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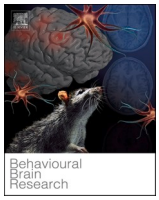

Research report

# Exploring transgenerational inheritance in epigenotypes of DAT heterozygous rats: Circadian anomalies and attentional vulnerability

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ABSTRACT

Dopamine (DA) is mainly involved in locomotor activity, reward processes and maternal behaviors. Rats with KO gene for dopamine transporter (DAT), coding for a truncated DAT protein, are in hyperdopaminergic conditions and thus develop stereotyped behaviors and hyperactivity. Our aim was to test the prior transgenerational modulation of wild and truncated alleles as expressed in heterozygous DAT rats: specifically, we addressed the possible sequelae due to genotype and gender of the ancestors, with regard to behavioral differences in $F_1$, $F_2$, $F_3$ rats. We studied non-classical DAT heterozygotes (HETs) based on two specular lines, with putative grand-maternal vs. grand-paternal imprinting. MAT females ($F_1$; offspring of KO male and WT female) mated with a KO male to generate MIX offspring ($F_2$). Specularly, PAT females ($F_1$; offspring of KO female and WT male) mated with a KO male to generate PIX offspring ($F_2$). Similarly to PAT, we obtained MUX ($F_2$; HET offspring of MAT sire and KO dam); we also observed the $F_3$ (MYX: HET offspring of KO male and MUX female, thus with DAT-KO maternal grandmother like also for PIX). We studied their circadian cycle of locomotor activity and their behavior in the elevated-plus-maze (EPM). Locomotor hyper-activity occurs in $F_1$, the opposite occurs in $F_2$, with MYX rats appearing undistinguishable from WT ones. Open-arm preference emerged in PIX and MIX rats. Only MAT and MYX rats showed a significant vulnerability for ADHD-like inattentive symptoms (duration of rearing in the EPM; Viggiano et al., 2002). A risk-taking profile is evident in the $F_2$ phenotype, while inattentiveness from $F_1$ progeny tends to be transferred to $F_3$. We hypothesize that DAT-related phenotypes result from effective inheritance through pedigree of imprints that are dependent on grandparents, suggesting a protective role for gestation within a hyperdopaminergic uterus. For major features, similar odd ($F_1$, $F_3$) generations appear opposed to even ($F_2$) ones; for minor specific features, the phenotype transfer may affect the progenies with a male but not a female DAT-KO ancestor.

## 1. Introduction

Dopamine (DA) is a neurotransmitter belonging to catecholamines, and it is involved in reward-based decision making, habitual responses, emotional and endocrine regulation. Dopaminergic neurons of the mesolimbic pathway are implicated in behaviors inherent to gratification [1]: the peculiarity lies in the fact that an increase in DA levels occurs not exclusively in consequence of a positive event but also in response to cues or stimuli that anticipate future gratification. In the prefrontal cortex (PFC), DA is implicated with attention processes, executive functions and working memory [2]. Specific DAergic nuclei of the hypothalamus are subserving the daily locomotor activity cycle and maternal behavior [3].

DA is transported back into the presynaptic cell through the membrane dopamine transporter (DAT), which belongs to the group of Na+ / Cl- dependent transporters [4]. DAT has been found in the motor, prefrontal, anterior cingulate, and visual cortexes, but is mostly found in the mesocortical and mesolimbic pathways [5] of the forebrain. DAT belongs to the SLC6 family of transporters and allows DA elimination from the synaptic cleft in response to physiological demands [6]. Using

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biotechnological approaches and molecular techniques, it was possible to insert a stop codon into the SLC6A3 gene that codes for the DAT protein, rendering it shorter and functionally silenced [7,8]. High levels of DA, via an altered expression of (social) gratification, tends to develop an implicit memory impairment and a repetitive pattern of habits. When DA is not transported back into terminals at synapses level, DAT-KO rats and mice manifest hyperdopaminergia, significant locomotor hyperactivity, stereotyped behaviors and circadian rhythm dysfunction [7,9,10]. The resulting alterations, in humans, can be associated with schizophrenia [11], substance abuse, obsessive-compulsive disorder (OCD) and attention-deficit / hyperactivity disorder (ADHD) [12], autism spectrum disorder [13], as well as Tourette's syndrome and Parkinson's disease [14,15]. Among these, ADHD is characterized by hyper-activity and inattention, resulting in dysfunctional behavior of the affected subject.

Environmental inputs may have molecular impacts and exert their modulatory effects at the level of genetic expression. Transgenerational inheritance is considered when there is a plasticity of phenotypes in the $F_2$ and $F_3$ [16] and if this is mediated by the germ line [17]. Environmental stimuli can affect an embryo/fetus while in gestation [16]: sequelae of neurotoxic and/or stressful exposures during pregnancy are widely studied. Environmental inputs also may occur when gametes are still developing, causing a transgenerational inheritance: mechanisms include impact on DNA methylation or acetylation and, at the level of chromosomes, modifications of histones and of chromatin structure via non-coding RNA [18].

As a result, in the maternal and/or paternal lineage, gametes may manifest epigenomic differences [19]. Altered phenotypes can manifest themselves at the $F_2$ or $F_3$, developing the so-called transgenerational behavioral change [20]. No one to our knowledge has ever carried out epigenetic studies investigating what happens during maturation of gametes in DAT KO rats or mice. Therefore, we have chosen to focus on sequelae of having DAT KO ancestors in the pedigree. Normally, developmental studies focus on classical HETxHET breeding, actually never observing what might happen to $F_1$ and $F_2$ progeny if the initial parents (father and/or mother) are KO. We propose in fact two sources of variability, depending on whether the initial father or mother in the lineage is KO for DAT. In the first case, a high level of dopamine in the testes can generate consequences in the spermatozoa, impacting on future offspring; if conversely the KO ancestor is the initial mother, then whole pregnancy of $F_1$ (while gonads develop which will actually conceive $F_2$) occurs in a hyperdopaminergic uterus. Recently, we obtained non-classical acronyms to denote the various heterozygous (HET) lineages for the DAT gene [8,21].

In the present study, we explored transgenerational inheritance, based on two specular breeding lines. As for $F_1$ groups, MAT subjects (maternal line) are the offspring of a KO male and a WT female, while PAT subjects (paternal line) are the offspring of a WT male and a KO female [22]. We hypothesized that alleles may receive some epigenetic marking ("imprints") while segregating apart during the meiosis, and / or additional modifications within either the epididymis or the uterus. A key difference between MAT and PAT rats is the following: for PAT rats, prenatal hyperdopaminergia is tapping onto the egg then conveying into a PAT zygote; for MAT rats, the epithelial cells of the epididymis, in their KO father, can determine the transgenerational inheritance of paternal traits [23].

It has been observed [24] how the presence of epigenetic alterations in primordial germ cells (PGCs), also including maturation of egg cells in the uterus, develop a transgenerational inheritance. Infants of dams with elevated dopamine levels had themselves significantly elevated levels, for instance [25,26]. We wondered whether differential effects could be found in DAT-HET progeny when the WT egg cell was fertilized by a KO spermatozoon versus when a WT spermatozoon fertilized the KO egg cell. To verify the effects, likely resulting from the imprinted alleles, we proceeded to follow the maternal and paternal lines, with different combinations in subsequent generations.

Our own previous studies allow us to propose how the $F_1$ (MAT and PAT) should exhibit hyperactive profiles while the $F_2$ (MUX, MIX and PIX) should conversely display significantly hypoactive profiles [29,30]. In a previous study [30], indeed, we observed opposite divergences in locomotor activity of DAT-HET rats between $F_1$ and $F_2$. However, circadian locomotion at $F_3$ turned out to be the same as it was at $F_0$. While in that study the initial ancestor was a **male** DAT-KO, presently we extended the study by adding also the progeny of a **female** DAT-KO: when bred with a WT sire, we obtained PAT rats, and hence PIX rats. Also, when bred with a $F_1$ MAT male, we obtained MUX ($F_2$) and hence MYX ($F_3$) rats. Since the latter are at $F_3$, we also carried out a separate analysis including only MYX vs. WT, to confirm or dismiss whether the $F_3$ phenotype is drawn back at $F_0$-like levels, as we previously observed [30]. Aiming at the characterization of ADHD symptoms, in relation to the impact of intrauterine dopamine in the offspring of KO mothers, we chose to test (i) hyperactivity, through the analysis of circadian cycles of activity, and (ii) inattention, through the duration of rearing behavior within EPM test.

Subjects were exposed during the adolescent period to testing of circadian locomotor cycles, according to epigenotype. In order to address the role of genotype and / or of fostering, MAT and PAT versus WT and foster-WT rats were included in the first analysis. The second analysis involved $F_2$ and $F_3$ rats (PIX, MIX, and MYX epigenotypes; shown in Table 1) combined and compared to WT controls, to address (i) the role of hyperdopaminergic grandmother's uterus and / or (ii) anomalous dam parenting.

## 2. Methods

The Animal Welfare Survey Board (OBA ISS), on behalf of the Italian Ministry of Health, approved the experimental procedures (formal license 1008/2020-PR issued to WA, veterinary supervision by G. Panzini). The experimental procedures were carried out in strict compliance with European regulations (European Community Council Directive 2010/63/EEC) and the guidelines of the Italian legislation. In addition, according to the principles of the 3Rs, we minimized the number of subjects and the suffering of the animals involved in the experiment.

We continued the biased and non-classical reproduction approach by breeding the DAT-KO male now with a $F_1$ heterozygous rather than WT female. In the $F_2$ generation, PIX offspring (from PAT dam and KO sire)

**Table 1**
Epigenotypes: abbreviations' legend [8].

| Abbreviation | Explanation |
|---|---|
| WT = wild-type | Most frequent genotype, here for dopamine transporter (DAT), in the natural population |
| KO = knock-out | Homozygote knocked-out, here for the DAT gene: due to infertile KOxKO breeding, is usually obtained as sibling of other DAT-HET epi-genotypes |
| HET = Heterozygous | Inheriting one healthy and one knocked-out allele from each parent |
| MAT = maternal | Heterozygous specimens, offspring of a KO male and a WT female |
| PAT = paternal | Heterozygous specimens, offspring of a WT male and a KO female |
| MIX = mixed with KO siblings - "imprinted (?) maternal allele" | Heterozygous offspring of a MAT female and a KO male. "M stands for healthy maternal grandMother" |
| PIX = mixed with KO siblings - "imprinted (?) maternal allele" | Heterozygous offspring of a PAT female and a KO male. "P stands for healthy maternal grandFather" |
| MUX = mixed, and born from KO uterus | Heterozygous offspring of a KO female (like for PAT) and a MAT male |
| MYX = mixed, and its dam in KO uterus | Offspring from KO male and MUX female, that is in turn heterozygous offspring from a KO female and a MAT male (i.e.: like for PIX, the maternal grandmother was KO and the dam was born from KO uterus) |

and MIX offspring (from KO sire and MAT dam) present inverted grandparents: our breeding strategy is thus resulting in the "atypical" allele (i.e., WT but carrying putative epigenetic marks derived from the encounter with a KO gonad-produced gamete carrying the KO allele) originating by maternal grandmother or maternal grandfather (shown in Fig. 1), respectively. We also inverted parents at $F_1$ by breeding a KO female and a MAT male, to obtain the MUX progeny. Such MUX offspring (sire is MAT and dam is KO) is a sort of $F_2$ for the factor "gestation within hyperdopaminergic uterus": MUX subjects in this are similar to PAT ones, but with a heterozygous rather than WT sire. The $F_3$ HET rats here consist of MYX subjects, that is, the offspring of KO sire and a dam now being MUX (in turn, the offspring of MAT sire and KO dam [8]: MYX are therefore like PIX but on next generation, with HET rather than WT maternal grand-dad). In all cases, within the KO dam, high levels of DA (resulting from the lack of reabsorption) interfere with the hormone prolactin (PRL) resulting in little or no milk [27,28]. Therefore, to avoid death, PAT and MUX newborn subjects are given at birth to WT dams, for adoption.

All rats were maintained, according to an inverted light-dark cycle (lights off at 8 am and on at 8 pm), in a room with controlled temperature and humidity (T 21± 1° C; relative humidity 60 ± 10%) and with food (pellets, Mucedola SrL, Italy) and water ad libitum. Breeding pairs consisted of one male and two females. At birth, rats were culled to 5±1 males and 3±1 females. The number of male rats used is 12–15 per group, coming from six individual dams per group; in order to avoid the well-known litter effect, no more than two male siblings were used per litter. In the case of PAT and MUX, the newborn pups from KO dams were fostered to WT dams by replacing own pups of the latter. In the case of MIX, PIX and MYX, their KO siblings were discarded at weaning.

We used a naturalistic approach in order to gain a thorough understanding of how social interaction occurs in nature, rather than creating "gender" groups of only males or only females [31,32]. Therefore, we induced random encounters among pairs of rats: within a quadruplet, two yoked pairs rotated with exchange of males ("swap"), where half of the encounters were M/M and half were M/F. This allowed to analyze the social interactions with a wider repertory, as different types of interaction (that contribute to behavioral variability) are proposed by same-sex (M/M) and mixed (M/F) yoked pairs. The locomotor activity of adolescent female rats is typically more pronounced [33,34] due to sex-related hormonal changes, that also have a major impact on sensory (i.e. olfactory) capacities [35]. This in turn affects the social signals that the male rats perceive.

Behavioral data were analyzed by Repeated Measures ANOVA (RM - ANOVA), using StatView software (SAS Institute Inc.). The significance level was set at $p \leq 0.05$, and post hoc analysis was performed using Tukey's HSD test. All figures report the mean value and standard error of the mean.

### 2.1. Experiment 1 (circadian activity cycles)

#### 2.1.1. Subjects

The experimental cohort (72 rats) consisted of two control groups ("true" WT offspring from WT dam and WT sire, either left with the natural dam or fostered to an adoptive WT dam); $F_1$ heterozygotes, MAT (offspring from KO sire and WT dam) and PAT (offspring from KO dam and WT sire, adopted at birth by a WT dam). Then, two groups of $F_2$ heterozygotes namely MIX, (i.e., offspring from heterozygous MAT dam and KO sire: healthy-but-vulnerable allele from maternal grandmother with putative imprints from maternal grandfather) and PIX (i.e. offspring from heterozygous PAT dam and KO sire: healthy-but-vulnerable allele from maternal grandfather with putative imprints from maternal grandmother; lastly, the $F_3$ heterozygotes (MYX: offspring from KO sire and MUX dam that is, in turn, heterozygous offspring from a KO female and a MAT male, WT allele already vulnerable from the maternal grandfather with additional, putative imprint from maternal grandmother).

#### 2.1.2. Procedure

Male rats were marked on the tail with a blue marker at weaning [post-natal day (PND) 22±1], and were alternatively planned to encounter between them or with a female, according to a protocol [22] with a total duration of 29 days and interchange of pairs every three days. This schedule occurred during adolescence, between PND 25 and PND 54. The very same pairs of subjects were repeatedly formed again and again through exchange ("swap") of the male per yoked pair.

#### 2.1.3. Apparatus

Continuous detection of locomotor activity is a fundamental tool for assessing the conditions the subject manifests in a given context. We used a grid of sensors positioned above each cage at an angle of ±50° and anchored to a plate at the back of the cage. Each sensor was connected to a control unit that routed readings to the computer via the parallel interface. Data collection was performed using Activiscope software (Technosmart, Guidonia, Italy). Raw data on locomotor activity were recorded 24/7 for approximately one month. After each male "swap" we used only three days of readings with the removal of the fourth day (when present): this allowed homogenous scoring from each new pair of subjects to interact.

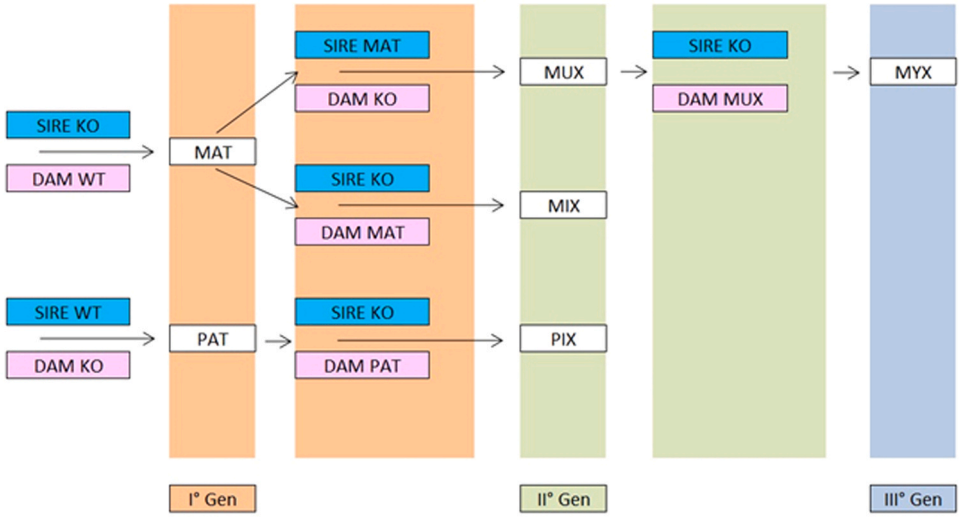


**Fig. 1.** Genealogy of DAT HET subjects.

Upon completion of data recording and export, we made various triplets composed of the 3 days immediately following an exchange ("male swap"). Any fourth day of the same pair that did not fit into the analysis was eliminated. To group all locomotor detections made on the very same pair of subjects, the three relevant triplets of days were identified and placed contiguously, resulting in nine adjacent days (consisting of three triplets of days) taken on that pair of animals. From the resulting nine days, the average day (expressed by each pair, formed by the 24-hour points) was calculated, which always began at 8 a.m. and ended at 7 a.m. of the next day. The average days thus calculated formed a matrix of as many rows as were the rat pairs by 24 columns, and were entered into the StatView file for final analysis.

#### 2.1.4. Analysis of circadian rhythms

The factorial design (2×2x24) was subdivided as follows and applied into two separated ANOVAs, aimed to verify the comparisons for both generation steps: first, the $F_0$ vs. $F_1$, then, $F_2$ vs. $F_3$. "Within" factor always represents the 24-hourly time points of the average day. In the **first** ANOVA for $F_0$ controls and $F_1$ rats, the two "Between" factors represent the type of maternal cares versus genotype. In the **second** ANOVA with $F_2$ and $F_3$ rats, the two "Between" factors represent the type of maternal care versus maternal grandmother in the pedigree. When analyzing $F_0$ controls and $F_1$, therefore, the 2×2 design combined a "genotype" factor (shown in Fig. 2), with WT controls (valid for both natural and fostered WTs) Vs. HET (valid for both MAT and PAT HETs), and a "maternal-care" factor (shown in Fig. 3), with natural dam (valid for both WT and MAT) Vs. foster dam (valid for WT and PAT both fostered to a WT dam).

The $F_2$ vs. $F_3$ ANOVA is somewhat more complicated for the sake of transgenerational inheritance. We hypothesized that the previous foetal development of dams in a hyperdopaminergic uterine environment could affect their own offspring: the factor termed "maternal grandmother" (shown in Fig. 4) compared both MYX and PIX, whose dams were MUX and PAT (therefore, with grand maternal KO uterus for their own dams), to both MIX and WT (with WT maternal grandmother). Then, as second factor in the 2x2 desigtn, note that MUX and MAT rats were having altered parental behavior towards their MIX and MYX pups whereas, conversely, fully normal caring was expressed from a wild type dam or also in the case of the PAT dam [29]. We thus used a "Parental-care Quality" factor (shown in Fig. 5) giving account for the putative difference between the "vulnerable" caring (from a MAT or MUX parent on MIX and MYX pups) and the "healthy" reference cares, in this case by WT or PAT dams on WT and PIX rats (Fig. 4). Therefore, a "maternal grandmother" factor, where the grandmother could be KO or WT, interacted with a "parental caring quality" where the maternal cares could be either vulnerable or healthy.

### 2.2. Experiment 2 (elevated plus maze)

#### 2.2.1. Subjects

The first experimental cohort (36 rats) consisted of two control groups (WT offspring from WT dam and WT sire, left with natural dam or adopted from non-natural WT dam); two groups of $F_1$ heterozygotes namely MAT (KO sire and WT dam) and PAT (WT sire and KO dam); two groups of $F_2$ heterozygotes namely MIX (offspring from heterozygous MAT dam and KO sire) and PIX (offspring from heterozygous PAT dam and KO sire). The second experimental cohort (18 rats) consisted in $F_3$ heterozygotes namely MYX (KO sire and MUX dam) and a new parallel control group of WT (WT sire and WT dam).

#### 2.2.2. Apparatus and procedure

The elevated plus maze (EPM), belonging to the broad category of ethological tests, is relative to the emotional impact of anxiety [36], and exploits rats' spontaneous aversion to elevated and open areas. The maze has two closed arms (in a cross shape) made of plexiglass: it is black for the underneath (including the floor) and transparent for the walls of the closed arms, which have no top cover. The two open and two closed arms (50 cm) extend from a central platform (15 cm), all placed about 50 cm above the room floor. The rats were initially placed with the four arms in the center and facing a closed arm, so that they move freely for 15 minutes. The facility-room light was dim, white and indirect. The test was conducted from 10:30 am to 3:30 pm in the same experimental room where rats were already housed. Between subjects, the EPM was cleaned using a mixture of water and alcohol (50%) and then with dry cloths.

All sessions were recorded using of the SONY DCR-SX21E camera, positioned laterally at a high (>45°) angle, suitable for recording the entire labyrinth. The recordings were analyzed using The Observer XT 10 software (Noldus, NL) with respect to spatiotemporal and behavioral parameters [37]. The former, included the total duration and number of entries into the closed and open areas, as well as in the central area. The behavioral measures, intervening in each relevant area, were: exploration, rearing, wall rearing, self-grooming, sniffing, immobility, head dipping, and stretched-attendance posture (SAP). These last two behavioral items, typical of the EPM, are related to expressions of risk

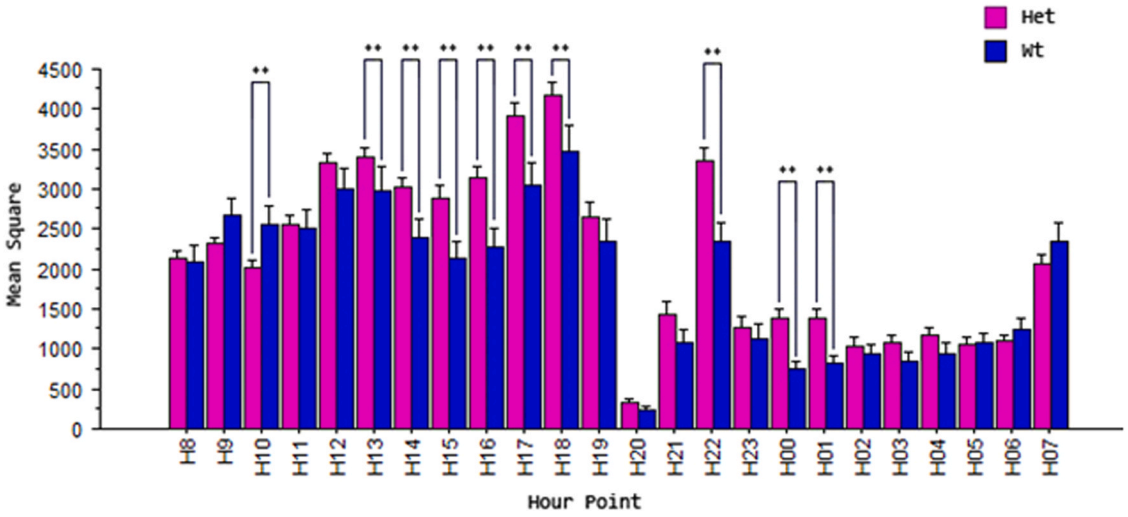


**Fig. 2.** Daily average of locomotor activity in hourly counts for groups of rats collapsed by genotype: HET (purple color denoting MAT and PAT together) and WT (blue color). The circadian cycle was monitored for a period of one month (PND 25 to PND 54; pairs of rats with swap of males every 3 / 4 days, see methods) and then the daily average was calculated. N = six pairs of rats from three swapping quadruplets per group, see Methods. Asterisks refer to statistically significant differences ($P < 0.05$). When p-value is less than 0.01, it is flagged with two asterisks (**). For details see Table 1.

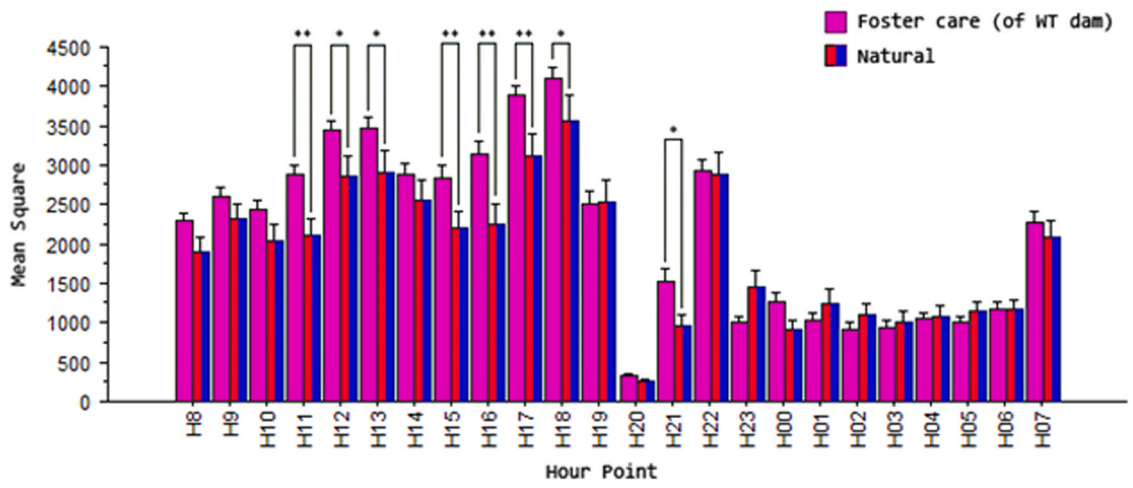


**Fig. 3.** Comparison of same circadian locomotor cycles of Fig. 2 between rats now collapsed for maternal care: we compared a foster dam (purple color denoting PAT and foster-WT) with a natural dam (red/blue color denoting MAT and WT). The circadian cycle was monitored for a period of one month (PND 25 to PND 54; pairs of rats with swap of males every 3 / 4 days, see methods). N = six pairs of rats from three swapping quadruplets per group, see Methods. Asterisks refer to statistically significant differences ($P < 0.05$). When p-value is less than 0.01, it is flagged with two asterisks (**). For details see Table 1.

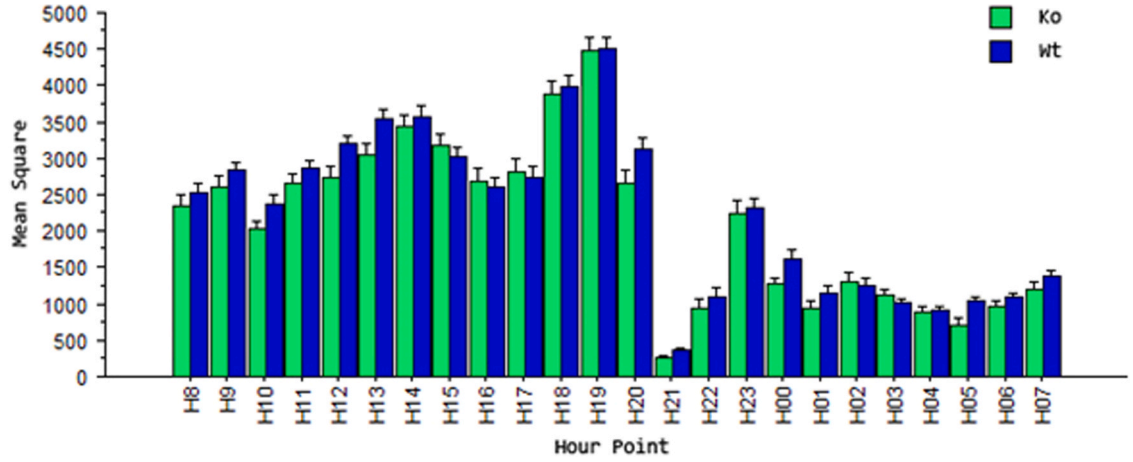


**Fig. 4.** Daily average of locomotor activity in hourly counts for groups of rats collapsed by maternal grandmother: we compared those rats whose maternal grandmother was KO (dark green color, denoting PIX from mother PAT and MYX from mother MUX) vs. those rats whose maternal grandmother was WT (blue color, denoting MIX from mother MAT and WT from mother WT). The circadian cycle was monitored for a period of one month (PND 25 to PND 54; pairs of rats with swap of males every 3 / 4 days, see methods). N = six pairs of rats from three swapping quadruplets per group, see Methods. For details see Table 1.

assessment [37].

#### 2.2.3. Analysis of EPM data

The ANOVA presents a 3×2×6 factorial design: the "between groups" factor is related to the six epi-genotypes, the two "within group" factors are related to the three areas of the EPM (Open, Center and Closed) in which given a behavior occurs, as well as formally compared items of behavioral ethogram (shown in Fig. 6). We first analyzed the time spent by each epigenotype in performing each behavior within the areas of the EPM (Open, Center and Closed). Then, we formally compared behavioral items as the attentional versus the risk-assessment ones (i.e. Rearing vs. Head Dipping; shown in Fig. 7).

Two ANOVAs were conducted, comparing the $F_1$ and $F_2$ with the control groups (i.e., MAT, PAT, MIX, PIX, WT, and W-WT) and, separately, the $F_3$ with the control group (MYX vs. WT). Finally, we have calculated the percentage between time spent in possible distraction (i.e., Rearing duration) and the totality of time spent in the closed arm, for each epigenotype versus control groups.

## 3. Results

### 3.1. Results of EXP. 1 (circadian activity cycles)

#### 3.1.1. Controls and first generation

*Higher locomotor activity in DAT-HET epigenotypes.* Considering (shown in Fig. 2) interaction of the circadian cycle with genetic factor ($F_{23,1150} = 5.234$; $P < .0001$) in its two levels (heterozygous vs. wild type; Tukey GF = 1150; K = 7; Threshold = 591.502), we observed that locomotor activity manifests higher levels in DAT-HET genotype. This was significant when the dark to light phase-change approached (from 2 p.m. to 6 p.m.). We found a typical level of locomotor activity (values ranging between 2000 and 4000) rising before the light turn on: the highest hyperactivity (over the value of 4000) is reached by the PAT epigenotype (5 p.m. and 6 p.m.) and to some extent by MAT epigenotype (6 p.m.). In the hours following the lighting on at the facility, the HET genotype still manifests a pronounced activity compared to WT controls, in particular at 10 p.m.

*Offspring of the two genotypes display reactions to adoption.* Considering (shown in Fig. 3) interaction of the circadian cycle with the epigenetic

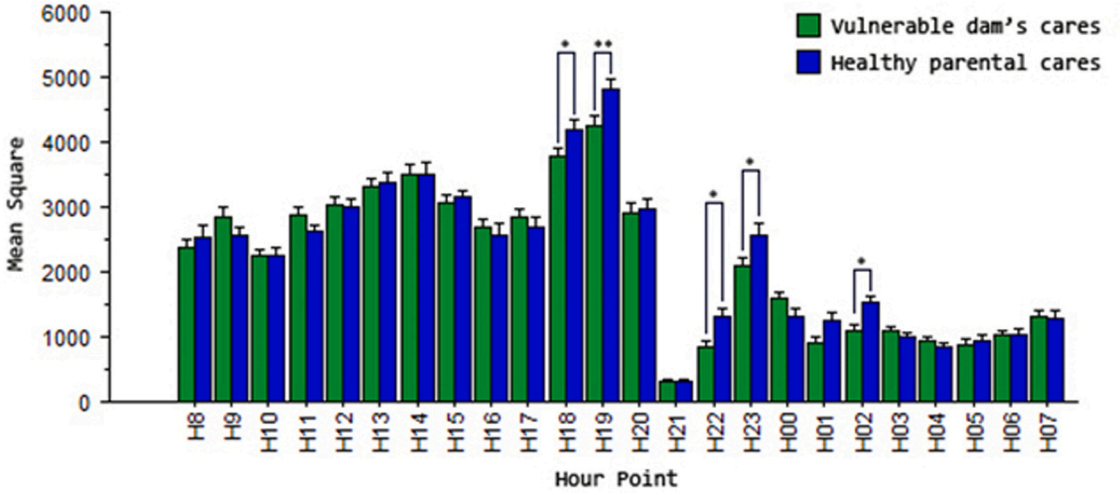


**Fig. 5.** Comparison of same circadian locomotor activity of Fig. 4 between rats now collapsed for parental care: we compared the vulnerable care group (green color, denoting MIX plus MYX cared from altered MAT and from MUX dams, respectively) vs. a reference parental care group (blue color, denoting PIX plus WT cared from WT-like and from WT dams, respectively). The circadian cycle was monitored for a period of one month (PND 25 to PND 54; pairs of rats with swap of males every 3 / 4 days, see methods). N = six pairs of rats from three swapping quadruplets per group, see Methods. Asterisks refer to statistically significant differences ($P < 0.05$). When p-value is less than 0.01, it is flagged with two asterisks (**). For details see Table 1.

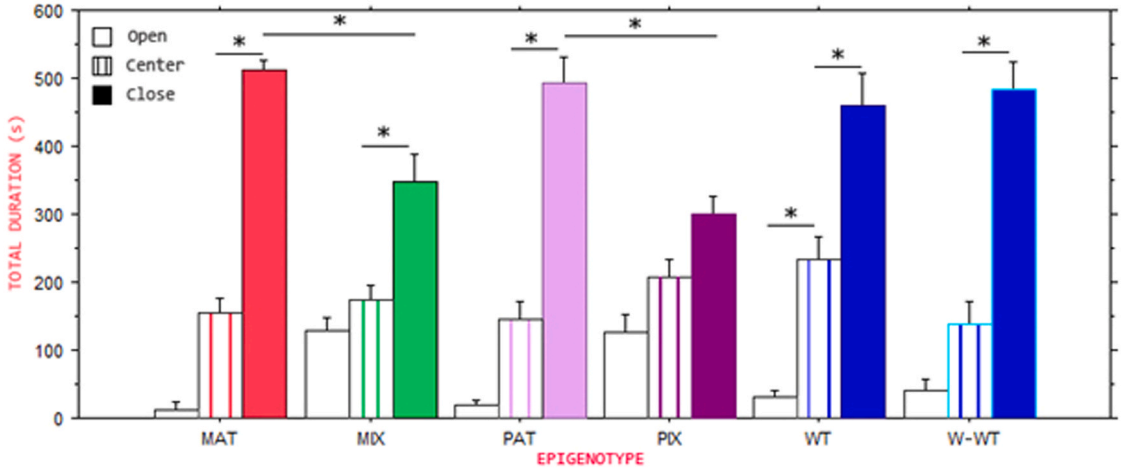


**Fig. 6.** Time (s) spent by the MAT (offspring of KO sire and WT dam), MIX (offspring of KO sire and MAT dam), PAT (offspring of WT sire and KO dam), PIX (offspring of KO sire and PAT dam) and control groups, WT (offspring from both WT parents) and W-WT (offspring fostered to a non-natural WT dam), in the open, center and closed arms. The time spent in each area among the different epigenotypes significantly depends on the $F_1$ vs. $F_2$ to which each HET subject belongs: note that there is statistical significance (p-value <.0001) between MAT vs. MIX and PAT vs. PIX in the closed arm. Accordingly, both MIX and PIX spend much more time in the open space and less time in the closed space. Asterisks refer to statistically significant differences ($P < 0.05$). When p-value is less than 0.01, it is flagged with two asterisks (**). Cohort 1, n = 6 per group; Cohort 2, n = 9 per group. For details see Table 1.

factor ("maternal-care" $F_{23,1150} = 3.893$; P <.0001) in its two levels (natural vs. foster dam's care; Tukey GF = 1150; K = 7; Threshold = 505.443; 591.502), we observed that locomotor activity is significantly elevated in both PAT and foster-WT, namely those pups with maternal care by the foster dam, when compared to those with the biological WT dam (both MAT and WT). This pattern is also observed in the middle of the day (11 a.m. - 1 p.m.). This evidence in relation to the previous one, suggests that the genotype and the fostering are simply additive, and act on a common element of all pups (that is, an awareness of the coming phase change in the circadian cycle).

### 3.1.2. *Second- and third-generation DAT-HET rats*

*There is no "KO grandmother" effect for both MYX and PIX.* We observed (shown in Fig. 5) a clear parental care x hour effect ($F_{1,1564} = 2.262$; P =.0006): locomotor activity appears significantly elevated in offspring receiving the care of healthy parents (WT and PIX) compared with offspring in which the maternal care is actually vulnerable (MIX and MYX; Tukey GF = 1564; K = 7; Threshold = 398.291; 466.106). This somewhat depressed behavior is observed during the pre-phase change (6 p.m. and 7 p.m.) and immediately after (10 p.m., 11 p.m., and even 2 a.m.).

The ANOVA shows the overall grandmother x parental care x hour interaction ($F_{23,1564} = 12.209$; P <.0001): the profile of locomotor activity is affected by the KO grand-maternal uterus in the lineage and only in combination with a vulnerable caring by the MAT or MUX parent. A significant difference in activity is observed in the hours preceding the phase change and in the hours immediately following (Tukey GF = 1564; K = 10; Threshold = 603.792; 696.994). Indeed, it is evident that MYXs (Grandmother KO, Vulnerable parenting) have higher locomotor intensity in comparison with MIXs (Grandmother WT, Vulnerable parenting); the same is true for WTs (Grandmother WT; Healthy parenting) compared with PIXs (Grandmother KO, Healthy parenting).

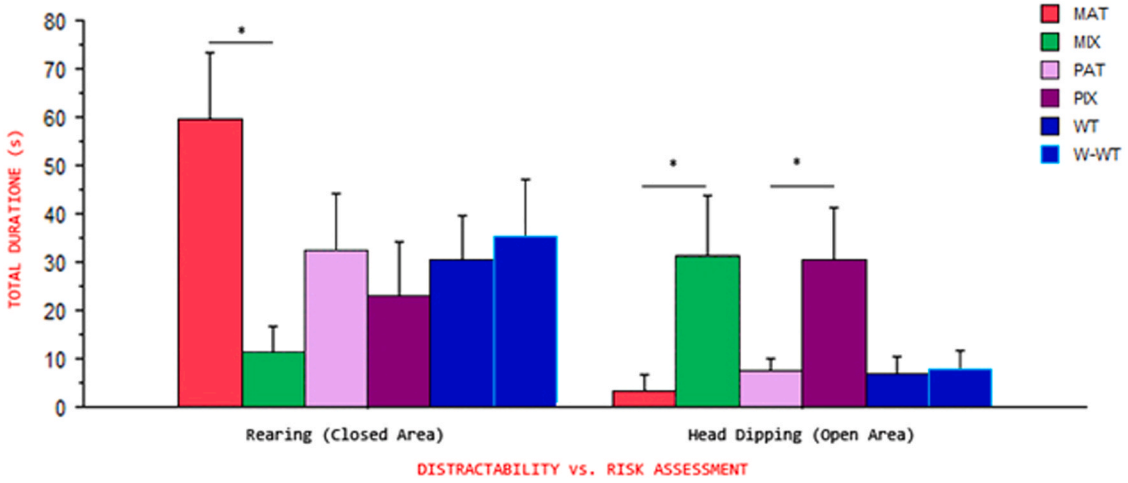


**Fig. 7.** Time (s) spent by $F_1$ epigenotypes (MAT and PAT), $F_2$ epigenotypes (MIX and PIX), and control groups (WT and W-WT) in two forms of behavior, which are valid indexes of distractibility vs. risk assessment, respectively. The $F_1$ (MAT) possesses a statistically significant higher level of duration of rearing (in closed areas) than the $F_2$ (MIX). Epigenotypes MIX and PIX, in open areas, are confirmed to have more "risky" head - dipping behavior. Asterisks refer to statistically significant differences ($P < 0.05$). When p-value is less than 0.01, it is flagged with two asterisks (**). Cohort 1, n = 6 per group; Cohort 2, n = 9 per group. For details see Table 1.

The common aspect of PIXs and MIXs relates to their belonging to the $F_2$, which was indeed expected to show some depressive tendency [29]. Conversely, MYXs, who are on the next generation (i.e., $F_3$), develop a WT-like recovery of circadian behavior similarly to what was seen for $MIX^2$s [29]. PIXs and MIXs, while exhibiting a generalized decrease in activity, also seem to show this behavior in the dark phase.

The "grandmother x hour" factor (shown in Fig. 4) does not show any significant interaction ($F_{1,1564} = 1.714$; $P > .01$). The birth of the current dam (PAT or MUX) in the hyperdopaminergic uterus of the maternal grandmother (a condition proper to both PIX and MYX) is not significantly relevant to locomotor activity, neither in the middle of the day nor at the dark/light phase change (Tukey GF = 1564; K = 7; Threshold = 521.476; 610.265).

### 3.2. Results of EXP. 2 (elevated plus maze)

#### 3.2.1. Time spent by DAT-HET rats in EPM areas depends on their epigenotype

Time spent in each area is significantly dependent on the epigenotype group to which each subject belongs ($F_{16,60} = 5.714$; $P < .0001$). A classical profile is found in the WT: these subjects spent more time in the closed area than in the central one; also, they spent more time in the central area than in the open one (Tukey GF = 60; K = 12; Threshold = 146.777). Within the groups (Fig. 6), we observed that MATs and PATs, like the two control groups, spent much more time in the closed arms than in the center position; MIXs were just above the threshold, while PIXs did not. MIX and PIX have little, or no significant difference of time spent in the close versus center and open areas, thus showing more swagger; indeed, MIXs and PIXs spent significantly less time in the closed arms (and more in the open ones) than MATs and PATs. The $F_3$ (MYX) did not differ (shown in Table 2) from the WT control group for time spent in either of the three areas ($F_{2,32} = .6387$; P-Value = .909).

**Table 2**
Time (s) spent by the F3 HET rats of MYX epigenotype (offspring of KO sire and MUX dam; the latter is the progeny of MAT sire and KO dam) and the WT control group (cohort 2). As expected, MYX and WT are not significantly different (F1,32=.455; P =.6387). Cohort 1, n = 6 per group; Cohort 2, n = 9 per group. For details see Table 1.

| AREA | MYX | WT |
|---|---|---|
| OPEN | 84.4 ± 26.9 | 102.2 ± 36.6 |
| CENTER | 192.9 ± 21.7 | 228.9 ± 29.1 |
| CLOSED | 420.7 ± 29.8 | 392.1 ± 26.4 |

#### 3.2.2. MIX and PIX epigenotypes (at $F_2$) display risk-taking behaviour

We observed that both items for the risk assessment behaviors (stretched attend and head dipping, when formally compared) occur in the central area and do not differ ($F_{5,30} = .694$; $P = .6317$). For open-arm performance, which may indicate risk taking, head dipping was higher in MIX and PIX epigenotypes than in MAT and PAT subjects ($F_{5,30}$; F-Value = 1.948; P =.1157), confirming that MIX and PIX are more fearless than the other groups (Threshold = 11.149). The cautious form of risk assessment (stretched attend) manifest similarities over the various groups ($F_{5,30} = 1.123$; $P = .3695$). We thus observed (Fig. 7) how the head-dipping behavior (the most dangerous form of risk assessment) designates the $F_2$ rats as more swaggering and risk takers.

#### 3.2.3. The distractibility behaviour, measured by rearing duration, is greater for MAT than for MYX and is absent in PAT and PIX epigenotypes

Relative to the distractibility item, namely closed-arm rearing, there is a statically significant effect ($F_{5,30} = 3.888$; $P = .0078$) of the epigenotype group (Tukey GF = 30; Threshold = 29.271): specifically, an increased rearing was typical of MAT vs. MIX. In the $F_3$, the comparison in closed arms shows a greater propensity to rearing behavior for MYXs compared to the control group, which however did not reach significance ($F_{1,16} = .380$; $P = .5465$; Tukey GF = 16; Threshold = 18.092). To standardize for time spent within areas, we have been comparing the percentage indes, namely duration of rearing in closed arms versus the total elapsed time in closed arms (shown in Table 3): we found that there were no statistically significant differences from controls, including MYX who just missed the threshold ($F_{1,16} = .814$; $P = .3802$), with the exception of MAT rats reaching a percent of 11.6 ± 2.6 (Tukey Threshold = 6.480 Vs. MIX rats). Although failing to reach significance, the $F_3$ (MYX) tends to present this trait, and so did the $F_1$ (MAT). Noteworthy, the $F_2$ of equal lineage (MIX) is clearly less distracted and poorly ADHD-like. Although MATs are $F_1$ heterozygotes like PAT and MIX are $F_2$ heterozygotes like PIX, we observed that the lineage stemming from "P" asset, namely in descent from a KO (grand) dam, does not manifest these differences in attentiveness, as if they were protected from displaying ADHD phenotype.

**Table 3**
Average percent proportion (%) of the duration for "Rearing" behavior out of the total sum of time elapsed in the closed arms, by epigenotypes of Cohort 1 (F1 and F2 vs. control groups, n=6) and by epigenotypes of cohort 2 (F3 vs. control group, n=9). * Only MAT rats have significant higher values ($p<.05$). For details see Table 1.

| Cohort 1 | REARING / TOTAL CLOSED (%) |
|---|---|
| MAT | 11.6 ± 2.6 * |
| MIX | 3.4 ± 1.7 |
| PAT | 7.1 ± 2.5 |
| PIX | 7.4 ± 3.8 |
| WT | 6.9 ± 2.1 |
| W-WT | 7.4 ± 3.0 |
| **Cohort 2** | **REARING / TOTAL CLOSED (%)** |
| MYX | 9.7 ± 2.1 |
| WT | 6.9 ± 2.1 |

## 4. Discussion

DAT-HET rats exhibit a range of behaviors that broadly resemble WT rats. The major notable difference is that HETs have a tendency toward asocial behavior, [69] whereas WTs exhibit a notable prosocial activity [71]. Conversely, the DAT-KO rat provides a phenotype with hyperactivity and stereotyped behavior. It was found that the offspring with KO father could exhibit behaviors associated mainly with OCD [68,70]. Instead, by examining the offspring of a KO mother, our present goal was to explore the potential ADHD - like profile in terms of hyperactivity (circadian rhythms) and inattention (EPM, duration of rearing). Present research characterized a model for ADHD in relation to the transgenerational impact, of elevated intra-uterine dopamine, in the $F_1$, $F_2$ and $F_3$ offspring of DAT-KO mothers.

The behavioral variability of rats and specifically of the attentive phenotype has been studied in the literature [38–40]. Repertoire of behaviors such as methylphenidate reaction, inattention, hyperactivity and impulsivity have been hypothesized [41] to be focal points in ADHD. In the study by Viggiano et al. [42] a line of rats (NHE) was used to study the symptoms belonging to ADHD, including rearing as an association to the attention being drawn from external stimuli. It has also been found that [43] the high concentration of DAT [44] determines a high behavioral functionality. It has consistently been observed that the attention deficit of NHE rats [45] is associated with the hyper functioning mesocorticolimbic system, like it happens in human ADHD.

In order to investigate the behavioral phenotype of HET rats for DAT, which can be considered more sensitive to environmental impact (because of epigenetic modulation of one only wild allele), the EPM tests were carried out. This was chosen on the basis of literature and the pharmacological validation [46] and allowed us to study the motor, socio-emotional, attentional, and impulsive variables associated with ADHD / OCD. In the present study, we detected the transgenerational epigenetic changes in DAT heterozygous rats of three generations, possessing one wild-type and one mutated allele, but inherited according to specular lineages [22]. They developed neurochemical and behavioral dysfunctions not always consistent with haplo-insufficiency in the DAT gene [47,48]. In the present study, we observed intercurrent differences among offspring of specific grandparents. Two lineages of $F_1$ derive from a swap of a WT and a KO parent, and offspring is termed MAT or PAT depending on the wildtype parent [8]. The $F_2$ progeny, i.e., epigenotypes termed MIXs and PIXs, derive from a KO sire and these MAT and PAT used as dam. It is evident, thus, that they have swapped grand-parents.

The inversion of parents, used for PAT to be compared to MAT, was further realized also at $F_2$, for purpose of comparison: once adults, male MAT subjects ($F_1$ rats possessing the healthy maternal allele) were also mated themselves with a female KO, giving birth to $F_2$ subjects called MUX. Both MIX and PIX / MUX have a KO and a WT maternal grandparent: MIX and MUX have a KO grandfather and a WT grandmother but the opposite is true for PIX. MUX and MYX share lineage features with PAT and PIX, but additionally MUX (like PAT) develop in a hyperdopaminergic uterus (i.e., their own dam is KO). Like PAT rats, MUXs have the wild-type allele from the sire (asset "P"). The clearly depressed MUX phenotype was already observed [29] while MIX rats only displayed asocial tendencies [49]. The $F_3$ (MYX progeny), here studeid for the first time, results from mating a MUX female and a KO male.

### 4.1. Circadian cycles in DAT-HET epigenotypes

Transgenerational sequelae were studied on circadian cycles. Due to the reversed light/dark cycle, lights in the enclosure are turned on at 8 p.m. (lit phase, rest period when locomotor activity should be low) and turned off at 8 a.m. (dark phase, when wake period locomotor activity should be high) [50]. Our hypothesis was that the mutation of the DAT gene in heterozygosity could result in transgenerational changes across these three generations, as a function of the initial ancestor: original hyperdopaminergia residing in the epididymis or the uterus of the KO grandparent implied that epigenetic marks could be subsequently transmitted via the sperm on the egg, plus the uterine hyperdopaminergia of the (grand) dam.

In the $F_1$ (MAT and PAT) there is high locomotor activity. We found specifically that both $F_1$ HET epigenotypes (less for MAT and more for PAT) possessed elevated locomotor activity (compared with the WT control group) in the last dark hours before the light change (2–6 p.m.). It follows how PAT activity is higher than MAT activity, consistent with the early reports of preferential allele expression [21]. On the $F_1$, the mutated allele from the sire matures in the hyperdopaminergic epididymis before the zygote of a MAT is conceived. On the contrary, in the PAT epigenotype, the egg cell (as well as embryo and fetus) mature within the hyperdopaminergic uterus and these putative marks may explain allele imprinting [22]. The activity cycles, on the other hand, were observed to be reduced in the $F_2$. First of all, when collapsing PIX+MYX vs. MIX+WT, no difference emerged, denoting that when the current dam had being gestated within a hyperdopaminergic uterus of a KO grand-dam, this entails no sequelae to the current offspring. When collapsing MIX+MYX vs. PIX+WT, the former pool of epigenotypes has a reduced activity. While PIX and WT have had normal postnatal cares, as PAT maternal behavior is like that of a WT [22], the profile of cares expressed by MAT and MUX dams is altered [29] and this means that circadian data may be explained by a fully post-natal factor. Its impact is however relatively small, confirming that inherited genetic asset is stronger than maternal impact [21].

Combining these data it turns out that MIX < MYX and PIX < WT. PIX and MIX are similarly showing a general downward rearrangement in activity, just concentrated in the dark phase: in fact, they show a peak of locomotor activity just before the phase - change and a subsequent lack of rebound in the first hours following light onset (which was typical of MAT and PAT, though). The common aspect of PIX and MIX is that they are at $F_2$, whereas MYX are at $F_3$ and develop an overall behavior similar to WT. Furthermore, the fact that circadian locomotor cycles relative to the $F_3$ (MYX) do not differ from WT controls and hence exhibited normalized activity compared with the $F_2$ (PIX and MIX), is consistent with reported data [30] in which the opposite effects were present in the first two generations, and disappeared in the third. A lower-activity trend was found in MIX relative to PIX, who differed for ancestors (grandfather was KO for MIX but grandmother was KO for PIX). Such modulation is probably revealing the fact that activity cycles are tendentially depressed by $F_2$ (vulnerable phenotype), yet more in MIX (WT maternal grandmother) compared to PIX (KO maternal grandmother). On the other hand, MYXs are peculiar: despite these are rats whose grandmother was planned to be KO, and additionally have anomalous postnatal cares by a MUX dam, they possess a behavior similar to WTs in agreement with the study of Pepe [30]. As such, they are driven primarily from belonging to the $F_3$, with little or no effect recited by neither postnatal cares nor allele imprinting in the

grand-mother's egg.

### 4.2. Inattention and risk-taking in DAT-HET epigenotypes

In the second experiment, together with the two control groups (W-WT and WT), we subjected HET rats at the $F_1$ (MAT and PAT), $F_2$ (MIX and PIX), and $F_3$ (MYX) to the Elevated Plus Maze (EPM). We compared the time spent by these rats in the three areas (open, middle, and closed arms) of the EPM. It was observed that the $F_2$ rats possessed a greater propensity to stay in the open arms (and less in the closed ones), therefore manifesting proclivity to risky behavior. Analyzing the risk-assessment profile, no differences emerged for stretched attend (cautious behavior). As for head dipping (a potentially more dangerous behavior), we found that both MIX and PIX, at the $F_2$, were more swaggering than all the other epigenotypes. Previous data showed that MAT rats were more anxious and fearful, although on a different task [47]: therefore, the $F_2$ rats display an apparently opposite profile than the first-generation one, while the third generation returns to control levels (present $F_3$ MYX in exp. 2). Therefore, a robust profile emerged consistently out of activity cycles and EPM data. However, one item displayed a different profile: this happened for the duration of "Rearing". Such a behavior is associated [43,51] with distractibility, a key symptom of attention deficit \ hyperactivity disorder (ADHD).

We observed, specifically for the rearing behavior in the closed arm of the EPM, a propensity for asset "M" to show vulnerability to attentional anomalies: the $F_1$ (MAT) rats have high level of this ADHD-like symptom, whereas the $F_2$ (MIX) turns out to be distracted to an extremely small extent (11.6% vs. 3.4%). Distractibility is apparently modulated when the diseased allele is marking the healthy one, but only if the first is coming via the sperm of a KO male ancestor and not when coming via the egg of a KO female ancestor. After swapping the ancestors, indeed, progeny is equivalent to controls both at $F_1$ and $F_2$, (PAT and PIX), manifesting no variation at all in rearing (7.1% vs. 7.4% of total time spent in closed areas). An intermediate profile is instead present in the MYX rats: they present a slightly but not significantly higher value than the WT (9.7% vs. 6.9%; shown in Table 2). In agreement with a paternal role, this may originate in having had a MAT grandfather (asset "M") and thus trans-gerationally inheriting the ADHD-like phenotype.

Since rats and mice exhibit significant neurobehavioral similarities to humans, they are frequently employed as animal models to investigate ADHD [52–57]. Additionally, DAT KO mice show hyperactivity and learning problems that are comparable to those observed in human ADHD [58]. A study on the link between ADHD symptoms and the rat animal model was carried out by Kamimura et al. [59]: using a variety of behavioral tests, including the recording of nocturnal and diurnal activity, they established that impulsivity and hyperactivity in rats can be linked to symptoms that exist in ADHD among developing humans.

### 4.3. Translational value and limitations

Our study investigated transgenerational changes across $F_1$, $F_2$ and $F_3$ to verify what already found in the previous study [30]. $F_1$ (MAT and PAT) and $F_2$ (MIX and PIX) DAT-HET epigenotypes were subjected to the EPM test, therefore limiting the analysis to anxiety and/or risky behavior. In future studies, additional tests for cognitive assessment (such as the Y-maze and Novel Object Recognition) and for social-stimulus selection (such as SPT or EPT) [44] will be included. We carried out a pilot study to verify the sequelae of sperm- and/or egg-cell maturation under hyper-dopaminergia that were recently predicted [8]: it is clear that a molecular evaluation of epigenetic marks is presently missing. In our next replication study, a complete ex-vivo investigation will be carried out, including DNA methylation.

The results issuing out from our research on the animal model will be useful for the comprehension of a wide range of phenomena in humans [60]. In the latter species, DAT1 gene exists in two major forms, the "9 R" (protective) and "10 R" (conferring the vulnerability to ADHD or OCD) VNTR alleles [61]. In fact, the present preclinical work can inform similar clinical studies [61] like those in which we analyzed the epigenetic mark in children with "9/9" sire and "10/10" dam compared to children with specular parents.

In the first experiment (circadian cycles), the social interactions of adolescents rats are playful (the prepuberal male does not have yet the instinct to reproduce and the female has not had first menstrual cycle yet). For EPM, only males were used as experimental subjects, since females had reached pubertal status and oestrus would greatly alter daily locomotion. A clear limitation is that we only studied prepuberal or male experimental subjects, and we are hence not able to divide the analysis by including the female gender. Another limitation is that our rat study investigated possible transgerational epigenetic changes in the DAT-HET population over three generations, only in some out of the possible genotypes [8]: specifically, by choosing to explore the subsequent offsprings with KO sires, we introduced a limitation of completedness in the choice of experimental subjects. A subsequent study could exploit a greater number of possibile crossings (namely, starting from PAT and MAT then obtaining subsequent offsprings with WT sires). This would allow to further increase the analysis of DAT-HET behavioral variability. An additional constraint of the present paper is its solely behavioral and preliminary nature, as it lacks measurements at the molecular level.

## 5. Conclusion

In the rat model, a potential correlation may exist between heightened dopaminergic activity in the uterus and fetal distress, a distinctive occurrence in certain pregnant women. Elevated DA levels are associated with increased uterine contractile activity in females [62,63]. This might determine lower fetal oxygenation between contractions [64]. Such dysfunction may culminate in hypoxic-ischemic conditions (HIE) in the newborn [65]. It is noteworthy that, when pregnancy occurred in DAT knockout (KO) females, some discernible protective consequences were seemingly found on the immediate offspring and their subsequent generation. As a whole, the $F_1$ exhibits hyperactivity, while the $F_2$ displays hypoactivity and an inclination towards risk-taking behavior. Distractibility may have its origins from the allele coming by a male KO progenitor, while pregnancy in the KO uterus seemingly had no sequelae.

In conclusion, phenotypes associated with the dopamine transporter (DAT) may not arise solely from a specific genotype but rather emerge also as a consequence of intricate inheritance patterns: alleles may be epigenetically edited while flowing within the pedigree, and the observed phenotype is particularly dependent on the modulating involvement of (male) grandparents [66–68].

## Statement of ethics

The Animal Welfare Survey Board (OBA ISS), on behalf of the Italian Ministry of Health, approved the experimental procedures (formal license - 1008/2020-PR issued to WA, veterinary surveillance by G. Panzini). The experimental procedure stook place under strict compliance with the European regulations (directive of the European Community Council 2010/63/EEC) and with the Italian law guidelines. Furthermore, according to 3Rs principles, we minimized the number of subjects and the suffering of the animals involved in the experiment.

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## CRediT authorship contribution statement

**Walter Adriani:** Supervision, Methodology, Conceptualization. **Antonella Gigantesco:** Writing – review & editing, Supervision. **Giuseppe Curcio:** Writing – review & editing, Supervision. **Fabiana Festucci:** Formal analysis, Data curation. **Concetto Puzzo:** Writing – original draft, Investigation, Formal analysis, Data curation.

## Author contributions

**Concetto Puzzo** performed the experiments, analyzed the data and wrote original draft preparation. **Fabiana Festucci** analyzed the data. **Antonella Gigantesco** and **Giuseppe Curcio** commented and supervised the writing of a final version. **Walter Adriani** performed conceptualization of the pedigree approach.

## Declaration of Competing Interest

The authors have no conflicts of interest to declare.

## Data availability

All data generated or analysed during this study are included in this article. Individual data are not publicly available but can be shared with interested researchers upon reasonable request. Further inquiries can be directed to the corresponding author.

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