Cellular and Molecular Life Sciences (2018) 75:4357–4370
https://doi.org/10.1007/s00018-018-2876-y

ORIGINAL ARTICLE

Melatonin receptors limit dopamine reuptake by regulating dopamine transporter cell-surface exposure

Abla Benleulmi-Chaachoua[1,2,3] · Alan Hegron[1,2,3] · Marine Le Boulch[1,2,3] · Angeliki Karamitri[1,2,3] · Marta Wierzbicka[4,5] · Victoria Wong[4,5] · Igor Stagljar[4,5] · Philippe Delagrange[6] · Raise Ahmad[1,2,3] · Ralf Jockers[1,2,3]

Received: 11 February 2018 / Revised: 26 June 2018 / Accepted: 16 July 2018 / Published online: 24 July 2018

Abstract

Melatonin, a neuro-hormone released by the pineal gland, has multiple effects in the central nervous system including the regulation of dopamine (DA) levels, but how melatonin accomplishes this task is not clear. Here, we show that melatonin MT_1 and MT_2 receptors co-immunoprecipitate with the DA transporter (DAT) in mouse striatal synaptosomes. Increased DA re-uptake and decreased amphetamine-induced locomotor activity were observed in the striatum of mice with targeted deletion of MT_1 or MT_2 receptors. In vitro experiments confirmed the interactions and recapitulated the inhibitory effect of melatonin receptors on DA re-uptake. Melatonin receptors retained DAT in the endoplasmic reticulum in its immature non-glycosylated form. In conclusion, we reveal one of the first molecular complexes between G protein-coupled receptors (MT_1 and MT_2) and transporters (DAT) in which melatonin receptors regulate the availability of DAT at the plasma membrane, thus limiting the striatal DA re-uptake capacity in mice.

Keywords Melatonin · Dopamine · Melatonin receptor · DAT · Synaptosome

Introduction

The dopamine (DA) transporter DAT is a key regulator of the extrasynaptic DA availability. This transporter mediates DA re-uptake into the nerve terminals, thus preventing dopaminergic hyperactivation. DAT is located on dopaminergic neurons of the ventral tegmental area, the limbic system, in the prefrontal cortex, and highly expressed in substantia nigra projections ending in the striatum formation [1]. DAT is the unique transporter responsible of DA clearance from the synaptic cleft.

DAT is down-regulated by addictive compounds such as cocaine and amphetamine analogues that cause an over-activation of the dopaminergic circuit, accompanied by enhanced locomotion and reward feelings [2]. Moreover, disruptions of dopamine clearance are associated with a broad spectrum of neuropsychiatric disorders such as Parkinson's disease (PD), schizophrenia, and attention-deficit hyperactivity disorder [3–5].

DAT surface expression is one of the limiting factors of the rate of DA uptake. At the level of nerve terminals, DAT expression is maintained in equilibrium between the cell surface and endosomal vesicles [6]. This equilibrium may be modulated by post-translational modifications as well as by

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00018-018-2876-y) contains supplementary material, which is available to authorized users.

Abla Benleulmi-Chaachoua and Alan Hegron contributed equally.

✉ Ralf Jockers
ralf.jockers@inserm.fr

1 Inserm, U1016, Institut Cochin, 22 Rue Mechain, 75014 Paris, France

2 CNRS, UMR 8104, 22 Rue Mechain, 75014 Paris, France

3 University of Paris Descartes, Sorbonne Paris Cité, 12 Rue de l'École de Médecine, 75006 Paris, France

4 Donnelly Centre, Department of Biochemistry, Faculty of Medicine, University of Toronto, 160 College Street, Toronto, ON M5S 3E1, Canada

5 Department of Molecular Genetics, Faculty of Medicine, University of Toronto, 160 College Street, Toronto, ON M5S 3E1, Canada

6 Pôle d'Innovation Thérapeutique Neuropsychiatrie, Institut de Recherches Servier, 125 Chemin de Ronde, 78290 Croissy, France

protein–protein interactions [7]. Reduced DAT expression has been associated with schizophrenia [8] and is observed in the dopamine transporter deficiency syndrome that is characterized by early infantile-onset progressive parkinsonism dystonia [9].

DAT is known to be phosphorylated in response to the activation of several kinases such as PKC, PI3 K, MEK1/2, and p38. DAT also undergoes ubiquitination prior to its down-regulation via endocytosis downstream of PKC activation. The target residues of phosphorylation and ubiquitination are mainly located in the N-terminal domain of DAT, the main regulatory domain of the transporter [10–12]. Moreover, DAT activity can be reversed into DA efflux at the cell surface prior to endocytosis as a result of the phosphorylation of N-terminal serine residues upon PKC activation [13], which can be mediated by amphetamine treatment [14, 15]. Furthermore, DAT was reported to interact with several membrane and cytosolic proteins [16–19]. Some proteins such as GPR37 diminish DA uptake by decreasing the cell-surface expression of DAT in mouse synaptosomes [20], others, like the Parkin protein and the DA D2 receptor and the kappa-opioid receptor, increase the uptake by enhancing the cell-surface expression of DAT [21–23].

The DA availability at the synaptic cleft is a result of a release and uptake process. Several studies report the role of the neuro-hormone melatonin in the regulation of DA availability at different regions in the brain [24, 25]. Melatonin is a neuro-hormone synthesized by the pineal gland during the night phase and has been shown to regulate various processes including neuronal functions [26, 27]. Most of these effects are mediated by two melatonin receptors MT_1 and MT_2 belonging to the G protein-coupled receptor (GPCR) super-family [28]. Melatonin deregulations either at the level of hormone secretion, receptor polymorphisms or expression are associated with a large spectrum of diseases and disorders such as circadian rhythm abnormalities, Parkinson's and Alzheimer's diseases [29, 30], attention-deficit hyperactivity disorder [31], schizophrenia [32], and type 2 diabetes [33].

In this study, we evaluate the possible functional and physical interaction between the melatonin and the dopamine system on the level of two membrane-bound components of these systems, namely, the melatonin receptors and DAT. Experiments were performed in vitro by transfection the different protein components and in vivo by detecting DA uptake in striatal synaptosomes and monitoring amphetamine-induced locomotor activity.

Materials and methods

Compounds

Dihydroxyphenylethylamine-[2,5,6,7,8-3H]-dopamineNET116000), [*N*-methyl-3H]WIN-35428 (NET10330) and 2-[^{125}I]-iodomelatonin (NEX2360) were purchased from Perkin Elmer, S77834 was a generous gift from Servier, Nitrocefin (484400) and melatonin (CAS 73-31-4) from Calbiochem, dopamine (H8502) and Nomifensine (N1530) from Sigma-Aldrich.

Cell culture and transfection

HEK293T cells were grown in complete medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 4.5-g/L glucose, 100-U/mL penicillin, 0.1-mg/mL streptomycin, and 1-mM glutamine) (Invitrogen, CA). Transient transfections were performed using JetPEI (Polyplus Transfection, France), according to manufacturer's instructions.

DNA constructs

Myc- and Flag-hDAT were kindly donated from Dr M.E.A. Reith laboratory from New York University School of Medicine—USA [34]; ΔN1-55-Flag-hDAT and YFP-hDAT from Dr T.S. Shippenberg laboratory from the National Institute on Drug Abuse; Baltimore—USA [35]; and β-Lac-hDAT plasmid was from Dr A. Salahpour (University of Toronto) [36].

Membrane Yeast two-hybrid (MYTH) screen

The MYTH screens were done as outlined previously [37]. In short, baits were transformed into a *Saccharomyces cerevisiae* strain by the lithium acetate/single-stranded carrier DNA/polyethylene glycol method as described by [38]. Subsequently, a prey cDNA library in the NubG-X orientation was transformed into these yeast strains containing MT_1 receptors by the same method. Putative interactors were selected by growth on synthetic dropout media lacking the amino acids tryptophan, leucine, adenine, and histidine (SD-WLAH). Prey plasmids were isolated using a commercial DNA isolation kit modified for yeast, followed by transformation and additional amplification in *Escherichia coli*. Final purified plasmids were subjected to sequencing and BLAST analysis, to identify specific interactors. To eliminate spurious interactors, the prey plasmids were once again transformed into yeast harboring MT_1 or an artificial bait (the transmembrane segment

of the human T-cell-surface glycoprotein CD4 linked to Cub-TF) and interaction was assessed. Any preys that also interacted with the artificial bait were deemed promiscuous and discarded.

Immunoprecipitation and Western blot

Striata from male mice (8–12 weeks) were homogenized in 7-ml ice-cold 5-mM HEPES, 0.32-M sucrose, pH 7.4 supplemented with protease inhibitors: leupeptine (1 mg/ml), pepstatin (1 mg/ml), benzamidine (2 mg/ml), AEBSF (1 mg/ml), and phosphatase inhibitor: 2-mM Na3VO4, 10-mM NaF (buffer H) and grind in a potter and centrifuged (800×*g*; 7 min; 4 °C). The supernatant was then centrifuged (9200×*g*; 10 min; 4 °C). The resultant pellet was resuspended in and then centrifuged (10,000×*g*; 17 min; 4 °C). The pellet obtained contained synaptosomes and was solubilised 4 h in the same buffer with 0.5% Brij 96 (Sigma, P6136) at 4 °C on wheel and centrifuged (14,000×*g*; 1 h; 4 °C). The soluble fraction was subjected to immunoprecipitation using G protein Sepharose beads (Sigma) with monoclonal antibodies (2 µg/ml) raised against the carboxyl-terminal tail of mMT_1 or mMT_2 (manuscript in preparation). Pull-down experiments were performed with streptavidin beads (GE Healthcare Life Sciences) in IP buffer (TEM buffer supplemented with 75-mM Tris pH 7.5, 2-mM EDTA, and 12-mM $MgCl_2$ with protease inhibitors) overnight. Samples were denatured overnight at room temperature in SDS-PAGE loading buffer (62.5-mM Tris/HCl, pH 6.8, 5% SDS, 10% glycerol, and 0.5% bromophenol blue) and the protein separated by SDS-PAGE. Immunoblot analysis was performed with the indicated antibodies (rabbit anti-DAT antibody (1:800) (AB5802) Millipore preferentially recognizing the mature DAT form and rabbit (H-80) Santa-Cruz (SC14002) preferentially recognizing the immature form and immunoreactivity was revealed using secondary antibodies coupled to 680 or 800 nm fluorophores using the Odyssey LI-COR infrared fluorescent scanner (ScienceTec, France).

BRET measurement

For BRET donor saturation curves, 30,000 HEK293T cells were seeded in 96-well white Optiplates (Perkin Elmer Life Sciences) with 0.125 ng of MT_1-Rluc or 1.25 ng of MT_2-Rluc or 0.083 ng of CCR5-Rluc and 0–125 ng of DAT-YFP plasmids. Cells were then incubated 48 h at 37 °C before BRET measurements. Luminescence and fluorescence were measured simultaneously using plates and were read on the Mithras LB 940 with 480 ± 10 nm (Rluc) and 540 ± 20 nm (YFP) emission filters and BRET ratios calculated as above.

Synaptosomal preparation

Synaptosomes from striatum, hypothalamus, and cortex of male mice (8–12 weeks) were prepared as described in [39]. Briefly, tissues were homogenized in a buffer containing 320-mM sucrose, 5-mM HEPES, and protease inhibitors. After centrifugation at 2000×*g* for 8 min, the supernatant was centrifuged at 30,000*g* for 30 min. The resulting synaptosomal pellet was kept at 4 °C and used for subsequent analysis within 4 h.

DA uptake and β-CFT binding

HEK293T cells were transfected in poly-L-Lysine-coated 24 well plates. 48-h post-transfection, cells were washed with uptake buffer (5-mM Tris-HC1, 7.5-mM HEPES, 120-mM NaC1, 5.4-mM KCI, 1.2-mM $CaC1_2$, 1.2-mM $MgSO_4$, 5-mM D-glucose, and 1-mM ascorbic acid; pH 7.2) and incubated for 5 min with 20 nM of [^{3}H]-Dopamine. The uptake was terminated by two washes with ice-cold uptake buffer, and lysed in 0.5 ml of 1% SDS. Measurement was performed using scintillation counter. For synaptosomal DA uptake, 100 µg of proteins were subjected to DA uptake test for 5 min. Uptake was stopped by the addition of 3 ml of ice-cold uptake buffer, followed by rapid filtration through Whatman filters and two additional rinses. The DA uptake kinetic test was performed in the presence of a fixed concentration of [^{3}H]-dopamine (20 nM) and increasing DA concentrations as indicated. β-CFT binding was performed in the presence of 4 nM of [^{3}H]WIN-35428 for 30 min at room temperature. Non-specific binding was assessed with 25 nM of nomifensine.

B-lactamase assay

The β-lactamase assay was performed as previously described in (33) with minor modifications. Briefly, cells were plated into individual wells of a poly-D-lysine-coated 96-well plate. The assay was performed 24 h after plating. Cells were treated or not with PMA (1 µM) for 30 min at 37 °C and then incubated with the β-lactamase substrate nitrocefin. Absorbance at 492 nm was measured immediately and then every 5 min for 30 min in a microplate spectrophotometer. The reaction rate (slope of the curve in the linear range) was taken as the readout for this assay.

^{125}I-MLT binding

The assay was performed as previously described [40].

Homogeneous time-resolved fluorescence (HTRF)-based cAMP assay

cAMP levels were determined in HEK293T cells by *HTRF* using the "cAMP femto2" kit (Cisbio, Bagnols-sur-Cèze, France) as previously described [41].

Hyperactivity induced by D-Amphetamine

Activity of male mice (8–12 weeks) was measured in eight open fields (actimeters) which are Plexiglas transparent open-box (42-cm L, 42-cm W, and 40-cm H). The distance travelled (horizontal activity) was recorded with infrared photobeams detection systems (Acti-track, LSI Leticca, Panlab). Open fields were placed in four independent compartments, under a dim light (10 Lux). Within each compartment, open fields were visually isolated from the experimental room.

Animals were individually placed in the open fields and allowed to move freely for a 90-min session. Their distance travelled was recorded all long, and at T30 min, D-Amphetamine (3 mg/kg) or vehicle was injected intraperitoneally to mice. The data collected were the spontaneous locomotor activity (SLA) taking in account the distance travelled during the 30-min period before the injection of D-Amphetamine or its vehicle and the distance travelled during the 60-min period following the injection.

Immunofluorescence

HeLa cells were fixed with 4% formaldehyde and permeabilized with 0.2% Triton X-100. Cells were incubated with anti-myc rabbit polyclonal (Santa-Cruz sc-789) or anti-myc mouse monoclonal (Santa-Cruz sc-40) antibodies or anti-HA mouse monoclonal (Biolegend MMS-101P) or anti-HA rabbit polyclonal (Cell Signaling #3724) antibodies, followed by staining with TRITC or FITC-conjugated secondary antibodies (Biotium) in the presence of DAPI. Images were taken with a microscope ZEISS Observer Z1 with the lense PLAN FLUOR 100 × and analyzed by Image J software.

Statistical analysis

Results were analyzed by PRISM (GraphPad Software). Data are expressed as mean ± SEM of at least three experiments. One-sample *t* test and one-way ANOVA test with Dunnett's multiple comparisons test were applied for statistical analysis.

Results

Identification of DAT/melatonin MT_1 receptor complexes with MYTH

To identify proteins interacting with the human melatonin MT_1 receptor, we applied the recently modified split-ubiquitin Membrane Yeast Two-Hybrid (MYTH) technique [37]. Full-length receptors were N-terminally tagged with the yeast mating factor α to encourage plasma membrane localization and C-terminally tagged with half of ubiquitin (Cub) fused to an artificial transcription factor comprising LexA and VP16. Successful cell-surface expression of melatonin receptors in yeast was monitored by immunofluorescence microscopy. Screens were performed in duplicate against recombinant NubG-prey of human fetal brain cDNA library, a tissue know to express MT_1 receptors [42]. Forty-seven high-quality interactions were retained after deleting false-positive candidates interacting with an artificial bait, the transmembrane segment of the human T-cell-surface glycoprotein CD4 linked to Cub-TF [43]. Among these proteins was DAT that interacted with MT_1 receptors (2 clones with 330/620 amino acids) (Table 1).

Melatonin MT_1 and MT_2 receptors interact with DAT in mouse striatum and HEK293T cells

To confirm the existence of DAT/melatonin receptor complexes in the mouse brain, we isolated the synaptosomal fraction of the hypothalamus, striatum, and cortex from C57BL/6 mice. In agreement with the previous reports, our pilot experiments showed the highest DAT expression and ^{3}H-DA uptake in the striatum followed by the cortex and hypothalamus (Fig. 1a, b) [44]. Expression of melatonin receptors was monitored in 2-[^{125}I]-iodomelatonin (^{125}I-MLT) binding experiments and found to be highest in the hypothalamic followed by the striatal and the cortical

Table 1 MT1 receptor interacts with DAT in MYTH screening

Bait	Prey	MYTH
Melatonin Receptor-Cub-TF	Ost1-NubG	–
Melatonin Receptor-Cub-TF	Ost1-NubI	+++
Artificial Bait-Cub-TF	Ost1-NubG	–
Artificial Bait-Cub-TF	Ost1-NubI	+++
Melatonin Receptor-Cub-TF	NubG-DAT	+++
Artificial Bait-Cub-TF	NubG-DAT	–

Interaction results of the melatonin receptor or artificial baits when tested against negative control, positive control or DAT preys by MYTH

– no interaction, +++ interaction, Ost1-NubG (negative) and -NubI (positive) Control preys

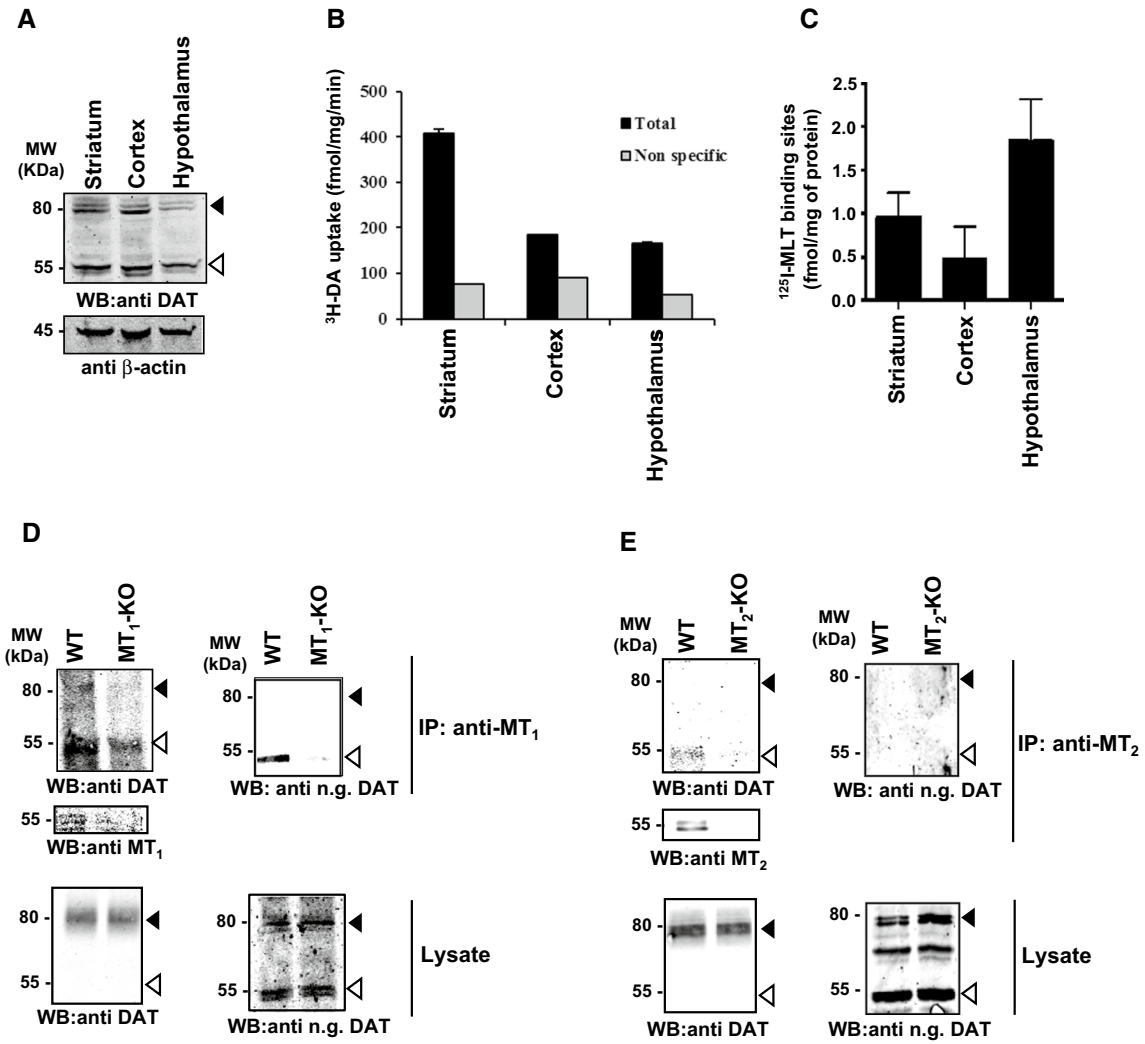

Fig. 1 DAT is expressed in the striatum, hypothalamus and cortex and interacts with MT_1 and MT_2 in mice striatal synaptosomes. **a** Western-blot analysis on 30 µg of synaptosomal fraction of mice striatum, cortex and hypothalamus using anti-DAT antibodies. The blot shows that the mature (black arrow head) and the immature (white arrow head) forms of DAT are present at various levels in all three brain regions. **b** ^{3}H-DA uptake assay on 100 µg of synaptosomes from different brain regions as indicated, in the presence or absence of 25 nM of nomifensine to define the non-specific uptake. **c** Number of specific 2-[^{125}I]-iodomelatonin binding sites in presynaptic membranes from rat striatum, hypothalamus and cortex. **d**, **e** Co-IP between MT_1 and DAT in striatal synaptosomes of WT and MT_1-KO (**d**) or MT_2-KO (**e**) mice using anti-MT_1 (**d**) or anti-MT_2 (**e**) antibodies for IP and antibodies against DAT (left panels) or non-glycosylated DAT (n.g. DAT) (right panels) for WB detection. Similar results were obtained in two additional experiments. Black arrow head, mature form of DAT; white arrow head, immature form of DAT

synaptosomes (Fig. 1c). Based on these findings, the striatum was used for the following experiments. Immunoprecipitation of MT_1 and MT_2 from lysates of striatal synaptosomes with monoclonal antibodies recognizing specifically the mouse MT_1 and MT_2 revealed the presence of both receptors in precipitates of WT but not mice with targeted deletion of MT_1 or MT_2 (MT_1-KO; MT_2-KO), respectively (Fig. 1d, e, middle panels). Western blotting of precipitates with anti-DAT antibodies, recognizing predominantly the mature form migrating at a molecular weight of 80 kDa over the immature non-glycosylated 55 kDa form [21] showed a strong band at 55 kDa but none at 80 kDa suggesting an enrichment of the immature form of DAT in MT_1 precipitates and to a lesser extend in MT_2 precipitates (Fig. 1d, e, left upper panels). As the 55 kDa form of DAT was largely undetectable in lysates with this antibody, we wanted to confirm the identity of the DAT band in precipitates with a second antibody recognizing predominantly the immature form (anti n.g. DAT) (SC14002). The 55-kDa band was once again detected with this antibody in MT_1 precipitates validating the interaction of MT_1 with the immature form of DAT (Fig. 1D, right upper panel). We were unable to

confirm the 55-kDa band in MT_2 precipitates, most likely because the amount of precipitated DAT was below the detection limit of this antibody (Fig. 1e, right upper panel).

To further confirm these interactions, we performed bioluminescence resonance energy transfer (BRET) donor saturation experiments in cells transfected with the *Renilla* luciferase (Rluc) energy donor fused to the C-terminus of MT_1 and MT_2 and the yellow fluorescent protein (YFP) energy acceptor fused to the N-terminus of DAT. The chemokine receptor CCR5-Rluc fusion protein was included as a negative control and the respective MT_1 and MT_2 homodimer combinations as positive controls. The co-expression of a fixed amount of Rluc fusion proteins and increasing amounts of YFP-DAT resulted in the expected hyperbolic curve [45, 46] reaching an asymptote indicating specific interactions between DAT and MT_1 and MT_2, similar to the previously reported MT_1 and MT_2 homodimers [47] (Fig. 2a, b). Colocalization of DAT with MT_1 and MT_2 in transfected HeLa cells further confirmed the close proximity of DAT and melatonin receptors (Fig. 2c; Supplementary Fig. 1A). Taken together, co-IP, BRET, and colocalization experiments provide evidence that DAT interacts with MT_1 and MT_2 in striatal synaptosomes and transfected cells.

Melatonin receptors decrease dopamine uptake through DAT

To explore potential consequences of melatonin receptors on DAT function, we measured DA uptake in HEK293T cells expressing DAT in the absence or presence of receptors. Expression of MT_1 and MT_2 decreased DA uptake by 59 ± 5.9 and $26 \pm 2.8\%$ ($n = 3$), respectively (Fig. 3a). In agreement with the previous reports, co-expression of GPR37 decreased DA uptake by $14 \pm 1.6\%$ ($n = 3$) [20], while the presence of another GPCR, the CCR5 receptor, and the 4-transmembrane-spanning endospanin-1 protein, known to regulate the cell-surface expression of other membrane receptors [48], did not affect the DAT-promoted DA uptake (Fig. 3a). Expression of comparable quantities of DAT and successful expression of the various GPCRs and endospanin-1 was confirmed in total cell lysates by (Fig. 3a inset, Supplementary Fig. 1B). Pre-incubation with melatonin (1 µM) for 30 min did not modulate the inhibitory effect of MT_1 and MT_2 receptors on DA uptake in HEK293T cells and striatal synaptosomes (Fig. 3b, c). The absence of effect of melatonin was confirmed in concentration–response experiments in striatial synaptosomes (Supplementary Fig. 1C). Shorter or longer pre-incubation times with melatonin (1 µM) were also without effect (not shown).

Inactivation of $G_{i/o}$ and $G_{q/11}$ proteins, known to be activated by melatonin receptors [49], with pertussis toxin (PTX) or YM-254890, respectively, was unable to abolish the inhibitory effect of melatonin receptors on DA uptake arguing against the involvement of constitutive receptor activity in this effect (Supplementary Fig. 1D, E). This was further confirmed by treating HEK293 cells with the melatonin receptor inverse agonist S77834 [50], which was also unable to elevate the inhibition of DA uptake (Fig. 3b). S77834 was similarly ineffective in modifying the basal DA uptake in striatal synaptosomes of WT mice (Fig. 3c). Successful inhibition of constitutive MT_1 and MT_2 receptor activity by S77834 under these conditions was shown in the cAMP accumulation assay (Supplementary Fig. 1F).

The N-terminus of DAT (amino acids 1–62) plays a key role in DAT function, because it undergoes various post-translational modifications leading to DAT down-regulation [10, 12]. It serves as a protein–protein interaction platform for syntaxin 1A [51], synaptogrin-3 [52] and possibly also for dopamine D2 receptors [22, 35]. Pull-down experiments between MT_1 and MT_2 receptors and a DAT mutant deleted of the first 55 amino acids [35] (ΔN-Flag-DAT) showed that the N-terminal DAT domain is not necessary for the interaction with melatonin receptors (Supplementary Fig. 1G). Consistently, a similar inhibition pattern of DA uptake by melatonin receptors was also observed for the ΔN-Flag-DAT mutant at similar DAT expression levels (Fig. 3d, Supplementary Fig. 1H).

To confirm our in vitro finding, we measured DA uptake in striatal synaptosomes of MT_1-KO and MT_2-KO mice. DA uptake was readily measurable in synaptosomal preparations of WT mice containing all the necessary elements for the transport of neurotransmitters thus reliably reflecting in vivo conditions [39]. DA uptake was doubled in MT_1-KO and in MT_2-KO mice (Fig. 3e). This enhanced DA transport capacity is not accompanied by increased DAT expression levels as monitored by western blot in synaptosomal lysates (Fig. 3e).

Altogether, these results show that melatonin receptors inhibit DA uptake through DAT in transfected HEK293T cells and mice striatal synaptosomes and that this effect is not regulated by melatonin receptor activation or constitutive activity and does not involve the regulatory N-terminal domain of DAT.

Melatonin receptors decrease DAT cell-surface expression

Regulation of DAT-promoted DA uptake may be due to either a modulation of the affinity of DA for DAT or to a modification of DAT cell-surface expression [21]. Kinetic analysis of DAT-mediated DA uptake showed that K_m values were similar in HEK293T cells expressing DAT alone (1.57 ± 0.15 µM) or co-expressing DAT and MT_1 (1.07 ± 0.49 µM) or MT_2 (1.49 ± 0.31 µM) (Fig. 4A). K_m values were comparable to those measured in previous studies [53]. Maximal DA uptake rates (V_{max}) were

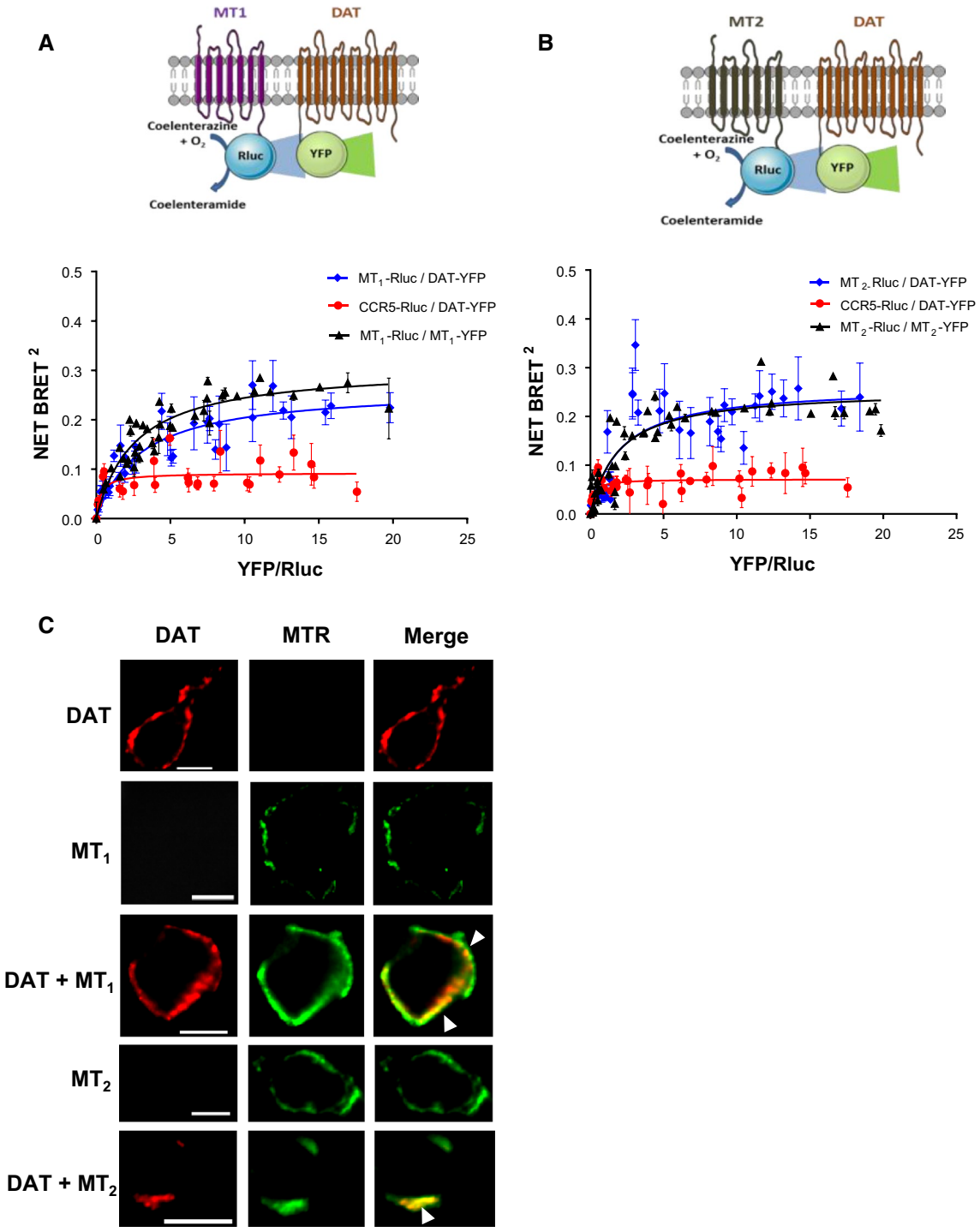

Fig. 2 DAT interacts and colocalizes with MT_1 and MT_2 in transfected cells. **a**, **b** BRET donor saturation experiments with HEK293T cells transfected with a fixed amount of MT_1-Rluc (**a**), MT_2-Rluc (**b**), or CCR5-Rluc (negative control) (**a**, **b**) and increasing quantities of YFP-DAT (**a**, **b**), MT_1-YFP (**a**) or MT_2-YFP (**b**). The saturation curves were obtained from three to five independent experiments. **c** HeLa cells expressing Myc-DAT and Flag-MT_1 or Flag-DAT and Myc-MT_2 were permeabilized and colocalization of receptors and DAT (white arrow heads) evaluated by immunofluorescence microscopy using the appropriate anti-tag antibodies. Scale bars represent 5 µm. Experiment was performed at least two other times with same results. *MTR* MT_1 or MT_2

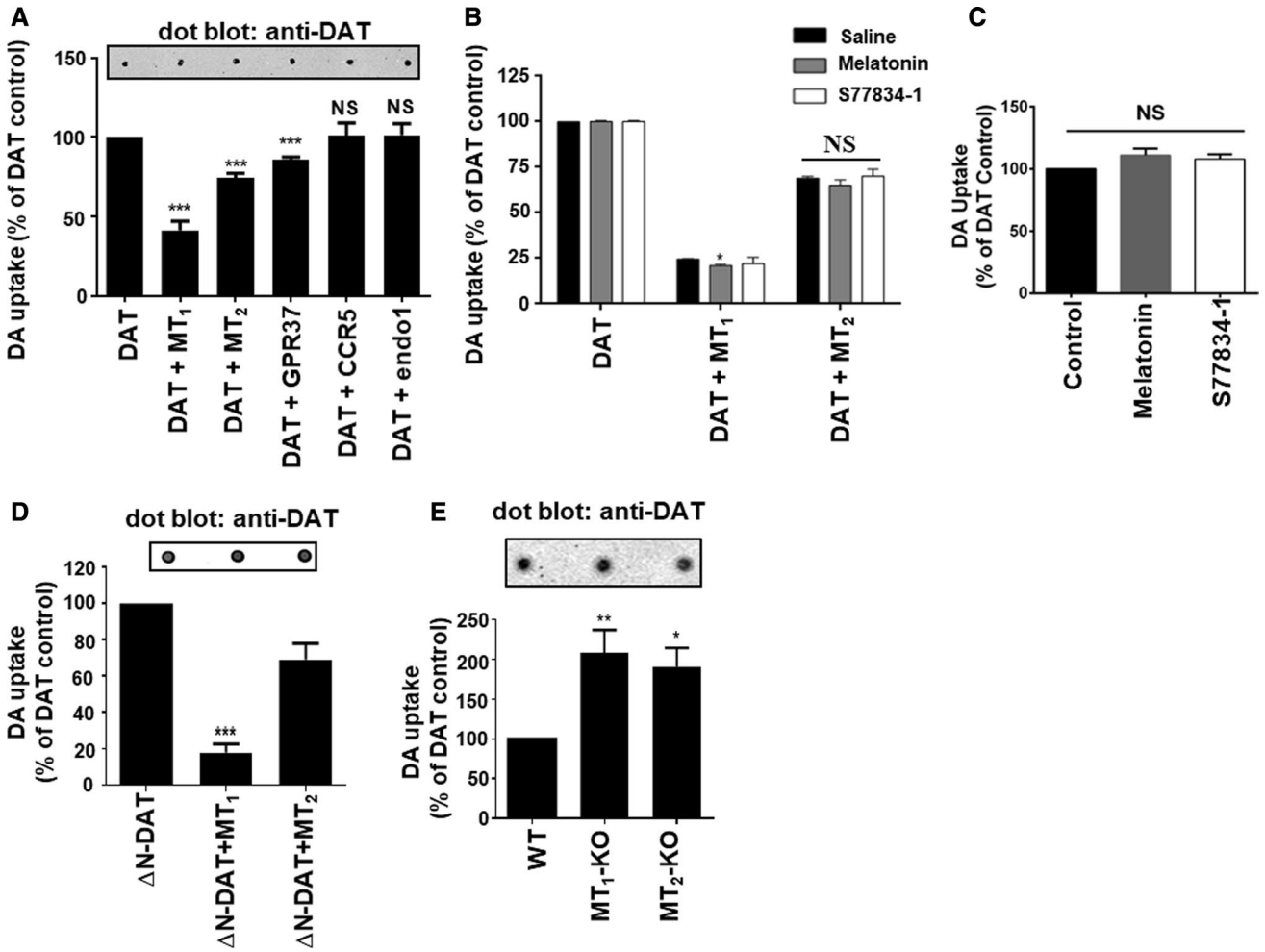

Fig. 3 MT_1 and MT_2 constitutively decrease DA uptake in HEK293T cells and striatal synaptosomes. **a** DA uptake in HEK293T cells expressing Flag-DAT alone or with Flag-MT_1, Myc-MT_2, Myc-GPR37, HA-CCR5 or Myc-Endospanin1 (Endo1). Inset: dot-blot analysis of DAT expression with anti-DAT antibodies. **b**, **c** DA uptake in HEK293T cells expressing Flag-MT_1 or Myc-MT_2 (**b**) or on 100 µg of striatal synaptosomes of WT mice (**c**) pre-incubated or not with melatonin or the inverse agonist S77834-1 (1 µM each, 30 min). **d** DA uptake in HEK293T cells expressing ΔN-Flag-DAT alone or with Flag-MT_1 or Myc-MT_2. **e** DA uptake on 100 µg of striatal synaptosomes of the indicated mice strains. Dot-Blot analysis of the synaptosomal fractions using anti-DAT antibodies. Data are expressed as relative specific uptake compared to DAT alone and are presented as mean ± SEM; $n = 3$ (**a**–**c**) or $n = 5$ (**d**). *NS* not statistically different. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.005$, with respect to vehicle (one-way ANOVA followed by multiple comparison post hoc test)

significantly decreased in cells co-expressing DAT with MT_1 (78.39 ± 14.17 fmol/mg/min) or MT_2 (138.20 ± 12.43 fmol/mg/min) as compared to cells expressing DAT alone as expected (222.20 ± 9.38 fmol/mg/min) ($N = 3$; p value < 0.001). Altogether, these results exclude an effect of melatonin receptors on the affinity of DA for DAT.

To assess the impact of melatonin receptors on DAT cell-surface expression, we determined the number of DAT at the cell surface with the radiolabeled cocaine analogue β-CFT [*N*-methyl-^{3}H]WIN-35428. A drop of 50–70% in WIN-35428-binding sites was observed in cells co-expressing either MT_1 or MT_2 (Fig. 4b). Similar effects were observed in cells co-expressing DAT and GPR37 recapitulating previously reported results (Fig. 4b) [20]. Expression of the negative control receptor CCR5 had no influence on the number of WIN-35428 binding sites (Fig. 4b). For all conditions, the total amount of expressed DAT did not change (Fig. 4b, inset). Collectively, these data suggest that melatonin receptors limit the number of DAT expressed at the cell surface.

Melatonin receptors retain DAT in the biosynthetic pathway

Decreased DAT cell-surface expression might result from higher internalization or lower export of DAT in the presence of melatonin receptors. DAT internalization is known to be dependent on protein kinase C (PKC) activation, directly by

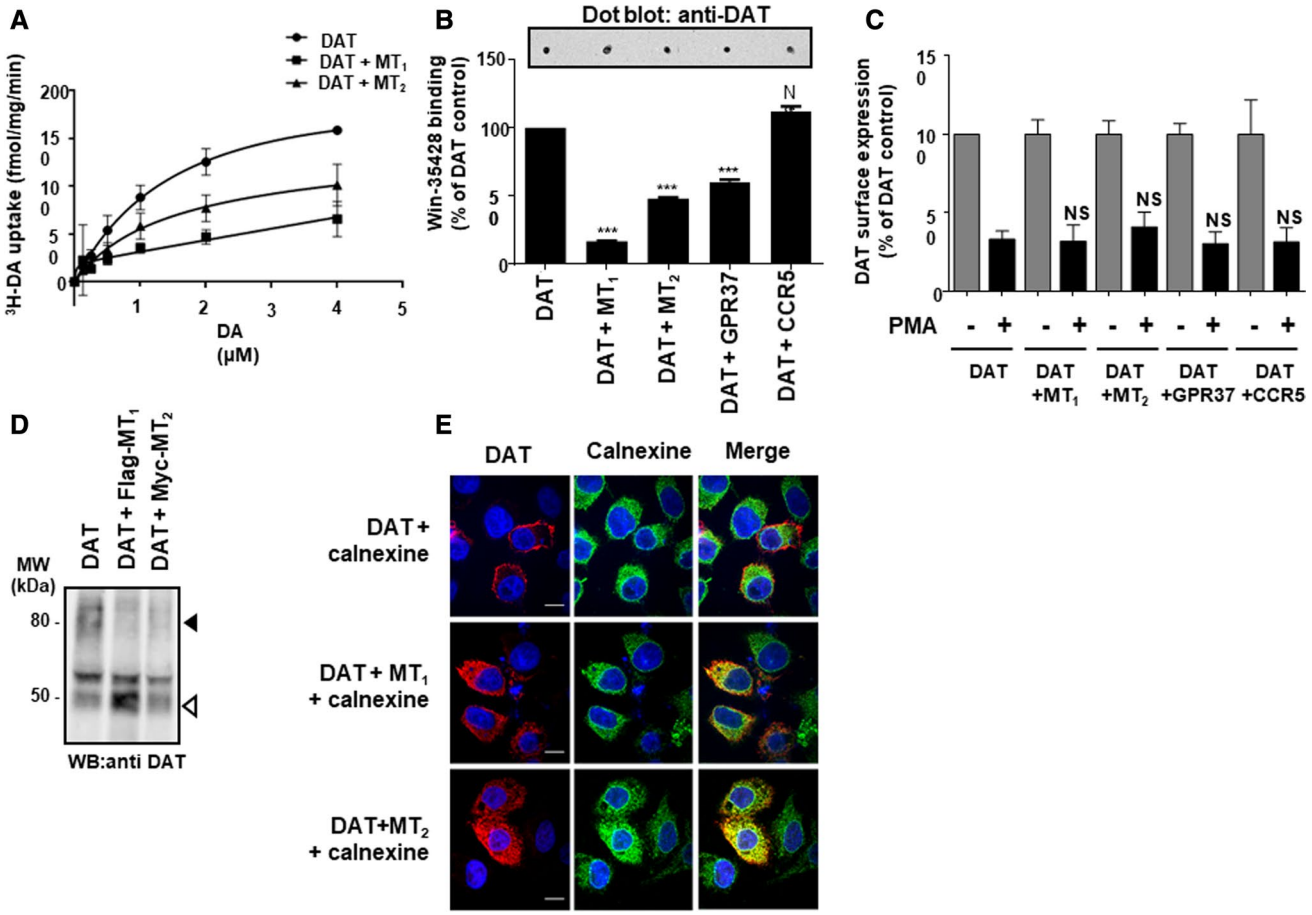

Fig. 4 MT_1 and MT_2 do not affect the affinity of DAT for DA but decrease DAT cell-surface expression. **a** DA uptake kinetics were determined in HEK293T expressing DAT with the indicated receptors by incubating cells with 20 nM of ^{3}H-DA and increasing concentrations of unlabeled DA. Michaelis constants (K_m) are: 1.57 ± 0.15 µM (DAT alone), 1.07 ± 0.49 µM (DAT + MT_1), 1.49 ± 0.31 µM (DAT + MT_2); $n = 3$. **b** Number of DAT expressed at the surface of HEK293T cells was determined with the tritiated cocaine analogue [*N*-methyl-^{3}H]WIN-35428 (4 nM). Non-specific binding was measured in the presence of nomifensine (25 µM). Data are presented as % of control (DAT alone) and are the mean ± SEM; $n = 3$. Inset: dot-blot analysis of cell fractions with anti-DAT antibodies. **c** β-lac-DAT fusion protein was expressed alone or with the indicated proteins and pre-treated or not with PMA (1 µM, 30 min). Data are presented as % of in the absence of PMA; $n = 3$–6. **d** Western-Blot analysis revealing the 80 kDa glycosylated form of DAT (mature) and the 50 kDa non-glycosylated form of DAT (immature, intracellular) in HEK293T cells expressing DAT with or without Flag-MT_1 or Myc-MT_2. Similar results were obtained in two additional experiments. **e** HeLa cells expressing Myc-DAT and Flag-MT_1 or Flag-DAT and Myc-MT_2 were permeabilized and the colocalization of DAT and calnexine (Endoplasmic Reticulum marker) monitored by immunofluorescence microscopy. Scale bars represent 5 µm. Experiment was performed at least 2 other times with the same results. *NS* non-significant, ***$P < 0.005$ using **b** one-sample *t* test or **c** one-way ANOVA test with Dunnett's multiple comparisons test

phosphorylating DAT or indirectly by mediating its ubiquitination [7]. Treatment of HEK293T cells for 30 min with the potent PKC activator, phorbol myristate ester (PMA) [54] caused the expected decrease of DAT cell-surface expression of 60% and this effect was not significantly modified by the co-expression of melatonin receptors (Fig. 4c).

To explore the influence of melatonin receptors on the retention of DAT in the biosynthetic pathway, cell lysates were analyzed by western blot for the presence of the immature, intracellular (55 kDa) DAT form and the mature, cell-surface-located (80 kDa) form. In the presence of melatonin receptors, an enrichment of the immature DAT form was observed, consistent with the retention of DAT in intracellular compartments under this condition (Fig. 4d). Before further investigations on where this retention occurs, we checked the presence of Golgi and endoplasmic reticulum in our synaptosomal preparations using antibodies against 58 K marker and calnexine, respectively (Supplementary Fig. 3). Then colocalization of DAT with calnexine was only observed in cells expressing MT_1 or MT_2 further confirmed the intracellular retention of DAT in the presence of melatonin receptors (Fig. 4e). No colocalization was observed with the 58 K Golgi marker and the GM130 *cis*-Golgi marker (Supplementary Fig. 2A, B).

Taken together, co-expression of melatonin receptors with DAT decreases its cell-surface expression by retaining DAT in the endoplasmatic reticulum of the biosynthesis pathway. This observation is also consistent with the co-immunoprecipitation of the immature, intracellular form of DAT with melatonin receptors in the striatum.

Mice lacking melatonin receptors are less sensitive to amphetamine-induced locomotion

MT_1-KO and MT_2-KO mice show increased DA uptake capacity in striatal synaptosomes. Therefore, we predicted decreased synaptic DA concentrations and decreased locomotor activity in these mice. Thus, we measured amphetamine-induced locomotor activity in WT and KO mice (Fig. 5a–c). Whereas basal locomotor activity in all three mouse strains was similar, amphetamine-induced locomotor activity was decreased by half in MT_1-KO and MT_2-KO mice compared to WT mice. Amphetamine showed a similar affinity for DAT in striatal synaptosomes prepared from WT and MT_1-KO and MT_2-KO mice excluding the possibility that reduced amphetamine-induced locomotor activity is not due to decreased affinity of amphetamine for DAT (Fig. 5d).

Discussion

In this study, we report that melatonin receptors inhibit DA uptake by diminishing the cell-surface expression of DAT in a complex containing melatonin receptors and DAT. This conclusion is supported by in vitro studies in HEK293T cells and in vivo studies in melatonin receptor KO mice including co-immunoprecipitations, DA uptake and locomotor activity.

Initially, DAT was identified as a potential binding partner of the MT_1 receptor in an MYTH screen suggesting the binary character of the interaction and arguing towards the complex formed between MT_1 and DAT. The high sequence homology of MT_1 with MT_2 prompted us to consider also the second melatonin receptor for which we observed indeed similar results as for MT_1 receptors: formation of protein

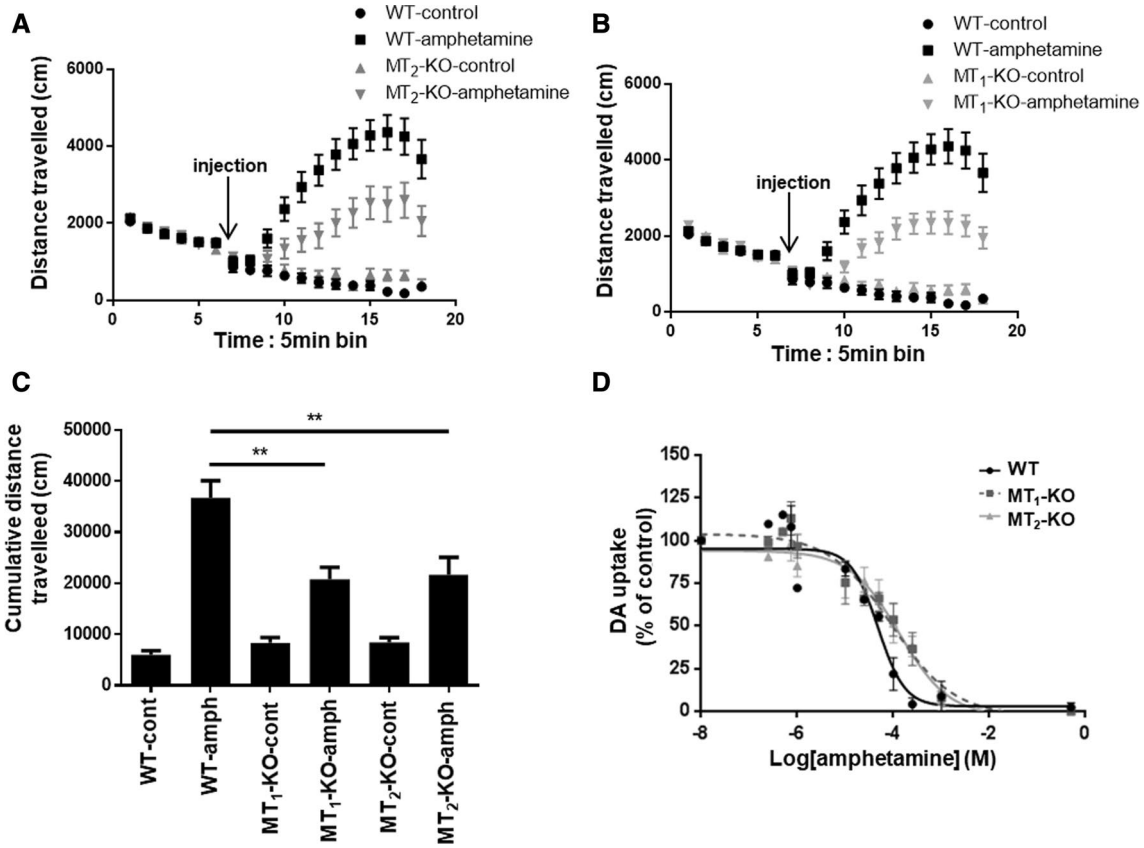

Fig. 5 Melatonin receptor KO mice are less sensitive to amphetamine than wild type mice. **a**–**c** D-amphetamine was injected (3 mg/kg) in WT, MT_1-KO or MT_2-KO mice (arrow on the graph) and the distance travelled by the mice was recorded during 60 min. **c** presents cumulative distance travelled during 60 min after D-amphetamine injection. Data are presented as mean ± SEM; $N = 3$. **d** ^{3}H-DA (20 nM) uptake in striatal synaptosomes of the indicated mice strains in the presence of increasing D-amphetamine concentrations. $LogIC_{50}$ values are -4.32 ± 0.10 (WT), -4.03 ± 0.15 (MT_1-KO), -3.87 ± 0.14 (MT_2-KO); $N = 3$. $^{**}P < 0.01$, using one-way ANOVA test with Dunnett's multiple comparisons test

complexes and a decrease in DA uptake, although to a lesser extent. K_m values for DA were similar suggesting unmodified DA transport capacity of DAT in the presence of melatonin receptors. In contrast, maximal transport levels were significantly reduced. Stimulation of MT_1 and MT_2 with a saturating concentration of melatonin did not modify this effect. The inability of the melatonin receptor inverse agonist S77834 to prevent the inhibitory effect of MT_1 and MT_2 on DA uptake excludes also the involvement of constitutive receptor activity in this effect. Both, melatonin and S77834, were also ineffective in altering the DA uptake in isolated striatal synaptosomes. Similarly, the absence of effect of melatonin and S77834 argues against a direct allosteric effect of melatonin receptors in the common complex. Taken together, melatonin receptors inhibit the maximal DA uptake capacity of the DAT independently of the activation state of melatonin receptors.

Regulation of DAT surface expression has been previously identified as a limiting parameter that regulates DA uptake. Our results indicate that the decreased DA uptake capacity in the presence of MT_1 and MT_2 is caused by the retention of DAT in intracellular compartments. This is supported by the retention of DAT in the ER of HEK293T cells co-expressing MT_1 or MT_2, the enrichment of the immature, intracellular form of DAT under these conditions and the co-immmunoprecipitation of the immature 55 kDa form of DAT with melatonin receptors in the striatum. Although the precise molecular species of DAT engaged with its interacting partners was not always reported, in many cases, the mature form of DAT seems to be involved. Interestingly, co-immunoprecipitation experiments between snapin and DAT in the striatum suggest the involvement of the immature form of DAT, similar to our findings [55]. The interaction with the non-glycosylated form of DAT is interesting as DAT activity depends not only on its membrane expression but also on its glycosylation status as shown in vitro and by the differential vulnerability of midbrain DA cells in Parkinson's disease [56].

The dopamine D2 receptor has been shown to increase DA uptake as a consequence of enhanced DAT cell-surface expression possibly involving reduced membrane endocytosis and increased in membrane exocytosis [22, 35]. Furthermore, in striatum, activation of the kappa-opioid receptor increased the V_{max} for DAT-mediated DA transport and DAT surface expression most likely by forming a molecular protein complex [23]. In the case of the ubiquitin ligase, E3 Parkin increased DAT cell-surface localization is achieved but by an alternative mechanism. Actually, correctly folded DAT is stabilized by oligomerization during biosynthesis in the endoplasmic reticulum, a process that is also necessary for efficient translocation of DAT to the cell surface [57]. Importantly, incorrectly folded DAT protomers have dominant negative effects on the WT in these oligomers and inhibit the cell-surface delivery of DAT. Parkin is shown to enhance the selective ubiquitination (and thus degradation) of the miss-folded and/or unglycosylated DAT and cause the increase in the cell-surface expression of mature forms of DAT.

The striatum expresses abundant amounts of DAT. The expression and function of melatonin receptors is much less established in this brain region. Uz et al. demonstrated in single-cell PCR experiments that the mRNA coding for the MT_1 receptor localizes all over the central dopaminergic system including the ventral tegmental area, the substantia nigra and the striatum. Localization of mRNA in the substantia nigra strongly suggests that the protein is expressed in the neuron terminals projecting in the striatum confirming our result [58]. Our finding of ^{125}I-MLT-binding sites in the striatum (this report and [59]) confirms these results on the protein level. Interestingly, MT_1 is part of a presynaptic protein network including synapsin, SNAP25 and 47, the voltage-gated calcium channel Cav2.2, Munc-18, rabphilin, and snapin to which DAT has now to be added [59].

Our study shows that MT_1 and MT_2 have a negative role on the capacity of DAT for DA reuptake. The presence of melatonin receptors could thus protect neurons from intensive DA reuptake. DA is known to form reactive oxygen species through enzymatic degradation. Elevated rate of intra-neuronal DA would thus increase the degeneration of the dopaminergic neurons characterized in several neurodegenerative illnesses as in Parkinson's disease, where melatonin receptors are concomitantly down-regulated [29, 60]. Intriguingly, the effect of MT_1 and MT_2 on DA uptake appears to be completely independent on stimulation of these receptors by melatonin or any constitutive receptor activity. This conclusion is further supported by the observation that ip injection of melatonin had no significant effect on amphetamine-induced locomotor activity in rats [61].

Melatonin represents the most reliable readout of the circadian rhythm of the biological master clock. According to our results, variation of melatonin levels along the circadian cycle is unlikely to impact on DAT function and the formation of the melatonin receptor DAT complex. In contrast, variation of MT_1 and MT_2 receptor levels might be important as they are submitted to a circadian regulation due to a negative feedback loop that exists between melatonin and the expression of its receptors [62]. This regulatory mechanism might be just one component of a complex network of circadian regulation of the dopaminergic system that involves circadian variation of DA levels itself, dopamine D2 and D4 receptors and diurnal rhythms in quinpirole-induced locomotor behaviors [63–65]. In turn, DA has also been shown to regulate major components of the central circadian clock mechanism like Per2 expression in the dorsal striatum [66]. Melatonin has been suggested to modify the dopaminergic system through additional, receptor-independent,

mechanisms. Long-term treatment with melatonin prevented DAT down-regulation in a rotenone-induced PD model [67] and a model of amphetamine-induced hippocampal damage [68], most likely through its antioxidant properties.

Taken together, our findings establish a new relationship between melatonin receptors and the control of extracellular DA levels by regulating the DA transport capacity of DAT, particularly in the striatum, as indicated by the enhanced DA uptake in striatal synaptosomes of MT_1-KO and MT_2-KO mice. Given the fact that DAT activity is considered to reflect the general state of dopaminergic circuit, and DAT alterations go together with dopaminergic deregulations in many diseases such as Parkinson's disease [3] and depression [69], our findings warrant further investigation.

Acknowledgements We thank Drs. Michel Bouvier and Bianca Plouffe (University of Montréal) for continues support and advice in BRET experiments, respectively, Anne-Sophie Journé (Institut Cochin) for her implication in mice breeding and expert advice in BRET and Dr. S. Doly (Institut Cochin) for critical comments on the manuscript. This work was supported by Grants from the Fondation Recherche Médicale (Equipe FRM FRM DEQ 20130326503 to R.J.), Inserm, CNRS and the Who am I? laboratory of excellence No. ANR-11-LABX-0071 funded by the French Government through its "Investments for the Future" program operated by The French National Research Agency (ANR) under Grant No. ANR-11-IDEX-0005-01.

Author contributions AB, AH, MLB, AK, MW, VW, and RJ performed research and/or analyzed data; RJ, AB, and AH wrote the manuscript; IS and PD reviewed and/or edited the manuscript and contributed to discussion; PD and RJ managed the project.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

1. Bjorklund A, Dunnett SB (2007) Fifty years of dopamine research. Trends Neurosci 30:185–187
2. O'Neill B, Tilley MR, Han DD, Thirtamara-Rajamani K, Hill ER, Bishop GA, Zhou FM, During MJ, Gu HH (2014) Behavior of knock-in mice with a cocaine-insensitive dopamine transporter after virogenetic restoration of cocaine sensitivity in the striatum. Neuropharmacology 79:626–633
3. Ishibashi K, Oda K, Ishiwata K, Ishii K (2014) Comparison of dopamine transporter decline in a patient with Parkinson's disease and normal aging effect. J Neurol Sci 339:207–209
4. Laakso A, Bergman J, Haaparanta M, Vilkman H, Solin O, Syvalahti E, Hietala J (2001) Decreased striatal dopamine transporter binding in vivo in chronic schizophrenia. Schizophr Res 52:115–120
5. Dougherty DD, Bonab AA, Spencer TJ, Rauch SL, Madras BK, Fischman AJ (1999) Dopamine transporter density in patients with attention deficit hyperactivity disorder. Lancet 354:2132–2133
6. German CL, Hanson GR, Fleckenstein AE (2012) Amphetamine and methamphetamine reduce striatal dopamine transporter function without concurrent dopamine transporter relocalization. J Neurochem 123:288–297
7. Eriksen J, Jorgensen TN, Gether U (2010) Regulation of dopamine transporter function by protein-protein interactions: new discoveries and methodological challenges. J Neurochem 113:27–41
8. Markota M, Sin J, Pantazopoulos H, Jonilionis R, Berretta S (2014) Reduced dopamine transporter expression in the amygdala of subjects diagnosed with schizophrenia. Schizophr Bull 40:984–991
9. Ng J, Zhen J, Meyer E, Erreger K, Li Y, Kakar N, Ahmad J, Thiele H, Kubisch C, Rider NL, Morton DH, Strauss KA, Puffenberger EG, D'Agnano D, Anikster Y, Carducci C, Hyland K, Rotstein M, Leuzzi V, Borck G, Reith ME, Kurian MA (2014) Dopamine transporter deficiency syndrome: phenotypic spectrum from infancy to adulthood. Brain 137:1107–1119
10. Lin Z, Zhang PW, Zhu X, Melgari JM, Huff R, Spieldoch RL, Uhl GR (2003) Phosphatidylinositol 3-kinase, protein kinase C, and MEK1/2 kinase regulation of dopamine transporters (DAT) require N-terminal DAT phosphoacceptor sites. J Biol Chem 278:20162–20170
11. Miranda M, Wu CC, Sorkina T, Korstjens DR, Sorkin A (2005) Enhanced ubiquitylation and accelerated degradation of the dopamine transporter mediated by protein kinase C. J Biol Chem 280:35617–35624
12. Miranda M, Dionne KR, Sorkina T, Sorkin A (2007) Three ubiquitin conjugation sites in the amino terminus of the dopamine transporter mediate protein kinase C-dependent endocytosis of the transporter. Mol Biol Cell 18:313–323
13. Cowell RM, Kantor L, Hewlett GH, Frey KA, Gnegy ME (2000) Dopamine transporter antagonists block phorbol ester-induced dopamine release and dopamine transporter phosphorylation in striatal synaptosomes. Eur J Pharmacol 389:59–65
14. Khoshbouei H, Sen N, Guptaroy B, Johnson L, Lund D, Gnegy ME, Galli A, Javitch JA (2004) N-terminal phosphorylation of the dopamine transporter is required for amphetamine-induced efflux. PLoS Biol 2:E78
15. Sitte HH, Freissmuth M (2015) Amphetamines, new psychoactive drugs and the monoamine transporter cycle. Trends Pharmacol Sci 36:41–50
16. Torres GE (2006) The dopamine transporter proteome. J Neurochem 97(Suppl 1):3–10
17. Kivell BM, Kahlig K, Galli A, Javitch JA, Shippenberg TS (2004) Regulation of dopamine transporter function and cell surface expression by kappa opioid receptors. In: 34th annual meeting of the society for neuroscience, San Diego
18. Bolan EA, Shan L, Devi LA, Javitch JA, Shippenberg TS (2004) Regulation of dopamine transporter function by kappa-opioid receptors: role of protein trafficking. In: 34th annual meeting of the society for neuroscience, San Diego
19. Pei L, Lee F, Mirecki A, Zou S, Zhang H, Vukusic B, Liu F (2004) Regulation of dopamine transporter function by dopamine D2 receptors. In: 34th annual meeting of the society for neuroscience, San Diego
20. Marazziti D, Mandillo S, Di Pietro C, Golini E, Matteoni R, Tocchini-Valentini GP (2007) GPR37 associates with the dopamine transporter to modulate dopamine uptake and behavioral responses to dopaminergic drugs. Proc Natl Acad Sci USA 104:9846–9851
21. Jiang H, Jiang Q, Feng J (2004) Parkin increases dopamine uptake by enhancing the cell surface expression of dopamine transporter. J Biol Chem 279:54380–54386
22. Lee FJ, Pei L, Moszczynska A, Vukusic B, Fletcher PJ, Liu F (2007) Dopamine transporter cell surface localization facilitated by a direct interaction with the dopamine D2 receptor. EMBO J 26:2127–2136
23. Kivell B, Uzelac Z, Sundaramurthy S, Rajamanickam J, Ewald A, Chefer V, Jaligam V, Bolan E, Simonson B, Annamalai B, Mannangatti P, Prisinzano TE, Gomes I, Devi LA, Jayanthi LD,

Sitte HH, Ramamoorthy S, Shippenberg TS (2014) Salvinorin A regulates dopamine transporter function via a kappa opioid receptor and ERK1/2-dependent mechanism. Neuropharmacology 86:228–240
24. Zisapel N, Egozi Y, Laudon M (1982) Inhibition of dopamine release by melatonin: regional distribution in the rat brain. Brain Res 246:161–163
25. Escames G, Acuna Castroviejo D, Vives F (1996) Melatonin-dopamine interaction in the striatal projection area of sensorimotor cortex in the rat. NeuroReport 7:597–600
26. Dubocovich ML, Delagrange P, Krause DN, Sugden D, Cardinali DP, Olcese J (2010) International Union of Basic and Clinical Pharmacology. LXXV. Nomenclature, classification, and pharmacology of G protein-coupled melatonin receptors. Pharmacol Rev 62:343–380
27. Jockers R, Delagrange P, Dubocovich ML, Markus RP, Renault N, Tosini G, Cecon E, Zlotos DP (2016) Update on melatonin receptors. IUPHAR review. Br J Pharmacol 173:2702–2725
28. Zlotos DP, Jockers R, Cecon E, Rivara S, Witt-Enderby PA (2014) MT1 and MT2 melatonin receptors: ligands, models, oligomers, and therapeutic potential. J Med Chem 57:3161–3185
29. Adi N, Mash DC, Ali Y, Singer C, Shehadeh L, Papapetropoulos S (2010) Melatonin MT1 and MT2 receptor expression in Parkinson's disease. Med Sci Monit 16:BR61–67
30. Brunner P, Sozer-Topcular N, Jockers R, Ravid R, Angeloni D, Fraschini F, Eckert A, Muller-Spahn F, Savaskan E (2006) Pineal and cortical melatonin receptors MT1 and MT2 are decreased in Alzheimer's disease. Eur J Histochem 50:311–316
31. Bendz LM, Scates AC (2010) Melatonin treatment for insomnia in pediatric patients with attention-deficit/hyperactivity disorder. Ann Pharmacother 44:185–191
32. Monteleone P, Natale M, La Rocca A, Maj M (1997) Decreased nocturnal secretion of melatonin in drug-free schizophrenics: no change after subchronic treatment with antipsychotics. Neuropsychobiology 36:159–163
33. Karamitri A, Renault N, Clement N, Guillaume JL, Jockers R (2013) Minireview: toward the establishment of a link between melatonin and glucose homeostasis: association of melatonin MT2 receptor variants with type 2 diabetes. Mol Endocrinol 27:1217–1233
34. Chen N, Reith ME (2008) Substrates dissociate dopamine transporter oligomers. J Neurochem 105:910–920
35. Bolan EA, Kivell B, Jaligam V, Oz M, Jayanthi LD, Han Y, Sen N, Urizar E, Gomes I, Devi LA, Ramamoorthy S, Javitch JA, Zapata A, Shippenberg TS (2007) D2 receptors regulate dopamine transporter function via an extracellular signal-regulated kinases 1 and 2-dependent and phosphoinositide 3 kinase-independent mechanism. Mol Pharmacol 71:1222–1232
36. Lam VM, Beerepoot P, Angers S, Salahpour A (2013) A novel assay for measurement of membrane-protein surface expression using a beta-lactamase. Traffic 14:778–784
37. Snider J, Kittanakom S, Damjanovic D, Curak J, Wong V, Stagljar I (2010) Detecting interactions with membrane proteins using a membrane two-hybrid assay in yeast. Nat Protoc 5:1281–1293
38. Gietz RD, Woods RA (2006) Yeast transformation by the LiAc/SS Carrier DNA/PEG method. Methods Mol Biol 313:107–120
39. Gainetdinov RR, Fumagalli F, Wang YM, Jones SR, Levey AI, Miller GW, Caron MG (1998) Increased MPTP neurotoxicity in vesicular monoamine transporter 2 heterozygote knockout mice. J Neurochem 70:1973–1978
40. Guillaume JL, Daulat AM, Maurice P, Levoye A, Migaud M, Brydon L, Malpaux B, Borg-Capra C, Jockers R (2008) The PDZ protein mupp1 promotes Gi coupling and signaling of the Mt1 melatonin receptor. J Biol Chem 283:16762–16771
41. Cecon E, Chen M, Marcola M, Fernandes PA, Jockers R, Markus RP (2015) Amyloid beta peptide directly impairs pineal gland melatonin synthesis and melatonin receptor signaling through the ERK pathway. FASEB J 29:2566–2582
42. Thomas L, Purvis CC, Drew JE, Abramovich DR, Williams LM (2002) Melatonin receptors in human fetal brain: 2-[I-125] iodomelatonin binding and MT1 gene expression. J Pineal Res 33:218–224
43. Huang X, Dai FF, Gaisano G, Giglou K, Han J, Zhang M, Kittanakom S, Wong V, Wei L, Showalter AD, Sloop KW, Stagljar I, Wheeler MB (2013) The identification of novel proteins that interact with the GLP-1 receptor and restrain its activity. Mol Endocrinol 27:1550–1563
44. Meister B, Elde R (1993) Dopamine transporter mRNA in neurons of the rat hypothalamus. Neuroendocrinology 58:388–395
45. Mercier JF, Salahpour A, Angers S, Breit A, Bouvier M (2002) Quantitative assessment of beta 1 and beta 2-adrenergic receptor homo and hetero-dimerization by bioluminescence resonance energy transfer. J Biol Chem 277:44925–44931
46. Couturier C, Jockers R (2003) Activation of the leptin receptor by a ligand-induced conformational change of constitutive receptor dimers. J Biol Chem 278:26604–26611
47. Ayoub MA, Levoye A, Delagrange P, Jockers R (2004) Preferential formation of MT1/MT2 melatonin receptor heterodimers with distinct ligand interaction properties compared with MT2 homodimers. Mol Pharmacol 66:312–321
48. Couturier C, Sarkis C, Seron K, Belouzard S, Chen P, Lenain A, Corset L, Dam J, Vauthier V, Dubart A, Mallet J, Froguel P, Rouille Y, Jockers R (2007) Silencing of OB-RGRP in mouse hypothalamic arcuate nucleus increases leptin receptor signaling and prevents diet-induced obesity. Proc Natl Acad Sci USA 104:19476–19481
49. Brydon L, Roka F, Petit L, deCoppet P, Tissot M, Barrett P, Morgan PJ, Nanoff C, Strosberg AD, Jockers R (1999) Dual signaling of human Mel1a melatonin receptors via G(I2), G(I3), and G(Q/11) proteins. Mol Endocrinol 13:2025–2038
50. Devavry S, Legros C, Brasseur C, Delagrange P, Spadoni G, Cohen W, Malpaux B, Boutin JA, Nosjean O (2012) Description of the constitutive activity of cloned human melatonin receptors hMT(1) and hMT(2) and discovery of inverse agonists. J Pineal Res 53:29–37
51. Binda F, Dipace C, Bowton E, Robertson SD, Lute BJ, Fog JU, Zhang M, Sen N, Colbran RJ, Gnegy ME, Gether U, Javitch JA, Erreger K, Galli A (2008) Syntaxin 1A interaction with the dopamine transporter promotes amphetamine-induced dopamine efflux. Mol Pharmacol 74:1101–1108
52. Egana LA, Cuevas RA, Baust TB, Parra LA, Leak RK, Hochendoner S, Pena K, Quiroz M, Hong WC, Dorostkar MM, Janz R, Sitte HH, Torres GE (2009) Physical and functional interaction between the dopamine transporter and the synaptic vesicle protein synaptogyrin-3. J Neurosci 29:4592–4604
53. Giros B, el Mestikawy S, Godinot N, Zheng K, Han H, Yang-Feng T, Caron MG (1992) Cloning, pharmacological characterization, and chromosome assignment of the human dopamine transporter. Mol Pharmacol 42:383–390
54. Huff RA, Vaughan RA, Kuhar MJ, Uhl GR (1997) Phorbol esters increase dopamine transporter phosphorylation and decrease transport V_{max}. J Neurochem 68:225–232
55. Erdozain AM, De Gois S, Bernard V, Gorgievski V, Pietrancosta N, Dumas S, Macedo CE, Vanhoutte P, Ortega JE, Meana JJ, Tzavara ET, Vialou V, Giros B (2018) Structural and functional characterization of the interaction of Snapin with the dopamine transporter: differential modulation of psychostimulant actions. Neuropsychopharmacology 43:1041–1051
56. Afonso-Oramas D, Cruz-Muros I, Alvarez de la Rosa D, Abreu P, Giraldez T, Castro-Hernandez J, Salas-Hernandez J, Lanciego JL, Rodriguez M, Gonzalez-Hernandez T (2009) Dopamine transporter glycosylation correlates with the vulnerability of

midbrain dopaminergic cells in Parkinson's disease. Neurobiol Dis 36:494–508
57. Sorkina T, Doolen S, Galperin E, Zahniser NR, Sorkin A (2003) Oligomerization of dopamine transporters visualized in living cells by fluorescence resonance energy transfer microscopy. J Biol Chem 278:28274–28283
58. Uz T, Arslan AD, Kurtuncu M, Imbesi M, Akhisaroglu M, Dwivedi Y, Pandey GN, Manev H (2005) The regional and cellular expression profile of the melatonin receptor MT1 in the central dopaminergic system. Brain Res Mol Brain Res 136:45–53
59. Benleulmi-Chaachoua A, Chen L, Sokolina K, Wong V, Jurisica I, Emerit MB, Darmon M, Espin A, Stagljar I, Tafelmeyer P, Zamponi GW, Delagrange P, Maurice P, Jockers R (2016) Protein interactome mining defines melatonin MT1 receptors as integral component of presynaptic protein complexes of neurons. J Pineal Res 60:95–108
60. Haavik J, Almas B, Flatmark T (1997) Generation of reactive oxygen species by tyrosine hydroxylase: a possible contribution to the degeneration of dopaminergic neurons? J Neurochem 68:328–332
61. Palaoglu S, Palaoglu O, Akarsu ES, Ayhan IH, Ozgen T, Erbengi A (1994) Behavioural assessment of pinealectomy and foetal pineal gland transplantation in rats: Part II. Acta Neurochir (Wien) 128:8–12
62. Poirel VJ, Masson-Pevet M, Pevet P, Gauer F (2002) MT1 melatonin receptor mRNA expression exhibits a circadian variation in the rat suprachiasmatic nuclei. Brain Res 946:64–71
63. Akhisaroglu M, Kurtuncu M, Manev H, Uz T (2005) Diurnal rhythms in quinpirole-induced locomotor behaviors and striatal D2/D3 receptor levels in mice. Pharmacol Biochem Behav 80:371–377
64. Ikeda E, Matsunaga N, Kakimoto K, Hamamura K, Hayashi A, Koyanagi S, Ohdo S (2013) Molecular mechanism regulating 24-hour rhythm of dopamine D3 receptor expression in mouse ventral striatum. Mol Pharmacol 83:959–967
65. Gonzalez S, Moreno-Delgado D, Moreno E, Perez-Capote K, Franco R, Mallol J, Cortes A, Casado V, Lluis C, Ortiz J, Ferre S, Canela E, McCormick PJ (2012) Circadian-related heteromerization of adrenergic and dopamine D(4) receptors modulates melatonin synthesis and release in the pineal gland. PLoS Biol 10:e1001347
66. Hood S, Cassidy P, Cossette MP, Weigl Y, Verwey M, Robinson B, Stewart J, Amir S (2010) Endogenous dopamine regulates the rhythm of expression of the clock protein PER2 in the rat dorsal striatum via daily activation of D2 dopamine receptors. J Neurosci 30:14046–14058
67. Lin CH, Huang JY, Ching CH, Chuang JI (2008) Melatonin reduces the neuronal loss, downregulation of dopamine transporter, and upregulation of D2 receptor in rotenone-induced parkinsonian rats. J Pineal Res 44:205–213
68. Leeboonngam T, Pramong R, Sae-Ung K, Govitrapong P, Phansuwan-Pujito P (2018) Neuroprotective effects of melatonin on amphetamine-induced dopaminergic fiber degeneration in the hippocampus of postnatal rats. J Pineal Res. https://doi.org/10.1111/jpi.12456
69. Hsiao MC, Lin KJ, Liu CY, Schatz DB (2013) The interaction between dopamine transporter function, gender differences, and possible laterality in depression. Psychiatry Res 211:72–77