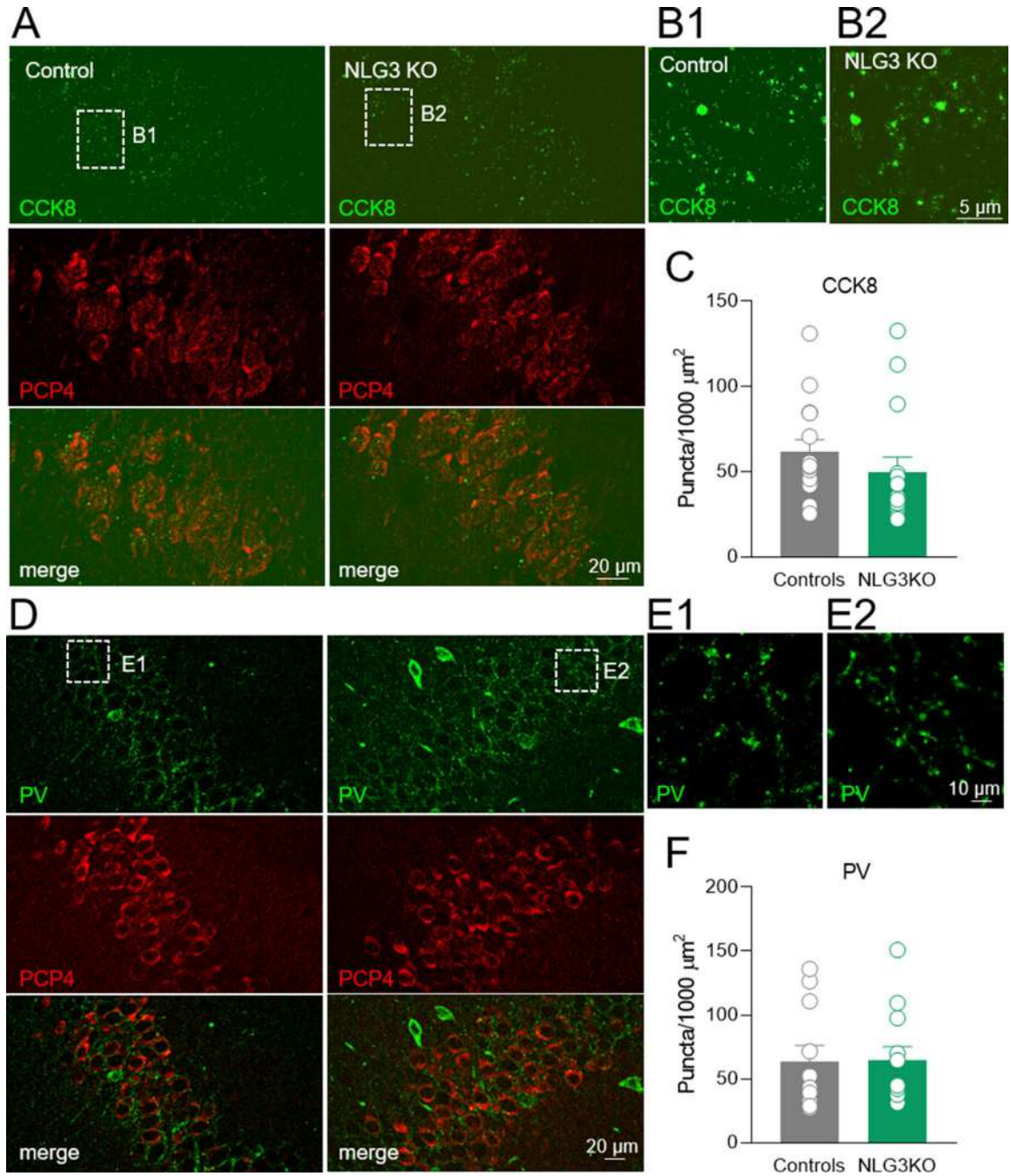

FIGURE 8 | Loss of NLG3 does not significantly affect GABAergic innervation from CCK and PV positive interneurons in the CA2 hippocampal region. **(A)** Double immunostaining labeling the presynaptic CCK terminals (CCK8) and CA2 principal neuron (PCP4) in control and NLG3 KO mice. **(B1–B2)** insets of **(A)** showing higher magnification of CCK positive terminals in control and NLG3 KO mice. **(C)** Summary plot showing the number of CCK positive puncta in 1,000 µm^2 (n = 15 from three animals per genotype). Open circles represent values from CA2 hippocampal single section analyzed, and bars are mean ± SEM. p = 0.07; Mann–Whitney test. **(D–F)** as for **(A–C)** but for PV positive interneurons (n = 11 from three controls and n = 12 from three NLG3 KO mice).

that particular genotype and therefore cannot be attributed to the anesthetic used. LFPs represent summed synaptic activity located in small volume around the recording site, related to the strength of functional connectivity between neurons (54). Consistent with STA data, the observed modifications in theta and gamma power probably reflect aberrant functional connectivity between the hippocampus and associated networks (55–57). However, while STA defects were confined to the CA2 region of the hippocampus, changes in theta and gamma power occurred in both CA2 and CA3 hippocampal areas. This is in accord with the recent observation that both CA2 and CA3 hippocampal regions contribute to generate gamma oscillations

(58). Changes in theta and gamma power have been often found in the EEG/MEG of autistic children during their resting state (59–63) or during sustained attention (64). Interestingly, as in the present experiments, a strong reduction in gamma power associated with a dysfunction of GABAergic signaling was detected in the CA3 region of the hippocampus of mice lacking the adhesion molecule NLG4, exhibiting behavioral deficits reminiscent of those found in ASDs (65). However, unlike the present experiments, in NLG4 knock-out mice the oscillatory activity pharmacologically induced by kainate *in vitro* was highly dependent on perisomatic inhibition generated by PV+ interneurons.

As in the present experiments, abnormal oscillatory activity may arise from elevation in the cellular balance of excitation and inhibition, *via* an increased excitatory and a decreased inhibitory activity (66). The E/I balance represents a critical condition for the correct functioning of neuronal networks, and it is essential for nearly all brain functions, including representation of sensory information and cognitive processes (67). In the present study, the E/I imbalance accounts for the selective enhancement of cell excitability recorded in *in vivo* experiments from the CA2 hippocampal region. It is commonly accepted that network oscillations generated by the interplay between excitation and inhibition involve the activity of PV+ interneurons mediating the feedforward and feedback inhibition (68). Unexpectedly, a more detailed analysis into perisomatic inhibition, using toxins known to affect presynaptic calcium rise *via* VDCC, has unveiled a clear reduction in the probability of GABA release from CCK+ interneurons in the CA2 hippocampal region. Interestingly, in contrast with the present data, a detailed analysis of unitary inhibitory postsynaptic currents between CCK+ interneurons and principal cells in the CA1 region of the hippocampus revealed an increased probability of GABA release probably consequent to impairment of a tonic endocannabinoid signaling (23), suggesting that, in mice lacking NLG3, changes in GABAergic signaling are cell and circuit specific. Although PV+ and CCK+ interneurons target the same domains of pyramidal cells, the functional role of the latter in network synchronization is still unclear. By comparing, in anesthetized rats, spike time during oscillations, Klausberger et al. (69) suggest that these interneuron subtypes contribute differently to network activity. While PV expressing interneurons provide more stereotyped oscillatory entrainment of the entire network, CCK+ interneurons might shape the activity of subgroups of principal cells forming neuronal assemblies.

In conclusion, our data from NLG3 mice strongly suggest that alterations in CA2 network dynamics may account for the behavioral deficits, similar to those observed in autistic children. It should be stressed however that, while social memory is likely to be processed by the CA2 region of the hippocampus, sociability might involve other brain structures including the basolateral complex of the amygdala, which projects to the ventral hippocampus (52).

Whatever the origin of behavioral deficits reported here, our results further highlight the crucial role of an altered $GABA_A$-mediated neurotransmission in neurodevelopmental disorders (67, 70). Thus, interfering with GABAergic signaling may be considered as a key strategy for the treatment of some forms of ASDs that involve genetic modification/deletions of synaptic proteins.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and the supplementary files.

ETHICS STATEMENT

This study was carried out in accordance with principles of Basel declaration and recommendations of Italian Animal Welfare legislation (D.L. 26/2014) that implemented the European Committee Council Directive (2010/63 EEC). The protocol was approved by local veterinary authorities, the EBRI ethical committee and the Italian Ministry of Health (authorization number: 1084/PR15).

AUTHOR CONTRIBUTIONS

MG and EC designed the research with the contribution of all authors. DP performed behavioral experiments. BM and DP performed slice electrophysiology. BM and AP performed analysis of *in vivo* data. MG performed *in vivo* electrophysiology and immunohistochemistry. PZ performed WB. All authors participated in the interpretation of data. EC and MG wrote the manuscript with comments and contributions from all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2019.00513/full#supplementary-material

FIGURE S1 | Lack of NLG3 protein in the hippocampus of NLG3 KO mice. Immunoblot analysis of NLG3 expression in the hippocampus of controls and NLG3 KO mice. Western blot detecting actin was used as loading control.

FIGURE S2 | Enhanced locomotor activity in NLG3 KO mice. **(A)** Examples of track plots showing locomotor activity during the open field task in a control (left) and in a NLG3 KO mouse (right). **(B)** Scatter plots showing the total distance traveled by control (gray, n = 10) and NLG3 KO mice (green, n = 9). **(C)** Scatter plot showing the velocity in control (gray, n = 10) and in NLG3 KO mice (green, n = 9). **(D)** Scatter plots showing the time spent by control (gray, n = 10) and NLG3 KO mice (green, n = 9) in the inner and outer zones, in the open field test. Open circles represent values from single animals, and bars are mean ± SEM.

FIGURE S3 | Enhanced high gamma occurrence in NLG3 KO mice. **(A)** Summary plots showing theta (left), low gamma (middle), and high gamma (right) occurrence frequency in the CA2 region of control (gray, n = 17) and NLG3 KO mice (green, n = 14). **(B)** Same as **(A)** but for the CA3 area (control: n = 15; NLG3 KO, n = 11). Bars are SEM. $^*p < 0.05$, Mann-Whitney test.

FIGURE S4 | No changes in the short-term GABAergic plasticity in NLG3 KO mice. **(A)** Sample traces of five consecutive IPSCs evoked at 20 Hz in CA2 pyramidal cells by stimulation of GABAergic inputs in the pyramidal layer in control and in NLG3 KO mice before (grey and green, respectively) and after (black) application of ω-Ctx-GVIA 1 μM. **(B)** Scatter plots showing mean IPSC amplitudes (± SEM) evoked at 20 Hz in CA2 principal cells in control (n = 5) and in NLG3 KO mice ($n = 4$) before (grey and green, respectively) and after (black) application of ω-Ctx-GVIA 1 μM. Open circles are mean ± SEM.

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BRIEF RESEARCH REPORT
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Application of the Scale for the Assessment of Feeding Interaction (SVIA) to Children With Autism Spectrum Disorder

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Background and Objectives: Feeding problems occur more frequently among children with Autism spectrum disorder (ASD). The aim of this study was to analyse eating difficulties of ASD children through the direct observation of the caregiver-child co-regulation system.

Methods: We compared 60 ASD children with a control group of 50 typically developing Italian children on the Scale for the Assessment of Feeding Interaction (SVIA). The Brief Autism Mealtime Behaviour Inventory (BAMBI) was used to define the presence of an eating disorder.

Results: The ASD group showed higher scores on all dimensions of the SVIA compared to the control group. The SVIA and the BAMBI showed significant correlations. In a second step, the ASD sample was divided into two subgroups, children with and without feeding difficulties. The comparison between the ASD subgroups with the control group on the SVIA scales showed significant differences on all dimensions. Finally, significant differences emerged between the two ASD subgroups in three SVIA dimensions.

Conclusion: These data suggest the importance of direct observation of feeding in the assessment of children with ASD. The SVIA seems to be able to point out some feeding difficulties in these subjects and to discriminate ASD with and without an eating disorder. Critical aspects of the application of SVIA to autistic children are discussed.

Keywords: autism spectrum disorder, feeding disorder, mother-child co-regulation, direct observation, scale for the assessment of feeding interaction, brief autism mealtime behavior inventory

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INTRODUCTION

In early childhood, feeding is a precious moment of interaction (1). During feeding, children begin to recognise signals in their social (i.e., external) environment (vocalisations, glances, gestures, mimicking facial expressions) and internal signals of hunger/satiety (2–4). Studies on breastfeeding sequences reveal the intersubjective nature of nutrition: when an infant temporarily stops sucking, mothers use this break to talk to or touch him. This turn-taking—characterising early feeding interactions—is considered the first form of 'dialogue' between adult and infant (5).

In families with typically developing children, mealtimes are an opportunity to structure daily routines, which support social learning. Children spontaneously observe and imitate the actions of adults, who, in turn, adapt their actions and language to facilitate the child's learning (6). This caregiver-child regulation system lays the foundation for future social skills (7–9), subsequent self-regulation skills (10) and is recognised as one of the predictors of developmental outcomes (1, 11, 12).

Autism spectrum disorders (ASDs) are a group of neurodevelopmental disorders that include a wide range of complex developmental disabilities. These include impairments in social interactions, difficulties with language and communication, repetitive and ritualised behaviours, restricted interests, behavioural inflexibility and impaired sensory processing (13). In particular, children with ASD tend be less accurate with identification of facial expressions (14) and they typically display problems with the quality and quantity of joint attention (15), besides presenting reduced eye contact and poor integration of eye contact with verbal and non-verbal communication (16). Children with ASD also can show a limited or immature motor repertoire and significant imitation deficits (17, 18). They often present characteristics including stereotyped movements, adherence to routines, resistance to change and intense preoccupations (13).

Early in life, in subjects with ASD, co-regulation processes between mother and child can be affected. Hirschler-Guttenberg et al. (19) analysed how children with ASD regulate both positive and negative emotions during free play with their mothers at home by micro-coding the behaviour of children. Observations indicated that pre-schoolers with ASD were less socially engaged and less compliant than typically developing peers. Similarly, a recent study by Guo et al. (20) found that mother-infant with ASD-dyads pass more time in mismatched emotion-engagement states (e.g., child negative/mother positive). Furthermore, children with ASD spent more time engaged exclusively with objects than children without ASD.

The caregiver–child relationship at mealtime has been studied mainly in the context of eating disorders or other feeding-related risk factors (21–27). To date, no studies have been conducted that examine the feeding relationships of children affected by ASD. The feeding of subjects with ASD has mainly been investigated through indirect observation methods (28). These studies have highlighted the strong association between feeding difficulties and autism. Feeding problems occur more frequently among children with ASD than typically developing children (29) or children with other disabilities (30). Food selectivity is the most common feeding disorder among the ASD population. These children show high rates of food refusal, a high frequency of singular food intake and a limited repertoire of accepted foods, with a tendency to maintain food restrictions over time (31, 32). Selectivity concerns sensory characteristics of foods (flavour, odour, colour, texture and temperature) (29, 33, 34). Additionally, children with ASD have been shown to have many eating rituals (32, 35, 36), as well as aggressive mealtime behaviours (37). Ritualistic and repetitive patterns of behaviour are commonly believed to contribute to food selectivity (32).

The new *Diagnostic and Statistical Manual of Mental Disorders*—5th edition (DSM-5) diagnostic criteria for avoidant/restrictive food intake disorder (ARFID), a childhood feeding disorder, encompasses some of the problems seen among children with ASD. The mechanisms of eating/feeding disorders in populations with ASD differ from those reported in children with feeding disorders without ASD; research has only begun to explore this difference. The meta-analysis by Sharp et al. (38) recommended including an assessment of feeding problems as part of routine medical evaluations, as well as developing assessment methods to identify empirically supported treatments for feeding problems in ASD.

The causes of eating/feeding problems in ASD are complex and include a combination of biological and environmental factors (32). Atypical feeding behaviours can impact the family feeding process and influence how families are forced to organise their mealtimes. For families of children with ASD, mealtimes can be overly focused on meal preparation and the sensory experiences of the child, which significantly limits opportunities for meaningful shared experiences (39). Therefore, the need exists to study ASD feeding disorders through direct observation methods, with a focus on the dyadic co-regulation system.

The aim of the present study was to analyse eating difficulties of children with ASD through direct observations of the caregiver-child co-regulation system. We compared ASD children with a typically developing Italian control group on the Scale for the Assessment of Feeding Interaction (SVIA), a measure of interactive behaviours that identifies relational models between parents and children during feeding sessions (Ammaniti M., Lucarelli L., Cimino S., D'Olimpio F. Scala di valutazione dell'interazione alimentare madre-bambino – SVIA; unpublished manuscript). We hypothesised that the clinical group would show higher scores on all the dimensions of the SVIA compared tothe control group. Moreover, in the ASD group, we analysed the relation between the SVIA and the Brief Autism Mealtime Behaviour Inventory (BAMBI), a self-reporting measure for the evaluation of mealtime behavioural problems in ASD children (40).

In a second step, we divided the ASD group into two subgroups, a subgroup with eating difficulties and a subgroup without eating difficulties, as assessed by the BAMBI. We hypothesised higher scores on the SVIA in both ASD groups with and without eating disorderscompared to the control group. Additionally, we hypothesised that ASD children with an eating disorder would show higher scores on the SVIA dimensions than ASD children without an eating disorder.

MATERIALS AND METHODS

Participants

The participants included 60 families with children diagnosed with ASD or with a "high risk" of autism and 50 families of typically developing children.

The ASD sample was recruited from the Child Neuropsychiatry Unit of the Policlinico Umberto I, in Rome, Italy. The inclusion criteria in the study were: be affected by ASD following the

DSM-5 criteria and be between 18 and 48 months of age. Exclusion criteria were: the presence of a known genetic disorder (e.g., Rett syndrome, Fragile X syndrome, Down syndrome), a medical disorder (e.g., epilepsy) or a history of environmental exposure (e.g., valproate, foetal alcohol syndrome, very low birth weight).

All parents provided written informed consent. The children were aged 20–44 months (mean ± SD = 32.27 ± 5.55). The sample included 49 males and 11 females. Diagnoses of ASD were based on the DSM-5 criteria (13). 38 children were assessed by modules 1 or 2 of the Autism Diagnostic Observation Schedule (ADOS) and 22 children were assessed by the toddler module (41). All ADOS-2 comparison scores were in the range of moderate or severe autism (module 1or 2) or of "high risk" of autism (toddler module). All children underwent a cognitive evaluation. In particular, 20 children were assessed through the Leiter International Performance Test-Revised (Leiter-R) (42); the average total IQ at the Leiter-R was 109.95 (SD = 14.45). The other 40 children, for whom a structured testing was not possible, were assessed through the Griffiths Mental Developmental Scale-Extend Revised (GMDS-ER) (43) and all had a development quotient below 24 months.

No child had first-degree relatives with ASD.

The control group was recruited from different kindergarten classes in Rome. The chief teachers of the schools were contacted in order to plan the recruitment of the participants in the study among their pupils with normal development and without relationship or communication problems. All parents provided written informed consent and stated that they never had access to child mental health services. Four recruited children were excluded from the study for the evidence of a slight language disorder. Ultimately, the control group included 34 males and 16 females, aged 18–43 months (mean ± SD = 29.88 ± 8.05).

Procedures

The study was approved by the Ethics Committee of the Department of Dynamic and Clinical Psychology, Sapienza University of Rome, Italy. In the clinical group, all procedures were part of the assessment for ASD. The control group was assessed at home, after being recruited from the kindergarten classes. Parents signed a consent form prior to enrolment. Extensively trained clinicians conducted the assessments, which consisted of a videotaped parent-child interaction during mealtime.

Measures

All mothers and their infants were observed using an Italian adaptation of the Feeding Scale – Scale for the Assessment of Feeding Interaction (Ammaniti M., Lucarelli L., Cimino S., D'Olimpio F. Scala di valutazione dell'interazione alimentare madre-bambino – SVIA unpublished manuscript), which can be applied to children between the ages of 1–36 months. This scale measures interactive behaviours and identifies relational models between parents and children during feeding sessions. Prior to taking part in the study, mothers of ASD children were instructed to bring foods that they would usually offer to their infants at home and to bring food for themselves, if they were accustomed to eating with their infants. The SVIA consists of 41 items distributed among 4 subscales (described below in detail): affective state of the mother, interactional conflict, food refusal behaviour of the child and affective state of the dyad. Each item received a score on the following Likert scale: 0 (*none*), 1 (*a little*), 2 (*pretty much*) and 3 (*very much*). A global rating was obtained for each subscale.

1) The *affective state of the mother* subscale (15 items, overall score from 0 to 45) refers to the possible difficulties of the caregiver in showing positive affect as well as the frequency and quality of expressed negative affect. It also evaluates the mother's ability to interpret the child's signals and facilitate reciprocal and empathic exchanges. Higher scores in this subscale indicate difficulties in expressing positive feelings and in correctly interpreting the infants' needs.
2) The *interactional conflict* subscale (16 items, overall score from 0 to 48) evaluates both the presence and intensity of conflict within the dyad. This subscale score is high when, for example, the mother forces the child to eat or when she directs the meal according to her own emotions and intentions rather than following the communicative feedback of the child. High scores also result from the child showing behaviours of distress and avoidance in response to intrusiveness of the mother.
3) The *food refusal behaviours of the child* subscale (4 items, overall score from 0 to 12) includes items that only concern the child. This subscale explores the feeding patterns of the child, with high scores indicating food refusal, poor nutritional intake and difficulty in behavioural state regulation, such as irritability, hyper-excitability, being easily distracted, showing opposition and negativity.
4) The *affective state of the dyad* subscale (6 items, overall score from 0 to 18) evaluates the quality of affect in the mother–child interaction. A high score indicates negative involvement within the dyad, in which emotions of anger and hostility prevail. In this situation, the caregiver does not facilitate the child's autonomous initiatives by, for example, exerting constant control during mealtime. As a consequence, the child is intensely reactive, showing distress.

Mother–infant interactions during feeding were recorded for at least 20 min. Two trained psychologists, blind to the infants' diagnosis, rated the videotaped feeding interactions (Ammaniti M., Lucarelli L., Cimino S., D'Olimpio F. Scala di valutazione dell'interazione alimentare madre-bambino – SVIA; unpublished manuscript).

Children's feeding difficulties were investigated with the BAMBI (40). The BAMBI is an 18-item parental report questionnaire that was designed to capture mealtime behaviours in children with ASD. The BAMBI is scored on a 1–5 Likert scale (1 indicating the behaviour "never" occurs at mealtime, 5 indicating the behaviour "always" occur at mealtime). Reversed scoring is used for four of the items rating positive mealtime behaviours. A total score (ranging from 18 to 90) is calculated from the sum of all items, with

higher scores reflecting more mealtime behavioural problems. The BAMBI examines specific problem behaviours seen in populations with ASD. As such, it has strong potential for clinicians to assess feeding problems in children with ASD. The BAMBI was recently used in a large study on nutrition in 256 children with ASD, showing strong associations between BAMBI scores and repetitive and ritualistic behaviours, sensory features, as well as externalising and internalising behaviours (44). In the present study, we used the BAMBI cut-off score of 34 suggested by DeMand et al. (45), who investigated the psychometric properties of the BAMBI scale in a large, representative ASD sample (ages 2–11 years). These authors identified 4 factors: food selectivity, disruptive mealtime behaviours, food refusal and mealtime rigidity.

Statistics

Quantitative data are summarised by means ± standard deviation (SD) and range. To compare ASD and control groups on the SVIA dimensions and to analyse the specific role of gender, a series of one-way analysis of covariances (ANCOVAs) were performed, including gender as a covariate. The Pearson's linear correlation coefficient, r, was used to assess the association between the SVIA dimensions and the BAMBI total score. Finally, the ASD group was divided into two subgroups (based on the BAMBI cut-off score of 34) and one-way ANOVAs were performed to compare these two ASD groups with the control group. Specifically, Bonferroni *post hoc* analyses were applied to test differences among the three groups.

The number of subjects enrolled in the control and ASD groups (1. control, n = 50; 2. ASD, n = 60) allowed for detecting differences of small size (Cohen's d = 0.54) at a two-tailed significance level = 0.05 and a power = 0.80. When performing pairwise comparisons between control subjects and ASD subjects with and without eating disorders, at a two-tailed significance level = 0.0167 (to take into account multiple comparisons by Bonferroni's correction) and a power = 0.80, the numbers of subjects in the three subgroups (1. control, n = 50; 2.1. ASD with eating disorders, n = 13; 2.2. ASD without eating disorders, n = 44) are sufficient to detect differences of small/medium size (Cohen's d = 1.03, 0.68, and 1.05, for comparison 1 vs 2.1, 1 vs 2.2, and 2.1 vs 2.2, respectively).

Statistical analyses were conducted using the Statistical Package for Social Science (SPSS) version 24.A p-value < 0.05 was considered significant for all analyses.

RESULTS

As shown in **Table 1**, ANCOVA analysis between the ASD group and control group on the SVIA dimensions showed higher scores on all the SVIA dimensions in the ASD group, than the control group. No effect of gender, included as a covariate, was found ($p = 0.975$).

In the ASD group, Pearson's correlation coefficients between the BAMBI score and the following dimensions of SVIA were statistically significant, even if the strength of the association was weak/moderate: Interactive conflict (r = 0.29, $p = 0.03$), Food refusal (r = 0.31, $p = 0.02$) and Affective state of the dyad (r = 0.27, $p = 0.04$). A feeble and non-statistically significant correlation was found between the BAMBI score and the Affective state of the mother dimension (r = 0.16, $p = 0.246$).

In a second step, the ASD sample was divided into two subgroups based on the BAMBI scores: 13 children with feeding difficulties (i.e., BAMBI score equal to or higher than the cut-off of 34) and 44 children without feeding difficulties (i.e., BAMBI score below the cut-off of 34). Three mothers did not fill out the questionnaire and their data were not included in the comparison. We compared these two subgroups with the control group on the SVIA scales and significant differences emerged on all dimensions. Specifically, *post hoc* analyses (Bonferroni) showed significant differences between both ASD subgroups, with and without an eating disorder, and the control group on all the SVIA dimensions ($p < 0.001$ for all).

Finally, significant differences emerged between the two ASD subgroups in three SVIA dimensions (Interactive conflict, Food refusal and Affective state of the dyad), but not in the Affective state of the mother dimension (**Table 2**).

TABLE 1 | Analysis of covariances between ASD and control groups on SVIA dimensions.

SVIA dimension	ASD group n = 60		Control group n = 50		F	p
	Mean	SD	Mean	SD		
Affective state of the mother	9.52	3.53	2.95	2.56	120.18	<0.001
Interactional conflict	8.79	4.04	4.17	2.45	49.92	<0.001
Food refusal	5.52	2.31	2.12	1.47	80.73	<0.001
Affective state of the dyad	3.06	1.96	1.29	1.29	29.64	<0.001

TABLE 2 | ANOVA between ASD subgroups with and without eating disorder on SVIA dimensions.

SVIA dimension	ASD group with eating disorder n = 13		ASD group without eating disorder n = 44		Control group n = 50		F	p
	Mean	SD	Mean	SD	Mean	SD		
Affective state of the mother	10.52	3.08	9.25	3.73	2.95	2.56	58.80	0.013
Interactional conflict	11.20	3.70	8.17	3.96	4.17	2.45	31.03	0.003
Food refusal	7.02	2.10	5.02	2.20	2.12	1.47	48.10	0.005
Affective state of the dyad	4.34	1.94	2.68	1.84	1.29	1.29	21.07	0.613

DISCUSSION

The aim of this study was to deepen our understanding of the patterns of food regulation in children with ASD, within the caregiver-child relationship. In particular, we evaluated the applicability of the SVIA in a group of children with ASD.

The comparison between groups on the SVIA showed higher scores on all the SVIA dimensions in the ASD group than the control group (no effect of gender, used as a covariate, emerged). These data were in line with the results of previous studies about the relation between autism and feeding disorders (29). Specifically, ASD children showed higher scores on the SVIA Food refusal dimension, compared to the control group; ASD children often got up and wandered without a specific purpose, likely as a result of the greater attention they paid to objects than to people. Furthermore, the presence of stereotyped behaviour led these children to physically isolate themselves, a behaviour which did not support food interactions.

Moreover, ASD children showed higher scores on the SVIA Affective state of the mother and Interactional conflict dimensions; the communicative deficits in ASD children affect the co-regulation processes between mother and child (19). Indeed, the caregivers of the clinical group showed more difficulties in expressing positive feelings and in correctly interpreting the children's needs. In our study, ASD children showed reduced spontaneous initiation of interactions. Finally, ASD children showed higher scores on the SVIA Affective state of the dyad, as the clinical group had more difficulties in forming autonomies than the control group, possibly due to motor impairment. In these circumstances, mothers of ASD children were less likely to encourage engagement and attention towards the shared purpose of eating, than the mothers of the control group. As a consequence, the dyads of the clinical group showed more behaviours of distress, as well as negative involvement.

Regarding the relations between SVIA and BAMBI, in the ASD group, SVIA dimensions and the BAMBI score showed significant, though moderate correlations, except in the Affective state of the mother dimension. Several items of this subscale refer to the possible difficulties of the caregiver in showing positive affect, as well as the frequency and quality of expressed negative affect. Regarding the caregivers, these data could be explained as the effect of the parent's mutual adaptation to the child's communicative difficulties. We know from previous studies that children with ASD can send "unrecognisable signals." Parents of our ASD children were prone to fail in this primary task, which could be related to the fact that the study was conducted during a diagnostic assessment phase, when the child's problems are not yet well known. In other words, this result could be related to the effects of the child's atypical communication on the dynamic characteristics of the co-regulation system, not to the eating disorder in ASD.

However, given the cross-sectional design of the study, we cannot rule out that the difficulties of the mother in regulating negative affect expression, as well as difficulties in expressing positive feelings and in correctly interpreting the infants' needs could be related to individual characteristics of the mothers themselves.

Based on the increased probability of children with ASD developing an eating disorder (29), as well as the similarities between the DSM-5 criteria for ARFID and eating difficulties in children with ASD (13), we hypothesised that ASD children with eating disorders would show higher scores on the SVIA dimensions than ASD children without eating disorders. Our results partially confirmed these hypotheses, showing higher scores in the group of ASD children with eating disorders, than the control group, on the SVIA Affective state of the mother, Interactional conflict and Food refusal dimensions.

In fact, the data showed that the mothers of ASD children with eating disorders seemed to exhibit more intrusive interactions than mothers of ASD children without an eating disorder. The caregiver did not facilitate the child's autonomous initiatives by exerting a constant control ("Mother waits for infant to initiate interactions"; "Mother distracts or allows infant to distract during feeding"). As a consequence, the mother-ASD child-dyads showed a high interactive conflict during mealtime and the levels of distress were higher than in the mother-non-ASD child-dyads.

The main limitations of this study include the small sample size, the use of a version of the BAMBI not validated in Italy for the assessment of eating disorders and the different contexts in which the observations of feeding interactions were conducted.

The main strength of the study is that the direct observation of feeding in the assessment of children with ASD was employed, but some critical reflection can be proposed about this application. The SVIA seems to be able to point out some feeding difficulties in these subjects and to discriminate ASD with and without eating disorder. The application of the SVIA also allowed for the evaluation of autistic children in an ecological context, enriching existing clinical information and providing applicable possibilities for improving relationships. In children under 30 months of age, the area of restricted and repetitive behaviours may be more evident during mealtimes. In particular, the child may exhibit more mannerisms triggered by contact with food and feeding routines.

Mealtimes promote regular interactive exchanges and create repeated social learning opportunities for children with ASD. In this context, children with ASD, like typically developing children, begin to experience the sharing of affection and reciprocal communication with their caregivers. The information obtained by SVIA in this study was shared with parents in order to support social reciprocity and communication exchanges.

At the same time, from a clinical point of view, the application of the SVIA highlighted some critical aspects of this instrument in a group of children with autism. In particular, the ASD children, with or without a feeding disorder, tended to have high scores in the areas of social interaction and emotional regulation of the SVIA, which cannot be explained as avoidance of the relationship or as an effect of the parent's inability to respond appropriately to the child's signals. The limited variability of facial expressions and the atypical eye contact of children with ASD (14, 16), that is recognised in many items of the SVIA, may have led to such pathological

scores. These results are in line with studies on the peculiarity of expression and regulation of emotions in parent-child dyads with ASD (46). These data need to be interpreted in light of the neurodevelopmental and communicative deficits of children with ASD.

In conclusion, the identification of behavioural patterns regarding feeding has fundamental implications for early interventions. In the future, it will be important to adopt a multidimensional model in evaluating autistic children with food difficulties. Future models should include children's biological, psychological and social maturation factors, considering the specific symptomatology/functioning of autism and the developmental patterns of the relationship between caregivers and children with ASD.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Ethic Committee of the Department of Dynamic and Clinical Psychology, Sapienza University of Rome, Italy, with written informed consent from all parents. All parents gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethic Committee of the Department of Dynamic and Clinical Psychology, Sapienza University of Rome, Italy.

AUTHOR CONTRIBUTIONS

EC and MT contributed to the conception and design of the study, performed the statistical analysis, and wrote the first draft of the manuscript. GP and CA assessed the subjects and organized the database. FCh revised the statistical analysis. FCa wrote the sections of the manuscript and revised the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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Neural Processing of Dynamic Animated Social Interactions in Young Children With Autism Spectrum Disorder: A High-Density Electroencephalography Study

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Background: Atypical neural processing of social visual information contributes to impaired social cognition in autism spectrum disorder. However, evidence for early developmental alterations in neural processing of social contingencies is scarce. Most studies in the literature have been conducted in older children and adults. Here, we aimed to investigate alterations in neural processing of social visual information in children with autism spectrum disorder compared to age-matched typically developing peers.

Methods: We used a combination of 129-channel electroencephalography and high-resolution eye-tracking to study differences in the neural processing of dynamic cartoons containing human-like social interactions between 14 male children with autism spectrum disorder and 14 typically developing male children, aged 2–5 years. Using a microstate approach, we identified four prototypical maps in both groups and compared the temporal characteristics and inverse solutions (activation of neural sources) of these maps between groups.

Results: Inverse solutions of the group maps that were most dominant during free viewing of the dynamic cartoons indicated decreased prefrontal and cingulate activation, impaired activation of the premotor cortex, and increased activation of parietal, temporal, occipital, and cerebellar regions in children with autism spectrum disorder compared to their typically developing peers.

Conclusions: Our findings suggest that impairments in brain regions involved in processing social contingencies embedded in dynamic cartoons are present from an early age in autism spectrum disorder. To the best of our knowledge, this is the first study to investigate neural processing of social interactions of children with autism spectrum disorder using dynamic semi-naturalistic stimuli.

Keywords: ASD, high-density EEG, source imaging, eye-tracking, frontal, cingulate, parietal, cerebellum

INTRODUCTION

Autism spectrum disorder (ASD) is a heterogeneous neurodevelopmental disorder characterized by social and communication deficits, repetitive behaviors, and restricted interests, with a prevalence of approximately 1 in 59 in the US (1). ASD is currently defined as a single entity by the *Diagnostic and Statistical Manual of Mental Disorders* (*DSM-5*) (2), where the term spectrum refers to the heterogeneity in the range and severity of symptoms among others.

Despite the heterogeneity in ASD symptoms, deficits in social cognition have consistently been reported in individuals on the spectrum at all ages and have been suggested to represent a core deficit in ASD (3). These deficits start from a very early age and can lead to inadequate social experiences required for "social learning," as well as insufficient "cognitive learning" (4, 5). Individuals with ASD generally exhibit abnormalities in eye contact and the processing of biological motion and facial information (6, 7), and struggle attributing social meaning to visual stimuli when they are ambiguous (8) compared to their typically developing (TD) peers. As such, deficits in orienting to people and the resultant reduction in or lack of social interactions may be a hallmark of autism (9).

As the child develops, these insufficiencies in social cognition are thought to result in impaired development of brain regions responsible for processing social information, and impaired cognitive development, such as the fusiform gyrus (FG), amygdala, superior temporal cortex, anterior temporal cortex, temporo-parietal junction, medial prefrontal cortex, anterior cingulate cortex (ACC), precuneus, inferior frontal cortex, and inferior parietal lobule (IPL) [for reviews, see Frith (10), Pelphrey et al. (3), and Schaer et al. (11)].

Neuroimaging studies have revealed a different organization and functioning at the large-scale brain level in ASD. Functional studies of social cognition have, for example, highlighted robust differences in activation of these regions between individuals with ASD and their TD peers (12–14). Structural abnormalities have also been found in several of these brain regions including reduced grey matter in the ACC and temporal cortex of individuals with ASD aged 8–50 years (15), and in the temporal cortex and IPL of adults with ASD (16, 17). Network abnormalities have also been reported in several brain regions including the frontal lobes (18). However, to date, most functional brain studies using social stimuli have been conducted in school-aged children, adolescents and adults, whereas putative deficits in neural processing of social cognition in toddlers and preschoolers, at the time of ASD diagnosis, have been poorly examined.

Eye-tracking is a powerful tool for measuring the social component of visual perception and has been used to quantitatively confirm the clinically observed reduction of interest in social cues and interactions in individuals with ASD (19–21). Another powerful tool for studying social cognition is high-density electroencephalography (EEG), which is used to study the brain's electro-cortical activity at a large-scale level. It is particularly useful in infants and children because it is non-invasive, can be informative regardless of communication ability, and requires less physical adjustment to the equipment in comparison to other neuroimaging techniques such as magnetic resonance imaging (22, 23). EEG studies have shown that an abnormal pattern of brain activity in response to faces versus objects is present early in life in ASD (24–26) and that this abnormal pattern is also present in 10-month-old infants at risk for autism, suggesting that abnormality in face versus object processing is an early indicator of risk for developing ASD (27). Recently, by combining high-density EEG and eye-tracking, we found that directed functional connectivity alterations of social brain networks is a core component of atypical brain development at early stages of ASD (28, 29).

Here, we used a combination of high-density EEG and eye-tracking in children with ASD aged 2–5 years and compared them to age-matched TD children, to investigate alterations in neural processing of semi-naturalistic cartoons that explicitly depict human-like social interactions. Analysis of the EEG data was performed using a microstate analysis technique, which is a data-driven, reference-free approach [for a review, see Michel and Koenig (30)]; EEG microstates are short-lasting (~100 ms) periods of stable topographies of the electric potentials in the ongoing EEG (31). Typically, only a few archetypical maps represent the majority of the broad-band resting EEG, reproducible within and across subjects (32). EEG microstate analysis thus allows the parsing of the ongoing broad-band EEG into a limited number of distinct quasi-stable states, reflecting short-lasting coordinated activation of large-scale brain networks, continuously alternating between each other (33, 34). The analysis of the temporal dynamics of the microstate time series, their individual presence and duration, as well as their source localization offers a new way of looking at brain network dynamics in the ongoing EEG during different mental and cognitive states (30, 35–37).

We hypothesized that children with ASD would exhibit differences in activation of brain regions that are typically specialized in social information processing compared to their TD peers, such as, the FG, amygdala, superior temporal cortex, anterior temporal cortex, temporo-parietal junction, medial prefrontal cortex, ACC, precuneus, inferior frontal cortex, and IPL. Subsequently, we also used eye-tracking gaze data to divide the ASD group according to their gaze behavior, to conduct an exploratory subgroup analysis which aimed to investigate whether autistic children with control-similar (CS) gaze patterns (CS ASD) showed significant differences in the way their brain processed social information from children with control-dissimilar (CD) gaze patterns (CD ASD). We hypothesized that activation of the abovementioned regions, involved in social information processing, would be affected by visual exploration

Abbreviations: ANOVA, Analysis of variance; ACC, anterior cingulate cortex; ACG, anterior cingulate gyrus; ADOS, Autism Diagnostic Observation Schedule; ASD, autism spectrum disorder; BA, Brodmann area; CD, control-dissimilar; CS, control-similar; DLPFC, dorsolateral prefrontal cortex; DMPFC, dorsal medial prefrontal cortex; EEG, electroencephalography; FG, fusiform gyrus; GEV, global explained variance; GFP, global field power; IPL, inferior parietal lobule; ITG, inferior temporal gyrus; LAURA, Local Autoregressive Average; MFG, middle frontal gyrus; MTG, middle temporal gyrus; SFG, superior frontal gyrus; TD, typically developing.

patterns of children with ASD, with greater differences in neural activation expected between CD ASD and TD than between CS ASD and TD children. Although this exploratory subgroup analysis was expected to yield interesting results, these were considered to be preliminary due to the small sample size of the ASD subgroups. Hence, interpretation of the results from the subgroup analysis was exercised with caution, and future studies of larger sample size are recommended for consolidation of results.

METHODS

Participants

High-density EEG and eye-tracking data were successfully collected from 46 children aged 2–5 years, of whom 21 had a confirmed diagnosis of ASD (2 females) and 25 were TD (10 females). Given the well-documented gender bias in ASD (38, 39), and that we only had EEG data from 2 females with ASD, this paper solely investigated male participants, and data from female participants (ASD = 2, TD = 10) were excluded from the analysis, resulting in 19 ASD and 15 TD male children. Following data inspection, five participants with ASD and one participant with TD were excluded due to unrepairable noisy signal. Thus, the final group included in the EEG analysis was composed of 14 participants with ASD (mean age 3.3 ± 0.8 years) and 14 age-matched TD participants (mean age 3.2 ± 0.9 years).

Participants with ASD were recruited through French-speaking parent associations and specialized clinical centers. All participants in the ASD group had received a clinical diagnosis of ASD prior to their inclusion in the research protocol, and none of them had any known neurogenetic conditions such as Fragile X, Rett, or Phelan McDermid syndromes, or neurofibromatosis. As a part of the research protocol, diagnosis was confirmed using either the Autism Diagnostic Observation Schedule—Generic (ADOS-G) (40) or the Autism Diagnostic Observation Schedule, second edition (ADOS-2), the latter including a toddler module that defines concern for ASD (41). ADOS assessments were administered and scored by trained psychologists who met requirements for research reliability. ADOS-G scores were then transformed according to Gotham and colleagues' algorithm (42), and ADOS-2 toddler module scores were transformed into standardized calibrated severity scores according to Esler and colleagues' method (43), to facilitate comparison of scores from different modules. On a scale of 10, the mean severity score for the ASD group in the current study was 7.4 ± 1.9.

TD participants were recruited through announcements in the Geneva community. All TD participants were screened for neurological/psychiatric deficits and learning disabilities and family history of ASD prior to their inclusion in the research protocol. For all participants, a telephone interview and a medical development history questionnaire were conducted prior to their first visit to the research center. TD children also underwent ADOS-G or ADOS-2 evaluations in order to ensure typical development and exclude any signs of ASD. All TD children had a minimal ADOS severity score of 1, except for one child who had a score of 3 but did not belong on the spectrum, since scores of 1–3 receive a non-spectrum ADOS classification (42). Researchers ensured that the parents or legal guardians of participants understood the study protocol and gave their informed consent for participation and publication of results prior to their inclusion in the study. The study protocol conforms with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and was approved by the Local Research Committee, the Commission Centrale d'Ethique de Recherche (CCER) in Geneva, Switzerland.

Stimuli and Procedure

The stimulus of interest in this study was an animated cartoon movie named *Trotro*, which depicts the donkey Trotro engaging in human-like social interactions with his parents or friends or playing with toys across various scenes using both body language and spoken French (44) (**Figure 1**). Presentation of stimuli was controlled by Tobii Studio software v. 3.2 (Tobii® Technology, Sweden). Participants were seated approximately 65 cm away from the recording screen. A five-point calibration was performed using a built-in Tobii mechanism using child-friendly animations. The testing room had no windows, and lighting conditions were kept constant. All participants watched four 2- to 3-min movies of the Trotro animated cartoon. The movies were organized in two blocks, separated by a short break. The stimuli of interest (Trotro) were interspersed with another set of stimuli that were intended to analyze biological and non-biological motion processing. All participants watched the same movies in the following order: Block 1: 1) Trotro movie 1: Trotro amoureux (Trotro is in love); 2) non-biological motion (part 1); 3) Trotro movie 2: Trotro et la boite des secrets (Trotro and the box of tricks); 4) biological motion (part 1). Block 2: 5) Trotro movie 3: Trotro part en vacances (Trotro goes on holidays); 6) non-biological motion (part 2); 7) Trotro movie 4: L'anniversaire de Nana (Nana's birthday); 8) biological motion (part 2). Here, we investigated data collected during the four Trotro movies, with a total duration of 11 min 11 s. The data concerning biological and non-biological motion stimuli will be published separately.

EEG Data Collection and Analysis

EEG Data Acquisition and Pre-processing

High-density EEG was continuously recorded with a 129-channel Hydrocel Geodesic Sensor Net® (Electrical Geodesics Inc., Eugene, OR, USA). The EEG data were acquired with a sampling rate of 1,000 Hz and a recording reference at the vertex; impedances were kept below 30 kΩ. Electrodes located on the cheeks and on the nape were excluded, and 110 electrodes were retained for further analysis, which was performed using the free academic Cartool software v. 3.60 (http://sites.google.com/site/cartoolcommunity, Geneva, Switzerland) (45). The two runs of EEG data were concatenated and digitally filtered between 1 and 40 Hz, using a second-order Butterworth filter with a −12 db/octave roll-off. The filter was computed linearly with two passes, one forward and one backward, in order to eliminate phase shifts, and with poles calculated each time to the desired cut-off frequency. Subsequently, an additional notch filter was applied to eliminate 50 Hz noise. Data contaminated by oculomotor artifacts were

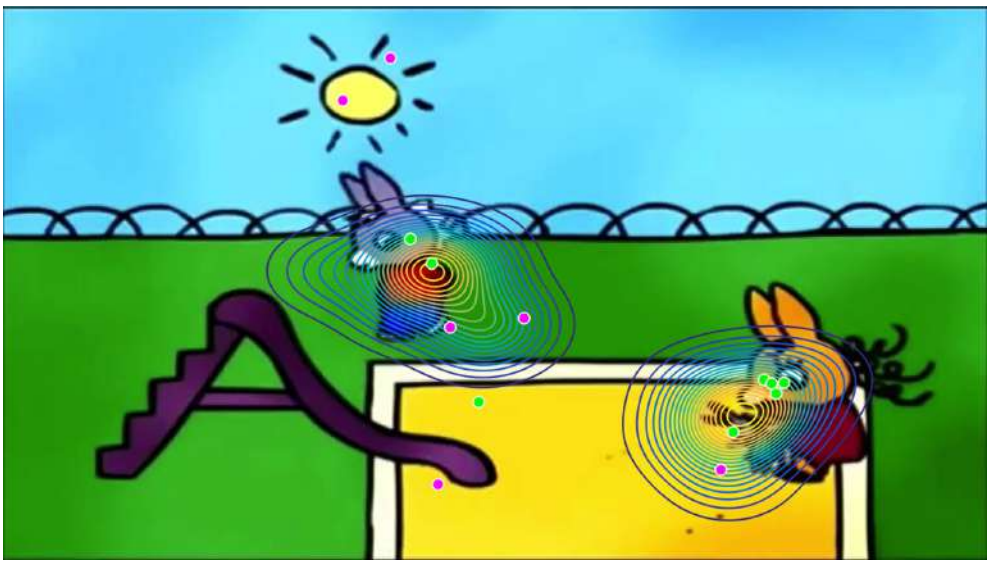

FIGURE 1 | A video still from a "Trotro" cartoon film illustrating the position of the "norm" in gaze, which was established using a kernel density distribution estimation on gaze data from a group of 26 typically developing children aged 2–5 years (represented here with contour plots). For this particular frame, the gaze of typically developing children was split into two foci of interest, centered on the main characters. The gaze of each child with autism spectrum disorder (ASD) in this sample is demonstrated with individual dots. Green dots represent gaze coordinates of control-similar (CS) children with ASD (n = 8), while pink dots represent gaze coordinates of control-dissimilar (CD) children with ASD (n = 6). It is important to note that the subgrouping into CS and CD children with ASD is based on average gaze data from the entire movie. Therefore, it follows that for this specific frame, two CD ASD children, whose gaze patterns were, on average, far from the "norm" over the course of the movie, were focusing closer to the center of attention of TD children than one CS ASD child.

excluded using an infomax independent component analysis implemented in Matlab (46–48). For each participant, channels exhibiting substantial noise were interpolated using a 3-D spline interpolation procedure (49). On average, 15.5 channels were interpolated for each participant. Data were re-referenced to the common average reference, downsampled from 1,000 to 125 Hz, and reduced to the local maxima of the global field power (GFP), in order to improve signal-to-noise ratio (33, 45, 50).

EEG Microstate and Source Analyses

For each participant, the topographic maps at GFP peaks from the four Trotro cartoon conditions were submitted to a k-means spatial cluster analysis (45, 50, 51), to identify templates of the most dominant topographic maps present during free viewing of Trotro cartoons, using a weighted optimum of 11 selection criteria (52). Only segments that were free of muscular artifacts as identified by visual inspection were considered for the k-means analysis. Following the individual-level cluster analysis, the dominant topographic maps from each subject were submitted to a second group-level k-means cluster analysis, which identified the most dominant topographic maps (the microstate maps) for each participant group. To assess whether the microstate maps from each group differed topographically, an unpaired topographic analysis of variance (TANOVA) was conducted for each pair of maps (e.g. ASD Map 1 and TD Map 1) (53), and a Pearson's spatial correlation analysis was carried out on the two groups of maps.

In order to estimate the temporal characteristics of the dominant maps within each group, spatial correlation was conducted between the microstate maps and the actual topographic maps at each time point of each participant's artifact-corrected EEG data from the Trotro cartoon conditions. This resulted in each participant's data at each time point being assigned to one of the microstate maps with which it correlated best (45, 51). To ensure that data segments were not artificially interrupted by noise during low GFP, temporal smoothing was conducted with a window half size of 3 and strength (Besag factor) of 10 (45, 50). The labeling process allowed for the computation of the following temporal parameters of each of the microstate maps: global explained variance (GEV), mean duration, time coverage, and frequency of occurrence. The GEV is an estimate of the explained variance of a given map, weighted by the GFP. The mean duration is the average duration (in ms) of EEG data segments that were assigned to a given group map, whereas the time coverage is the percentage of total time in individual EEG data that is represented by a given microstate map. The frequency of occurrence represents the number of times per second that a given microstate map occurs in the individual EEG data. In order to determine the most dominant maps within each group and compare these between groups, a one-way analysis of variance (ANOVA) with temporal parameters as the dependent variables and group map (1, … n) as the within-subjects factor was performed using Statistica software v. 13 (Dell Inc., Tulsa, OK, USA). The results and post hoc tests were Bonferroni-corrected for multiple comparisons.

For source localization, all time points that were assigned to a given microstate map were concatenated for each subject. A linear distributed inverse solutions was then computed for each time point using the Local Autoregressive Average (LAURA) regularization approach, described in Grave de Peralta et al. (54)

and Grave de Peralta et al. (55), and implemented in Cartool software (45). Five thousand solution points were constrained to and equally distributed in the grey matter using the Montreal Neurological Institute (MNI) template brain for toddlers aged 33–44 months with consideration of skull thickness (Locally Spherical Model with Anatomical Constraints, LSMAC) (56–56). The source maps were then averaged across all time points for each subject and for each microstate map separately (59).

In order to calculate differences in neural activation between ASD and TD, the sources underlying the microstate maps that had the highest temporal parameters were compared using a non-parametric unpaired randomization test with an exhaustive evaluation of all possible permutations of the subjects (more than 16,000 iterations) (60). The probability threshold of this randomization test was set to $P < 0.01$. In order to determine the direction of the difference (i.e. ASD > TD vs. TD> ASD), unpaired t-tests were performed, and the t-values were thresholded to the *P*-values of the permutation test ($P < 0.01$). Brain regions with significant differences in activation between the two groups were identified by consensus labeling by researchers trained in neuroanatomy.

RESULTS

Dominant Group Maps

The group-level k-means cluster analysis identified four dominant topographic maps for each participant group, which explained 80.44% of the total variance for the ASD group and 77.43% of the total variance for the TD group (**Figure 2**). A Pearson's spatial correlation analysis revealed that each of the four dominant microstate maps for the ASD group was highly correlated with one of the microstate maps for the TD group. TANOVA analysis showed no significant differences in topography of the corresponding maps between groups ($P > 0.05$), and (**Table 1**).

Temporal Dynamics

Within each participant group, a one-way ANOVA with temporal parameters as the dependent variables and microstate map (1, 2, 3, 4) as the within-subjects factor revealed significant differences in each of the four temporal parameters between maps. For the ASD group, there were significant differences between maps in GEV ($F_{3, 39} = 50.22$, $P < 0.001$, **Figure 3A**), mean duration ($F_{3, 39} = 31.58$, $P < 0.001$), time coverage ($F_{3, 39} = 26.36$, $P < 0.001$, **Figure 3B**), and frequency of occurrence ($F_{3, 39} = 21.40$, $P < 0.001$). Bonferroni-adjusted post hoc tests revealed that Map 3 had significantly higher temporal parameters than all other maps ($P < 0.001$), and Map 4 had higher mean duration ($P = 0.007$), time coverage ($P = 0.02$), and frequency of occurrence ($P = 0.02$) than Map 2. Within the TD group, Map 3 had the highest GEV ($F_{3, 39} = 46.06$, $P < 0.001$, **Figure 3C**), mean duration ($F_{3, 39} = 32.30$, $P < 0.001$), time coverage ($F_{3, 39} = 21.78$, $P < 0.001$, **Figure 3D**), and frequency of occurrence ($F_{3, 39} = 19.24$, $P < 0.001$). Bonferroni-adjusted *post hoc* tests revealed that Map 3 had significantly higher temporal parameter estimates than all other maps ($P < 0.001$). Map 4 had significantly higher GEV compared to Map 1 ($P = 0.02$), higher mean duration compared to Map 1 ($P < 0.001$) and Map 2 ($P = 0.006$), higher time coverage compared to Map 1 ($P = 0.004$) and Map 2 ($P = 0.01$), and higher frequency of occurrence compared to Map 1 ($P = 0.004$) and Map 2 ($P = 0.01$).

TABLE 1 | Pearson's spatial correlation coefficients between autism spectrum disorder (ASD) and typically developing (TD) group map pairs.

	TD Map 1	TD Map 2	TD Map 3	TD Map 4
ASD Map 1	**0.88***	0.56*	0.40*	0.58*
ASD Map 2	0.22	**0.99***	0.64*	0.01 NS
ASD Map 3	0.66*	0.37*	**0.98***	0.52*
ASD Map 4	0.16 NS	0.50*	0.61*	**0.84***

**Correlations significant at P < 0.003 (Bonferroni-adjusted), NS: P > 0.05.*

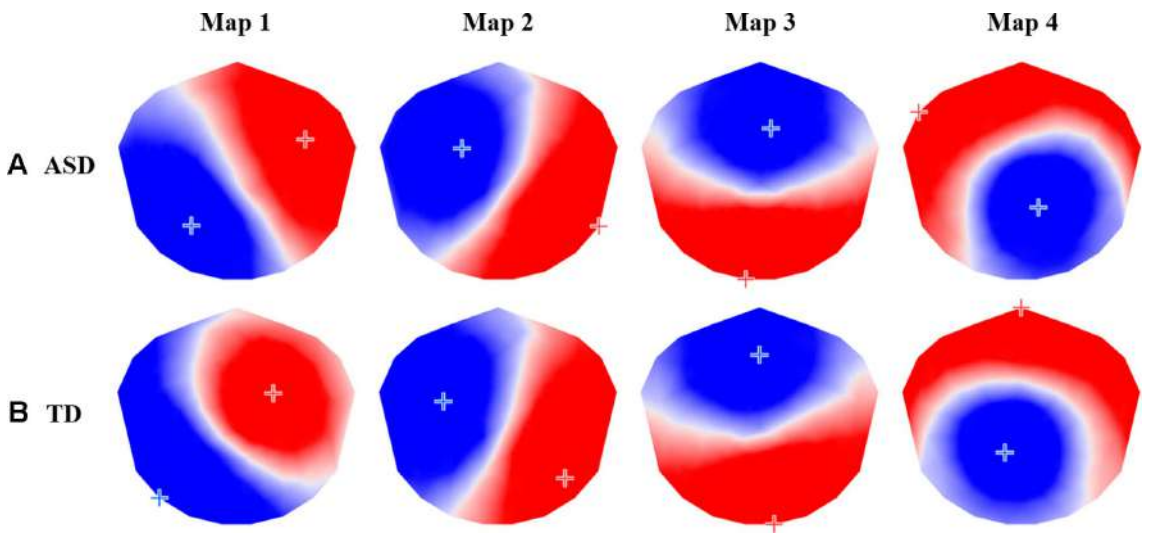

FIGURE 2 | The four prototypic maps identified by the group-level k-means cluster analysis for **(A)** autism spectrum disorder (ASD) and **(B)** typically developing (TD) participants.

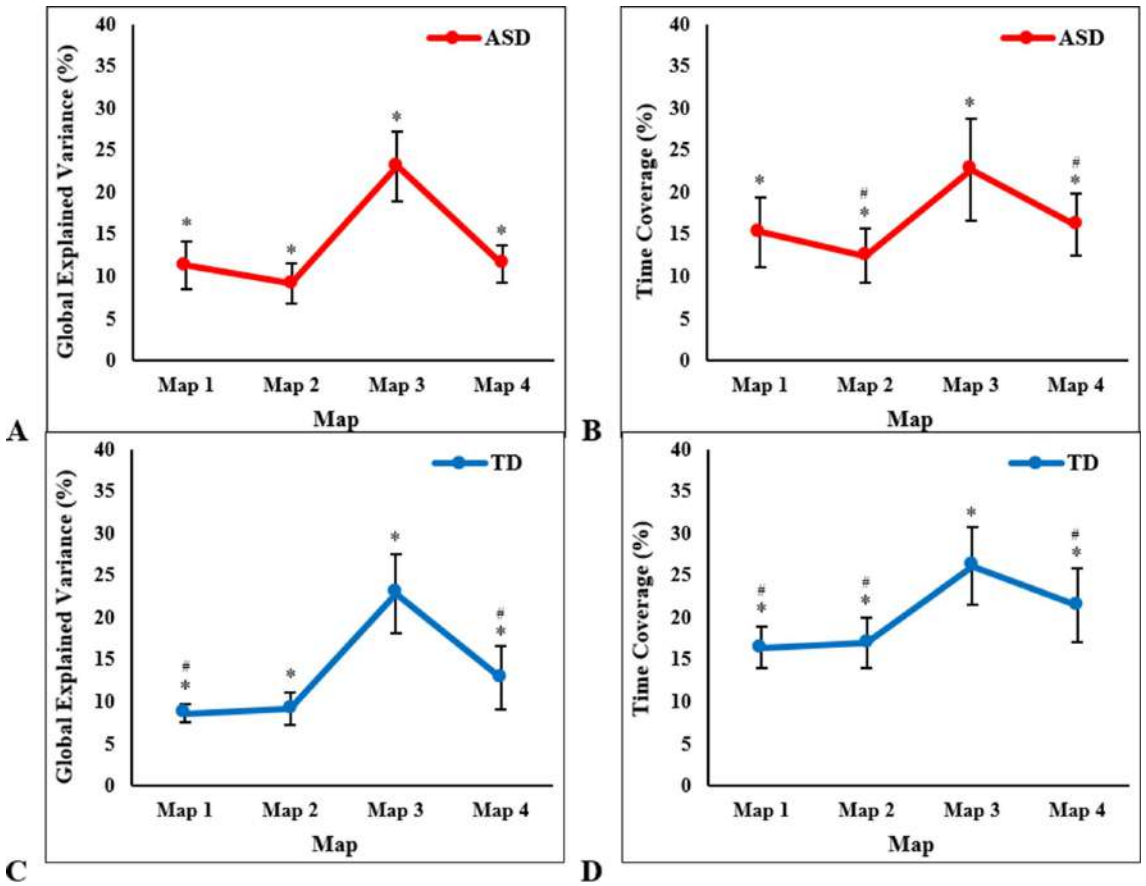

FIGURE 3 | One-way ANOVA results showing significant between-map differences in children with autism spectrum disorder (ASD), in mean (± SD): **(A)** global explained variance and **(B)** time coverage; and in typically developing (TD) children, in mean (± SD): **(C)** global explained variance and **(D)** time coverage. *Significant *post hoc* tests with Map 3, #significant *post hoc* tests with Map 4, at $P < 0.003$ (Bonferroni-adjusted).

For both groups, Map 3 had the highest temporal parameters, followed by Map 4. These maps were the most present and accounted for the majority of the variance in the data. Since these maps were very similar and spatially highly correlated between groups, the inverse solutions for Map 3 and Map 4 were computed for each group at the single subject level and subsequently statistically compared between groups in order to establish group differences in the neural activation patterns of these quasi-similar maps.

Source Localization of Group Differences

Non-parametric permutation tests of the source localization between the two groups showed significant differences in EEG activation ($P < 0.01$) for the sources of Map 3 and Map 4. For Map 3, the ASD group showed decreased activation of the left and right middle frontal gyrus (MFG, Brodmann area (BA) 9 and 8, respectively) and right superior frontal gyrus (SFG, BA 8), and increased activation of the left IPL (BA 40), compared to the TD group (**Table 2**, **Figure 4**). For Map 4, the ASD group exhibited decreased activation of the right MFG (BA 6), left and right SFG (BA 9 and 6, respectively), and left medial frontal gyrus (BA 10)/anterior cingulate gyrus (ACG, BA 42), compared to the TD group. On the other hand, the ASD group showed increased activation of the left anterior cerebellum (culmen), left posterior cerebellum (pyramis, declive), left IPL (BA 40), left FG (BA 37), right middle temporal gyrus (MTG, BA 21), right inferior temporal gyrus (ITG, BA 21), and left SFG/MFG gyri (BA 6) compared to the TD group in Map 4 (**Table 3**, **Figure 5**).

Subgroup Analysis With Respect to Gaze Pattern

Simultaneously to EEG data acquisition, high-resolution eye-tracking was performed on all participants. From the TD group,

TABLE 2 | Source localization of group differences for Group Map 3 showing differences in activation in brain regions, thresholded at P < 0.01, between the autism spectrum disorder (ASD) group (n = 14) compared to the typically developing (TD) group (n = 14) during free viewing of "Trotro" cartoons. The t-values of the unpaired t-test at the corresponding solution point are given.

	Talairach Coordinates	Brain Region	t-value
ASD < TD	23, 19, 48	Right superior frontal gyrus, BA 8	–2.90
	23, 18, 42	Right middle frontal gyrus, BA 8	–2.85
	–50, 18, 28	Left middle frontal gyrus, BA 9	–2.81
ASD > TD	–37, –54, 45	Left inferior parietal lobule, BA 40	3.26

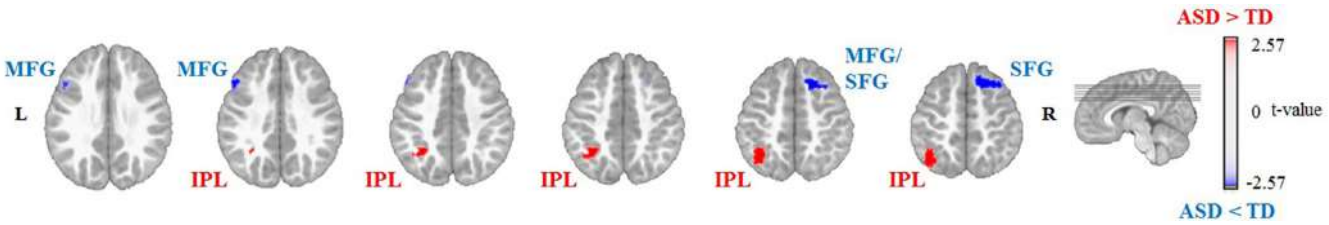

FIGURE 4 | Source localization of group differences for Group Map 3 showing decreased activation of the left and right middle frontal gyrus (MFG) and right superior frontal gyrus (SFG), and increased activation of the left inferior parietal lobule (IPL) in the autism spectrum disorder (ASD) group (n = 14) compared to the typically developing (TD) group (n = 14) during free viewing of "Trotro" cartoons. The t-values of the unpaired t-test thresholded to $P < 0.01$ of the randomization test are plotted. L, left; R, right.

TABLE 3 | Source localization of group differences for Group Map 4 showing differences in activation in brain regions, thresholded at P < 0.01, between the autism spectrum disorder (ASD) group (n = 14) compared to the typically developing group (n = 14) during free viewing of "Trotro" cartoons. The t-values of the unpaired t-test at the corresponding solution point are given.

	Talairach coordinates	Brain region	t-value
ASD < TD	30, 0, 56	Right middle frontal gyrus, BA 6	–2.83
	23, 12, 55	Right superior frontal gyrus, BA 6	–3.31
	–16, 51, 27	Left superior frontal gyrus, BA 9	–3.60
	–10, 50, 7	Left middle frontal gyrus, BA 10	–3.12
	–10, 43, 7	Left anterior cingulate gyrus, BA 42	–3.03
ASD > TD	–23, 0, 62	Left middle frontal gyrus, BA 6	4.30
	–21, 9, 55	Left superior frontal gyrus, BA 6	3.47
	–52, –54, 42	Left inferior parietal lobule, BA 40	4.34
	64, –17, –8	Right middle temporal gyrus, BA 21	2.78
	64, –10, –14	Right inferior temporal gyrus, BA 21	2.99
	–30, –50, –6	Left fusiform gyrus, BA 37	3.04
	–23, –58, –24	Left anterior cerebellum, culmen	3.46
	–37, –71, –17	Left posterior cerebellum, declive	3.41
	–23, –71, –29	Left posterior cerebellum, pyramis	2.80

a normative gaze distribution was obtained by applying a kernel density distribution estimation on gaze data at each frame of the Trotro movies. Following this, the probability of proximity to this pre-established "norm" was computed frame-by-frame for each participant with ASD. According to this analysis, the ASD group was mean-split into two subgroups according to the similarity of their average gaze with the TD group. The first subgroup had gaze patterns more similar to the "norm," referred to as the CS ASD subgroup, and the second subgroup had gaze patterns dissimilar to the "norm," referred to as the CD ASD subgroup (**Figure 1**). Given the small sample size of the ASD subgroups (n = 8 and n = 6, respectively), the separate analysis of the source patterns comparing the subgroups with the TD group is reported in the **Supplementary Material** only.

DISCUSSION

The current study used high-density EEG and eye-tracking to investigate differences in neural activation in the brains of

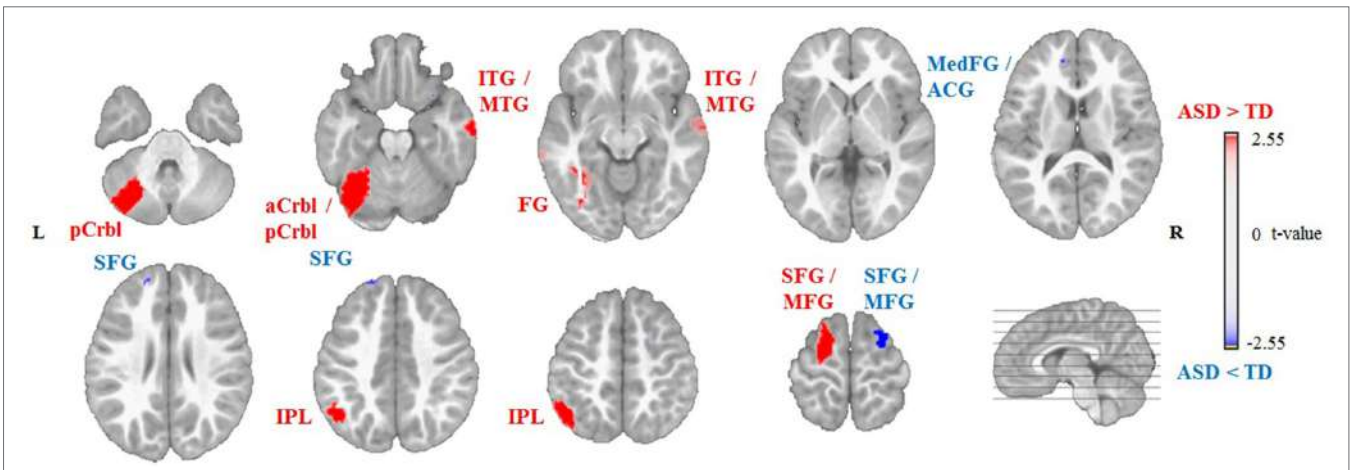

FIGURE 5 | Source localization of group differences for Group Map 4 showing decreased activation of the left medial frontal gyrus (MedFG)/anterior cingulate gyrus (ACG) complex, left and right superior frontal gyrus (SFG), and right middle frontal gyrus (MFG), and increased activation of the left anterior (aCrbl) and posterior cerebellum (pCrbl), left fusiform gyrus (FG), right inferior and middle temporal gyri (ITG/MTG), left inferior parietal lobule (IPL), and left SFG/MFG gyri in the autism spectrum disorder (ASD) group (n = 14) compared to the typically developing (TD) group (n = 14) during free viewing of "Trotro" cartoons. The t-values of the unpaired t-test thresholded to $P < 0.01$ of the randomization test are plotted. L, left; R, right.

children with ASD and their TD peers during free viewing of dynamic semi-naturalistic stimuli containing social interactions. Using an EEG microstate approach, we identified four dominant topographic maps that had significantly different temporal dynamics from one another for each group and were very similar and spatially highly correlated between the groups. Two of the four microstate maps (Maps 3 and 4) that had the highest temporal parameters in both groups were compared in the inverse space to reveal group differences in neural activation during viewing of the Trotro cartoons. Notably, children with ASD showed decreased prefrontal and cingulate activation, impaired activation of the premotor cortex, and increased activation of parietal, temporal, occipital, and cerebellar regions, compared to their TD peers. To the best of our knowledge, only one recent study with ASD participants (aged between 5 and 18 years) and TD participants (aged between 5 and 15 years) using an EEG microstate approach has been published. Results from this study revealed the presence of four dominant topographic maps in the resting-state EEG, with statistically significant differences between the individuals with ASD and their TD peers among the temporal parameters evaluated. However, only temporal parameters were compared, and no source localization was performed (61).

Subsequent analyses comparing subgroups within the ASD group with the TD group (see **Supplementary Material**) revealed decreased activation of the prefrontal, premotor, and cingulate regions in CD ASD but not CS ASD, compared to TD children. There was a contrasting increase in activation in the premotor cortex of CS ASD children, compared to TD children. Increased temporal activation was found only in the CD ASD subgroup, whereas increased activation of the cerebellum and parietal cortex was found in both ASD subgroups in comparison to the TD group. Most of the brain regions reported in the current study, where group differences in neural activity were present, have roles in social and non-social executive functioning (3, 10, 11). Given the social nature of the semi-naturalistic stimuli presented, the results are interpreted in the context of social cognition.

The Prefrontal, Premotor, and Cingulate Cortices

We reported decreased activation within the frontal cortex of children with ASD compared to TD children. For Map 3, this decreased activation was found in the left dorsolateral prefrontal cortex (DLPFC, within MFG, BA 9) and in the right premotor cortex (within the SFG/MFG, BA 8). Similarly, for Map 4, children with ASD exhibited decreased activation of the left DLPFC (within the SFG, BA 9) and the right premotor cortex (within the MFG and SFG, BA 6). Additionally, for Map 4, the ASD group showed decreased activation of the left dorsal medial prefrontal cortex (DMPFC, within the medial frontal gyrus, BA 10), compared to children with TD. These results would suggest the presence of alterations within these areas in children with ASD while viewing the social scenes. Recently, using directed functional connectivity analyses, based on electrical source imaging, we found altered connectivity in the theta frequency band within several of the frontal and the cingulate regions while children with ASD were watching movies of biological motion (28). This would suggest that alterations in the spatio-temporal EEG microstates and the directed functional connectivity are already present at early developmental stages in ASD.

The DMPFC is involved in self-referential activities (62), considering the mental states of others, and "theory of mind" (63, 64), and has a general role in social cognitive processing (65). The DMPFC is a region likely to develop abnormally in ASD, possibly resulting in individuals with ASD showing atypical activation of this region on social–cognitive tasks (13). Children with ASD have been shown to have a disturbance of dopaminergic activity within this region (66), and adults with ASD have been shown to have decreased grey matter density within the DMPFC (67). In addition to decreased activation within the DMPFC in Map 4, we reported reduced activation of the ACC (within the ACG, BA 42), compared to TD children. This finding is in line with the existing literature; an activation likelihood estimation meta-analysis of 50 neuroimaging studies of social cognition in children and adults with ASD highlighted the cingulate cortex as one of the main regions with decreased activity compared to TD children and healthy adults (12). The ACC is an important region within the "social brain" and is involved in goal-directed behaviors, including control of saccadic movements during visual orienting (68). Structurally, children and adults with ASD have been reported to have reduced grey matter (15) and white matter (69) volumes within the ACC. Impairments in the functioning of the ACC and DMPFC, together, are thought to be involved in atypical social orienting, and social cognition and may be a substrate for this in ASD (13, 68).

The DLPFC plays an important role in higher cognition, including executive functioning, and has reciprocal connections with the ACC and with regions involved in motor control, such as the premotor cortex and basal ganglia, and in higher-order sensory processing, such as the parietal and temporal cortices (70). The premotor cortex is involved in the selection of movements and in encoding the intention to perform certain movements based on external visual cues (71). Our sample of children with ASD exhibited decreased activation of the left and right premotor cortex (BA 6), but they also exhibited increased activation in the left SFG/MFG gyri (BA 6), compared to the TD group, suggesting a general dysfunction in the premotor cortex of children with ASD. The reduction in activation of the DLPFC and ACC, coupled with dysfunctional activation of the premotor cortex, may suggest an impaired ability to appropriately process the dynamic motion stimuli presented by the Trotro cartoons in our sample of children with ASD. Furthermore, decreased activation of both the ACC and DMPFC in these children during free viewing of the Trotro cartoons suggests a deficit in detecting and/or understanding the social content of the cartoon movies.

The subgroup analysis (see **Supplementary Material**) indicated that impaired activation of the premotor cortex was present in both subgroups in opposing directions; while the CS ASD subgroup showed increased activation of the premotor cortex, the CD ASD subgroup exhibited decreased activation of this region, compared to TD children. This finding suggests that increasing activation of the premotor cortex may be part of the mechanism by which CS ASD children establish gaze patterns

that more closely follow moving targets on the screen, in a similar manner to TD children.

The Parietal Cortex

In contrast to the reduced activation of the frontal, premotor, and cingulate cortices, increased activation of the left IPL was observed in our group of children with ASD compared to those with TD, regardless of their gaze patterns. A magnetoencephalography (MEG) study performed during rest using a source-space approach found altered coherence within parietal regions; however, the ASD group in this study only included adolescents (72). The IPL is an integral part of the mirror neuron system; it is connected with the ventral premotor cortex and plays a fundamental role in processing visual and somatosensory information (73, 74). The mirror mechanism is involved in understanding others' actions and intentions and is located in the same areas that are involved in goal-directed actions, the parieto-frontal network. In brief, the mirror mechanism entails that the motor system is involved not only in producing movements but also in cognitive functions such as observing motor behaviors, and these observations consequently result in motor activation, as if the observer is mirroring the action being executed by someone else (75). The "direct matching hypothesis" maintains that activation of the mirror neuron system upon observing an action is essential to understanding the goal of that action (76). The parieto-frontal circuit involved in the mirror mechanism is thought to be dysfunctional in ASD (16, 75, 77, 78). Our study shows increased activation of the IPL in children with ASD compared to those with TD. A recent activation likelihood estimation meta-analysis reports stronger effects in the IPL of individuals with ASD compared to TD individuals (78). Therefore, our finding of increased IPL activation in children with ASD, regardless of their gaze patterns, may suggest a dysfunction of the mirror neuron system upon observing actions of the animated Trotro donkey characters, which is evident from a young age.

The Cerebellum

The cerebellum has long been recognized for its fundamental role in movement coordination and balance; however, it is also considerably involved in a variety of other functions including cognition, emotion, and perception, such as motion perception [for a review, see Baumann et al. (79)]. Moreover, the cerebellum has an important role in oculomotor control and has connections to regions within the prefrontal and parietal cortices, which are involved in visuospatial attention (80). Thus, it is thought to be essentially involved in controlling covert visual attention (80–82).

Although we found that our group of children with ASD, specifically the CD ASD subgroup (see **Supplementary Material**), showed increased activation within the left anterior and posterior cerebellum, compared to the TD group, these findings should be interpreted with care. Using MEG and an object recognition task, Peiker and colleagues (83) have shown that perceptual integration deficits observed in adults with ASD are related to alterations in the connectivity between the left cerebellum and right posterior superior temporal sulcus. Moreover, increased motor activation of the cerebellum but decreased cerebellar attention activation have been previously reported in adults with ASD during motor and attention tasks, respectively (83). It is possible that our ASD group may suffer from more pronounced cerebellar impairments than TD children and that this has a functional implication on their ability to control their eye movements through the cerebellum, preventing them from assembling visual details into an entire and integrated percept. Over-activity of the cerebellum in the ASD group may suggest an impairment in the ability of the cerebellum to maintain the accuracy of saccades onto visual targets within the Trotro cartoon. Alternatively, as reduced processing of the irrelevant context has been previously reported in older children and adults with ASD (85), increased activation of the cerebellum may be due to the lack of ability of the ASD group to distinguish the socially relevant from irrelevant information in the cartoons.

The Anterior Temporal Lobe

Anterior portions of the fusiform, inferior, and middle temporal gyri form part of the anterior temporal lobe (86), which is thought to play an important role in storing and retrieving social knowledge (87). These regions form part of the social brain, which has been reported to be altered in ASD. Our results showed that children with ASD exhibited increased activation of the right MTG (BA 21), ITG (BA 21), and left FG (BA 37), compared to TD children. However, the subgroup analysis (see **Supplementary Material**) indicated that this increase in activation might only be present in the CD ASD and not CS ASD children, when compared to TD children. Thus, increased activation of these regions may reflect an impairment in the function of the anterior temporal lobule to appropriately process social information from the cartoon stimuli, possibly explaining the deviance of gaze of the CD ASD children from socially relevant parts of the cartoon scenes.

Limitations

This study has several limitations. The sample sizes are small (ASD and TD, n = 14), mainly due to the difficulty of collecting EEG data that are free from movement artifacts from a pediatric population. Owing to the small sample size, the subgroup analysis that took gaze behavior into account was underpowered (CS ASD n = 8, CD ASD n = 6). The results of the subgroup analysis (see **Supplementary Material**) are insightful and interesting; however, they need to be consolidated by future studies with larger sample sizes.

While direct evidence that scalp EEG can capture subcortical signals has recently been produced (88), whether EEG can detect subcortical signals is still a matter of debate. Therefore, it is important to consolidate the present findings, particularly those in deeper brain regions, with future studies using methodologies of higher spatial resolution such as functional magnetic resonance imaging.

CONCLUSIONS

A combination of frontal and parietal processing is thought to be optimal for understanding social situations; however, these areas, amongst several others, have been shown to be

dysfunctional in ASD. Our findings suggest that from a young age, children with ASD, especially those with gaze patterns that diverged from the gaze patterns of TD children, exhibited abnormalities in neural activation during free viewing of dynamic social stimuli, including reduced activation of frontal and cingulate regions and increased activation of inferior parietal, temporal, and cerebellar regions. These results suggest that children with ASD, particularly those with CD gaze patterns, process the visual stimuli differently and fail to detect the social information. Eye-tracking and high-density EEG may be a promising combination that could aid in diagnostic differentiation of different ASD subtypes in the future, possibly leading to more targeted treatment interventions.

DATA AVAILABILITY

The datasets for this manuscript are not publicly available because analysis of these data is ongoing as part of a longitudinal study, and results are expected to be published in the future. When all data has been published, requests to access the datasets should be directed to Dr Marie Schaer, marie.schaer@unige.ch.

AUTHOR CONTRIBUTIONS

RKJ conducted data collection and analysis and the writing of this manuscript, and is the primary and corresponding author. TAR was involved in experimental design, data collection, and analysis, and contributed feedback on the written manuscript. NK conducted participant recruitment and participated in data collection and in the design of the novel eye-tracking analysis method used in the current study. HFS was involved in data collection and contributed feedback on the written manuscript. MF conducted participant recruitment and participated in experimental design and data collection. AC and MIT provided intellectual input on EEG data analysis. CMM was involved in experimental design and provided intellectual input on all aspects of the EEG data analysis and the written manuscript. MS is the principal investigator and was involved in experimental design, participant recruitment, and providing intellectual input on data analysis and feedback on the written manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2019.00582/full#supplementary-material

SUPPLEMENTARY MATERIAL: SUBGROUP ANALYSIS ACCORDING TO GAZE PATTERNS

Method: Eye-tracking Data Collection and Analysis

Simultaneously to EEG data acquisition, high-resolution eye-tracking was performed on all participants using a Tobii TX300 system (Tobii® Technology, Sweden) with a sampling rate of 300 Hz. A data-driven method developed by our group was used to define age-appropriate dynamic "norms" of visual exploration of the complex social scenes presented in the "Trotro" cartoon movies (89). For the calculation of the normative gaze distribution, gaze data from the 14 TD children in the EEG analysis, as well as an additional 12 TD children who did not have EEG data but had good eye-tracking data, were combined resulting in a larger sample size of 26 TD children (mean age 3.4 ± 1.2 years), which was appropriate for defining the "norm". Normative gaze distribution was obtained by applying the kernel density distribution estimation on gaze data at each frame of the "Trotro" movies (90). Following this, the probability of proximity to this pre-established "norm" was computed frame-by-frame for each participant with ASD from our sample. For each participant with ASD, these values were averaged across the four "Trotro" cartoon conditions to obtain a single mean value that described the probability of proximity for that participant to the average gaze pattern of the TD group. Higher values of this measure represented a visual exploration pattern that was similar to that of the TD group, while lower values represented a pattern that was dissimilar or further away from that of the TD group. The gaze patterns differed for each child in the ASD group; some following a pattern close to that of the TD children and some following a completely different pattern. Therefore, a mean-split of the average proximity to the "norm" was used for the ASD group in order to establish two subgroups which did not significantly differ in mean age (t-test, $P = 0.86$) or ADOS severity scores (t-test, $P = 0.30$). The first subgroup had gaze patterns more similar to the "norm", referred to as the control-similar (CS) ASD subgroup (n = 8, mean age 3.3 ± 0.8 years, mean ADOS severity 6.9 ± 1.9), and the second

subgroup had gaze patterns dissimilar to the "norm", referred to as the control-dissimilar (CD) ASD subgroup (n = 6, mean age 3.3 ± 0.8 years, mean ADOS severity 8.0 ± 2.0) (**Figure 1**). The sources of the microstate maps 3 and 4 of these subgroups were then separately compared to the TD group by unpaired randomization tests.

Results: Source Localization of Subgroup Differences

Control-Similar (CS) ASD vs. TD

In comparison to the TD group, the CS ASD group exhibited increased activation of the left IPL (BA 40) for Map 3 (**Supplementary Figure 1**) and Map 4 (**Supplementary Figure 2**), and of the left posterior cerebellum (declive), and left SFG/MFG (BA 6) for Map 4 only.

Control-Dissimilar (CD) ASD vs. TD

For Map 3, the CD ASD group showed decreased activation of the left MFG (BA 9) and right SFG (BA 8), and increased activation of the right MTG (BA 21), right ITG (BA 21), right FG (BA 19), right precentral gyrus (BA 6), and left IPL (BA 40), compared to the TD group (**Supplementary Figure 3**). For Map 4, the CD ASD group showed decreased activation of the left medial frontal gyrus (BA 10)/ACG (BA 32), left SFG (BA 9) and right MFG (BA 6), and increased activation of the left and right anterior cerebellum (culmen), left posterior cerebellum (declive), right posterior cerebellar tonsil, right MTG (BA 21), right ITG (BA 21), left and right FG (BA 37) and left IPL (BA 40), in comparison to the TD group (**Supplementary Figure 4**).

SUPPLEMENTARY FIGURE 1 | Source localisation of group differences for Group Map 3 showing increased activation of the left inferior parietal lobule (IPL) in the control-similar (CS) autism spectrum disorder (ASD) group (n = 8) compared to the typically developing (TD) group (n = 14) during free viewing of "Trotro" cartoons. The t-values of the unpaired t-test thresholded to $P < 0.01$ of the randomization test are plotted. L, Left; R, Right.

SUPPLEMENTARY FIGURE 2 | Source localisation of group differences for Group Map 4 showing increased activation of the left posterior cerebellum (pCrbl), left inferior parietal lobule (IPL), and left superior and middle frontal gyri (SFG/MFG) in the control-similar (CS) autism spectrum disorder (ASD) group (n = 8) compared to the typically developing (TD) group (n = 14) during free viewing of "Trotro" cartoons. The t-values of the unpaired t-test thresholded to $P < 0.01$ of the randomization test are plotted. L, Left; R, Right.

SUPPLEMENTARY FIGURE 3 | Source localisation of group differences for Group Map 3 showing decreased activation of the left middle frontal gyrus (MFG) and right superior frontal gyrus (SFG), and increased activation of right inferior temporal gyrus (ITG), middle temporal gyrus (MTG), fusiform gyrus (FG), precentral gyrus (PCG), and left inferior parietal lobule (IPL) in the control-dissimilar (CD) autism spectrum disorder (ASD) group (n = 6) compared to the typically developing (TD) group (n = 14) during free viewing of "Trotro" cartoons. The t-values of the unpaired t-test thresholded to $P < 0.01$ of the randomization test are plotted. L, Left; R, Right.

SUPPLEMENTARY FIGURE 4 | Source localisation of group differences for Group Map 4 showing decreased activation of the left medial frontal gyrus (MedFG)/anterior cingulate gyrus (ACG), left superior frontal gyrus (SFG) and right middle frontal gyrus (MFG), and increased activation of the left and right anterior (aCrbl) and posterior cerebellum (pCrbl), right middle temporal gyrus (MTG), right inferior temporal gyrus (ITG), left and right fusiform gyrus (FG), and left inferior parietal lobule (IPL) in the control-dissimilar (CD) autism spectrum disorder (ASD) group (n = 6) compared to the typically developing (TD) group (n = 14) during free viewing of "Trotro" cartoons. The t-values of the unpaired t-test thresholded to $P < 0.01$ of the randomization test are plotted. L, Left; R, Right.

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Neurodevelopmental Disorders and Adaptive Functions: A Study of Children With Autism Spectrum Disorders (ASD) and/or Attention Deficit and Hyperactivity Disorder (ADHD)

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Introduction: Autism spectrum disorder (ASD) and attention deficit and hyperactivity disorder (ADHD) are the two most common neurodevelopmental disorders observed in childhood. The DSM-5 accepts a combined diagnosis of ADHD and ASD, while the DSM-IV did not. The aim of this study was to identify and evaluate the adaptive profile of children and adolescents with a diagnosis of comorbid ADHD and ASD, in comparison with adaptive functioning in subjects with a diagnosis of only ASD or ADHD.

Materials and Methods: Ninety-one children (77 boys, 14 girls), aging from 3.1 to 13.4 years (mean age: 8.3 ± 7.2), who met the criteria for a diagnosis of ASD and/or ADHD were enrolled. A neuropsychological evaluation involving cognitive and adaptive assessment was conducted using the Autism Diagnostic Observation Schedule – Second Edition (ADOS-2), the Conners' Parent Rating Scale – Revised: Long Version (CPRS-R), the Wechsler Intelligence Scale – Fourth Edition or the Griffiths Mental Developmental Scales – Extended Revised, the Vineland Adaptive Behaviour Scale – Second Edition (VABS-II).

Conclusion: As to the adaptive skills in the three groups evaluated, a worse general profile was ascertained in the ASD and in ASD plus ADHD groups in comparison with respect to the ADHD-only group. With VABS-II evaluation, we found significant differences among the three groups across all domains and combined scores: Communication ($F = 18.960$; $p < 0.001$), Socialization ($F = 25.410$; $p < 0.001$), Daily Living Skills ($F = 19.760$; $p < 0.001$), Motor ($F = 9.615$; $p < 0.001$), and Adaptive behavior composite [ABC] ($F = 29.370$; $p < 0.001$). Implications of neurodevelopmental double diagnosis such as ASD plus ADHD are discussed.

Keywords: autism spectrum disorders (ASD), attention deficit hyperactivity disorder (ADHD), adaptive function, children, comorbidity

INTRODUCTION

Autism spectrum disorder (ASD) and attention deficit and hyperactivity disorder (ADHD) are the two most common neurodevelopmental disorders observed in childhood (1, 2). According to the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5) (1), ASD is a disorder characterized by deficits in social communication as well as restricted, repetitive patterns of behaviors. In the child population, ASD prevalence has been estimated to be about 1%, but a recently published US study put it at 1.68% (3). Approximately 30% of children with ASD undergo a regression of development with variable course that maybe associated with epileptic abnormalities in an undetermined percentage (4, 5).

Attention-deficit/hyperactivity disorder (ADHD) is one of the most common mental disorders affecting children. Symptoms of ADHD include inattention, hyperactivity, and impulsivity. Current estimated prevalence is 5% of children and 2.5% in adults. ADHD is often first identified in school-aged children when it leads to disruption in the classroom and/or difficulties with school duties. It is more common among boys than girls (DSM-5) (1).

Impairment of social competences in neurodevelopmental disorders is common and needs to be thoroughly addressed. In recent years, there has been increasing interest in the diagnostic overlap and similarities between ADHD and ASD (6–11). Evidence indicated that both disorders co-occur with a high frequency, in 20–50% of children with ADHD meeting criteria for ASD and in 30–80% of ASD children meeting criteria for ADHD. (12). The co-occurrence of ASD and ADHD was found to increases with age, appearing in school age children more clearly, with severity of ASD and ADHD symptoms and with lower IQ (8, 13). Moreover, the increase in inattention and impulsivity concomitant with increases in ASD severity may be able to predict the severity of challenging behaviors and social skills deficits in toddlers and should be carefully evaluated in this population (14–16). In addition individuals with ASD frequently experience additional psychiatric comorbiditties (17).

As to adaptive functions, some studies found that children with ASD+ADHD showed a more severe impairment in adaptive functioning and a poorer quality of life than children with ASD alone (18) while other studies found varying profiles depending on cognitive level and age (19, 20).

A specific social-communication core deficit, associated with restricted and repetitive behaviors (RRBs) is the hallmark of ASD. In contrast to the DSM-IV (APA 1994), the DSM-5 (1) allows a combined diagnosis of ADHD and ASD. Both ASD and ADHD develop from interactions among multiple genetic and environmental factors, which have an effect on complex neurobiological systems already during prenatal life. These interactions are likely to be involved in the distinct developmental trajectories, clinical characteristics, and outcomes of the two disorders (21).

Children with ADHD frequently display peculiar social difficulties. Social competences in ADHD are thought to be related to self-regulation difficulties, low social skills adaptive level, and attentional issues, which can impact the overall ability to process social information. Children diagnosed with predominantly inattentive ADHD (PI) are more passive, less aggressive, less assertive, and less knowledgeable of appropriate social behavior than those diagnosed with combined ADHD (CB). Children with PI are more likely than typical children to be socially neglected, whereas children with CB are more likely to be socially rejected (6). Children with ADHD may have low social impact; their isolation and/or intrusive approaches to other children can be mistaken for unawareness of social rules, as in ASD (22). At the neuropsychological level, both ADHD and ASD present difficulties in executive function (EF), even if EF deficits might differ between the two disorders. Inhibitory dysfunction is characteristic of ADHD, while in ASD central coherence and theory of mind deficits play a major role (23–25). Studies investigating the potential influence of ADHD on ASD have reported contrasting results regarding its influence on autistic symptoms severity (26, 27). Furthermore, several studies have noted that children suffering from both disorders generally present a more severe psychiatric burden. It was observed that children with both ASD and ADHD were more likely to have conduct problems or anxiety or depression symptoms than children with ASD only (28–30). Neurophysiological investigation using event-related potentials (ERPs) on these conditions detected a dissociation between disorders on the basis of distinct stages of emotion processing (31, 32). Further investigations demonstrated that children with ASD and ASD + ADHD showed reduced theta and alpha power on quantitative electroencephalographic studies compared to children without ASD e.g. controls and ADHD (33).

In research on interventions, children with ASD+ADHD undergoing social skills training failed to improve, as opposed to children with ASD only and children with ASD and anxiety disorder (34).

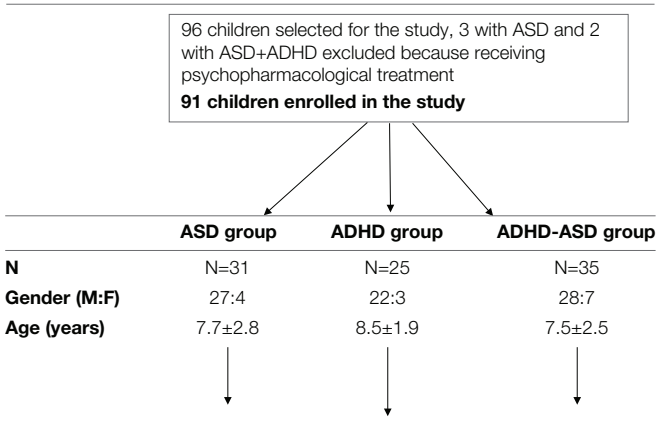
The main aim of this study was to identify and evaluate the adaptive functions of children and adolescents with a diagnosis of ASD+ADHD, in comparison with adaptive functioning in subjects with a diagnosis of ASD or ADHD.

PARTICIPANTS

This cross-sectional study included 91 children (77 boys, 14 girls), ranging from 3.1 to 13.4 years (mean age: 8.3 ± 7.2), who met the criteria for a diagnosis of ASD and/or ADHD (**Table 1**). The children were consecutively recruited between January 2016 and December 2017 among those referred to our outpatient clinic for assessment and diagnosis of developmental difficulties, and were enrolled in the study according to the clinical features that were ascertained during evaluation and that pointed to the mentioned diagnostic domains.

All children underwent a full clinical evaluation, including medical history, clinical observations, and assessment. Diagnosis of ASD was based on DSM-5 criteria. Impairments in intentional communication, eye contact engagement, shared attention behavior, use of gestures, and restrictive and repetitive behaviors were assessed and detailed. Diagnoses were also confirmed by using the Autism Diagnostic Observation Schedule – Second Edition (ADOS-2) (35).

TABLE 1 | Flow chart of the protocol of the study.



ADOS-2 for ASD diagnosis in n= 31+35; CPRS-R to quantify ADHD n= 25+35; WISC-IV for subjects > 6 years or the Griffiths Mental Developmental Scales – Extended Revised for subjects < 6 years; the Vineland Adaptive Behaviour Scale – Second Edition (VABS-II) to rate adaptive functioning

Diagnosis of ADHD was based on DSM-5 criteria. Overall, clinical criteria evaluation contributed to defining the diagnostic classification. In addition to the ADHD features of hyperactivity, impulsivity, and/or inattentive problems, relevant social deficits – if present – were also thoroughly described. We also used the Conners' Parent Rating Scale – Revised: Long Version to confirm the ADHD diagnoses n = 25 (CPRS-R) (Conners 2001).

Exclusion criteria included (1) genetic and neurodevelopmental disorders of known etiology (such as tuberous Sclerosis, fragile X syndrome, and chromosomal abnormalities), (2) serious chronic diseases, (3) significant sensory or motor impairment, (4) presence of seizures, and (5) use of psychoactive drugs as possibly interfering with the clinical profileAccording to the diagnostic criteria, 31 children have been identified as ASD only (ASD group), 25 as ADHD only (ADHD group), and 35 as ADHD and ASD (ASD+ADHD group). Characteristics of the groups are described in **Table 2**.

After complete description of the study to parents and/or guardians, written informed consent for data acquisition and clinical examination was obtained according to procedures approved by the local ethics committee. The research was conducted according to the rules of the Helsinki Declaration regarding good clinical practice and ethics.

The study was approved by our local ethics committee.

MEASURES

All children were examined by our research team. The neuropsychological evaluation involving cognitive and adaptive assessment was conducted by means of a diagnostic protocol in order to identify the main clinical and developmental features and to depict a comprehensive profile of the children enrolled in the study. The protocol included the administration of (1) the ADOS-2 for measuring the severity of autistic symptoms (35) to children with ASD symptoms n = 31 + 35; (2) the CPRS-R to quantify the severity of ADHD symptoms (Conners 2001); (3) the Wechsler Intelligence Scale – Fourth Edition (WISC-IV) (36) for subjects >6 years or the Griffiths Mental Developmental Scales – Extended Revised (GMDS-ER; Griffiths) for subjects < 6 years to evaluate intellectual functioning; (4) the Vineland Adaptive Behaviour Scale – Second Edition (VABS-II) (37) to rate adaptive functioning.

TABLE 2 | Demographic and clinical characteristics of the study groups.

	ASD group	ADHD group	ADHD-ASD group	p
N	31	25	35	
Gender (M:F)	27:4	22:3	28:7	0.625
Age (years)	7.7 ± 2.8	8.5 ± 1.9	7.5 ± 2.5	0.300
IQ	81.4 ± 18.7	103.3 ± 17.0	76.5 ± 20.6	<0.001
VABS-II				
Socialization	71.5 ± 11.2	88.4 ± 7.5	68.6 ± 13.0	<0.001
Communicativon	75.3 ± 11.8	87.3 ± 8.7	71.0 ± 10.0	<0.001
Daily living skills	73.6 ± 8.4	89.4 ± 12.8	70.9 ± 13.6	<0.001
Motor skills	84.5 ± 11.7	95.3 ± 12.1	80.7 ± 14.4	<0.001
ABC	71.9 ± 8.2	86.8 ± 8.0	69.4 ± 10.6	<0.001
CPRS-R (ADHD Index)				
PI		74.3 ± 10.5	78.4 ± 10.7	0.163
PH		68.7 ± 13.2	68.2 ± 12.0	0.893
CB		73.9 ± 12.0	75.6 ± 9.6	0.631
PI : CB		5:20	11:24	0.324
ADOS-2				
ADOS value	16.5 ± 4.9		15.2 ± 4.5	0.244
CSS (comparison severity scores)	7.8 ± 1.7		7.6 ± 1.6	0.622

Quantitative variables presented as mean ± standard deviation. VABS-II, Vineland Adaptive Behaviour Scale, second edition; CPRS-R: Conners' Parent Rating Scale-Revised: Long Version; ADOS-2: Autism Diagnostic Observation Schedule- Second Edition. ABC, Adaptive Behaviour Composite; PI, Prevalent inattentive clinical presentation; PH, Predominantly Hyperactivity/Impulsivity clinical presentation; CB, Combined clinical presentation; CSS, Calibrated Severity Score. Quantitative variables presented as mean ± standard deviation.

The ADOS-2 was administered by experienced and board certified examiners to determine the severity of ASD symptoms. It is a semi-structured, standardized assessment instrument designed to obtain information about social-communication development and repetitive and restricted interests in children. This tool is considered the gold standard for ASD evaluation and is widely used in clinical practice. The ADOS-2 diagnostic algorithm yields classifications of ASD versus non-ASD children and a calibrated severity score (CSS) for the algorithm total that provides further information, including the severity of the disorder.

The CPRS-R is a tool for obtaining parental reports of childhood behavior problems that contains summary scales supporting ADHD diagnosis and quantifying ADH severity. This scale has a seven-factor model composed of the following factors: Cognitive Problems, Oppositional, Hyperactivity–Impulsivity, Anxious–Shy, Perfectionism, Social Problems, and Psychosomatic. It has good internal reliability, high test–retest reliability, and effective discriminatory power. In addition, the CPRS-R includes a corresponding factor structure with the Conners Teacher Rating Scale – Revised and comprehensive symptom coverage for ADHD and related disorders. Three types of ADHD are now recognized: predominantly inattentive (PI), predominantly hyperactive–impulsive (PH) and combined (CB).

To evaluate intellectual functioning and determine the IQ, we administered – according to the age of the child – the WISC-IV or the GMDS-ER. Both scales provide a value that represents the subject's general intellectual ability. These measures are standardized by chronological age, with a mean of 100 ± 15. In this study, we considered IQ indicators to be the Full Scale Intellectual Quotient (FSIQ) for the WISC-IV and the developmental quotient (DQ) for the Griffiths Scales.

The VABS-II is a semi-structured parent interview used to obtain parent ratings of children's adaptive functioning across three domains: Communication, Socialization, and Daily Living skills. Standard scores were obtained for each domain and combined to provide an Adaptive behaviour composite (ABC) standard score. VABS-II scores have a mean of 100 ± 15, with lower scores indicating more severe impairment.

STATISTICAL ANALYSIS

Comparisons between groups were examined, as appropriate, by means of one-way analysis of variance (ANOVA) followed by post-hoc Welch two-sample t-test and Tukey contrasts for multiple comparisons of means, as well as by means of Pearson's chi-squared test, Kruskal-Wallis rank sum test, and Wilcoxon rank sum test with continuity correction. Linear regression model with ABC as outcome variable was used to model several covariates. Variables were entered according to a procedure of forward selection. The first variable entered into the equation was the one with the largest correlation with ABC. The next variables were added according to the largest change in the R 2 statistic, until no more change occurred. The chosen model was the one with the largest R2. Goodness-of-fit statistics are shown: Multiple R 2, Adjusted R 2, F-statistics, standard error of the estimate, and p-value. An alpha level of 0.05 was used for all statistical analyses. Results, if not otherwise specified, are given as means ± SDs. All statistical analyses were performed using the *R* Language and Environment for Statistical Computing program (http://www.R-project.org; accessed October 2018).

RESULTS

As shown in **Table 2**, mean age and gender ratio did not differ between the three groups ($F = 1.221$; $p = 0.300$ and $\chi^2 = 0.939$; $p = 0.625$). IQ was different among groups ($F = 15.140$; $p < 0.001$). The post-hoc analysis showed that IQ was significantly higher in the ADHD group, compared to the ASD ($t = 4.232$; $p < 0.001$) or to the ASD+ADHD group ($t = 5.317$; $p < 0.001$), while there was no significant difference between the ASD and the ASD+ADHD group ($t = 1.048$; $p = 548$).

In terms of parent ratings of children's adaptive functioning, measured by means of the VABS-II, we found significant differences among the three groups across all domains and combined scores: Communication ($F = 18.960$; $p < 0.001$), Socialization ($F = 25.410$; $p < 0.001$), Daily Living Skills ($F = 19.760$; $p < 0.001$), Motor ($F = 9.615$; $p < 0.001$), and ABC ($F = 29.370$; $p < 0.001$) (**Table 3**). Subsequent post-hoc analyses showed that higher mean scores always depended on ADHD either alone or combined, while there were no statistically significant differences between the groups that presented with ASD.

Considering clinical presentations in the two groups presenting ADHD features, in the ADHD-only group we observed 20 CB and 5 PI presentations; in the ASD+ADHD group, 24 CB and 11 PI presentations. The difference between the two groups was not statistically significant ($\chi^2 = 0.974$; $p = 0.324$).

In terms of ADHD symptom severity, the mean score of the Conners Index was 76.7 ± 7.2 in the ADHD group and 79.6 ± 10.9 in the ASD+ADHD group. The difference was not statistically significant ($W = 376$; $p = 0.360$). Overall, none of Conners indexes showed relevant differences between the ASD+ADHD and the ADHD-only group (**Table 2**).

ADOS-2 total scores were similar in the two groups with ASD features (ASD group: 16.5 ± 4.9; ASD+ADHD group: 15.1 ± 4.5) with no statistically significant differences between the two groups ($t = 1.177$; $p = 0.244$).

TABLE 3 | *Post-hoc* analyses for VABS-II values.

Domains	ASD vs. ADHD		ASD+ADHD vs. ADHD		ASD+ADHD vs. ASD	
	t	p	t	P	t	p
Socialization	5.663	<0.001	6.782	<0.001	1.028	=0.561
Communication	4.344	<0.001	6.061	<0.001	1.700	=0.211
Daily living skills	4.969	<0.001	5.992	<0.001	0.946	=0.612
Motor skills	3.067	<0.01	4.314	<0.001	1.165	=0.477
ABC	6.086	<0.001	7.291	<0.001	1.108	=0.512

VABS-II, Vineland Adaptive Behaviour Scale, second edition; ABC, Adaptive Behaviour Composite.

In a linear regression model, higher VABS-II ABC scores were negatively associated with age and ASD diagnosis, and positively associated with IQ. There was no significant association with gender (**Table 4**).

DISCUSSION

Research focusing on co-occurring ADHD and ASD has been directed primarily on origins and clinical characteristics and with minor effort on interventions. Children with ADHD and ASD experience more difficulty in daily situations as compared to those with only one disorder. Co-occurrence of ADHD and ASD is associated with a lower quality of life and poorer adaptive functioning as compared to children with ASD only (38). In addition, co-occurring ADHD and ASD may be less responsive to standard treatments for either disorder than individuals with only one form of the disorders. At present there are few reports regarding developmental trajectories when ADHD and ASD co-occur and it may be important to examine whether early ASD treatment can influence the stability of ADHD symptoms and vice versa (22).

As to the adaptive skills, in the current study a worse general profile was ascertained in ASD and in ASD+ADHD groups with respect to the ADHD-only group in all VABS-II domains (Communication, Daily Living Skills, and Socialization) and ABC. Slightly lower scores not statistically significant were found in the combined group with ASD+ADHD in comparison to ASD group detected with respect to all the VABS-II domains: Communication, Daily Living Skills, and Socialization (37).

Other studies compared adaptive profiles in the three groups of children with ASD, ADHD, and ASD+ADHD and demonstrated the following findings (19, 20). In the study of Ashwood et al. (19), all children had a normal intellectual level representing a selected population and the combined group ASD+ADHD had the worst performance in adaptive evaluation with lowest scores among the three groups enrolled in this research. Further, it raised the question of whether intellectual abilities and social cognition are partly independent and act in different skill domains in ASD profiles. All the children with ASD+ADHD had a cognitive level in the normal range nonetheless demonstrated relevant adaptive difficulties supporting the hypothesis of distinct domains of neurodevelopment within the single child.

Also in the study by Turygin et al. (20), adaptive scores were lowest in the combined group, ASD+ADHD, and children with ASD resulted to be the more impaired among the three groups of study. However, this difference was not found to be significant between the combined and ASD group similar to the findings of the current study. Toddlers with co-occurring ASD+ADHD may represent a group that demonstrates greater early deficits in functioning compared to those with ASD that deserve further studies and follow-up monitoring. As to the cognitive level in the current study a higher range of intellectual abilities was found for the ADHD-only group, the other two groups presented IQ scores between the mean and borderline range of value (ASD: IQ about 81 and ASD+ADHD: IQ about 76). The wider range of IQ level in this sample represents more reliably the ASD population, in which differences in IQ scores are usually observed. Importantly it has been reported that children with ASD+ADHD with lower cognitive level have more severe social impairment, and greater delays in adaptive functioning than children with ASD only (39). In conclusion children with ASD+ADHD demonstrated a more severe adaptive impairment in comparison to children with ASD only even if not reaching statistic significance.

As an additional remark and a future direction in the evaluation of adaptive skills in ASD it is important to note that minimal clinically important differences (MCIDs) on VABS-II scores have not been rigorously established in ASD. To fill that gap a large multisite study has been carried out and lower VABS-II standardized score MCID estimates were observed for younger and more cognitively impaired children. This should be taken in account when evaluating adaptive functions in ASD concomitant to intellectual disability alone or combined with ADHD (40–42,).

There are some limitations in the current study that should be mentioned. Adaptive functions have been detailed in three different clinical groups, but the lack of a typically developing control group is the first limitation to be noted. A second limitation pertains to the relatively small sample size that may have influenced the between-group differences reported as to adaptive and cognitive ability and to the effect of IQ on adaptive function that may be underestimated. A higher number of participants would likely reflect more accurately these differences between the groups. Lastly this is a cross sectional evaluation of the three clinical groups and longitudinal studies of adaptive functioning in ASD+ADHD are strongly needed in order to increase the understanding of the development, change, and stability of symptoms over time and to identify protective and worsening factors of these conditions.

Children with ASD+ADHD had a greater treatment need which could imply additional treatments for both school and community services (26, 43, 44).

When symptoms are not managed, they may lead to more severe psychiatric comorbidity as well as poorer school, family, and cognitive outcomes among this population

TABLE 4 | Linear Regression Model. Outcome variable: Adaptive Behaviour Composite (ABC) of the VABS-II.

	Beta	SE	t	p
(Intercept)	77.567	5.952	13.032	<0.001
Age	–0.871	0.373	–2.336	<0.05
ASD diagnosis	–12.387	2.533	–4.890	<0.001
ASD+ADHD diagnosis	–14.218	2.592	–5.484	<0.001
IQ	0.164	0.048	3.453	<0.001
Gender	0.487	2.723	0.179	=0.858

Overall model: p < 0.001, Multiple R2: 0.505; Adjusted R2: 0.482. F-statistics: 21.7; Residual standard error: 8.46 on 85 degrees of freedom (1 observation missing). SE, Standard Error; VABS-II, Vineland Adaptive Behaviour Scale, second edition.

and so specific attention is warranted to readily provide appropriate intervention.

ETHICS STATEMENT

The study is carried out in accordance with the recommendations of Good Clinical Practice (GCP) guidelines, with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki and its later supplements and local legal requirements. The IRB is the Institutional Review Board, at the University Hospital of Siena approved the study procedure and all study documents.

AUTHOR CONTRIBUTIONS

VS contributed to the design of the study, recruit and made multiple assessments of the children. FB, MS and AP contributed to the evaluation and recruitment of the children. LG participated in the design and the statistical analysis of the study. RC contributed in writing of the article and revised the methods of recruitment and selections of the study groups.

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Sex Differences in Social Adaptive Function in Autism Spectrum Disorder and Attention-Deficit Hyperactivity Disorder

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Background: Social-communication difficulties, a hallmark of ASD, autism spectrum disorder (ASD) are often observed in attention – deficit/ hyperactivity disorder (ADHD), although are not part of its diagnostic criteria. Despite sex differences in the prevalence of ASD and ADHD, research examining how sex differences manifest in social and communication functions in these disorders remains limited, and findings are mixed. This study investigated potential sex differences with age in social adaptive function across these disorders, relative to controls.

Method: One hundred fifteen youth with ASD, 172 youth with ADHD, and 63 typically developing controls (age range 7–13 years, 75% males) were recruited from the Province of Ontario Neurodevelopmental Disorder (POND) Network. Social adaptive function was assessed using the Adaptive Behavior Assessment System-Second Edition (ABAS-II). The proportions of adaptive behaviors present in each skill area were analyzed as a binomial outcome using logistic regression, controlling for age, and testing for an age-by-sex interaction. In an exploratory analysis, we examined the impact of controlling for core symptom severity on the sex effect.

Results: Significant sex-by-age interactions were seen within ASD in the communication ($p = 0.005$), leisure ($p = 0.003$), and social skill areas ($p < 0.0001$). In all three areas, lower scores (indicating poorer function) were found in females compared to males at older ages despite females performing better at younger ages. There were significant differences in the sex-by-age interactions in the social and leisure domains between those with ASD and typically developing controls, with typically developing females showing better scores at older, compared to younger, ages. There were also significant differences in the sex-by-age interactions between ASD and ADHD on the social and leisure domains, as females with ADHD consistently scored higher on social skills than males across all ages, unlike

those with ASD. Sex differences across age in the social domains for ADHD were similar to those in the typically developing group.

Conclusion: Sex differences in social and communication skill areas were observed between ASD and ADHD, and typically developing controls, with females with ASD performing worse than males at older ages, despite an earlier advantage. These findings reinforce the need to take a developmental approach to understanding sex differences which may have diagnostic, prognostic, and treatment implications.

Keywords: autism spectrum disorder, sex differences, attention-deficit hyperactivity disorder, neurodevelopmental disorders, social-communication behaviours

INTRODUCTION

Autism spectrum disorder (ASD) and attention-deficit/hyperactivity disorder (ADHD) are common neurodevelopmental disorders. Autism spectrum disorder is characterized by deficits in social communication, as well as restricted and repetitive behaviors and interests (1). Attention-deficit/hyperactivity disorder (ADHD) is characterized by inattention, hyperactivity, and impulsivity that interfere with function (1, 2). According to both Canadian and US surveillance studies, the prevalence of ASD is about 1.5% [National Autism Spectrum Disorder Surveillance System Report (3)] (4). The prevalence of ADHD is estimated at 5–7% (5). Even though the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5) (1) diagnostic criteria for these disorders appear to show little symptom overlap, the two disorders frequently co-occur. The prevalence of ADHD in individuals with ASD has been reported in the range of 30 to 80%, while ASD is estimated to occur in 20% to 50% of individuals with ADHD (6–8). Moreover, overlapping behavioral traits have been reported in both youth with ASD and those with ADHD, including inattention, hyperactivity, social impairment, and repetitive behaviors (9–13).

Both ASD and ADHD are characterized by a male predominance (14, 15). In epidemiological studies, the male to female ratio in ASD ranges from 1.33:1 to 16:1 (16, 17). The most recent male to female ratio in ASD was reported to be 4:1 (4). The sex ratio varies by cognitive ability, with higher ratios (10:1) in individuals with higher cognitive abilities (IQ) but lower ratios (2:1) in individuals with comorbid intellectual disability (16, 18). In children with ADHD, male to female ratio estimates range from 10:1 to 3:1 in clinical and community samples, respectively (14).

In the context of prominent sex differences in the prevalence of ASD and ADHD, it is important to understand how such sex differences may interact with specific symptom domains. For example, males with ASD have been reported to exhibit more repetitive behaviors than females (19–21) while females with ADHD have been found to have less inattention, hyperactivity/impulsivity, and fewer total ADHD symptoms than males with ADHD (22). A better understanding of as yet underexplored sex differences in symptom domains across ASD and ADHD may help us elucidate the biological underpinnings of these disorders, characterize possible sex-specific profiles, and potentially influence the development of treatments.

Social-communication difficulties, a hallmark of ASD, are often observed in ADHD although not part of its diagnostic criteria. Interpersonal difficulties, peer rejection, and social problems are prominent in ADHD (23, 24). Greater impairments in peer relations (25) and poor friendship quality and stability (26, 27) have been reported. Children with ADHD have few reciprocated friendships, are rated by peers as less-preferred socially, (25) and are more likely to be disliked by their peers compared to typically developing children (28, 29). A systematic review by Kok et al. (30) on social skills in children with ADHD reported social deficits in females with ADHD compared to typically developing female peers. Specifically, females with ADHD experienced less positive peer interactions, and lower rates of friendship participation and stability compared to same-aged typically developing females. Martel et al. (31) reported significant deficits on the social problems domain of the Child Behavior Checklist in children with ADHD compared to controls. Studies using both measures of autistic traits and more global measures of social deficits continue to identify social impairments in ADHD (31–35). Using an autism criteria checklist (32), children with ADHD presented with deficits in the desire to interact with others had problems with non-verbal communication and poor eye contact and had difficulty forming relationships.

Evidence of sex differences in social-communicative abilities is mixed in both ASD and ADHD (5, 36, 37). In the case of ASD, some studies have reported that males with ASD had more social-communication deficits than females (38–42), while other studies have found no sex differences (21, 43–47), and yet another few studies have reported more social difficulties in post-pubescent girls than boys with ASD (48, 49). In the case of ADHD, most of the research has focused on males (50), making it difficult to characterize the role of sex differences. Studies of sex differences in peer functioning among children with ADHD are few and have yielded contradictory results (5, 36). Studies of community samples have shown that females were more likely than males with ADHD to be rejected and disliked by peers; however, studies of clinical samples reported that males had more parent-reported peer problems than females (51, 52), and yet others have found no differences (24, 53).

These inconsistencies across studies could be the result of power issues stemming from small samples of females, variability in measures used, as well as possible changes in symptoms across development. Of note, there are limited studies examining sex differences across age. To date, McLennan et al. (49) study is the

only longitudinal study that has explored sex-specific trajectories in ASD symptoms, where females were found to be more impaired than males in ratings of social function and reciprocal friendships after age 10.

In typically developing children, quantitative and qualitative research has suggested that females engage in more prosocial behavior (54), express greater concern regarding others' feelings (55, 56), and spend more time in dyadic interactions than males (57, 58). Also, females usually have tighter and more intimate social networks and peer relationships than males that involve higher peer attachment (58). Moreover, formation of intimate social groups and group affiliations increases more during adolescence for females than for males (59). Age effects have also been noted, with more improvements in associative play at age 3–4, cooperative play at 4–5 and social interactions with peers at ages 5–6 in females than in males (57).

In summary, research in sex differences in social-communication function in ASD and ADHD is inconclusive. Inconsistencies may be due to variations in methodology, power issues due to smaller female samples, possible changes in skills, and symptoms across development, or may reflect a real lack of robust sex differences. Moreover, most ASD and ADHD research in this area has not included a typically developing control group, making it difficult to determine whether the observed male to female differences are a reflection of typical sex effects across development.

The aim of the current study is to understand the pattern of potential sex differences in social adaptive function in ASD and ADHD and compare them to typically developing controls.

Note that this is a cross-sectional study, and as such any age-by-sex interactions are only suggestive of changes with age. For ease of communication, we occasionally use terms such as "increase," "improve," or "decline," but acknowledge that our findings are not based on longitudinal data.

METHOD

Participants

The present study included children between the ages 7 and 13 years with diagnosis of ASD or ADHD, and typically developing (TD) controls. The data were accessed from the Province of Ontario Neurodevelopmental Disorders (POND) Network database, a research network across five Ontario universities and hospitals (Holland Bloorview Kids Rehabilitation Hospital, the Hospital for Sick Children; McMaster University and the Offord Centre; the Lawson Health Research Institute; and Queen's University). Typically developing controls were volunteers from the community with no first degree relative with a neurodevelopmental disorder. This study was specifically reviewed and approved by an ethics committee. Written and informed parental consent was obtained for all participants under the age of 16.

Measures: Diagnosis of ASD was supported by the Autism Diagnostic Observation Schedule-2 (ADOS-2) (60), and the Autism Diagnostic Interview-Revised (ADI-R) (61). Diagnosis of ADHD was confirmed using the parent interview for child symptoms (PICS) (62). Participants' parents completed the Adaptive Behavior Assessment System-Second Edition (ABAS-II) parent-report measure (63). Intellectual ability (IQ) was estimated using a Wechsler scale (Wechsler Abbreviated Scale of Intelligence (WASI-I or-II) (64, 65), Wechsler Intelligence Scale for Children-4th edition (WISC-IV) (66), or the Stanford Binet Intelligence Scales (67), when a Wechsler scale was not appropriate.

Adaptive Behavior Assessment System-Second Edition (ABAS-II) (63)

The ABAS-II parent-report measure was used in the present study to assess social and communication functions for children diagnosed with ASD, ADHD, and typically developing controls. This parent-report measure assesses an individual's daily adaptive functioning. The measure consists of 10 skill areas: communication, community use, functional academics, home living, health and safety, leisure, self-care, self-direction, social, and work skills. Parents or guardians were asked to assess how often their child engages in a particular activity using a 4-item Likert scale (0—is not able, 1—never when needed, 2—sometimes when needed, 3—always when needed). The present study examined scores on the communication, leisure, and the social skill areas of the ABAS-II questionnaire. We selected the social, communication, and leisure areas, as they are arguably the most relevant to a child's ability to adapt to broadly conceptualized social demands of day–day life. To capture this concept in a way that is not overly cumbersome to the reader, we used the term "social adaptive function" throughout. The ABAS-II communication skill area consists of 24 items and assesses pragmatic language and listening skills. The leisure skill area consists of 22 items that assesses the individual's ability and frequency to plan and organize leisure and/or recreational activities, while the social skill area consists of 23 items assessing peer interaction and ability to form friendships. The test–retest reliability coefficients of the adaptive domains range from 0.80 and 0.90s. The inter-rater reliability coefficients of the GAC (General Adaptive Composite—which is derived from the sum of scaled scores from the 10 skill areas and is thought to represent a comprehensive estimate of an individual's overall adaptive functioning) are 0.91 (ages 10–21), and the average corrected reliability coefficients of the skill areas of each performance level ranges from 0.78 to 0.98 (63). The ABAS-II is a measure, with norms from the general population, which assesses social and communication adaptive functions across a broad range, and is not designed to assess social-communication deficits that are specific to ASD or to any other specific disorder. As the present study includes a cross-disorder analysis, we selected this measure to ensure that the same construct is measured across disorders. Moreover, the ABAS-II measures adaptive or "real-world" social and communication functions (rather than skills or deficits), which provides an index of an individual's competency in everyday contexts.

Measures Used in Exploratory Analyses

To assess whether trait severity (estimated by number of symptoms) is associated with observed sex differences in social adaptive function, the following parent-reported questionnaires were available: the Social-Communication Questionnaire (SCQ; 68) to estimate social and communication deficits (items testing for repetitive behavior on the SCQ were removed from the analysis), Strength and Weakness of ADHD Symptoms and Normal

Behavior Rating Scale (SWAN) (69) to estimate hyperactivity and impulsivity symptoms, and the Repetitive Behavior Scale-Revised (RBSR) to estimate repetitive behaviors.

Analytic Plan

ABAS-II item scores were transformed into dichotomous variables. To accomplish this, scores of 0 or 1 were converted to "0" (corresponding to the absence of a skill), and scores of 2 or 3 were converted to "1" (i.e., the presence of that skill). We then analyzed the proportion of behaviors present in each skill area as a binomial outcome using logistic regression, controlling for age and sex. The advantage of dichotomizing reduces the variability due to parental expectation of appropriate frequency of the skills, which increases our confidence that a particular skill is present or absent. All analyses were performed using SAS 9.4 (2002–2010 by SAS Institute Inc., Cary NC, USA). We first examined the age effect across males and females within each group (ASD, ADHD, and controls) by including an age × sex interaction in the model. Where the interaction was significant, we estimated the sex effect across a range of integer ages to facilitate interpretation of the interaction effect. Where the interaction was not significant, we reported the overall sex effect. We then used the estimated coefficients from the final models to predict the proportion of symptoms at ages 8, 9, 10, 11, and 12 to provide scenarios for graphical representation to help interpret the impact of age-by-gender interaction terms and, when the interaction was not significant, to show the effect of age across both males and females. The graphs (**Figures 1**–**4**) display the probability of getting a score of 1 on any individual item, which also corresponds to the expected proportion of adaptive behaviors present. For example, seen in **Figure 1**, boys scored positively (i.e., positive score of 1, indicating presence of adaptive behavior) on 64% of the items

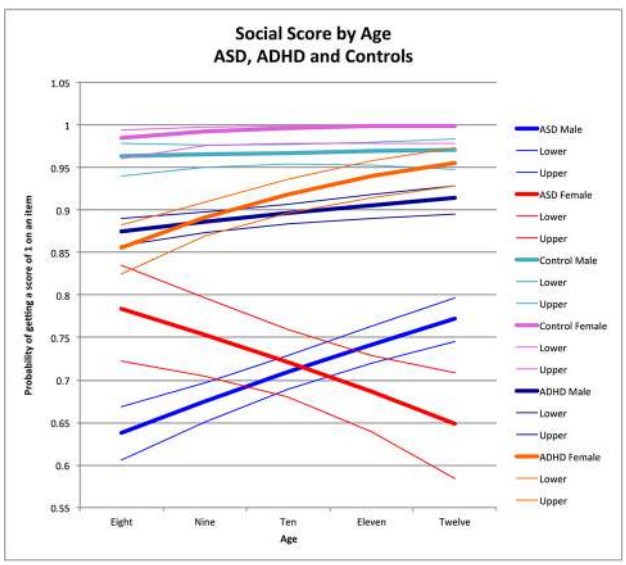

FIGURE 1 | Social score by age: ASD, ADHD, and controls. This graph depicts the probability of obtaining a positive score of 1 on an individual item in the social skill area (indicating skill is present) across ages in ASD (where males are in blue while females are in red), in ADHD (where males are in dark blue and females are in orange), and in typically developing controls (where males are in light blue and females are in pink). Dx, diagnosis; ASD, Autism Spectrum Disorder; ADHD, Attention Deficit Hyperactivity Disorder.

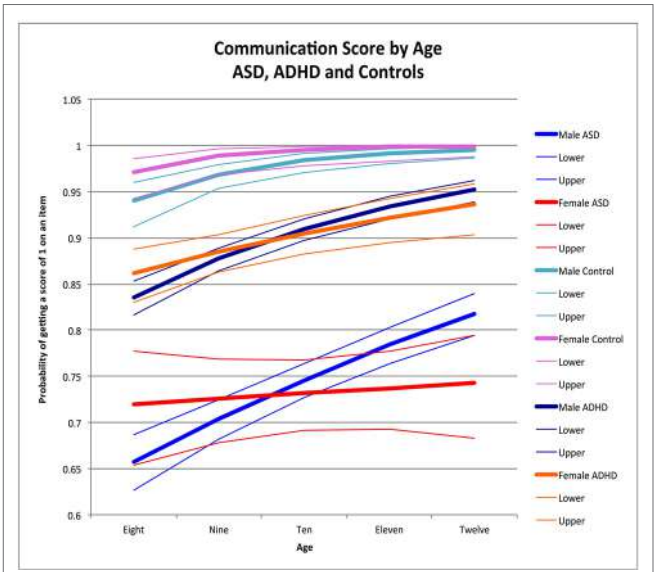

FIGURE 2 | Communication score by age: ASD, ADHD, and controls. This graph depicts the probability of obtaining a positive score of 1 on an individual item in the communication skill area (indicating skill is present) across ages in ASD (where males are in blue while females are in red), ADHD (where males are dark blue while females are orange), and in typically developing controls (where males are in light blue and females are in pink). Dx, diagnosis; ASD, Autism Spectrum Disorder; ADHD, Attention Deficit Hyperactivity Disorder.

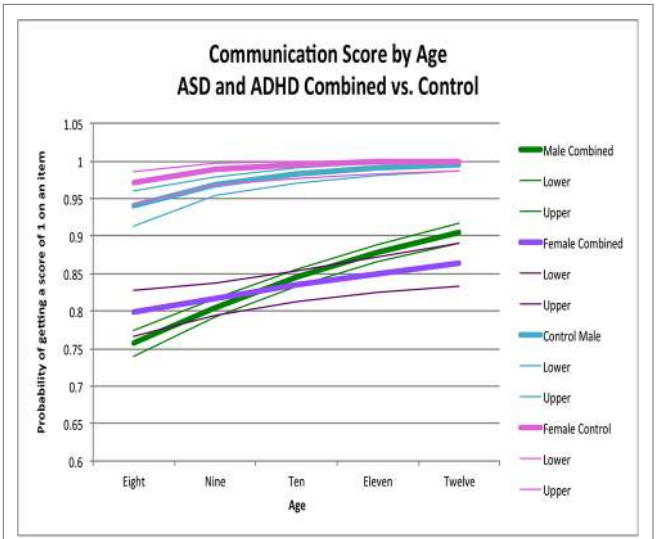

FIGURE 3 | Communication score by age: ASD and ADHD combine versus control. This graph depicts the probability of obtaining a positive score of 1 on an individual item in the communication skill area (indicating skill is present) across ages in ASD and ADHD combined model (where males are in green while females are in purple) and in typically developing controls (where males are in light blue and females are in pink). Dx, diagnosis; ASD, Autism Spectrum Disorder; ADHD, Attention Deficit Hyperactivity Disorder.

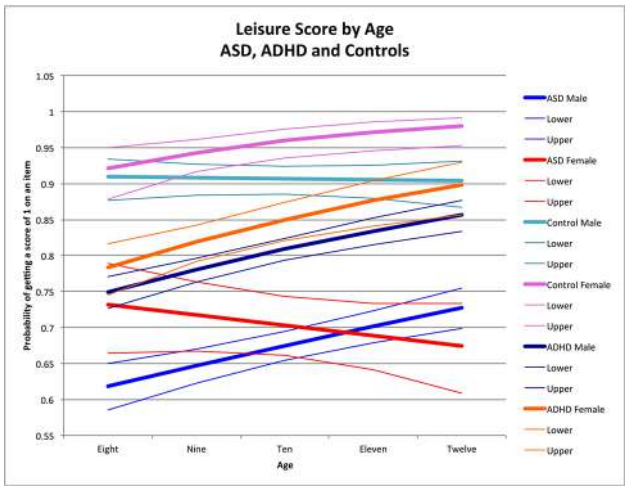

FIGURE 4 | Leisure score by age: ASD, ADHD, and controls. This graph depicts the probability of obtaining a positive score of 1 on an individual item in the leisure skill area (indicating skill is present) across ages in ASD (where males are in blue while females are in red), ADHD (where males are dark blue while females are orange), and in typically developing controls (where males are in light blue and females are in pink). Dx, diagnosis; ASD, Autism Spectrum Disorder; ADHD, Attention Deficit Hyperactivity Disorder.

in the social skill area whereas girls scored positively on 78% of the items.

We then compared diagnostic groups to each other and to controls to determine if sex effects differed between them. Where the age-by-sex effect was significant within one or both diagnoses (dx), a three-way interaction was included in the combined model to test for differences in the age-by-sex effect between the different groups. Where this interaction was significant, the sex effect between diagnoses was then evaluated at different points across the age range to characterize the impact of the three-way interaction. Where the three-way interaction was not significant, the overall sex effect was evaluated using a sex-by-diagnosis (dx) interaction.

If no significant three-way interaction and age-by-sex-by-diagnosis (dx) interaction was found between ADHD and ASD, then both groups were combined into one model for the purpose of comparing to controls.

Exploratory Analysis

To determine whether symptom severity, as approximated by symptom count, influenced sex differences in ASD and ADHD, items of the symptom-/trait-based measures that assess social and communication deficits, inattention and hyperactivity/impulsivity, and repetitive behaviors (SCQ, SWAN, RBSR) were then added to the models. Scores on the SWAN and RBSR were dichotomized to correspond to presence/absence of a symptom, to be comparable to the SCQ. We explored the proportion of variability in the sex effect within and across disorders that was accounted for by the number of symptoms, by inspecting sex-by-age-by-diagnosis interactions in each domain before and after controlling for symptom counts. We examined the effect of adding trait measures to the ASD and ADHD models separately, both visually and by looking at the change in significance level for the sex-by-age interaction where significant. As there are no objective criteria for characterizing the magnitude of the change in sex-by-age effects, we only report these effects qualitatively with a focus on overall trends and not on individual changes.

RESULTS

Study Sample

A total of 350 children were included in the analyses. Sample information and demographics are reported in **Table 1**. The overall age range was 7–13 years, and 75% of the sample was male. No significant differences were noted between males and females in IQ within ASD, ADHD, and controls.

Main Group Differences

Overall, typically developing children outperformed children with ADHD, and both groups outperformed children with ASD across all three domains on the ABAS-II (raw scores—communication skill area $F = 100.8$, $p < 0.0001$; leisure skill area $F = 80.1$, $p < 0.0001$; social skill area $F = 94.6$, $p < 0.0001$) (see **Table 2** for the pairwise comparisons). Females outperformed males on the communication, leisure, and social skill areas in both the ADHD and control groups but not in the ASD group (please see **Table 1** for the demographic information as well as **Supplemental Tables 4**, **5**, and **6** for the mean scores of male and female participants across age).

Sex Differences in Social, Communication, and Leisure Skills in ASD, ADHD, and Controls

Social Skill Area

Older children obtained higher social scores than younger children among males with ASD (male OR for age = 1.18 $p < 0.0001$) (**Figure 1**; note that y-axis depicts the probability of getting a score of 1 on an individual item) and children with ADHD across both sexes (female OR for age = 1.11, $p = 0.002$; male OR for age = 1.35, $p < 0.0001$) (**Figure 1**). In ADHD, there was a significant difference in the effect of age between the two sexes in ADHD ($\chi^2 = 5.47$, $t0.02$); specifically, both sexes had better performance across age, but the magnitude was greater in females (**Table 3**; **Figure 1**). There was a significant negative effect of age in females with ASD (female OR for age = 0.85, $p < 0.0001$) resulting in a significant difference between males and females with ASD ($\chi^2 = 1.18$, $p < 0.0001$) (**Figure 1**). There was no significant effect of age in male and female controls (male OR for age = 1.6, $p = 0.6$; female OR for age = 2.1, $p = 0.07$). Sex-by-age interactions between participants with ASD and controls reached statistical significance (sex-by-age-by-diagnosis interaction: $\chi^2 = 5.37$, $p = 0.02$).

TABLE 1 | Sample characteristics and information.

	ASD			ADHD			Controls		
n	115			172			63		
Comorbid ADHD or ASD	6			2					
Males (%)	**93 (81)**			**128 (74)**			**41 (65)**		
				Mean (SD)					
	Male	**Female**	**T-test**	**Male**	**Female**	**T-test**	**Male**	**Female**	**T-test**
Age	10.1 (1.8)	10.1 (1.7)	t=0.00 p>0.9	9.6 (1.7)	9.3 (1.5)	t=1.03 p=0.3	9.9 (1.6)	9.9 (1.8)	t=0.00 p>0.9
IQ	85.6 (24.1)	88.3 (20.9)	t=0.48 p=0.6	102.4 (16.2)	98.3 (15.6)	t=1.5 p=0.1	108.9 (12.3)	113.2 (10.3)	t=1.40 p=0.2
ABAS communication score	4.7 (3.1)	4.8 (3.5)	t=0.13 p=0.9	7.6 (2.9)	8.7 (3.2)	t=2.1 p=0.04	10.9 (2.3)	12.2 (2.1)	t=2.20 p=0.03
ABAS leisure score	5.7 (2.8)	6.0 (3.0)	t=0.45 p=0.7	7.8 (2.9)	9.4 (3.0)	t=3.12 p=0.002	10.7 (2.5)	13.0 (1.9)	t=3.77 p=0.0004
ABAS social score	3.4 (3.0)	4.0 (3.8)	t=0.80 p=0.4	6.7 (3.5)	8.4 (3.5)	t=2.77 p=0.006	9.8 (3.1)	12.3 (1.8)	t=3.47 p=0.001
SWAN scores	8.5 (5.2)	7.6 (5.3)	t=0.72 p=0.5	10.6 (5.0)	9.4 (5.0)	t=1.37 p=0.2	0.2 (0.6)	0.4 (1.7)	t=0.68 p=0.5
SCQ scores	18.1 (7.1)	17.0 (7.3)	t=0.65 p=0.5	7.0 (5.0)	6.2 (4.8)	t=0.92 p=0.4	3.0 (2.3)	1.8 (1.4)	t=2.23 p=0.03
RBSR scores	8.9 (8.0)	9.2 (8.7)	t=0.16 p=0.9	3.5 (4.9)	3.8 (5.5)	t=0.34 p=0.7	0.1 (0.5)	0.3 (0.9)	t=1.14 p=0.3
ABAS GAC	66.9 (15.2)	66.3 (16.2)	t=0.16 p=0.9	80.2 (14.4)	84.0 (14.2)	t=1.51 p=0.1	96.6 (11.9)	108.6 (11.6)	t=3.84 p=0.0003

SWAN, SCQ, and RBSR scores are totals after dichotomizing the individual item scores into 0 (for absent) and 1 (for present). ABAS-II GAC scores are standardized total scores which summarizes performance across all skill areas on the ABAS-II, except for Work.

SD, standard deviation; ABAS, Adaptive Behavior Assessment System-Second Edition; SCQ, Social Communication Questionnaire; SWAN, Strengths and Weaknesses of ADHD symptoms and Normal Behaviour Rating Scale; RBSR, Repetitive Behavior Scale-Revised; GAC, General Adaptive Composite.

When comparing participants with ADHD to controls, the three-way interaction did not reach statistical significance (sex-by-age-by-diagnosis interaction: $\chi^2 = 1.25$, $p = 0.3$). However, there was a significant sex-by-diagnosis interaction as a result of a strong female advantage in controls compared to females with ADHD across all ages ($\chi^2 = 6.04$, $p = 0.01$; see **Figure 1**)

The sex-by-age interaction in ASD was also significantly different from the sex-by-age interaction in ADHD (sex-by-age-by-diagnosis: $\chi^2 = 24.94$, $p < 0.0001$) with better performance in older children than younger children among females with ADHD, but the opposite effect in ASD, where older females performed more poorly than younger females (**Figure 1**).

TABLE 2 | Pairwise comparisons.

Communication

	Mean difference	Standard error	Confidence interval	Significance
Control vs. ASD	18.83*	1.38	15.5–22.1	p<0.0001
Control vs. ADHD	8.51*	1.30	5.4–11.6	p<0.0001
ADHD vs. ASD	10.32*	1.06	7.8–12.9	p<0.0001

Leisure

	Mean difference	Standard error	Confidence interval	Significance
Control vs. ASD	16.31*	1.30	15.8–22.8	p<0.0001
Control vs. ADHD	9.09*	1.22	5.1–11.8	p<0.0001
ADHD vs. ASD	7.22*	1.00	8.1–13.5	p<0.0001

Social

	Mean difference	Standard error	Confidence interval	Significance
Control vs. ASD	19.27*	1.46	13.2–19.4	p<0.0001
Control vs. ADHD	8.45*	1.38	6.1–12.03	p<0.0001
ADHD vs. ASD	10.815*	1.13	4.8–9.6	p<0.0001

Based on estimated marginal means.

**The mean difference is significant at the 0.05 level, adjustment for multiple comparisons: Bonferroni.*

Communication Skill Area

Older children demonstrated higher communication performance than younger children among males with ASD (male OR for age = 1.24, $p < 0.0001$) (**Figure 2**), male controls (male OR for age = 1.96 $p < 0.0001$) (**Figure 2**), and males and females with ADHD (male OR for age = 1.41, $p < 0.0001$; female OR for age = 1.24, $p = 0.002$), with no significant age-by-sex effect in ADHD ($\chi^2 = 2.57$, $p = 0.1$). In female controls, there was no significant effect of age (female OR for age 2.63, $p = 0.5$), possibly due to ceiling effects occurring after age 9. Similarly, there was no effect of age in females with ASD (female OR for age = 1.03, $p = 0.6$), but there was a significant sex-by-age interaction ($\chi^2 = 8.07$, $p = 0.005$). No significant age-by-sex effect emerged in controls ($\chi^2 = 0.56$, $p = 0.5$) (**Figure 2**).

Sex-by-age patterns and the main effects of sex ($\chi^2 = 0.003$, $p > 0.9$) ($\chi^2 = 0.31$, $p = 0.6$) were similar between ASD and ADHD (**Figure 3**; **Table 3**); we thus combined these groups for

TABLE 3 | Age effects and sex by age interactions for ASD, ADHD, and controls.

	ASD	ADHD	Control
Social skill area	***OR (95% CL)***	***OR (95% CL)***	***OR (95% CL)***
	p value	***p value***	***p value***
Male age effect	1.18 (1.12; 1.24) <.0001	1.11 (1.04; 1.20)	1.06 (0.85; 1.32)
		0.002	0.6
Female age effect	0.85 (0.75; 0.95) 0.0001	1.35 (1.17; 1.57)	2.12 (0.93; 4.84)
		0.0001	0.07
Sex × age interaction χ^2; p value	26.03; <.0001	5.47; 0.02	2.50; 0.1
Diagnosis x sex × age interaction		24.94;	
ADHD vs. ASD		0.0001	
χ^2; p value			
Diagnosis x sex × age interaction		5.37;	
ASD vs. control		0.02	
χ^2; p value			
Diagnosis x sex × age interaction		1.25;	
ADHD vs. control		0.3	
χ^2; p value			
Leisure skill area	***OR (95% CL)***	***OR (95% CL)***	***OR (95% CL)***
	p value	***p value***	***p value***
Male age effect	1.13 (1.08; 1.19) <0.0001	1.19 (1.13; 1.26) < 0.0001	0.98 (0.86; 1.13) 0.8
Female age effect	0.93 (0.83; 1.05)	1.25 (1.11; 1.41)	1.43 (1.11; 1.85)
	0.2	0.0002	0.006
Sex × age interaction χ^2; p value	8.97,	0.58,	6.35,
	0.003	0.5	0.01
Diagnosis x sex × age interaction		6.91;	
ADHD vs. ASD		0.009	
χ^2; p value			
Diagnosis x sex × age interaction		12.29;	
ASD vs. control		0.0005	
χ^2; p value			
Diagnosis x sex × age interaction		3.93;	
ADHD vs. control		0.05	
χ^2; p value			
Communication skill area	***OR (95% CL)***	***OR (95% CL)***	***OR (95% CL)***
	p value	***p value***	***p value***
Male age effect	1.24 (1.17; 1.30) < 0.0001	1.41 (1.30; 1.52) < 0.0001	1.96 (1.42; 2.69) < 0.0001
Female age effect	1.03 (0.92; 1.16)	1.24 (1.08; 1.42)	2.63 (1.29; 5.37)
	0.6	0.002	0.5
Sex × age interaction χ^2; p value	8.07,	2.57,	0.56,
	0.005	0.1	0.5
Diagnosis x sex × age interaction		0.31;	
ADHD vs. ASD		0.5786	
χ^2; p value			
Diagnosis x sex × age interaction		1.41;	
ASD vs. control		0.2340	
χ^2; p value			
Diagnosis x sex × age interaction		1.09;	
ADHD vs. control		0.2996	
χ^2; p value			

OR, odds ratio; odds ratio of greater than 1 indicates more adaptive behaviors at older ages, while odds ratio less than one indicates fewer adaptive behaviours at older ages.

further analyses. When ASD and ADHD were combined into one model, there were significant sex-by-age effects across the pooled sample (χ^2 = 11.22, p = 0.0008). Specifically, females had significantly better scores than males at younger ages (i.e., age 8, OR = 1.27, 95% CI = 1.02-1.58 p = 0.03) whereas males had significantly better scores than females at older ages (i.e., age 12, OR = 0.67, 95% CI = 0.51–0.89 p = 0.005) (**Figure 3**). Sex-by-age effects (χ^2 = 1.61, p = 0.4) and main effects of sex (χ^2 = 2.05, p = 0.4) for this combined ASD+ADHD group were not significantly different than controls in the communication skill area (**Figure 3**).

Leisure Skill Area

Older children obtained higher leisure scores than younger children among males with ASD (male OR for age = 1.13, p < 0.0001) (**Figure 4**), female controls (female OR for age = 1.43 p = 0.006), and both males and females with ADHD (male OR for age = 1.19, p < 0.0001; female OR for age = 1.25, p = 0.0002) (**Figure 4**), with no significant age-by-sex effect in ADHD (χ^2 = 0.58, p = 0.5). There was no age effect in male controls (male OR for age = 0.98, p = 0.8) or in females with ASD (female OR for age = 0.934, p = 0.2) resulting in significant sex-by-age effects in controls (χ^2 = 6.35, p = 0.01) and ASD (χ^2 = 8.97, p = 0.003). Notably,

these age-by-sex interactions were in opposite directions across groups, yielding a significant three-way interaction (age-by-sex-by-diagnosis) characterized by better performance with age in females for the control group, but poorer performance with age in females with ASD ($\chi^2 = 12.29$, $p = 0.0005$) (**Figure 4**). In ADHD, males and females both had better performance with age, but males consistently scored more poorly than females on leisure skills at all age points ($\chi^2 = 0.58$, $p = 0.5$). Notably, although females and males in both groups had better skills with age, the sex differences with age increased more in controls than those with ADHD (sex-by-age-by-diagnosis interaction: $\chi^2 = 3.93$, $p = 0.05$) (**Figure 4**).

The sex-by-age interactions were significantly different between ASD and ADHD (sex-by-age-by-diagnosis interaction: $\chi^2 = 6.91$, $p = 0.0086$) with better scores at older ages than younger ages in females with ADHD but poorer scores at older ages for females with ASD (**Figure 4**).

Exploratory Analyses

Trait scores from the SWAN, SCQ, and RBSR were highly significant predictors of communication, leisure, and social adaptive abilities (all OR's < 1, *p*'s < 0.0001) with higher trait scores associated with significantly lower ABAS total scores, across diagnostic groups.

In the combined sample of all participants with neurodevelopmental conditions, previously reported significant diagnosis by sex-by-age interactions remained significant after controlling for SCQ, SWAN, and RBSR (see **Supplementary Table 1**). However, within ASD, a previously reported significant sex-by-age interaction in the communication domain was no longer significant after controlling for SCQ ($\chi^2 = 2.78$, $p = 0.1$), with a trend noted also in the leisure domain (original sex-by-age interaction in the leisure domain was $\chi^2 = 8.98$, $p = 0.003$ and after controlling for SCQ, sex-by-age interaction was $\chi^2 = 5.10$, $p = 0.02$) (**Supplementary Table 2**). Please see **Supplementary Table 3** to see the influence of SCQ, SWAN and RBSR on sex differences in ADHD for social skill area. For instance as seen in **Supplemental Figures 1–6**, when RBSR was added to the models, sex differences were virtually unchanged with lines representing the log (odds ratio) for sex overlapping those without RBSR in the model. However, we noted changes in the log (odds ratio) after controlling for SCQ in both ASD and ADHD (**Supplemental Figures 1–6**) with the largest changes noted for ASD communication domain. Changes to the log (odds ratio) when adding SWAN to the model were generally smaller than those seen when SCQ was added to the model, with minimal changes to the sex effect.

DISCUSSION

This study is the first to our knowledge to examine sex differences in social adaptive function across ASD, ADHD, and typically developing controls. Controls outperformed (i.e., higher expected proportion of adaptive behaviors present) both ADHD and ASD groups, with ASD males and females performing worse on adaptive function in all three skill areas. We found that social adaptive function was better or stable across age points in all but the girls with ASD, whose social performance was significantly poorer at the older time points when compared to the younger time points. When compared to males with ASD, females with ASD had poorer function at older ages, despite better performance at younger ages. Sex differences in children with ASD and ADHD were similar to each other in the communication skill area, with females having significantly better scores than males at younger ages, while males had significantly better scores than females at older ages. In the leisure area, both females and males with ADHD had higher scores at older compared to younger ages with females having better scores compared to males across all ages. Finally, exploratory analyses revealed that the severity of the social deficit in children with ASD partially accounted for sex differences in performance on the ABAS-II communication, and potentially leisure skill areas.

The present findings suggest a different trajectory for social adaptive function in females than males with ASD. Our findings are consistent with the only longitudinal study to date to examine sex differences in social abilities in ASD (49). In this study, females with ASD showed less impairment in early social behaviors using the ADI-R (i.e., social imitative, play, seeking, and offering comfort) than males, but greater social impairments (i.e., poor friendships) in adolescence and adulthood. Holtmann et al. (48) and Lord et al. (70) also found social difficulties in adolescent females compared to males. These findings suggest that social deficits may start to emerge for girls when social situations become more complex and when social pressures increase in adolescence, as girls may rely more on communication and interpersonal skills compared to males (49), a conceptualization consistent with the DSM-5 articulation of social deficits as social demands exceeding capacity. Another possibility is that there was a cohort effect, wherein the 8-year-old girls had access to better social skills training programs than the 12-year-old girls early in their development. It also remains possible that other symptoms (e.g., anxiety) may have started to interfere with social function in older girls, but these were not examined in the current study. This issue emphasizes the need for qualitative and quantitative research that examines male and female social and communication functions in multiple contexts and diverse/complex situations over time with typically developing peers, to determine unique challenges that females with ASD experience over time. Of note, there were no significant sex differences in ASD in communication, leisure, or social skill performance when collapsed across age, which is consistent with previous studies that found no significant sex differences in social and communication abilities in children within the age ranges 7–12 (43, 44, 47). This highlights the critical importance of examining age effects when exploring diagnostic group differences in behavioral and functional domains across neurodevelopmental conditions. The current findings regarding ASD were not consistent with some previous literature that reported that adolescent females had fewer social difficulties than males (38, 40–42). However, these studies included mostly older adolescents and adults, did not examine age effects, and included smaller samples.

Our findings for the ADHD group were in line with the current hypothesis and were consistent with some past research

that showed more social-communication problems in children with ADHD compared to controls (30, 71). The findings are also consistent with some exiting literature suggesting more peer problems in males relative to females using parent-reported measures (51, 52). However, other studies have reported that females with ADHD were more likely than males to be reported by teachers as being rejected by peers (53, 72) while others found no differences (24, 73). Discrepancies possibly stemmed from use of diverse array of measures and constructs, as well as informants in addition to potential true differences in behavior across settings. Furthermore, some studies either recruited children with no formal diagnosis of ADHD who reported symptoms consistent with ADHD (72, 73) or had children diagnosed with ADHD using the DSM-III criteria (24, 53) and as such may have included a somewhat different population than later studies.

Some limitations of this study are as follows: (1) We may have been underpowered for some comparisons as only 25% of the sample were females, and we had a relatively small typically-developing control group. (2) In addition, parent-reported measures were used to assess social adaptive function, which may be influenced by parental biases and expectations. More importantly, parent reports may miss a lot of nuances in their child's lived experiences. Additional assessments using structured clinical interviews and observational measures would have been desirable to provide a richer understanding of the symptoms and behaviors of children in the sample. Moreover, self-reported measures for older children may be beneficial in understanding the unique needs and perspectives of older males and females. Our method of dichotomizing the item scores may have mitigated some of the variability due to parental expectation of appropriate frequency of skills. However, we acknowledge that this results in some loss of the variability that would be available by examining the full range of item scores. Both strategies have strengths and limitations, and we acknowledge the limitation. (3) The present study did not control for IQ, but we do note that IQ differences between males and females in the present study were not significant (see **Table 3.1**). (4) Most importantly, this study employed a cross-sectional design and does not account for potential heterogeneity in trajectories. We acknowledge that our findings are limited by the cross-sectional nature of this study. A longitudinal design is required to confirm our findings and determine both the onset of symptom manifestation differences between males and females, as well as individual trajectories over time (45). (5) Finally, we recognize that the dichotomy between ASD and ADHD is not as definitive as suggested (particularly given the co-occurrence of ADHD and ASD in the current sample), but this dichotomy was necessary for group comparison purposes.

Clinical Implications

Our study highlights the importance of considering potential sex differences in social adaptive function within and across neurodevelopmental disorders. Understanding such differences will ultimately be critical in both improving the diagnostic/prognostic process, and accounting for variability in presentations in males and females (74). A potential implication of the present findings pertains to treatment planning. The particular pattern observed in females with ASD suggests a female-specific trajectory in social communication, that may imply that social interventions may be needed earlier than might be expected given their apparent competence early on, or potentially that different social interventions may be appropriate for females, although our data does not speak to that. Furthermore, the present study provides a foundation upon which future studies can be built. There is an urgent need for longitudinal studies examining sex differences over time in social adaptive function, given the considerable heterogeneity in this population.

CONCLUSION

This study examined sex differences in social and communication functions in children with ASD and ADHD compared to typically developing children. Our findings confirm social adaptive function deficits in both ASD and ADHD, with both male and female children with ADHD showing improvements with age, whereas females with ASD had poorer function at older ages, despite an early advantage. Findings will enhance our understanding of sex differences in social adaptive function across disorders, both informing our understanding of underlying biology and in identifying/addressing unique needs for males and females with developmental disorders.

DATA AVAILABILITY

All datasets analyzed for this study are included in the manuscript and supplementary files. Also, all data will be available through a public domain release in the last quarter of 2019.

ETHICS STATEMENT

This study was reviewed and approved by the Holland Bloorview Kids Rehabilitation Hospital Research Ethics Board. Written and informed parental consent was obtained for all participants under the age of 16.

AUTHOR CONTRIBUTIONS

TM contributed to conceptualization, did data analysis, and is primarily responsible for manuscript preparation. AD contributed to the data analysis and manuscript. JB participated in the design, of the study, co-supervised data analytic approaches, and revised and edited manuscript. EA supervised all procedures in this study and manuscript. XL, EK, SG, RN, JC and RS made substantial contributions to the conception, acquisition of the data for the work, and revised and edited the manuscript. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2019.00607/full#supplementary-material

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Evaluation of Altered Functional Connections in Male Children With Autism Spectrum Disorders on Multiple-Site Data Optimized With Machine Learning

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No univocal and reliable brain-based biomarkers have been detected to date in Autism Spectrum Disorders (ASD). Neuroimaging studies have consistently revealed alterations in brain structure and function of individuals with ASD; however, it remains difficult to ascertain the extent and localization of affected brain networks. In this context, the application of Machine Learning (ML) classification methods to neuroimaging data has the potential to contribute to a better distinction between subjects with ASD and typical development controls (TD). This study is focused on the analysis of resting-state fMRI data of individuals with ASD and matched TD, available within the ABIDE collection. To reduce the multiple sources of heterogeneity that impact on understanding the neural underpinnings of autistic condition, we selected a subgroup of 190 subjects (102 with ASD and 88 TD) according to the following criteria: male children (age range: 6.5–13 years); rs-fMRI data acquired with open eyes; data from the University sites that provided the largest number of scans (KKI, NYU, UCLA, UM). Connectivity values were evaluated as the linear correlation between pairs of time series of brain areas; then, a Linear kernel Support Vector Machine (L-SVM) classification, with an inter-site cross-validation scheme, was carried out. A permutation test was conducted to identify over-connectivity and under-connectivity alterations in the ASD group. The mean L-SVM classification performance, in terms of the area under the ROC curve (AUC), was 0.75 ± 0.05. The highest performance was obtained using data from KKI, NYU and UCLA sites in training and data from UM as testing set (AUC = 0.83). Specifically, stronger functional connectivity (FC) in ASD with respect to TD involve ($p < 0.001$) the angular gyrus with the precuneus in the right (R) hemisphere, and the R frontal operculum cortex with the pars opercularis of the left (L) inferior frontal gyrus. Weaker connections in ASD group with respect to TD are the intra-hemispheric R temporal fusiform cortex with the R hippocampus, and the L supramarginal gyrus with L planum polare. The results indicate that both under- and over-FC occurred in a selected cohort of ASD children relative to TD controls, and that these functional alterations are spread in different brain networks.

Keywords: autism spectrum disorders, children, resting-state fMRI, functional connectivity, machine learning, ABIDE

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INTRODUCTION

According to the *Diagnostic and Statistical Manual of Mental Disorders*, fifth edition (DSM-5) (1) autism spectrum disorders (ASD) are a heterogeneous set of neurodevelopmental disorders characterized by deficits in social communication and social interaction and the presence of restricted, repetitive behaviors. Updated data on the prevalence of ASD in the United States (Centers for Disease Control and Prevention—CDC) (2) identified 1 in 59 children (1 in 37 boys and 1 in 151 girls) as having ASD. The exact etiopathogenesis of idiopathic ASD is not yet fully established: however, recent evidences point to an interaction between genetic liability and environmental factors in producing early alteration of brain development (3). In this framework, some recent studies have used pattern classification techniques to analyze structural and functional neuroimaging data, in order to highlight brain signatures able to distinguish ASD subjects from controls (4).

Among neuroimaging techniques, resting-state functional magnetic resonance imaging (rs-fMRI) allows to collect brain functional connectivity (FC) data from individuals not engaged in any specific task (5), and thus it is particularly suited to extract information on the functional brain organization of young or non-cooperative or low-functioning ASD subjects (6). In particular, recent rs-fMRI investigations have provided crucial evidence on the disruption of functional networks in individuals with ASD (7–9). However, rs-fMRI findings of subjects with ASD suggested conflicting patterns of FC, with the presence of over FC, under FC and a combination of both (10). Most studies focused on adolescents and adults, where under FC in subjects with ASD has been predominantly observed, and usually found to be related to social impairment (11, 12). The under-FC pattern involves several brain areas, including the salience network, the default mode network (DMN), and language-related regions (11, 13, 14). Conversely, studies carried out on young children have demonstrated that there is an over-FC pattern, detected at whole-brain level and in subsystems (15), in particular in the default mode, salience, frontotemporal, motor and visual networks (16).

The inconsistent results obtained on adults, adolescents and children suggest that the alteration of FC could be partly ascribed to age. Since ASD has an early developmental origin, it is necessary to focus on childhood to be sure that no age-related compensatory mechanisms have already happened (15). Due to the possible age dependence of FC alterations in ASD, it is important to select a specific age range for the cohort of subjects involved in research studies (17). Furthermore, it has been observed that sex impact on both structural (18–20) and functional (21) brain organization in subjects with ASD. Another factor to consider is eye status during scan, which may introduce FC alterations, in particular at local level (22).

Several investigations analyzed the FC with machine learning methods (17–23). These tools are able to learn relevant differences between a group of subjects affected by a specific condition and a control group of subjects with typical development from a dataset (training set) and make predictions on unknown observations (testing set). As a general rule, the greater the number of subjects used in the training phase, the higher the reliability and generalization ability of the classifier. Large data samples are difficult to acquire in a single site, thus they are often obtained by collecting data from multiple sites. In this case, a classifier is trained on a more representative cohort of subjects, therefore, in principle, it can make more general predictions. However, additional sources of variability may affect multicenter analysis, e.g. slightly different acquisition protocols or participant instructions during image acquisition (23), and it has been observed that classification accuracy for multi-site analysis is lower than single-site results (24). Moreover, the site-dependent information encoded in multi-site data may lead a classifier to learn to distinguish categories of subjects according to confounding parameters instead of relying on differences between subjects related to the diagnostic classes.

We explored in this study the FC of subjects with ASD, exploiting the potential of machine-learning approaches to highlight subtle differences between the FC profile of subjects with ASD and controls.

MATERIALS AND METHODS

Sample Composition

We selected a sample of subjects with ASD and controls for our analysis within the publicly-available data sample collected within the Autism Brain Imaging Data Exchange (ABIDE) initiative[1] (25). The main selection of subjects was carried out on participants' age: specifically, we focused our analysis on children in the age range of 6.5 to 13 years to reduce the impact of developmental changes during puberty. Several sites contributing to the ABIDE I collection recruited participants below 13 years of age, except Caltech, CMU and SBL (**Figure 1A**).

In addition to age, participants were chosen according to sex, eye status during the scan, and the available number of subjects with the selected characteristics at each site. Only male children were selected since the male sample size is larger than female across sites (**Figure 1B**), in line with the epidemiology of ASD (26). Scans with open eyes were chosen because they are more numerous and with a low risk of sleep during acquisition time, that can represent an additional source of variability that is difficult either to monitor or prevent (22) (**Figure 1C**).

After the previous selections, only the four most populated remaining sites were analyzed (n = 190; ASD = 102 and TD = 88) (**Figure 1D**): Kennedy Krieger Institute (KKI), NYU Langone Medical Center (NYU), University of California, Los Angeles (UCLA), University of Michigan (UM). Furthermore, the groups of subjects from KKI (ASD = 18, TD = 24), NYU (ASD = 33, TD = 23), UCLA (ASD = 23, TD = 21), UM (ASD = 28, TD = 20) were age-matched.

More details about the impact of selection criteria on the classification performance are reported in Supplementary Materials.

The mean and standard deviation values of age, full scale intelligence quotient (FIQ), ADOS Gotham total and ADOS Gotham severity scores (27) are reported for each site for ASD and TD groups of subjects in **Table 1**. The distributions of clinical and demographic variables are reported in **Figure 2**. The selected site parameters, in terms of vendor, scan duration (28) and diagnostic category are reported in **Table 2**.

[1]http://fcon_1000.projects.nitrc.org/indi/abide/

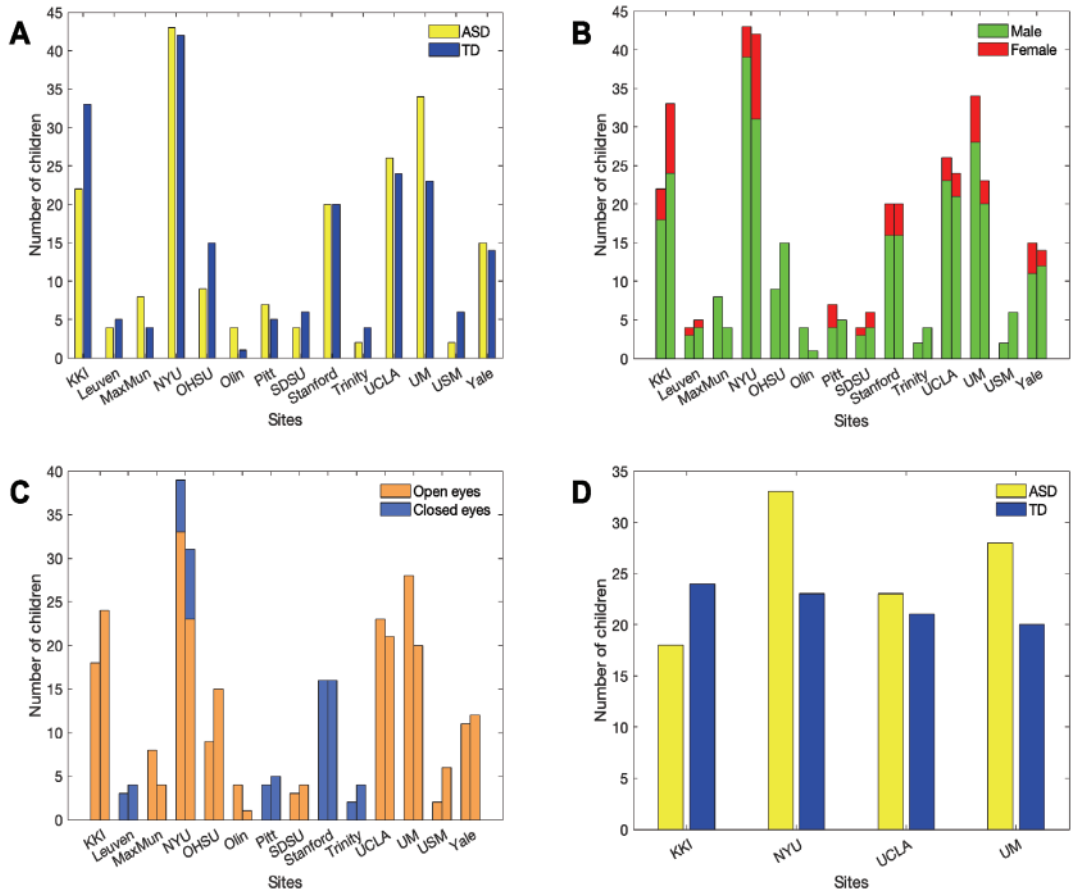

FIGURE 1 | Number of children per ASD and TD groups (yellow = ASD, blue = TD) before **(A)** and after adopting selection criteria regarding sex **(B)**, eye status during scan **(C)** and sample size at each site **(D).**

Resting-State fMRI Data

We analyzed the preprocessed data available on the ABIDE preprocessed homepage (29, 30), using the Configurable Pipeline for the Analysis of Connectomes (CPAC) (31), that includes slice-timing, motion correction, intensity normalization, nuisance signal removal (e.g. tissue signals, low-frequency drifts), and registrations. Band-pass filtering and global signal regression strategies were chosen as processing strategies to reduce the impact of physiological noise and global signal, that includes non-neuronal components and fluctuations in neuronal activity (32). Both anatomical and functional atlases were chosen to derive the FC measures; in particular, we chose Harvard-Oxford (HO) and Automatic Anatomical Labeling (AAL) as anatomical templates and Craddock-200 (CC) as functional templates to extract time series from brain regions (33). The timeseries and the information about labels of regions for each atlas are reported on the ABIDE preprocessed homepage in the pipeline section. Further analysis was conducted using the functional Power template obtained by brain-wide graph analysis (34). For this analysis, we extracted the time series from the preprocessed functional images since they are not directly available on the ABIDE preprocessed homepage.

Functional Connectivity Analysis

For each atlas, the Pearson correlation was calculated between the time series of pairs of regions to obtain a NxN correlation matrix for each subject, where N indicates the number of regions of the selected atlas.

The correlation values were normalized according to Fisher transformation (35), where the number of timepoints is taken into account:

$$Z = \frac{1}{2}\sqrt{n-3}ln\left(\frac{1+r}{1-r}\right)$$

where n is the number of timepoints of time series and r indicates the Pearson correlation values. From each symmetric FC matrix obtained we used $\frac{N(N-1)}{2}$ non-redundant values as features for the machine learning classification.

Machine Learning Based Classification

Supervised binary classification of ASD and TD classes was carried out with Support Vector Machines (SVM) (36), which

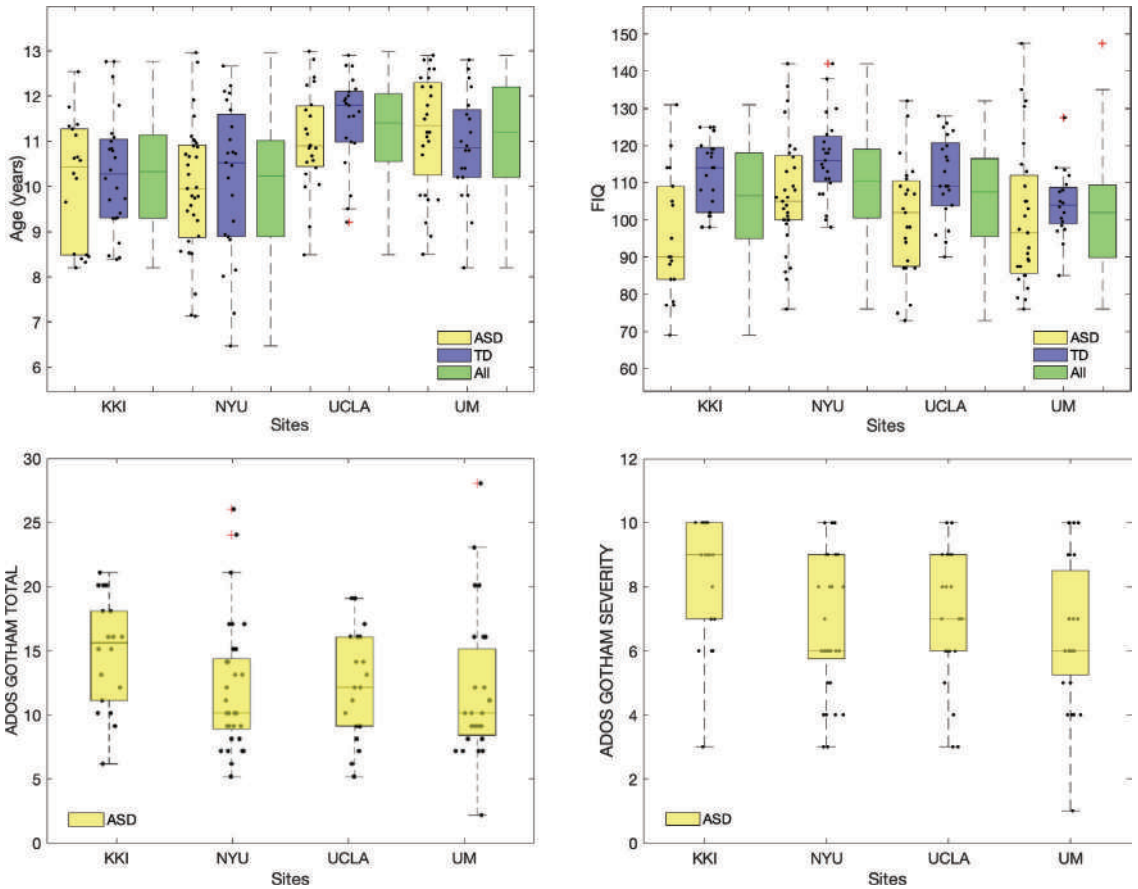

FIGURE 2 | Distributions of age, FIQ, ADOS Gotham total and ADOS Gotham severity scores across sites. Top row: age and FIQ distributions are reported for ASD, TD and all subjects together; bottom row: ADOS total (left) and ADOS Gotham severity (right) scores are shown for children with ASD. Points representing each single subject were overlaid to the box plot. A small random noise has been added on x axis label for each subject in order to make all points visible.

are able to handle noisy and correlated features and can provide better results with respect to other classifiers on dataset with small samples and large number of features (37).

SVM classifiers are able to separate distributions of data in two classes (i.e. ASD and TD) through a hypersurface, described a function according to selected kernel (38). This separation surface is learned from the training set and allows to make predictions on testing set, composed by unknown data. A linear-kernel support-vector-machine (L-SVM) was chosen as it has been demonstrated to provide a more robust performance with respect non-linear kernel SVM when the number of features is large with respect to the number of training cases (39). In addition, L-SVM provide a direct way to interpret the findings: the separating hyperplane is a linear function defined by the weight vectors and an offset. The weights associated with each feature express the direction along which the normalized pairwise correlations differ between two classes: higher weights correspond to more discriminating features; the weight signs allow to identify whether a connection is stronger or weaker in ASD than TD subjects.

Machine-learning based classifiers were implemented to optimize the evaluation of possible altered functional connections in ASD. In particular, the L-SVM classification was carried out on the FC features derived for each parcellation scheme to choose the optimal one among the AAL, HO, CC and Power atlases. In order to reduce the effect of site-specific sources of variability (23), a leave-site out cross-validation scheme was performed: each training set was composed by all the sites except one, that was left out for validation. The classification performance was evaluated in terms of the area under the ROC curve (AUC) (40).

Significant Connections

In order to identify the most significant connections able to discriminate between ASD and TD children, a permutation test was carried out on the entire dataset of children. This non-parametric technique allows to assign statistical significance to the classification.

A L-SVM classifier was trained on the data after 10,000 permutations of the class labels. The absolute values of the obtained weights were compared with the ones of the classifier trained on the correct labels (38, 39). Through this procedure probability maps were generated and thresholded at three different p-values ($p < 0.01$, $p < 0.005$, $p < 0.001$) in order to identify the most discriminating functional connections and to visualize them at different significance levels.

TABLE 1 | Dataset composition and sample characteristics in KKI, NYU, UCLA and UM sites.

Sites	Subject group, mean ± std [range]		Statistical test	
	ASD	TD	Statistic	p-value
	Age (Years)		t-Test (t)	
KKI	10.1 ± 1.4 [8.2–12.5]	10.3 ± 1.3 [8.4–12.8]	–0.51	0.62
NYU	10 ± 1.4 [7.1–13]	10.2 ± 1.7 [6.5–12.7]	–0.52	0.61
UCLA	11 ± 1.1 [8.5–13]	11.5 ± 1 [9.2–12.9]	–1.37	0.18
UM	11.2 ± 1.3 [8.5–12.9]	10.9 ± 1.2 [8.2–12.8]	0.92	0.36
	FIQ		Mann-Whitney Test (z)	
KKI	95 ± 17 [69–131]	112 ± 10 [98–125]	–3.17	<0.001*
NYU	108 ± 16 [76–142]	117 ± 11 [98–142]	–2.58	0.01*
UCLA	100 ± 16 [73–132]	111 ± 11 [90–128]	–2.4	0.02*
UM	101 ± 20+ [73–132]	105 ± 9 [85–127]	–1.49	0.14
	ADOS Gotham total			
KKI	15 ± 4 [6–21]			
NYU	12± 5 [5–26]			
UCLA	12 ± 4+ [5–19]			
UM	12 ± 6+ [2–28]			
	ADOS Gotham severity			
KKI	8 ± 2 [3–10]			
NYU	7 ± 2 [3–10]			
UCLA	7 ± 2+ [3–10]			
UM	7 ± 2+ [1–10]			

ASD, autism spectrum disorder; TD, typical developmental control; std, standard deviation; FIQ, full scale intelligence quotient.
t, two group independent t test statistic between ASD and TD groups mean values.
z, two group independent Mann-Whitney test statistic between ASD and TD groups median values.
**Significant differences between mean (or median) ASD and TD groups.*
+Missing values from some UCLA and UM sites ASD children were removed in calculating the mean and the standard deviation of parameters.

TABLE 2 | KKI, NYU, UCLA and UM characteristics in terms of vendor, scan duration and the diagnostic categories.

Sites	Scanner	Time scan (min)	Participants	
			TD	ASD
KKI	Philips	6.33	24	18
NYU	Siemens Allegra	5.9	23	33
UCLA	Siemens Trio Tim	5.8	21	23
UM	GE	9.8	20	28

ASD, autism spectrum disorder; TD, typical developmental control.

Depending on the weight signs, the alteration in FC are recognized as over FC, in correspondence to positive model weights, and under FC, in correspondence to negative model weights.

Functional alterations in terms over FC and under FC were analysed in the Mesulam subsystems (25, 41), including the connections both between and within heteromodal, unimodal, paralimbic, limbic, primary, and subcortical regions.

Statistical Methods and Analysis Tools

Statistical tests were conducted on age and FIQ values to evaluate the matching between the cohorts of ASD and TD children in each site. Specifically, t-test was conducted on age values and Mann-Whitney U-test on not normal FIQ values. The normality of the distributions of age and FIQ values was evaluated by Shapiro-Wilk test.

Furthermore, statistical differences across sites were evaluated through one-way ANOVA, which was applied on the normally-distributed age values, and Kruskal-Wallis test, which was applied on nonnormally-distributed FIQ and ADOS scores, the latter standardized according to Gotham algorithms (27). In particular, we analyzed the ADOS Gotham total score, which is related to social affect and restricted repetitive behaviour, and the ADOS Gotham severity score, which captures the calibrated autism symptom severity. The statistical tests results were corrected using Bonferroni method for multiple comparison correction.

In order to evaluate the significant functional connections different between each site and the other sites combined together, a Mann-Whitney U-test was carried out. In particular the analysis was conducted only on the control children to avoid to include confounding effects related to the disorder. The p-values obtained were corrected using Benjamini-Hochberg false discovery rate (FDR), taking account for the number of false discovery ($q \leq 0.05$) (42).

Possible correlations between functional connections showing the most significant group differences and autism symptom severity and overall level of functioning have been investigated according to Spearman rank correlation coefficient. Specifically, the relationships between FIQ and FC values were evaluated in ASD and TD groups separately.

Functional connectivity analysis, classifications, permutation test and cerebral maps representation, and the study of correlations between altered FC values and clinical scores were carried out with Matlab 2017a (The MathWorks, Inc.). In particular, in-house built scripts and functions were developed, and, for the SVM classifier training, the *fitcsvm* matlab function has been used, with the default choice of the *c*

parameter – the parameter that regulates the trade-off between having zero training errors and allowing for misclassifications – for the linear-kernel SVM, to avoid running optimization of hyperparameters, which would have required and additional nested cross validation.

Effect of Site and Other Confounding Parameters

The impact of the site and of the other confounding parameters (e.g. sex, eye status) on the performance in the ASD vs. TD machine-learning based classification was evaluated and reported in the Supplementary Materials. A statistical comparison among the FC maps of TD children obtained at the four different sites was also carried out to highlight the impact of the acquisition site on FC information (see **Supplementary Materials**).

RESULTS

Sample Analysis

T-test analysis on age and Mann-Whitney analysis on FIQ values in each site showed that ASD and TD groups are only age-matched whereas no dataset is matched on FIQ, except for the UM sample (**Table 1**). The results of one-way ANOVA and Kruskal-Wallis analyses carried out for each participant's parameter showed that there are significant differences between two or more sites according to age and FIQ. Multiple comparisons, using Bonferroni correction, were conducted for each parameter to identify which sites were different according those parameters. Both KKI and NYU samples showed statistically significant differences from UCLA and UM samples according to age, whereas only the NYU sample was different from the UM sample according to FIQ (**Table 3**).

Functional Connectivity Measures

The FC was evaluated for all children of the four sites using the AAL, HO, CC, and Power atlases (**Figure 3A**). For each child we identified the possible null rows/columns in the FC matrix due to null time series. When the HO, the CC and the Power atlases were applied, null time courses were obtained in some cerebral regions (**Figure 3B**). Specifically, in the temporal, frontal and parietal lobes, close to brain edges. Subjects with at least one null row/column in the FC matrix were identified in the datasets related to HO, CC, and Power atlases and the critical regions were highlighted for each parcellation scheme. These regions are shown in **Figure 3B**, where they are represented as spheres positioned in the centroid of each atlas region with a radius proportional to the number of subjects (n) presenting that critical region. In order to avoid removing regions that may be potentially interesting for ASD diagnosis, we decided to remove the subjects from HO (n = 3) and CC (n = 3) dataset. Regarding Power atlas, since the number of subjects containing critical regions was too high (n = 130), we decided to remove the regions and not the subjects, leaving 230 regions for the classification analysis. Therefore, multisite analysis was conducted on 190 subjects with AAL and Power atlases and on 187 subjects with HO and CC.

The connectivity analysis was carried out on cortical and subcortical regions, excluding the cerebellum. Since Harvard-Oxford atlas does not already have cerebellum areas, we removed them in the other atlases we used. Cerebellar areas were identified through the corresponding labels for the AAL and through the label generated from the overlap between AAL and CC for CC templates; in Power atlas, the cerebellum was identified from the corresponding MNI coordinates. After the previous selections the number of regions were reduced to 90 for AAL, 110 for HO, 184 for CC and 230 for Power atlases, respectively.

TABLE 3 | One-way ANOVA/Kruskal-Wallis analysis for each participant's parameter: age, FIQ, ADOS Gotham total, ADOS Gotham severity. The Bonferroni correction for multiple comparisons has been used. The tests on age and FIQ values have been conducted on the cohorts of subjects including both ASD and TD children of each site.

Variable	N	Statistical test		Group
		Statistic	p value	
Age (years)	48	F = 10.23	<0.001*	KKI-UCLA, KKI-UM, NYU-UCLA, NYU-UM
FIQ	47	χ^2 = 9.51	0.02	NYU-UM
ADOS Gotham total	99	χ^2 = 7.15	0.07	
ADOS Gotham severity	99	χ^2 = 8.05	0.05	

Correlations Between Altered FC Values and Clinical and Cognitive Measures

We tested the possible correlations of the four functional connections showing the most significant group differences with autism symptom severity and the overall level of functioning, and we found the following significant results in terms of Spearman ρ: negative correlations of the FC between the R hippocampus and temporal fusiform cortex with The ADOS total ($\rho = -0.21$, $p = 0.04$) and ADOS severity scores ($\rho = -0.24$, $p = 0.02$); a positive correlation of the FC between L inferior frontal gyrus and R frontal operculum cortex with the FIQ ($\rho = 0.196$, $p = 0.049$).

Classification

The L-SVM classification was carried out on the FC measures of the whole dataset using four different atlases. The classification performances are compared in terms of the mean AUC obtained in the leave-one-site-out cross-validation scheme.

The best performance was obtained using the HO atlas, as shown in **Table 4**. The classification results obtained according to the leave-one-site-out cross-validation scheme of data derived with the HO atlas are reported in more detail in **Table 5**, where, in addition to the AUC, also the sensitivity, specificity and accuracy values are shown. In particular, the highest performance was obtained using FC patterns from KKI, NYU, and UCLA samples in the training phase (n = 139) and leaving out the patterns of the UM sample for the validation (n = 48), as shown in **Table 5**.

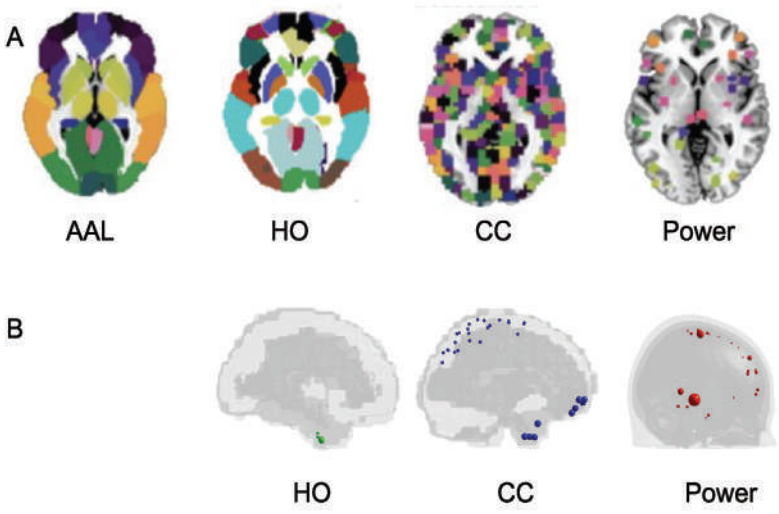

FIGURE 3 | Parcellation schemes used in this analysis **(A)**: Automated Anatomical Labeling (AAL), Harvard-Oxford (HO), Craddock (CC) and Power atlases used in functional connectivity analysis. Regions with null time series obtained in implementing the HO, CC, and Power atlases on data **(B)**. Critical regions are represented as spheres positioned in the centroid of each atlas region, with a radius proportional to the number of subjects presenting that critical region.

TABLE 4 | ASD vs. TD classification performance: the impact of using different parcellation schemes in the leave-one-site-out cross-validation scheme is shown in terms of mean and standard deviation of AUC. For each atlas, the number of descriptive features (m) is reported.

	Atlas, mean ± std			
Classification	**AAL** (m = 4005)	**HO** (m = 5995)	**CC** (m = 16836)	**POWER** (m = 26335)
AUC (%)	72 ± 3	75 ± 5	70 ± 10	64 ± 6

AUC, area under the ROC curve; std, standard deviation; AAL, Automated Anatomical Labeling; HO, Harvard-Oxford; CC, Craddock.

Significant Connections

The functional connections that contribute the most to the discrimination between subjects with ASD and controls were obtained through a permutation test applied to the dataset of children from all sites together (KKI, NYU, UCLA, and UM), including 187 subjects. The list of relevant functional connections between brain regions are reported in **Tables 6** and **7**, and they are shown in **Figure 4** where over-FC and under-FC patterns are highlighted for different thresholds on p values. The altered functional connections are represented in axial (**Figure 4A**), coronal (**Figure 4B**) and sagittal (**Figure 4C**) views. In the top row of each panel the functional connections which are significantly stronger in ASD relative to TD are depicted, whereas in the bottom row the functional connections which are significantly weaker in ASD relative to TD are shown. Each region is represented as sphere positioned in the region centroid, with a radius proportional to the number of connections involving that region and coloured according to the membership in the six functional Mesulam divisions: heteromodal, unimodal, limbic, paralimbic, subcortical, and primary. This representation facilitates the considerations regarding altered connections in and between functional brain areas.

DISCUSSION

The goal of this study was to highlight through machine-learning based techniques possible alterations in the FC of children with ASD in the age range of 6.5–13 years, available within the ABIDE cohort. Several selection criteria were adopted to focus our investigation on a more homogeneous sample of subjects, and thus to reduce the possible sources of variability. Specifically, age, sex, and eye status of the participants are known factors that may introduce heterogeneity in FC. Consequently, this study was focused on male children, in a limited age range, whose rs-fMRI scans were acquired with open eyes. Furthermore, only the four most populated sites were considered. The FC analysis was carried out using different atlases, and machine-learning classifiers were implemented to select the parcellation scheme with the best discrimination performance. Notably, the choice of atlas has an impact on classification performance for two reasons: as the functional signals of the voxels are averaged within a brain parcel, both the region location and its size affect the signal information content and the noise level. The use of the anatomical HO atlas led to a better classification performance with respect to the use of AAL, CC and Power atlases (see **Table 4**). The use of the anatomical HO atlas led to a better classification performance with respect to the use of AAL, CC and Power atlases (see **Table 4**). This result can be explained in terms of a trade-off between the conflicting needs of averaging the functional signals over a non-too-large brain parcels, while keeping acceptable the number of features to classify. A parcellation scheme with a limited number of parcels would generate a manageable number of features to classify, thus avoiding the classifier overfitting problem. By contrast, averaging the functional signal over brain regions that are too large can cause the weakening or disappearance of the signal itself. This

TABLE 5 | ASD vs. TD classification performance obtained for the Harvard-Oxford atlas. The classification performances are reported in terms of sensitivity, specificity, accuracy and AUC for each site left out as validation set in the cross-validation scheme. The mean and standard deviation of all figures of merit over the four sites are also reported (the mean AUC and its standard deviation are also shown in **Table 4**).

	L-SVM				
	Leave one site out				
Classification	**KKI**	**NYU**	**UCLA**	**UM**	**mean ± std**
Sensitivity (%)	67	48	83	79	69 ± 16
Specificity (%)	75	83	61	75	74 ± 9
Accuracy (%)	71	63	73	77	71 ± 6
AUC (%)	71	75	72	83	75 ± 5

AUC, area under the ROC curve; std, standard deviation.

TABLE 6 | List of significantly stronger (ASD > TD) functional connections in ASD children from KKI, NYU, UCLA, UM, obtained for $p < 0.01$, $p < 0.005$, and $p < 0.001$. Beside the Harvard-Oxford labels of the regions defining the connections, lowercase letters are reported in reference to the visual representation of each connection shown in **Figure 4**.

	Significant connections				
Harvard-Oxford regions			**Mesulam subsystems**		**p-value**
		ASD > TD			
R Angular Gyrus (b)	–	R Precuneus Cortex (p)	Heteromodal	Heteromodal	<0.001
L Inferior Frontal Gyrus (pars opercularis) (h1)	–	R Frontal Operculum Cortex (f)	Heteromodal	Unimodal	<0.001
R Inferior Frontal Gyrus (pars triangularis) (h2)	–	R Middle Temporal Gyrus (anterior division) (k1)	Heteromodal	Heteromodal	<0.005
R Precentral Gyrus (o)	–	L Inferior Temporal Gyrus (anterior division) (i1)	Primary	Unimodal	<0.005
R Parahippocampal Gyrus (posterior division) (l2)	–	R Parietal Operculum Cortex (m)	Paralimbic	Unimodal	<0.005
R Amygdala (a)	–	L Inferior Temporal Gyrus (temporo-occipital part) (i3)	Limbic	Unimodal	<0.01
R Inferior Frontal Gyrus (pars opercularis) (h1)	–	R Lateral Occipital Cortex (inferior division) (j1)	Heteromodal	Unimodal	<0.01
L Inferior Temporal Gyrus (temporo-occipital part) (i3)	–	R Lateral Occipital Cortex (inferior division) (j1)	Unimodal	Unimodal	<0.01
R Lateral Occipital Cortex (superior division) (j2)	–	L Frontal Medial Cortex (e)	Unimodal	Paralimbic	<0.01
R Inferior Temporal Gyrus (temporo-occipital part) (i3)	–	R Parahippocampal Gyrus (anterior division) (l1)	Unimodal	Paralimbic	<0.01
L Inferior Frontal Gyrus (pars triangularis) (h2)	–	R Temporal Fusiform Cortex (posterior division) (u)	Heteromodal	Unimodal	<0.01
R Precentral Gyrus (o)	–	R Temporal Fusiform Cortex (posterior division) (u)	Primary	Unimodal	<0.01
R Lateral Occipital Cortex (inferior division) (j1)	–	L Frontal Operculum Cortex (f)	Unimodal	Unimodal	<0.01
R Superior Temporal Gyrus (posterior division) (r)	–	L Supracalcarine Cortex (s)	Unimodal	Unimodal	<0.01
L Subcallosal Cortex (q)	_	L Supracalcarine Cortex (s)	Paralimbic	Unimodal	<0.01

R, right hemisphere; L, left hemisphere.

trend can be appreciated in the classification performances shown in **Table 4**. In particular, the FC values derived according to the HO atlas have higher discriminating power with respect to those of the AAL atlas, which is characterized by 30% fewer regions, and thus the signals are averaged over larger brain areas and have lower specificity in describing brain functioning. When the CC and Power atlases are implemented, the expected increase in the performance with the increase in the number of parcels does not hold anymore. Despite CC and Power are defined according to functional parcellation schemes and thus potentially more informative than the structural-atlas based ones, the excessive increase in the number of features makes the classifier overfit data and lose its generalization capability, causing the decrease in classification performance instead of the expected increase. For these reasons, the best compromise, at least for the data sample considered in this study, was the implementation of the HO atlas. The binary classification results between subjects with ASD and controls in a leave-one-site-out cross-validation scheme achieved an average accuracy of 0.71 ± 0.06 and an average AUC of 0.75 ± 0.05. The best performance was obtained when the training was carried out on FC patterns of children from KKI, NYU, and UCLA, and the classifier performance was evaluated on data from the UM site, reaching an AUC of 0.83. Within the cross-validation scheme, large variability in the results obtained on the different left-out samples were detected (e.g. AUC in the 0.71–0.83 range), as a result of the differences in demographic, clinical and possible data acquisition variability across sites. This variability suggests caution in interpreting the results, and requires their dedicated replication on larger homogeneous cohorts of subjects. The difference across the sites is also evident in the statistical analysis conducted on the average FC matrices between one site and the others combined together (**Figure S1**). Lot of functional connections in UM control children differ significantly from KKI, NYU and UCLA combined together, probably for different site parameters, linked to scanning protocol used. Specifically, as shown in **Table 2**, the scan duration of time series in UM is longer than the KKI, NYU and UCLA same parameter. Nielsen et al. (24) demonstrated that the scan duration is linked to the classification accuracy, since the longer the time series,

TABLE 7 | List of significantly weaker (ASD < TD) functional connections in ASD children from KKI, NYU, UCLA, UM, obtained for $p < 0.01$, $p < 0.005$, and $p < 0.001$. Beside the Harvard-Oxford labels of the regions defining the connections, lowercase letters are reported in reference to the visual representation of each connection shown in **Figure 4**.

Significant connections					
Harvard-Oxford regions			**Mesulam subsystems**		**p-value**
ASD < TD					
R Hippocampus (g)	–	R Temporal Fusiform Cortex (posterior division) (u)	Limbic	Unimodal	<0.001
L Supramarginal Gyrus (anterior division) (t)	–	L Planum Polare (n)	Unimodal	Unimodal	<0.001
L Middle Temporal Gyrus (anterior division) (k1)	–	R Middle Temporal Gyrus (posterior division) (k2)	Heteromodal	Heteromodal	<0.005
R Precentral Gyrus (o)	–	L Angular Gyrus (b)	Primary	Heteromodal	<0.005
R Inferior Temporal Gyrus (posterior division) (i2)	–	L Angular Gyrus (b)	Unimodal	Heteromodal	<0.005
R Precuneus Cortex (p)	–	R Temporal Fusiform Cortex (posterior division) (u)	Heteromodal	Unimodal	<0.005
R Cuneal Cortex (d)	–	L Frontal Operculum Cortex (f)	Unimodal	Unimodal	<0.005
L Cingulate Gyrus (anterior division) (c1)	–	L Cingulate Gyrus (posterior division) (c2)	Paralimbic	Paralimbic	<0.01
R Precuneus Cortex (p)	–	L Parahippocampal Gyrus (anterior division) (l1)	Heteromodal	Paralimbic	<0.01
R Cingulate Gyrus (posterior division) (c2)	–	R Temporal Fusiform Cortex (posterior division) (u)	Paralimbic	Unimodal	<0.01
L Cingulate Gyrus (posterior division) (c2)	–	R Temporal Fusiform Cortex (posterior division) (u)	Paralimbic	Unimodal	<0.01

R, right hemisphere; L, left hemisphere.

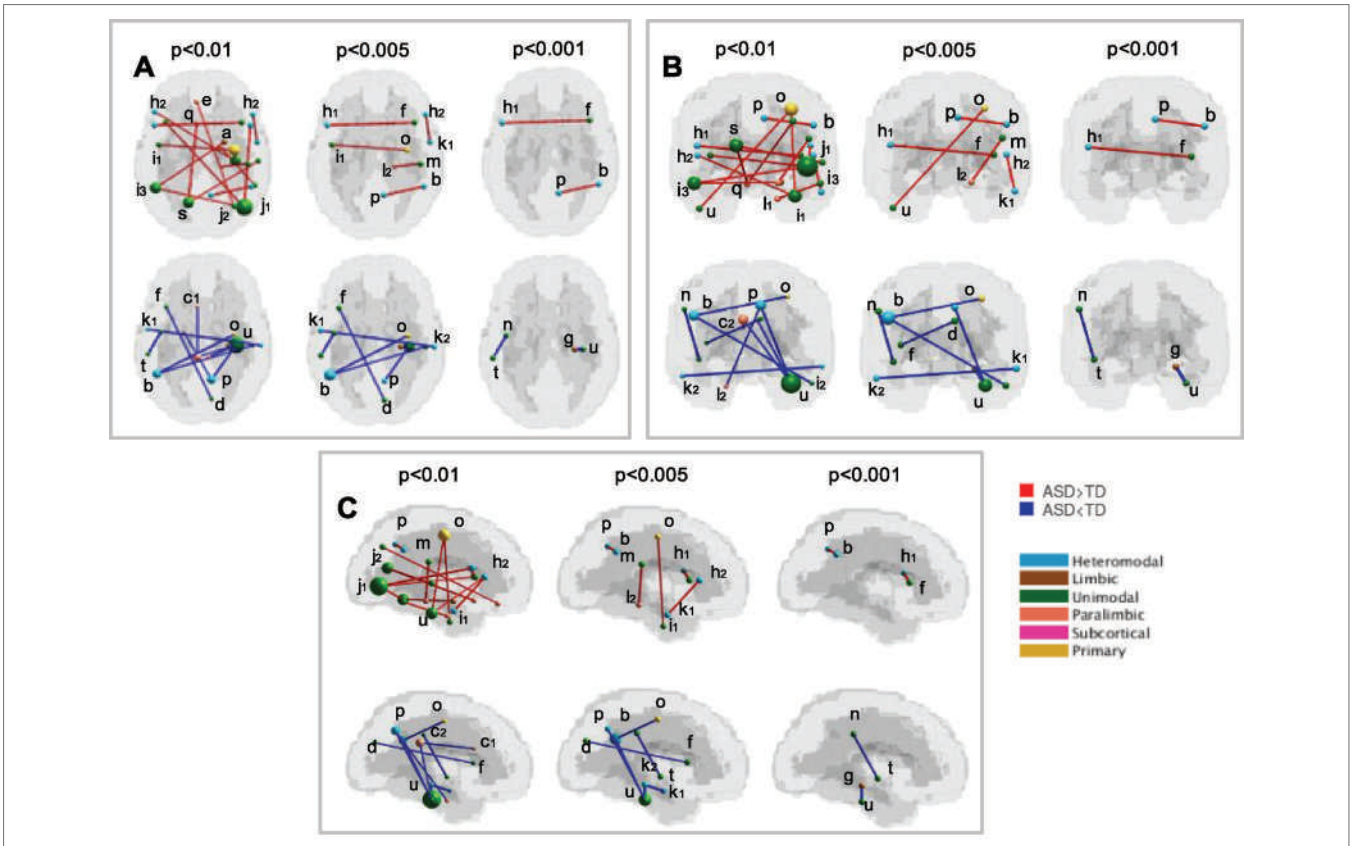

FIGURE 4 | Significant functional connections in the discrimination between subjects with ASD and typical controls obtained using the HO atlas. Altered connections are shown in axial **(A)**, coronal **(B)** and sagittal views **(C)**. In each view, the over-connectivity (top row) and under-connectivity (bottom row) patterns in ASD children in and between the functional Mesulam divisions are shown for different thresholds on significance levels ($p < 0.01$, $p < 0.005$, and $p < 0.001$). The membership of each region to one of the Mesulam division is highlighted by color code applied to a sphere positioned in the region centroid, whose radius is proportional to the number of altered connections involving that region. Lowercase letters are reported to indicate each region, in reference to the reference to the results reported in **Tables 6** and **7**.

the better the performance achieved. The best performance we obtained on the UM site data in the cross-validation scheme is also consistent with the following interpretation: the composition of the samples collected at each single site is not equivalent in terms of the information it provides on the ASD condition. Moreover, none of the four samples we considered is large enough to represent the entire population of children with ASD, which is intrinsically extremely heterogeneous in terms

of etiopathogenesis (43), neuroanatomical alterations (44), and phenotypic expression (45). In addition, if the population with ASD was sufficiently represented by these data samples, we would have obtained a lower standard deviation in the leave-one-site-out cross-validation results.

Our rs-fMRI analysis identified both over- and under-FC patterns in the ASD group relative to controls. This result could be interpreted from a developmental perspective (46, 47), considering that both children – in which generally over-FC prevails (15, 48) – and preadolescents/early adolescents – in which under-FC is more frequently reported (9, 11) – are present in our sample. However, other recent studies suggest the coexistence of over- and under-FC in the brain of subjects with ASD, independently of their age (49, 50). The absence of the adult population did not allow us to verify this hypothesis in our sample.

Specifically, we detected increased FC within DMN (between the angular gyrus and the precuneus) in ASD individuals compared with controls, in line with some previous investigation (16, 48). Interestingly, a study that investigated age-related changes in FC by dycothomizing their sample into younger (6- to 9-year olds) and older subjects (10- to 17-year olds) identified reduced FC between DMN nodes in the older group only, and that this FC in the DMN increased with age in the TD controls, but not in the ASD children, providing support for the "developmental disconnection model" of ASD (51).

A stronger connection between anterior and posterior areas of the brain (e.g. middle temporal and inferior frontal; lateral occipital, and frontal operculum) was detected in our sample. Strikingly, the opposite pattern – under-connection between anterior and posterior areas of the brain for ASD subjects – was identified in a recent investigation that has applied deep learning algorithms to the ABIDE dataset (52). Unlike the current study, Heinsfeld and colleagues (52) did not restrict the analysis to a limited age range: therefore, the opposite direction of correlation between antero-posterior regions could be partly ascribable to the different ages of the samples (adults vs. children).

Among the brain regions in which an increase in FC was detected, it is important to consider the inter-hemispheric connections between the inferior frontal gyri – IFG – (i.e. L pars opercularis with R frontal operculum), since these areas are critical for speech expression, that is frequently impaired in ASD individuals, but also for higher-level social cognitive abilities, such as theory of mind and empathy, typically compromised in ASD. Interestingly, even if all the participants of the current study fall into a near-average FSIQ, we detected a significant positive correlation between the level of cognitive functioning as measured by full-scale intelligence quotient and increased left-right IFG FC. Other studies have also found correlations between cognitive abilities and FC in ASD. For example, Reiter et al. (53) found significant under-FC within the DMN and the visual ventral stream in lower-functioning ASD children compared with matched higher-functioning ASD, while Linke et al. (54) showed that reduced interhemispheric connectivity between auditory cortical areas was correlated with lower verbal IQ. Conversely, some investigations did not report any impact of IQ levels on FC results [9, 55 (i.e. Weng 2010 and Salmi 2013)].

An opposite pattern compared to what we have identified, and thus characterized by weak FC in IFG and other language-related brain regions, has been observed in toddlers with ASD, and was correlated with impairment in expressive language ability (56). Under-FC involving interhemispheric Broca's area was also reported in adolescents with ASD and clear comorbid language impairment (14), suggesting a role of altered FC in communication deficits of subjects with ASD. Of note, in a recent whole-brain meta-analysis of rs-fMRI investigations in ASD, the IFG is one of the few brain regions in which resting-state activity was increased (57).

Although with a lower statistical significance ($p < 0.01$), increased FC is also displayed within the temporal cortex of subjects with ASD -between the R inferior temporal gyrus and the R parahippocampal gyrus-. Crucially, an increased local FC in these regions was found in high-functioning adolescents with ASD and was correlated with higher core ASD symptom severity (58). Moreover, a similar pattern of local functional over-FC in posterior brain regions including the parahippocampal gyrus was reported in a mixed group of children and adolescents with ASD (59). This regional pattern of over-FC in posterior brain areas involved in visual processing is consistent with preference for local over global visual processing repeatedly observed in individuals with ASD (60, 61).

Importantly, among the under-FC findings, we observed lowered FC between R hippocampus and R fusiform cortex. In line with this finding, the fusiform and the hippocampus – together with the amygdala – belong to the facial memory regions, i.e. structures that are implicated in the memory for faces, an ability particularly impaired in subjects with ASD (62). Alterations in the fusiform–hippocampal cortex emerged also from studies investigating the anatomy (63), the structural connectivity (64), and the FC (65) of individuals with ASD relative to TC. Moreover, insofar as brain–behaviour relationship is concerned, the reduced connectivity between the hippocampus and the fusiform cortex in the ASD group is related to ASD symptom severity (assessed by the Autism Diagnostic Observation Schedule, total and calibrated severity scores, with higher scores indicating greater impairment). Therefore, our results support an impaired connectivity in the brain systems underlying social cognitive skills that is more pronounced in children with more severe ASD core symptoms, suggesting a direct involvement of FC abnormalities in the ASD pathophysiology. Further, the weaker connection between supramarginal gyrus (part of the inferior parietal lobule) and planum polare (part of the superior temporal gyrus) contributed most to differentiating ASD from TD controls. Notably, these regions belong to the DMN, which has been suggested to be involved in social cognition, theory of mind (66, 67), self-evaluation, and introspection, and whose disruption has been consistently reported in subjects with ASD (7, 68, 69). Therefore, reduction in resting state FC in regions of the DMN might underlie some of the core features associated with ASD. Not unexpectedly, other pivotal hub of the DMN, such as the middle temporal gyrus, the parahippocampal gyrus, the posterior cingulate gyrus, the precuneus, and the angular gyrus are part of the weaker connections we found in children with ASD.

Among the weaknesses of the present study is the limited number of subjects in the sample. We focused on children in the age range of 6.5–13 years, thus strongly reducing the number of subjects available in the ABIDE preprocessed sample. In addition, due to the possible additional heterogeneity factors related to gender and eyes status, we restricted the analysis to males whose scans were acquired with open eyes. Only the four more populated sites satisfying all these conditions were considered for the analysis. The choice of applying narrow selection criteria-thus restricting the analysis to a sample of less than 200 subjects-derives from the need of reducing the heterogeneity factors only to those intrinsically related to the ASD condition. In this framework, we could not assess the impact of sex on altered functional connections, due to the exiguous number of female children in the ABIDE cohorts, and to the unbalanced amount of subjects with ASD and controls at each site (see **Figure 1A**). We provided in the Supplementary Materials a confirmation of the fact that the ASD vs. TD discrimination ability of the classifier increases when increasingly stringent selection criteria are applied. The augmented classification performance in the proposed cross-validation scheme corresponds to better generalization capability of the classifier, which is consistent with a reduced heterogeneity in the multisite cohort.

Despite the restriction criteria adopted on the whole sample, the remaining four cohorts show demographic characteristics that are significantly different across sites, as shown in the ANOVA and Kruskal-Wallis analyses carried out on ASD and TD children (**Table 3**). The different characteristics of the cohorts become visible in the ASD vs. TD discrimination results reported separately on each site in the leave-one-site-out cross-validation scheme (see **Table 5**).

In addition to the characteristics of the population analyzed at each site, other effects may have had an impact on the classification results. To demonstrate the strength of the impact of the site provenience on the classification, we reported in the Supplementary Materials the 4-class L-SVM classification performance of the FC patterns of the TD of the four sites, which reaches an accuracy of 0.94. Since site-related heterogeneity factors play an important role in classification results, it might be appropriate to restrict multisite analyses only to sites that present similar characteristics, for example in terms of scan time duration and scanner vendors. Other approaches could be to consider the site as covariate and regress out the multisite variability from the analysis, or to use advanced techniques to filter out site heterogeneity (70). Other multisite trials, related to other brain diseases, recommend a standardization procedure across sites, including, for example, post-acquisition corrections of image artifacts (71).

A possible limitation of this study, which is related to the size of the sample we considered, is the risk of overfitting during the classifier training. The number of FC features derived using a parcellation atlas with N regions scales as $\sim N^2$ thus a compromise should be achieved between the desired granularity of the signal localization and the risk of overfitting, which affects the classifier training when the number of features exceeds the number of available cases. As the latter risk affects all the classification experiments in our analyses, regardless the atlas we used, we adopted the linear-kernel SVM classifiers, which have demonstrated robust generalization performances even in case of small training sets with respect to the number of features (39). Feature selection criteria could also be considered to reduce the risk of overfitting; however, better results are not always guaranteed, due to global effects that may influence the FC (23). Whole-brain feature selection approaches based on L-SVM recursive feature elimination (SVM-RFE) may be attempted (72).

Provided these limitations, it is straightforward that the significant altered connections we found are specific of this data sample and therefore not generalizable to female population, to low-functioning individuals, and to subjects with a different age-range. Our analysis suggests the need to collect more populated data samples, which have to be properly stratified in order to reduce the known sources of heterogeneity that may affect the investigation.

CONCLUSION

In conclusion, the use of machine learning techniques has allowed the identification of few significant altered functional connections in children with ASD with respect to controls. Despite an average performance of AUC = 0.75 is achieved in ASD vs. control classification in the leave-one-site-out cross-validation scheme, the classification performances obtained on each single site are highly variable, with AUC values in the 0.71–0.83 range. In particular, for one of the samples (UM), subjects with ASD and controls can be very effectively differentiated (AUC = 0.83) by using the FC patterns learned on the other three sites.

In multisite retrospective studies, selecting sites with similar scanning protocol and restricting the FIQ and age ranges of participants is a prerequisite to limit the impact of confounding factors in the results of the analysis. Nevertheless, these restrictions do not guarantee that the populations represented at each site contribute similar information to the analysis, especially in the case of limited numerosity of the sample and highly heterogeneous conditions.

Despite these considerations, the present study highlighted a set of functional connections that are altered in children with ASD with respect to TD controls. Both over- and under-FC patterns have been detected, confirming the coexistence of mixed FC findings not only in ASD subjects in a wide age range (73), but also within a selected, homogeneous sample of ASD children.

DATA AVAILABILITY

Publicly available datasets were analyzed in this study. This data can be found here: http://fcon_1000.projects.nitrc.org/indi/abide/

ETHICS STATEMENT

The publicly available data resource ABIDE preprocessed (http://fcon_1000.projects.nitrc.org/indi/abide/) has been used in this analysis.

AUTHOR CONTRIBUTIONS

SC and AR designed the study; GS, PB, EF, LP, and PO carried out data processing and analysis; FM and SC interpreted the results; GS, AR, and SC drafted the manuscript; GS edited the manuscript; all authors revised and approved the content of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2019.00620/full#supplementary-material

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Autism Spectrum Disorders and Perinatal Complications—Is Oxidative Stress the Connection?

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Background: Autism spectrum disorders (ASD) are complex psychiatric disorders, with gene environment interaction being in the basis of their etiology. The association of perinatal complications and ASD is well established. Recent findings suggested that oxidative stress and polymorphism in genes encoding antioxidant enzymes might be involved in the development of ASD. Glutathione transferases (GSTs) have an important role in the antioxidant defense system. We aimed to establish whether the predictive effects of prenatal and perinatal complications (as possible oxidative stress inducers) on ASD risk are dependent on GST polymorphisms.

Methods: The study included 113 ASD cases and 114 age- and sex group-matched healthy controls. All participants were genotyped for GSTA1, GSTM1, GSTT1, and GSTP1 polymorphisms. The questionnaire regarding prenatal and perinatal risk factors and complications was administered for all the subjects in the study.

Results: The evaluated perinatal complications as a group significantly increased the risk of ASD [odds ratio (OR) = 9.415; p = 0.000], as well as individual perinatal complications, such as prematurity (OR = 11.42; p = 0.001), neonatal jaundice (OR = 8.774; p = 0.000), respiratory distress syndrome (OR = 4.835; p = 0.047), and the use of any medication during pregnancy (OR = 2.413; p = 0.03). In logistic regression model, adding GST genotypes did not modify the significant effects found for prematurity and neonatal jaundice as risk factors in ASD. However, there was a significant interaction of GST genotype with medication use during pregnancy and the use of tocolytics during pregnancy, which was predictive of ASD risk only in carriers of *GSTM1-null*, as opposed to carriers of *GSTM1-active* genotype.

Conclusion: Specific perinatal complications may be significant risk factors for ASD. *GSTM1* genotype may serve as a moderator of the effect of some prenatal factors on the risk of ASD such as using medication during pregnancy. It may be speculated that different oxidative stress-related genetic and environmental factors could lead to development of ASD. Apart from etiological mechanisms, possible therapeutic implications in ASD are also discussed.

Keywords: autism, perinatal complications, oxidative stress, prematurity, glutathione transferase

INTRODUCTION

The increasing prevalence of autism spectrum disorder (ASD) has led to an increase in interest for environmental factors and their potential influence (1). By definition, environmental risk factors are those non-genetic factors that lead to development of a disorder in individuals with a genetic susceptibility (2). Recognizing the impact of environmental factors, as well as gene–environment interactions in persons at risk, may be of great importance for prevention and treatment of ASD (3, 4)

Multiple studies explored the effect of prenatal and perinatal factors on the risk of ASD. They explored various factors, using different criteria, and getting different results (5).

Maternal age was found to be significant in several large studies, (6, 7), while the most recent study showed a different finding—the risk for ASD might increase if the mother is younger (8). It is argued that maternal age might have a direct effect on ASD risk—possibly by epigenetic changes (9), and also might increase the risk for perinatal complications in general (10). The results for paternal age and the risk of ASD were more consistent—a larger number of studies showed it might be significant risk factor (6, 7, 11–13), although there are studies with different results (5).

The effect of medication during pregnancy is well established (8, 13, 14). The studies exploring the use of medication during pregnancy and the risk of ASD were mostly oriented towards mood stabilizers and antidepressants (15, 16). There are only few studies exploring the effect of other medication during pregnancy. A meta-analysis done in 2009 also showed that the use of medication during pregnancy increased risk of ASD, 1.46 times. Also, the study by Dodds et al. confirmed that the risk for ASD increases 2.66 times with prescribed medication (in this study mostly lithium, antihypertensives, antidepressants, and anticoagulants) (17).

Prematurity was also identified as a significant risk factor for ASD (5, 12, 13, 18). Surprisingly, a recent large study did not prove prematurity to be a significant risk factor for this group of disorders (8). Asphyxia at birth as well as respiratory distress syndrome (RDS) have also been established as ASD risk factors (5, 12, 17, 19, 20). Several studies confirmed low birth weight (LBW) to be a significant ASD risk factor as well (21, 22), but other results were conflicting (5). Conflicting results were also found for intracranial hemorrhage (12, 17, 19). Significant findings were shown for neonatal jaundice and risk for ASD as well (5, 23–25), while several studies did not show this significance (8, 11).

Oxidative stress is proposed to be important in the etiology of ASD, and it might be the underlying mechanism by which prenatal and perinatal complications possibly contribute to ASD development (3). The transition from fetal to neonatal phase is a great stress for the newborn due to a significant increase in the production of free radicals. Mature and healthy newborns overcome these changes in oxygen concentration, but the problem may occur when intrauterine development is derranged in some way (26). Increased levels of oxidative stress were found in newborns with RDS (26, 27). In prematurity, one of the most common types of brain injury is diffuse white matter injury (DWMI), and studies have shown that it is oxidative stress related (28, 29). Recent studies have shown oxidative distress in children with hyperbilirubinaemia, manifested as decreased levels of paraoxonase and increased levels of malondialdehyde (30, 31). Also, it was found that markers of oxidative stress in newborns with hyperbilirubinemia [decreased glutathione (GSH)] tend to normalize after phototherapy and lowering in bilirubin concentration (32).

The effect of parental age on ASD also might be explained by the effects of oxidative stress (33). The spermatosoids of older men have a higher level of DNA damage, due to higher sensitivity to oxidative stress—offspring of older men may have more DNA fragmentation that lead to neuropsychiatric disorders (33). Also, older women might have lower capacity for homocystein cycle, leading to a decreased antioxidant defense in the embrion. This series of events also migh lead to neuropsychiatric disorders in the offspring (33).

It has been suggested that certain genetic polymorphisms might make children more vulnerable to perinatal complications (29). Studies that explored the oxidative stress as the basis of the gene–environment interaction in ASD also pointed out to the possible role of glutathione transferases (GSTs) in ASD development, especially regarding their important role in the antioxidant defense system (33–36). Several studies have proposed the significant association between GST polymorphisms and ASD risk, either independent (37) or in interaction with environmental factors, such as exposure to lead, mercury, and aluminum (35, 36). Our recent results have shown that *GSTM1 active* genotype decreases the risk of ASD, while *GSTA1 CC* genotype increases susceptibility to ASD. The combination of *GSTM1 active* and *GSTT1 active* as well as combination of *GSTT1 active* and *GSTP1 llelle* genotypes decrease the risk for ASD, while a higher risk of ASD was observed if combination of *GSTM1 active* and *GSTP1 llelle* was present (38).

In this study, we explored the frequency of specific prenatal and perinatal complications in patients with ASD and healthy controls, determining their effect on the risk for ASD. We further aimed to establish whether the predictive effects of prenatal and perinatal complications are affected by the most common GST polymorphisms (GSTA1, GSTM1, GSTP1, and GSTT1), in order to explore the possible complex multifactorial etiological pathway for developing ASD.

MATERIALS AND METHODS

Study Population

This was a case–control study, involving 113 ASD patients (92 males, 21 females, 9.36 ± 5.88 years old) and 114 age- and sex group-matched controls. The inclusion criterion for the case group was any of the ASD diagnosis. The diagnosis was made using the ICD-10 criteria (39), confirmed by a child psychiatrist with experience in diagnosing and treatment of ASD. The evaluation was performed in a clinical interview with a parent and examination of a child. Besides clinical assessment, the diagnosis was confirmed by the Autism Diagnostic Interview—Revised (ADI-R) (40), conducted by a trained child psychiatrist.

The control group was recruited from the Urology and Orthopedic Department of University Children's Hospital, Belgrade, Serbia. Control subjects were diagnosed with unintentional injuries (fractures) and urogenital tract disorders (phimosis, chryptorchismus, penal curvature), and were recruited consecutively, at the same time as the cases. The exclusion criteria for the controls were presence of a neurological or psychiatric disorders as well as any kind of developmental delays in personal or family history. The difference in age and sex distribution within the group level was not statistically significant.

Instruments

Autism Diagnostic Interview—Revised (ADI-R) (40). ADI-R is a standardized semi-structured parent/caregiver interview, used for the assessment of signs of ASD. The description of each item, given by the parent/caregiver, is made for childhood (ever) and current behavior. Specific items regarding social reciprocity, communication, and restricted, repetitive, and stereotyped behavior (RRSB) are used to create the scores for these three domains (ADI-R A, ADI-R B, and ADI-R C score, respectively). Higher scores mean greater impairment—more severe symptoms. In this study, the interview was performed by certified child psychiatrists.

Sociodemographic and exposure questionnaire was created for the current study and was administered to parents of cases and controls. Aside from the basic sociodemographic information, the questionnaire examined different prenatal exposures, as well as perinatal complications in participants of the study (parental age, parity, infections, smoking, alcohol intake during pregnancy, prematurity, neonatal jaundice, RDS, intracranial hemorrhage, etc.). It comprised questions regarding both the presence and quantity of specific exposure/complication. The questionnaire is shown in the **Supplemental Material**.

DNA Isolation

Total DNA was isolated from 200 µl of the whole peripheral blood using QIAamp DNA Blood Mini Kit (Qiagen, Chatsworth CA, USA) and QIAamp Mini spin columns with a small chance of sample-to-sample cross-contamination. In the first step, optimized detergent buffers and enzyme Proteinase K (600 mAU/ml, 40 mAU/mg protein) were used to lyse samples and stabilize DNA. In the second step, DNA was adsorbed onto the QIAamp silica membrane during a brief centrifugation. The lysate buffering conditions are adjusted to allow optimal binding of the DNA to the QIAamp silica membrane. In the following two steps, the DNA bound to the QIAamp membrane was washed without affecting DNA binding. Purified DNA was eluted from the QIAamp Mini spin column in a concentrated form in AE Buffer. Isolated DNA, free of protein, nucleases, and other contaminants or inhibitors, was stored at −20°C for later use. DNA concentration and purity were determined spectrophotometrically at 230, 260, 280, and 320 nm using GeneQuant pro (Biochrom, Cambridge, England).

GST Genotyping

Genotyping was performed blinded to the case–control status, and blinded quality control samples were inserted to validate genotyping identification procedures. Concordance for blinded samples was 100%. All assays performed contained positive and negative controls.

The analysis of the SNP GSTA1 -69C > T (rs3957357) was performed using polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) according to the method by Ping et al. (41). A 400 bp fragment was amplified in a reaction mixture containing primers, MasterMix, and water (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and subjected to the PCR protocol indicated in the **Table 1**. For RFLP analysis, 5 µl of PCR product was digested overnight at 37°C with 2 U of restriction enzyme EarI and 1xTango Buffer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) in total volume of 15 µl. DNAse free water was used as the negative control. Digested products (GSTA1*CC: 400 bp, GSTA1*CT: 400 bp + 308 bp + 92 bp and GSTA1*TT: 308 bp + 92 bp) were separated on 3% agarose gel (125 V constant, 0.27 A, 50 W) and stained with SYBR® Safe DNA Gel Stain (Invitrogen Corporation, Carlsbad, CA, USA) and visualized on GL200 Camera (Gel Logic Imaging System, Kodak) or on Chemidoc (Biorad, Hercules, CA, USA) (**Figure 1**).

TABLE 1 | The primer sequences, PCR conditions, and restriction enzymes.

Polymorphism	Primer sequences	PCR protocol
*GSTA1**C-69T	F, 5′-GCATCAGCTTG CCCTTCA -3′, R, 5′-AAACGCTGTCA CCGTCCTG -3′	Denature: 94°C for 3 min Followed by 94°C for 30 s Annealing: 56°C for 30 s Extension: 72°C for 30 s #cycles: 30 Final extension: 72°C for 10 min Restriction enzyme: Eam1104I incubation at 37°C overnight
*GSTP1*Ile105Val	F, 5′-ACCCCAGGGCTC TATGGGAA-3′, R, 5′-TGAGGGCACAAG AAGCCCCT-3′	Denature: 95°C for 10 min Followed by 94°C for 30 s Annealing: 59°C for 30 s Extension: 72°C for 30 s #cycles: 29 Final extension: 72°C for 10 min Restriction enzyme: Alw26I incubation at 37°C overnight
GSTM1	F, 5′-GAACTCCCTGAAAA GCTAAAGC-3′, R, 5′-GTTGGGCTCAAATA TACGGTGG-3′	Multiplex PCR: Denature: 94°C for 3 min Followed by 94°C for 30 s Annealing: 59°C for 30 s Extension: 72°C for 45 s #cycles: 30 Final extension: 72°C for 4 min
GSTT1	F, 5′-TTCCTTACTGGTCCT CACATCTC-3′, R, 5′-TCACGGGATCATG GCCAGCA-3′	
CYP1A1	F, 5'-GAACTGCCACTT CAGCTGTCT-3' R, 5'-CAGCTGCATTTG GAAGTGCTC-3'	

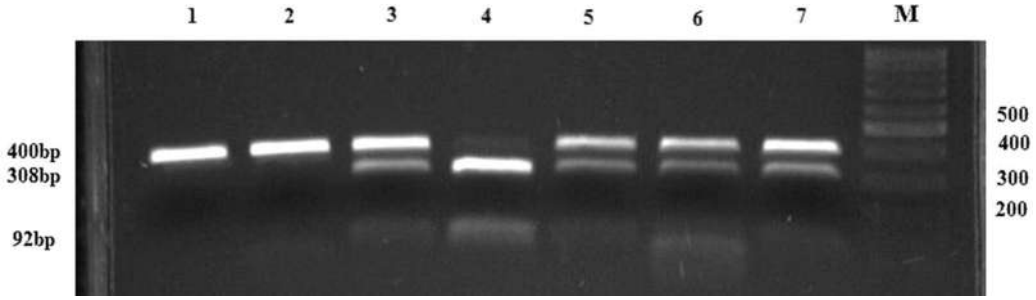

FIGURE 1 | PCR-RFLP restriction products of the *GSTA1* gene. Lanes 1 and 2 represent PCR products of *GSTA1**CC genotype (400 bp bands); lanes 3, 5, 6, and 7 represent PCR-RFLP restriction products of *GSTA1**CT genotype (400 bp, 308 bp, 92 bp bands); Lane 4 comprises RFLP-PCR restriction products of *GSTA1**TT genotype (308 bp, 92 bp bands); M, DNA marker; N, negative control without a DNA content.

GSTP1 Ile105Val polymorphism was analyzed using the PCR–RFLP method by Harries et al. (42). Briefly, amplification was conducted using primers presented in **Table 1**. The amplification was performed by denaturing at 95°C for 10 min, followed by 29 cycles at 94°C for 30 s, annealing at 59°C for 30 s and 72°C for 30 s. The final extension was done at 72°C for 10 min. The amplification 176 bp products were digested by 10 U of restriction endonuclease Alw261 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 37°C overnight and eletrophoresed on 3% agarose gel. The presence of restriction site resulting in two fragments (91 and 85 bp) indicated variant allele (Val/Val), while presence of only 176 bp fragment indicated Ile allele (Ile/Ile). In case of heterozygous genotype (Ile/Val), all three fragments were present (**Figure 2**).

The DNA sequences of GSTM1 and GSTT1 were analyzed by multiplex PCR in Mastercycler gradient thermal cycler (Eppendorf, Hamburg, Germany) according to the method by Abdel-Rahman et al. (43). The multiplex PCR technique used to detect homozygous deletions of GSTM1 and GSTT1 included primers for GSTM1, GSTT1, and CYP1A1 housekeeping gene, used as an internal control for amplifiable DNA (**Table 1**). Isolated DNA (~50 ng) was amplified in a total volume of 25 µl reaction mixture containing 7.5 pmol of each primer, 12.5 µl of MasterMix (0.05 U/µl Taq DNK polymerase, 4 mmol $MgCl_2$, 0.4 mmol of dNTP) and water (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Amplified PCR products (GSTM1: 215 bp, GSTT1: 481 bp, CYP1A1: 312 bp) were electrophoresed (125 V constant, 0.27 A, 50 W) on 2% agarose gel, stained with SYBR® Safe DNA Gel Stain (Invitrogen Corporation, Carlsbad, CA, USA), and visualized on GL200 Camera (Gel Logic Imaging System, Kodak) or on Chemidoc (Biorad, Hercules, CA, USA) (**Figure 3**). Since the assay does not distinguish heterozygous or homozygous wild-type genotypes and therefore detects the presence (at least one allele present, homozygote or heterozygote) or the absence (complete deletion of both alleles, homozygote) of the genotype, the active genotype was detected according to presence of the particular band (GSTM1-active: 215 bp, GSTT1-active: 481 bp) and the absence of these bands was indicative of the null genotypes.

Statistical Analysis

Beside descriptive statistics, the study included the tests of statistical difference of control variables between the case and the control group (χ^2 or t test depending on the variable type). The χ2 test was also used for the assessment of possible genotype departure from Hardy–Weinberg equilibrium. Series of univariate logistic regressions were conducted for all explored perinatal and prenatal factors in order to produce univariate odds ratios (OR). These were followed by a two-step multivariate logistic regression model of ASD risk. The first step included

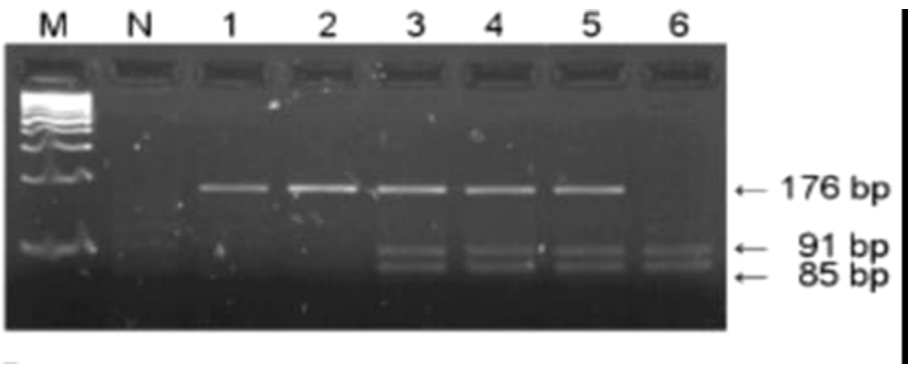

FIGURE 2 | PCR–RFLP restriction products of the *GSTP1* gene. Lanes 1 and 2 represent products of wild-type (Ile/Ile) genotype, lanes 3, 4, and 5 represent heterozygous (Ile/Val) while lane 6 indicates homozygous (Val/Val) genotype; M, DNA Q2 marker; N, negative control.

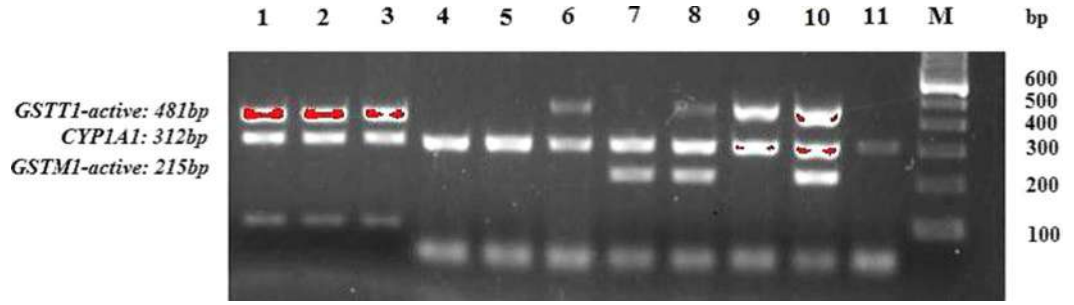

FIGURE 3 | PCR products of the *GSTM1* and *GSTT1* genes. Lanes 8 and 10 comprise PCR products of combined *GSTT1-active/GSTM1-active* genotype (481 bp and 215 bp bands, respectively); lanes 1, 2, 3, 6, and 9 comprise PCR products of *GSTT1-active/GSTM1-null* genotype (481 bp bands); Lane 7 represents PCR products of *GSTT1-null/GSTM1-active* genotype (215 bp bands); Lines 4, 5, and 11 indicate *GSTT1-null/GSTM1-null* genotype; 312 bp band represents the *CYP1A1* housekeeping gene, used as internal control for amplifiable DNA; M, DNA marker.

prenatal and perinatal predictors found in univariate analyses, which were the most present in the sample. In this step, we were also controlling for familial factors (parental age) due to its possible effect on perinatal complications in general and its link to oxidative stress (10, 33). In the second step, these predictor effects were adjusted for the GST genotypes. Since one variable effect (use of medication during pregnancy) lost its significance after the second step, a moderation effect of GST genotypes on this variable was analyzed using moderation analyses [based on ordinary least square regression within path analysis method, using bootstrapping confidence intervals (CIs) - macro PROCESS] (44). The moderation analysis was conducted for the use of tocolytics during pregnancy as well. As effect size indicators, we used OR (with the 95% CI), as well as Cox & Snell and Nagelkerke R^2. The probability level of ≤0.05 was considered statistically significant.

Ethical Standards

The study was approved by the Ethics Committee of the Institute of Mental Health, University Children's Hospital and Faculty of Medicine, University of Belgrade, Serbia, and has been performed in accordance with the principles of good research practice. Prior to participation in the study, parents/caretakers signed the informed consent.

RESULTS

Basic sociodemographic characteristics of ASD cases and controls are shown in **Table 2**. There were no differences in age and sex between the case and the control group (p = 0.120 and p = 0.731, respectively). There were no differences in maternal age (p = 0.465) or paternal age (p = 0.159) as well. Finally, there were no significant differences in maternal and paternal education between ASD cases and controls (p = 0.100 and p = 0.793, respectively).

Perinatal complications of ASD cases and respective controls are shown in **Table 3**. Comparing to control group, the group of ASD cases had higher frequency of medication use during pregnancy (p = 0.030, OR = 2.413; CI: 1.35–4.32), particularly the tocolytics (p = 0.029; OR = 2.467; CI: 1.098–5.546). Also, significant differences were shown for perinatal complications. Having any perinatal complication raised the risk of ASD 9.415 times (p = 0.000; OR = 9.415; CI: 4.870–18.203). Prematurity

TABLE 2 | Sociodemographic factors in ASD cases and controls.

Variable	Cases (n = 113)	Controls (n = 114)	t	X^2	P
Child's age (years), X ± SD	9.36 ± 5.88	10.62 ± 6.33	−1.562	/	0.120
Child's sex, n (%)					
Male	92 (81)	95 (83)	/	0.144	0.731
Female	21 (19)	19 (17)			
Maternal age at birth	28.45 ± 4.79	27.93 ± 5.42	0.731	/	0.465
Paternal age at child's birth	32.93 ± 6.32	31.69 ± 6.12	1.415	/	0.159
Mother's educations					
Elementary school	10 (9.9)	5 (4.9)			
High school	43 (42.6)	58 (56.3)	/	4.602	0.100
More than high school	48 (47.5)	40 (38.8)			
Father's education					
Elementary school	8 (8.1)	8 (7.8)			
High school	57 (57.6)	64 (62.1)	/	0.464	0.793
More than high school	34 (34.3)	31 (30.1)			

TABLE 3 | Prenatal and perinatal complications in the case and the control group—descriptives and univariate analyses.

Variable	Cases	Controls	X^2/t	Sig.	Univariate logistic regression OR	Sig.
Use of medication during pregnancy (any)	Yes 48 (47.1%) No 54 (52.9%)	Yes 28 (36.8%) No 76 (73.1%)	8.968	**0.03**	OR = 2.413; CI: 1.35–4.32	0.030
Use of tocolytics during pregnancy	Yes 21 (20.8%) No 80 (79.2%)	Yes 10 (9.6%) No 94 (90.4%)	4.987	**0.026**	OR = 2.467; CI: 1.098–5.546	0.029
Perinatal complication (any)	Yes 67 (65.0%) No 36 (35.0%)	Yes 17 (16.5%) No 86 (83.5%)	50.254	**0.000**	9.415; CI: 4.870–18.203	0.000
Prematurity	Yes 19 (18.4%) No 84 (81.6%)	Yes 2 (1.9%) No 101 (98.1%)	15.324	**0.000**	11.42; CI: 2.586–50.455	0.001
Low birth weight (less than 2800 gr)	Yes 11 (10.7%) No 92 (89.3%)	Yes 6 (5.8%) No 97 (94.2%)	1.603	0.205		
Perinatal asphyxia	Yes 4 (3.9%) No 99 (96.1%)	Yes 1 (1.0%) No 102 (99.0%)	1.845	0.369		
Intracranial hemorrhage	Yes 6 (5.8%) No 97 (94.2%)	Yes 2 (1.90%) No 101 (98.1%)	2.081	0.279		
Neonatal jaundice	Yes 50 (48.5%) No 53 (51.5%)	Yes 10 (9.7%) No 93 (90.3%)	37.626	**0.000**	8.774; CI: 4.11–18.725	0.000
Respiratory distress syndrome	Yes 9 (8.7%) No 94 (91.3%)	Yes 2 (1.9%) No 101 (98.1%)	4.706	**0.030**	4.835; CI: 1.018–22.957	0.047
Paternal age at child's birth	32.93 ± 6.32	31.69 ± 6.12	1.415	0.159		
Maternal age (at child's birth)	28.45 ± 4.79	27.93 ± 5.42	0.731	0.465		

(p = 0.001; OR = 11.42; CI: 2.586–50.455), neonatal jaundice (p = 0.000, OR = 8.774; CI: 4.11–18.725), and RDS (p = 0.047, OR = 4.835; CI: 1.018–22.957) were significantly more present in the case group. There were no significant differences in the frequency of LBW (p = 0.205), perinatal asphyxia (p = 0.209), or intracranial hemorrhage (p = 0.169) between cases and controls. No differences were observed in the parental age either.

In order to explore relative effects of perinatal complications that appeared as significant predictors in univariate analyses, we performed a two-step multivariant logistic regression of ASD risk as a dependent variable. In the first step, we included prematurity, neonatal jaundice, RDS, and use of medication during pregnancy as predictors, controlling for maternal and paternal age at birth. The regression model was significant (X^2 = 56.533, p = 0.000; Cox & Snell R^2 = 0.256, Nagelkerke R^2 = 0.342), with significant effects adding to the risk of ASD for all perinatal predictor variables except RDS (**Table 3**). In the second step, we explored the effects of the same predictors, adjusting not only for parental age, but for GST genotypes as well. This model was also significant (X^2 = 62.995, p = 0.000; Cox & Snell R^2 = 0.281, Nagelkerke R^2 = 0.375), with prematurity and neonatal jaundice keeping their significant predictive effects, and RDS effect not showing significance again. However, after controlling for *GST* genotypes, the predictive effect of use of medication during pregnancy became insignificant (**Table 4**), leading towards the hypothesis of possible moderation by GST genotype.

A line of moderation analyses were conducted with ASD status as outcome, use of medication during pregnancy as a predictor, and each GST genotype as a moderator, controlling for other perinatal factors (neonatal jaundice, prematurity, RDS), parental age at birth, and all the other GST polymorphisms in each analysis. We found significant effect of interaction between *GSTM1* genotype and medication use during pregnancy on the risk of ASD. Therefore, GSTM1 genotype was a significant moderator of the effect of medication use during pregnancy on ASD risk. The use of medication was significantly predictive of the higher ASD risk only in carriers of *GSTM1-null* genotype, whereas among carriers of *GSTM1-active* genotype, the predictive effect of medication use was not significant (**Table 5**).

Moderation analyses were also conducted with ASD status as outcome, use of tocolytics during pregnancy as a predictor, and GST genotypes as moderators, with controlling for the same factors as in previous moderation analyses. A significant effect of interaction between *GSTM1* genotype and the use of tocolytics during pregnancy on the risk of ASD was also found. As for the use of all medications, the use of tocolytics was predictive of the higher ASD risk only in carriers of *GSTM1-null* genotype (**Table 6**).

TABLE 4 | Two-step multivariate logistic regression model of the ASD risk with prenatal and perinatal factors as predictors.

	Step one: Controlling for parental age			Step two: Controlling for parental age and GST genotypes		
	Wald	Sig.	OR	Wald	Sig.	OR
Prematurity	4.930	**0.026**	6.093	5.043	**0.025**	6.722
Neonatal jaundice	25.548	**0.000**	8.453	24.972	**0.000**	8.814
Respiratory distress syndrome	0.549	0.459	1.926	0.236	0.627	1.545
Use of medication during pregnancy	4.175	**0.041**	2.080	3.565	0.059	2.007

TABLE 5 | Significant GSTM1 null moderation of the effects of medication use during pregnancy on ASD development (with parental age, neonatal jaundice, prematurity, RDS, and other GST genotypes as covariates; *p < 0.05; ** p < 0.01).

Predictor to outcome	Interaction effect of *GSTM1* genotype with use of medication during pregnancy B (LLCI-ULCI)	*GSTM1-active* conditional effect B (LLCI-ULCI)	*GSTM1-null* conditional effect B (LLCI-ULCI)
Use of medication during pregnancy to ASD status	−1.580 (−3.120 to −0.039)*	−1.060 (−1.1720 to 0.960)	1.474 (0.404 to 2.544)**

TABLE 6 | Significant GSTM1 null moderation of the effects of tocolytic use during pregnancy on ASD development (with parental age, neonatal jaundice, prematurity, RDS, and other GST genotypes as covariates; *p < 0.05; **p < 0.01).

Predictor to outcome	Interaction effect of *GSTM1* genotype with use of tocolytics during pregnancy B (LLCI-ULCI)	*GSTM1-active* conditional effect B (LLCI-ULCI)	*GSTM1-null* conditional effect B (LLCI-ULCI)
Use of tocolytics during pregnancy to ASD status	−2.792 (−5.208 to −0.376)*	−0.598 (−0.532 to 1.412)	2.732 (0.860 to 4.604)**

DISCUSSION

The present study explored the prenatal factors and perinatal complications in individuals with ASD, as well as their possible interaction with genetic polymorphisms in GSTs. Our findings have shown that prematurity, neonatal jaundice, RDS and use of medication during pregnancy were significantly more frequent in ASD group. After performing the multivariant logistic regression analysis, exploring the relative effects of individual complications, the findings showed that prematurity, neonatal jaundice, as well as the use of medication during pregnancy had a significant effect, raising the risk of ASD development. When adjusted for GST genotypes, we found no evidence of change in the effect significance for prematurity and neonatal jaundice as risk factors in ASD. However, the effect of medication use during pregnancy was moderated by GSTM1 genotype. It was significantly predictive of ASD risk only in carriers of *GSTM1-null*, whereas among carriers of *GSTM1-active* genotype no significant relationship was found between the medication use and the ASD risk. The same finding was reached when we explored the effect of tocolytics—their use was significantly predictive of ASD risk only in carriers of *GSTM1-null* genotype.

Prematurity is recognized as a significant risk factor for ASD, after controlling for other perinatal complications, as well as GST genotypes in our study. This is in line with the existing data (5, 12, 18), although our study showed even higher risk. On the other hand, a study by Burstyn et al. in which the cut-off was also set at 37th week failed to confirm this association (21). As it was already mentioned, DWMI is one of the most common brain injury in prematurely born children, and is associated with oxidative stress and decreased cognitive abilities, as well as with behavioral and psychological difficulties (28, 29). *In vitro* and animal studies have shown that oxidative stress affects apoptosis and leads to decrease in myelinization and oligodendrocyte differentiation (29).

We also confirmed that neonatal jaundice is a significant risk factor for ASD, which is in concordance with several studies. Neonatal jaundice is the result of immaturity of the liver and its functions, as well as increased fetal erythrocytes degradation, while the accumulated bilirubin might potentially lead to brain damage (25). Interestingly, it has been proposed that oxidative stress might be among the primary causes of eritrocyte membrane impairment with consequent hyperbilirubinaemia. Indeed, increased superoxide dismutase (SOD) and glutathione peroxidase (GPX) activities were found in children with neonatal jaundice. A study by Raicevic et al. (2014) explored the levels of oxidative stress markers and bilirubin in children who had fetal distress during labour and it showed significantly lower eritrocyte count and significantly higher bilirubin levels. They proposed that oxidative distress might cause higher erythrocytes degradation due to fetal distress caused by other perinatal complications or primarily decreased activity of antioxidant enzymes, while increased levels of bilirubin might also act harmfuly to neonatal brain (45). Other studies have also suggested that oxidative stress is the mediator of neurotoxic effect of bilirubin (46). It has been shown that neonate carriers of *GSTM1-null* genotype are at high risk to develop pathologic hyperbilirubinemia and may have higher bilirubin levels (47). Glutathione S-transferases can act as intracellular binding proteins for nonsubstrate ligands, including bilirubin and bilirubin conjugates, thus decreasing the efflux of bilirubin into plasma. Specifically, polymorphisms in GSTM1 and GSTT1 genes may affect their ligandin functions in bilirubin transport. Our findings have shown that neonatal jaundice leads to a higher increase in risk for ASD than in recent studies. This could partially be explained by recall bias, since it seems that recalling to perinatal complications is higher in parents of children with developmental difficulties. In our group of ASD individuals, the significant effect of neonatal jaundice has not changed after controlling for genotypes.

Regarding RDS, our result showed almost five-fold increased risk of ASD, which is in agreement with literature data (17, 19). However, susceptibility for ASD development in children with RDS was somewhat lower than in our study (17, 19). A recent meta analysis also recognized RDS as a significant risk factor for ASD (48). The RDS is significantly more frequent in prematurely born children (26), but is also associated with different perinatal

complications and oxidative stress. Moreover, RDS is also related to oxidants/antioxidants imbalance, since increased markers of oxidative stress were found in newborns with this condition (26, 27). In our study, after controlling for other perinatal complications, the effect of RDS became insignificant in the development of ASD.

Similarly to a previous study, we showed an increased risk of ASD development in relation to using any medication in pregnancy (49). In our study, most of the used medications were tocolytics, progesterone, antibiotics, and benzodiazepines. When we stratified the study group according to specific drugs, only tocolytics were recognized as a significant risk factor for ASD. The tocolytics used in our sample were hexoprenaline and phenoterole, beta-2 adrenergic agonists, of which the exact mechanism how they can contribute to ASD risk is not clear yet. Animal studies that explored the effect of prolonged treatment with phenoterole confirmed the increased production of free radicals; however, the studies were oriented towards cardiomyocites, and not neurons (50). *In vitro* studies, on the other hand, showed that phenoterol might be considered as a substrate for peroxidase, further producing reactive metabolytes, although its main detoxification metabolic pathway is *via* conjugation (51).

To our knowledge, our study represents the first comprehensive analysis of prenatal and perinatal complications in conjunction with oxidative stress-related gene interactions in the development of ASD. So far, this mechanistic link has been evaluated in animal models of psychiatric disorders such as schizophrenia, but not specifically in ASD (52).

A recent study conducted within a student population suggested that an adverse intrauterine and/or early life environment, accompanied by the cumulative exposure to perinatal complications, correlate with externalizing problems particularly in childhood and adolescence. This was further accompanied by increased levels of lipid peroxidation, thus pointing out to the role of oxidative distress on psychopathology in this vulnerable life period (53).

When it comes to oxidative stress-related gene interactions, our study suggests that *GSTM1* genotype moderates the effect of medication use during pregnancy on the risk of ASD, specifically for the use of tocolytics. Namely, significant predictive value on ASD risk was observed for children carriers of *GSTM1-active* genotype whose mothers used any medication during pregnancy, and specifically tocolytics, as well. This finding might be significant, since studies have shown higher risk of ASD in children whose mothers used beta-adrenergic agonists as tocolytics during pregnancy (54). To our knowledge, this finding represents the first oxidative stress-specific gene–environment interaction shown in children with ASD. This seems plausible having in mind that GSTs participate in conjugation of endogenous and exogenous xenobiotics, including medications, thus decreasing their toxicity and facilitating their excretion from the body (55). Due to deletional polymorphism, individuals that lack GSTM1 isoenzyme activity might have altered capacity for detoxification of substrate drugs, but also decreased antioxidant capacity (56, 57). In this line, it might be speculated that children carriers of *GSTM1-null* genotype are more vulnerable to drug side-effects due to impaired detoxification and/or antioxidant capacity. This further implies that different oxidative stress-related genetic and environmental factors might, in conjunction, lead to development of ASD.

It is also important to emphasize that, while the effect of medication during pregnancy is significantly moderated by *GSTM1* genotype, the effect of neonatal jaundice and prematurity increases the risk of ASD, independently of the GST genotype status. The independent effect of neonatal jaundice might be explained by the fact that different GST classes bind bilirubin with differential affinity (58). On the other hand, preterm children have decreased antioxidant defense mechanisms, due to the fact that the physiological maturation of antioxidant capacity occurs at the end of gestation (59). Therefore, preterm children might be more sensitive to neonatal oxidative stress due to immaturity of the antioxidant system, possibly regardless of their genotype.

Finally, it should be noted that the effect of perinatal complications on the ASD risk explored in our sample might be moderated by polymorphisms of other oxidative stress genes.

In our study, there were no case–control differences for LBW, perinatal asphyxia, and intracranial hemorrhage. Several studies have confirmed birth weight lower than 2,500 g to be a significant risk factor for ASD (21, 22). In our study, the criterion for LBW was 2,800 g, which is a somewhat higher cut-off, and might be an explanation for the difference. Also, the study by Haglund and Kallen showed that LBW increased the risk for autism and not for Aspergers syndrome (22). Our study included the whole autism spectrum. On the other hand, study by Mamidala et al. showed no significant correlation of LBW and ASD, which is in corcordance with our study (5). We did not find the association between risk of ASD and perinatal asphyxia. The available data are rather inconsistent, although several studies suggested the association (5, 12, 20). In our study group, perinatal asphyxia was present in 3.9% cases and 1% of controls, reaching four-fold increased ASD risk; however, this perinatal complication was not recognized as a significant risk factor. The incidence of perinatal asphyxia is 5–10 in 1,000 live born children, so it can be assumed that in a large sample its role in ASD susceptibility might reach statistical significance (60). Intracranial hemorrhage was present in 5.8% cases and 1.9% controls, reaching three-fold increased OR for developing ASD, still statistically insignificant. The literature data on this prenatal factor are also conflicting. Until now, two studies showed an increased risk of ASD, when this complication was studied individually (17) or together with cerebral oedema and convulsions (19), while Duan et al. defined the complication as intrapartal craniocerebral injury and did not find the association with ASD development (12).

There are several limitations of our study. The first is related to the relatively small sample size, since larger sample could have offered possibility to include more predictors in the multivariate analyses. Furthermore, several factors that were assessed in the study could not be explored in terms of multivariate effects due to low frequences within the groups of subjects. Also, the case–control study design does not provide possibility for causal conclusions, which could be better provided by a

longitudinal design. Finally, a significant limitation is indeed the retrospective assessment of prenatal and perinatal predictor variables, which could have resulted in incorrect information due to the recall bias.

On the other hand, our study showed significant findings regarding the influence of perinatal complications on the risk of ASD, mostly confirming the findings of previous studies on this matter. Further, by controlling for GST genotypes, we also tested the hypothesis that some of the prenatal and perinatal complications have significant effect on ASD only in individuals at genetic risk, in terms of oxidative stress. A very significant result of the moderating effect of GSTM1 genotype on the effect of medication use during pregnancy offers some therapeutic possibilities to individuals at risk. Several studies explored application of antioxidant therapy in autism, such as N-acetyl cysteine, methyl B12, and omega-3 fatty acids, however with conflicting results (61–63). The overall suggestion is that not all individuals with ASD would benefit from antioxidant therapy. Indeed, only children with oxidative stress-specific susceptibility, confounding effect of prenatal and perinatal risk factors, and/or oxidative stress-related genetic polymorphisms might be the target population.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, to any qualified researcher.

ETHICS STATEMENT

This study was carried out in accordance with the principles of good research practice with written informed consent from all subjects or subject's parent/caregiver. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of the Institute of Mental Health, University Children's Hospital and Faculty of Medicine, University of Belgrade, Serbia.

AUTHOR CONTRIBUTIONS

VM-M, MP-M, MP-E, TP, and TS designed the study. TP, MM-V, and VM-M performed the statistical analysis. VM-M, MP-M, MM-V, and MD recruited and screened the participants. MP-M diagnosed the patients. AS-R performed the genetic analyses. VM-M, MP-M, MP-E, AS-R, ME, MM-V, and RG performed the literature search and wrote the manuscript. DL-T, TP, and TS gave critical comments to the manuscript. All of the authors approved the final manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2019.00675/full#supplementary-material

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SYSTEMATIC REVIEW
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Meta-Analysis of Sex Differences in Social and Communication Function in Children With Autism Spectrum Disorder and Attention-Deficit/ Hyperactivity Disorder

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Background: Sex differences in the prevalence of neurodevelopmental disorders such as autism spectrum disorder (ASD) and attention-deficit/hyperactivity disorder (ADHD) are well documented, but studies examining sex differences in social and communication function remain limited and inconclusive.

Objectives: The objective of this study is to conduct a meta-analysis of sex differences in social-communication function in children with ASD or ADHD and typically developing controls.

Methods: Using PRISMA, a search was performed on Medline and PSYCHINFO on English-language journals (2000–2017) examining sex differences in social and communication function in ASD and ADHD compared to controls. Inclusion criteria: 1) peer reviewed journal articles, 2) diagnosis of ASD or ADHD and controls, 3) age 6–18 years, 4) measures of social–communication function, and 5) means, standard deviations, and sample sizes reported in order to calculate standardized mean differences (SMD).

Results: Eleven original/empirical studies met inclusion criteria for ASD and six for ADHD. No significant sex differences were found between ASD and controls in social (SMD = –0.43; $p = 0.5$; CI: –1.58–0.72), or communication function (SMD = 0.86; $p = 0.5$ CI; –1.57––3.30) and between ADHD and controls in social function (SMD = –0.68: $p = 0.7$, CI: –4.17–2.81). No studies evaluated sex differences in communication in ADHD. Significant heterogeneity was noted in all analyses. Type of measure may have partially accounted for some variability between studies.

Conclusions: The meta-analysis did not detect sex differences in social and communication function in children with ASD and ADHD; however, significant heterogeneity was noted. Future larger studies, controlling for measure and with

adequate numbers of female participants are required to further understand sex differences in these domains.

Keywords: autism spectrum disorder, sex differences, attention-deficit/hyperactivity disorder, meta-analysis, neurodevelopmental disorders, social function

INTRODUCTION

Rationale

Autism spectrum disorder (ASD) and attention-deficit/hyperactivity disorder (ADHD) are neurodevelopmental disorders, affecting multiple aspects of behavior and cognition (1). Sex differences in prevalence are well documented, but how such sex differences interact/impact core symptom domain phenotypes remains unclear. Given the potential implications for both understanding biology and developing effective interventions, understanding such interactions is critical.

ASD is characterized by deficits in social communication, and repetitive/restricted behaviors, and occurs in approximately 1.5% of children (2, 3). ADHD is characterized by difficulties in attention, hyperactivity, and impulsivity, and has a prevalence of 5–7% in children (4). Comorbidity among these disorders has been reported to be high. The prevalence of comorbid ADHD is reported to be between 30 and 80% in individuals with ASD (5, 6) whereas the presence of ASD is estimated to range between 20 to 50% of individuals with ADHD (7–9). There is also consistent evidence of overlapping behavioral traits, such as inattention, hyperactivity, inhibitory control and other executive functions, repetitive behavior, and social deficits across these disorders, although such symptoms are not always a part of core symptom domains for a specific disorder (6, 10–14).

Both ASD and ADHD are characterized by male predominance. The male to female ratio in ASD has been reported to range from 1.33:1 to 16:1 (15–18). IQ has been reported to influence male to female ratios, with higher ratios (10:1) in individuals with higher IQs and lower ratios (2:1) in individuals with comorbid intellectual disability (15, 19). In ADHD, the male to female ratio is reported to vary between 6:1 (clinical samples) and 3:1 (community samples). Considering the differences in the prevalence of these disorders in males and females, it is important to understand how core symptom presentation may vary by sex.

Social communication deficits are a core symptom of ASD (1), but have also been reported in ADHD. For example, studies have found children with ADHD to have impairments in peer relations and poor friendship quality and stability (20, 21). Some research has argued that social difficulties in children with ADHD may result directly from ADHD symptoms (22, 23) rather than reflecting qualitative impairments in social–communicative function that are characteristic of ASD (24). However, in contrast to this hypothesis, several authors report the presence of social and communicative profiles in ADHD that are qualitatively similar to those associated with ASD (25–27). For example, studies that use the Child Communication Checklist and Social Responsiveness Scale have found that children with ADHD are impaired in a similar manner to many children with ASD (28, 29), suggesting that social–communication impairment in ADHD may not entirely result from ADHD symptoms alone as suggested by Huang-Pollack et al. (22); Tseng and Gau (23). Even though social deficits are seen across these neurodevelopmental disorders, and may indeed have similar presentations, it is unclear how/whether sex differences in prevalence and onset observed in these disorders influence severity of social and communication deficits. Investigating such differences will help us understand the experiences and the unique manifestations/needs of males and females diagnosed with different neurodevelopmental disorders.

There have been relatively few studies in ASD examining sex differences in social–communication function, and findings have been inconsistent. Some studies found that females diagnosed with ASD engaged in significantly more social/peer interaction and had better communication skills compared to males (30–34), while others found no significant differences between males and females (18, 35–37), and some reported that adolescent females had more social–communication difficulties than males (38, 39). Previous systematic reviews have attempted to synthesize inconsistent results and have found no significant differences in social communication function in males and females with ASD. However, these reviews did not include studies with a control group against which to compare findings (40, 41).

Similarly, evidence for sex differences in social–communication function in ADHD remain inconsistent. Most of the literature on ADHD has focused mostly on males and there is limited information on peer relation and social interaction difficulties in females with ADHD (42). While some studies have documented more deficits in peer interaction in males than females (43, 44), other studies found that females were more likely to be rejected/disliked by peers than males (45, 46). Furthermore, a few studies have reported no sex-related differences in social functioning (47–49). To date, the meta-analyses by Gaub and Carlson (50) and Gershon (51) are the only meta-analyses that have examined sex differences in social functioning in children/adolescents with ADHD. Even though both meta-analyses concluded that there were no differences between males and females with ADHD with respect to social/peer functioning, the analyses lacked typically developing control groups, and were performed more than 15 years ago. Thus, some of the study participants were diagnosed with ADHD based on DSM III criteria, but most importantly no studies from the last 15 years were included.

In summary, although sex differences are well documented in the prevalence of neurodevelopmental disorders, and social deficits are observed across such disorders, there is limited research examining how such sex differences may influence social and communication function. Previous attempts at

synthesizing available evidence did not include typically developing control (TD) groups, making it unclear whether observed sex differences are similar to those found in the general population or are specific to a condition. Thus, this meta-analysis will attempt to examine whether there are sex differences in social–communication function between children with ASD and ADHD and controls.

Research Objective

This study will review the current literature in order to examine potential sex differences in social–communication function in children with ASD and ADHD compared to typically developing controls.

METHODS

Study Design

The current study is a meta-analysis of the literature that will examine sex differences in social and communication function in children diagnosed with ASD and ADHD compared to controls, followed by a meta-analysis of a subgroup of studies to summarize and quantitatively compare sex differences in social and communication function between children with these developmental disorders and controls.

Search Strategy

A search was performed using OVID Medline and PsychINFO databases for relevant articles in September 2017, on sex differences in social and communication function in ASD and ADHD, using the keywords listed in **Table 1**. Key search terms and Medical Subject Headings terms (MeSH-used for indexing articles) for Medline and PsychINFO for neurodevelopmental disorders, sex differences and social and communication behaviors were selected with the assistance of an academic librarian (PW). During development of key search terms and MeSH headings, the key words, "social" and "communication" were found to produce a more extensive and broader search as these terms captured a wide range of types of social and communication skills, such as social pragmatic skills, verbal and nonverbal communication.

TABLE 1 | Key search term and search strings used for the databases OVID Medline and OVID PSYCHINFO.

Category	Search Terms
Neurodevelopmental Disorders	1. child development disorders, pervasive/ or Asperger syndrome/ or autism spectrum disorder/ or exp autistic disorder/ 2. exp Child Development Disorders, Pervasive/ 3. Attention Deficit Disorder with Hyperactivity/ 4. autis*.mp. 6. attention deficit.mp. 7. (attention adj3 disorder*).mp. 8. hyperactivit*.mp. 9. All above
Sex Differences	10. Sex Factors/ 11. (sex adj3 factor*).mp. 12. (sex adj3 differ*).mp. 13. (male* adj3 female*).mp.14. (boy or boys).mp. 15. (girl or girls).mp. 16. (male* adj3 differ*).mp. 17. (female* adj3 differ*).mp.18. human sex differences/ 19. (gender adj3 differenc*).mp. 20. (gender adj3 profile*).mp.21. sex characteristic*.mp. 22. All above
Social Behavior and Communication	23. (social or COMMUNICATION).mp.
MEDLINE Search Strings including limits	24. 9 and 22 and 2325. limit 24 to (year = "2000 -Current" and "all child (0 to 18 years)" and English and humans and journal article)
PSYCHINFO Search Strings including limits	24. 9 and 22 and 2325. limit 24 to (journal article and english and human and year = "2000–current")

**represents the truncation symbol for PsychINFO and MEDLINE databases.*

Participant/Comparators

The inclusion criteria were: 1) peer reviewed journal articles, 2) published in English between the year 2000 and 2017, 3) males and females in the sample, 4) diagnosis of ASD or ADHD by DSM criteria and typically developing controls, 4) age range of 6–18 years old, 5) sex differences between the diagnostic group (i.e., ASD or ADHD) and controls tested using measures of social–communication function, and 6) means, standard deviations, and sample sizes reported in order to calculate standardized mean differences (SMD).

Systematic Review Protocol

Title and abstract of articles were screened for inclusion criteria by two raters (TM, MM). A third rater was consulted in case of discrepancies (EA). In addition, articles were excluded if they were off topic, descriptive studies, did not provide mean scores, standard deviations, and sample sizes for social or communication function for males and females, and/or did not include a typically developing control group. Authors of excluded articles were contacted to request data on control groups for the inclusion in the analysis, but none provide the requested information.

Data Extraction

We used the Quality Assessment Tool for Cohort and Cross-Sectional Studies to assess the quality of the studies (please see **Supplemental Tables 1** and **2**). Variables extracted for analysis included mean age and standard deviation, sample sizes of males and females with a developmental disorder and controls, type of measure used to assess social and/or communication function, mean scores and standard deviations for females and males on these measures.

Data Analysis

Random-effects meta-analyses were performed using the "metafor" package in R (52, 53; R Project for Statistical Computing, RRID: SCR_001905) for measures of social and communication function in ASD and social function in ADHD. We used a random-effects model to account for variance within and between studies caused by sampling error and other artefacts (54). Standardized mean sex differences for social and communication function were calculated

in ASD and for social function in ADHD. We then calculated SMD between groups. Social and communication function were analyzed separately since some studies tested these individually or only tested for one of these. Where tests for heterogeneity were significant, a mixed-effects model was used to test for the effect of the moderator "Measure" (test/instrument used), as well as "Age" [age of participant-categorized into child (6–12 years); child/adolescent (for studies including both children and adolescents); adolescent (12–18 years)]. ASD groups were entered into the analysis first. Positive effect sizes represent females outperforming males more the in ASD relative to controls, while negative effect sizes represent males outperforming females more in ASD relative to controls. Where multiple measures of the same symptoms were used within one study, we report on measures that were commonly used in other studies. A few studies had more than one measure that assessed social and/or communication behaviors. For the ADHD articles, some articles used more than one measure to assess social function (55–57). To maintain consistency across all studies, measures were selected if they assessed social behavior and if they were parent reports (i.e. My Child—55; Social Adjustment Inventory for Children and Adolescents—56; Quality of Play Questionnaire—57). For ASD social function, we found that three studies had reported both the total scores and social communication domain scores of the SRS (58–60). To determine whether we should report the effect size of the total score versus the social communication domain score, the effect sizes of the SRS total scores and social communication domain scores were plotted on to a forest plot and were compared. As both were found to have similar effect sizes and to stay consistent with studies that publish total scores, we decided to use the SRS total scores. Additionally, given that two of the ADHD studies (56 and 61) used community rather than clinic samples, we ran the analyses with and without them. All R scripts for all analyses were borrowed from Dr. Laura Hull (40) and slightly modified with her permission. The R-Script used in the present study is available upon request.

RESULTS

Study Selection and Characteristics

The initial database search identified 2,105 results (**Figure 1**). Of the 2,105 studies found, 1,805 were excluded based on title review, which led to 300 articles available for abstract review. From those, articles were excluded if they were off topic (n = 109) or were descriptive studies (n = 10). Of the remaining 181 articles, 164 articles were excluded after a thorough examination of the data provided (123 articles did not provide mean scores, standard deviations, and sample sizes for social or communication function/deficit for males and females, 36 articles did not include a typically developing control group while 5 articles did not report social–communication scores on any measures). Only 17 original/empirical studies met the inclusion criteria; 11 studies measuring social–communication function in ASD and 6 studies measuring social function in ADHD. **Figure 1** provides a detailed overview of this selection process. A summary of the quality of the studies included is seen in **Supplemental Tables 1** and **2**. All studies were cross sectional in nature. All but two studies represented clinical samples, which are associated with high risk of bias. Study demographics for ASD and ADHD are presented in and **Tables 2** and **3** respectively.

Standardized mean sex differences for social and communication function were calculated in ASD (**Tables 4** and **5**), and for social function in ADHD (**Table 6**). SMD were then computed between groups using the "metafor" package in R software (52, 53; R Project for Statistical Computing, RRID: SCR_001905), to yield pooled SMDs; the pooled SMDs are represented in the forest plots in **Figures 2–4**. Please note that since higher scores represent more impairment in some measures but better abilities in others, signs on scores were changed to ensure higher scores indicate less impairment on all measures. A positive effect size indicates that females outperformed males.

Synthesized Findings

ASD Social Domain

Main Effects

Table 4 displays the measures used to assess social function, male and female individual mean scores, and the calculated SMD between males and females in ASD and TD groups. No significant sex differences in social function in ASD compared to TD were found (**Figure 2**) (SMD = −0.43, p-value = 0.5). Of note, no significant sex differences were noted in social function within ASD (Online Resource 1, **Supplemental Figure 1**) (SMD = 0.13, p = 0.6) or within TD (Online Resource 1, **Supplemental Figure 1**) (SMD = 0.24, p = 0.1) either. Significant heterogeneity was found in this analysis [$Q(df = 9) = 345.45$, $p < 0.0001$], therefore, measure and age were included in the model.

Effect of Measure

Measure was not significant in the random effect model [$QM(df = 5) = 0.14$, $p = 0.7$].

Effect of Age

Age was also found not to be significant [$QM(df = 3) = 5.88$, $p = 0.1$].

ASD Communication Domain

Main Effects

Table 5 displays the measures used to assess communication function, male and female individual mean scores, and the calculated SMD between males and females in ASD and TD groups. A random-effects meta-analysis revealed no significant sex differences between ASD and TD (**Figure 3**) (SMD = 0.86, p = 0.5). Of note, no significant sex differences were found within ASD (SMD = 0.25, p = 0.3) or TD (Online Resource 1, **Supplemental Figure 2**) (SMD = 0.019, p = 0.9) either. Significant heterogeneity was found in this analysis [$Q(df = 2) = 155.66$, $p < 0.0001$], therefore, moderators of measure and age were individually evaluated.

Effect of Measure

Measure was found to be significant in the random effect model [$QM(df = 2) = 7.58$, $p = 0.02$]. The resulting mixed-effects meta-analysis found significant variation in sex differences for

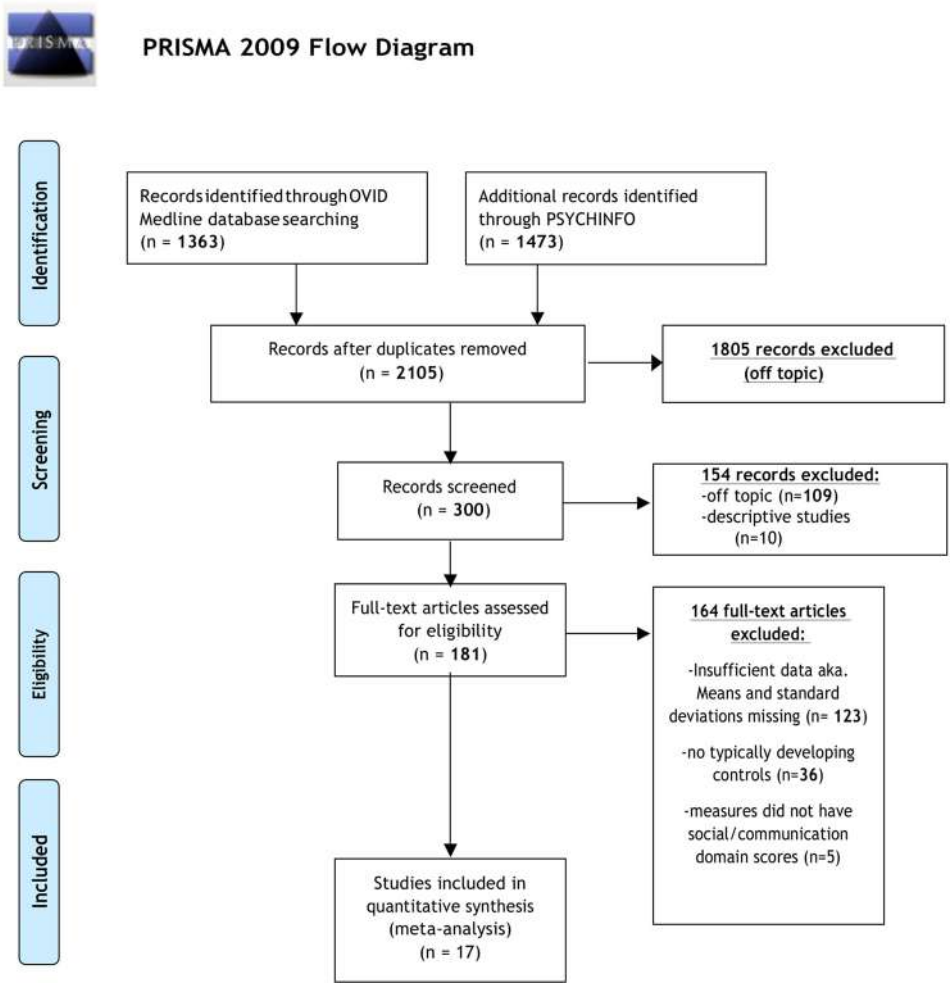


FIGURE 1 | PRISMA flow diagram displaying article selection process. Flow chart from: (62).

communication function between ASD and TD groups only for the Autism Diagnostic Interview-Revised (ADI-R)–Nonverbal Communication ($p = 0.006$) in one study. However, the test for residual heterogeneity after including "measure" as a moderator remained significant [QE($df = 1$) = 28.23, $p < 0.0001$], suggesting that other moderators may still be at play.

Effect of Age

Age was found not to be significant in the model [QM($df = 2$) = 0.27, $p = 0.9$].

ADHD Social Domain

Main Effects

Table 6 displays the measures used to assess social function, male and female individual mean scores, and the calculated SMD between males and females in ADHD and TD groups. A random-effects meta-analysis revealed no significant sex difference in social function between ADHD or TD (**Figure 4**) (SMD = −0.68, $p = 0.70$). Of note, there were no significant sex differences in social function within ADHD (SMD = −0.038, $p = 0.84$) and TD (Online Resource 1, **Supplemental Figure 3**) (SMD = 0.11, $p = 0.42$) either. Significant heterogeneity was found in this analysis [$Q(df = 5) = 2{,}316.76$, $p < 0.0001$], therefore, moderators of measure and age were included in the model.

Effect of Measure

Measure was found to be significant [QM($df = 5$) = 5.48, $p = 0.019$]. The resulting mixed-effects meta-analysis found a significant variation in sex differences for social function between ADHD and TD groups using the Social Adjustment Inventory for Children and Adolescents–Activity with peers ($p = 0.024$) in one study but not for the rest of the measures (*Child Behavior Checklist*–Social Problems ($n = 2$), Children's Depression Inventory–Interpersonal Problems ($n = 1$), My Child–Parent Empathy ($n = 1$), Quality of Play–Conflict Scale ($n = 1$)]. Still, the test for residual heterogeneity was significant [QE($df = 1$) = 571.57, $p < 0.0001$] indicating that other moderators, not included in the model, may still be influencing the effect.

TABLE 2 | Autism spectrum disorder (ASD) demographic information.

Author				ASD							
	IQ Measure Used	IQ*	Age Range	Mean Age (SD)	Female (n)	Male (n)	Total (n)	Mean Age (SD)	Female (n)	Male (n)	Total (n)
Cholemkery et al. (59)	–Hamburg–Wechsler Intelligence Test for children–WIE or the CFT 20-R for adults	-ASD: 102.15 (SD 16.23),-TD:105.32 (SD 11.62)	6–18 Child/adolescent	12.28 (3.03)	17	43	60	11.18 (3.32)	18	24	42
Cholemkery et al. (60)	–Hamburg–Wechsler Intelligence Test for children–WIE or the CFT 20-R for adults	–ASD: 100.6 (SD 15.2)–TD group is 103.4 (SD 14.5)	6–18 Child/adolescent	12.5 (2.7)	8	47	55	11.9 (2.9)	10	45	55
Head et al. (32)	Not reported	70 or above	10–16 Child/adolescent	13.73(1.97)	25	25	50	12.00 (1.84)	25	26	51
Horiuchi et al (63)	WISC-III or WISC-IV	–Full IQ: 88.3 (20.1), range: 40–132–28 had an intellectual disability	4–16 Child/adolescent	7.92 (3.28)	44	129	173	7.92 (3.28)	44	129	173
May et al. (58)	WISC-IV or WASI	70 or above	7–12 Child	12.96 (1.11)	32	32	64	12.67(0.89)	30	30	60
Park et al. (64)	Korean version of the Leiter International Performance Scale	50 or above–No significant sex difference in ASD (p = 0.8) and TD (p = 0.4)	4–15 Child/adolescent	M: 8.36 (2.79) F: 8.17 (3.37)	20	91	111	M: 8.94 (1.59) F: 8.31 (2.21)	25	26	98
Sedgewick et al. (34)	WASI	Not reported	12–16 Adolescent	M:13.10(1.0) F:13.6(1.1)	13	10	23	M:14.0(1.1) F:14.0(0.11)	13	10	23
Solomon et al. (37)	WASI	–Range from 76 to 145 in ASD and 98–139 in TD–No significant sex difference (did not report stats)	8–18 Child/adolescent	M:12.45(3.72) F:12.0(3.42)	20	20	40	M:12.53(3.32) F:11.42(2.37)	19	17	36

ASD, autism spectrum disorder; TD, typically developing controls; SD, standard deviation; M, males; F, females; WIE, Wechsler Intelligence Test; CFT 20-R, revised Culture Fair Intelligence Test; WISC, Wechsler Intelligence Scales for Children; WASI, Wechsler Abbreviated Scales of Intelligence.
**IQ information is limited to what was reported in the studies.*

Effect of Age

Age was not found to be a significant moderator [QM(df = 3) = 0.19, p = 0.98].

Community Versus Clinic Sample

Graetz (61) and Biederman (56) were the only studies that used community samples instead of clinic samples. When the meta-analysis was conducted excluding the community samples, no significant sex differences emerged [SMD: –0.113; p = 0.8106; confidence interval (–1.04–0.81)].

DISCUSSION

Summary of Findings

This study examined potential sex differences in social and communication function in neurodevelopmental disorders (i.e., ASD and ADHD) and typically developing groups. The meta-analysis found no evidence of sex differences between ASD and TD groups in social or communication function. Still, with only three studies examining sex differences in communication between ASD and TD, the strength of evidence remains limited. There were no studies examining sex differences in communication function for ADHD. We found no sex differences between ADHD and TD groups in social function. However, the type of measure may partly explain some of the heterogeneity across studies in the domain of communication in ASD and social in ADHD, although only a single study in each disorder was found to be a significant source of heterogeneity and as such other unreported characteristics of these studies such as population characteristics and social economic status may have been responsible for the finding. In summary, there were no sex differences found in social–communication function between ASD and TD and ADHD and TD. However, the choice of measure across studies may have influenced results in some domains but this effect was only seen in one study in each case. Also, given there was significant residual heterogeneity, the variability between studies could have been caused

TABLE 3 | ADHD demographic information.

Author	IQ Measure	IQ	ADHD							
			Age Range	Mean Age (SD)	Female (n)	Male (n)	Total (n)	Mean Age (SD)	Female (n)	Male (n)	Total (n)
Biederman et al. (56)	Wechsler intelligence test–Full scale IQ	–80 or greater	6–17 Child/ adolescent	M:12.6(4.7)F: 13.6(4.4)	25	73	98	M:13.4(5.5) F:13.7(5.5)	235	244	479
Graetz et al. (61)	Not reported	Not reported	6–13Child/ adolescent	M:9.2(2.4) F:8.9(2.4)	26	76	102	M:9.6(2.3) F:9.5(2.3)	1,075	976	2,051
Marton et al. (55)	WISC-IV or Wechsler Intelligence Scale for Children	–80 or greater–ADHD 103.6 (SD = 12.8)– TD was 112.0 (SD = 12.5)	8–12 Child	10.08 (1.39)	14	36	50	10.20 (1.46)	12	30	42
Skogli et al. (65)	WASI–Full scale IQ	–70 or greater– Female controls were significantly higher than males and females with ADHD [$F(3,126) = 4.6, p = 0.004$)	8–17 Child/ adolescent	11.2	37	43	80	11.9	18	32	50
Rucklidge and Tannock, (66)	Wechsler Intelligence Scale for Children-Full Scale IQ	–80 or greater	13–16 Adolescent	M:14.80(1.22) F:14.68(1.51)	24	35	59	M:14.80(1.22)F: 15.60(1.04)	28	20	48
Mikami and Lorenzi (57)	Wechsler Intelligence Scale for Children-fourth edition	–Verbal IQ 75 or greater–Verbal IQ between ADHD and TD groups were significantly significant $F(1,121) = 18.94$, $p < 0.01$)	6–10 Child	M:8.24(1.14) F:8.19(1.44)	21	42	63	M:8.33(1.28) F:8.10(1.07)	20	42	62

ADHD, attention-deficit/hyperactivity disorder; TD, typically developing controls; SD, standard deviation; M, males; F, females; WISC-IV, Wechsler Intelligence Scales for Children–Fourth Edition ; WASI, Wechsler Abbreviated Scales of Intelligence.
**IQ information is limited to what was reported in the studies.*

by other factors (e.g. socio-economic status, population characteristics).

Several biological theories have attempted to describe/ explain sex differences in developmental disorders. According to Eme (67), the sex least frequently affected by the developmental disorder (females) is relatively more severely affected. Eme (67) explained this using two types of models 1) the polygenetic multiple-threshold model, which suggests that females require a higher genetic/environmental load to be affected, 2) constitutional variability model, which proposes that greater genetic variability in males produces higher rates of less severe manifestations of disorders, while females are more likely to be affected in cases where there is a pathological event (e.g. brain damage). This theory is also consistent with other models used to explain sex differences in ASD such as the Genetic Variability Model (68) and Liability Threshold Model (69). The extreme male brain theory (70) suggests that both males and females with ASD present with an "extreme male" profile of good systematizing abilities at the expense of empathizing abilities, so that fewer sex differences in social communication may be predicted (30, 70). Our findings would partially support the extreme male brain theory, as we found no differences between ASD males and females, although we also did not find sex differences in social function and communication in controls. The latter, although consistent with previous systematic reviews in typically development (70, 72), would not be consistent with the extreme male brain theory. Still several limitations of the identified studies preclude strong conclusions.

To explain potential sex differences in ASD, a few social theories have articulated possible scenarios. Holtmann (38) developed a term called the "interpreting bias" which is the difference between observed and expected behaviors. Holtman (38) suggested that despite comparable levels of ASD traits in males and females on direct measures, parents with children with ASD may expect more socially sophisticated behaviors in their daughters than in their sons, and hence will report more social impairment in their girls than in their boys. Similarly, Crick and Zahn-Waxler (73) reported that girls with ASD were perceived by parents as having a greater level of social impairment, despite comparable symptoms reported and directly observed on the ADI-R and Autism Diagnostic Observation Schedule. Another possible explanation about sex differences in ASD is the increase in social demand/complexity with age may differ between boys and girls. McLennan (39) found more impairment with age in girls but not boys, and suggested that as the child transitions into adolescence, social situations may get more diverse and complex for females, as peer activities in typical girls and young women become mostly dependent on communication and interpersonal skills compared to boys who may have other social options that are less verbal and less intensely interactive (e.g. spectator sports and competitive play). Thus, social

TABLE 4 | Sex differences in social function for ASD and TD.

Authors	Social Measures	Community vs. Clinic samples	Age	ASD			TD		
Social				**Female (SD)**	**Male (SD)**	**SMD (95% CI)**	**Female (SD)**	**Male (SD)**	**SMD (95% CI)**
Cholemkery, (59)	SRS Total	Clinic	6–18Child/ adolescent	−113.00(24.2)*	−92.95(24.98)*	−0.80(−1.40, −0.20)	−22.94(12.75)*	−20.33(12.54)*	−0.20(−0.80,0.41)
Cholemkery, (60)	SRS Total	Clinic	6–18Child/ adolescent	−111.90 (25.70)*	-94.50 (26.30)*	−0.65(−1.40,0.10)	−22.20(15.40)*	−18.8(12.50)*	−0.26(−0.90,0.40)
Head et al. (32)	The Friendship Questionnaire	Clinic	10–16Child/ adolescent	76.76 (13.97)	61.48 (15.64)	1.01(0.43,1.60)	84.84 (9.91)	74.76 (12.15)	0.89(0.32,1.47)
Horiuchi et al. (63)	SDQ-Prosocial	Clinic	4–16Child/ adolescent	4.30(2.80)	4.28(2.50)	0.01(−0.33,0.35)	6.02(2.00)	5.71(2.00)	0.15(−0.19,0.50)
May et al. (58)	SRS Total	Clinic	7–12Child	−97.41(31.77)*	−99.97(22.71)*	0.09(−0.40,0.58)	−23.17(16.49)*	−27.30(20.42)*	0.22(−0.29,0.73)
Park et al. (64)	ADI-R Social Subscale	Clinic	4–15Child/ adolescent	−8.55 (4.43)*	−10.25 (3.83)*	0.43(−0.06,0.92)	−1.00(1.22)*	−1.28 (1.46)*	0.20(−0.35,0.75)
Sedgewick et al. (34)	SRS-2 Total	Clinic	12–16Adolescent	−72.00(32.39)*	−103(27.76)*	0.98(0.11,1.85)	−43(13.18)*	−40.00(26.16)*	−0.15(−0.97,0.68)
Solomon et al. (37)	SRS Total	Clinic	8–18Child/ Adolescent	−103.85(27.64)*	−104.60(32.04)*	0.02(−0.60,0.64)	−18.11(18.79)*	−62.12(60.81)*	0.98(0.29,1.67)

Table displays, measures that assess social abilities, age, mean scores, and standard deviations for females and males, and calculated standardized mean differences between females and males in autism and typically developing controls.

ASD: autism spectrum disorder; TD, typically developing controls; SD, standard deviation; SMD, standardized mean difference; CI, confidence interval; SRS, Social Responsiveness Scale; SDQ, Strengths and Difficulties Questionnaire; ADI, Autism Diagnostic Interview–Revised

**Please note, that since higher scores represents more impairment in some measures, while other measures had higher scores mean less impairments, to maintain consistency among the measures, signs on the male and female mean scores were changed to ensure higher scores means less impairment for all measures.*

TABLE 5 | Sex differences in communication function for ASD and TD.

Authors	Communication Measures	Community vs. Clinic	Age	Autism			TD		
				Female (SD)	**Male (SD)**	**SMD (95% CI)**	**Female (SD)**	**Male (SD)**	**SMD (95% CI)**
May et al. (58)	Children's Communication Checklist (2nd Edition)–General Communication Composite	Clinic	7–12 Child	36.75 (15.05)	33.19 (16.00)	0.23(−0.27,0.70)	80.60 (22.94)	78.63 (19.78)	0.09(−0.42,0.60)
Park et al. (64)	ADI-R nonverbal communication subscale	Clinic	4–15 Child/ adolescent	−17.75 (8.20)*	−22.31(6.16)*	0.69(0.20,1.18)	−1.80 (2.33)*	−1.50 (1.90)*	−0.14(−0.70,0.40)
Solomon et al. (37)	Children's Communication Checklist (2nd Edition)–General Communication Composite	Clinic	8–18 Child/ adolescent	76.00 (14.93)	80.95 (24.55)	−0.24(−0.90,0.40)	113.05 (16.20)	111.00(16.37)	0.12(−0.53,0.80)

Table displays, measures that assess communication abilities, age, mean scores, and standard deviations for females and males, and calculated standardized mean differences between females and males in autism and typically developing controls.

ASD, autism spectrum disorder; TD, typically developing controls; SD, standard deviation; SMD, standardized mean difference; CI, confidence interval.

** Please note, that since higher scores represents more impairment in some measures, while other measures had higher scores mean less impairments, to maintain consistency among the measures, signs on the male and female mean scores were changed to ensure higher scores means less impairment for all measures.*

deficits may become more evident in girls as they transition to adolescence compared to boys. Another key social factor that has been reported to influence sex differences relates to gender specific expectations related to play and social roles. Despite similar amounts of socializing, Kuo et al. (74) found that males with ASD tended to play video games, whereas females with ASD mostly talked with their friends, suggesting that these skills may allow females with ASD to maintain closer and more empathetic friendships, ultimately to interact as expected by their nonautistic female peers. However, our

TABLE 6 | Sex differences in social function for ADHD and TD.

Authors	Social Measures	Community vs. Clinic	Age	ADHD			TD		
Social				**Female (SD)**	**Male (SD)**	**SMD (95%CI)**	**Female (SD)**	**Male (SD)**	**SMD(95% CI)**
Biederman et al. (56)	Social Adjustment Inventory for Children and Adolescents score–Activity with peers	Community	6–17 Child/ adolescent	−2.70 (0.60)*	−2.10 (0.80)*	−0.79(−1.30,−0.30)	−1.60 (0.60)*	−1.80 (0.70)*	0.31(0.10,0.50)
Graetz et al. (61)	Child Behaviour Checklist-Teacher's Report Form–Social problem	Community	6–13 Child/ adolescent	−4.00 (3.10)*	−4.80 (3.10)*	0.26(−0.20,0.70)	−1.20 (1.60)*	−1.10 (1.60)*	−0.06(−0.20,0.00)
Marton et al. (55)	Index of Empathy for Children andAdolescents–Child Empathy	Clinic	8–12 Child	72 (10.6)	68.49 (8.97)	0.37(−0.30,1.00)	78.58 (5.24)	73.23 (6.89)	0.81(0.12,1.50)
Skogli et al. (65)	Child Behaviour Checklist–Social Problems	Clinic	8–17 Child/ adolescent	−60.00 (7.40)*	−60.40 (9.20)*	0.05(−0.40,0.50)	−50.30 (0.50)*	−50.50 (1.50)*	0.16(−0.40,0.70)
Rucklidge et al. (75)	Children's Depression Inventory–Interpersonal Problems	Clinic	13–16 Adolescent	−54.67(12.10)*	−50.76(10.84)*	−0.34(−0.90,0.20)	−48.68 (10.01)*	−44.55 (2.70)*	−0.52(−1.10,0.10)
Mikami et al. (57)	Quality of Play Questionnaire-Conflict Scale	Clinic	6–10 Child	−0.91(0.81)*	−0.69(0.70)*	−0.04(−0.40,0.30)	−0.19(0.32)*	−0.16(0.20)*	0.12(−0.40,0.70)

Table displays, measures that assess social abilities, age, mean scores, and standard deviations for females and males, and calculated standardized mean differences between females and males in ADHD and typically developing controls.
ADHD, attention-deficit/hyperactivity disorder; TD, typically developing controls; SD, standard deviation; SMD, standardized mean difference; CI, confidence interval.
**Please note, that since higher scores represents more impairment in some measures, while other measures had higher scores mean less impairments, to maintain consistency among the measures, signs on the male and female mean scores were changed to ensure higher scores means less impairment for all measures.*

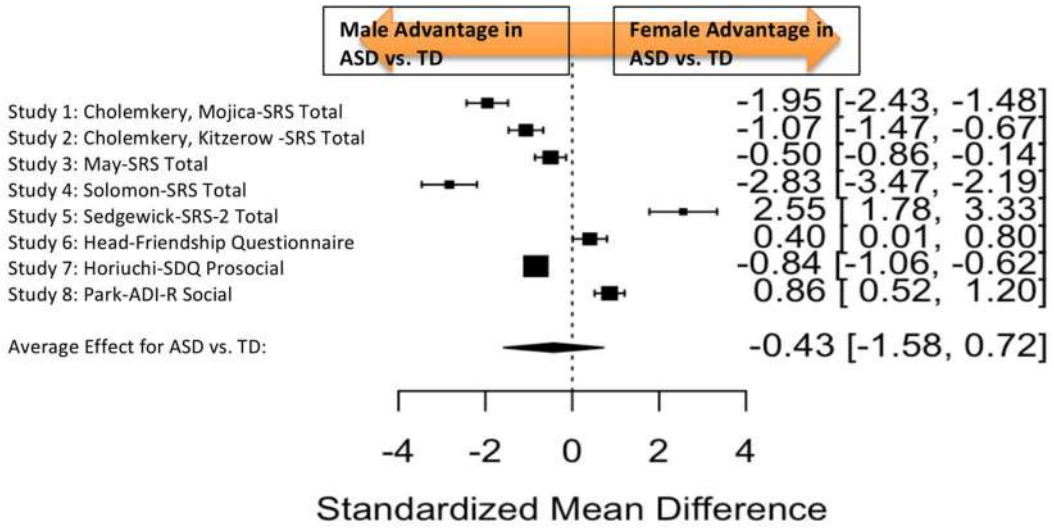
study results cannot at this point inform these theories as we found no consistent sex differences.

In addition, significant heterogeneity was observed among studies. There are several reasons why there may be discrepancies among studies examining sex effects in ASD and ADHD:

1. Measurement issues: Variability in measures that may be capturing unique constructs, or have differences in psychometric properties. For example, Marton et al. (55) used the parent reported measure "My Child" which assesses only empathic ability, while Skolgi et al. (65) and Graetz et al. (61), used the Child Behavior Checklist Social Problems domain which surveys a broader range of social problems. In the ASD communication domain, the ADI-R assesses social and communication symptoms relevant to ASD while the Children's Communication Checklist-2 assesses communication skills such as language structure, pragmatic skills and communication skills that are not diagnostic specific.
2. Population differences: Most studies included clinical samples, which may be subject to referral and identification bias. Studies of clinical samples may include more severe cases and/or symptoms that draw more attention, potentially influencing the expression of ASD and ADHD in males and females in the results (76). In fact, a meta-analysis by Gaub and Carlson (50) found that clinic referred females significantly differed from nonreferred females with ADHD, such that clinic-referred females exhibited more severe symptoms and disruptive behaviors. Moreover, girls are more likely to have inattentive symptoms/subtype (77), which may go less noticed and be less likely to lead to a referral and/or ADHD diagnosis compared to the other subtypes. Even though the present study used a random effects model to account for such variances, most studies in this meta-analysis are from clinic populations, and so it is possible that sex differences were examined in children who had more disruptive and severe symptoms.

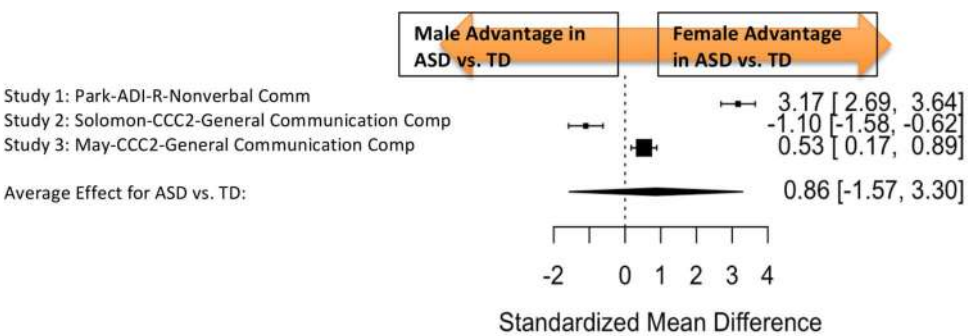
Limitations

There were certain limitations in this study. A key limitation was the small number of studies identified. According to Hunter and Schmidt (54), a meta-analysis based on a small number of studies is more susceptible to second-order sampling errors, which may inflate the observed variance. Moreover, several of the studies had very few females included, and may be underpowered to detect sex differences. Further, the choice of measure was identified to be a potential confounding variable, albeit only in single studies, but the residual heterogeneity remained significant, indicating that



Forest plot of standardized mean difference (SMDs) for social function in each study and average effect, drawn in R using "metafor" package (Viechtbauer, 2010; R Foundation for Statistical Computing, Vienna, Austria) and Dr. Laura Hull's scripts were reused to calculate SMD. Rectangles represent effect sizes (SMD), with, lines are 95% confidence interval and diamond is the average effect. The wider the diamond, the wider the confidence interval. Positive effects indicates more of a female advantage in ASD relative to controls, while a negative effect indicates more male advantage in ASD relative to controls.

FIGURE 2 | Meta-analysis of studies comparing sex differences in social abilities between ASD and controls. Forest plot of standardized mean difference (SMDs) for social abilities in each study and average effect, drawn in R using "metafor" package (48; R Foundation for Statistical Computing, Vienna, Austria) and Dr. Laura Hull's scripts were reused to calculate SMD. Rectangles represent effect sizes (SMD), with, lines are 95% confidence interval and diamond is the average effect. The wider the diamond, the wider the confidence interval. Positive effects indicates more of a female advantage in ASD relative to controls, while a negative effect indicates more male advantage in ASD relative to controls.



Forest plot of standardized mean difference (SMDs) for communication function in each study and average effect, drawn in R using "metafor" package (Viechtbauer, 2010; R Foundation for Statistical Computing, Vienna, Austria) and Dr. Laura Hull's scripts were reused to calculate SMD. Rectangles represent effect sizes (SMD), with, lines are 95% confidence interval and diamond is the average effect. The wider the diamond, the wider the confidence interval. Positive effects indicates more of a female advantage in ASD relative to controls, while a negative effect indicates more male advantage in ASD relative to controls.

FIGURE 3 | Meta-analysis of studies comparing sex differences in communication abilities between autism spectrum disorder (ASD) and controls. Forest plot of standardized mean difference (SMDs) for communication abilities in each study and average effect, drawn in R using "metafor" package (48; R Foundation for Statistical Computing, Vienna, Austria) and Dr. Laura Hull's scripts were reused to calculate SMD. Rectangles represent effect sizes (SMD), with, lines are 95% confidence interval and diamond is the average effect. The wider the diamond, the wider the confidence interval. Positive effects indicates more of a female advantage in ASD relative to controls, while a negative effect indicates more male advantage in ASD relative to controls.

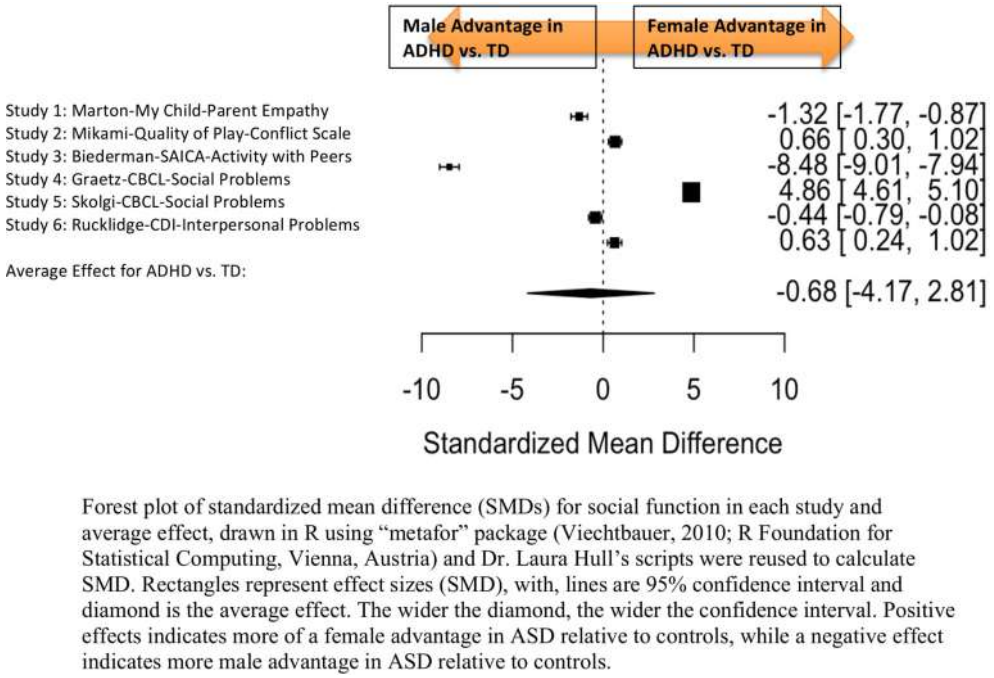


FIGURE 4 | Meta-analysis of studies comparing sex differences in social abilities between attention-deficit/hyperactivity disorder (ADHD) and controls. Forest plot of standardized mean difference (SMDs) for social abilities in each study and average effect, drawn in R using "metafor" package (48; R Foundation for Statistical Computing, Vienna, Austria) and Dr. Laura Hull's scripts were reused to calculate SMD. Rectangles represent effect sizes (SMD), with, lines are 95% confidence interval and diamond is the average effect. The wider the diamond, the wider the confidence interval. Positive effects indicates more of a female advantage in autism spectrum disorder (ASD) relative to controls, while a negative effect indicates more male advantage in ASD relative to controls.

there were other confounding variables that were influencing sex differences. Previous studies have implicated IQ, ethnicity, and comorbidities (18), as well as other social and biological factors, (genetic influences, social/cultural environments; 76, 78) in interacting with potential sex differences but we had no access to such data. Also, since most of these studies used parent reported measures, results may have been influenced by parental expectations or biases (i.e., the "interpreting bias" described by 38). Moreover, there was some variability in the types of constructs evaluated by measures used in the meta-analysis; while the majority of measures evaluated deficits, other measures may have measured skills. However, there was no evidence that in this set of studies, sex differences were different across the two constructs.

Future research investigating sex differences across neurodevelopmental disorders should include large cohorts with adequate numbers of female participants with neurodevelopmental disorders and consistent use of measures. Longitudinal designs should be employed to examine sex differences over time. Other moderators such as cognitive abilities, socio-economic status, ethnicity and comorbidities should be explored. Additionally, examining sex differences in community samples would be important in understanding whether there are variations in reported sex differences between clinical versus community samples. Lastly, other biological markers (e.g., genetics, brain) of sex differences should be evaluated.

Implications

Understanding potential sex differences in social and communication outcomes across neurodevelopmental disorders is critical in elucidating the biology of these disorders. In addition, this study suggests that other unidentified factors including potentially IQ and population characteristics may explain the significant heterogeneity observed across the studies and should be included in future studies.

CONCLUSIONS

The present study did not identify significant sex differences in social communication between ASD, ADHD, and controls. However, the limited number of studies, small female samples, and heterogeneity of measures/tools used, suggests that conclusions may not be drawn with confidence until larger longitudinal studies that address these issues. We argue that the overlap on the social–communication domains between the two disorders is not well characterized in the current literature and can only be resolved when participants with ASD and ADHD are recruited in single cohorts and evaluated by similar measures to understand whether there are systematic differences in the types of social–communication deficits observed or whether there are overlapping subgroups across both disorders with unique patterns of deficits.

DATA AVAILABILITY STATEMENT

The R-Script used in the present study is available upon request.

AUTHOR CONTRIBUTIONS

TM contributed to conceptualization, did data analysis, and is primarily responsible for manuscript preparation. AD contributed to the data analysis and manuscript. JB participated in the design, of the study, cosupervised data analytic approaches, and revised and edited manuscript. NM helped with the data analysis and revised manuscript. P-YW is the librarian on the study and assisted with the search, selection, and the process of the systematic review and has revised manuscript. AI has significantly contributed to the manuscript preparation. EA supervised all procedures in this study and manuscript. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2019.00804/full#supplementary-material

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REVIEW
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Executive Function in Autism Spectrum Disorder: History, Theoretical Models, Empirical Findings, and Potential as an Endophenotype

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This review presents an outline of executive function (EF) and its application to autism spectrum disorder (ASD). The development of the EF construct, theoretical models of EF, and limitations in the study of EF are outlined. The potential of EF as a cognitive endophenotype for ASD is reviewed, and the Research Domain Criteria (RDoC) framework is discussed for researching EF in ASD given the multifaceted factors that influence EF performance. A number of executive-focused cognitive models have been proposed to explain the symptom clusters observed in ASD. Empirical studies suggest a broad impairment in EF, although there is significant inter-individual variability in EF performance. The observed heterogeneity of EF performance is considered a limiting factor in establishing EF as a cognitive endophenotype in ASD. We propose, however, that this variability in EF performance presents an opportunity for subtyping within the spectrum that can contribute to targeted diagnostic and intervention strategies. Enhanced understanding of the neurobiological basis that underpins EF performance, such as the excitation/inhibition hypothesis, will likely be important. Application of the RDoC framework could provide clarity on the nature of EF impairment in ASD with potential for greater understanding of, and improved interventions for, this disorder.

Keywords: executive function, autism spectrum disorder, neurobiology, excitation/inhibition, GABA, endophenotype

PREFACE

Autism spectrum disorder (ASD) is a neurodevelopmental condition defined by difficulties in social communication and interaction, as well as restricted, repetitive patterns of behavior, interests, or activities (1). The social communication domain includes difficulties in reciprocal social interaction (2); deficits in non-verbal social communication (3, 4); and impairments in ability to develop, maintain, and understand relationships (5). Symptoms associated with the restricted and repetitive behavior domain manifest across motor, verbal, non-verbal, and sensory modalities (6). Observed behaviors in the restricted and repetitive domain may include motor stereotypies, echolalia, insistence on sameness, ritualized behaviors, narrow interests, and hyper- or hypo-reactivity to sensory stimuli (1).

A number of cognitive models (5, 7) have been proposed to explain difficulties observed across the life span in ASD (8, 9). One such model, the executive dysfunction hypothesis, focused on explaining the atypical executive function (EF) processes in ASD (10, 11). This model developed following observation of difficulties in set shifting (ability to shift mindset to new concepts), response inhibition (ability to inhibit a dominant response), and working memory (retaining and updating information in short-term memory) (12). Early research focused on set shifting (13) and its relationship to stereotypic and repetitive behaviors (14). Findings were interpreted to show a link between cognitive rigidity and the perseverance to routines and stereotypies observed in ASD (15). Increasingly, however, research implicates a broader influence of EF on the ASD phenotype. These include impacts of EF on social cognition (16, 17), mental health (18), disability (19, 20), and lifelong functioning outcomes (21). Overall, findings on EF in ASD suggest a broad impairment (22, 23) that is characterized by marked heterogeneity (24). The study of EF in ASD has focused primarily on investigating discrete EF constructs or domains (25). This is in contrast to the wider range of EF models developed in response to neurotypical development (26–28).

This paper presents a discussion of EF research in ASD, an overview of EF models drawn from typical and atypical development, and their potential contribution to the study of ASD. Factors that may moderate research outcomes of EF in ASD are also discussed. These include measurement issues of the EF construct, moderator influences on EF, and differences in the developmental trajectory of EF in ASD. Finally, a research model based on the efficacy of EF as an endophenotype is proposed within the research framework of Research Domain Criteria (RDoC) (29).

CONCEPTUALIZATION OF EXECUTIVE FUNCTION

The term EF was first proposed in the mid-20th century to explain functions associated with the frontal cortex (30). Frontal lobes were of interest following case studies, such as Phineas Gage (31), where it was observed that frontal lobe damage was associated with impairment of discrete functions, such as planning, organization, and self-regulation, even though general intellectual functioning remained mostly intact. This observation and subsequent case studies (32) led to conclusions that the frontal lobes have a primary role in organizing higher-order functions (33). Much of the subsequent research of EF focused primarily on the frontal lobes and functions associated with them (34, 35).

EF has been broadly defined as the overarching regulation of goal-directed, future-oriented, higher-order cognitive processes (28, 36–38). Although there is general agreement on the broad concept of EF, the theoretical models and processes that may underpin it vary considerably. Models of EF draw on different theoretical paradigms and include cognitive, clinical, behavioral, and neurobiological frameworks (39). This has, in part, contributed to the divergent frameworks of theorized models and mechanisms (38). In this paper, we present an overview of EF models and distinguish between them based on the level of analysis and measurement of the EF construct. Models are classified based on behavioral, cognitive, neuroanatomical, and neural measurement frameworks. **Tables 1** and **2** summarize key features of these EF models and associated measurement tools.

Cognitive and Behavioral Models of EF

A number of cognitive models of EF have discriminated between automatic and controlled cognitive processes (78) that are regulated by discrete attentional systems. Models focusing on attentional control included those proposed by Baddeley (79), Posner (42), and Shallice (80). Executive attention was attributed a regulatory role that facilitated focus on salient cues and regulated EF processes (**Table 1**).

Many researchers adopted a fractionated approach in order to distinguish between individual EF processes or domains (EFs) (12, 81) (**Table 2**). The number of discrete EFs reported on in the literature has ranged from 2 (82) to more than 30 (38). The three most commonly reported or core EFs are set shifting, response inhibition, and working memory (12, 38). Different levels of complexity have been proposed for EFs. For example, it is suggested that the three core EFs above, contribute to the higher-order EFs such as reasoning, planning, and problem solving (81). The Delis–Kaplan model (50) was developed in response to clinical observations of functions sensitive to frontal lobe damage and proposed nine EFs (**Table 1**). Until recently, most research focused on the study of a combination of the above core and higher order EFs. These are broadly referred to as cool EFs, defined as EF processes that are conducted independently of contextual framework or affective and motivational influences (83).

More recently, a distinction has been drawn between cool EFs and other cognitive processes, defined as hot EFs (84). Hot EFs are defined as the cognitive processes mediated by affective and motivational demands (76). They represent goal-oriented behaviors, moderated by personal appraisal of the affective or motivational significance of the stimuli. Hot EFs are increasingly studied in ASD cohorts (85) and are particularly relevant for this group because of their likely influence on behavioral regulation (86). Behavioral regulation is an integral component of models proposed by Stuss (28, 87), Barkley (37), and Gioia (48). Each of these models adopts a multifactorial approach that integrates cool and hot EFs as well as behavior regulatory control to varying degrees.

The model proposed by Stuss (28, 87) integrates cool EFs (task setting and monitoring) and non-EFs frontal lobe processes (energization, behavioral/emotional self-regulation, and metacognition). Energization refers to processing speed when completing cognitive tasks. Behavioral/emotional self-regulation is in part dependent on activation of EFs (task setting and monitoring). Metacognition has a higher-order supervisory role in integrating all EFs and non-EFs processes towards goal attainment.

TABLE 1 | Summary of EF models.

EF model	EF construct(s)	EF mechanism	Neurobiological underpinnings	Predictions	Interventions
Unifactor models					
Working memory (40)	Central executive Central executive fractionated to component parts of – Focused attention – Divided attention – Attention switching – Interface with long-term memory (episodic buffer)	Attentional focus, storage, and decision making Central executive regulates information control to the working memory component process of the phonological loop and visuospatial sketchpad Information integrated in episodic buffer and interfaced with long-term memory	Baddeley (40) noted that this has been guided by observations of patients with neurobiological damage but does not specify distinct neurobiological mechanisms	Model viewed as a homunculus approach predicting complex behavior regulation Impaired mechanisms would lead to broad behavioral dysregulation	Working memory assessment system for children with a practical guide for cognitive interventions (40, 41)
Attentional control (42)	Executive attention	Fractionation of attentional system into components of – Orienting – Alerting – Cognitive Cognitive attention responsible for regulation of cognitive functions		Impaired mechanisms would lead to broad behavioral dysregulation	Cognitive remediation programs to improve attentional control
Supervisory Attentional system (43)	Executive attention Inhibitory control	Distinction is made between routine or habituated actions versus non-routine actions Non-routine actions require the individual to disengage from habituated behavior patterns and make a novel response The supervisory attentional system exerts supervisory control in novel situations where routine or previously learned behaviors must be inhibited		Impaired mechanisms would lead to broad behavioral dysregulation including perseverative behaviors, distractibility, and apathy due to disrupted inhibitory control (44)	Cognitive remediation programs to improve attentional control
Multifactorial models					
Unity and diversity (12)	Common factor (response inhibition) Set shifting Updating/working memory	Maintain and manage goals Task switching Updating and replacing irrelevant information in working memory	Genetic underpinning of EF common factor (45) Frontal lobe involvement for common EF factor; prefrontal cortex and basal ganglia circuitry for shifting factor; basal ganglia mediated updating process (46) Mediated by GABA/glutamate neural mechanisms (47)		Pharmacological interventions targeting GABA and cognitive interventions addressing the specific cognitive mechanisms

(Continued)

TABLE 1 | Continued

EF model	EF construct(s)	EF mechanism	Neurobiological underpinnings	Predictions	Interventions
Fractionated models of EF					
Diamond's model of EF_1 Delis–Kaplan model of EF_2	Set shifting$_{1, 2}$ Response inhibition$_{1,2}$ Working memory$_{1,2}$ Planning$_1$ Problem solving$_{1,2}$ Reasoning$_{1,2}$ Fluency$_2$ Categorical processing$_2$ Verbal abstraction$_2$	Regulation of discrete EF cognitive processes	Neurobiological underpinnings not specifically defined in the model but supported by findings of neuroanatomical localization of discrete domains and functional connectivity between brain regions The Delis–Kaplan model draws on observations of patients with prefrontal lobe injuries, and emphasis is therefore on the prefrontal lobes	Impairment in discrete EF processes	Cognitive remediation interventions addressing each EF domain Pharmacological interventions addressing neural substrates
Models linking EF and behavioral regulation					
Stuss' model of EF (28)	Task setting Task monitoring	EFs interacting with non-EF domains of: Energization Behavioral/emotional self-regulation Metacognition	Task setting: left lateral frontal cortex Task monitoring: right lateral frontal cortex Behavioral/emotional regulation: orbitofrontal cortex Energization: superior medial prefrontal cortex Metacognition: frontal poles	Impairment in EF processes of task setting and monitoring leading to specific deficits and overall dysregulation due to association with behavioral/emotional self-regulation	Cognitive remediation interventions targeting EF processes and potentially pharmacological interventions targeting underpinning neural mechanisms
Barkley's model of EF	"the use of self-directed actions so as to choose goals and to select, enact and sustain actions across time towards those goals usually in the context of others often relying on social and cultural means for the maximization of one's long-term welfare as the person defines that to be" (37)	Mediated by cognitive processes that tap into traditional definitions of EF Self-directed attention (self-awareness and monitoring) Self-restraint (inhibition) Self-directed sensing (non-verbal working memory) Self-directed speech (verbal working memory) Self-directed emotions and motivations Self-directed play (planning and problem solving)	Development of five EFs draws on Luria's model and observations of patients with prefrontal lobe injuries (37)	Impaired regulation of each of the domains leading to overall difficulties in goal attainment	Intervention strategies may be addressing distinct underlying cognitive components of each of the self-management domains
Gioia's model of EF	Self-regulation of behavior based on "selection, initiation, execution and monitoring of cognition and behaviour," p.1 (48)		"Frontal systems" regulation of EF processes Emphasis on the regulatory control by the frontal lobes of cortical and subcortical areas, p.3 (48)	Impaired regulation of each of the domains	Intervention strategies may be addressing distinct underlying cognitive components of each of the self-management domains

(Continued)

TABLE 1 | Continued

EF model	EF construct(s)	EF mechanism	Neurobiological underpinnings	Predictions	Interventions
Neurobiological models of EF					
Luria's model	Complex information processing	Functional integration of three brain functional units First and second functional units: responsible for alertness and sensory information processing Third functional unit: responsible for regulation and execution of behavior	First and second functional units controlled by parietal, temporal, and occipital lobes Third functional unit regulated by the frontal lobes	"Frontal lobe syndrome" (49) Disinhibition Inability to follow sequence of action Repetitive motor movements	
E/I hypothesis		GABA/glutamate balance	Neural circuitry cortical and subcortical areas	Impairment in discrete EFs depending on neuroanatomical localization	Pharmacological interventions

EF, executive function; E/I, excitation/inhibition; GABA, γ-aminobutyric acid.

Barkley's model (88) is defined by five EF factors that regulate behavior towards achieving future goals (37). The five EF factors were empirically derived from behavioral ratings, primarily in cohorts with attention deficit hyperactivity disorder (ADHD). They are described as an individual's ability to manage time, organize and problem-solve, exercise restraint, self-motivate, and regulate emotion (37). The five EF factors are surmised to be influenced by external (cultural/societal factors) and intra-individual processes (88).

Gioia and associates (48) utilized the umbrella definition of self-regulatory process of EF that involves the "selection, initiation, execution and monitoring of cognition and behaviour" (p. 1). Within this framework, they developed a behavioral assessment that utilizes self- and/or informant ratings and draws on cool EFs (e.g. response inhibition, set shifting, and working memory) and behavioral control (e.g. emotional control).

Neurobiological and Neural Models of EF

Alexander Luria was one of the first researchers to introduce a model based on neurobiological processes (26) suggesting the broader engagement of various brain regions. In this model, frontal lobes were conceptualized as the regulatory area directing complex problem solving. Damage to the frontal lobes was associated with the frontal lobe syndrome (49), characterized by disinhibition, inability to follow a sequence of instructions, and repetitive motor movements.

Advancements in neuroimaging techniques have placed increasing focus on neuroanatomical localization of EF processes primarily within frontal cortical regions. Localization of cool EF processes has been associated primarily with the dorsolateral prefrontal cortex (PFC), while the top–down processes that regulate hot EFs are linked to the orbitofrontal or ventromedial prefrontal cortex. Some cognitive models also propose specific neuroanatomical correlates of EF. For example, for Stuss' model (87) it was proposed that the task setting and monitoring EFs are localized in the left and right lateral frontal cortex, respectively, while behavioral/emotional regulation corresponds with the localization of hot EFs in the orbitofrontal cortex. Energization is reported to be mediated by the superior medial prefrontal cortex, while metacognition is guided by the frontal poles (87).

The identification of these regional contributions, while valuable, does not encapsulate the broad cortical systems that are being recognized as significant in the neural processes that underlie EF processes (89). Building on the neuroanatomical localization of EF, connectivity models focus on neural circuitry between cortical regions and may present a more integrated approach in the study of EF.

Neuroimaging studies identified that discrete EFs are linked to broader brain networks including the areas within the prefrontal cortex. For example, set shifting was associated with activity of the lateral prefrontal cortex, anterior cingulate cortex, and inferior parietal lobule (52). Set switching task was associated with involvement of the prefrontal cortex and frontoparietal areas of the brain (52, 90). An extended brain network connectivity between dorsal and ventral brain networks was observed in fluency tasks including activation in the inferior frontal gyrus and left dorsolateral prefrontal cortex (34). A differentiation between dorsal and ventral brain networks was observed between phonemic and semantic fluency tasks, respectively (61). Similarly, extended brain network involvement is reported during completion of planning tasks including activation of the dorsolateral prefrontal cortex, the anterior and posterior cingulate areas, and the parietal cortex (91). Activation of frontal regions during working memory tasks included activation of the bilateral superior and middle frontal gyri, bilateral frontal polar regions, and precuneus gyrus (92).

At the neurochemical level of analysis, a number of neurotransmitters have been linked to EF processes. A comprehensive review (93) summarized the role of four neurotransmitter systems in EF. Dopamine (DA) was reported to influence cool EF constructs (set shifting, response inhibition) and to moderate hot EF reward processes. Norepinephrine (NA) circuits were associated with a number of EF cognitive processes (including response inhibition and set shifting likely

TABLE 2 | The definition and assessment measures of discrete EF domains.

EF domain	Neuropsychological and experimental task measures
Set shifting/concept formation Set shifting or concept formation is defined as the capacity to shift between mental processes to form new concepts and identify the conceptual relationships shared by stimuli (12, 50). Other commonly used terminology for set shifting includes concept formation and cognitive or mental flexibility (25). Theorized mechanisms for set shifting have included switching between mental processes. It has been argued, however, that set switching (51) represents a distinctly different EF component that needs to be differentiated from set shifting.	Wisconsin Card Sorting Test (WCST) (52) Intra/Extra Dimensional Shift (IED)—CANTAB (53) Sorting test—D-KEFS (50) Dimensional Change Card Sort test (DCCS) (54) DCCS—NIH Cognition ToolBox (55) Flexible Item Selection Task (FIST) (56). Set Shifting test—CogState (https://www.cogstate.com/) Rule Shift Cards test—BADS (57) Temporal Judgement test—BADS (57)
Mental flexibility/set switching Set switching has been defined as the capacity to switch between mental processes (multiple tasks, operations, or mental sets) in response to changing demands (51, 58). It is distinct from set shifting, where the focus is on identifying novel relationships.	Trails Making Test (Trails B) (59). Trails Making Test—D-KEFS (50)
Fluency Fluency is defined as the capacity to generate verbal and non-verbal stimuli including ideas, designs (50), and words (60). Verbal fluency is a frequently studied measure of executive functioning (34) and is distinguished into phonemic (generativity for unrelated words) and semantic fluency (generativity for semantically related words or categories) (61). There is some debate as to whether phonemic and semantic fluency represent EF (36, 60) or language processes (62). However, a number of studies supported by neuroimaging findings (34) suggest that verbal fluency is reliant on core EF processes (63, 64).	Controlled Oral Word Association Test (COWAT) (65) Verbal Fluency test—D-KEFS (50) Design Fluency test—D-KEFS (50). 20 Questions Test—D-KEFS (50) Word Context test—D-KEFS (50) Proverb test—D-KEFS (50)
Planning Planning is defined as the capacity to execute a sequence of actions so that a desired goal is achieved (36).	Tower of Hanoi (66) Tower of London (67) One Touch Stockings (OTS)—CANTAB (53) Stockings of Cambridge (SOC)—CANTAB (53) Action Programme Planning test—BADS (57) Key Search test—BADS (57) Zoo Map test—BADS (57) Modified Six Elements test—BADS (57)
Response inhibition Response inhibition primarily refers to the ability to inhibit a previously learned or prepotent response (12). Two additional components contribute to inhibition: resistance to distractor interference and resistance to proactive interference (68). Resistance to distractor interference refers to the ability to process a target stimulus while ignoring irrelevant information presented at the same time, while resistance to proactive interference refers to the ability to efficiently process distractors from recently activated memory stimuli. Some research classifies resistance to proactive interference as a working memory process.	Stroop test (69) Color-Word Interference Test—D-KEFS (50) Go/no-go task (70) Hayling test (71) Eriksen flanker task (72). Stop Signal Task—CANTAB (53) Flanker Inhibitory Control and Attention test—NIH Cognition ToolBox (55) Go–No Go Test—CogState (https://www.cogstate.com/)
Working memory The concept of working memory is sometimes used interchangeably with short-term memory (STM), although different processes relate to each. Working memory refers to the capacity to store and dynamically manipulate information in temporary STM (36).	Letter sequencing task (73) Digits Backwards—Wechsler Memory Scale (74) Spatial Working Memory (SWM)—CANTAB (53). Spatial Span (SSP)—CANTAB (53) List Sorting Working Memory Test—NIH Cognition ToolBox (55) n-back task (75) One Back test—CogState (https://www.cogstate.com/) Two Back test—CogState (https://www.cogstate.com/)
Hot EF Top–down processes activated in situations with motivational and emotional significance (76).	Affective Go/No-go (AGN)—CANTAB (53) Cambridge Gambling Task (CGT)—CANTAB (53) Information Sampling Task (IST)—CANTAB (53) Iowa Gambling test (77)

(Continued)

TABLE 2 | Continued

EF domain	Behavioural rating measures
Emotional/personality change, motivational change, behavioral change, cognitive change	Dysexecutive Questionnaire (DEX)—BADS (57)
Global executive composite Behavioral Regulation Index—initiate, organization of materials, plan/organize, task monitor, working memory Metacognition Index—emotional control, inhibit, self-monitor, shift	Behavioral Rating Inventory of Executive Function (BRIEF) (48)
Self-management in time, self-organization/problem solving, self-restraint, self-motivation, self-regulation of emotion	Barkley Deficits in Executive Functioning Scale (BDEFS) (37)

BADS, Behavioural Assessment of the Dysexecutive Syndrome; CANTAB, Cambridge Neuropsychological Test Automated Battery; D-KEFS, Delis–Kaplan Executive Function System.

due to influence of NA on arousal and attentional systems. Serotonin (5-hydroxytrypatamine [5-HT]) modulated response inhibition, through its action in the orbitofrontal cortex. Finally, the cholinergic system mediated set shifting and was proposed to also interact with a number of other neural circuits for a more complex integration of EF processes.

The role of γ-aminobutyric acid (GABA) is increasingly linked with mediating processes associated with neural circuitry in the prefrontal cortex. GABA is the primary inhibitory neurotransmitter in the mature brain, working with excitatory glutamate to create an excitation/inhibition (E/I) balance thought to reflect the activity of the cortex. More excitation is theorized to represent greater activity, while greater inhibition suggests decreased cortical activity (94). Increased GABA (compared to glutamate) within the lateral PFC has been associated with better ability to select between competing tasks (95). Improved working memory performance under increased memory load was associated with higher GABA concentration in the dorsolateral PFC. A recent study (pre-print) (47) attributed a key role to GABAergic genetic contributions to the common EF factor (45), using a large sample in a genome-wide association study (GWAS). This study highlighted the role of the excitatory/inhibitory balance in EF, especially the role of GABA-mediated inhibition.

The models described above reflect the divergent approaches taken in the study of EF in normative literature. In ASD, however, focus has been primarily on comparing diagnostic groups with autism and other cohorts on performance on discrete EF constructs. The executive dysfunction hypothesis discussed below sums a large part of empirical research of EF in ASD. It may reflect efforts to identify discriminating profiles between different groups. Novel approaches to the study of EF in ASD have focused on brain connectivity and neurotransmitter imbalance with limited evaluation of other EF models.

EXECUTIVE FUNCTION AND AUTISM SPECTRUM DISORDER

Executive Dysfunction Hypothesis

Early studies of EF in ASD were summarized in a review by Pennington and Ozonoff (10). Executive dysfunction was proposed as a model for understanding behavioral problems in ASD, including impaired theory of mind (ToM). Their review of research studies across neurodevelopmental disorders suggested that discrete EFs (set shifting, response inhibition, and working memory) might be appropriate cognitive markers for differentiating between ASD and ADHD.

Empirical findings on EF deficits in ASD were subsequently formalized in the executive dysfunction hypothesis (25) proposed in an effort to review and integrate the extant literature of EF in ASD. The review focused on four EFs: planning, mental flexibility, inhibition, and self-monitoring, assumed to represent the core EF domains. The executive dysfunction hypothesis suggested impairment on distinct EF domains, supporting a fractionated model of EF. In addition to identifying impairment in EFs, the review also highlighted considerable variability in EF performance between studies and within cohorts.

Since the introduction of the executive dysfunction hypothesis, there has been a proliferation of studies investigating cool EFs in ASD; these have been synthesized in a number of meta-analyses. Findings in the extant literature of executive dysfunction and heterogeneity in EF performance complement the observations made by Hill (25) and Pennington (10).

A meta-analysis on cognitive flexibility (96) indicated life span impairment in ASD. The study adopted a broad definition of cognitive flexibility and combined research on set shifting, set switching, and inhibitory control. A meta-analysis in children and youth investigating the components of response inhibition, prepotent response inhibition and interference control, identified age related differences (68). Impairment in prepotent response inhibition attenuated with increasing age, whereas difficulties in interference control persisted across the life span. An investigation of working memory (97) in children and young adolescents revealed impairment across both verbal and spatial working memory. There were no age-related differences; however, a larger effect size was observed for spatial compared to verbal working memory, suggesting greater difficulties in the spatial domain for youth with ASD. Planning is considered a key EF in adaptive behavior, and a meta-analysis reported impairment in planning for individuals with ASD (98). Planning difficulties were independent of moderator influences of age, intellectual functioning, and assessment type. The meta-analyses described above confirm impairment in discrete EFs; however, it remains uncertain whether these are underpinned by a common mechanism or whether discrete EFs are differentially impaired in ASD.

Two recent meta-analyses (22, 23) investigated cool EFs in ASD across multiple EF domains and thus address this question. Broad impairment in EF was observed both in children and youth (22) and across the life span (23). In the (22) meta-analysis, impairment in response inhibition and planning was less prominent compared to deficits in flexibility (set switching and set shifting), generativity/fluency, and working memory. Impairment across all of the above domains was identified in the (23) meta-analysis. Both studies suggest that an underlying common pathway may influence EF processes in ASD.

The meta-analyses described above also identified substantial heterogeneity in EF performance, despite consideration of a number of moderator variables. Hot EFs may be contributing to the unexplained heterogeneity, particularly in the view that they are independent of cool EF processes (86, 99). Comparable to most research of cool EFs in ASD, the study of hot EFs principally adopted the fractionated approach investigating discrete domains. Impairment has been observed in tasks associated with affective decision making and delay discounting (85, 100). Given the limited studies completed to date, it is unclear whether hot EFs could alone explain the heterogeneity observed in EF performance in ASD.

Atypical Brain Connectivity

Throughout the ASD literature, there have been consistent findings of atypical functional connectivity, though this has varied between over-connectivity and under-connectivity (101). The regions impacted in ASD include areas encompassed by the default, salience, and executive control networks (102) and in cortical–subcortical circuitry (103). When connectivity is investigated for distinct EFs, there are reported differences in the circuitry associated with working memory (104) and response inhibition (105), with atypicalities reported to persist across the life span (106).

The Excitation/Inhibition Hypothesis (E/I)

The E/I model (107) examines observed behavior in ASD at the neural level. The E/I model focuses on the action of glutamate and GABA and the balance between the two. The model suggests that an imbalance between neural excitation (driven by glutamate) and neural inhibition (driven by GABA) in brain circuits contributes to ASD symptomatology (108) and associated impairment in perceptual, motor, and cognitive systems (107, 109). The links between ASD, EF, and the E/I hypothesis have not been extensively investigated. The observed reductions in GABA concentration and GABA receptors in the frontal lobes (110) suggest a likely influence of GABA on frontal lobe processes, including EF. For example, greater concentrations of GABA in the frontal lobe have previously been associated with superior cognitive performance (111). It is theorized that reductions in frontal GABA may be contributing to the broad EF difficulties in ASD. Furthering this hypothesis is tentative support that GABA may relate to response inhibition processes (112). Evidence that the E/I imbalance can be shifted with pharmacological interventions, and that this shift is accompanied by a normalization of functional connectivity patterns in the frontal regions (113), suggests a potential intervention strategy for ASD that may lead to improvements in cognitive processes, including EF.

Moderating Influences on EF in ASD

Moderator variables and other mediating factors (e.g. measurement of EF construct) may contribute to the observed variability of EF findings in ASD. A number of these factors are discussed below.

Measurement of EF

The validity and reliability of EF measures may significantly moderate observed performance. Validity refers to the extent that the EF assessment tool accurately taps the theorized EF construct (36, 114). Reliability refers to the consistency of the EF assessments to measure the EF construct (36). Research in EF has been criticized for lacking valid and reliable measures. The main criticism relates to the lack of task purity in the tools utilized to measure EF (10, 64). It has been demonstrated that EF assessment tools likely measure multiple EF and non-EF processes, thus challenging their efficacy to assess distinct EFs.

Measurement of EF has traditionally focused on neuropsychological assessments sensitive to frontal lobe damage (50, 115). Assessment tools, however, including classic measures such as the Wisconsin Card Sorting Test (WCST) (115) are not pure measures of the underlying EF, e.g., set shifting (52). Experimental tasks have also been utilized as likely purer measures of discrete EFs (12). More recently, development of behavioral rating scales (37, 116) aimed to provide more ecologically valid assessments of EF (37, 117, 118) focusing on executive regulation of everyday behaviors. Studies in ASD demonstrated a significantly larger effect size for behavioral rating scales compared to neuropsychological and experimental measures (23). These findings suggest that behavioral measures may better capture EF processes and are more ecologically valid (118).

Developmental Trajectory of EF

An overview of the developmental trajectory of cool EFs in neurotypical development and in ASD is presented in **Figure 1**. In typical development, maturation of EFs begins in infancy and continues throughout childhood and adolescence and into early adulthood (119, 120). The rate of improvement for individual components, with the exception of fluency, begins to taper at about age 12 (119), with most EFs reaching their peak in late adolescence/in the early 20s (120).

In ASD, there is evidence of executive dysfunction across development for discrete EF domains (e.g. working memory, set shifting/switching, fluency) (22, 68, 121) with some support of improvements in EF ability over time (122). Developmental research of hot EFs in ASD is limited. Recent research found no significant age-related changes in ASD in the neurotypical comparison group (100). This contrasts with other research in neurotypical development that suggests a variable developmental trajectory (76, 99).

The variability in peak developmental periods for distinct EFs may be contributing to some of the heterogeneity observed in EF performance in ASD. The use of mixed age groups in ASD research mask these differences and could contribute to variability observed between studies.

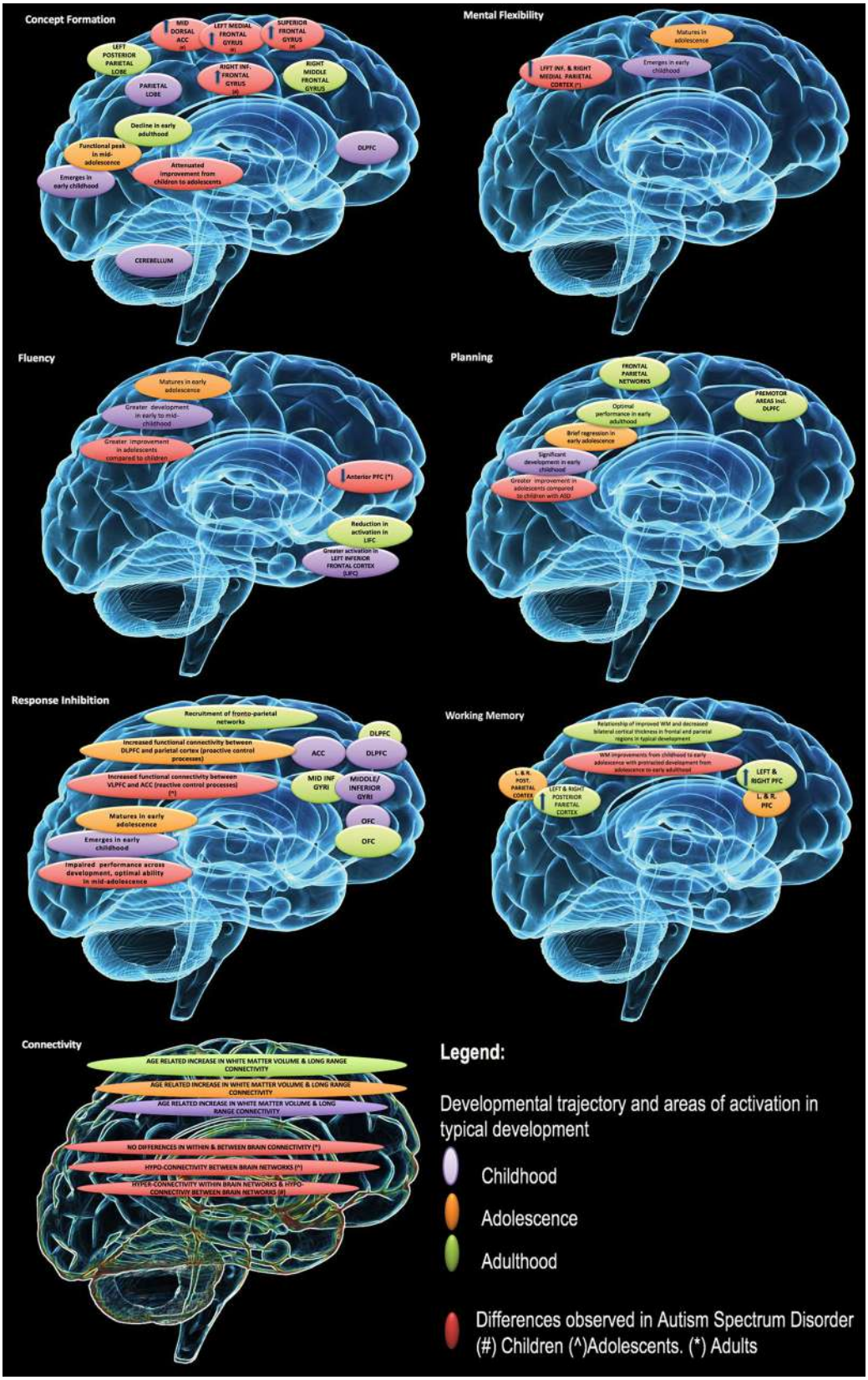

FIGURE 1 | Developmental changes in executive function and associated impairment in autism spectrum disorders (ASD). Reproduced with permission from (23).

Moderator Variables of EF

General Intellectual Functioning

Early research on EF developed partly based on observations that higher cognitive processes (e.g. planning, concept formation) may be impaired despite intact intellectual functioning. Despite this general observation, there is some empirical support that intellectual ability may moderate performance on neuropsychological assessments of EF (123). This is pertinent in the study of ASD, where differences between specific indices of intelligence (verbal, perceptual, and full-scale intelligence scales) have been reported for the clinical subgroups of autistic disorder and Asperger's syndrome (124).

Sample Characteristics and Task Characteristics

Each study sets its own criteria to define eligibility and to enroll ASD participants, creating a lack of consistency between studies. These differences include ASD diagnosis (as per earlier *Diagnostic and Statistical Manual of Mental Disorders* [*DSM*] classifications), choice of comparison groups, age, and criteria matching the ASD cohort to the comparison group. The diagnostic criteria for ASD have broadened significantly since the first inclusion of autism in the *DSM-III* (125). In *DSM-5* (1), discrete diagnostic categories (autistic disorder, Asperger's syndrome) have been merged into a single spectrum, facilitating uniformity in the diagnostic selection criteria (but likely introducing greater heterogeneity). Prior to the introduction of the *DSM-5*, a number of studies were comprised of mixed diagnostic classifications (126–129), while some studies included the informal classification of high functioning autism (HFA) (130). HFA defined ASD cohorts with no intellectual disability (IQ greater than 70). However, inclusion criteria on level of intellectual functioning ranged between studies from borderline (131), low average (129), to average (132). This could have contributed to greater variability in intellectual and executive functioning and may in part explain differences between studies.

Most studies have utilized standardized diagnostic assessments of ASD (Autism Diagnostic Observation Schedule [ADOS], Autism Diagnostic Interview [ADI]) and *DSM*-based diagnostic criteria. Some studies may utilize screening assessments (132, 133) or classification criteria not drawn from the *DSM* (134). These factors may also contribute to the variability in EF performance.

Selection of comparison control groups also varies between studies. Although most studies include neurotypical comparison groups, there have also been comparisons conducted with non-affected siblings (135, 136) or clinical groups only (137–139).

The type of assessment, whether it is a psychometric test, an experimental task, or a behavioral rating scale, is an important moderating factor in the discussion of EF. It has been suggested that behavioral rating scales capture different underlying mechanisms (140) compared to performance-based tasks and therefore should not be utilized as substitute measures of EF performance. In particular, self-ratings on behavior may reflect individual's motivation for goal setting, achieving personal goals, and their personal expectations in relation to these goals (140). By comparison, neuropsychological assessments and experimental tasks are performance-based measures that measure EF within the designed parameters of the task. Research in ADHD (141) and ASD (20) lends some support that different cognitive mechanisms may underpin these measures. For example, low correlations were reported between performance measures and a behavioral rating scale of EF (142).

Administration format (traditional versus computerized presentations of test material) may also moderate EF performance. There is evidence that individuals with ASD perform better on computerized administration in comparison to traditional administration of EF tests (118, 143), although this is not unequivocal (144). Further, the presentation format of the test stimulus (verbal versus visual stimuli) and participant response format (motor versus verbal response) may be important moderators. This is particularly relevant to ASD research, as there is some support for superior performance in individuals with ASD in visuoperceptual tasks requiring attention to detail (145).

Sex Differences

ASD is a neurodevelopmental condition that occurs more in males, currently with about three males diagnosed to every one female (146). A number of theories have been proposed to explain this difference. These are based on genetic and/or neurobiological differences between males and females as described, for example, in the imprinted-X liability model (147), the male brain theory (148), and the female protective effect theory (149, 150). There is growing interest in identifying the characteristics that might differentiate male and female individuals with ASD, including EF performance. However, comparisons of males and females with ASD on neuropsychological assessments and self-/informant appraisals of EF have been limited. Some research findings (151–153) suggest differences between males and females with ASD on EF performance, while others report no differences (154, 155). One potential confounding factor is that not all studies included sex-matched neurotypical control groups. Sex differences in cognitive performance observed in neurotypical populations may also be present between females and males with ASD. These, however, will not be identified in ASD cohorts without comparisons to sex-matched neurotypical controls.

Co-Morbid Conditions and Affective States

The presence of co-morbid ADHD may influence EF performance in ASD, and this was particularly evident in inhibition (156). Other co-morbid conditions (e.g. depression, anxiety) have a high prevalence in individuals with ASD (157) and may have a moderating role on EF. In particular, the influence of anxiety (158) and stress (159) on EF has been well documented. Overall, research to date suggests a moderating effect of anxiety on cognitive function in non-clinical samples of highly anxious individuals (160–163). In ASD, anxiety negatively correlated with test performance on neuropsychological assessments of concept formation (18). Anxiety was also shown to correlate with impaired performance on neuropsychological measures of inhibition, mental flexibility, and shifting (164). The links

between affective states and EF highlight the importance of investigating their role in ASD research.

Heterogeneity of EF

The preceding discussion highlighted that observed executive impairment in ASD is characterized by heterogeneity with a range of contributing of factors. A research framework that can utilize EF as a marker and facilitate classification of ASD into distinct subtypes could contribute to diagnostic and intervention strategies for this group. Using cognitive and neuroimaging measures, three ASD subtypes were identified in a recent study based in part on performance on response inhibition tasks (165). A second study (166) showed that performance measures of cognitive flexibility distinguish between children with and without ASD. Interestingly, however, extension of the above study to brain connectivity circuits of cognitive flexibility did not identify subtypes at the neural level (167). The authors suggested that a dimensional approach might be more appropriate for some cognitive processes. The RDoC framework (29) incorporates a dimensional approach and can evaluate EF across cognitive and neural measures. We discuss below the efficacy of EF as an endophenotype for ASD and propose that the RDoC framework can advance research of EF in ASD.

EXECUTIVE FUNCTION AS A COGNITIVE INTERMEDIATE PHENOTYPE

Endophenotypes, or intermediate phenotypes (168), are characteristics that present vulnerabilities in a particular population, linking genes, brain processes, and observed behavior. Endophenotypes may encompass neurocognitive functions (136, 169), making EF a likely candidate. Criteria that must be satisfied for considering a marker as an endophenotype include: the marker must be associated with the illness/disorder in the population; it must be heritable; and it must present at higher rates within affected families than the general population (170).

The wealth of empirical findings linking EF with the broader ASD phenotype (in particular, the diagnostic clusters as defined in the *DSM-5*) support its potential as an endophenotype. Early reviews of the literature (171, 172) and empirical studies reported a correlation between neuropsychological (129, 173, 174) and behavioral measures (175) of executive impairment with severity of repetitive behaviors. This relationship was reported for specific EF domains, such as cognitive flexibility, response inhibition, and working memory. Another study suggested that EF deficits were specific to repetitive but not restricted behavior patterns (15). These findings led to theories that linked restricted and repetitive behavior symptoms to EF, suggesting that EF constructs can differentiate within behavioral clusters in ASD. A number of studies show that EF influences ToM performance in ASD (17, 176) and may influence the social communication cluster. The ToM model (5) was one of the prominent cognitive explanations for impaired social cognition in ASD. It proposed that impaired ability to attribute mental states to self and others contributes to a range of deficits including those observed in the social communication cluster (177). Recent research also indicated that ToM may predict disability (178). Support of a putative link between EF and ToM includes findings that reduced working memory moderated social communication skills (179). In summary, there is evidence that EF influences both diagnostic clusters of ASD (1) and would be a valuable endophenotype for targeted interventions.

EF has measurable behavioral outcomes (37, 142) and is linked to genetic (168) and neurobiological (180) processes. For example, functional imaging studies have demonstrated that neuropsychological assessments of EF are linked with activation of brain areas including frontoparietal (168) and frontal cortical areas (52). Further, genetic influences account for about half of the variability in EF performance (45, 168). The neural substrates of GABA and glutamate present a neural link for the EF (common factor) which has a genetic basis but may be measured with cognitive tasks (47). Lastly, there is empirical support that EF difficulties in relatives of probands with ASD are at a higher rate than the general population (181).

In summary, research on EF indicates that it satisfies the definition of endophenotypes and supports its role as an endophenotype for ASD.

A RESEARCH FRAMEWORK AND FUTURE RESEARCH DIRECTIONS

We suggest that a model of EF in ASD that bridges the pathway from genetics to neural circuitry and to the observed EF phenotype may better capture the heterogeneity of EF in ASD. The unity and diversity model (12, 45, 46) provides a link for an integrated research framework for EF. The common EF factor could contribute to quantifying heterogeneity in EF performance in ASD. Complementing the above, investigation of core cool EFs (set shifting and working memory), hot EFs/behavioral regulation, and affective states in a single research framework can further advance the study of EF in ASD.

The RDoC framework (29) provides research guidelines that may resolve a number of the limitations observed in ASD research. The RDoC approach advocates a focus on a dimensional research framework. It is guided by research across "systems"-based domains that are evaluated by different levels of measurement (extending from the molecular/genetic level to the observed behavioral phenotype) (182).

The guiding principles of the RDoC framework (183) focus on: a dimensional systems approach, behavior–brain relationships, and multiple levels of analysis (molecular, circuit behavior, symptom). These principles align with the study of EF and ASD, creating a framework to guide this complex research area. Further, the RDoC framework can be adapted to reflect key characteristics of neurodevelopment (developmental trajectories/sensitive periods) (183, 184) and can be particularly relevant to the study of neurodevelopmental conditions, including ASD.

The RDoC framework presently consists of six systems domains: negative valence systems, positive valence systems, cognitive systems, social processes, arousal and regulatory systems, and sensorimotor systems. Each system is characterized

by different constructs that are evaluated across distinct units of analysis (or measurement): genes, molecules, cells, circuits, physiology, behavior, self-report, and paradigms. Research of EF in ASD brings together a number of these systems and specifically the "positive valence systems," "negative valence systems," and "cognitive systems."

The positive valence systems are responsible for responses to positive motivational situations or contexts, such as reward seeking. The negative valence systems are responsible for responses to aversive situations or context, such as fear, anxiety, and loss, and the cognitive systems domain is responsible for cognitive processes.

The positive valence systems domain presents a framework for integrating the relationship between hot EFs and behavioral regulation. Complementing these, the negative valence systems domain captures the contribution of anxiety in ASD (157) and its moderating role in EF outcomes (158). Within the cognitive systems domain, the constructs of cognitive control and working memory reflect the EF factors of the unity and diversity model (12). These can evaluate the contribution of cool EFs in ASD. Investigated together, these three systems would provide researchers with a common language facilitated by a consensus on the specific components under each unit of analysis. Furthermore, such an integrated approach would provide greater opportunity to identify subtype profiles within ASD. Targeted intervention strategies can then be tailored to each profile with primary focus on the domains of the cognitive, positive, and negative valence systems. A summary of the proposed integrated framework is presented in **Figure 2**.

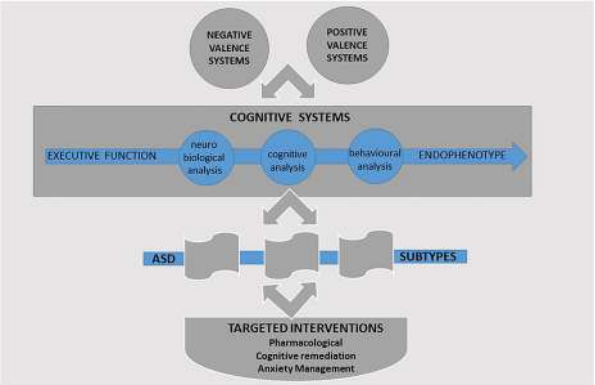

FIGURE 2 | A research framework for the study of executive function (EF) in ASD.

CONCLUSION

EF is an important factor in the study of ASD and with great potential as an endophenotype. Despite the plethora of theoretical models, there is conceptual confusion in EF research that would benefit from a unified research methodology. The findings of broad EF impairment in ASD are an important step, as they unify much of the research on cool EFs and highlight that differences are likely guided by genetic variability in EF processes. The application of the RDoC framework has potential to improve our understanding of EF in ASD and elucidate the mechanisms responsible. RDoC presents a framework to integrate research obtained from diverse measures (neuropsychological tests, experimental tasks, behavioral ratings) to characterize the relevant circuitry and investigate additional factors (e.g. hot EFs) and moderators (e.g. anxiety). Taken together, the RDoC approach presents new opportunities for profiling ASD subtypes and for targeted assessments and interventions.

AUTHOR CONTRIBUTIONS

ED and AG contributed to the conception and planning of the review. ED conducted the literature search and provided the initial draft. ED, MD, and AG wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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Few Differences in the Externalizing and Criminal History of Young Violent Offenders With and Without Autism Spectrum Disorders

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Autism spectrum disorders (ASDs) are known to be associated with an increased risk of aggression and challenging behavior. In this study, we have mapped the externalizing history of a nationally representative cohort of young violent offenders with ASD, compared with offenders without ASD. Two hundred and sixty-nine violent offenders were assessed for prevalence of ASD, and participated in a thorough assessment of previous externalizing problems and criminal history. Twenty-six offenders met consensus clinical DSM-IV criteria for ASD and they were compared to offenders without ASD from the same cohort. Overall, we found a very high prevalence of externalizing and antisocial behaviors in the history of these offenders and there were few differences between the groups. Placements in foster homes were overrepresented in the ASD group and the ASD-offenders had significantly more often been diagnosed with a neurodevelopmental disorder (i.e. ASD or ADHD) by a clinician before the study. At index conviction, ASD offenders were overrepresented in sex crimes with a child victim. Though offenders without ASD had more previous convictions, in particular drug crimes, we found no difference in terms of total number of prosecuted crimes. Substance use disorders were more common among offenders without ASD. The ASD offenders scored higher compared to the non-ASD offenders on the Affective facet of the Psychopathy Checklist-Revised (PCL-R) but there were no differences in terms of total PCL-R scores. Our results provide important knowledge of the developmental history of offenders with ASD. Though this is a small and atypical phenotype it poses significant challenges to the criminal justice system and we need to understand more of it to be able to prevent these individuals from committing crimes but also to provide a fair judicial treatment, to assess exculpatory factors and improve our forensic treatment models.

Keywords: autism spectrum disorder, externalizing behaviour, attention deficit disorder with hyperactivity, conduct disorder, psychopathy, crime, violence

INTRODUCTION

Violent and criminal behavior in individuals with Autism Spectrum Disorder (ASD) has often been considered a sensitive topic to discuss, particularly in individuals with so-called high-functioning ASD (i.e., without a general intellectual disability) (1). Their lack of reciprocity, social naivety, compulsivity and resistance to change has been raised as possible spectrum characteristics that could explain instances of criminal behavior. However, researchers have expressed worries that reports of serious offences may lead to unnecessary anxiety on the part of parents and to stigmatization of people with ASD (2). There is a lack of research which try to disentangle the empirical background of persistent violent and criminal behavior among individuals with ASD in the general population (e.g. 3, 4).

Almost two decades ago, Moffitt and colleagues (5) suggested a common neurodevelopmental basis for ASD and childhood-onset antisocial behavior. In later support of this, twin studies have shown that the same genetic and environmental factors that are linked to ASD also influence the development of oppositional and conduct problems (6) and autism-like social interaction problems are implicated as among the strongest predictors of conduct problems (7). In a study of childhood arrestees, delinquent behavior was positively associated with autism symptoms, even after adjustment for externalizing disorders, i.e. ADHD and conduct disorder (CD) (8). Studying a large population-based record-linkage cohort, Heeramun and colleagues (4), replicated this increased risk of violent offending among individuals with ASD, but contrary to Geluk and colleagues, when controlling for ADHD and CD this relationship disappeared. Other studies have shown that persistently disruptive children often have autism behavioral traits, and as many as one third might meet criteria for an ASD (9, 10), but only in a small minority of these children, their ASD is detected by the psychiatric services. Similarly, in clinical groups of children with ASD, conduct problems are common, though seldom noted in their medical record. Particularly atypical autism seems to be overrepresented in children with CD (e.g., 11).

Childhood psychosocial adversities and maladjustment have previously been linked to a number of negative outcomes over the life time, including antisocial behaviors (12, 13). Among children with ASD, Howlin and Clements (14) found abuse to be associated with more behavioral difficulties, including aggression, compared to typically developed children. Similarly, Allely et al. (15) have described the presence of a broad range of adverse contextual factors among individuals with ASD who commit serious violent crimes.

Certain psychiatric disorders can increase the risk of violent behavior (16–18), with a particularly strong relationship between substance abuse and repeated violent criminality (e.g. 19). Likewise, this relationship seems to correspond for individuals with ASD (3, 20). Substance use-related problems have traditionally been considered rare in ASD, but recently a review pointed to large variability in different studies (21) and Butwicka and colleagues (22) even showed an increased risk for substance use related problems in a population-based cohort study, without a link to ADHD. From earlier studies in clinical settings (e.g. 23), we know that substance use disorder (SUD) rates can be substantial, and that comorbid ADHD could be a contributing factor to increased SUD in ASD.

Among adults with ASD, there are numerous case reports of serious and persistent offending behavior (e.g., 24–27). Though there are some reports of non-violent criminality (e.g., 28), violent crimes seem clearly overrepresented in the literature. Within the category of violent crime and ASD, arson (e.g., 29, 30) and sexual offence (e.g. 31–33) have gained specific attention. However, the quality of research in this area is generally low and often hampered by the fact that the prevalence of ASD, most probably due to clinical practice and diagnostic criteria, has risen dramatically over the last 20 years, making long term follow-up difficult. Several authors have noted that we need to find out more about the relation between ASD and criminal behavior (34, 35).

To summarize, there has been a vivid debate on the criminal propensity of individuals with ASD and a striking difference in results between population-based studies and criminal cohort studies. In cases where ASD is connected to criminal behavior there are different views as to which kinds of antisocial acts these individuals commit. To address this issue, the present study compares violent offenders with and without ASD on a range of measures of externalizing and criminal behavior over their life-course in an effort to map the developmental history of aggressive and antisocial behaviors in ASD.

METHODS

Procedure

The study was approved by the Research Ethics Committee at Lund University. All inmates in the participating prison facilities received oral and written information about the study from a prison staff and those that agreed to participate in the study provided written informed consent.

Participants were consecutively assessed according to a pre-set protocol. The clinical assessments, which were performed by experienced clinical psychologists who had a special training in the instruments used, were conducted during a full day session. Before the assessment started, the psychologist had read all file information, including prison health care journals, detailed reports on previous living circumstances and criminal history, and incidents during ongoing sanction, available from the Swedish Prison and Probation Service.

The participants were also given the opportunity to receive feedback on the preliminary results from the assessments. Participants showing indications of severe psychopathology were given the opportunity to be referred to the prison's psychiatrist for further assessment and treatment. A small monetary compensation for time spent in the study was provided (SEK 200, approximately $22).

Participants

Participants (n = 269) consisted of male violent offenders recruited from the Development of Aggressive Antisocial Behavior Study (DAABS). The DAABS is a nationally representative cohort of

all young adult male offenders (aged 18–25 years) convicted of hands-on violent (including sexual) offenses and imprisoned in one out of nine prisons (low to high security levels) in the Western Region of the Swedish Prison and Probation Service between March 2010 and July 2012. The participation rate was 71%. Detailed descriptions of the cohort are provided in previous publications (36–38). In the total DAABS cohort, 26 participants (10%) met criteria for an ASD at the clinical assessment (Autistic disorder n = 2, Asperger´s disorder n = 18, Pervasive developmental disorder not otherwise specified n = 6). The remaining 243 participants did not have an ASD and were used as a comparison group in this study. There were no statistically differences between the groups in terms of age (ASD: 21.6 years (19.0-25.9), non-ASD: 22.2 years (18.6-25.9), p = 0.090). Two clinical assessments were prematurely ended, because of the participants' clinical conditions, and on some variables there was insufficient or opposing information, which resulted in missing data.

Diagnostic Evaluation

Participants were assessed for lifetime and current psychiatric disorders by a structured interview protocol based on the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I) and the SCID-II. For the disorders not covered in the SCID (e.g. developmental disorders, impulse control disorders and sexual disorders), an amendment including a lifetime DSM-IV (39) symptom checklist of individual criteria or symptom definitions was added.

For assessment of ASD, the Asperger Syndrome/high functioning autism Diagnostic Interview (ASDI), (40) was used, which is a combined interview and observation schedule for clinical assessment. All participants were also asked about the presence of atypical sensory perception, a commonly reported symptom in ASD. When possible, a collateral interview (the Autism-Tics. ADHD and other Comorbidities inventory/A-TAC, 41) was performed to obtain the developmental history of the participants. In many cases it turned out extremely difficult to get in touch with their families and it was only in a minority of cases an interview could be performed. The A-TAC was used in six ASD cases (23.1%) and in thirty (12.3%) of the non-ASD cases. For participants potentially meeting diagnostic criteria for an ASD-disorder, the team tried to perform an in-depth autism spectrum examination, including either a "Diagnostic Interview for Social and Communication disorders" (the DISCO), (42) with parents/caregivers or an "Autism Diagnostic Observation Schedule" (ADOS), (43) with the participant. The DISCO was used in one ASD case (3.8%) and the ADOS in three cases (11.5%).

Final diagnostic decisions were based on all the available information, provided by the files, registers, clinical and collateral interviews, self-rating questionnaires and the clinical impression of the respondent during the 6–7 hours assessment, in consensus by the clinical psychologist and a senior clinician and researcher (EB or BH), in accordance with the LEAD-principle (44). Comorbidity between ADHD and ASD was allowed in order to account for overlap between the two conditions. In accordance with the DSM-IV, age at onset for symptoms of CD was specified and categorized as early onset (age ≤10) or late onset (age >10).

Previous Externalizing and Criminal Behaviors

Detailed information on the psychosocial background, including externalizing behavior, and criminal history but also previous placements in foster homes and institutions was collected by a structured protocol. In Sweden, out of home placements could be the result of either a destructive behavior of the child or serious maltreatment and abusive conditions in their homes, not seldom a combination of both. The protocol also covered in-depth information on the index offence, divided into the following categories; murder/manslaughter, robbery, assault, sexual offence with adult victim, sexual offence with child victim, and other violent crimes.

Since the availability of official and objective crime data on the participants could vary at the time of assessment, complete official register-based criminal history was collected from the Crime Register, held by the National Council of Crime Prevention, from age of criminal responsibility until the index conviction at inclusion in the DAABS The collected data included all offences defined in the Swedish Penal Code (45:700), Narcotic Drugs Punishment Act (46:64) and the law on punishment of certain traffic offences (47:649), including court convictions, order of summary punishment and omission of prosecution.

Criminal history, including information from the crime register, was divided into six categories: violent offenses (e.g. murder/manslaughter, assault, unlawful threat, robbery, and arson) following Falk and colleagues (19), and the remaining, i.e. sexual offenses, drug-related offenses, property offenses (collapsing theft and vandalism, excluding robbery), traffic violations, and fraud following the definitions of the National Council of Crime Prevention (48). All crimes included attempted and aggravated forms.

Psychopathy

Psychopathy was measured through the Psychopathy Checklist-Revised (PCL-R, 49), consisting of 20 items rated on a three-point scale (0 = does not apply, 1 = may apply or applies in some respects, 2 = does apply), with total scores ranging from 0 to 40. Though a cut-off score of 30 is often used for assessment and research purposes, Cooke and colleagues (1999, 2005) have proposed a cut-off of 25 point for European prisoners. The offenders were assessed based on all information available from interviews, observations, and files. Analyses utilized the four-facet structure (Interpersonal, Affective, Lifestyle, Antisocial).

Statistical Analysis

Data were analyzed using SPSS 25 (SPSS, Chicago, IL, USA) software using two-tailed p-values. The level of significance was set at $p < 0.05$. Descriptive data were expressed in terms of mean values and standard deviations. Between-group differences for categorical data were analyzed using χ^2 with Fisher´s exact test when $n < 5$, Phi-values and odd ratios are also reported.

For continuous data, we calculated effect sizes using standard mean differences (Cohen's *d*) for t-test comparisons. Due to the exploratory nature of the study, multiple comparisons were allowed with no adjustments.

RESULTS

Background and Index Conviction Characteristics

As seen in **Table 1**, placements in foster homes during upbringing were overrepresented in the ASD-offenders (OR = 2.275, CI = 0.990–5.224, p < .05), but not in institutions. The ASD-offenders had significantly more often been diagnosed with a neurodevelopmental disorder (i.e. ASD or ADHD) by a clinician before participating in the DAABS (ADHD: OR = 4.417, CI = 1.836–10.624, p < .001; ASD: OR = 18.968, CI = 4.236–84.947, p < .001).

In terms of index conviction, only sex crime against a child victim differentiated ASD offenders from non-ASD offenders (OR = 4.200, CI = 1.216–14.503, p < .05) among the six violent index crime categories.

Externalizing Disorders

Among the early onset externalizing disorders identified at the DAABS assessment (**Table 2**), we found no differences between the groups. As adults, only substance use disorder (OR = 0.368, CI = 0.148–0.912, p < .05) differed between the two groups and was less common in the non-ASD group.

Table 2 also reports official data from the national Crime Register, including the index conviction at inclusion in the study. The non-ASD offenders had more convictions (SMD = 0.42, CI = −0.10–2.10, p < .05) but there was no difference in terms of number of crimes. When looking at the specific kinds of crimes, the only crime category that stood out was drug crimes (SMD =

TABLE 1 | Background and index crime characteristics.

	ASD N (%)	No ASD N (%)	Phi	OR (95% CI)
Placements foster home	11 (42.3)	59 (24.4)	0.121*	2.275 (0.990–5.224)
Placements institutions	10 (38.5)	93 (38.6)	−0.001	0.995 (0.433–2.285)
Previously diagnosed neurodevelopmental disorder				
ADHD	10 (38.5)	30 (12.4)	0.216***	4.417 (1.836–10.624)
ASD	5 (19.0)	3 (1.2)	0.313[a]***	18.968 (4.236–84.947)
Murder/manslaughter	2 (7.7)	11 (4.6)	0.043[a]	1.742 (0.365–8.327)
Robbery	11 (42.3)	87 (36.1)	0.038	1.298 (0.571–2.951)
Assault	8 (30.8)	108 (44.8)	−0.084	0.547 (0.229-1.307)
Other violent crimes	9 (34.6)	109 (45.2)	−0.063	0.641 (0.275–1.495)
Sex with adult victim	0 (0)	16 (6.6)	−0.083[a]	0.934 (0.903–0.966)
Sex with child victim	4 (15.4)	10 (4.1)	0.149[a]*	4.200 (1.216–14.503)

[a]*Fisher's Exact Test; ***p < .001, *p < .05.*

TABLE 2 | Externalizing disorders and previous convictions.

	ASD N (%)	No ASD N (%)	Phi (φ)	OR (95% CI)
Conduct disorder, any onset	22 (88.0)	187 (77.3)	0.076	2.157 (0.622–7.477)
Conduct disorder, childhood onset	10 (38.5)	63 (26.0)	0.091	1.894 (0.810–4.432)
ADHD, childhood	19 (73.1)	150 (62.2)	0.083	1.921 (0.740–4.988)
ADHD, adulthood	13 (50.0)	102 (42.3)	0.057	1.476 (0.647–3.369)
ADHD and conduct disorder, any	16 (66.7)	128 (53.1)	0.078	1.766 (0.728–4.281)
Antisocial personality disorder	13 (52.0)	156 (64.5)	−0.075	0.597 (0.261–1.366)
Substance use disorder, any	18 (69.2)	208 (86.0)	−0.136*	0.368 (0.148–0.912)
	ASD M (SD)	**No ASD M (SD)**		**SMD[a] (95% CI)**
Age at first conviction	17.1 (2.1)	17.0 (2.3)		0.04 (−1.15–0.97)
Number of convictions	3.0 (1.8)	4.0 (2.4)		0.42 (−0.10–2.10)*
Number of crimes total	13.5 (10.1)	18.5 (16.4)		0.32 (−2.33–12.47)
Number of violent crimes	4.2 (4.3)	5.3 (4.5)		0.26 (−0.91–3.23)
Number deadly violent crimes	0.0 (0.2)	0.1 (0.2)		0.04 (−0.09–0.11)
Number of sex crimes	0.5 (2.0)	0.2 (0.8)		0.2 (−0.74–0.20)
Number of drug crimes	1.6 (3.1)	3.8 (4.6)		0.48 (0.07–4.21)*
Number of property crimes	2.9 (3.1)	3.4 (4.9)		0.09 (−1.76–2.66)
Number of traffic crimes	0.7 (2.0)	2.5 (5.3)		0.35 (−0.57–4.19)
Number of fraud crimes	1.0 (2.2)	0.7 (1.6)		0.15 (−1.05–0.53)

[a]*Cohen's d; *p < .05.*

0.48, CI = 0.07–4.21, p < .05), where non-ASD offenders were more often represented.

Psychopathy

In **Table 3**, PCL-R scores are presented. The only facet where there was a significant difference (SMD = 0.53, CI = −2.24 to −0.15, p < .05) was the Affective facet.

DISCUSSION

There is a stark difference between population-based studies of individuals with ASD, studies which does not seem to show an increased risk of criminal offending, and the heterogenous literature on identified offenders, where ASD is clearly overrepresented. The prevalence of ASD in prisons and other secure institutions is considerable. Over the last 20 years, several papers have been published showing that between 10 and 20 percent meet criteria for ASD in these settings (36, 50–54). There are studies showing that risk factors for persistent criminality and violence seem to be the same for individuals with as without ASD (e.g. 4, 55, 56) but there have been few studies on the antecedents of adult violent criminality in terms of life history of externalizing and criminal behavior in offenders with ASD.

The present study compared violent offenders with and without ASD on a range of externalizing behaviors, violence and crime-related variables. We found few differences between the groups. In terms of characteristics of the index offence, the ASD group was overrepresented among the sex offenders with a child victim, an offender category where a link to ASD has previously been suggested (e.g. 57). However, we found no differences in terms of life-time convictions for sexual offences. The overall picture of criminal history among these offenders pointed to more similarities than differences. In this context it is crucial to remember that these 26 individuals with ASD seem to represent a very complex clinical phenotype. The whole cohort was characterized by a heavy burden of childhood adversities and an early onset of psychosocial problems, as described in earlier publications (38). The ASD group stood out with an even higher percentage of foster home placements (42% vs. 24%). In a Swedish context, this kind of out-of-home placements mirrors either severe problems with harmful and destructive behavior in these children or serious maltreatment and destructive conditions in their homes, not seldom a combination of both. We know that children with disabilities, including ASD, are at heightened risk of maltreatment (58, 59), which in turn is associated with elevated levels of hyperactivity, aggression, and temper tantrums.

Also, the ASD subjects, as well as the non-ASD subjects in this study, were characterized by massive comorbidity, in neurodevelopmental as well as clinical disorders. There are several studies that links the risk of offending in the ASD group to the simultaneous presence of ADHD (4, 60). In our cohort, three out of four ASD individuals (73.1%) met criteria for childhood ADHD, a finding which has been reported previously (36). Childhood onset conduct problems, perhaps the strongest predictor for future criminality, was also very common among the ASD offenders (38.5%) and two-thirds of all ASD subjects (66.77%) had a combination of ADHD and CD.

It is noteworthy that many of the ASD offenders had previously been recognized for their abnormal developmental, and, in most cases, received an ADHD diagnosis, but in only 19% of the cases an ASD diagnosis. A late diagnosis of ASD have been linked to several contextual factors, including adversity (61) and low socio-economic status (62). We also know that an early identification of ASD is critically important to improve health, level of functioning and wellbeing but we need more studies to find out if it affects the risk of criminality as well.

Lifetime prevalence of SUDs was very high in both groups. Almost 70% of the ASD offenders met criteria for a substance use disorder, though the risk for developing these disorders was even higher in the non-ASD group. As far as we know, the study by Långström and colleagues (3) is the only one that has linked SUDs to an increased risk of violent offending in ASD. Of course, this study cannot replicate these findings but it is valuable to describe the high prevalence of SUDs among these young violent offenders with ASD.

In the official crime register, it was evident that though the non-ASD group had more convictions (p < .05), no difference was found in terms of total number of crimes, violent, sexual, property, traffic or fraud crimes. The non-ASD group was convicted of more drug crimes, which correlates to their higher prevalence of substance use disorders.

The relationship between ASD and psychopathy has been discussed for some time. Frith (63) suggested deficient empathy as a key component in both ASD, and psychopathy, but later research seem to favor the hypothesis that individuals with ASD primarily lack cognitive empathy, while "psychopaths" are low in emotional empathy (1). Despite this interest, there are few empirical studies of individuals representing the so called "double-hit", i.e. ASD individuals that have additional impairments in empathy and response to distress of other people that are not part of the ASD core symptomatology itself (64). In our study we found scores in both groups comparable to other European prison studies (65) and total scores did not differ between the two groups. However, we noticed a higher score on the Affective facet in the ASD group (p < .05). This facet describes lack of remorse or guilt, shallow affect, callous traits or lack of emotional empathy and failure to accept responsibility for their own actions. In a sample of adolescents with ASD, Carter Leno and colleagues (66) found callous-unemotional traits above the cut-off in 51% of their study group and it was associated with the same deficit in fear recognition previously reported in typically developing samples. This high score was not related to the amount of conduct problems in their group. We still don´t know if cognitive

TABLE 3 | Scores on the Psychopathy Checklist-Revised.

	ASD M (SD)	No ASD M (SD)	SMD[a] (95% CI)
PCL-R total score	17.7 (7.5)	17.7 (6.9)	0.02 (–3.12–3.36)
Interpersonal facet	1.2 (1.6)	1.0 (1.4)	0.19 (–0.93–0.39)
Affective facet	4.3 (2.2)	3.1 (2.2)	0.53 (–2.24 to –0.15)*
Lifestyle facet	6.0 (2.9)	6.5 (2.6)	0.21 (–0.66–1.76)
Antisocial facet	5.6 (2.8)	6.4 (2.9)	0.29 (–0.51–2.15)

[a]*Cohen's d; *p < .05.*

impairments associated with ASD, e.g. deficient theory of mind, increase the risk of developing these traits, perhaps in the presence of maltreatment and suboptimal parenting. In our study, we did not measure the presence of alexithymia, i.e. impaired ability to reflect on and report own emotions. It is possible that alexithymia could mediate some of the variance in the Affective facet.

An obvious limitation in the current study was the small sample and the large number of analyses performed, increasing the risk of type I and II errors. We justify these analyses with the importance of describing this particularly sensitive phenotype in order to be able to identify these individuals much earlier in their development. Relevant and fine-grained data will not be possible to collect through population-based studies and this study represents 20% of the underlying prison population of Sweden (38). Conducting clinical assessments in a prison setting also presents certain challenges, and in many cases parents were not able to give a developmental history of the participants. We applied the LEAD principle in all our diagnostic assessments, still considered to represent the gold standard for psychiatric assessments, and used multiple sources of information about the health and functioning of the participants, present as well as historical.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Ethics committee at Lund University (Dnr: 2009/405). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BH and EB conceived and designed the study. EB, MW, and BH obtained the data. BH drafted the initial manuscript with contributions from SB, MW, AT and EB. BH did all the analyses. Finally, all the authors critically revised the manuscript and approved the final version.

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REVIEW
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Sensory Abnormalities in Autism Spectrum Disorders: A Focus on the Tactile Domain, From Genetic Mouse Models to the Clinic

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Sensory abnormalities are commonly recognized as diagnostic criteria in autism spectrum disorder (ASD), as reported in the last edition of the Diagnostic and Statistical Manual of Mental Disorder (DSM-V). About 90% of ASD individuals have atypical sensory experiences, described as both hyper- and hypo-reactivity, with abnormal responses to tactile stimulation representing a very frequent finding. In this review, we will address the neurobiological bases of sensory processing in ASD, with a specific focus of tactile sensitivity. In the first part, we will review the most relevant sensory abnormalities detected in ASD, and then focus on tactile processing deficits through the discussion of recent clinical and experimental studies. In the search for the neurobiological bases of ASD, several mouse models have been generated with knockout and humanized knockin mutations in many ASD-associated genes. Here, we will therefore give a brief overview of the anatomical structure of the mouse somatosensory system, and describe the somatosensory abnormalities so far reported in different mouse models of ASD. Understanding the neurobiological bases of sensory processing in ASD mouse models may represent an opportunity for a better comprehension of the mechanisms underlying sensory abnormalities, and for the development of novel effective therapeutic strategies.

Keywords: autism, somatosensory, touch, mouse, behavior

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INTRODUCTION: ALTERED SENSORY REACTIVITY IN ASD

Individuals diagnosed with autism spectrum disorder (ASD) show deficits in social interaction and communication (developing, understanding and maintaining relationships) and repetitive/stereotyped behaviors, with different degree of severity, and sensory issues (1). However, it is interesting how typical cognitive difficulties of ASD are often associated with alterations in the perception of the external world. It is estimated that about 90% of individuals diagnosed with ASD have atypical sensory experiences (2). Differences in the sensory profile of ASD subjects are confirmed across lifespan (3, 4) and cross-culturally (5). Indeed, this trait is nowadays recognized in the DSM-V as hyper/hypo reactivity to sensory stimuli, demonstrating its primary importance in the description of the syndrome.

The correlation among autism and sensory deficits is not new. Formerly, (6) one of the first to describe autism, included different atypical sensory behaviors in his analysis (including heightened sensitivity to noise and touch, attraction to visual patterns and spinning objects, finger-stimming in front of the eyes) although considering them as a secondary phenomenon that occurs in parallel to the primary phenomenon (6).

(7) were the first instead to describe, in a case report study, a group of children who were particularly reactive to "unusual sensitivities" (low intensities of stimulation) in several sensory modalities (7). They hypothesized that an early developmental onset of sensitivity to sensory stimuli would cause social withdrawal in childhood.

Based on the Freud's "protective barrier against stimuli" (Reizschutz), they proposed that these children eventually succeed in building defending strategies to protect themselves from sensory overload, which would result in developmental distortions typical of autistic conditions. Eveloff described different behavioral difficulties faced by autistic children (8). He hypothesized that altered sensory processing is the effect of the lack of early experiences of environmental stimuli therefore interfering with the development of self-representations in autism. Psychologist Lorna Wing noted the "detail-oriented" behavior of autistic children, showing that they have significantly more sensory processing abnormalities than both typically developing (TD) children and children with Down's syndrome (9). She was the first to suggest to include abnormal sensory perceptual features as a proper diagnostic tool into 'basic impairments in autism'. However, this was not included in the first diagnostic criteria for autism by DSM in 1980 (10). Ornitz extended the concept of autism as a sensory and information processing disorder (11). He suggested that autism could be highlighted in young children looking at abnormal behaviors caused by perceptual differences (12). Nonetheless, there have been detractors of this theory and the strength of sensory features in autism has been put under scrutiny during the past decades. As an example, Richer strongly argued against the sensory theory of autism stating it was "incoherent and instable" (13). He, instead, stated that the autistic children's incompetence in language and symbol use was mainly due to their avoidance behavior in communication interactions. Similarly, Rutter proposed language deficits as the base of autistic syndrome, distancing himself from the sensory theory (14). Another line of research came in parallel from the field of occupational therapy (OT). (15) formulated the theory of sensory integration (SI) dysfunction to describe several neurological disorders including autism. This theory tried to relate sensory processing deficits with behavioral abnormalities and had the merit to define SI in terms of behavioral responses identifying for example tactile defensiveness and fight-or-flight reactions. More recently, OT researchers have suggested to consider the use of the term "sensory processing disorder" as comprising three primary groups (sensory modulation disorder, sensory discrimination disorder and sensory-based motor disorder) and the subtypes found within each group (16). However, sensory processing disorder is not still considered as a disorder *per se*. With Rutter, the theory of "social-perception" took hold in 1983 (17). He concluded the sensory symptoms found in the autistic population were the result of deficits in social cognition. It is not the processing of a sensory stimulus per se that creates difficulties in the autistic subject, but rather the processing of stimuli of emotional nature (i.e., those that possess a social content). Finally, in 2013 sensory processing deficits were included for the first time among the international diagnostic criteria of autism in the revision of Diagnostic and Statistical Manual of Mental Disorders (DSM-V) (1). From a clinical point of view, sensory deficits are documented already in the 6th month of life of infants later diagnosed with autism (18, 19). This gives us a dual information, firstly that sensory symptoms anticipate social and communication deficits (19), and secondly that abnormal sensory traits could be predictive of the autistic condition (20). This appears strikingly evident when considering that not only the vast majority of individual diagnosed with autism experience atypical reactivity to sensory stimuli (2, 21), but also that this affect every sensory modality: smell (22, 23), taste (24), audition (25), vision (26), and touch (27, 28). It seems clear that understanding the neurobiological bases underlying these sensory processing deficits represents a new challenge for ASD research, specifically aiming to identify early biomarkers and novel possible therapeutic strategies for these disorders.

Here, we will describe sensory defects in ASD, specifically focusing on altered tactile sensitivity. An in-depth analysis of somatosensory defects detected in mouse lines harboring mutations in ASD-relevant genes will also be presented. The aim of this review is to highlight the contribution of animal model studies in our understanding of the neurobiological bases of altered sensory sensitivity in ASD.

SMELL, TASTE, AUDITORY, AND VISUAL DEFICITS IN ASD

Smell

The sense of smell in ASD is poorly investigated. Nonetheless, a parent-report study pointed out how the most pronounced sensory symptom to dissociate ASD children from children with other developmental disorders are de facto taste and smell abnormal responses (29). Furthermore, it has been reported that almost 40% of ASD children with sensory abnormalities exhibit an altered smell and taste perception (30). Children and adolescents aged between 10 and 18 years showed impaired olfactory identification, but typical odor detection (31). Another study with ASD children aged from 5 to 9 years showed no differences in olfactory identification compared with controls, however older children were less accurate than younger ones at identifying odors (32). In the follow-up study (5 years later), the same ASD individuals had developed odor identification impairments (33). A more recent study confirmed that ASD children present impaired odor identification but normal odor detection compared to control participants (22). However, a clear picture of how and when altered olfaction occurs in the ASD cascade has not yet emerged (22). Considering the possible

influence of language in the common odor task, Rosenkrantz and coworkers suggested to use olfactory sniffing as a language and task-free measure of autism and its severity. Since sniffs are automatically modulated (vigorous sniffs for pleasant and truncated sniffs for unpleasant odors), these authors found that children with autism had a profound altered sniff response, sniffing equally regardless of odor valence (for example taking vigorous sniffs of rotten fish odor) compared to typically developing controls (23). These authors also found that this difference persisted despite equal reported odor perception and allowed for 81% correct ASD classification based on the sniff response alone. Moreover, they found that increasingly aberrant sniffing was associated with increasingly severe ASD, proposing it as a novel ASD biomarker (23).

Taste

There are few studies that deal with taste processing in ASD. However, it has been reported that children with ASD eat a smaller variety of food (e.g., less vegetables, fruits, dairy) regardless of texture, and refuse more food than typical developing children (34, 35). Bennetto and colleagues found a lower accuracy in taste discrimination for sour and bitter tastes, but similar identification for sweet and salty tastes, in adolescents (10–18 years) with ASD compared with control peers (31). Similar results were found in adults, except for sweet taste which was also impaired in those adults with ASD (24). A possible explanation for these taste identification differences in ASD might stem from the restricted diets of ASD patients that could alternatively explain why adolescents and adults are less accurate in identifying tastes (24). Abnormalities at the level of peripheral receptors and their transduction cascades could lead to taste impairments (36). Another view focuses on central rather than peripheral mechanisms (31). Indeed, central areas such as the thalamus, insula and cingulate cortex are involved in taste discrimination (37), and areas including the thalamus have been shown to be reduced in size in individuals with ASD (38). Thus, the difference in taste processing might be the result of atypical activity in these areas. However, further investigation is needed to understand whether ASD is associated with taste sensory defects at a peripheral or central level.

Audition

Sensory processing abnormalities have also been observed in the auditory domain. Indeed, children with autism often show difficulty in discerning two occurring tones when presented closely (39). In addition, a delayed evoked neural response compared to TD children have also been documented (40, 41). This latency has been observed in response to pure tones as well as to complex social stimuli (for example sound produced by speech) (42) and has been proposed as predictive of autism symptom severity (43). Although evidences for sensory processing deficits are more and more abundant in ASD literature, there are several reports that highlight enhanced perceptual strengths in response to specific sensory stimuli. As an example, individuals with ASD show superior abilities in pitch discrimination and in categorization compared to controls (25). In an effort to bring together all these findings, it has been suggested that perceptual capabilities may be subject to the nature and complexity of the sensory stimuli, with impairments associated with more complex stimuli and enhancements seen more often with simple stimuli (44, 45).

Vision

Over the years, there have been many studies investigating different aspects of visual perception in ASD. Defective retinal function has been described in ASD patients (46–49). Enhanced visual evoked potentials (VEP) in response to high spatial frequencies have been found in visual brain areas of ASD children, while unaffected control children generally responded to visual stimuli with low spatial frequency (50). Other studies showed that visual perception in ASD is more detail-oriented, suggesting that primary visual processing might contribute to social and communication deficits in ASD (51–54). It is generally accepted that individuals with ASD "see" and process the world differently, having a strong detail oriented ability in expense to global processing (55). ASD individuals are faster in detecting single details in Embedded Figure Tasks (EFT- a task in which participants are asked to find a target figure hidden in a larger image), being less susceptible to distractors (56–60). Moreover, gaze patterns from individuals with autism show a preference for scene regions of high pixel-level saliency compared with object-level saliency or semantic-level saliency scenes in passive viewing of naturalistic scenes (61). This means that they favor regions of the scene that are related with contrast, color, or orientation (pixel-level) rather than related with size, density, or contour complexity of objects (object-level) or related with text, tools, or faces (semantic-level). How individuals with ASD have this detail-oriented visual ability is still under debate. Moreover, further complexity is given from the fact that in autism, basic measures of visual sensitivity such as visual acuity (59, 62), contrast discrimination (63, 64), and orientation processing (65, 66) are all comparable with normal developing children. Conversely to the static stimuli, ASD individuals show atypical processing of dynamic visual stimuli (67, 68). Indeed, ASD subjects show an impaired global motion perception in discerning the direction of a cloud of moving dots (69, 70), even though the detection thresholds for local motion appear to be typical (71), or ever superior in ASD (72, 73).

TACTILE SENITIVITY IN ASD

The typical description of sensory processing abnormalities falls in the terminology of "over-responsiveness", "under-responsiveness", and "failure to habituate". Over-responsiveness, also called hyper-sensitivity, refers to children being more "reactive" to sensory stimulation compared to controls (74, 75), often associated with negative emotion or active avoidance of stimulation. However, the terminology used in clinical reports and questionnaires often fails in separating "over-responsiveness" from "impaired habituation". Moreover, it is unclear whether this refers to hyper-excitability of sensory cortex or the expression of negative emotions to tactile stimulation. Conversely, under-responsiveness, also called hypo-

sensitivity, is characterized by reduced reactivity to sensory stimulation and sensory seeking (75). Both over- and under-responsiveness then fall under the general term of tactile defensiveness (76), which describes both abnormal emotional responses to tactile stimulation as well as withdrawal/avoidance of a stimulation.

The vast majority of studies investigating tactile dysfunction have traditionally focused on parent and teacher reports and questionnaires. These studies, although informative, lack objectivity in the strict sense since they are based on subjective assessments of both behavioral and emotional responses to touch (77). Only recent works addressed the study of tactile abnormalities through a psychophysics approach, aiming to reduce the degree of subjectivity and to highlight neurophysiological underpinnings of this phenomenon.

As reported in a recent review (77), a number of studies have described tactile abnormalities using sensory profiles and parents reports (78–81). By using two parent-report measures, the Short Sensory Profile (SSP) and the Autism Diagnostic Interview-Revised (ADI-R), and a clinical observation with the Autism Diagnostic Observation Scale (ADOS), Rogers et al. compared sensory profiles in toddlers with ASD and typically developing controls and with other groups of developmental delay such as Fragile-X children and children with Down syndrome. They found significantly elevated levels of sensory symptoms in children with autism compared with both children with typical development and those with delayed development of the same mental age. In particular, children with autism obtained significantly higher scores of tactile sensitivity and auditory filtering than children in the developmental delay and controls. Moreover, they observed a correlation between abnormal sensitivity and adaptive behaviors. They also found no meaningful relationships between social-communicative scores and sensory scores in children with mixed developmental delays, or the typically developing children. The explanation of the authors is that since sensory symptoms are not in general a peculiarity of autism, they could represent an additional primary impairment rather than an autism-specific impairment. Moreover, they found that sensory scores (including tactile scores) did not correlate with either developmental levels or with ratio IQ scores for any group except the children with Fragile-X syndrome. Increased sensory scores were associated with clinical diagnosis rather than with IQ or immature developmental levels (78). Other tests including the Infant/Toddler Sensory Profile (ITSP), Infant-Toddler Social and Emotional Assessment (ITSEA), Autism Diagnostic Interview-Revised (ADI-R), and Autism Diagnostic Observation Schedule-Generic (ADOS-G), revealed that toddlers with ASD show higher under responsiveness (described as low registration by the authors) and stimulus avoidance as well as low frequency of seeking behaviors compared to IQ-, age-matched controls (79). Foss-Feig and coworkers investigated both under- and over-responsiveness to tactile stimuli in children with ASD through three measures of sensory processing: Tactile Defensiveness and Discrimination Test-Revised (TDDT-R), the Sensory Experiences Questionnaire (SEQ), and the Sensory Profile (SP). They reported that heightened levels of tactile seeking behavior were associated with more severe levels of social and repetitive behaviors. Additionally, heightened levels of hypo-responsiveness to tactile stimuli were associated with more severe levels of social and non-verbal communication impairments as well as increased repetitive behaviors. Conversely, over-responsiveness was not correlated with any of core symptoms of ASD (80). Data extracted from experimenter-reports of over-responsiveness, parent-reports of tactile symptoms, and self-reports of pleasantness of texture, showed that children with ASD have superior over-responsiveness scores compared to controls. Moreover, they observed a positive correlation between over-responsiveness and parent-report of tactile symptoms and between over-responsiveness and social impairments. Conversely, pleasantness ratings were inversely related with impaired communication (81). However, the contribution of ASD comorbidities such as intellectual disability (ID) and/or language impairments might have a role in defining the responses to studies involving self-reports and have to be considered. An individual with ASD and ID may have difficulties in describing to the experimenter the sensations generated by the stimulus, adding complexity to the interpretation and replication of studies. A risk of an imbalanced picture of ASD may arise and a selection bias for intellectual disability has been reported as issue in ASD research (82, 83). Most of studies on tactile processing so far have focused on children, however there are also studies (4, 84) showing that abnormal sensory processing is also present in adults.

These studies, although informative indicators of the tactile abnormalities in ASD, appear to be inconsistent with respect to pattern of response, correlation among measures, and diagnostic terms. In addition, different types of reports were used in different studies. All such aspects render these studies difficult to be compared; moreover, they do not always correlate to clinical observation, nor they provide indicators of possible cortical dysfunctions.

More recently, researchers have preferred a psychophysics approach to study tactile functionality in ASD in a more objective modality. Some of these studies have shown how detection of tactile stimuli is impaired in both adults and children with ASD (for example in vibration detection; (85), so in line with previous reports. However, other studies showed that tactile detection is normal in autism (57, 86, 87). It is possible to speculate that these differences result from the different type of stimulation used (i.e., flutter, vibration, sinusoidal, or constant) as well as its location. Although these works have the merit of bringing a greater objectivity to the study of tactile abnormalities in ASD, it remains unclear whether underlying sensory mechanisms are altered, or it is the emotional response to sensory input that leads to issues in filtering of the signal resulting in hyper/hypo-responsiveness.

Imaging studies have also tried to investigate the underlying neural mechanism of abnormal tactile sensitivity in ASD. Since tactile stimuli are part of the somatosensory world and as such rely on subcortical and cortical brain regions, researchers focused on possible differences in these brain areas between ASD and TD

control subjects. Coskun and colleagues were among the first to report abnormalities in the sensory map organization of ASD individuals. Using magnetoencephalography (MEG) recordings, these authors examined the cortical responses to passive stimulation of the thumb and index finger of dominant hand as well as the lip from ASD and TD controls. They found a different cortical representation of the thumb and the lip in ASD individuals compared to TD controls (88), namely the distance between the cortical representations of these two body parts was significantly larger in the autism group than in TD subjects. Moreover, in the cerebral cortex, the thumb is typically closer to the lip than the index finger; this was not observed in ASD individuals. However, as found in a successive study by the same group (89), the variability of the evoked potential as a response to passive stimulation of the thumb and index finger did not differ between controls and adults with ASD. Conversely, other authors showed a lower amplitude of contralateral cortical S1 response to tactile stimulation in children with ASD (27). Although these studies provide us with useful indications of cortical function in autism, discrepancies exist across studies. Moreover, the variability in neural responses appears to be higher in ASD (90, 91). A possible explanation could be sought in the type of stimulation involved (i.e., passive vs. active) as well as in the high heterogeneity of ASD (2). In addition, a limit of these studies lies in the complexity to compare findings in children with those obtained in adolescents and adults.

Several studies suggest that ASD pathogenesis might involve an imbalance between excitation and inhibition (E/I imbalance). This hypothesis is supported by several lines of evidence showing that the γ-aminobutyric acid (GABA) system is altered in ASD, and that may relate to alterations in sensation and symptoms in both animal models and humans. A pivotal role of GABAergic dysfunction in ASD was first hypothesized in early 2000s by Hussman (92) and Rubenstein and Merzenich (93), even if the key role of GABA in shaping neural response to tactile stimulation (94, 95), as well as in brain development and cortical plasticity (96, 97), was known from many years. Several genetic, neuropathological, and neuroimaging studies showed that GABAergic dysfunctions occur in ASD (98), and defective GABAergic neurotransmission has been suggested as a potential candidate in sensory deficits in ASD (99). In the tactile domain, a study investigating tactile detection thresholds in TD children was the first to report that tactile sensitivity was associated with *GABRB3* genetic variation in typically developing children (100), confirming findings from animal model studies. The *GABRB3* gene, coding for the β3 subunit of the GABA receptor channel, is one of the many candidate genes to be associated with autism (101, 102). Moreover, GABA levels were shown to be reduced in the sensorimotor cortex and positively correlated with worsened detection thresholds in children with ASD; in addition, GABA levels were not correlated with adaptation or frequency discrimination as for TD children (103).

Taken together, these results suggest that altered inhibition could explain some of the behavioral features of tactile abnormalities in ASD. Studies performed in appropriate mouse models contributed to better understand the neurobiological bases of tactile abnormalities in ASD.

UNDERSTANDING THE IMPACT OF SOMATOSENSORY FUNCTION IN SHAPING SOCIAL BEHAVIOR

Altered sensory processing has revealed to be an important feature for the clinical description of ASD. As discussed above in the review, sensory dysregulation encompasses multiple modalities (vision, hearing, touch, olfaction, gustation) and arises early in the progression of ASD. There is evidence that this could impact social functioning. It has been proposed that sensory stimuli and social behaviors may have a reciprocal influence on each other throughout development (104). This idea is reinforced form findings of early abnormal sensory sensitivity to stimuli predicting later joint attention and language development (18) and higher levels of social impairment in adults with ASD (105).

Touch is considered one of the most basic ways to sense the external world (106) and has been reported to have a significant role in role in several social aspects such as communication (107), developing social bonds (108), and overall physical development and connectivity of brain areas (109, 110). For this reason, skin has been proposed by some authors as "social organ" (111).

It has been suggested that irregularities in touch and tactile perception may be associated with broad levels of social dysfunction in ASD. For example, as described earlier in the paper, touch seeking behaviors have been found to predict levels of social impairment, and tactile hypo responsivity was associated with both poorer social functioning and nonverbal communication skills (80). Differences in tactile processing and tactile preference behaviors in ASD have also been reported in early infancy (112). Furthermore, lack of social touch can lead to higher levels of anxiety, stress, and depression (113), aspects which are commonly seen in ASD population (114, 115). Moreover, atypical touch during infancy can develop into critical deficits later in life, specifically in regards to attachment. While individuals with ASD are capable of forming a secure attachment to their caregivers, they tend to be less securely attached than their typically developing peers (116). In addition, individuals with ASD who have secure attachments tend to have less socially severe symptoms than individuals with ASD who are not securely attached, suggesting symptom severity and overall level of functioning could impact the strength of attachment (117).

Touch is also important in developing social bonding. Oxytocin, the neuropeptide primarily involved in social bonding, has long known to be released in response to positive tactile stimuli (touch, warmth, odors) (118). In individuals with ASD, oxytocin abnormalities have been found in plasma levels (119), in the gene that encodes for the oxytocin receptor, OXTR (120), as well as in oxytocin receptors (121). However, the

behavioral and neural effects of oxytocin were negatively correlated with ASD-like traits, suggesting these effects to be diminished in individuals exhibiting low social and emotional abilities associated with autistic traits (122). Future research should look further into the importance of tactile perception in shaping social aspects, as well as its impact on other social domains not previously explored.

Theoretical models have been proposed to integrate sensory and social features of ASD. One model that tried to explain altered sensory functioning in ASD is the "temporal binding hypothesis" (123). This theory lays on the assumption that sensory stimuli that occur in close temporal proximity are more likely to be integrated and so to be perceived as a whole; thus, timing information is crucial to binding and integrating associated stimuli (124). The possibility of an extended "temporal binding window" in individuals with ASD which may give rise to alterations in sensory processing has been proposed (125). Indeed, a longer temporal binding window could create a blurred, unpredictable sensory environment, as unrelated stimuli become bound together. Ideally, throughout development important social cues may fail to become integrated or salient. Thus, according to this theory an extended temporal binding window could negatively impact social behavior in ASD through altered binding of social cues.

Another theory is the "intense world theory" which offers a neurological mechanism for how the sensory and social features of ASD may be related (126, 127). This theory proposes an excessive functioning of neural circuits as the base of sensory and social impairments. Thus, such neural circuits are hyper-reactive, hyper-plastic, and generally up-regulated. This would create an intense world, a fragmented world (with focus on individual components of the environment), and an aversive world. Low level sensory perception is enhanced (intense world) and coupled with deficits in sensory integration (fragmented world). Throughout development, this could lead to an over-specialization for perceiving primary sensory cues at the expense of the ability to navigate in a socially complex world (127). In this way, the intense world theory explains both the unique sensory and the social features of ASD and offers a mechanism for how an up-regulation in primary sensory perception results in social avoidance and withdrawal.

Another theory focus on "atypical hierarchical information processing" as base of sensory and social functioning defects in individuals with ASD. Since we live in a world buzzed with stimuli, in order to adequately perceive and operate in it, humans use both incoming sensory information (bottom-up processes) and inference from prior experience and context (top-down processes) (128). It has been suggested that under-utilization of top-down processes such as context or experience (129) or an over-reliance on bottom-up sensory perception (130) is characteristic of perception in ASD. At the neural level, this profile may reflect hyper-activation of primary sensory cortices, decreased prefrontal activity, and reduced neural habituation during sensory processing (131). According to this theory, this information processing profile may inhibit social functioning as the interpersonal world demands strong central coherences, integration of context, and utilization of prior knowledge. Thus, over-functioning of bottom-up sensory processing coupled with under-utilizing top-down perception in ASD could explain both enhanced sensory processing and inefficient social functioning in this population.

When discussing dysfunctions of the somatosensory system, it is important to consider the sensory processing cascade in its entirety. Starting from the periphery (i.e., the skin, where the mechanical stimuli are transduced in electrical signals), moving to the intermediate stations (i.e., spinal cord and/or brainstem, where the electrical signals are delivered by means of neuronal ascending pathways), reaching subcortical and cortical brain areas (i.e., primary somatosensory cortex and other higher function somatosensory processing areas, where integration/codification of the information occurs), sensory information can undergo more or less severe modifications. Indeed, abnormal development or interaction in any of these steps could ideally lead to abnormal sensory processing. Moreover, since proper tactile perception is of importance in early development as well as in forming social and physical relationships (132), a possible relation between tactile abnormalities and social behaviors could be a matter of fact. For this reason, when assessing the behavioral outcomes of relevant social/sensory task performed by mouse models of ASD, it is at least necessary, when possible, to correlate the behavioral response to a potential neurobiological defect. Indeed, even though humans and animals have evolved under different evolutionary pressures making social behaviors much harder to compare, molecular and cellular functions are strongly conserved and so appear to be mostly comparable.

However, what must be kept in mind is that social behaviors not a unitary behavior with a unique neurobiological basis, but rather different aspects of social behavior show different neural substrates. Moreover, the modulation of environmental cues, the type of sensory stimulation, and the role of conspecific actions in shaping the social response add complexity to our understanding of social behavior in animals (included humans) (133).

In recent years, social neuroscience has made great progress in identifying the neural substrates of social behaviors, and the brain processes linked to social interactions in disease have received considerable attention. In humans, social cognition differentiates between social perceptual processes (devoted to the detection and the analysis of social stimuli like a face), social attribution processes (involved in the inference of other's and one's mental states from behavior), and social categorization processes (involved in the process by which individuals are placed into groups based on common characteristics like gender and ethnicity). On the neural level, the social perceptual processes include the primary sensory areas as well as more specialized regions like the fusiform face area (FFA) and the temporo-occipital associative cortex (V5 and extra-striate body area, EBA). The social attribution processes include the premotor cortex, the superior temporal sulcus (STS) and the temporo-parietal junction (TPJ). The social categorization

processes encompass the medial/dorsolateral prefrontal cortex and the anterior cingulate cortex. Instead, the emotional content and the motivational appraisal of social stimuli appear to be mediated by the amygdala, the orbitofrontal cortex, and the hippocampus (134, 135). Although one should be always cautious in comparing social behaviors in humans and in mice, it interesting how brain regions such as the amygdala and the hippocampus have been also related to social circuits for behavioral decision in mice (133).

How does social behavior relate to sensory stimulation processing? This question is far from being fully answered, however what we can say is that the sensory inputs (coming from other individuals or from the environment), whether they are olfactory, visual, or somatosensory, are processed and integrated over time with social internal states and transformed into behavioral outputs that in turn provide sensory cues to the other individual forming a feedback loop (133). As an example, somatosensory stimulation is critical for both mating (during copulation) and parenting (tactile stimulation of pups affects the development of normal behaviors) (136, 137).

The hypothesis that peripheral nervous system dysfunctions (namely, dysfunctions of the sensory system) could contribute to ASD pathophysiology has a recent history. Although somatosensory abnormalities in humans and rodents have long been reported, still little is known about their role in ASD. Further efforts are necessary to unravel the neural correlates of social behaviors, and their relationship with sensory processing abnormalities could be of help in describing the social impairments found in ASD.

STUDYING THE NEUROBIOLOGICAL BASIS OF ASD THROUGH MOUSE MODELS

It has long been known that ASD has a high degree of heritability: studies on monozygotic twins revealed a peak of concordance of 90% compared to 10% of dizygotic twins and siblings (138, 139). However, only recent efforts and technological advancements in genetics made it possible to identify a plethora of gene variants associated with ASD. These variants have been found in several hundreds of different genes and cover the entire spectrum of mutations, from single-nucleotide variants (SNVs) to copy number variants (CNVs), including inherited as well as *de novo* mutations (140, 141). Several genetic mutations in ASD have been associated with genes coding for proteins involved in synaptic functions, such as *SHANK* (142), *CNTNAP* (143, 144), *NLGN* (145, 146), and *NRXN* (147). Some examples of CNVs associated with ASD include chromosomal loci 15q11-q13 (148), 16p11.2 (149), and the *UBE3A* (150), *NRXN1* (147), and *CNTN4* (151) genes. In adding complexity to the understanding of ASD pathophysiology, a subset of single gene mutations associated with ASD are also responsible for other neurodevelopmental disorders, including *FMR1* in fragile X syndrome, *TSC1* in tuberous sclerosis, and *MECP2* in Rett syndrome. The tremendous progress made in identifying all these genes associated with ASD has subsequently resulted in the generation of several ASD mouse models, through which it is possible to infer the effect of single mutations, thus advancing our understanding of the biological bases underpinning this complex syndrome. A multitude of mouse models have been generated by knockout and knockin mutations in ASD candidate genes. In developing new mouse models it is important to consider different aspects such as face validity (i.e., resemblance to human symptoms), construct validity (i.e., similarity to the causes of the disease), and predictive validity (i.e., expected responses to treatments that are effective in the human disease), with the best animal model keeping together the three validity criteria (152).

Given the complex phenotypic and genetic heterogeneity of ASD, developing a mouse model keeping together all these aspects represents a challenge for every researcher. Nonetheless, according to the Simons Foundation Autism Research Initiative (SFARI) Gene database (http://gene.sfari.org/, as of October 29, 2019) there are up to now 264 genetic, 42 pharmacologically induced and 4 inbred mouse models of ASD.

Since the diagnosis of ASD is mainly given by the analysis of behavioral aspects rather than physiological criteria, and being the mice, like humans, a social species displaying an extensive variety of social behaviors, neuroscientists tried to develop and refine behavioral paradigms that could be relevant to the human condition. The symptoms however may be uniquely humans and are often highly variable among individuals, so it appears clear that designing mouse behavioral assays relevant to autistic symptoms represents a unique challenge. However, different behavioral paradigms have been developed considering the two core symptoms of human disorder (social/communication defects and repetitive behaviors) and revealed to be qualitatively efficient and reproducible. For a detailed discussion of these experimental approaches, the reader is referred to the comprehensive review by Silverman et al. (153).

Beyond the central vs peripheral dysfunction dichotomy in ASD, it is interesting how sensory impairments in ASD do not only correlate to tactile processing defects but rather represent a complex multifaceted sensory phenomenon that encompass also other sensory systems. A brilliant example comes from the recent work by Goel and colleagues (154) focused on the sensory processing of *Fmr1*$^{-/-}$ mice in the visual domain. These mice exhibit a delayed learning in a visual discrimination task, an impairment similar to the human deficit in visual perception in FXS individuals. The reduced number of orientation-selective pyramidal cells of the primary visual cortex (V1) might represent the neural correlate of this defect. In targeting the visual cortex, the authors also found a reduction in the functional output of parvalbumin (PV) neurons (a subclass of GABAergic interneurons) in *Fmr1*$^{-/-}$ mice, as compared to wild-type controls. Surprisingly, when a DREADD (Designer Receptors Exclusively Activated by Designer Drugs) strategy was used to restore PV activity and orientation tuning in V1, *Fmr1*$^{-/-}$ mice accelerated learning in the visual task. Other studies focusing on the visual domain has also been carried out on *En2*$^{-/-}$ (155), BTBR (156), and *SERT-Ala56* knockin mice (157).

ORGANIZATION OF THE MOUSE AND HUMAN SOMATOSENSORY SYSTEM

The somatosensory system in mammals conveys sensory information from receptors located in the skin, muscle, and joints to the brain. In mice, the somatosensory system is dominated by the input coming from the facial vibrissae: the neuronal representation of whiskers in the primary somatosensory cortex (the barrel field) occupies more than two thirds of its total area (158). The anatomical and functional organization of the somatosensory system is highly conserved and is based on two major ascending components: the dorsal column system and the trigeminal system. The first-order sensory neurons are the dorsal root ganglion cells and the trigeminal ganglion cells that collect information from the receptors located in the body and the face, respectively. The whisker pad of mice is highly innervated: a single whisker follicle is sheathed in a complex capsular structure which receives up to 200 axonal projections (159). Moreover, the nerves of a single whisker do not connect with the adjacent follicle (160). The dorsal root ganglion (DRG) neurons send their central processes to make synapse in the spinal cord while the trigeminal ganglion cells make synapse in the hindbrain. The main hindbrain nucleus receiving afferents from the whisker system is the spinal trigeminal nucleus (Sp). The Sp can be divided in the oral, interpolar and caudal part (Sp5O, Sp5I, Sp5C), forming the largest nucleus of the mouse hindbrain. The whisker macro representation starts to be appreciable at the level of the hindbrain in concrete structures called "barrelettes" (161). The spinal cord and hindbrain nuclei in turn project to specialized somatosensory nuclei of the thalamus: the ventral posterior group (VP). The initial anatomical separation of the two systems is interrupted at the level of the thalamus, which represents a relay station for all sensory stimuli. The VP region of the thalamus is subdivided into a large medial portion (VPM), which receives afferents from the trigeminal system, and a smaller lateral portion (VPL) which instead receives afferents from the limbs and the trunk. The size of each subdivision of VP is proportional to the number of afferents, so the VPM appears to be larger than the VPL. Moreover, even from the VPM it is possible to appreciate a representation of individual facial whiskers, the so called "barreloids" (162). Somatosensory processes also terminate in clusters of heterogeneous thalamic nuclei (the posterior group, Po) lying medial, dorsal, and caudal to VPM. The largest component of the Po forms the medial subdivision (PoM), which also receives inputs from the whisker pad providing a parallel source of information to the primary somatosensory (S1) cortex (163). In rodents, two further clusters of nuclei have been identified in this region of the thalamus: the reticular nucleus of the thalamus (Rt) and the zona incerta (ZI). These two clusters do not receive somatosensory input from the brainstem or spinal cord but being packed with GABAergic neurons and strongly projecting to the VP, they are thought to play an important role in modulating the output of VP (164). All somatosensory stimuli converge onto the primary (S1) and secondary (S2) somatosensory cortices. S1 is dorsolateral in the rostral part of the neocortex, whereas S2 is located laterally to S1. The primary somatosensory cortex in mice is dominated by the barrel field (S1BF), containing the representation of single facial whiskers. In 1970, Woolsey and Van Der Loos were the first to report these distinct anatomical structures named "barrels" (165). Further division of the S1 are the forelimb area (S1FL), the trunk area (S1Tr), and the hindlimb area (S1HL), with each of these areas characterized by a thick condensed layer IV. **Figure 1** schematically reports the organization of the somatosensory pathways in mice.

As compared to the mouse, the human somatosensory system presents important similarities and differences. Somatosensory receptors located in the skin are essentially the same, and the anatomy of the ascending pathway organization is maintained in both species. The organization of somatosensory cortex found in mice is comparable to that found in mammals with relatively little expansion of the neocortex (166). Much of somatosensory cortex in these mammals is represented by two distinct systematic representations of the contralateral body surface, named the first (primary) representation, or S-I, and the second representation, or S-II (167). The larger S-I represents the body from tail to mouth in a mediolateral cortical sequence, while the smaller S-II has a head-to-tail mediolateral (or dorsoventral) cortical sequence (168). Instead, somatosensory cortex in higher primates (including humans) contains more subdivisions than somatosensory cortex in non-primates. Experiments on the organization of anterior parietal cortex in macaque monkeys defined S-I as a broad region including cytoarchitectonic areas 3 (3a and 3b), 1, and 2 of Brodmann, though Kaas argues that only area 3b should be considered primary somatosensory cortex (168, 169). Area 3b, indeed, forms a complete representation of the body surface. In mice, two whiskers that are adjacent to each other on the animal's face are represented in adjacent cortical barrels, and the barrel field constitutes a topographic map. Similarly, a topographical organization of the somatosensory cortex (the so called homunculus) is present in humans (170). As for the cortical representation of the whiskers in mouse and rat, the homunculus is a topographic map because neighboring sites on the skin are represented at neighboring sites in the cortex. The whiskers are the critical touch organ in rats and mice, whereas in humans and other primates the fingertips are their equivalent. Each fingertip is innervated by axons from 250–300 sensory neurons (a comparable number as the whisker) and because individual axons terminate in multiple receptor structures, the density of mechanoreceptors is remarkably high (over 1,000 per cm^2). One important way in which fingerprint touch differs from whisker touch is that primates manipulate objects with our hands whereas rodents do not manipulate objects with their whiskers. This difference is evident when comparing the mechanism for sensing texture. For mice and rodents in general, the firing rate of neurons in barrel cortex differ from rough to smooth surface (171). In primates, the perception of coarse textures is based on the difference in firing rate between adjacent slowly adapting neurons(172); the perception of fine surfaces is based on vibrations in the skin, transduced by rapidly adapting Pacinian receptors (173). Finally, important differences have been found in the structure of supragranular layers 2 and 3 of the mouse and human somatosensory cortex (174). **Figure 2** schematically reports the somatotopic representation of the mouse and human primary somatosensory cortex.

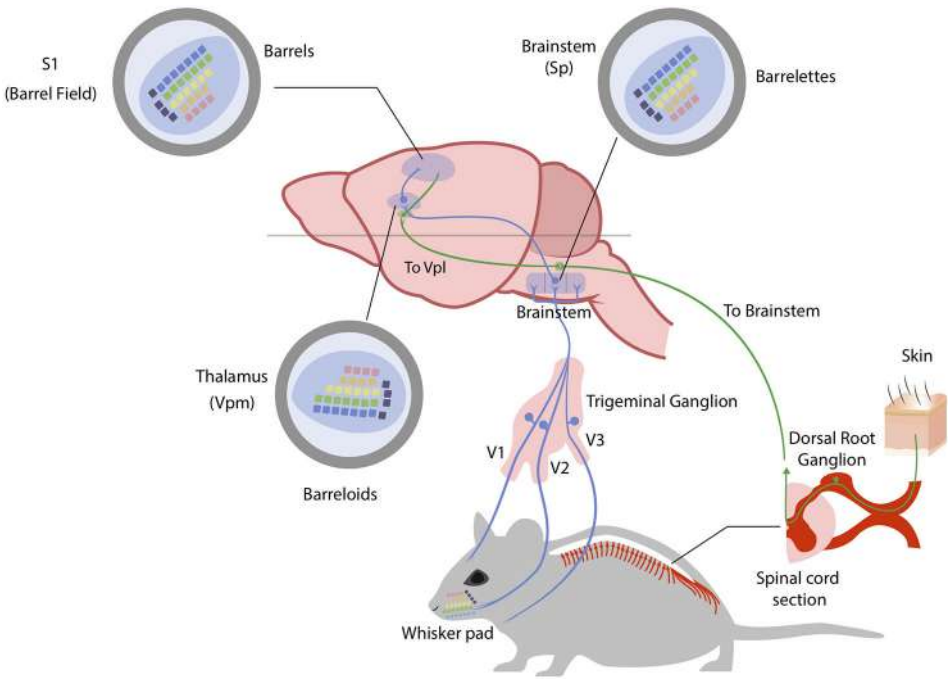

FIGURE 1 | The mouse somatosensory system. Somatosensory stimuli coming from the head region of the mouse are conveyed to the brain through trigeminal ganglion neurons. Neuronal fibers are depicted in blue (for trigeminal ganglion pathway) and green (for anterior and lateral spinothalamic pathways). The ophthalmic (V1), maxillary (V2) and mandibular (V3) branches of trigeminal ganglion process region-specialized somatosensory information with the maxillary branch (V2) innervating the whiskers. Here whiskers are indicated and color-coded to best follow their brain representations (whisker pad). Trigeminal ganglion neurons project to brainstem nuclei (spinal trigeminal nuclei – Sp) where they form an inverted neuronal representation of single whiskers (barrelettes). Trigeminothalamic fibers in turn project to the ventral posteromedial nucleus in the thalamus (Vpm) where again single whiskers are represented and shifted in orientation (barreloids). Finally, thalamocortical axons from the Vpm reach the primary somatosensory cortex (S1) in the barrel field, forming the final neuronal representation of single whiskers (barrels). Somatosensory stimuli coming from the body of the mouse are instead conveyed to the brainstem through dorsal root ganglia (DRG) neurons. The main difference in this system is the fact that somatosensory stimuli are conveyed to the ventral posterolateral nucleus of the thalamus (Vpl) before reaching the sensory cortex. See text for references.

SOMATOSENSORY ABNORMALITIES IN MOUSE MODELS OF AUTISM-RISK GENES

The scope of this review is to focus on sensory abnormalities in genetic mouse models of ASD (that is, mice bearing mutations in ASD-relevant genes). Alternative, non-genetic models such as maternal immune activation and valproate exposure during pregnancy have revealed to be valuable tools to study ASD-like phenotypes in rodents (175, 176). These studies contributed to the neurobiological investigation of sensory abnormalities in ASD (177, 178), but will not be reported in detail in this review.

ASDs are generally thought to be caused by defective brain development, and most studies traditionally focused solely on brain alterations. However, emerging evidences from mouse studies suggest that at least some aspects of the disorder are linked to defects in the peripheral nervous system that communicates sensory information to the brain. **Table 1** summarizes the most relevant mouse models of autism-risk genes, described in this section, which display somatosensory system defects at central or peripheral level. For an extensive summary ASD-relevant mouse strains showing somatosensory deficits, the reader is referred to the AutDB database (http://autism.mindspec.org/autdb). A brilliant example of central sensory processing defects comes from the work of Michaelson and colleagues (179). Starting from the finding of touch-related sensory processing defects (i.e., blunted responses to painful touch-related stimuli and/or tactile seeking behavior as well as tactile aversive behaviors) in *SYNGAP1* heterozygosity in humans, they found that *Syngap1* heterozygosity causes touch-related deficits in cortical circuit activation in mice, namely a reduced whisker related activation of receptive fields in the primary somatosensory cortex. Moreover, alteration of whisker evoked activation was found to be whisker-dependent. Particularly, neurons in the layer 2/3 of the somatosensory cortex were less active in heterozygous mice compared to WT in live calcium imaging experiments. Interestingly, these deficits in touch-related cortical circuits were associated with reduced whisker-evoked synaptic potentials in layer 2/3 and anatomical irregularity of layer 4 neurons. This is interesting because neurons of layer 2/3 of the somatosensory cortex integrate bottom-up sensory signals originating in the periphery with information arriving from higher cortical areas (186), whereas neurons in layer 4 receive the bulk of sensory-related

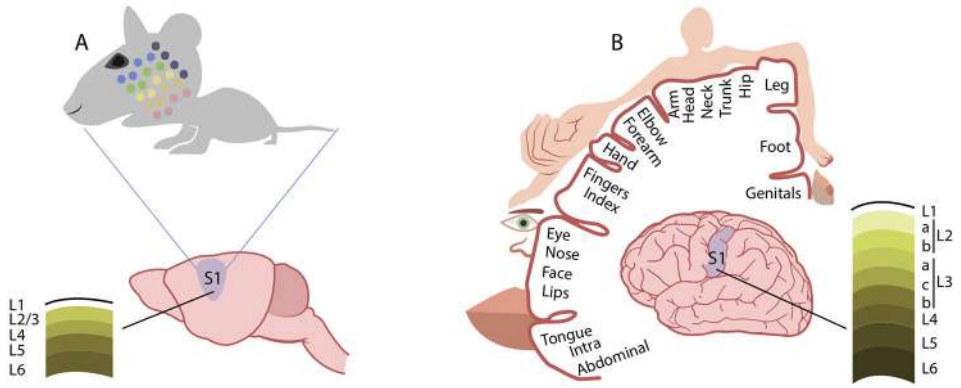

FIGURE 2 | Comparison of cortical somatosensory representation in mice and humans. Distorted representation of body areas in the mouse **(A)** and human **(B)** primary somatosensory cortex (S1). In both species, S1 somatosensory maps reflect the extent of cortical areas devoted to the processing of sensory information from different parts of the body. In mice, the altered proportions of the head and whisker pad with respect to other body regions mirrors the extent of innervation from these areas. Similarly, in humans, the cortical somatosensory representation is enlarged for those regions, such as the hands and the lips, that are densely innervated by sensory fibers. Conversely, the structure of supragranular layers 2 and 3 markedly differs between the mouse **(A)** and human **(B)** somatosensory cortex. See text for references.

TABLE 1 | Somatosensory deficits in mouse models for autism-risk genes.

Mouse model	Behavioral test	Measure	Phenotype	Age at testing	Sex	Reference
Syngap1$^{+/-}$	Tactile novel object recognition test (NORT)	Time spent exploring the novel object	Increased in HET	6-8 weeks	M/F	179
	Go/no go task involving whisker deflection	Correct answers reporting whisker deflection	Decreased in HET			
Fmr1$^{-/-}$	Tactile defensiveness assay in head-restrained mice	Withdrawal/habituation to whisker stimulation	Increased in KO	PD 14-16 and 35-41	M/F	180
Shank2$^{-/-}$	Electronic von Frey Apparatus	Mechanical withdrawal threshold	Increased in KO	>2 months	n.s.	181
	Hot plate test	Latency to first reaction	Increased in KO			
	Von Frey filaments for allodynia after neuropathic or inflammatory pain	Positive response (licking, biting, withdrawal)	Decreased in KO			
Cntnap2$^{-/-}$	Calibrated von Frey hairs	Mechanical withdrawal threshold	Decreased in KO	8-16 weeks	M/F	182
	Hot plate test	Latency to withdrawal	Decreased in KO (53°C)			
	Pain sensitivity to capsaicin and formalin	Duration of response (licking, biting, paw lifting)	Increased in KO			
Mecp2 KO, *Skank3B* HET, and *Fmr1* KO	Texture specific novel object recognition	Preference for the novel object	Decreased in all models	6-8 weeks	M/F	183
Mecp2 KO, *Skank3B* HET, and *Fmr1* KO	Tactile prepulse inhibition assay (T-PPI)	Air puff response	Increased in all models	6-8 weeks	M/F	183
Mecp2 cKO in DRG	Texture specific novel object recognition	Preference for the novel object	Decreased in cKO	6-8 weeks	M/F	183
	T-PPI	Air puff response	Increased in cKO			
Gabrb3 HET and *Gabrb3* cKO in DRG	Texture specific novel object recognition	Preference for the novel object	Decreased in both models	6-8 weeks	M/F	183
	T-PPI	Air puff response	Decreased in both models			
Shank3B HET and *Shank3B* cKO in DRG	T-PPI	Air puff response	Increased in both models	6-8 weeks	M/F	184
	Texture specific novel object recognition	Preference for the novel object	Decreased in Shank3B HET			
En2$^{-/-}$	Whisker nuisance test	Scoring of avoidance behaviors	Increased in KO	3-6 months	M/F	185

cKO, conditional knockout; DRG, dorsal root ganglia; F, female; HET, heterozygous; KO, knockout; M, male; NORT, tactile novel object recognition test; n.s., not specified; PD, postnatal day; T-PPI, tactile prepulse inhibition assay.

information arriving from subcortical areas (187). So, ideally these defects could represent the neurological basis of tactile behavior abnormalities. Indeed, the authors found that *Syngap1* heterozygous mice were unable to discriminate among objects that differ for the texture in the NORT test. Thus, a possible correlation among circuitry dysfunctions and tactile behavior deficits could be a matter of fact.

This possibility is supported by the research carried out by He and colleagues on the *Fmr1* knockout mouse model of ASD (180). Fragile X syndrome (FXS), in which transcriptional silencing of the *Fmr1* gene leads to loss of the fragile X mental retardation protein (FMRP), represents one of the most common single-gene cause of autism [from 1% to 6% of cases; (188)], and the vast majority of FXS individuals show tactile impairments (189). The *Fmr1* knockout mouse model of FXS exhibits behavioral deficits analogous to human symptoms and, as reported by He and colleagues, also shows tactile defensiveness measured as avoidance motor response in a whisker stimulation test in both juvenile (P14-P16) and adult (P35-41) mice. Moreover, the authors reported that in young mice only a reduced fraction of neurons of layer 2/3 of the barrel field were responsive to whisker stimulation in a time-locked manner and showed impaired adaptation to repeated whisker stimulation, suggesting that this could represent the explanation for the observed behavioral over-reactivity (180).

Another study focused on behavioral aspects of sensory processing, showing reduced nociception and chronic pain in *Shank2*$^{-/-}$ mice (181), as an extension of tactile hyposensitivity found in ASD individuals. These authors reported basal tactile sensitivity impairment in *Shank2*$^{-/-}$ mice as compared to WT, namely a higher basal mechanical threshold (the force applied when the mouse withdraws its paw) by using an electronic Von Frey apparatus (used to assess withdrawal responses in rodents). Moreover, the authors found a reduced sensitivity in *Shank2*$^{-/-}$ mice to chronic neuropathic pain (i.e., induced by nerve ligation) as well as inflammatory pain (i.e. induced by antigens injection) suggesting that these alterations could be due to defect both at the brain level than at peripheral level. Indeed, peripheral synaptic dysfunctions in the spinal cord, as well as central somatosensory cortex defects could explain these impaired responses in *Shank2*$^{-/-}$ mice.

Recent studies indicate that peripheral alterations of tactile sensitivity in mouse models of autism-risk genes might contribute to social and sensory behavior defects relevant for ASD. One example comes from the work of Dawes and colleagues on the *Cntnap2*$^{-/-}$ mouse (182). They found that loss of *Cntnap2* resulted in pain related hypersensitivity (as tested through the Von Frey apparatus and the pinprick application) in mice. Since *Cntnap2* was found to be expressed in dorsal root ganglion neurons (DRG), the authors measured primary sensory neuron activity *in vivo* through calcium imaging and *in vitro* through patch-clamp technique to assess if *Cntnap2* could impact neuronal excitability. They showed that DRG neurons were significantly hyper-responsive to sensory stimulation showing larger increase in intracellular calcium concentration and significantly lower rheobases (defined as the smallest injected current that elicit an action potential) compared to WT. Moreover, they found from *in vivo* extracellular recording of DRG neurons that loss of *Cntnap2* leads to dorsal horn neuron hyper excitability, in line with the behavioral assays.

In line with these findings, Orefice and colleagues showed that mice harboring mutations in *Mecp2*, *Gabrb3*, *Shank3*, and *Fmr1* genes exhibit aberrant tactile sensitivity, as detected by abnormal behavioral responses to skin or whisker stimulation (183). When compared with control wild-type littermates, all these mutant mice failed to distinguish between smooth and rough-textured objects in the texture novel object recognition test (NORT), indicating impairments in skin-based texture discrimination. In addition, this study tested sensorimotor gating and skin sensitivity using the tactile prepulse inhibition test (PPI), which consists in delivering puffed air onto the back of mice and evaluating whether this prepulse could inhibit a subsequent startle response to a loud stimulus. Interestingly, all mutant mice tested showed an enhanced response compared to controls. Further testing additionally demonstrated that this exaggerated response was elicited by air puff alone, suggesting an abnormal hypersensitivity to tactile stimulation (183). In order to explore the neuronal basis of this tactile deficit in *Mecp2* mutant mice, the authors deleted *Mecp2* in different body areas, namely from forebrain excitatory neurons, from the neurons caudal to cervical level 2 (including the spinal cord and the peripheral sensory system), and from the sensory ganglia (including trigeminal ganglia). Sensory testing through NORT and air PPI revealed that somatosensory deletion of *Mecp2* alone leads to aberrant tactile sensitivity. The authors then tested the hypothesis that GABA imbalance could have a role in impaired tactile sensitivity in *Mecp2* and *Gabrb3* mutant mice: deletion of *Mecp2* or *Gabrb3* in peripheral somatosensory (dorsal root ganglia, DRG) neurons caused mechanosensory dysfunction through loss of $GABA_A$ receptor-mediated presynaptic inhibition of inputs to the CNS (183). More recently, using a similar approach, the same authors found that acute treatment with $GABA_A$ receptor agonist selectively acting on mechanosensory neurons reduced tactile over-reactivity in six different ASD mouse models, both genetic and environmental (184). Moreover, chronic treatment of two genetic mouse lines, namely *Mecp2* and *Shank3* mutants, improved multiple ASD-associated behavioral phenotypes such as tactile over-reactivity, anxiety-like behaviors and social impairments. These results strongly support the hypothesis that peripheral somatosensory circuit dysfunctions could contribute to social deficits in ASD.

The idea of GABA imbalance in explaining the somatosensory defects reported in mouse models of ASD (see above, *Studying the Neurobiological Basis of ASD Through Mouse Models*) comes from the studies on the *Gabrb3* gene, which encodes one subunit of GABA receptors on postsynaptic neurons and is associated with ASD (102). Mice heterozygous for this gene show a reduced startle response. In addition, an increased tactile sensitivity and a reduced sensorimotor processing were reported for *Gabrb3* heterozygous male mice (190), and a reduced expression of

Gabrb3 was found in *Mecp2* deficient mice (191). A similar approach was used by our laboratory in describing the somatosensory defects of *En2* mutant mice (185). Genetic studies (192–194) and expression analyses on post-mortem brain tissues (195–197) indicated that deregulated expression of the human *EN2* gene is linked to ASD. Accordingly, *En2*$^{-/-}$ mice are considered a reliable model for investigating the neurodevelopmental basis of ASD. Indeed, *En2*$^{-/-}$ mice show ASD-like behaviors (198), and a lower expression of *Fmr1* (199) accompanied by anatomical defects common to *Fmr1* knockout mice (200). We reported that *En2*$^{-/-}$ mice have a significantly reduced synchronization in somatosensory-auditory/associative cortices and dorsal thalamus, suggesting the presence of aberrant somatosensory processing in these mutants. Indeed, when tested in the whisker nuisance test (201, 202) *En2*$^{-/-}$ mice showed hyper-responsiveness to repetitive whisker stimulation. In line with our findings of primary somatosensory cortex functional hypo connectivity, sensory hyper-responsivity in *En2*$^{-/-}$ mice was accompanied by a reduced activation of primary somatosensory cortex showed by a decreased c-Fos immunoreactivity in layer IV. Interestingly, whisker stimulation under anesthesia also resulted in reduced *c-fos* mRNA expression in the *En2*$^{-/-}$ mice primary somatosensory cortex, corroborating the data obtained following whisker stimulation in freely moving animals. Our hypothesis is that this disruption of sensory processing in *En2*$^{-/-}$ mice is likely due to impaired function of GABAergic signaling, since *En2*$^{-/-}$ mice present a reduced number of GABAergic interneurons in the hippocampus and somatosensory cortex (203). In addition, altered electrophysiological and behavioral markers of sensory processing can be rescued by pharmacologically enhancing GABAergic signaling in ASD mouse models (98). Further efforts are needed to reveal the anatomical networks by which GABAergic deficits impact somatosensory processing in mice models of ASD. Our current work focuses on exploring the potential somatosensory defects in different sensory areas such as the trigeminal ganglion, the thalamus and the somatosensory cortex trying to extend these findings to other mouse models of ASD.

Together, these findings reinforce the need of studying sensory features of ASD in mouse models and suggest that tactile impairment in mice, akin to human ASD tactile abnormalities, could be explained through sensory processing defects in the peripheral and central nervous system.

CONCLUSIONS

In this review, we have discussed evidences of sensory impairments found in ASD. Abnormal sensory processing in autism represents a common feature and is recognized as a diagnostic criterion. It encompasses many aspects of all sensory systems, leading to both central and peripheral defects. Mouse models of autism-risk genes recapitulate sensory impairments found in autistic individuals and represent a valuable tool to study the cellular and molecular mechanism underlying sensory behaviors. We have addressed the organization of mouse somatosensory system to introduce the most recent findings on tactile sensitivity in genetic mouse models of ASD as well as studies on aberrant sensory processing in somatosensory and other sensory domains. Further efforts are needed to effectively link the sensory abnormalities and social features of ASD to the intrinsic multifaceted nature of sensory dysfunctions in ASD.

ETHICS STATEMENT

Experiments performed in our laboratory and reported in this review were approved by the University of Trento Animal Welfare Committee and Italian Ministry of Health.

AUTHOR CONTRIBUTIONS

All authors contributed to draft the manuscript. LB wrote the manuscript. GP and YB edited the manuscript. GP and YB provided funding.

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Received Cradling Bias During the First Year of Life: A Retrospective Study on Children With Typical and Atypical Development

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A population-level left cradling bias exists whereby 60–90% of mothers hold their infants on the left side. This left biased positioning appears to be mutually beneficial to both the mother and the baby's brain organization for processing of socio-emotional stimuli. Previous research connected cradling asymmetries and Autism Spectrum Disorders (ASD), entailing impairment in socio-communicative relationships and characterized by an early hypo-lateralization of brain functions. In this explorative study, we aimed to provide a contribution to the retrospective investigations by looking for early behavioral markers of neurodevelopmental disorders such as ASD. We hypothesized that an atypical trajectory in maternal cradling might be one of the possible signs of an interference in mother-infant socio-emotional communication, and thus of potential neurodevelopmental dysfunctions. To this aim, we examined photos depicting mother-child early cradling interactions by consulting family albums of 27 children later diagnosed with ASD and 63 typically developing children. As regards the first half of the first year of life, no differences were shown between maternal cradling-side preferences in typical and ASD groups, both exhibiting the left-cradling bias in the 0–3 months period, but not in the 3–6 months period. However, our results show dissimilar patterns of cradling preferences during the second half of the first year of life. In particular, the absence of left-cradling shown in typical mothers was not observed in ASD mothers, who exhibited a significant left-cradling bias in the 6–12 months age group. This difference might reflect the fact that mother-infant relationship involving children later diagnosed with ASD might remain "basic" because mothers experience a lack of social activity in such children. Alternatively, it may reflect the overstimulation in which mothers try to engage infants in response to their lack of responsiveness and social initiative. However, further investigations are needed both to

distinguish between these two possibilities and to define the role of early typical and reversed cradling experiences on neurodevelopment.

Keywords: Autism Spectrum Disorders, infant-holding bias, brain lateralization, retrospective investigation, neurodevelopment, epigenetics, behavioral markers, mother-infant relationship

INTRODUCTION

In contrast to right biased motor actions associated with motor sequences and environment-directed behaviors (1, 2), cradling behavior is associated with a bias to the left side of the body whereby an infant is held by an agent (usually the mother) close to her body by using arms and hands (3, 4), as shown in **Figure 1**. Indeed, 60–90% of mothers hold their infants to the left of the vertical midline of their body (5) almost independently of their handedness (6, 7), positioning the head against the chest and/or over the shoulder in their left peripersonal hemispace, and almost always bearing the weight using the left arm. Research shows that the left-cradling bias is strong and fairly stable in the first 18 months of life of the child for mothers. After this period, it was initially shown that left-cradling behavior starts to decline to the point that it is replaced, in some cases, by a right-cradling preference by the time the child is 2 or 3 years old (8). However, in recent longitudinal studies, Scola and colleagues (9) found a slight decrease of left cradling only after 19 months from delivery in mothers, and Todd and Banerjee (10) showed that it was strongest when babies were aged less than 12 weeks.

FIGURE 1 | Graphic representation of left-cradling behavior (courtesy of Rocco Cannarsa).

When a female cradles/holds an infant on the left side, the infant's face is positioned on the left of her visual field and the visual information is processed dominantly by the right hemisphere of the brain, believed to be specialized for the perception and expression of emotion (11, 12). Manning and Chamberlain (13) suggested that, from the mother's point of view, the left-cradling bias facilitates the monitoring of her infant's well-being cues through her left visual and perhaps auditory fields (14) by providing a direct communication projecting to her right hemisphere, specialized for recognizing emotional facial expressions (12, 15). On the other hand, given that many studies showed that newborns are endowed with a predisposition to attend face-like stimuli (16, 17), left-sided cradling would allow the infant to receive the more salient emotional information by means of a constant access to the left side [i.e., the most expressive side (18)] of the mother's face (19).

Besides sleeping and being fed, the newborn life experience is nestled in a close relationship with the adult caregiver (in most cases, the mother), very often expressed in the context of cradling behavior. It would thus be reasonable to propose that cradling is a major framework for most of the neonate's early social and communicative experiences, which provide the epigenetic foundations for the development of later social and communicative abilities (20, 21). In this regard, a growing line of research on behavioral genetics questioned about whether and to what extent changes to the phenotype—especially as regards the occurrence of neurodevelopmental disorders—are under the epigenetic control of imprinting processes not yet fully understood (22).

Using chimeric face tasks, many studies (23–25) have demonstrated that the left-cradling bias is predicted by a typical right-hemispheric specialization in the perception of emotions [see ref (26) for a thorough examination of leftward perceptual and emotional asymmetries]. Therefore, the left bias has been assumed to be associated with better recognition of emotional stimuli presented to the left visual and auditory fields, which are under right-hemispheric control (14). Specifically, Huggenberger and collaborators (27) suggested that cradling side preference is determined by a management of cognitive resources during monitoring emotional signals from the infant face. Vervloed, Hendriks, and van den Eijnde (28) also investigated the effects of the "received" lateral cradling bias, showing that healthy individuals who had been held in the right arm during childhood exhibited in turn a significantly reduced

left-bias for emotional faces compared to those who had been held in the left arm. Additionally, Hendriks, van Rijswijk, and Omtzigt (19) suggested that reduced or sub-optimal exposure to face information during infancy (due to a reversed lateral cradling position, i.e., on the right side) might have consequences for the ability to recognize faces and facial expressions later in life. This is likely to occur because the early infant exposure to faces is extremely important not only for fostering the bonding between newborn and caregiver (17), but also for later visual cognitive development (29, 30). Indeed, both male and female observers seem to show an experience-dependent bias of the right hemisphere for the female face, possibly because of the greater incidence of left cradling during the early stages of development, as suggested by refs (31) and (32). Furthermore, studies on non-human vertebrates seem to confirm the presence of an evolutionary right-hemispheric predisposition to process social stimuli to the benefit of an infant's left-sided positioning during interactions with the mother (33) [see ref (34) for a review].

Pileggi and colleagues (35), assuming that the left-cradling bias is fostered by instinctive and right-hemisphere-localized attachment processes that allow individuals to relate to others, found that left-cradling bias is absent in children with Autism Spectrum Disorders (ASD), a population characterized by chronic and severe impairment in empathizing competencies and social relations (36). These findings were corroborated by Fleva and Kahn (37), who showed a negative correlation between left-cradling bias and the presence of autistic traits in adults, and by Malatesta and colleagues, who showed positive correlations between left-cradling bias and both empathy (38) and secure attachment (39). In this regard, it should be pointed out how, compared with typically developing individuals, those with autism are not biased to facial information from the left visual field, as shown by various studies using both eye-tracking and chimeric faces [e.g., see refs (40, 41)]. These studies showed decreased right-hemispheric dominance for emotion processing in this population, different from the patterns of lateralization usually shown by typically developing individuals.

Much evidence has shown that decreased cerebral lateralization is associated with impaired cognitive functions, and it can also emerge behaviorally as mixed handedness [e.g., see ref (42)], given the crucial role that functional asymmetries play during cognitive tasks that require the use of both hemispheres. Hemispheric specialization provides the individuals with several advantages, such as the capacity to exploit in parallel the competences of the left and right hemispheres, to decrease the duplication of execution across hemispheres, and to reduce the initiation of simultaneous and incompatible responses (2, 43). In fact, the existence of a link between glitches in the typical separation of hemispheric functions during brain development and the occurrence of several mental disorders has been hypothesized, as in the case of the communicative shortcomings shown by patients with schizophrenia (44) or other instances of emotion dysregulation disorders in humans and animals (see ref (45) for a review). With regard to this, Forrester and colleagues (46) assessed handedness as a marker of cerebral lateralization in different manual activities both in typical and autistic children, considering that reduced hemispheric specialization in motor behaviors might be an early marker of alterations in brain architecture related to autism onset. Indeed, the study showed that within the context of object manipulation and self-directed behaviors, children diagnosed with autism demonstrated decreased hand dominance compared with their typically developing counterparts. Moreover, Knaus and collaborators (47) showed that ASD is associated with atypical language laterality in adolescents. Specifically, autistic children are characterized by an early hypo-lateralization of brain function compared to typically developing children.

Although Autism Spectrum Disorder (ASD) etiology is still unclear, we now know that such disorders have strong heritable and genetic underpinnings (48) involving 300–500 different genes (49). Remarkably, in their study on relatives, Manning and Denman (50) found that women's left cradling passed down to subsequent daughters and granddaughters, thus revealing genetic influences (through the female line) on lateral cradling tendencies. Along with cradling-side preferences, developmental instability (which in turn has been related to reduced left-cradling tendencies) seems to be passed down from mother—but not father—to children (51), suggesting that genetic and environmental [see also ref (52)] stressors could alter typical cradling asymmetries. Interestingly, a recent study showed that elevated levels of prenatal amniotic oestrogens (which could represent a hormonal stressor) are an important predictor of ASD in boys (53).

To date, data gathered hint at the importance of investigating associations between observations of cradling behavior received by the caregiver and later incidence of ASD, the early detection of which would have crucial implications for therapeutic success of clinical intervention (20, 21). Currently, autism is usually not diagnosed until a child is at least 3 years old, with a mean diagnosis age of 5.7 years (54, 55). Therefore, most recent research used both prospective [e.g., the early observation of newborns "at risk" to develop autism because of previously affected siblings (56)] and retrospective [e.g., analyzing home-movies from the first months of life of autistic children, and their caregivers (57, 58)] methodologies in order to diagnose the condition earlier. These studies indicated that autistic symptoms involve not only social communication and repetitive behaviors, but also influence to some extent motor capacities and the regulation of attention and emotion (59). Analogously, previous findings seem to endorse the opinion that empathy (37, 38), social attachment (35, 39), and emotion lateralization (13, 14) strongly affect early lateral cradling preferences in females. Moreover, a recent study conducted by Forrester and colleagues (60) suggested interesting associations between left-cradling bias and enhanced social processing abilities in (typically developing) 5–6 years old children.

Cradling evidence seems to converge towards a link between reversed cradling behavior, decreased handedness, and atypical development (21). An examination of the cradling bias as a possible early behavioral marker of later typical or atypical

development of the child seemed desirable at this point. Thus, we hypothesized that an atypical developmental trajectory in maternal cradling, indicating an interference in socio-emotional communication between mother and infant, might be one candidate epigenetic behavioral marker of ASD in children, arising, and already observable in the first hours after delivery.

We present a retrospective longitudinal study capitalizing on the cradling-side preferences assessed from pictures belonging to family albums. It is rather reasonable to expect that most parents keep a rich collection of images depicting their children since immediately after birth, often including photos depicting the children being cradled. This appeared to be a good proxy for measuring cradling side preference in a sample of mothers of atypically developing children, especially because the retrospective nature of such a survey would reflect the expression of cradling behavior in the months preceding the diagnosis, in the assumption that—*a posteriori*—any behavior could account as a potential marker predicting the later development of the disorder.

The "family photo album" methodology is not new, as witnessed by Manning (61), who examined many photographs from his colleagues' family albums in which they were cradling their infants. He examined photos dividing them according to the age of the cradled child and found that the left-cradling percentage in females was strongest (the figure was between 60 and 70%) when the children were 0-3 months old. In the other age groups (3–6 months, 6–12 months, 1–2 years, > 2 years), females exhibited only a non-significant tendency to cradle on the left, the left-cradling bias decreasing after the third month after child birth. These findings are consistent with Todd and Benerjee's (10) recent reports.

METHODS

Participants

Mothers (age range at the time of evaluation: 29–50; M = 40.52; SD = 5.05) of 63 typical children (age range at the time of evaluation: 1.4–16 years; M = 8.44; SD = 3.41) and mothers (age range at the time of evaluation: 27–55; M = 38.59; SD = 6.12) of 27 children diagnosed with ASD (age range at the time of evaluation: 1.9–16 years; M = 4.78; SD = 3.43) took part in the study. Mothers in the typical group were recruited from pediatrics practices and primary and secondary schools of Italian regions Molise, Abruzzo and Marche. Participants in the atypical group were recruited from all over the country among parents whose children had been diagnosed with ASD at "Stella Maris IRCCS" of Pisa (Italy). Only participants with a certified diagnosis of ASD according to medical certification were recruited in the atypical group. All mothers participating in the study provided written informed consent to participate in the study by signing an authorization form. Neither invasive nor risky procedures were involved, and the data were analyzed anonymously. The study was carried out in accordance with the principles of the Declaration of Helsinki and following the approval of the Italian "National Institute of Health" ("Istituto Superiore di Sanità") ethical committee (Ethical Committee Approval Number: PRE 469/16).

Procedure

Mothers of children were approached by the experimenter under the supervision of psychologist/doctor/teacher, depending on the context in which they were recruited: schools or pediatrics practices in the case of the typical/control group; in the waiting rooms of "Stella Maris IRCCS" in the case of the atypical/experimental group.

Once recruited, mothers were asked to fill in a take-home survey concerning their child in which they were required to indicate preliminary information about both the child (sex; diagnosis; birth order; handedness) and themselves (age; handedness). Then, participants were asked to consult their family photo albums, specifically seeking photographs in which mothers were cradling their children, and to make a single entry on a first grid, for photos in which the child was under 12 months of age, or on a second grid, for photos in which the child was over 12 months of age. Using the baby's head as a reference point, participants were required to indicate the side on which the child was being held in each photo, taking note of the age (in years and months) of the baby at the time of capture.

RESULTS

We collected data from 1,667 photos (range per participant: 3–101; M = 26.46; SD = 20.86) in which mothers were cradling their typical children (N = 63; control group) and 543 photos (range per participant: 0-51; M = 20.11; SD = 13.08) in which mothers were cradling their children later diagnosed with ASD (N = 27; experimental group). Two mothers belonging to the atypical group did not provide any photos in which they were cradling their children.

In order to trace a cradling trajectory both in typical and in atypical development of children, we carried out an analysis splitting age groups on the basis of Manning's (61) photo-categories. We examined the following categories of photos collected per age group of the child: 0–3 months; 3–6 months; 6–12 months; 1–2 years. **Table 1** shows the distribution of photos in each age group:

Within each age group, only participants who provided at least 4 maternal cradling photos were included in the data analysis. Then, a cradling laterality quotient (CLQ) was computed for each participant as $\frac{right\ \ photos - left\ \ photos}{right\ \ photos + left\ \ photos}$

TABLE 1 | Number of collected photos depicting mothers cradling their typical (control group) and atypical (ASD; experimental group) per age group of the child.

Child development [N]	0–3 months (mean; SD)	3–6 months (mean; SD)	6–12 months (mean; SD)	1–2 years (mean; SD)
Typical [62]	**390** (6.19; 5.63)	**262** (4.19; 5.03)	**336** (5.33; 4.85)	**380** (6.03; 6.75)
Atypical (ASD) [27]	**166** (6.15; 6.26)	**67** (2.48; 2.46)	**119** (4.41; 5.03)	**139** (5.15; 6.68)

with participants scoring from -1 (all left photos) to +1 (all right photos). Data were analyzed with SPSS Statistics Version 20 (Armonk, NY, USA).

Age Group 0–3 Months

Thirty-seven participants of the typical group and 18 participants of the atypical group provided at least 4 maternal cradling photos in which infants were aged 0–3 months. The CLQ of mothers of typical children significantly differed from 0, showing a left-cradling bias (N = 37; M = -0.231 [61.55% of left cradling]; SD = 0.616; $t_{(36)}$ = -2.287; p = 0.028; d = -0.376; CI = -0.437, -0.26), and a similar pattern (albeit not significant) was observed for mothers of ASD children (N = 18; M = -0.208 [60.42% of left cradling]; SD = 0.442; $t_{(17)}$ = -2.002; p = 0.062; CI = -0.428, 0.011). Lateral cradling preferences in mothers of typical and ASD children did not differ significantly ($t_{(53)}$ = -0.143; p = 0.887).

Age Group 3–6 Months

Twenty-four participants of the typical group and seven participants of the atypical group provided at least four maternal cradling photos in which infants were aged 3–6 months. The CLQ of mothers of typical children significantly differed from 0, showing a right-cradling bias (N = 24; M = 0.245 [37.75% of left cradling]; SD = 0.573; $t_{(23)}$ = 2.099; p = 0.047; d = 0.428; CI = 0.004, 0.487), and a similar pattern (albeit not significant) was observed for mothers of ASD children (N = 7; M = 0.195 [40.25% of left cradling]; SD = 0.553; $t_{(6)}$ = 0.930; p = 0.388; CI = -0.317, 0.706). Also in this case, lateral cradling preferences in mothers of typical and ASD children did not differ from one another ($t_{(29)}$ = -0.208; p = 0.837).

Age Group 6–12 Months

Thirty-five participants of the typical group and 14 participants of the atypical group provided at least four maternal cradling photos in which infants were aged 6–12 months. The CLQ of mothers of typical children did not differ from 0, showing a slight and no significant left-cradling bias (N = 35; M = -0.059 [52.95% of left cradling]; SD = 0.679; $t_{(34)}$ = -0.514; p = 0.61; CI = -0.292, 0.174); in contrast, mothers of ASD children showed a strong left-cradling bias (N = 14; M = 0.426 [71.29% of left cradling]; SD = 0.543; $t_{(13)}$ = -2.933; p = 0.012; d = -0.67; CI = -0.740, -0.112). Although the control and the experimental group showed a different pattern, this difference did not reach statistical significance ($t_{(47)}$ = -1.801; p = 0.078).

Age Group 1–2 Years

Thirty-four participants of the typical group and 12 participants of the atypical group provided at least four maternal cradling photos in which infants were aged 1–2 years (i.e., between the 12th and the 24th month of child's age). Both the CLQ of mothers of typical children (N = 34; M = -0.061 [53.05% of left cradling]; SD = 0.602 $t_{(33)}$ = 0.588; p = 0.561: CI = -0.150, 0.271) and that of mothers of ASD children (N = 12; M = 0.073 [53.65% of left cradling]; SD = 0.589; $t_{(11)}$ = 0.431; p = 0.675; CI = -0.301, 0.448) did not differ from 0, showing no lateral cradling preference for this age group. Moreover, no difference was observed between the control and the experimental group ($t_{(44)}$ = 0.063; p = 0.95).

Figure 2 depicts the mixed cross-sectional longitudinal trajectory of received maternal left cradling in the first two years of life of both groups of children.

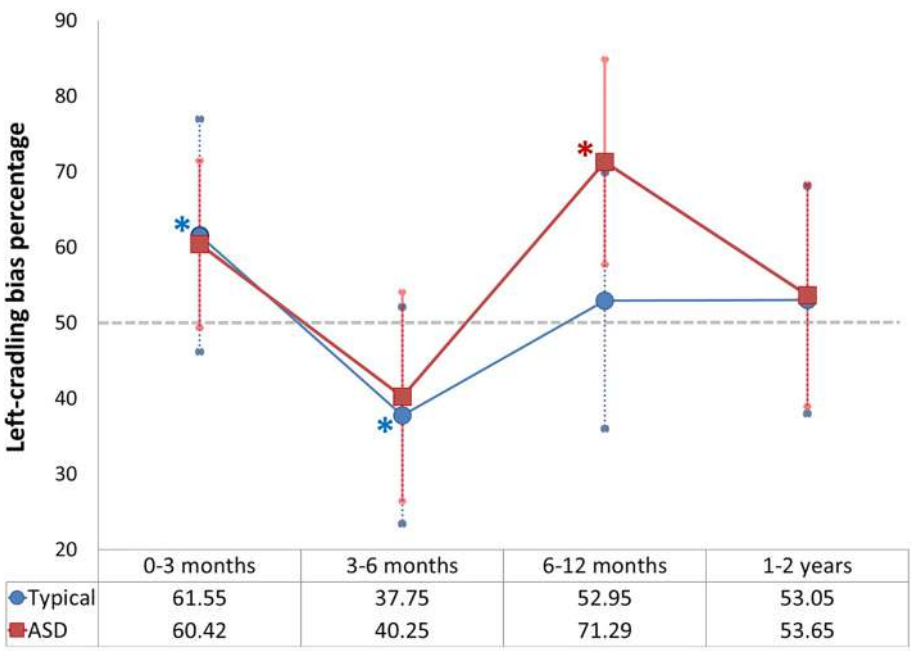

FIGURE 2 | Left-cradling percentage based on cradling laterality quotient (CLQ) of mothers of typical and Autism Spectrum Disorders (ASD) children in all age groups (the asterisks indicate significance of $p < 0.05$ [in blue as regards typical children; in red as regards ASD children]; the grey dashed line indicates the chance level [50%]; error bars indicate standard deviations).

DISCUSSION

The aim of this explorative study was to describe a retrospective longitudinal trajectory of maternal cradling side preference for children diagnosed with ASD—compared with that of typically developing children—in the first two years of life. Since it is impossible, at present, to observe autistic children before the second year of life (due to age of diagnosis), we decided to carry out an "indirect retrospective observation" of mothers using family photos in which they were cradling their children. Mothers were required to provide the age of the child for each photo in order to depict the longitudinal temporal cradling trajectory, according to the age groups used by Manning (61).

No difference was found in lateral cradling preferences between the mothers of typical and autistic children in the first three months after delivery, that is the period in which left-cradling bias is particularly strong in healthy mothers (3, 4, 10) but not in mothers with affective symptoms such as stress, anxiety or depression (38, 62, 63). The left-cradling bias was clearly apparent from photos of the first age group (0–3 months) in both groups: significantly in typical children and trending towards significance in ASD children (probably due to the smaller sample size). In this regard, it is important to note that the photo laterality quotient is an index not coming from a direct observation, and is thus susceptible to many potential factors that might intervene on the bias detection. Indeed, photos can capture a given moment, but they might not be systematically indicative of the actual cradling behavior involving mother and child. However, scoring the family photo albums was successfully used by Manning (61), and also in the present study a left-cradling bias (61.55%) was observed in the first three months, which confirms the usefulness of this method to obtain information not accessible otherwise.

As shown by Manning (61) and, more recently, by Todd and Banerjee (10), after the third month of life of the child there is a remarkable decline of the left-cradling preference in mothers. The present data replicated such a decline from the 12th week, and also indicated a clear right-cradling bias observable in mothers of typical children in the 3–6 months age group. This right bias was also present in mothers of ASD children, albeit it was not significant. In this regard, it should be noted that only seven participants of the ASD group provided an acceptable number of maternal cradling photos for this age group, thus making this comparison the least reliable of the study.

Interestingly, in the second half of the first year of life (age group: 6–12 months), mothers of children with autism exhibited a strong and significant increase of left-cradling bias, whereas the mothers of typical children did not show any lateral preference. In the subsequent age group (1–2 years), data did not show any difference between groups.

In this respect, it should be noted how past research suggested that cradling lateral preferences might not be due exclusively to the right-hemispheric specialization for emotion processing (6, 64). Indeed, a significant relationship between hemispheric lateralization and cradling-side bias is observed only for "basic" holding relationships, in particular those in which the held or cradled element (e.g., a doll) does not provide a feedback in response to the holding side or position. On the other hand, "advanced" holding relationships are characterized by a considerable involvement between the cradling and cradled individuals (e.g., a mother with her infant) (6, 64). In this case, the mother could gradually adjust her lateral preference in response to the infant's activity, and there might be more room for the effect of affective or psychological factors [e.g., insecure attachment, lack of empathy, depression (38, 39)]. Thus, it could be speculated that mother-infant relationships involving children later diagnosed with ASD might remain "basic" because mothers experience a lack of social activity in such children. Actually, many retrospective and prospective studies have reported that infants later diagnosed with autism have social difficulties in reciprocal interactions with their caregiver that were present since the first months of life (65). Muratori and colleagues (66) showed that infants later diagnosed with autism, compared with children with typical development, exhibited significantly worse performance in tasks that required the ability to shift attention from non-social to social stimuli, e.g., the orienting-to-name ability that usually increases around the 9th month (67). The lack of socially motivated engagement becomes an early specific signal of autism by 12 months of age of child, with respect to other neurodevelopmental disorders (57). Furthermore, Dundas, Gastgeb and Strauss (68) showed a left bias for faces in typical children arising around 11 months, whereas children with high risk of autism did not show such a bias (69). Similarly, Jones and Klin (70) found that ASD children showed a developmental decline in eye fixation from about 2 until 24 months of age, despite appearing to begin at normative levels prior to this drop.

Parents of children later diagnosed with autism seem to perceive, long before diagnosis, the lack of responsiveness and social initiative of their infants. Indeed, they engage themselves increasingly more in a close relationship and stimulate their children more than parents of neurotypical children (71). Many investigations reported that mother-child relationships involving ASD children showed qualitative differences with respect to those involving typically developed children (72). Mothers of autistic children, actually, tend to engage more in physical contact with their infants and perform more high-intensity child-directed behaviors (73). In general, compared with parents of typical children, parents of autistic children show more positive strategies of parenting style, probably in order to improve the attachment with their children (74). This over-responsive engagement style may represent a reaction, implemented precisely in the second semester by parents, to the atypical development exhibited by ASD infants (75).

Such evidence seems to suggest that the significant increasing of the left-cradling bias we observed in mothers of ASD children (during the 6–12 months period) might be an unconscious outcome of the attempts carried out by parents, and especially by the mother, to recover their infants to a more vivid emotional activity. A body of work, indeed, indicates that the defining features of autism are not present at the first 6 months of age but begin to emerge later (76). For example, a decreasing vocalization

and an increasing of non-social babbling (77) and more frequent and longer repetitive movements (78) have been described as characterizing this period.

The present results corroborate the idea that left cradling might be considered as an early marker of the quality of the search for emotional closeness between the cradling and cradled individuals (or at least, in the present case, of the parents' efforts to improve such a "basic" relationship).

Although possible stressing factors linked to the mother seem to be involved in both ASD onset (53) and reduced left-cradling preferences (51, 52), the fact that these variables were not related in the present study suggests that they result from different causes.

Finally, although our findings should be considered as preliminary, above all because of the small sample, the results reported here might encourage further studies aimed at investigating whether atypical patterns of cradling-side preferences in children with ASD might reflect either: (i) differences in the nature of the mother-infant relationship ("basic" or "advanced") or (ii) the indirect overstimulation in which mothers try to engage infants in response to their lack of responsiveness and social initiative, and (iii) whether they can be used as a non-invasive behavioral marker for the earlier identification (already in the first year of the infant's life) of children at risk of ASD.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

ETHICS STATEMENT

All participants provided written informed consent to participate in the study by signing an authorization form. Neither invasive nor risky procedures were involved, and the data were analyzed anonymously. The study was carried out in accordance with the principles of the Declaration of Helsinki and following the approval of the Italian "National Institute of Health" ("Istituto Superiore di Sanità") ethical committee (Ethical Committee Approval Number: PRE 469/16).

AUTHOR CONTRIBUTIONS

GM, LT, DM, and MS conceived and created the experiment. GM, CA, FA, and FM conducted the experiment. LT, MS, FM, GF, FA, and GV supervised all the phases of the study. GM, DM, LT, and MS analyzed the results. GM wrote the paper. All authors reviewed the manuscript.469/16).

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2020.00091/full#supplementary-material

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OPINION
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Autism Spectrum Disorder Versus Autism Spectrum Disorders: Terminology, Concepts, and Clinical Practice

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INTRODUCTION

Autism spectrum disorder (ASD) is a behaviorally defined complex neurodevelopmental disorder. The diagnosis of ASD is based on observations and assessments of behavior using Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) (1) or International Classification of Diseases, 11th Edition (ICD-11) criteria (2). Though the DSM and ICD are quite useful in determining whether a given individual's behavior is consistent with a given diagnosis, it does not speak to the etiology or impact of co-occurring conditions on the behavioral phenotype or presentation. Genetic syndromes, defined mutations, and *de novo* copy number variations are reported to account for almost 10% to 20% of cases within ASD (3). While the revisions to the diagnostic criteria introduced a few years ago into DSM-5 (1) updated ASD from the conceptual and practical perspectives, some persistent confusion regarding terminology and the diagnosis of the condition in individuals with intellectual disability remains. The simplified diagnosis of ASD, which merged previous diagnoses into a single disorder, has led to its use in plural (autism spectrum disorders) for different purposes.

From DSM-IV to DSM-5: Diagnosis of Autism Spectrum Disorder

The Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) was released in 1994, at a time in which new knowledge on ASD was rapidly emerging. DSM-IV tried to systematize the different clinical entities associated with autistic features, including recently identified disorders, such as Rett syndrome (4). The category under which disorders with severe autistic features were grouped, pervasive developmental disorders included three disorders (i.e., autistic disorder, Asperger's disorder, PDD-NOS) with substantial clinical overlap. The category also included childhood disintegrative disorder and Rett syndrome, the latter a genetic disorder with initial descriptions of prominent autistic features (4). Despite text corrections on the PDD-NOS section in the subsequent DSM-IV revision (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition—Text Revision, DSM-IV-TR) (5), several major shortcomings were identified in the implementation of DSM-IV/DSM-IV-TR criteria (6). These included the consistency of the diagnosis of Asperger's disorder, frequently labeled as high-functioning autistic disorder; the adequacy of the use of the diagnosis of PDD-NOS for mild neurodevelopmental disorder and

Asperger's disorder for individuals with unusual behaviors but not severe autistic features. Validity of the pervasive developmental disorders category and the diagnosis of childhood disintegrative disorder were also raised over the years (1), as well as new knowledge on the phenotype of Rett syndrome differentiated this entity from autistic disorder (7). The fact that the Centers for Disease Control and Prevention (CDC) reported rates of ASD by grouping by pervasive developmental disorders category (8), rather than by individual diagnoses, also contributed to the revisions of the DSM-IV approach to diagnosis. DSM-5 introduced three major changes: 1. It merged all of the diagnoses that were under the pervasive developmental disorders category into a single disorder termed autism spectrum disorder. 2. It eliminated two diagnostic entities (childhood disintegrative disorder and Rett syndrome). 3. It merged DSM-IV's Social and Communication symptom domains into a single social communication (and interaction) domain. Thus, DSM-5 recognized the empirical evidence demonstrating the challenges of implementing previous diagnostic schemes and the increasing body of literature supporting ASD as a broad spectrum diagnostic entity (1, 6).

Autism Spectrum Disorder Versus Autism Spectrum Disorders

Prior to the introduction of the unitary diagnosis of autism spectrum disorder (ASD), the term autism spectrum disorders began to be applied to epidemiological studies (8) and, more commonly, in the basic science and genetics literature to refer to genetic disorders associated with prominent autistic features or with a relatively high proportion of individuals meeting ASD diagnostic criteria. Although the rationale for this disorder grouping is strong for research on molecular and neurobiological mechanisms in ASD, the specificity of the label and its clinical application are troublesome. While some autism spectrum disorders are characterized by a higher prevalence of ASD than the general population, this is usually not higher than 50% or severe autistic behaviors are only transient (e.g., Rett syndrome) (9). Moreover, there is broad overlap between the cellular processes underlying ASD and those responsible for intellectual disability and severe language impairment (10, 11). The clinical use of the term autism spectrum disorders is included in the diagnostic evaluation of certain genetic disorders with prominent autistic features in order to ensure that the child receives appropriate support services, including early intervention and behavior management. Proper neuropsychological evaluations are also useful in determining whether pharmacologic and non-pharmacologic therapies are appropriate as well as in guiding appropriate school/educational placement. As mentioned above, although Rett syndrome was included in DSM-IV's pervasive developmental disorders category (5), the DSM-5 Neurodevelopmental Disorders Working Group determined that, despite transient severe autistic features in Rett syndrome, there was no reason for selecting Rett Syndrome over the other genetic disorders associated with ASD. Thus, rather than creating an extremely long and rapidly obsolete list of disorders, the DSM-5 Neurodevelopmental Disorders Working Group decided it was better to consider the etiology of ASD (genetic or not) as a specifier (*Associated with a known medical or genetic condition or environmental factor*), which could further refine the diagnosis.

Another application of the term autism spectrum disorders is to emphasize that ASD is thought to be more than one disorder from the pathophysiological viewpoint. However, if ASD is considered a "broad" behavioral syndrome and, as DSM-5 stresses through its etiology specifier, an entity with multiple causes and mechanisms, we suggest that there is no need for a **plural** term. It does not add diagnostic value from the etiological viewpoint. If referring to heterogeneity in terms of cognitive or behavioral impairments, three key specifiers are also included in the DSM-5 ASD diagnosis: "With or without accompanying intellectual impairment," "With or without accompanying language impairment," and "Associated with another neurodevelopmental, mental, or behavioral disorder." The single broad entity of ASD as defined by the DSM-5 is supported by field trials establishing the reliability, sensitivity, and specificity of the diagnosis (12). Meanwhile, the term Autism Spectrum Disorders is reminiscent of the five diagnoses in DSM-IV, diagnoses that ultimately demonstrated low consistency. Thus, although to some extent cumbersome, we suggest that the use of ASD plus the abovementioned specifiers is a better (i.e., more clear and specific) alternative to the term autism spectrum disorders.

Autism Spectrum Disorder and Intellectual Disability

While the discussion about the term autism spectrum disorders underscores the strengths of DSM-5's definition of ASD and its associated recommendations, the guidelines appear to be inadequate for addressing social communication impairments associated with genetic disorders that often lead to various degrees of intellectual disability. In fact, it was recently noted that the difficulties in assigning an ASD diagnosis to an individual with a complex genetic syndrome were recognized many years ago by Leo Kanner (13). DSM-5 indicates that the diagnosis of ASD in intellectual disability is possible, as long as the autistic features cannot be explained by global intellectual or communication impairments: "social communication should be below that expected for general developmental level." This statement puts emphasis not only on the selectivity of the deficits but also on the social communication and interaction impairment of ASD, which has led to some diagnostic challenges. ASD's core symptoms also include the presence of restricted and repetitive behaviors, interests, and activities (RRBs). Although all three types of social communication and interaction deficits (i.e., deficits in social-emotional reciprocity; deficits in nonverbal communicative behaviors used for social interaction; deficits in developing, maintaining, and understanding relationships) are required for the diagnosis, two of the four types of RRBs (i.e., stereotyped or repetitive motor movements, use of objects, or speech; insistence on sameness, inflexible adherence to routines, or ritualized patterns or verbal nonverbal behavior; highly restricted, fixated interests that are abnormal in intensity or focus; hyper- or hyporeactivity to sensory input or unusual

interests in sensory aspects of the environment) are essential in achieving the high sensitivity and specificity of DSM-5's ASD criteria. Consequently, the best way to test the feasibility and adequacy of ASD criteria in intellectual disability is to apply them to well-defined groups. This is necessary because of the heterogeneity of cognitive impairment in the general population.

Evidence is emerging that the behavioral profile of ASD phenomenology is atypical in individuals with co-occurring genetic disorders (14–16). Two of the most common intellectual disability syndromes associated with ASD have been evaluated in terms of DSM-5 criteria, and they have revealed opposite diagnostic challenges. Wheeler and colleagues (17) reported that 86.4% of males and 61.7% of females with fragile X syndrome met DSM-5 criteria for RRBs but only 29.4% of males and 13.0% of females met criteria for the social communication and interaction domain, in contrast with previous reports of up to 60% males being diagnosed with ASD (18–20). By using the Autism Diagnostic Interview-Revised (ADI-R) along with DSM-5 criteria, we demonstrated that in Phelan-McDermid syndrome 90% individuals met the social communication and interaction criteria and 55% met the RRBs criteria (15). Nevertheless, the cohort did not demonstrate greater impairment in adaptive social skills than in adaptive communication skills in the Vineland Adaptive Behavior Scales, Second Edition (21), which raises questions about the validity of the ASD diagnosis in this disorder as it is not clear that the social communication deficits are below that expected for general developmental level. The discrepancies between these studies and previously reported prevalence figures can be analyzed in different ways. By comparing these DSM-5 analyses with the prevalence of ASD according to DSM-IV, an interpretation is that the lower figure in fragile X syndrome reflects DSM-5's lower sensitivity. However, this assumes that the accuracy of DSM-IV is greater or DSM-IV–based figures are the gold standard. Another interpretation is that other features of these genetic conditions lead to an over- or under-recognition of DSM-5 criteria. In support of the latter are studies reporting the complexity of behavioral and other associated impairments in individuals with fragile X syndrome and ASD diagnosis, in particular, the frequent anxiety co-morbidity (20, 22). **Table 1** lists cognitive and behavioral features that potentially lead to the overdiagnosis of ASD in fragile X syndrome and other genetic syndromes associated with intellectual disability (23).

Given that the core social and behavioral symptoms of ASD may present differently in an individual with co-occurring intellectual disability and/or genetic disorders, the applicability and validity of standard ASD screening and diagnostic assessments and their standard scoring systems in these populations should be considered. Derks and colleagues were able to identify a specific subset of questions on the Social Communication Questionnaire (SCQ) that discriminated individuals with ASD and intellectual disability from those with intellectual disability alone (24). Additionally, our group recently adapted two commonly used ASD screening instruments, the Social Communication Questionnaire (SCQ) and the Social Responsiveness Scale-2 (SRS-2), for the diagnosis of ASD in fragile X syndrome. Our findings illustrate the difficulties in differentiating autistic features from characteristic cognitive and behavioral impairments observed in individuals with fragile X syndrome and other forms of intellectual disability (23). The study demonstrated that many SCQ and SRS-2 items are not sensitive to DSM-5 ASD diagnostic status. Furthermore, eliminating these non-specific items only leads to a modest increase in accuracy of the diagnosis of ASD in individuals with fragile X syndrome (23). Thus, it seems that in the context of genetic syndromes, the overall diagnostic impression of ASD is confounded by language impairment and other abnormal behaviors characteristic of neurodevelopmental genetic syndromes. The use of behavioral instruments, such as the Aberrant Behavior Checklist in Down syndrome with and without ASD (25), has also supported this notion. At this point, it is unclear whether these studies in genetic syndromes are applicable to non-syndromic ASD with intellectual disability. Evaluations of DSM-5 versus DSM-IV ASD criteria have demonstrated a lower overall prevalence of ASD using DSM-5 criteria, but greater agreement between DSM-IV and DSM-5 among individuals with intellectual disability than in those with normal cognition (26).

Unquestionably, many individuals with intellectual disability have social communication and interaction impairments that require adequate diagnosis and treatment. Nonetheless, the exclusive use of the ASD label in this situation decreases the validity of the ASD diagnosis with negative implications for clinical practice and research. If RRB-like features are not present at DSM-5's diagnostic threshold, we propose to use the more appropriate diagnosis of Social (Pragmatic) Communication Disorder (1). Although this entity was delineated as a specific communication disorder affecting the social communication domain, it includes most of the features and functional implications of the social communication and interaction deficits in ASD. In the situation where social communication

TABLE 1 | Cognitive and behavioral features affecting social communication in intellectual disability.

Communication
• Concrete or inflexible thinking
• Difficulties with flow of conversation
• Difficulties with logical thinking
• Language disorder (e.g., reduced vocabulary)
• Stereotyped or repetitive speech*
• Reduced facial expression and gestures due to motor impairment (e.g., hypotonia, parkinsonism)
• Decreased pointing due to motor impairment (e.g., hypotonia or hypertonia, reduced hand function, poor motor coordination)
• Reduced eye contact (e.g., eye gaze avoidance)**
Behavior
• Anxiety, general, or social types
• Hyperactivity or impulsiveness
• Sensory over-reactivity
• Irritability
• Repetitive movements (e.g., body rocking, hand flapping)*
• Perseverative behavior*

Common behaviors in intellectual disability, which could be diagnostic RRBs for ASD and/or stereotypic movement disorder.

***Common behavior in fragile X syndrome with or without ASD; related to anxiety.*

deficits do not meet DSM-5 threshold, but the individual displays RRB-like features, we recommend using the label Stereotypic Movement Disorder (9). This entity was delineated with a focus on the frequently present repetitive movements and behaviors in intellectual disability, including genetic syndromes, such as fragile X syndrome. We hope future revisions of DSM will address this apparent over-diagnosis of ASD in intellectual disability and determine if the problem extends beyond genetic syndromes.

DISCUSSION

We conclude that the use of the term Autism Spectrum Disorders, to refer to genetic disorders associated with prominent autistic features, is not recommended. Use of the plural term. Use of the term Autism Spectrum Disorders is also problematic in clinical practice since DSM-5's framework takes into consideration the range of impairments and severity in ASD. We also suggest that the diagnosis of ASD is inaccurate in many individuals with intellectual disability, particularly in those with genetic syndromes where the social communication deficits are not below that which can be expected given the individual's developmental level. Rather than basing the diagnosis exclusively on the social communication and interaction impairments, we recommend employing other diagnostic entities in DSM-5, such as Social Communication Disorder or Stereotypic Movement Disorder as appropriate.

AUTHOR CONTRIBUTIONS

LO participated in the interpretation and drafting of results and drafted the manuscript. WK conceived of the study, participated in the interpretation and drafting of results, and drafted the manuscript.

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