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Dysbindin-1 modifies signaling and cellular localization of recombinant, human D_3 and D_2 receptors

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Non-standard abbreviations

AC, adenylyl cyclase; ARF6, ADP-ribosylation factor 6; BLOC-1, biogenesis of lysosome-related organelles complex 1; CHO, Chinese Hamster Ovary; CREB, cAMP response element-binding protein; D_{2L} receptor, D_{2long} receptor; D_{2S} receptor, D_{2short} receptor; DA, Dopamine; DTNBP1, dystrobrevin binding protein 1; ERK1/2, Extracellular signal-regulated kinases1/2; FCX, Frontal Cortex; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase ; GASP-1, GPCR-associated sorting protein1; GSK-3 β, Glycogen synthase kinase 3 beta; h, human; HEK293, Human Embryonic Kidney 293; MAPK, Mitogen-activated protein kinases; MβCD, Methylβcyclodextrine; p, phospho; PBS, Phosphate buffered solution; Ser, Serine; SPA, Scintillation proximity assay; TBST, Tris buffered saline with Tween; Thr, Threonine

Abstract

Dystrobrevin binding protein-1 (dysbindin-1), a candidate gene for schizophrenia, modulates cognition, synaptic plasticity and frontocortical circuitry and interacts with glutamatergic and dopaminergic transmission. Loss of dysbindin-1 modifies cellular trafficking of dopamine D_2 receptors to increase cell surface expression, but its influence upon signaling has never been characterized. Further, the effects of dysbindin-1 upon closely-related D_3 receptors remain unexplored. Hence, we examined the impact of dysbindin-1 (isoform A) co-expression on the localization and coupling of human D_{2L} and D_3 receptors stably expressed in CHO or SH-SY5Y cells lacking endogenous dysbindin-1. Dysbindin-1 co-transfection decreased cell surface expression of both D_3 and D_{2L} receptors. Further, while their affinity for DA was unchanged, dysbindin-1 reduced the magnitude and potency of DA-induced adenylate cylase recruitment/cAMP production. Dysbindin-1 also blunted the amplitude of DA-induced phosphorylation of ERK1/2 and Akt at both D_{2L} and D_3 receptors without, in contrast to cAMP, affecting the potency of DA. Interference with calveolin/clathrin-mediated processes of internalization prevented the modification by dysbindin-1 of ERK1/2 and adenylyl cyclase stimulation at D_{2L} and D_3 receptors. Finally, underpinning the specificity of the influence of dysbindin-1 on D_{2L} and D_3 receptors, dysbindin-1 did not modify recruitment of adenylyl cyclase by D_1 receptors. These observations demonstrate that dysbindin-1 influences cell surface expression of D_3 in addition to D_{2L} receptors, and that it modulates activation

of their signaling pathways. Accordingly, both a deficiency and an excess of dysbindin-1 may be disruptive for dopaminergic transmission, supporting its link to schizophrenia and other CNS disorders.

Introduction

Dysbindin-1, a widely-expressed protein encoded by the dystrobrevin binding protein 1 (DTNBP1) gene located on chromosome 6p22.3 (Benson *et al.* 2001, Talbot 2009), can generate up to 16 different species of mRNA following transcription and processing. Amongst these potential variants, the three main isoforms are full-length dysbindin-1A and two truncated versions, dysbindin-1B and dysbindin-1C. Several allelic variants in the DTNBP1 gene appear to be more common in schizophrenia (Straub *et al.* 2002, Riley *et al.* 2009, Voisey *et al.* 2010, Ayalew *et al.* 2012) and recent findings suggest a specific link with corticolimbic and cerebellar connectivity (Nickl-Jockschat *et al.* 2012), fronto-hippocampal gray matter volume (Trost *et al.* 2013), cognitive dysfunction (Varela-Gomez *et al.* 2015) and hallucinations (Cheah *et al.* 2015). Interestingly, in several though not all studies (Fung *et al.* 2011) of schizophrenic patients, reduced levels of dysbindin-1 and DTNBP1 gene expression were found in an isoform-dependent fashion in the hippocampus (Talbot *et al.* 2004), dorsolateral prefrontal cortex (Tang *et al.* 2009) and auditory association cortices (Talbot *et al.* 2011). Further, dysbindin-1 is implicated in the pathogenesis of diverse CNS disorders by virtue of its broad-based modulation of synaptic plasticity, cognition and neurotransmission (Papaleo *et al.* 2012, Zhang *et al.* 2012, Glen *et al.* 2014, Moran *et al.* 2014, Varela-Gomez et al. 2015).

Dysbindin-1 associates into complexes with multiple binding partners (Talbot 2009, Marley & von Zastrow 2010, Larimore *et al.* 2014, Papaleo et al. 2012, Lee *et al.* 2015) and regulates a large number of cellular processes including nuclear transcription (Fu *et al.* 2015), the activity of Calmodulin-dependent Kinase (Papaleo et al. 2012), dendritic spine dynamics (Jia *et al.* 2014), synaptic vesicle transport and release (Larimore et al. 2014), and receptor trafficking (Talbot 2009, Ghiani & Dell'Angelica 2011, Saggu *et al.* 2013). In addition, dysbindin-1 modulates glutamatergic transmission (Shao *et al.* 2011), including mGluR-ERK coupling (Bhardwaj *et al.* 2015), AMPA receptor-mediated currents (Orozco *et al.* 2014) and both the localization and activity of NMDA receptor subunits in hippocampus (Tang et al. 2009, Glen et al. 2014). Dysbindin-1 also affects the activity of inhibitory Parvalbumin-positive GABAergic interneurons that form oscillating networks

with corticolimbic pyramidal glutamatergic neurones (Carlson *et al.* 2011). Hence, disruption of dysbindin-1 in schizophrenia may participate in the asynchrony of pyramidal cell-GABAergic interneuron circuits in corticolimbic structures and the corresponding cognitive deficits, abnormal behaviors and perturbed mood (Lewis *et al.* 2012, Millan *et al.* 2012).

Not surprisingly, several studies have examined the interaction of dysbindin-1 with dopaminergic transmission (Papaleo et al. 2012). Indeed, dysbindin-1 regulates the trafficking of dopaminergic receptors, and knockdown of dysbindin-1 in HEK-293 cells decreased the cycling of internalised D_2 receptors to lysosomes, resulting in enhanced cell surface expression (Marley & von Zastrow 2010). In this light, it is interesting that dysbindin-1 co-immunoprecipitates with GASP-1, a protein involved in lysosomal receptor trafficking (Marley & von Zastrow 2010). Additionally, Ji *et al.* showed an increase in D_2 receptor cell surface expression - without a concomitant decrease of endocytosis - in cultures of cortical neurons from dysbindin-1 mutant mice compared to wild-type mice (Ji *et al.* 2009). This corroborates data from Iizuka and colleagues who found increased D_2 receptor density in neuroblastoma cells after treatment with siRNA against dysbindin-1 (Iizuka *et al.* 2007). Moreover, Papaleo and co-workers demonstrated alterations in the response of pyramidal neurons of the medial prefrontal cortex layers II/III to D_2 receptor activation in dysbindin-1 knockout mice. Further, sensitivity to D_2 agonist- and antagonist-induced behavioural effects were enhanced and reduced, respectively, consistent with a higher membrane density (Papaleo et al. 2012). Finally, dysbindin-1-deficient sandy mice display learning and memory deficits, hyperactivity and a decreased ability to habituate to a novel "environment", effects *potentially* linked to altered dopaminergic transmission though this remains to be proven (Cox *et al.* 2009). Underpinning the complex role of dysbindin-1, it shows an epistasis interaction with Catechol-O-Methyl transferase in that a mutation of dysbindin-1 *or* COMT alone was favourable for cognition whereas mutual disruption was deleterious (Papaleo *et al.* 2014).

Though the above observations support the notion that disruption of dysbindin-1 impacts dopaminergic transmission, an action potentially related to aberrant mood, many questions remain. *First*, the influence of dysbindin-1 upon *signaling* at D_2 receptors has not yet been characterized. *Second*, the potential modulation by dysbindin-1 of closely-related D_3 receptors has yet to be documented, so the relative contribution of D_2 *vs* D_3 sites to *in vivo* actions of dysbindin-1 *in vivo* remains uncertain. For example, evoked DA release in prefrontal cortex is diminished in dysbindin-1 knockout mice, yet how this relates to a putative interaction with D_2 *vs* D_3 autoreceptors remains unclear (Nagai *et al.* 2010). Further, like D_2 receptors, D_3 receptors are implicated in schizophrenia (Joyce & Millan 2005) yet they differentially control mood and cognition (Gross & Drescher 2012,

Millan et al. 2012, Nakajima *et al.* 2013, Watson *et al.* 2012). *Third*, all studies to date have evaluated the effects of dysbindin-1 *depletion* on dopaminergic transmission (Iizuka et al. 2007, Ji et al. 2009, Marley & von Zastrow 2010), and the effects of dysbindin-1 itself (increased expression) has *not* as yet been evaluated.

Fourth, the latter question is of increasing interest in view of the issue of gene dosage. Both deficient *and* excess activity of core proteins disrupts function, especially in developmental disorders (Millan 2013) and recent evidence suggests that gene dosage may also be important for dysbindin-1 (Larimore et al. 2014, Mullin *et al.* 2015). Moreover, transfection of dysbindin-1 into pyramidal cells disrupted signaling at NMDA receptors, which are hypoactive in schizophrenia (Marek *et al.* 2010, Jeans *et al.* 2011, Moghaddam & Javitt 2012, Snyder & Gao 2013) while dysbindin-1 overexpression in mice led to a hyper-responsiveness to psychostimulants and decreased activity of genes like Arc that are substrates of synaptic plasticity (Shintani *et al.* 2014). Finally, increased levels of dysbindin-1 have been linked to epilepsy (Zhang et al. 2012).

In light of the above, the present studies examined the influence of *increasing* dysbindin-1 expression upon the cellular localization of recombinant human D_2 *and* D_3 receptors co-transfected in CHO and SH-SY5Y cells. In addition, since D_3 and D_2 receptors couple (in a similar but not identical manner) to adenylyl cyclase, Akt/GSK-3β and ERK1/2 pathways (Missale *et al.* 1998, Cussac *et al.* 1999, Newman-Tancredi *et al.* 1999, Muly 2002, Beom *et al.* 2004, Neve *et al.* 2004, Beaulieu *et al.* 2007, Lan *et al.* 2009, Cho *et al.* 2010, Mannoury la Cour *et al.* 2011), we examined the impact of dysbindin-1 upon these multiple signaling pathways under the control of D_3 and D_{2L} receptors.

Materials and Methods

Antibodies and Chemicals

All antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA) if not mentioned otherwise. Mouse monoclonal and rabbit polyclonal antibodies recognise GAPDH (#2118) and dysbindin (#4611) respectively. The phosphorylation of Akt and GSK-3β was revealed using rabbit monoclonal antibodies raised against the phosphorylated forms of Akt (at Ser473 and Thr308 residues, (#4060) and (#4065), respectively) and GSK-3β (at Ser9) (#9322). Activated MAPK was detected with a mouse monoclonal antibody recognizing phosphorylated $pp42^{mapk}$ (ERK2) and $pp44^{mapk}$ (ERK1) forms (#4370) as described by Cussac *et al.* (1999). Further, secondary, horseradish peroxidase-linked anti-mouse and anti-rabbit IgG were used (#7076 and #7074, respectively).

Adenylyl Cyclase experiments were performed using an AlphaScreen® cAMP assay kit from Perkin Elmer. Plasmid cDNA extractions were undertaken using a Qiagen kit (#12143) according to the manufacturer's instructions. Dopamine chlorhydrate, methyl-β-cyclodextrine and forskolin were purchased from Sigma (Saint Quentin Fallavier, France).

Cell culture and transfection

If not otherwise specified, experiments were performed using CHO cells which are well-known not to express endogenous D_2 or D_3 receptors, and which likewise do not express dysbindin (see Fig. S1A and S1B) (Kanterman et al., 1991; Cussac et al, 1999; Newman-Tancredi et al, 1999). CHO cells stably expressing human D_{2L} or D_3 receptors (4.6 and 12 pmol/mg protein, respectively) were grown in HAM/F12 or DMEM as described by Cussac et al. (1999). CHO-D_{2L} and CHO-D_3 cell lines were transfected with either dysbindin-1A cDNA ("dysbindin-1") in a pIREShygro plasmid or an empty pIREShygro plasmid ("control") using a Polyplus jetPEI™ kit (Cat. No 101-40) according to the manufacturer's instructions. Human SH-SY5Y neuroblastoma cells were grown in RPMI medium supplemented with 2 mM L-glutamine, penicillin (20 units/ml), streptomycin (20 µg/ml), and 10% (vol/vol) heat-inactivated fetal bovine serum. Cells seeded on coverslips were transiently transfected with dysbindin-1A cDNA and/or hemagglutinin (HA)-tagged dopamine D_3 receptor cDNA, using FuGENE® 6 Transfection Reagent according to the manufacturer's instructions (Promega). Transfection was performed in serum-free medium. After 48h, cells were processed for immunocytochemistry.

CHO-D_{2L} cells were purchased from Euroscreen. CHO-D_3 cells were generated at the Institut de Recherches Servier. SH-SY5Y cells were obtained from the American Type Culture Collection.

Immunocytochemistry for dysbindin-1 protein and HA-tagged receptors.

Transfected SH-SY5Y cells were fixed with 4 % paraformaldehyde, rinsed three times in 0.1M phosphate-buffered saline (PBS 1X), then placed in PBS 1X solution with 0.75% horse serum + 0.03% Triton for 20–30 min at room temperature to block non-specific sites. Then cells were incubated with dysbindin-1 primary mouse monoclonal antibody (Santa Cruz Biotechnology, #sc-390626) and HA-probe primary rabbit polyclonal antibody (Santa Cruz Biotechnology, #sc-805), overnight at 4°C. The next day, coverslips were rinsed three times in PBS 1X and incubated with secondary antibodies (Alexa Fluor 594 Goat anti-mouse and

Alexa Fluor 488 Goat anti-rabbit, Invitrogen-Life Technologies, #A-21044 and A-11008, respectively) at 37°C for 2 h, followed by three washes with PBS 1X. After washing, coverslips were mounted using glycerol gelatin. Images were acquired using D-eclipse confocal fluorescence microscope (Nikon).

Kinase Phosphorylation

For determination of ERK1/2, Akt and GSK-3β phosphorylation, cells were grown in 6 well plates until 90% confluence. After 24h, cells were transferred into a 12-well dish and starved overnight in a serum-free medium after a washing step using the same medium. Kinase phosphorylation levels were determined after 5 minutes of incubation at 37°C with increasing concentrations of DA diluted in serum-free medium (Mannoury la Cour et al. 2011). For experiments using Methylβcyclodextrine (MβCD), a cholesterol-depleting agent inhibiting lipid raft and caveolae/clathrin mediated receptor endocytosis (Christian *et al.* 1997), cells were pre-treated with MβCD (at 10 mM) for 30 min at 37°C before stimulation by DA at a concentration inducing maximal stimulation (10 µM). At the end of period, 250 µl of Laemmli sample buffer containing 200 mM dithiothreitol (DTT) was added. Whole-cell lysates were then boiled for 5 minutes at 95°C, sonicated and stored at -80°C.

Western immunoblotting

Western immunoblotting was performed as described, using 20 µg protein/sample (Mannoury la Cour et al. 2011). Optical densities of Western immunoblots were analyzed using a MCID Basic program (Micro Computer Imaging Device) and band intensity determined by excluding background noise. Values reported in the graphs generated using Prism™ software were expressed as a percentage of maximal effect induced by DA (10 µM) and obtained by calculating the ratio between optical densities measured for each phospho-kinase and those of GAPDH, used as a loading control, of the corresponding well.

Intact cell [^{3}H]-Sulpiride and membrane [^{3}H]-Methylspiperone binding assays

For determination of receptor internalization, cells were transferred into a poly-D-lysine coated 24 well plate and starved overnight prior to the experiment. Cells were then treated with either 0.2 mM sodium metabisulfite (control) or with sodium metabisulfite (Ref. 7681-57-4, Sigma) plus 10 µM dopamine in HAM F-12 (GIBCO Ref. 21765) for CHO-D_{2L} cells or in DMEM (GIBCO Ref. 41965) for CHO-D_3 cells for 1h at 37°C. Stimulation was stopped by quickly cooling cells on ice and washing 3 times with ice-cold PBS. At low temperatures, receptor internalization is blocked (Maggio *et al.* 1995). Cells were then incubated for 3.5 h at 4°C with 0.5 ml of [^{3}H]-Sulpiride (NET775250UC,

Perkin Elmer), a highly hydrophilic radioligand that essentially detects cell surface dopamine receptors (Barbier et al., 1997; Namkung et al., 2009; Namkung and Sibley, 2004), diluted in PBS (final concentration, 6.4 nM). Non-specific binding was defined with 5 µM (+)-butaclamol. Cells were washed 3 times with ice-cold PBS and 250 µl of 1% Triton/ 5 mM EDTA in PBS and 2 ml of scintillation mixture (Perkin Elmer Scintillation agent PS) added to each well. Radioactivity was counted using a TopCount microplate scintillation counter (Perkin Elmer LifeSciences). Protein concentrations were adjusted using bichinchoninic acid (BCA) protein assay kit and measured at 562 nm. Influence of dysbindin on binding affinity of dopamine D_{2L} and D_3 receptors was calculated with the radioligand [^{3}H]-Methylspiperone (NET856250UC, Perkin Elmer) as described previously (Scarselli et al., 2000).

Adenylyl Cyclase experiments

Cyclic AMP (cAMP) levels were evaluated using the AlphaScreen® cAMP assay (Ref. 6760625D from Perkin Elmer) based on competition between endogenous cAMP and exogenously-added biotinylated cAMP. CHO-D_1, CHO-D_{2L} and CHO-D_3 cells were seeded into 6-well dishes and transfected after 24h with pIREShygro plasmid containing human dysbindin-1A cDNA using a Polyplus jetPEI™ kit mentioned above. After 24h, cells were transferred into 24-well dishes in order to attain > 90% confluence, washed with warm 0.9% NaCl solution, then stimulated by DA (CHO-D_1) or by DA in the presence of 10 µM forskolin (CHO-D_{2L}, CHO-D_3) for 15 min at 37°C in the dark. Thereafter, cells were put on ice and washed with cold 0.9% NaCl solution to stop the reaction. Cells were lysed in a buffer containing 5mM HEPES and 0.3% Tween-20. After 1h incubation at 4°C, anti-cAMP Acceptor beads and biotinylated-cAMP/streptavidin Donor beads were added to lysates transferred into white OptiPlates™-384 (Perkin Elmer) and incubated for 2h in the dark under agitation before measurements of cAMP levels using an EnSpire™ Alpha 2390 Multilabel Reader (Perkin Elmer).

Antibody-capture/Scintillation Proximity Assays (SPA) studies of coupling to $G\alpha_{i3}$

The influence of dysbindin-1 co-expression was evaluated on coupling of D_{2L} and D_3 receptors to $G\alpha_{i3}$ proteins. DA-induced activation of $G\alpha_{i3}$ subunits coupled to D_{2L} and D_3 receptors was assessed as described (Millan *et al.* 2004). Briefly, $G\alpha_{i3}$ protein activation was determined using a [^{35}S]GTPγS binding assay coupled to immunocapture using mouse anti-$G\alpha_{i3}$ monoclonal antibody (SA-281, Biomol, San Diego, CA) in 96-well optiplates (PerkinElmer Life Sciences, Boston, MA). Detection was performed by Scintillation Proximity Assay (SPA) with beads coated with secondary

anti-mouse antibodies (GE Healthcare, Vélizy, France). Radioactivity was counted on a TopCount microplate scintillation counter (Perkin Elmer LifeSciences) and data analyzed by non-linear regression using the program Prism (GraphPad Software Inc., San Diego, CA).

Cell viability

After MβCD treatment, CHO-D_{2L} and CHO-D_3 cells were trypsinized and centrifuged at room temperature for 5 min at 100 × g. After re-suspension in DMEM, aliquots were diluted 1 to 1 with 0.5% trypan blue dissolved in 0.9% NaCl and the percentage of stained cells over the total number of cells evaluated after 3 minutes incubation at room temperature.

Statistical analysis

Cell surface expression levels were analyzed using a one-way ANOVA with Bonferroni corrections for multiple comparisons. For receptor binding experiments and cAMP accumulation assays, means ± SEMs were determined. Signaling experiments were analyzed using non-linear regression with a variable slope model to generate sigmoidal dose-response curves fitting each replicate within the two treatments (with or without dysbindin-1). An F-test of residuals was performed to test the null hypothesis that the upper asymptote is the same in both experimental conditions (regardless of lower asymptote, midpoint, and gradient), as opposed to the alternative hypothesis that the final asymptotic levels of the dysbindin-1 condition differed from those of the control condition. All other experiments were analyzed using two-way ANOVA.

Institutional approval

These studies have been approved by the Institut de Recherches Servier and by the Department of Biotechnological and Applied Clinical Sciences of the University of L'Aquila.

Results

Co-expression of dysbindin-1 reduces D_{2L} and D_3 receptor cell surface expression in CHO cells.

No detectable endogenous dysbindin was seen in CHO-D_{2L} and CHO-D_3 cells (Supplementary Fig. S1A and B). Transfection of dysbindin-1 into CHO-D_{2L} and CHO-D_3 cells elicited a decrease of both D_{2L} and D_3 receptor levels at the plasma membrane of about 39% (**Fig. 1A**) and 41% (**Fig. 1B**), respectively, compared to their respective density measured in cells non-transfected with dysbindin-

1 (control). Prolonged exposure of CHO-D_3 cells co-expressing dysbindin-1 with DA elicited a further decrease of receptor expression level to about 64% (**Fig. 1B**). The amplitude of density loss was similar to that observed in CHO-D_3 cells not expressing dysbindin-1 after stimulation by DA (10 µm for 1 h). For D_{2L} receptors, one-hour incubation with DA alone only induced a slight decrease of their expression level at the cell surface (20%) (**Fig. 1A**). However, as for D_3 sites, in the presence of dysbindin-1, prolonged exposure of CHO-D_{2L} cells to DA elicited a further reduction of cell surface density (42%) (**Fig. 1A)** compared to the DA-stimulated CHO-D_{2L} cells not expressing dysbindin-1.

***Dysbindin-1 co-localises with dopamine D_3 receptors in* SH-SY5Y neuroblastoma cells.**

In SH-SY5Y cells transfected with dysbindin-1 only, diffuse immunostaining was seen throughout the cell (Fig. 2, upper panel). In cells transfected with HA-tagged D_3 receptor alone, using an anti-HA antibody punctiform labelling was observed primarily in the plasma membrane and its vicinity with a relative paucity in the cytosolic compartment. In these D_3 receptor transfected cells, endogenous dysbindin-1 was undetectable (Fig. 2, middle panel). Co-transfection of dysbindin-1 with HA-D_3R induced an intracellular redistribution of HA-D_3R labelling out of the plasma membrane such that it largely overlapped with dysbindin-1 immunostaining (Fig. 2, lower panel, merged).

Dysbindin-1 does not alter ligand binding affinity or potency in G_{i3}-protein activation.

Saturation binding experiments revealed the presence of a single and saturable binding site in CHO-D_{2L} and CHO-D_3 cells. In CHO-D_{2L} cells, the presence of dysbindin-1 had no significant effect upon affinity of [^{3}H]-methylspiperone for D_{2L} receptors with K_D values of 0.23 ± 0.05 and 0.22 ± 0.05 nM in control and dysbindin-1 co-expressing cells, respectively (**Table 1**). Similarly, no significant change of K_D values was seen in CHO-D_3 control compared to dysbindin-1-expressing cells (K_D = 0.58 ± 0.14 and 0.47 ± 0.08, respectively) (**Table 1**). In addition, measurement of [^{35}S]-GTPγS binding at $G\alpha_{i3}$-proteins showed that co-expression of dysbindin-1 had no significant effect on potency of DA for stimulating recruitment of $G\alpha_{i3}$-proteins by D_{2L} and D_3 receptors (**Supplementary Fig. S2A and S2B, Table 1**).

Dysbindin-1 decreases the efficacy of DA upon D_{2L} and D_3 receptor-mediated inhibition of forskolin-induced cAMP accumulation.

As the presence of dysbindin-1 reduced cell surface density of D_{2L} and D_3 receptors, this decrease was expected to affect $G_{\alpha i}$-protein mediated downstream signaling. In both cell lines, dysbindin-1 diminished the efficacy of DA-mediated inhibition of forskolin-stimulated cAMP accumulation with 33% and 58% reduced efficacy in CHO-D_{2L} (**Supplementary Fig. S3A**) and CHO-D_3 (**Supplementary Fig. S3B**) cells, respectively. In addition, dysbindin-1 reduced DA potency in both cell lines. In CHO-D_{2L} cells, EC_{50} values increased from 167.3 nM in control to 855.6 nM in dysbindin-1 co-expressing cells, respectively, and from 14.4 nM in control to 38.9 nM in the dysbindin-1 co-expressing CHO-D_3 cell line **(Table 1).** In contrast to D_{2L} and D_3 receptors, dysbindin-1A did not influence D_1 receptor-mediated increases in cAMP accumulation (**Supplementary Fig. S3C**).

Dysbindin-1 reduces D_{2L} and D_3-mediated phosphorylation of Akt and GSK-3β.

The influence of dysbindin-1 on recruitment of the Akt/GSK-3β pathway was examined by measuring phosphorylation of Akt (Ser473) and GSK-3β (Ser9). As previously reported, DA induced a concentration-dependent increase of p-Akt (Ser473) and p-GSK-3β (Ser9) in both CHO-D_{2L} and CHO-D_3 cells (**Fig. 3 and 4**) (Mannoury la Cour et al. 2011). In the presence of dysbindin-1, activation of D_{2L} receptors still elicited an increase in Akt and GSK-3β phosphorylation levels but with a significant reduction in maximal effect of DA of about 56% and 29% for p-S473-Akt (**Fig. 3A**) and p-S9-GSK-3β (**Fig. 4A**) respectively, as compared to control cells (**Table 2**). In contrast, dysbindin-1 co-expression did not change the pEC_{50} values of DA at D_{2L} receptors (**Fig. 3A and 4A, Table 2**).

In CHO-D_3 cells, dysbindin-1 induced a more pronounced decrease in DA efficacy. The phosphorylation levels of Akt (Ser473) and GSK-3β were reduced by 57% and 44%, respectively (**Fig. 4B and Table 2**). The potency of DA at D_3 receptors on Akt and GSK-3β phosphorylation was slightly but not significantly decreased by co-expression of dysbindin-1 (**Fig. 3B and 4B, Table 2**).

Dysbindin-1 reduces D_{2L} and D_3- mediated phosphorylation of ERK 1/2.

As expected from previous reports, DA induced a concentration-dependent increase of ERK1/2 phosphorylation level in both cell lines (**Fig. 5A and 5B**). Recruitment of the ERK1/2 pathway by D_{2L} and D_3 receptors was also affected by co-expression of dysbindin-1 with a reduction of the

influence of DA upon pERK1/2 levels by about 48% in CHO-D_{2L} cells (**Fig. 5A, Table 2**) and 71% in CHO-D_3 cells compared to control cells (**Fig. 5B, Table 2**). However, no change in DA potency was observed in either cell line after co-expression of dysbindin-1 (**Table 2**).

The cholesterol-depleting agent MβCD attenuates the dysbindin-1 induced decrease of D_{2L} and D_3 receptor signaling.

The potential involvement of receptor internalization in the dysbindin-induced decrease in D_{2L} and D_3 receptor signaling was evaluated using the cholesterol-depleting agent MβCD (Christian et al. 1997). As MβCD can reduce cell viability (Ulloth *et al.* 2007), its toxicity for CHO-D_{2L} and CHO-D_3 cells was tested by trypan blue exclusion. After 30 minutes exposure to MβCD, the percentage of cell death in CHO-D_{2L} (4.5 ± 1.5%) and CHO-D_3 (7.6 ± 1.7%) cells was not significantly different from untreated CHO-D_{2L} (2.5 ± 1.6%) and CHO-D_3 (4.0 ± 2.4) cells. This demonstrates a lack of toxicity under these conditions.

As shown above, dysbindin-1 attenuated the inhibition of forskolin-induced cAMP production by DA (10 μM) in both CHO-D_{2L} (**Fig. 6A**) and CHO-D_3 (**Fig. 6B**) cells. Pre-incubation with MβCD led to a decrease in DA-mediated inhibition of forskolin-stimulated cAMP production in control CHO-D_{2L} and CHO-D_3 cells (43% and 38%, respectively). Nevertheless, co-expression of dysbindin-1 in the presence of MβCD did not result in any further significant decrease of DA efficacy (**Fig. 6A and 6B**).

In CHO-D_3 cells, co-expression of dysbindin-1 significantly inhibited the DA-induced increase of pERK1/2 levels. MβCD attenuated by itself the effect of DA on pERK1/2 levels (76%) but prevented a further inhibition due to co-expression of dysbindin-1 (89%) (**Fig. 7B and Table 3**). In CHO-D_{2L} cells, MβCD caused a slight reduction (32%) of the DA-mediated increase in ERK1/2 phosphorylation and reversed the effect of dysbindin-1 co-expression (35%) (**Fig. 7A and Table 3**).

Discussion

Dysbindin-1 co-expression decreases D_2 and D_3 cell surface receptor density in CHO and SH-SY5Y cells.

Using the hydrophilic radioligand, [^{3}H]-Sulpiride (Barbier *et al.* 1997)**,** and a protocol previously described for quantification of plasma membrane-localised D_{2L} receptors (Namkung & Sibley 2004, Namkung *et al.* 2009b), dysbindin-1 co-expression led to a down-regulation of both D_{2L}

and D_3 receptor membrane density in CHO cells. These findings mirror the effects of siRNA-mediated knockdown of dysbindin-1, which increased cell surface expression of D_2, but not D_1, receptors in SH-SY5Y cells and primary cultures of rat cortical neurons (Iizuka et al. 2007). Similar results were observed herein with D_3 receptors for which dysbindin-1 co-expression reduced cell surface density even more markedly than for D_{2L} receptors. In addition, dysbindin-1 and D_3 receptors co-localised in the internal compartment of neuroblastoma SH-SY5Y cells, supporting a fundamental role of dysbindin-1 in D_3 receptor trafficking.

Knock-down studies of "Muted", which, along with its binding partner dysbindin-1, is an essential component of the biogenesis of lysosome-related organelles complex 1 (BLOC-1), also increased cell surface expression of D_2 receptors (Iizuka et al. 2007), consistent with the important contribution of BLOC-1 related proteins in the trafficking of GPCRs. Reflecting a role for BLOC-1, dysbindin-1 depletion inhibited D_2 receptor proteolysis after their internalization *without* significantly affecting ligand-induced endocytosis *per se* in HEK cells and cortical neurons (Ji et al. 2009, Marley & von Zastrow 2010). These data suggest that, in addition to regulating receptor internalization of D_{2L} and D_3 receptors directly, dysbindin-1 may interfere with their recycling to the plasma membrane by facilitating their trafficking to lysosomes. However, this hypothesis requires further elucidation.

Under control conditions, the magnitude of receptor internalization differed between D_{2L} and D_3 receptors, with the less pronounced decrease of D_{2L} receptor density (20%) versus D_3 sites (>60%) following prolonged DA exposure in good agreement with previous observations (Itokawa *et al.* 1996, Kim *et al.* 2004). Mirroring the proposal of Ji *et al.* (2009), this difference might reflect the more rapid recycling of D_{2L} vs D_3 receptors to the cell surface, thereby counteracting the effect of DA (Ji et al. 2009). Interestingly, a phosphorylation-independent association and dissociation with β-arrestin-2 is important for desensitization and re-sensitization of D_{2S} receptors in HEK293 cells (Cho et al. 2010). Despite the current absence of support for a similar role of β-arrestins in *CHO* cells, the rapid recycling of D_2 receptors to the cell surface after prolonged exposure to DA may imply a similar mechanism, reminiscent of the role of β-arrestins in the regulation of D_2 receptors in rat striatal neurons (Macey *et al.* 2004, Skinbjerg *et al.* 2009). The higher magnitude of DA-induced D_3 *vs* D_2 receptor internalization suggests a relatively slow rate of D_3 recycling to the plasma membrane after agonist stimulation, or that they are rapidly degraded following endocytosis. These remain to be further characterized.

As regards the influence of dysbindin-1, no significant difference in the magnitude of DA-induced internalization was seen between D_{2L} and D_3 receptors in cells co-expressing dysbindin-1. It is possible that dysbindin-1 promotes D_{2L} receptor trafficking from early endosomes to lysosomes thereby interfering with their rapid recycling to the plasma membrane: this would explain the further decrease of D_{2L} receptor density (60%) observed in DA-treated cells after co-expression of dysbindin-1. As regards molecular mechanisms involved in the fate of internalised D_{2L} sites, their redirection to lysosomes by dysbindin-1 likely involves a direct interaction with GASP1 (GPCR-associated sorting protein1), a protein that promotes the proteolysis of many GPCRs following endocytosis (Bartlett *et al.* 2005, Marley & von Zastrow 2010) and controls the degradation of D_2 receptors in HEK cells exposed to DA (Thompson & Whistler 2011). The D_{2L} receptors remaining in endosomes would, on the other hand, be rapidly recycled to the plasma membrane with the assistance of the small GTPase, ARF6 (ADP-ribosylation factor 6) (Cho *et al.* 2013). GASP proteins may also be involved in the regulation of D_3 receptors, though this remains to be demonstrated in CHO cells (Thompson & Whistler 2011).

Thus, despite a similar and negative impact of dysbindin-1 upon cell surface density of D_{2L} and D_3 receptors, molecular mechanisms involved in their cycling may subtly differ, paralleling differences in their signalling seen in other recombinant systems (Missale et al. 1998, Cussac et al. 1999, Lan et al. 2009, Namkung *et al.* 2009a, Cho et al. 2010). In addition, as experiments here were undertaken with the long isoform (D_{2L}), we cannot exclude that further differences might be found with the short receptor, D_{2S}.

Dysbindin-1 co-expression decreases multiple pathways of D_{2L} and D_3 receptor signaling.

Inasmuch as GPCRs couple to multiple transduction mechanisms *via* G-protein-dependent and independent mechanisms, acting both at the cell surface and even following internalization (Jong *et al.* 2009, Purgert *et al.* 2014), the hitherto-unaddressed question of the influence of dysbindin-1 on D_3 and D_{2L} receptor signalling is important. As regards the canonical $G_{i/o}$ adenylyl cyclase pathway, dysbindin-1 decreased the efficacy of D_{2L} and D_3 receptor-mediated attenuation of forskolin-induced cAMP accumulation. By analogy to the selective influence of dysbindin-1 upon D_2 vs D_1 receptor cell surface expression (Iizuka et al. 2007), the reduction of DA efficacy at both D_{2L} and D_3 expressing cells was specific in that D_1 receptor-mediated enhancement of cAMP accumulation was not affected by dysbindin-1 co-expression, confirming the minor influence of dysbindin on this receptor (Iizuka et al. 2007). As cAMP elicits phosphorylation of CREB (cAMP response element-

binding protein), by attenuating the influence of D_{2L} and D_3 receptor activation on AC dysbindin-1 should indirectly promote levels of phospho-CREB in both CHO-D_{2L} and CHO-D_3 cell lines. Accordingly, the present data mirror a previous study showing that siRNA knockdown of dysbindin-1 in rat cortical neurons amplifies the influence of the D_2 agonist quinpirole upon phosphorylation of CREB (Iizuka et al. 2007).

Underpinning the above observations with AC, dysbindin-1 reduced the DA-induced phosphorylation of several other cellular signals prototypically engaged by D_{2L} and D_3 receptors: *first*, Akt (Ser473) and its molecular target, GSK-3β, and *second*, pERK1/2. Collectively, these data suggest a broad-based influence of dysbindin-1 upon D_{2L} and D_3 receptor-mediated signal transduction. Intriguingly, however, there were differences in the impact of dysbindin-1 on signalling cascades.

Dysbindin-1 differentially affects agonist potency and efficacy for coupling to adenylyl cyclase compared to ERK1/2

The present observations are consistent with classical receptor-occupancy theory (Kenakin 1982) and studies of post-synaptic dopaminergic receptors (Meller *et al.* 1987) in suggesting that DA needs to occupy a substantial proportion of D_{2L} and D_3 receptors to maximally inhibit cAMP production and phosphorylation of ERK1/2, Akt and GSK-3β (Meller et al. 1987, Missale et al. 1998, Tadori *et al.* 2009). Accordingly, the decrease of receptor density elicited by dysbindin-1 will have reduced receptor reserve and the number of "spare receptors" leading to a reduction in the efficacy of DA (Kenakin 1982).

On the other hand, the relationship of GPCR to agonist *affinity* is not so straightforward. Indeed, while dysbindin-1 did not modify the potency of DA for induction of Akt/GSK-3β and ERK1/2 signaling, it induced a rightward shift of the concentration-response curve of DA for both D_{2L} and D_3 receptor-mediated inhibition of cAMP production. This blunted potency of agonist-recruitment of AC does not reflect a genuine "diminution" of agonist affinity since no alteration of DA potency was seen at the level of G-protein activation in [^{35}S]-GTPγS binding studies. In addition, supporting the idea that a change in EC_{50} values for DA may be related to the reduction of receptor density at the cell surface: 1), similar K_D values were measured in CHO-D_{2L} and CHO-D_3 cells co-expressing dysbindin-1 compared to control cells and 2), a single and saturable binding site was detected in both CHO-D_{2L} and CHO-D_3 cells co-expressing dysbindin-1. Corroborating this notion, previous studies on β-adrenoceptors reported a positive correlation between agonist potency and receptor number at the cell surface (Bouvier *et al.* 1988, Whaley *et al.* 1994). In addition, mirroring the

present data for AC, Kenakin and Morgan (1988) showed that changes in the receptor/G-protein ratio negatively influence both agonist affinity and efficacy leading to a rightward shift of agonist concentration-response curves, together with a decrease of maximal effects in a context of a receptor depletion (Kenakin & Morgan 1989).

In contrast to cAMP formation, dysbindin-1 did *not* significantly influence the potency of DA for D_2 and D_3 receptor-mediated activation of Akt/GSK-3β and ERK1/2, which are recruited partly through *non*-G-protein-dependent mechanisms involving β-arrestins, receptor-internalization and/or recruitment of Src kinases (Oak *et al.* 2001, Nair & Sealfon 2003, Kim et al. 2004, Beom et al. 2004, Wang *et al.* 2005, Mannoury la Cour et al. 2011). This involvement of *multiple* G-protein-dependent and independent mechanisms in the stimulation ERK1/2 and Akt/GSK-3β results in marked signal amplification that counteracts the loss of receptor density, consistent with a decrease of efficacy but not of affinity (Kenakin 2006).

Blunting of DA-induced phosphorylation of ERK1/2 by dysbindin-1 may be related to altered cellular localization of D_2 and D_3 receptors.

The cholesterol-depleting agent, MβCD, is widely used as an inhibitor of receptor internalization since it disrupts lipid rafts and caveolar endocytosis, as well as preventing the formation of clathrin-coated pits (Christian et al. 1997, Rodal *et al.* 1999). An important issue in the use of MβCD is possible cytotoxic effects and alterations of cell morphology (Hinzey *et al.* 2012), but control experiment suggested that this was not the case in the present studies (see Results).

MβCD treatment decreased both D_{2L} and D_3 receptor signalling in CHO cells, corroborating studies performed in HEK cells that showed a critical role of cholesterol-rich lipid rafts for D_2 receptor signalling (Sharma *et al.* 2013). In the presence of MβCD, dysbindin-1 induced no further decrease in efficacy of DA at D_{2L} and D_3 receptors. This absence of an additive effect of dysbindin-1 suggests that the impact of dysbindin-1 co-expression upon D_{2L} and D_3 receptor signaling requires lipid masks, cholesterol-rich microdomains and/or clathrin- and caveolar-dependent processes disrupted by MβCD treatment (Rodal et al. 1999, Subtil *et al.* 1999, Zidovetzki & Levitan 2007).

Interestingly, β-arrestins act as adapters between phosphorylated receptors and clathrin-coated pits, yet were *not* involved in the phosphorylation of ERK1/2 by D_{2L} receptors in CHO cells (Kim et al. 2004). Indeed, the D_{2L}-mediated increase of pERK1/2 is essentially driven through receptor tyrosine kinase (RTK) transactivation not requiring β-arrestin-regulated endocytosis (see

above) (Oak et al. 2001, Kim et al. 2004). Thus, the partial inhibitory effect of MβCD upon DA-induced ERK1/2 phosphorylation was observed in CHO-D_{2L} cells suggests that an agonist-dependent translocation of D_{2L} receptors in caveolae might be involved in the recruitment of ERK1/2. This would resemble what has been described with caveolin-2 for D_1 receptor-mediated AC activation (Yu *et al.* 2004).

In contrast to D_{2L} receptors, MβCD abolished DA-elicited ERK1/2 phosphorylation in control CHO-D_3 cells indicating a requirement for β-arrestin/clathrin-dependent or caveolae-mediated endocytosis for ERK1/2 activation. However, the exact mechanism by which D_3 receptors elicit the phosphorylation of ERK1/2 in CHO cells remains to be elucidated, in particular with regards to a potential influence of compartmentalization in their signaling (Sharma et al. 2013). The suppression of D_3 receptor-mediated ERK phosphorylation by dysbindin-1 also reinforces the connection between D_3 receptor internalization and recruitment of this signal.

Finally, further studies are needed to address the relevance to dysbindin-1-mediated control of D_{2L}/D_3 receptor signalling of receptor internalization and trafficking to lysosomes through the BLOC-1 complex.

General Discussion and Conclusions

Since these studies were performed *in vitro* and we looked at the effects of *increased* dysbindin-1 expression, it is challenging to *directly* relate them to schizophrenia where dysbindin-1 is dysfunctional. Nonetheless, together with findings outlined in the introduction, they provide a new dimension to collectively strong evidence that dysbindin-1 negatively controls the operation of D_2 receptors and, as revealed herein, D_3 receptors. This is intriguing since D_3 and D_2 receptors exert an opposite negative and positive influence respectively upon cognition (Lacroix *et al.* 2003, Millan *et al.* 2007, Loiseau & Millan 2009, Micale *et al.* 2010, Gross & Drescher 2012, Millan et al. 2012, Nakajima et al. 2013, Watson et al. 2012). Cognition is impaired in schizophrenia (and animal models thereof): this impairment *could* be related to increased cell surface expression of D_3 rather than D_2 receptors in FCX, a possibility justifying experimental validation. In addition, increased D_3 receptor levels in the striatum and forebrain of schizophrenic patients (Gurevich *et al.* 1997) are consistent with reduced dysbindin-1 expression, though any causal relationship likewise remains to be proven. Finally, as mentioned in the introduction, there are CNS disorders where dysbindin-1 levels are increased, and its overexpression disrupts NMDA receptor-mediated transmission and synaptic plasticity (Zhang et al. 2012, Jeans et al. 2011, Shintani et al. 2014).

To conclude, the present data adopt an approach complementary to previous work in showing that *increasing* dysbindin-1 expression modulates cell surface density of D_{2L} receptors, and they extend previous data in demonstrating an impact upon cellular signaling. Moreover, they demonstrate that similar, though not identical, dysbindin-1 mediated actions blunt the operation of D_3 receptors. Future studies should more clearly characterize the molecular substrates underlying the influence of dysbindin-1 upon D_3 and D_{2L} receptors, and more thoroughly explore the functional repercussions, in particular for schizophrenia and other CNS disorders in which dysbindin-1 is implicated.

ARRIVE guidelines have been followed:

Yes

=> if No, skip complete sentence

=> if Yes, insert "All experiments were conducted in compliance with the ARRIVE guidelines."

Conflicts of interest: none

=> if 'none', insert "The authors have no conflict of interest to declare."

=> otherwise insert info unless it is already included

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Figure 1: Reduction of D_{2L} and D_3 receptor cell surface expression in CHO cells in the presence of dysbindin-1, and following prolonged exposure to DA.

D_{2L} (A) and D_3 (B) receptor cell surface density were evaluated in the presence of dysbindin-1 or after 1h exposure to DA (dark and dark grey bars) compared to medium (white and light grey bars). Data represent the means ± SEM of at least 6 replicates from three independent experiments. Data were analyzed using one-way ANOVA and Bonferroni's multiple comparison test. NS= not significant; ** = $p<0.01$; *** = $p<0.001$. $F = 20.36$ in CHO-hD_{2L} cells and $F = 26.25$ in CHO-hD_3 cells.

Figure 2: Dysbindin-1 and dopamine D_3 receptor co-localise in SH-SY5Y cells.

SH-SY5Y cells were transfected with cDNA encoding dysbindin-1 or HA-D_3 receptors alone, or co-transfected with dysbindin-1 plus HA-tagged D_3 receptors. HA-tagged receptors and dysbindin-1 are labeled in green and red, respectively. Merged panels indicate co-localization of HA-D_3 or receptors and dysbindin-1. The scale bar corresponds to 50 µm.

Figure 3: Decrease of DA efficacy for Akt (Ser473) phosphorylation in dysbindin-1 expressing CHO-D_{2L} and CHO-D_3 cells.

Akt (Ser473) phosphorylation was evaluated in control and dysbindin-1 co-expressing CHO-D_{2L} (A) and -D_3 (B) cells after stimulation with increasing concentrations of DA. Akt phosphorylation levels in cell lysates were quantified by immunoblotting. Western blots are representative of experiments performed at least three times. A two-way ANOVA compared effects of DA on efficacy in control vs dysbindin-1 conditions: p-S473-Akt, **p=0.002 (*F*=19.84) in CHO-D_{2L} cells (A) and p-S473-Akt *** p=0.003 (F=32.91) in CHO-D_3 cells (B).

Figure 4: Dysbindin-1-induced decrease in DA efficacy for GSK-3β phosphorylation in CHO-D_{2L} and CHO-D_3 cells.

Phosphorylation levels of GSB-3β were quantified in cell lysates by immunoblotting after incubation of CHO-D_{2L} (A) and CHO-D_3 (B) cells with increasing concentrations of DA. Western blots correspond to representative experiments where GAPDH was used as a control for well-loading. For each curve, data are means ± SEM of at least three independent experiments. A two-way ANOVA compared DA effects on efficacy in control *vs* dysbindin-1 co-expression conditions: p-S9-GSK-3β ** p=0.009 (*F*=8.21) in CHO-D_{2L} cells and *** p=0.0001 (*F*=42.97) in CHO-D_3 cells.

Figure 5: Decreased efficacy for DA-induced phosphorylation of ERK1/2 in CHO-D_{2L} and CHO-D_3 cells co-expressing dysbindin-1.

CHO-D_{2L} (A) and CHO-D_3 (B) cells co-expressing dysbindin-1 or not (control) were incubated with increasing concentrations of DA. Levels of pERK1/2 were detected by immunoblotting in cell lysates. Western blots below each curve are representative of experiments performed at least three

times where GAPDH was used as a well loading control. For each curve, data are the means ± SEM of at least three independent experiments. A two-way ANOVA compared DA efficacy in control *vs* dysbindin-1 co-expression conditions: p-Thr202/Tyr204-ERK1/2 **** p<0.0001 (F=125.9) in CHO-D_{2L} cells and **** p<0.0001 (F=109.2) in CHO-D_3 cells.

Figure 6: Influence of MβCD on the inhibitory effect of dysbindin-1 on adenylyl cyclase activity

CHO cells stably expressing D_{2L} cells (A) and D_3 (B) receptors were treated with medium only or pre-incubated with MβCD. Forskolin (10 µM) was added to stimulate adenylyl cyclase before activation of D_{2L} and D_3 receptors by DA (10^{-5} M). Control cells (pIRES) treated with forskolin were defined as 100%. Two-way ANOVA was used to compare control and dysbindin-1 co-expressing cells. (ns, not significant; *p<0.05). F = 14.64 for CHO-D_{2L} cells and F = 12.13 for CHO-D_3 cells.

Figure 7: Influence of MβCD on the inhibitory effect of dysbindin-1 on ERK1/2 phosphorylation in CHO-D_{2L} and -D_3 cells

ERK1/2 phosphorylation levels were detected by immunoblotting in CHO-D_{2L} (A) and CHO-D_3 cells (B) pre-treated with MβCD and activated by DA (10^{-5}M). The pERK1/2 levels measured in control cells after DA stimulation (defined as 100 %). Histograms represent the compilation of data generated by at least 3 independent experiments. A two-way ANOVA compared DA-treated control (empty vector) to dysbindin-1 co-expressing cells: ns = not significant; **p<0.01; ***p<0.001). F = 6.5 for CHO-D_{2L} cells and 16.67 for CHO-D_3 cells.

Tables

Table 1: Influence of dysbindin-1 on [^{3}H]-Methylspiperone binding affinity (K_D) and on the potency (pEC_{50}) and efficacy (Emax) of DA for stimulation of [^{35}S]-GTPγS binding and cAMP production in CHO cells stably expressing D_{2L} and D_3 receptors. Data represent the means ± S.E.M. of at least 3 independent determinations ([a]p = 0.001; [b]p = 0.006; [c]p = 0.001; [d]p = 0.001).

	Binding		*[^{35}S]-GTPgS Binding*				*cAMP production*			
	Control	*Dysbindin-1*	*Control*		*Dysbindin-1*		*Control*		*Dysbindin-1*	
	K_D (nM)		pEC_{50}	Emax	pEC_{50}	Emax	pEC_{50}	Emax	pEC_{50}	Emax
hD_{2L}	0.23 ± 0.05	0.22 ± 0.05	6.22 ± 0.14	59 ± 7	6.20 ± 0.09	71 ± 5	6.78 ± 0.17	93 ± 7	6.07 ± 0.06[a]	62 ± 7[b]
hD_3	0.58 ± 0.14	0.47 ± 0.08	8.28 ± 0.16	42 ± 3	8.14 ± 0.11	46 ± 3	7.84 ± 0.06	96 ± 3	7.41 ± 0.04[c]	41 ± 5[d]
hD_1	ND	ND	ND	ND	ND	ND	8.18 ± 0.03	96 ± 5	8.20 ± 0.01	98 ± 6

Table 2: Potency and efficacy of DA for induction of Akt, GSK-3β and ERK1/2 phosphorylation in dysbindin-1-transfected *vs* control CHO-D_{2L} and CHO-D_3 cells. Data represent the mean ± S.E.M. of at least 3 independent determinations (*=p<0.05; ** = p<0.01; *** = p<0.001 ****=p<0.001).

		pERK1/2		pAkt(Ser437)		pGSKβ (Ser9)	
		pEC50	Emax(%)	pEC50	Emax(%)	pEC50	Emax(%)
hD_{2L}	Control	7.84 ± 0.05	96 ± 3	7.74 ± 0.12	98 ± 5	7.83 ± 0.15	87 ± 4
	Dysbindin-1	8.21 ± 0.13	48 ± 3****	8.00 ± 0.12	42 ± 3**	8.25 ± 0.23	58 ± 5**
hD_3	Control	7.12 ± 0.08	93 ± 4	7.37 ± 0.11	98 ± 6	7.60 ± 0.16	97 ± 4
	Dysbindin-1	7.52 ± 0.25	22 ± 4****	7.72 ± 0.18	41 ± 4***	7.30 ± 0.20	53 ± 3***

Table 3: Influence of MβCD on DA-induced ERK1/2 phosphorylation in dysbindin-1-transfected *vs* control CHO-D_{2L} and CHO-D_3. Values represent efficacies measured under basal conditions (no treatment) or after DA stimulation. They are expressed as a percentage of the maximal effect of DA (10 µM) under control conditions (100%). Data are the means ± S.E.M. of at least 3 independent determinations. For statistical analysis refer to Figure 7.

		pERK1/2			
		control	*Dysbindin-1*	*MβCD*	*Dysbindin-1 + MβCD*
CHO-hD_{2L}	No treatment	2 ± 1	3 ± 2	3 ± 2	4 ± 1
	DA (10 µM)	100 ± 2	40 ± 5	68 ± 3	65 ± 14
CHO-hD_3	No treatment	15 ± 8	9 ± 7	6 ± 3	68 ± 3
	DA (10 µM)	91 ± 5	26 ± 9	24 ± 4	11 ±9

Figure 1 Schmieg et al.

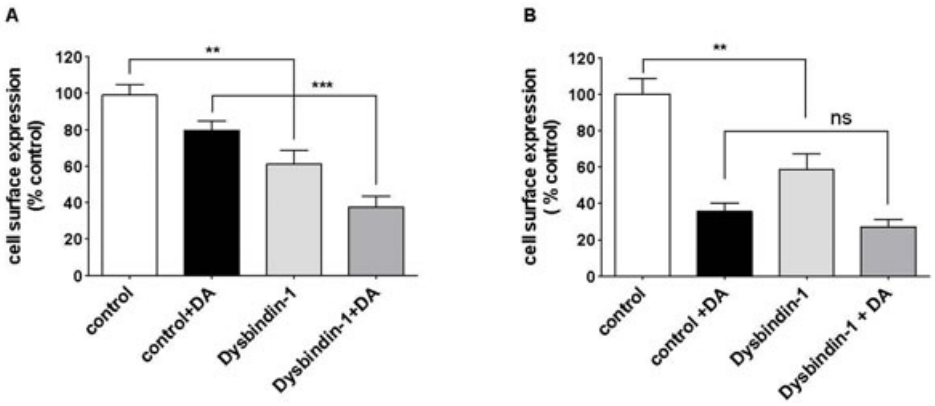

Figure 2 Schmieg et al.

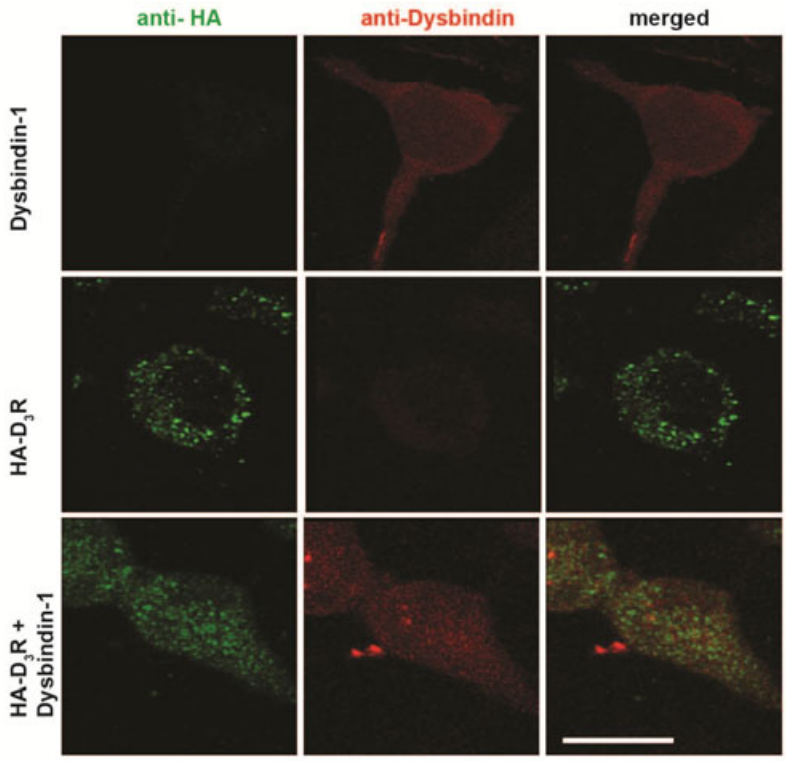

Figure 3 Schmieg et al.

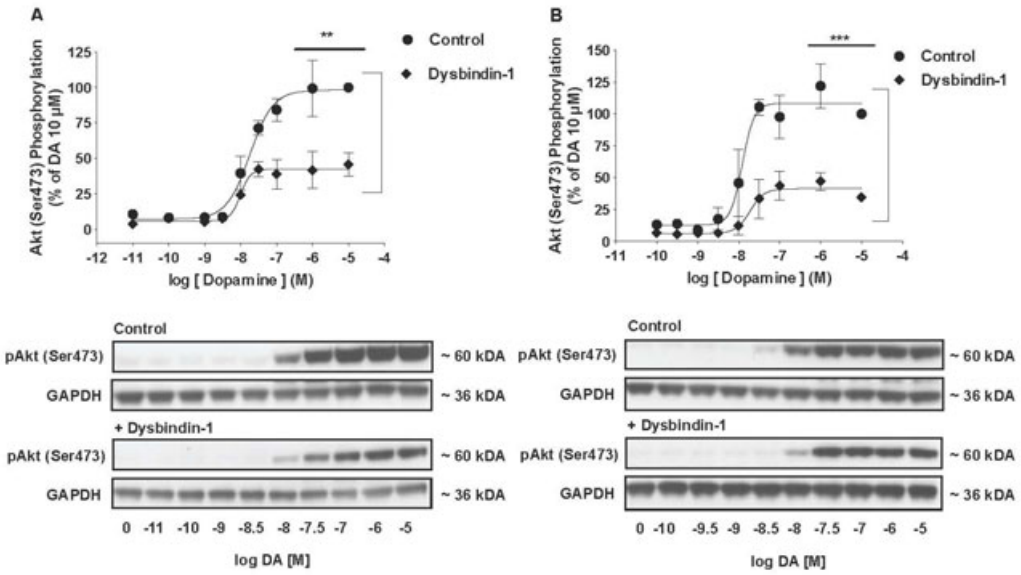

Figure 4 Schmieg et al.

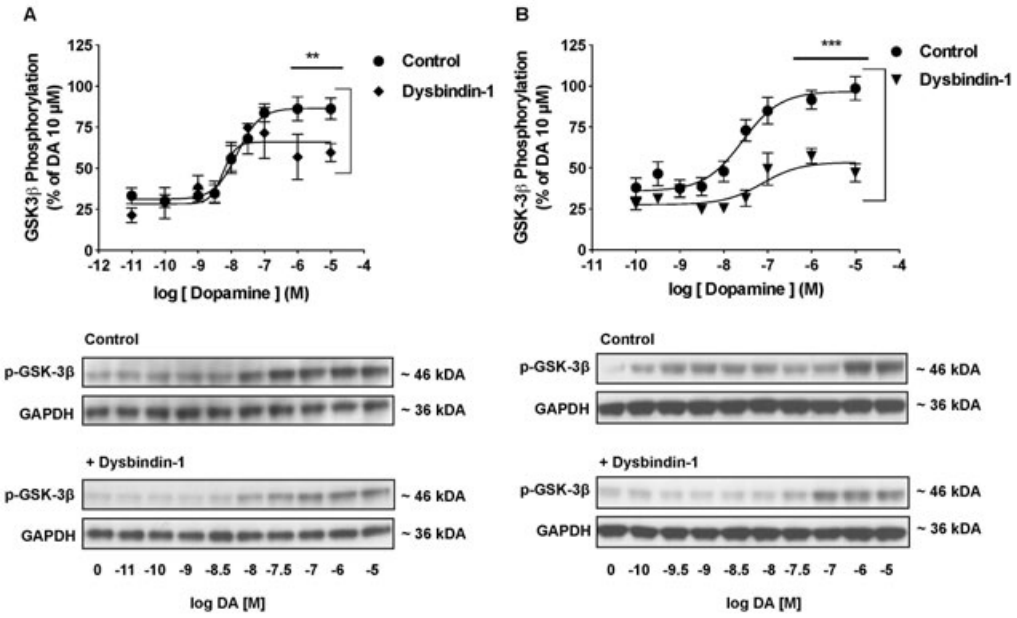

Figure 5 Schmieg et al.

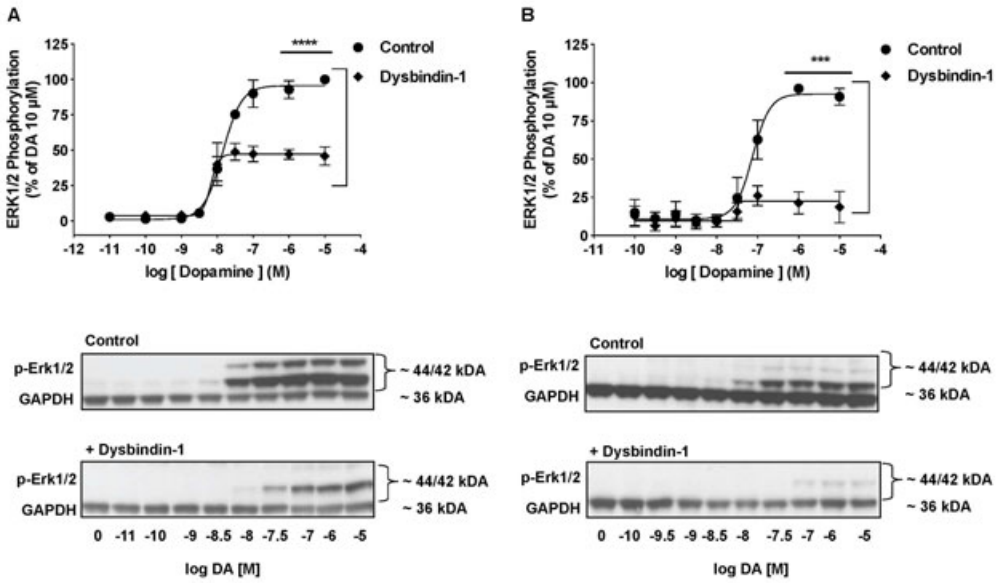

Figure 6 Schmieg et al.

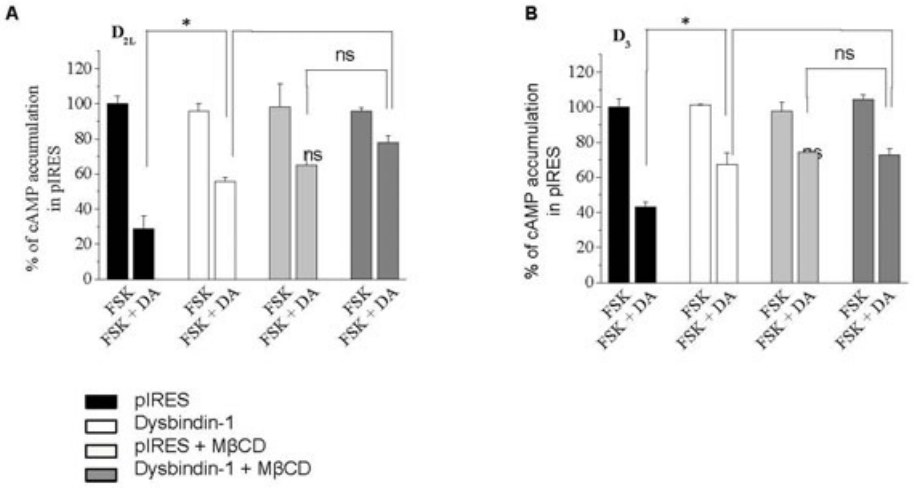

Figure 7 Schmieg et al.