

PRONOUNCED HYPERACTIVITY, COGNITIVE DYSFUNCTIONS AND BDNF DYSREGULATION IN DOPAMINE TRANSPORTER KNOCKOUT RATS

Abbreviated title: DOPAMINERGIC DYSREGULATION IN DAT-KO RATS

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Abstract

Dopamine (DA) controls many vital physiological functions and is critically involved in several neuropsychiatric disorders such as schizophrenia and attention deficit hyperactivity disorder (ADHD). The major function of the plasma membrane dopamine transporter (DAT) is the rapid uptake of released DA into presynaptic nerve terminals leading to control of both the extracellular levels of DA and the intracellular stores of DA. Here, we present a newly developed strain of rats (DAT-knockout, DAT-KO rats) in which the gene encoding the DAT has been disrupted by using zinc finger nuclease technology (ZFN). DAT-KO rats develop normally but weigh less than heterozygote and wild-type rats and demonstrate pronounced spontaneous locomotor hyperactivity. While striatal extracellular DA lifetime and concentrations are significantly increased, the total tissue content of DA is markedly decreased demonstrating the key role of DAT in the control of DA neurotransmission. Hyperactivity of DAT-KO rats can be counteracted by amphetamine (AMPH), methylphenidate and a few other compounds exerting inhibitory action on DA-dependent hyperactivity. DAT-KO rats also demonstrate a deficit in working memory and sensorimotor gating tests and show strong dysregulation in frontostriatal BDNF function. DAT-KO rats could provide a novel translational model for human diseases involving aberrant DA function and/or mutations affecting the DAT or related regulatory mechanisms.

Significance statement

Here, we present a newly developed strain of rats in which the gene encoding the DAT has been disrupted (DAT-KO rats). DAT-KO rats weigh less than controls and demonstrate

pronounced spontaneous locomotor hyperactivity. While striatal extracellular DA lifetime and concentrations are increased, the total tissue content of DA is markedly decreased demonstrating the key role of DAT in the control of DA neurotransmission. Hyperactivity of DAT-KO rats can be counteracted by amphetamine (AMPH), methylphenidate and a few other compounds exerting inhibitory action on DA-dependent hyperactivity. DAT-KO rats also demonstrate a deficit in working and show strong dysregulation in frontostriatal BDNF function. DAT-KO rats could provide a novel translational model for human diseases involving aberrant DA functions.

Introduction

Dopaminergic innervations are prominent in the brain and the dopamine (DA) system exerts modulatory control of motivation, reward, cognition and locomotion (Carlsson, 1987; Gainetdinov, 2008). Concentration of DA in the synaptic cleft is the primary determinant of DA signaling intensity. The key regulatory element of DA neurotransmission is the DAT, belonging to a family of plasma membrane transporters of solute carrier family 6 (*SLC6*). The DAT controls levels of extracellular DA and maintains DA stores by transporting released DA back into neurons. DAT is the well-established target of many drugs of abuse and neurotoxins (Saunders et al., 2000; Kahlig et al., 2006; Wheeler et al., 2015; Siciliano et al., 2016). Amphetamine (AMPH) and cocaine (COC) are psychostimulants that are able to induce euphoria and hyperactivity by increasing extracellular DA via interaction with the DAT.

The DAT knockout (KO) mouse model generated in 1996 by Giros and colleagues (Giros et al., 1996). DAT-KO mice display a distinct behavioural phenotype: they are hyperactive, display certain cognitive deficits and sleep dysregulation (Gainetdinov et al., 1999; Spielewoy et al., 2000). The hyperdopaminergic phenotype of DAT-KO mice have provided a simple model of hyperdopaminergic function in which the effects of various pharmacological agents affecting DA related functions and behaviours have been evaluated. In particular, they are extremely sensitive to D2 dopamine receptor blockers and antipsychotics such as haloperidol and show a paradoxical inhibitory response to psychostimulants AMPH and methylphenidate (MPH) (Gainetdinov et al., 1999; Spielewoy et al., 2000; Carboni et al., 2001).

DAT-KO mice have provided important information on the pathological consequences of aberrant DA function. DAT-KO mice are believed to best model Attention Deficit Hyperactive Disorders (ADHD) endophenotypes by demonstrating spontaneous hyperactivity, deficits in cognitive tests and anti-hyperkinetic responses to psychostimulants used in ADHD treatments (Gainetdinov and

Caron, 2000, 2001). At the same time, DAT-KO animals have provided numerous advances in understanding the pathology and pharmacology of other dopamine-related brain disorders, such as schizophrenia (Gainetdinov et al., 2001; Wong et al., 2012, 2015), bipolar disorder (Beaulieu et al., 2005, 2005), Parkinson's disease (Cyr et al., 2003; Sotnikova et al., 2005) and addiction (Rocha et al., 1998). Notably, several patients diagnosed with ADHD, bipolar disorder and parkinsonism (Vaughan and Foster, 2013; Hansen et al., 2014) have rare coding variants of the SLC6A3 DAT gene. Recently, DAT-KO mice were used to evaluate the efficacy of adenoviral therapy for Dopamine Transporter Deficiency Syndrome (DTDS) (Illiano et al., 2017), a newly recognized Parkinsonian-like condition with earlier hyperkinetic stage, whose symptomatology is directly caused by an impaired DAT functioning due to loss-of-function mutations found in SLC6A3 DAT gene (Kurian et al., 2009, 2011; Ng et al., 2014; Yildiz et al., 2017).

The recent progress in the development of gene editing approaches has made possible to perform such studies in genetically altered rats. Beyond obvious advantages of rat models, such as larger brain size for surgery and electrophysiological recordings as well as their closer physiological similarity to humans, rats have a much wider repertoire of well-established behavioural approaches to investigate cognitive functions that are critical for modelling neuropsychiatric conditions.

Here we present a new model of DAT deficiency, DAT-KO rats, generated by using zinc-finger nucleases (ZFN) technology (Geurts et al., 2009; Brown et al., 2013) used for the elimination of the DAT gene. The current study describes detailed neurochemical, behavioural, and pharmacological characterization of this model that could open new perspectives in understanding pathology and pharmacology of human diseases involving aberrant DA function and/or mutations affecting the DAT or DAT-related regulatory mechanisms.

Materials and methods

Animals

ZFN design, construction, in vitro validation, microinjection and founder selection were performed as previously described (Geurts et al., 2009; Carbery et al., 2010). The ZFN Target site was: CTCATCAACCCGCCACAGAcaccaGTGGAGGCTCAAGAG in the Exon 2 of Slc6a3 gene (NCBI Gene ID: 24898; Genomic NCBI Ref Seq: NC_005100.3; mRNA NCBI Ref Seq: NM_012694.2). The knockout rat lines were created in the outbred Wistar Han background at SAGE Labs. Adult littermate rats were housed by genotype in groups of 3 to 4 with free access to tap water and standard pellet food. They were kept at 22°C and on a 12/12 h light/dark cycle (lights on 0700–1900 h). All experiments were conducted in compliance with the Italian Ministry of Health (DL 116/92; DL 111/94-B) and European Community (86/609/EEC) directives regulating animal research. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Genotyping was performed by PCR followed by enzymatic digestion with BtsI MuiI (New England Biolabs (Milan, Italy)).

Real-Time PCR

Animals were sacrificed and PreFrontal Cortex (PFC), dorsolateral striatum (DLStr) and midbrain were dissected and dissociated for 15 minutes at 37°C with Pronase enzyme (Sigma) in Hank's Balanced Salt Solution (HBSS, Invitrogen). Brain samples were triturated with three glass pipettes of decreasing tip diameter and centrifuged at 900 rpm at room temperature for 5 minutes. To remove excess debris, cell pellets were resuspended in HBSS and filtered through a 70 µm mesh (BD Falcon, #352350). Cells-to-CT kit (Life Technology) was used to produce DNase I digested cell lysates and perform cDNA synthesis, according to manufacturer's instructions. cDNAs were

used for Taqman singleplex PCR. To prevent false positive originating from genomic DNA, we used negative control samples without reverse transcriptase. All reagents were supplied by Applied Biosystems. PCR master mix contained 1x Taqman Universal PCR Master Mix, 1x Gene Expression Assay mix, and 1µl cDNA for a total volume of 20µl. The following Gene Expression Assays were used: Drd2 (Assay ID Rn01452984_m1), Drd1a (Assay ID Rn00569454_m1), TH (Assay ID Rn00562500_m1); Gapdh (Assay ID Rn01775763_g1) and Hprt (Assay ID Rn01527840_m1). Samples were run in three replicates for each Gene Expression Assay. PCR reactions were carried out on a 7900 Thermal Cycler (Applied Biosystems) with 40 cycles of 95°C for 15 seconds and 60 °C for 1 minute. CT values for each gene were normalized to CT values for GAPDH and HPRT to obtain a relative expression level for each replicate and the three replicates were averaged together.

For BDNF analysis, an aliquot of total RNA of each sample (n=5 WT and n=5 DAT-KO rats) was treated with DNase to avoid DNA contamination. RNA was analyzed by a TaqMan qRT-PCR instrument (CFX384 real-time system, Bio-Rad Laboratories) using the iScript™ one-step RT-PCR kit for probes (Bio-Rad Laboratories). Samples were run in 384-well formats in triplicate as multiplexed reactions. Data were analyzed with the comparative threshold cycle ($\Delta\Delta C_t$) method using 36B4 as reference gene. The primer efficiencies were experimentally set up for each couple of primers.

Probes and primers for total BDNF, Creb, CaRF, Npas4 and 36B4 were purchased from Eurofins MWG-Operon (Ebersberg, Germany) and their sequences are shown below:

- total BDNF: forward primer 5'-AAGTCTGCATTACATTCCTCGA-3', reverse primer 5'-GTTTTCTGAAAGAGGGACAGTTTAT-3', probe 5'-TGTGGTTTGTTGCCGTTGCCAAG-3';
- Creb: forward primer 5'-AGATTCTAGTGCCCAGCAAC-3', reverse primer 5'-CTGTGCGAATCTGGTATGTTTG-3', probe 5'-TGTTCAAGCTGCCTCTGGTGATGT-3';
- Npas4: forward primer 5'-TCATTGACCCTGCTGACCAT-3', reverse primer 5'-AAGCACCAGTTTGTTCCTG-3', probe 5'-TGATCGCCTTTTCCGTTGTC-3';

- CaRF: forward primer 5'-GAGATGCACACACCATTCCA-3', reverse primer 5'-GTGTTGGCTCATTGGGTCT-3', probe 5'-CAGCCATCCAGCTCTTGTTGAAGA-3';
- 36B4: forward primer 5'-TTCCCACTGGCTGAAAAGGT-3', reverse primer 5'-CGCAGCCGCAAATGC-3', probe 5'-AAGGCCTTCCTGGCCGATCCATC-3'.

Probes and primers for BDNF exon IV and BDNF exon VI were purchased from Life Technologies (Milan, Italy) (BDNF exon IV: ID Rn01484927_m1 and BDNF exon VI: ID Rn01484928_m1). Thermal cycling was started with 10-min incubation at 50 °C (RNA retrotranscription) then 5 min at 95 °C (TaqMan polymerase activation). After this, 39 PCR cycles were run. Each PCR cycle consisted of heating the samples at 95 °C for 10 s to enable the melting process, then for 30 s at 60 °C for the annealing and extension reaction.

Protein extraction and western blotting

Protein extraction and preparation of samples for Western blot analysis were performed as described (Vecchio et al, 2017). Briefly, the tissues were dissected from freshly harvested brains. Brain samples were mechanically homogenized in RIPA buffer (50 mmol/l Tris-HCl, pH 7.4/150 mmol/l NaCl/1% Nonidet P-40/0.5% sodium deoxycholate/0.1% SDS; Sigma) plus protease inhibitor mixture (Roche 1873580), and protein concentration was measured using a BCA protein assay kit (Thermo Scientific). Protein extracts (25 µg) were separated by 10% SDS/PAGE and transferred to nitrocellulose membranes (GE Healthcare, Milan, Italy). Blots were immunostained overnight at 4°C with the following primary antibodies: Gapdh (FL-335): sc-25778, Santa Cruz Biotechnology, Inc., Heidelberg, Germany); DAT (C-20): sc-1433, Santa Cruz Biotechnology, Inc., Heidelberg, Germany). After washing, the membranes were incubated for 2 hours at room temperature with the appropriate secondary antibody (anti-mouse, anti-rabbit or anti-rat). Following secondary antibody incubations, membranes were washed and finally incubated with ECL detection reagent (Amersham RPN2232) for 5 minutes.

For BDNF analysis, medial prefrontal cortex (PFC) was dissected from a 2-mm section extending

from approximately bregma +5.16 to +3.24 (Paxinos and Watson, 2005) and dorsolateral striatum (DLStr) was dissected from a 2-mm section immediately caudal to the PFC section from n=5 WT and n=5 DAT-KO rats. Brain areas were immediately frozen on dry ice and stored at -80°C until being processed for molecular analysis. Protein extraction was performed as previously described (Caffino et al., 2017) with minor modifications. Briefly, PFC and DLStr were homogenized in a Teflon-glass potter in cold 0.32 M sucrose buffer pH 7.4 containing 1 mM HEPES, 0.1 mM EGTA and 0.1 mM PMSF, in presence of commercial cocktails of protease (Roche, Monza, Italy) and phosphatase (Sigma-Aldrich, Milan, Italy) inhibitors and then sonicated. The homogenate of DLStr was centrifuged at 800g for 5 min, the obtained supernatant was centrifuged at 13,000 g for 15 min obtaining a pellet (P2) and a supernatant (S2), referred as the cytosolic fraction. P2 pellet was resuspended in buffer containing 150 mM KCl and 1% Triton X-100 and centrifuged at 100,000 x g for 1 h. The resulting supernatant, referred as Triton X-100 soluble fraction (TSF), was stored at -20°C; the pellet, referred as PSD or Triton X-100 insoluble fraction (TIF), was homogenized in a glass-glass potter in 20 mM HEPES, protease and phosphatase inhibitors and stored at -20°C in presence of 30% glycerol. Total protein content was measured according to the Bradford Protein Assay procedure (Bio-Rad, Milan, Italy) using bovine serum albumin as calibration standard. Equal amounts of protein were run under reducing conditions on the criterion TGX precast gels (Bio-Rad Laboratories, Milan, Italy) and then electrophoretically transferred onto nitrocellulose membranes (Bio-Rad, Milan, Italy). Blots were blocked one hour at room temperature with 10% non-fat dry milk in TBS + 0.1% Tween-20 buffer, incubated with antibodies against the phosphorylated forms of the proteins and then stripped and reprobed with the antibodies against corresponding total proteins. The conditions of the primary antibodies were the following: mBDNF (1:1000, Icosagen, San Francisco CA, USA); anti phospho trkB Y706 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti total trkB (1:750, Santa Cruz Biotechnology); anti phospho CaMKII T286 (1:2000, Thermo Scientific, Waltham, MA, USA); anti total CaMKII (1:5000, Chemicon, Temecula, CA, USA); anti total PSD95 (1:4000, Cell Signaling Technology) and anti β -Actin (1:10000, Sigma-

Aldrich, Milano, Italy). Results were standardized using β -actin as the control protein, which was detected by evaluating the band density at 43 kDa. Immunocomplexes were visualized by chemiluminescence using the Chemidoc MP Imaging System (Bio-Rad Laboratories) and analyzed using the Image Lab software from Bio-Rad Laboratories. The activation of the proteins investigated were expressed as a ratio between the phosphorylated and the respective total forms.

In vivo microdialysis

In vivo brain microdialysis was performed in the right dorsal striatum (DStr) of freely moving rats (Carboni et al., 2001; Budygin et al., 2004) using concentric microdialysis probes (2 mm membrane length cut off 6000 Da; CMA-11, CMA/Microdialysis, Solna, Sweden). Stereotaxic coordinates for the position of the probes were chosen according to the atlas of Franklin and Paxinos (1997) and are relative to the bregma: AP 1.0; L 3.0; DV -6.6. Prior to fixation in stereotaxic apparatus, the animals were anesthetized with an oxygen/isoflurane mixture. The probes were implanted in the brain vertically through a small drilled aperture in the skull and fixed with dental cement. During implantation into the brain and for 1 h afterward, the dialysis probes were perfused with artificial cerebrospinal fluid (aCSF) (NaCl 147 mM, KCl 2.7 mM, CaCl₂ 1.2 mM, MgCl₂ 0.85 mM; CMA Microdialysis). 1 h after the operation, the animals were returned to their home cages.

Approximately 24 h after surgery, the dialysis probes were connected to a syringe pump and perfused with the aCSF at 1.0 μ l/min for 60 min (equilibration period). To reliably determine the basal extracellular DA levels in the striatum of freely moving rats a quantitative “low perfusion” rate microdialysis experiment was conducted (Gainetdinov et al., 2003). The perfusate was collected at a perfusion rate of 0.1 μ l/min every 90 min over a 6 h period into collection tubes containing 2 μ l of 1 M perchloric acid. To determine effect of AMPH (3 mg/kg, i.p.) on the extracellular DA level a “conventional” microdialysis approach was used. Dialysis probes were

connected to a syringe pump and perfused with aCSF at 1 μ l/min for at least 60 min for equilibration. Then perfusate was collected every 20 min, for 1 hour before injection and 2 hours after injection of AMPH.

HPLC

HPLC measurements were carried out as described before (Leo et al., 2014). Brain tissues were dissected from WT, KO and HET rats and homogenized in 40 volumes of 0.1 M HClO₄. Following centrifugation and filtration, the samples were analyzed by HPLC as described below. The protocol for sample preparation for the HPLC determination of tissue monoamines and their metabolites was performed as previously described. Measurements of DA, 5-hydroxytryptamine (5-HT) and metabolites in tissue samples and DA in microdialysis samples were performed by HPLC with electrochemical detection (ALEXYS LC-EC system, Antec Leyden BV, Netherlands) with a 0.7-mm glass carbon electrode (Antec; VT-03). The system was equipped with a reverse-phase column (3- μ m particles, ALB-215 C18, 1x150 mm, Antec) at a flow rate of 200 μ l/min. The mobile phase contained 50 mM H₃PO₄, 50 mM citric acid, 8 mM KCl, 0.1 mM EDTA, 400 mg/l octanesulfonic acid sodium salt and 10% (vol/vol) methanol, pH 3.9. The sensitivity of this method permitted the detection of fmol DA. Dialyzate samples (11 μ l) were injected into HPLC without any additional purification.

Fast Scan Cyclic Voltammetry (FSCV)

The brains were sectioned in cold carboxygenated artificial cerebrospinal fluid (aCSF) (126 mM

NaCl, 2.5 mM KCl, 1.2 mM NaH₂PO₄, 25 mM NaHCO₃, 2.4 mM CaCl₂, 11 mM D-glucose, 1.2 mM MgCl₂) on a VT1000S vibrating microtome (Leica Microsystems, Nussloch, Germany) at a thickness of 300 μ m. Coronal slices containing the dorsal striatum were allowed to recover for at least 1 h at room temperature in carboxygenated aCSF. For recordings, slices were superfused with 32 °C carboxygenated aCSF at a flow rate of 1 ml/min. FSCV recordings started 20 min after transfer to the slice chamber. Carbon fiber electrodes (7 μ m diameter, Goodfellow, Huntingdon, England) were made as previously described (Kawagoe *et al*, 1993; Kuhr and Wightman, 1986a). The carbon fibers were trimmed with a scalpel to 80-120 μ m under a microscope (Nikon). A carbon fiber microelectrode was inserted into the slice and a twisted bipolar stimulating electrode (Plastics One, Roanoke, VA) was placed on the surface of the brain slice ~200 μ m away. The potential of the working electrode was held at -0.4 V and scanned to +1.3 V and back at 300 V/s. Axonal DA release in the striatum was evoked by a single biphasic electrical pulse (1 ms long, 400 μ A) every 2 min through a stimulus isolator (AM-system, Carlsborg, WA). Data were filtered to reduce noise. Oxidation and reduction peaks were observed at ~ +0.65 V and -0.2 V (vs. Ag/AgCl reference) identifying DA as the released chemical. Electrodes were calibrated in a flow injection system using 1 μ M DA (Sigma Aldrich, St. Louis, MO, USA).

FSCV kinetic analysis

There were several established criteria for choosing which DA signals to use for analysis, two of these being that there should be no confounding electrical artefacts to interfere with the DA traces, and no pH shifts during recordings, which allow for a flat baseline before stimulation and provide the most uncontaminated DA dynamics as possible. Next, a 10:1 signal to noise ratio was used in order to guarantee that the actual signal was separated from background. All of these criteria insured accuracy in analysis. One to two recordings from each experimental group were excluded from analysis based on these criteria.

Data analysis was performed using Demon Voltammetry software described (Yorgason *et al.*,

2011a). Briefly, computations were based on user-defined positions on current traces for baseline (Pre-Stim cursor), peak (Peak Cursor) and return to baseline (Post-Stim cursor) positions. Half-life values were determined from exponential fit curves based on Peak cursor and Post-Stim cursor positions using a least squares constrained exponential fit algorithm (National Instruments, Milan, Italy) (Yorgason et al., 2011a). These measurements were performed on individual traces within each experiment. These numbers were then averaged within each experimental group (WT, KO and KO treated) and reported as mean \pm SEM. (n=4 for WT and KO control; n=6 for KO treated group). Half-life is considered to be a reliable measure for evaluating changes in striatal DA clearance *in vivo* and *in vitro*. This parameter accurately distinguishes differences in clearance rate similar to other established measures (Yorgason *et al*, 2011).

Locomotor activity in a novel environment

Locomotor activity was evaluated as described before (Sukhanov et al., 2014) by using an automated Omnitech Digiscan apparatus (AccuScan Instruments, USA) under illuminated conditions. The apparatus included four open field monitors, each consisting of a set of 16 light beams arrayed in the horizontal X and Y axes. The hardware detected beams broken by the animal, allowing the software to determine the location of the rat in the cage. Cages were divided into four compartments (20 cm x 20 cm). Animals were tested individually for defined periods with 5-min intervals. The total distance traveled was measured and expressed in terms of centimeters traveled by the animal. In addition, vertical activity as expressed in terms of the number of beam breaks. All rats were habituated to the test room for at least 1 hour prior to testing. Effects of drugs on locomotor activity in a novel environment were tested 30 min after placement of animals into locomotor activity monitor. d-AMPH hydrochloride, MPH hydrochloride, haloperidol (Sigma–Aldrich, Co., St. Louis, MO) and RO5203648 (F. Hoffmann-La Roche Ltd., Basel, Switzerland) were dissolved as described (Gainetdinov et al, 1999; Spielewoy et al, 2000; Revel et al., 2012).

For all behavioral experiments, drugs were administered intraperitoneally (i.p.) in a volume of 1 ml/kg. All solutions were made fresh daily.

24-hour spontaneous locomotor activity in a home cage

Rats were continuously monitored for spontaneous home-cage locomotor activity by means of an automatic device equipped with small passive infrared sensors placed on a standard rack over the top of each home-cage (ActiVScope system; TechnoSmart, Rome, Italy). These sensors (20 Hz) detected any movement of rats: scores were automatically divided into 60-min intervals.

Y maze spontaneous alternation test

To measure spontaneous alternation behavior and exploratory activity, a white plastic material Y-maze with arms 40 cm (long) by 6 cm (wide) with 13 cm walls was used. Each animal was tested in a single 8-min session, during which the animal was placed in the central platform and allowed free exploration of the maze. Spontaneous alternation, expressed as a percentage, refers to ratio of arm choices differing from the previous two choices to the total number of arm entries.

Startle and PPI test

Startle and Pre-Pulse Inhibition (PPI) testing were performed as described (Frau et al., 2016). The apparatus used for detection of startle reflexes (Med Associates, St Albans, VT, USA) consists of four standard cages placed in sound-attenuated chambers with fan ventilation. Each cage consists of a Plexiglas cylinder of 9 cm diameter, mounted on a piezoelectric accelerometric platform connected to an analogue-digital converter. Two separate speakers convey background noise and acoustic bursts. Both speakers and startle cages are connected to a main PC, which detects and

analyzes all chamber variables with specific software. Before each testing session, acoustic stimuli and mechanical responses were calibrated.

The test begins with a 5-min acclimatization period, with a 70-dB background white noise, which continues for the entire session. The acclimatization period is followed by three blocks, each consisting of a sequence of trials: the first and the third block consist of five pulse-alone trials of 115 dB. The second block consists of a pseudorandom sequence of 50 trials, including 12 pulse-alone trials, 30 trials of pulse preceded by 74, 78, or 82 dB pre-pulses (10 for each level of pre-pulse loudness), and 8 no-pulse trials, where only the background noise is delivered. Inter-trial intervals (i.e., the time between two consecutive trials) are randomly selected between 10 and 15 s. The % PPI was calculated using the following formula: $100 - (\text{mean startle amplitude for pre-pulse pulse trials} / \text{mean startle amplitude for pulse alone trials}) \times 100$.

Results

Generation of DAT-KO rats

Slc6a3 (DAT) KO rats were generated by Sigma Advanced Genetic Engineering Labs (SAGE[®] Labs; Sigma–Aldrich Co., St. Louis, MO). DAT-KO rats were created by using ZFN technology that produces a 5 bp mutation and an early stop codon (Fig. 1A). The targeted DNA to be deleted (*Slc6a3* exon 2) contained a specific nucleotide sequence substrate for restriction enzyme BtsIMutI. According to our genetically modified model, disruption of this specific sequence causes the loss of restriction site for BtsIMutI enzyme, resulting in wild-type rats (WT) maintaining all restriction sites on both alleles while heterozygous (DAT-HET) rats bear one truncated allele and an intact one. DAT-KO rats lose both restriction enzyme sites as predicted. (Fig. 1B). PCR primers were designed according to protocol and amplified DNA was digested as shown (Fig. 1B and C). In DAT-KO rats no detection of digestion product was observed, due to the loss of restriction site on both alleles. DAT-HET rats show both the digested DNA at lower molecular weight and mutated DNA, while WT DNA amplicon was completely digested by BtsIMutI, thus resulting in one single band at lower molecular weight (Fig. 1B and C). We also verified the absence of DAT protein in KO animals in western blot from striatal tissue (Fig. 1D).

While there are reasonable concerns for potential off-targeting effects of gene editing nucleases (including ZFN) technologies, there are little chances for these events in DAT-KO rats. First, the ZFN designs were generated using a proprietary algorithm by bioinformatics team at SAGE Laboratories that screens each design for the top 20 most homologous sequences, as well as for repeat elements, SNPs, and splice variants. In addition, used ZFN contains specifically engineered obligate heterodimer FokI cleavage domains that help guard against off-targeting by increasing specificity to cut at only the desired site. In any case, founder animals were breed back to WT Wistar Han rats (Charles River, France) for 6 generations that should be sufficient to cross away

any potential unintended off-target effects that may have occurred even with rigorous screening in the ZFN design and construction phase.

Wistar Han DAT-KO rat colony was kept under HET-HET breeding. Surprisingly, DAT-KO rats do not show a propensity to premature death as DAT-KO mice (Giros et al., 1996). They are viable as the WT controls (followed up to 5 months) and the colony follows the expected Mendelian ratio at age 4 months (1:2:1; Fig. 1E). Analysis of body weight from birth up to adulthood (4 months) revealed a major effect of genotype in both male and female populations (Fig. 1F) with DAT-KO animals showing lower weights compared to HET and WT siblings (Fig. 1F).

Neurochemical characterization of striatal DA transmission in DAT-KO rats

Analysis of DA dynamics by Fast Scan Cyclic Voltammetry

In order to evaluate consequences of DAT gene deletion on the extracellular dynamics of DA we employed Fast Scan Cyclic Voltammetry (FSCV) technique on brain slices (Jones *et al.*, 1998). First, we detected the kinetics of evoked DA release and clearance following single pulse (400 μ A, 1 ms, biphasic) stimulation in DStr slices prepared from DAT-KO, DAT-HET and WT littermates. The oxidation peak occurred at approximately +0.6 V and the reduction peak at approximately -0.2 V (Fig. 2A, insert), consistent with the electrochemical characteristics of DA (Bradaia et al., 2009; Leo et al., 2014)(Jones et al., 2006; John and Jones, 2007). The maximal amplitude of DA overflow evoked by single pulses in the DStr was not significantly different between genotypes but the clearance of released DA was markedly prolonged in DAT-HET and DAT-KO rats (Fig. 2A, B, C). We evaluated the uptake kinetics from an exponential fit curve using a least squares-constrained exponential fit algorithm (Yorgason et al., 2011) and quantified the half-life parameters for an

estimation of DA uptake rates. Under basal conditions, the time to clear released DA was 1.3, 10 and 50 seconds respectively in WT, DAT-HET and DAT-KO rats (Fig. 2B).

Next, we evaluated the effect of cocaine on evoked DA release and clearance. As might be expected, application of 3 μ M cocaine progressively prolonged an amplitude of striatal DA outflow and clearance (John and Jones, 2007) in WT and DAT-HET, but not in DAT-KO rats (Fig. 2C, D). Further, in order to test whether serotonin transporter (SERT) could provide extracellular DA clearance via promiscuous uptake, we applied 10 μ M fluoxetine, a selective SERT blocker. As presented (Fig. 2E), fluoxetine did not affect DA clearance in any of the genotypes. To evaluate if DA metabolizing enzymes can contribute to the clearance of DA in the absence of DAT we then tested the effect of the inhibition of Catechol-O-methyl-transferase (COMT) by 10 μ M tolcapone (Fig. 2F) and monoamine oxidase (MAO) by 10 μ M pargyline (Fig. 2G). COMT inhibition did not alter the kinetics of DA clearance in any genotype whereas inhibition of MAO did not affect released DA half-life in WT or DAT-HET animals but prolonged it in DAT-KO rats (Fig. 2F, G).

Analysis of extracellular DA levels by in vivo microdialysis

To evaluate the consequences of disrupted DA clearance on the basal extracellular DA levels, we applied a quantitative low perfusion rate microdialysis approach to freely moving animals that, unlike conventional microdialysis, provides a true measure of extracellular neurotransmitter concentrations (Bradaia et al., 2009; Leo et al., 2014). As expected, DAT-KO and DAT-HET rats showed an increased amount of basal extracellular DA levels in the striatum with about 8-fold and 3-fold increase over WT levels, respectively (Fig. 3A). Levels of DA metabolites DOPAC and HVA were elevated in DAT-KO rats only. Furthermore, to assess effects of AMPH on striatal DA release, we administered AMPH and monitored the extracellular levels of DA in the striatum of freely moving rats by a conventional microdialysis. As expected, AMPH produced a genotype-dependent effect (Fig. 3D, E, F). AMPH induced significant increase in extracellular DA in WT and

to a lesser degree in DAT-HET rats. At the same time, no effect was found in animals lacking the DAT.

Striatal tissue DA and metabolites

We next evaluated the total tissue content of monoamines and their metabolites in the striatum (Fig. 3G). Like in DAT-KO mice (Jones et al., 1998), we observed a pronounced reduction in total tissue DA levels in DAT-KO rats (around 13 fold; Fig. 3G) compared to control and HET animals. At the same time, the levels of an intraneuronal DA metabolite DOPAC and predominantly extracellular DA metabolite HVA, appeared to be significantly increased in DAT-KO rats.

Expression profile of selected DA-related genes in striatal samples

To evaluate the consequences of remarkable changes in the extracellular DA due to absence of re-uptake process, we evaluated by real time PCR the expression of genes critical for DA homeostasis. In the striatum, DAT-KO animals showed an increased TH mRNA expression (Fig. 3H), but decreased levels of both dopamine D1 (D1R) and dopamine D2 (D2R) receptors (Fig. 3I and J, respectively).

Behavioral Phenotyping of DAT-KO rats

Locomotor activity tests

It is well known, that alterations in DAT function cause pronounced changes in locomotor behavior by influencing the dopaminergic tone in the basal ganglia (Giros et al., 1996). We evaluated the locomotor activity of mutant rats in home cages during 24 hours and observed a significantly altered pattern of motor activity in DAT-KO animals that varied according to the light/dark cycle, increasing significantly during the night compared to WT and HET siblings (Fig. 4A). Locomotor

activity of DAT rats was also assessed in a novel environment in illuminated conditions using activity chambers. Total distance traveled and vertical locomotor activity counts were recorded in 1.5, 2.0, 2.5, 3.0 and 4.0 months-old animals (Fig. 4B, C). In each tested age, total distance traveled by DAT-KO rats was highly elevated comparing WT and DAT-HET animals, with a characteristic to DAT-KO mice perseverative locomotor pattern of activity (Ralph et al., 2001). DAT-KO rats also displayed prominent vertical activity at all tested ages (Fig. 4D, E).

Next, we evaluated effects of compounds that are known to suppress hyperactivity of DAT-KO mice (Gainetdinov, 2008). Among them, the most noticeable are psychostimulants AMPH and MPH used in the treatment of ADHD (Gainetdinov et al., 1999). Administration of AMPH (1, 2, 3, and 4 mg/kg i.p.) to DAT-KO animals had a paradoxical calming effect in hyperactive DAT-KO animals with the most significant effect starting from 2 mg/kg AMPH (Fig. 4F and G). In WT and HET rats, treatment with this psychostimulant produced a significant increase in locomotor activity (Fig. 4F and G). Similar results were obtained with MPH (Ritalin®) drug of choice in the treatment of ADHD. Administration of 1.5, 2.5 and 5 mg/kg MPH dose-dependently reduced the hyperlocomotion in DAT-KO animals (Fig. 4F and G) while inducing a strong increase in WT and DAT-HET rats. Recent studies have revealed that Trace Amine-Associated Receptor 1 (TAAR1) can regulate the DA system and affect dopamine-related behaviors. As it has been shown previously in DAT-KO mice (Revel et al., 2011), the partial TAAR1 agonist, RO5203648, effectively reduced the hyperlocomotor behavior of DAT-KO rats without significant effect in control and DAT-HET animals (Fig. 4F and G). We finally demonstrated that the administration of the typical antipsychotic drug, haloperidol (0.5 mg/kg, s.c.), potently reversed hyperlocomotion in the DAT mutant rat model as it does in DAT-KO mice (Gainetdinov et al., 1999). Haloperidol administration also reduced the locomotor activity of WT and DAT-HET rats (Fig. 4F and G)

Cognitive tests

To evaluate the consequences of DAT-deficiency related hyperdopaminergia on cognitive abilities of rats we first employed Y-maze task. The performance of the rats in the Y-maze task is expressed as a percentage and refers to ratio of arm choices differing from the previous two choices to the total number of arm entries. DAT-KO rats alternated between the arms of the maze significantly less than control indicating deficiency in working memory (Fig. 5A). Importantly, analysis of food consumption (*ad libitum*) revealed no difference in food intake between genotypes (data not shown). Finally, KO rats showed significant difference also in the startle response and pre-pulse inhibition test, used to assess sensorimotor gating functioning (Fig. 7B and C). DAT-KO rats showed an increased startle response amplitude and impaired pre-pulse inhibition compared to WT and DAT-HET rats. Two-way ANOVAs revealed significant differences both in startle response [$F(2,42) = 7.35, P < 0.01$] and pre-pulse inhibition [$F(2,42) = 21.11, P < 0.001$] (for significance level of single points see Fig. 7B and C). No differences were found between WT and DAT-HET rats (all $P > 0.05$).

Analysis of BDNF transmission in DAT-KO rats

In an attempt to investigate the putative mechanisms responsible for the cognitive impairment in DAT-KO rats, we evaluated the pattern of BDNF expression in the rat prefrontal cortex. Fig. XX shows that, in the homogenate, total BDNF mRNA levels are reduced in the prefrontal cortex of DAT-KO rats. In order to get further insights into the complex organization of the BDNF gene, we investigated the modulation of several transcripts whose expression is driven by separate promoters (Aid et al. 2007). We focused our analysis on BDNF exon IV, the most abundant transcript whose transcription is activity-dependent, and exon VI, primarily targeted to dendrites (Pruunsild et al., 2011). Interestingly, the analysis of these different BDNF transcripts revealed that such decrease could be ascribed to BDNF exon IV since no changes were seen in BDNF exon VI (Fig. 5 XXX). We then restricted our analysis to the regulation of promoter responsible for the transcription of BDNF exon IV, by investigating its main transcription factors. We found reduced gene expression

of the calcium responsive factor (CaRF) and neuronal Per-Arnt-Sim domain protein 4 (Npas4) with no changes in cAMP responsive element binding protein (Creb). Since the reduction of CaRF mRNA levels may suggest defective processes mediating calcium influx, we measured the Ca^{2+} /calmodulin-dependent protein kinase II (αCaMKII), known to be activated by calcium. As shown in fig. 5XY, the activation of αCaMKII is significantly reduced in the prefrontal cortex of DAT KO rats. We then measured BDNF protein levels in the prefrontal cortex and found it reduced (Fig. 5xyy). Similarly, we found a significant reduction in the activation of the high affinity BDNF receptor trkB.

We then measured mBDNF expression and signaling in rat dorsolateral striatum. Fig. 5X shows that lack of DAT induces the subcellular redistribution of the neurotrophin in this brain region, as mBDNF is increased in the cytosol of dorsolateral striatum while reduced in the post-synaptic density. Additionally, in the post-synaptic density of the dorsolateral striatum, we found reduced expression and phosphorylation of the high affinity BDNF receptor, trkB (Fig. 5x) suggesting a reduced BDNF-mediated signaling in DAT-KO rats. As the interaction between dopaminergic and glutamatergic systems in the striatum are important for cognitive processing, we examined the expression of a critical determinant of glutamate transmission, PSD-95, as an index of glutamate spine density, and found a reduced expression of PSD-95 in the striatum of DAT-KO rats compared to WT counterparts (Fig. 5G).

Discussion

The involvement of DA neurotransmission in several physiological and pathological conditions has led to intense studies on animal models of dopaminergic dysfunction. One such model, DAT-KO mice, has provided numerous advances in understanding the role of DA in these disorders (Giros et al., 1996; Gainetdinov, 2008). Here we describe the rat KO model of DAT deficiency that provides

certain advantages compared to the mouse model. Rat models offer many powerful advantages: the larger size means larger tissues and samples that can lead to a reduction in the number of animals required for a study. Moreover, the larger size of the rat allows surgeries that are much easier to perform, particularly in small brain areas. Rats are particularly far superior in comparison to mice when it comes to behavioural assays. They perform much more reliably and robustly than mice, and mouse behavioural assays typically require larger cohort sizes than those needed for rats due to increased variability. In the end, an animal model must be representative of human physiology and/or disease. The rat is inherently more translational than a mouse with closer physiology, richer behaviour, more robust and reproducible assay performance.

DAT-KO rats are viable and demonstrate the expected Mendelian ratio at 4 months of age. By comparison, DAT-KO mice showed a continuous decrease in survival rate starting from 4 weeks of age with about 35% of mutants demonstrating neurological dysfunctions and death due to postsynaptic neurodegeneration (Cyr et al., 2003). Like mutant mice, DAT-KO rats develop normally but weigh less than HET and WT control animals, an effect independent from food intake (Bossé et al., 1997).

To evaluate the neurochemical consequences of the absence of DAT on striatal DA transmission, we first measured real time extracellular DA dynamics following evoked release by using FSCV. As in DAT-KO mice (Jones *et al.*, 1998), DA persists for a protracted period in the synaptic cleft in DAT-HET and DAT-KO rats. The clearance of extracellular DA was about 40-fold slower in the striatum of DAT-KO compared to WT controls while decreased about 2-fold in DAT-HET rats. Extracellular half-life of evoked DA in the striatal slices of DAT-KO animals was not altered by cocaine, fluoxetine or COMT inhibitor tolcapone. At the same time it was somewhat prolonged by MAO inhibitor pargyline, indicating that while diffusion is the major mechanism available to eliminate DA from the extracellular space in the absence of DAT (Jones et al., 1998), there is some minor contribution of MAO in DA clearance as well (Benoit-Marand et al., 2000). As in DAT-KO mice (Jones et al., 1998), prolonged extracellular lifetime of released DA resulted in about 3 and 7-

fold increase in striatal basal extracellular levels of DA in DAT-HET and DAT-KO rats, respectively. At the same time, total tissue content of striatal DA was reduced 13-fold, indicating the critical contribution of DAT-mediated transport of DA in maintenance of intraneuronal stores of DA (Jones et al., 1998). The only difference with DAT-KO mice was a significant increase in total tissue concentration of DOPAC, presumably indicating a more prominent role of MAO in metabolism of DA in rats. As for cocaine in FSCV experiments, AMPH effects on striatal extracellular DA levels was absent in DAT-KO and reduced in DAT-HET rats, indicating the critical role of DAT in the effects of this psychostimulant. The absence of DAT and the persistent elevation of DA in the synaptic cleft produce profound variation in key DA-related genes. Like in DAT-KO mice (Giros et al., 1996) striatal mRNA levels of the two major DA receptors, D₁ (D₁R) and D₂ (D₂R) are significantly decreased. At the same time, striatal mRNA levels of the rate-limiting enzyme for DA biosynthesis, TH was increased in DAT-KO rats, while a small decrease was observed in DAT-deficient mice (Jaber et al., 1999).

The most prominent phenotype of DAT-KO rats is the hyperlocomotion determined by an increased DA tone in basal ganglia. DAT-KO rats showed an intense locomotor activity both in a new environment and in the home cages. The hyperlocomotion of homozygous KO rats persists during the lifespan, and as already seen in DAT-KO mice, is greater than the effect produced by DAT blocker drugs (Giros et al., 1996). As in DAT-KO mice, AMPH and MPH significantly reduced locomotor activity of DAT-KO rats while inducing the well-known increase in DAT-HET and WT animals (Gainetdinov et al., 1999). The psychostimulatory actions of AMPH and MPH are primarily dependent on a direct interaction of these compounds with DAT, leading to elevated DA (Fumagalli et al., 1998; Chen et al., 2006; Thomsen et al., 2009). Because DAT-KO rats lack the major target of AMPH action, the “calming” effect of psychostimulants suggests the involvement of a DAT-independent mechanism of action, possibly involving other monoamine transporters. Interestingly, we observed an inhibitory action of MPH at lower (about 10-fold) doses in rats in

comparison to mice (Gainetdinov et al., 1999) suggesting significant differences in pharmacodynamic or pharmacokinetic properties of this drug between these species. Hyperactivity and paradoxical inhibitory effects of psychostimulants used in the treatment of ADHD, could be a first indication of face and predictive validity of DAT-KO rats as an improved animal model for this disorder. Moreover, the ability of the partial TAAR1 agonist, RO5203648, to modulate the locomotor behaviour of DAT-KO rats supports the potential of TAAR1 as a target for therapeutic intervention in DA-related disorders. Previous data showed that activation of TAAR1 strongly regulates DA transmission and reduces DA-dependent behavioural effects of psychostimulants as well as hyperactivity of DAT-KO mice (Bradaia et al., 2009; Leo et al., 2014) likely via an interaction of TAAR1 with D₂R or with different neurotransmitter pathways, such as the glutamatergic (Espinoza et al., 2015).

As an indication of DA-related cognitive deficits, DAT-KO rats, like DAT-KO mice, show an impaired sensorimotor gating measured as reduced pre-pulse inhibition (PPI) of the acoustic startle reflex (Ralph et al., 2001). Notably, DA hyperactivation, induced by pharmacological interventions or genetic manipulation of DAT, causes PPI deficits in experimental animals similar to those observed in psychiatric disorders, such as schizophrenia and ADHD (Braff et al., 2001; Swerdlow et al., 2001); (Yamashita et al., 2006; Arime et al., 2012). DAT-KO rats show also a decreased Y-maze spontaneous alternation that indicated an impaired working memory function. Since intact dopaminergic signalling in the prefrontal cortex seems to be required for correct mnemonic function (Goldman-Rakic, 1996; Kesner and Rogers, 2004), we can postulate that absence of DA reuptake could impair the retention of short term memory information in DAT-KO rats. At least some of these dopaminergic effects may involve D₁ receptors (Lidow et al., 1991; Goldman-Rakic et al., 1992; Gaspar et al., 1995).

In an attempt to find a molecular correlate of the above shown cognitive deficit, we focused our attention on the neurotrophin BDNF, which plays a role in working memory (Egan *et al*, 2003) and whose expression was found to be developmentally dysregulated in the prefrontal cortex of

DAT-KO mice (Fumagalli *et al*, 2003). We found that DAT deletion in rats reduces total BDNF mRNA levels in the prefrontal cortex. Further, the analysis of the main different transcripts produced by the activation of the different promoters at 5'-UTR revealed that only BDNF exon IV is reduced. Since this transcript is tightly dependent on neuronal activity we suggest that, in these rats, activity-dependent gene expression is impaired. Moreover, the analysis of the transcription factors involved in the modulation of BDNF exon IV showed that the expression of the neuronal transcription factor NPAS 4, an immediate early gene selectively induced by neuronal activity (Sun and Lin, 2016), is reduced as well. Notably, the expression of calcium responsive factor CaRF, which also regulates the BDNF exon IV expression, is reduced suggesting reduced calcium influx, an evidence corroborated by the reduced activation of α CaMKII, a well-known intracellular calcium sensor required for CaRF activity (Zheng *et al.*, 2011). Given that both calcium influx and α CaMKII activation often represent the first stimulus for activity-dependent gene expression in neurons (Fields *et al.*, 2005), we suggest defective calcium influx as a potential mechanism for the impairment in the cortical activity-dependent gene expression in the prefrontal cortex of DAT KO rats.

Changes in BDNF mRNA levels were accompanied by a down-regulation of BDNF protein levels, suggesting that DAT deletion regulates also the translation of the neurotrophin. Of note, we also found reduced activation of the high affinity BDNF receptor trkB, suggesting that, in the prefrontal cortex of DAT KO rats, the BDNF-mediated transmission is impaired, an effect that may contribute to the impaired working memory function (Galloway *et al.*, 2008).

Our analyses also revealed that the removal of DAT altered the BDNF system also in the dorsolateral striatum, where it results in a redistribution of the neurotrophin. In the whole homogenate, we found increased expression of BDNF leading to the activation of trkB and its downstream intracellular signalling; however, the removal of DAT has led to increased BDNF expression in the cytosol while reducing its expression, together with its downstream signalling, in the post-synaptic density. This observation may have a relevant and functional effect, given the

well-established role of BDNF in the control of glutamatergic spine density (Young *et al*, 2015) and since reduced spine density was reported in striatal medium spiny neurons of DAT-KO mice (Berlanga *et al*, 2011). In fact, in line with the herein shown reduced BDNF expression, we also found a decreased expression of the marker of glutamatergic spines PSD-95, as previously observed in DAT-KO mice (Yao *et al*, 2004), suggesting potential structural rearrangements of glutamate neurons in the dorsolateral striatum.

Taken together, these observations indicate that DAT-KO rats represent an improved model of persistently increased dopaminergic transmission, that is presumably involved, at least in part, in endophenotypes of such disorders as schizophrenia, bipolar disorder, ADHD and Huntington's disease (Carlsson, 1987; Howes and Kapur, 2009; Gardoni and Bellone, 2015; Bonvicini *et al*., 2016). These rats have obvious advantages over mouse models by having increased survival rate, a larger size of the brain allowing investigations of smaller brain regions and being closer physiologically to humans. Since behavioural repertoire of rats is significantly more complex in comparison to mice, mutant rats could be particularly useful to evaluate the effects of novel therapeutics on cognitive functions.

Figure Legends

Figure 1. Generation of DAT knockout (KO) rats. **A)** DAT KO rats were generated by using zinc finger nuclease (ZFN) technology that produces a 5 bp deletion and an early stop codon. Green solid lines indicate exons 2 and 3, grey solid lines indicate introns; yellow boxes indicate DNA domains interaction sites; blue box indicates KO targeting site; red box indicate early stop codon generated after frameshift due to cleavage of 5 base pairs operated by ZFN. **B and C)** PCR primers were designed (black arrows). Wildtype (WT) DNA contains BtsIMutI restriction enzyme site (blue brackets). WT amplified DNA is fully digested into two low molecular weight bands (104 bp and 71 bp). Homozygote mutated DNA loses digestion sites on both alleles, therefore resulting in only

one final PCR product of 170 bp. Heterozygosis shows both digested DNA at lower molecular weight from the WT allele and mutated DNA at 170 bp. **D)** Western Blot analysis on striatal samples of DAT rats. DAT-KO rats show complete absence of DAT protein, whereas HET and WT still have DAT protein expression. **E)** Rats were bred following heterozygous (HET) male/HET female breeding scheme. 223 animals were born from 19 offsprings and we obtained 62 DAT^{+/+} (26.46%), 102 DAT^{+/-} (45.74%) and 59 DAT^{-/-} (27.80%), numbers very close to the ratio (1:2:1) expected in the absence of pre- and postnatal death. Infantile mortality is completely absent for all three genotypes up to 4 months. Population genotype distribution was analyzed by Chi-square test (Chi-square=1,7; DF=2; p=0,4275), showing that discrepancy of obtained data is not significant different from expected Mendelian distribution 1:2:1, resulting from mating of HET. **F)** Developmental phenotype. Both DAT-KO rats male and female develop normally but have lower weight in comparison to HET and WT rats.

Figure 2. Recording of electrically stimulated DA efflux in striatal brain slices from DAT rats. **A)**

A single biphasic stimulus (400 μ A, 1 ms) was used to evoke DA release in striatal brain slices from WT, HET and KO rats. The average time to clear released DA is 1.3, 10 and 50 seconds, respectively. Lower panel: Color plots represent the voltammetric currents (encoded in color in the z axis) plotted against the applied potential (y axis) and time (x axis). Upper Panel: Cyclic

voltammogram identifies the detected analyte as DA. **B)** Kinetics of evoked DA release in dorsal

striatum of DAT-WT, HET and KO. **C and D)** Effect of 3 μ M cocaine on evoked DA release.

Cocaine had no effect on evoked DA overflow in DAT-KO rats but induced the well know increase in DA release in both WT and HET (C) Cocaine-induces changes in half-life (D). **E)** 10 μ M of

Fluoxetine effect on half-life of evoked DA release. **F)** 10 μ M of Tolcapone effect on effect on half-life of evoked DA release. **G)** Pargyline effect on effect on half-life of evoked DA release.

Inhibition of MAOs by 10 μ M pargyline.

Figure 3. **A-C)** Quantitative low perfusion rate microdialysis showed an increased amount of released DA (**A**) in DAT-KO striata about 8-fold control values followed by a marked increase in both DA metabolites, DOPAC (**B**) and HVA (**C**). Results are the mean \pm SEM of six independent experiments. **E-F)** Effect of AMPH (3 mg/kg, i.p.) on extracellular DA measured by microdialysis in the striatum of freely moving rats. Results are the mean \pm SEM of six independent experiments. **G)** Neurochemical phenotype of DAT-KO rats. HPLC analysis on striatal samples showed 13-fold decrease in DA level along with increased metabolites (DOPAC and HVA). **H-J)** Molecular profile of selected DA related genes in striatal samples. **H)** TH mRNA expression is increased selectively in DAT-KO striatal samples (WT vs KO $p=0.0004$ and HET vs KO $p=0.0019$, $n=6$; one-way ANOVA) **I)** D1R mRNA are decreased in both HET and KO striatal samples. (WT vs HET $p=0.0056$ and WT vs KO $p=0.0004$; HET vs KO, $p=0.0489$, $n=6$; one-way ANOVA. **J)** D2R mRNA expression is reduced only in DAT-KO striatal samples (WT vs KO $p=0.0039$, HET vs KO, $p=0.0018$, $n=6$; one-way ANOVA.).

Figure 4. Basal locomotor activity of DAT-KO rats. **A)** Locomotor activity of adult animals was recorded in home cages for 24 hours. **B-D)** Traveled total distance and vertical activity of animals at different ages were assessed using an automated Omnitech Digiscan open fields for 2 hours. **C-E)** Additionally, total distance and vertical activity of 4 month-old animals were evaluated for 4 hours in the automated open fields. The results are expressed as the mean \pm SEM. * and # $P < 0.001$ (Bonferroni's test), relative to the corresponding WT (*) or HT (#) control groups. $N = 6-20$ per group. **F-G)** Drug effects on locomotor activity of DAT-KO rats. Before drug administration, the mice were habituated to the activity monitor for 30 min. After drug injection, the locomotor activity of the animals was recorded for additional 70 min. Total distance covered (**F**) and the vertical activity (**G**) for the 60 min period (from 40 min to 100 min) were used for subsequent analysis. The results are expressed as the mean \pm SEM. * $P < 0.05$ (Dunnet's test, t-test), relative to the

corresponding saline-treated control group. N = 6–19 per group, with exception of DAT-HET rats treated with haloperidol (n=4).

Figure 5. A) Spontaneous alternation test. To measure spontaneous alternation behavior and exploratory activity, a white plastic material Y-maze with arms 40 cm (long) by 6 cm (wide) with 13 cm walls was used. Each animal was tested in a single 8-min session, during the course of which the animal was placed in the central platform and allowed free exploration of the maze. Spontaneous alternation, expressed as a percentage, refers to ratio of arm choices differing from the previous two choices to the total number of arm entries. The percentage of alternation observed is strongly reduced in KO animals. Values are expressed as Mean \pm SEM of 8 rats/group. *P < 0.05, with respect to WT and HET rats **B)** Startle response and **C)** Pre-pulse inhibition of the startle response. The day of the test, rats were transferred in the experimental room under environmentally controlled conditions (sound proof and red dim lights) and after 30 min of habituation were positioned in the apparatus and startle response and pre-pulse inhibition assessed (see methods section). Rats were tested one once. Values are expressed as Mean \pm SEM of 8 rats/group. Block1, Block2 and Block3 indicate the three consecutive sequences of stimuli of the test. PP1, PP2 and PP3 indicate the three different intensities of the pre-pulse stimulus. Two-way ANOVA followed by Bonferroni's pair wise contrasts. *P < 0.05, **P < 0.01, ***P < 0.001 with respect to WT rats. **D-G)** Analysis of BDNF transmission in DAT-KO rats. **X)** Total BDNF (-12%, $t_{(8)}= 3.2$; p= 0.013) and BDNF exon IV (-20%, $t_{(8)}= 4.43$; p= 0.002) mRNA levels are reduced in the prefrontal cortex (PFC) of DAT-KO rats, while no changes were observed in the levels of BDNF exon VI (+2%, $t_{(8)}= 0.26$; p= 0.80). **X)** The analysis of the transcription factors involved in the modulation of BDNF exon IV revealed a significant reduction in the expression of Npas4 (-40%, $t_{(8)}= 5.41$; p= 0.0006) and CaRF (-21%, $t_{(8)}= 4.15$; p= 0.003) and no changes in Creb mRNA levels (+6%, $t_{(8)}= 0.65$; p= 0.53). **X)** Activation of the α CaMKII is reduced in the PFC homogenate of DAT-KO rats (-30%, $t_{(8)}= 3.30$; p= 0.011). **X)** The transcriptional changes of BDNF were paralleled by a reduction in the

mature form of BDNF (mBDNF) levels (-30%, $t_{(8)} = 3.12$; $p = 0.014$) and accompanied by reduced activation of trkB (-30%, $t_{(8)} = 2.38$; $p = 0.044$) in the PFC homogenate of DAT-KO rats. **X**) In the dorsolateral striatum (DLStr), mBDNF levels are increased in the homogenate and in the cytosol while reduced in the post-synaptic density (homogenate: +143%, $t_{(7)} = 9.56$; $p < 0.0001$; cytosol: +78%, $t_{(7)} = 2.59$; $p = 0.036$; post-synaptic density: -20%, $t_{(8)} = 2.53$; $p = 0.035$). **X**) trkB activation is reduced in the post-synaptic density of DLStr (-15%, $t_{(8)} = 2.38$; $p = 0.045$). **X**) PSD-95 is reduced in the post-synaptic density of DLStr of DAT-KO rats (-21%, $t_{(8)} = 2.57$; $p = 0.033$).

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