SUPPLEMENTARY INFORMATION

A C-terminal PDZ-domain sequence is required for striatal distribution of the dopamine transporter

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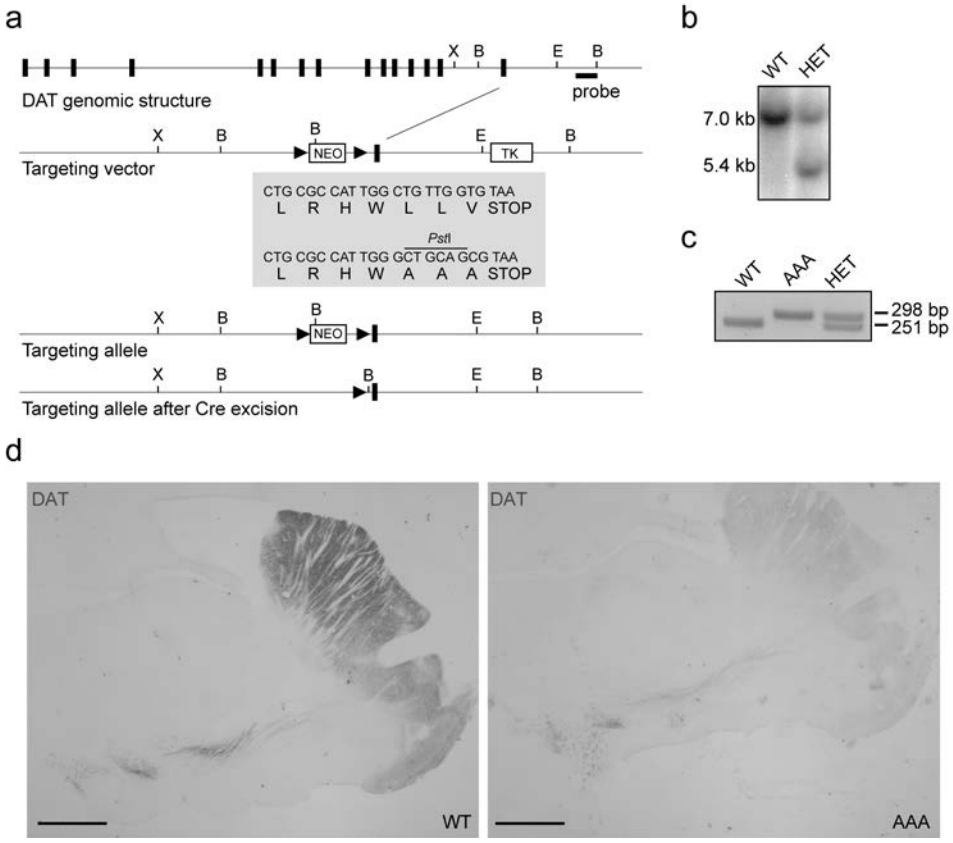
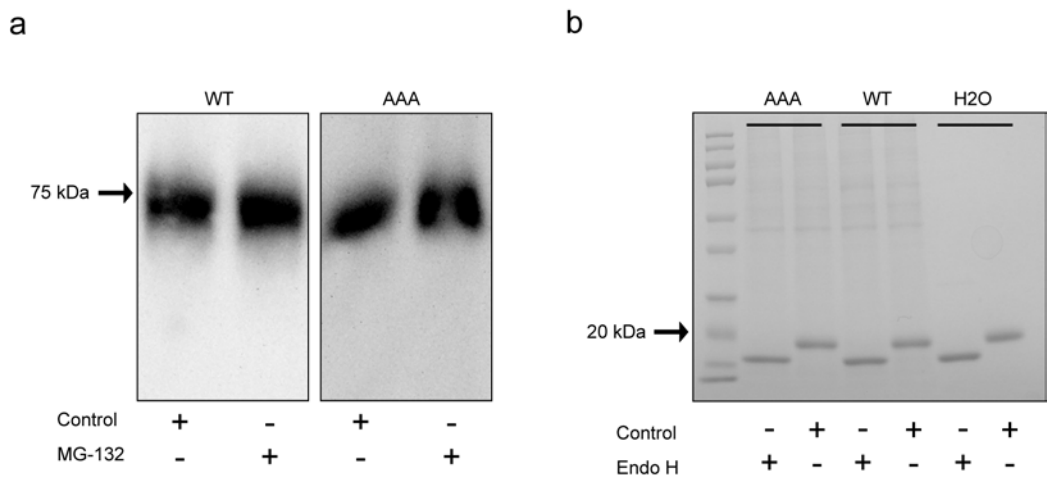


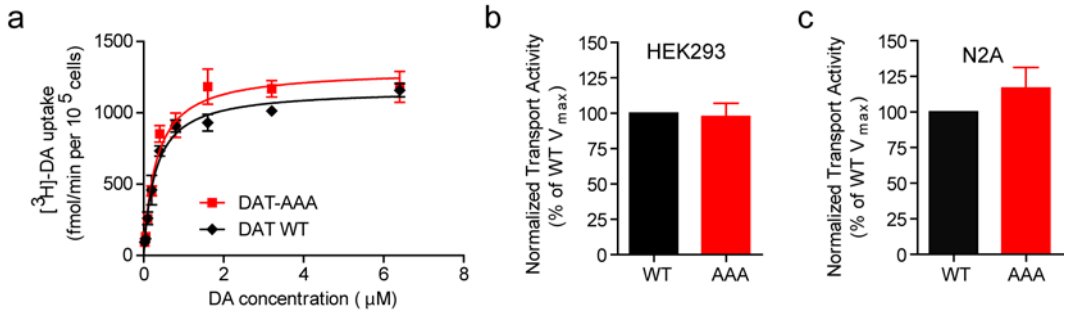
Supplementary Figure S1. Generation of DAT-AAA mice

(**a**) Gene targeting strategy. For detailed description of knock-in approach see **Supplementary Methods**. (**b**) Southern blot analysis of genomic DNA from mouse ES cells digested with *BamHI* show proper homologous recombination. The external probe used for southern blot hybridized with a 7 kb band WT and an additional 5.4 kb AAA band due to the *BamHI* site in the Neomycin-*lox*P region of targeted allele. Clones that undergone homologous recombination are heterozygous at the mDAT target locus with both WT and AAA alleles. (**c**) PCR genotyping analysis of mouse tail DNA preparations from WT, heterozygous and DAT-AAA mice. Genotyping were performed using the following primers: sense 5'- CCG TTG GTG CCC TCA AGG GTG GTA TCT ATC-'3, antisense: 5'- CTT TGT GAT CTC CCT GTC CCC GCT GTT G-'3. (**d**) Representative photomicrographs of DAT-immunoreactivity in a sagittal view showing midbrain projections radiating into the terminal fields of striatum from WT and DAT-AAA mice. The sagittal view emphasizes the massive loss of DAT-labeled fibers. Scale bars = 1 mm.



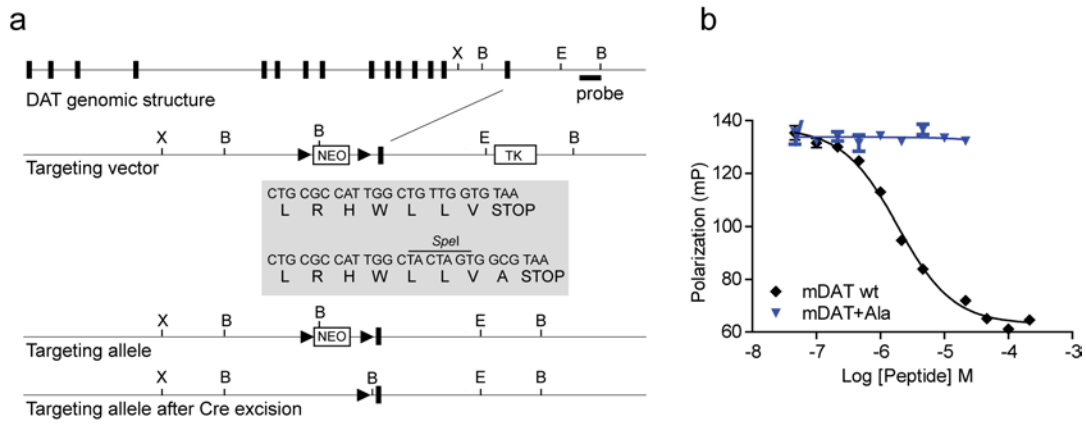
Supplementary Figure S2. Inhibition of proteosomal degradation and Endo H treatment control

(**a**) SDS-PAGE and immunoblotting of neuronal lysates from WT and DAT-AAA. Cultured dopaminergic neurons were treated with the 26S proteasome inhibitor, MG-132 (1μM, K_i= 4nM according to manufacture, Calbiochem, USA) for 4 hours (n=2). Longer treatments and higher MG-132 concentrations have been reported to cause loss of dopaminergic neurons[1]. We observed no effect of 26S proteasome inhibition on DAT degradation in neither WT nor DAT-AAA neurons. For both genotypes, DAT eluted as a single 70 kDa band irrespective of treatment. Immunoblottings were performed using rat anti-DAT monoclonal antibody (1:750, MAB369, Millipore, USA). (**b**) Positive Endonuclease H (Endo H) treatment control. Endo H activity was verified by adding 5μg Endo H substrate, RNase B (New England Biolabs, USA) to neuronal lysates (n=4, two from WT and two from DAT-AAA dopaminergic neurons), before Endo H (or control) treatment was carried out, according to manufacturer's protocol. Deglycosylation of RNaseB was visualized by Coomassie blue staining of 12% SDS gels. Samples with water instead of lysate were included to visualize the pure RNaseB bands.



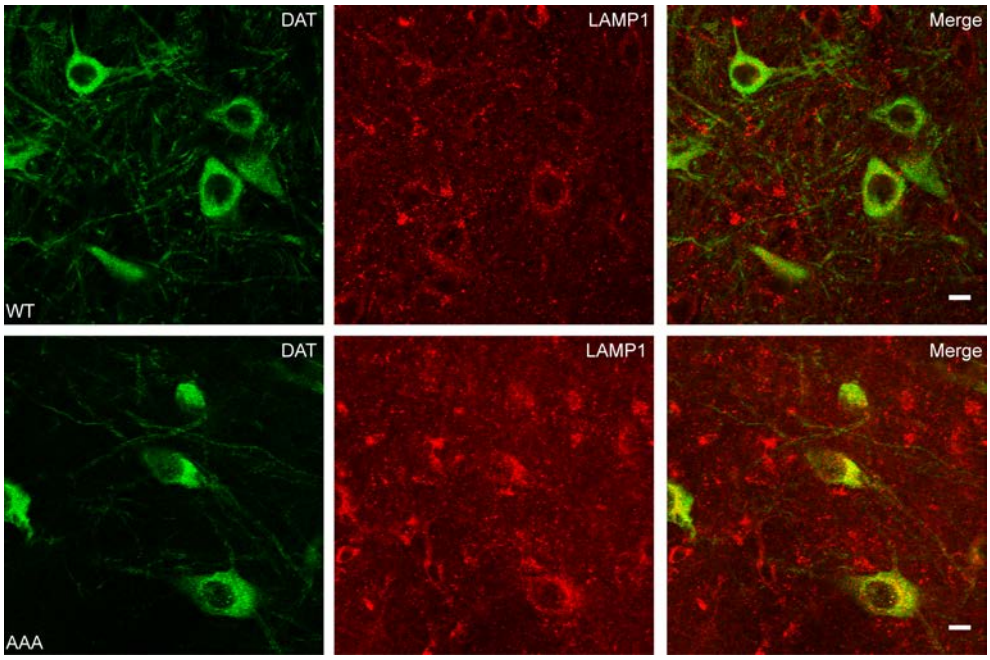
Supplementary Figure S3. Dopamine uptake in HEK293 and N2A cells

(**a**) [^{3}H]-dopamine saturation uptake in HEK293 cells with transient expression of WT or DAT-AAA. The curves are representative for three independent experiments. No difference was seen between WT and DAT-AAA expressing cells **(b)**. Uptake was carried out for 5 min with triplicate uptake determination at indicated concentrations of dopamine. WT V_{max} was 940 ± 176 fmol/10^5 cells. WT K_m: 0.47µM [0.32-0.62] and AAA K_m: 0.40 [0.32-0.48] (SE interval). Data are means ± s.e.m. of three experiments performed in triplicate, P>0.05, one sample t-test. (**c**) V_{max} for [^{3}H]-dopamine uptake (% of WT) in N2A cells with transient expression of WT or DAT-AAA. Experiments were carried out as [^{3}H]-dopamine uptake competition experiments (5 min uptake) with increasing concentrations of unlabeled dopamine[2]. V_{max} for WT was 240 ± 45 fmol/10^5 cells. WT K_m: 0.29 µM [0.29-0.39] and AAA K_m: 0.48 [0.39-0.59] (SE interval). Data are means ± s.e.m. of five experiments performed in triplicate, P>0.05, one sample t-test.



Supplementary Figure S4. Generation of DAT+Ala mice

(**a**) Gene targeting strategy. For detailed description see **Supplementary Fig. S1**. An alanine at the extreme DAT C-terminus (+Ala) was introduced into exon 15, including a silent restriction enzyme site (*SpeI*) to discriminate between WT and mutant alleles. (**b**) Fluorescence polarization competition binding of Oregon Green-labelled human DAT peptide (13 C-terminal residues, OG DAT C13) to the PDZ-domain protein (PICK1) with increasing concentration of indicated unlabelled peptides (corresponding to the 13-C-terminals residues of mDAT WT and DAT+Ala). Data are means ± s.e.m. (n=3). For the experiments, 40 nM OG DAT C13 was incubated with a fixed non-saturating concentration of purified PICK1 protein and competed with increasing amounts of unlabelled peptides. Following incubation for 15 min, fluorescence polarization values were determined as described[3].



Supplementary Figure S5. DAT-AAA partially co-localizes with a late endosomes/lysosomal marker

Midbrain sections immunostained for DAT (green, Alexa-488) and the late endosome/lysosomal marker LAMP1 (red, Alexa-568). Partial overlay between DAT and LAMP1 immunoreactivity is observed for both WT and DAT-AAA mice in agreement with postendocytic sorting of DAT to the lysosomal compartment upon constitutive internalization. Scale bars=10 µm.

Supplementary Table S1: Behavioural phenotype assessment of WT and DAT-AAA mice using SHIRPA primary screen

Parameter	WT (n = 16)	Het (n = 20)	DAT-AAA KI (n = 21)
Age (weeks)	14 (10-15)	11 (10-14)	14 (10-15)
Body weight (g)	28,5 ± 0,51	27,9 ± 0,41	29,3 ± 0,41
Body length (cm)	9,35 ± 0,06	9,13 ± 0,08	9,27 ± 0,06
Body position	3 (3-3)	3 (3-3)	3 (3-3)
Spontaneous activity	2 (2-3)	2 (2-3)	2 (2-3)
Respiration	2 (2-2)	2 (2-2)	2 (2-2)
Tremor	0 (0-0)	0 (0-0)	0 (0-1)
Transfer arousal	5 (4-5)	5 (3-5)	5 (4-5)
Palprebral closure	0 (0-0)	0 (0-0)	0 (0-0)
Piloerection	0 (0-0)	0 (0-0)	0 (0-0)
Startle response	1 (1-1)	1 (1-1)	1 (1-1)
Gait	0 (0-0)	0 (0-0)	0 (0-0)
Pelvic elevation	*3 (2-3)*	***2*** (2-3)***	*2 (2-3)*
Tail elevation	1 (1-1)	1 (1-1)	1 (1-2)
Touch-escape	*1 (1-2)*	***2**** (1-2)***	***2**** (1-3)***
Positional passivity	0 (0-1)	0 (0-0)	0 (0-0)
Trunk Curl	0 (0-0)	0 (0-0)	0 (0-1)
Limb grasping	0 (0-0)	0 (0-0)	0 (0-0)
Visual placing	3 (3-3)	3 (3-3)	3 (3-3)
Grip strength	2 (2-2)	2 (2-2)	2 (1-2)
Body tone	1 (1-1)	1 (1-1)	1 (1-1)
Pinna reflex	1 (0-1)	1 (0-1)	1 (0-1)
Corneal reflex	1 (1-1)	1 (1-1)	1 (1-1)
Toe pinch	2 (0-2)	1 (0-3)	1 (0-3)
Wire manoeuvre	1 (0-3)	1,5 (1-3)	2 (1-3)
Skin colour	1 (1-1)	1 (1-1)	1 (1-1)
Heart rate	1 (1-1)	1 (1-1)	1 (1-1)
Limb tone	1 (1-2)	2 (1-2)	2 (1-2)
Abdominal tone	1 (1-1)	1 (1-1)	1 (1-1)
Lacrimation	0 (0-0)	0 (0-0)	0 (0-0)
Salivation	1 (1-1)	1 (1-1)	1 (1-2)
Provoked biting	1 (0-1)	1 (0-1)	1 (0-1)
Righting reflex	0 (0-0)	0 (0-1)	0 (0-0)
Contact righting reflex	1 (1-1)	1 (1-1)	1 (1-1)
Negative geotaxis	0 (0-1)	0 (0-1)	0 (0-1)
Fear	0 (0-0)	0 (0-1)	0 (0-0)
Irritability	1 (0-1)	1 (0-1)	1 (0-1)
Aggression	0 (0-1)	0,5 (0-1)	0 (0-1)
Vocalisation	1 (0-1)	1 (0-1)	1 (0-1)
Locomotor activity	2591,50 ± 202,38	2978,63 ± 191,62	***3870,00 ± 216,65******

The primary SHIRPA screen[4] assesses various sensorimotor, cognitive and autonomic behaviours of the mice, based on observational assessments. The present phenotype assessment showed that mice were viable, fertile and displayed no major developmental defects. Importantly, DAT-AAA mice displayed no dwarfism as seen in DAT KO mice[5]; indicating preserved endocrine function. Differences were found in two parameters tested: 30 minutes locomotion and touch escape. In the 30 minutes locomotion test, DAT-AAA mice were significantly more active compared to WT mice ($P<0.001$, Bonferroni *post-hoc* t-tests following significant one-way ANOVA, $F(2,53)=10.09$, $P<0.001$). In the touch escape test, DAT-AAA mice had a faster flee response compared to WT ($P<0.01$, non-parametric Mann-Whitney test). No differences between the genotypes were seen in all other parameters tested. *$P<0.05$, **$P<0.01$ and *** $P<0.001$.

SUPPLEMENTARY METHODS

Site-directed mutagenesis. cDNA encoding hDAT was kindly supplied by Dr. Marc G. Caron (Duke University, Durham, USA) and the mDAT cDNA was kindly provided by Dr. Howard Gu (Ohio State University, Columbus, USA). Mutant transporters were generated by two-step PCR mutagenesis, using Pfu Polymerase (Stratagene) according to manufacturer's protocol. Restriction enzyme mapping and DNA sequencing was used to validate mutants.

Gene targeting strategy. The coding region of the mDAT gene constitutes 15 exons (indicated as black boxes) with exon 15 encoding the absolute C-terminus (-LRHWLLV). Mouse genomic DNA comprising a 9kb fragment of the mDAT gene was isolated from a Bacterial Artificial Chromosome library using *XmaI* and *EcoRV* restriction enzyme sites. The mDAT gene sequence upstream of exon 15 was ligated to a l*oxP*-flanked (arrow heads) neomycin resistance cassette conferring positive selection of homologous targeted embryonic stem (ES) cells. The region encompassing the AAA substitution in the absolute C-terminus was introduced downstream of the second *loxP*-site. To ensure proper homologous recombination, the herpes simplex virus thymidine kinase cassette was positioned in the 3' of the homology region downstream of the *EcoRV* site. Mutagenesis was performed in pBluescript II KS vector (Stratagene) using the Quickchange method and checked by sequence analysis. The AAA mutation was introduced into exon 15 including silent restriction enzyme sites (*PstI* for DAT-AAA) to discriminate between WT and mutant alleles. Targeting construct of the 9kb mDAT sequence was divided into a long arm of homology (~7kb in length) upstream of exon 15 and a short arm of homology (~2kb in length) including exon 15 and the downstream region. The targeting vector was linearized and electroporated into mouse ES cells (E14-derived from the agouti colored 129/Sv strain). Neomycin resistance cassette and herpes simplex virus thymidine

kinase cassettes were introduced upstream respectively downstream of the targeted exon to confer positive-negative selection of homologous targeted ES cells. ES clones resistant to G418 (geniticin sulphate) and the anti-viral drug FIAU (1-(2-deoxy, 2-fluoro-β-δ-arabinofuranosyl)-5-iodouracil) were checked by southern blot analysis and PCR analysis for correct homologous recombination. Positive clones were injected into B6D2/J blastocysts and transferred to uteri of pseudopregnants NMRI foster mothers to generate chimeras. Chimeric mice were identified by agouti coat color and bred with C57Bl/6J mice to generate heterozygous mutants. After germ-line transmission, the neomycin cassette was deleted by breeding mice carrying the heterozygous mutation to *EIIa-Cre* transgenic mice. B= *BamHI* site, X= *XmaI* site, E=*EcoRV* site.

Quantitative real-time PCR. Taqman® Reverse Transcription reagents (Applied Biosystems, Foster City, CA) were used to perform first strand synthesis on purified RNA according to the manufacturer's prescriptions. After synthesis, the cDNA was diluted twice with RNAse-free H_2O and 3 µl was used in a 20 µl quantitative RT-PCR reaction containing 1 x SYBR Green® Mix (Applied Biosystems) and 7.5 pmol of each primer. Samples were analysed on a DNA Engine Opticon (MJ Research, Waltham, MA). Quantitative PCR measurements were analysed by applying the
Pfaffl method[6].

Quantification of optical density (OD). Optical densitometry was applied for semi-quantification of DAT and TH immunoreactivity. Photomicrographs from striatum, substantia nigra, and VTA were acquired using a light microscope with identical settings. Mean values were calculated and values from DAT-AAA mice were normalized to levels of WT. Images were analysed using the ImageJ software.

Membrane preparations. Membranes were isolated from dissected striata from adult mice and from whole brains from mice pups (WT and DAT-AAA, P1-3). Tissue was homogenized in buffer containing 0.32 M sucrose, 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM *N*-ethylmaleimide (NEM) supplemented with protease inhibitor cocktail (Roche), though homogenization buffer for striatal membranes contained 10 mM Tris-HCl pH 7.5 and 1 mM EDTA. Homogenates were precleared by centrifugation at 1,000 *x g* for 10 min at 4^{o}C , and the supernatant was further centrifuged at 16,000 *x g* for 30 min. Obtained membranes were resuspended in homogenization buffer and the protein concentration was determined using BCA^{TM} Protein Assay Kit (Pierce). For whole brain homogenates, aliquots of 300 µg protein were centrifuged at 14,000 *x g* for 5 min at 4^{o}C, and the pellet was dissolved in loading buffer and incubated for 1 h at RT.

Striatal and neuronal culture lysate preparations. Striatal lysates derived from adult mice striata WT and DAT-AAA mice (for estimations of total TH expression) and whole-cell lysates from cultured dopaminergic neurons were prepared as described[7], though striata were lysed in a buffer containing 20 mM Tris-HCl pH 7.4, 300 mM NaCl, 2 mM EDTA/EGTA, 1% NP-40 and 2% Triton X-100 supplemented with protease inhibitors (Roche). Neuronal cultures treated with proteosomal inhibitor received 1 µM MG-132 (K_i= 4nM according to manufacture, Calbiochem, USA) for 4h before lysate preparation. Treatment of neuronal lysates with endoglycosidase H (Endo H) and/or Peptide: N-Glycosidase F (PNGase F) (both from New England Biolabs, USA) was carried out according to manufactures instructions (control treatments with denaturation buffer). Endo H activity was verified with RNase B (**Supplementary Fig. S2**).

Brain slice preparation and surface biotinylation. Experiments were performed as described[8] with minor modifications. Briefly, brains from WT and DAT-AAA mice were rapidly

removed and placed in ice-cold artificial cerebrospinal fluid (aCSF) containing 120 mM NaCl, 5 mM KCl, 30 mM glucose, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 25mM HEPES (pH 7.4). Coronal brain slices (300 μm) containing striatum were generated on a vibrating microtome (Leica VT1200) and allowed to recover for 20 min in aCSF at room temperature. Each striatal slice from WT and DAT-AAA mice were divided into its hemispheres and either placed 1h in oxygenated aCSF (95% O_2 and 5% CO_2) at 4˚C (total surface expression, no internalization) or at 37˚C (surface levels after 1h constitutive internalization). Slices were then incubated in oxygenated aCSF containing 1mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Thermo Scientific) for 45 min at 4˚C. Slices were rinsed twice in oxygenated aCSF (4˚C) for 10 min followed by quenching in 100 mM glycine 2 x 20 min in oxygenated aCSF (4˚C). Slices from respective genotype were pooled (six slices per mice), transferred to eppendorf tubes and placed on ice. Slices were mechanically disrupted using a pestle and homogenized in lysis buffer (1% Triton X-100, 0.1% SDS, 1 mM EDTA, 50 mM NaCl, 20 mM Tris, pH 7.5, and a cocktail of protease inhibitors). Homogenates were centrifuged for 10 min at 16,000 x *g* to remove debris. 60 μl of total lysates were stored for determining the total DAT input in each sample. Biotinylated proteins were precipitated from collected supernatants by overnight incubation at 4˚C with streptavidin beads (Thermo Scientific). Beads were then washed four times in lysis buffer and biotinylated proteins were eluted using 75μl 2X SDS loading buffer for 30 min at 37˚C. Streptavidin beads were removed by filtration, and DAT expression level in respective fractions was detected by SDS-PAGE and immunoblotting, as described below**.** Band intensities of biotinylated DAT were normalized to the band intensity of DAT in the respective input lysates.

Immunoblotting. Equal amounts of protein or membrane samples, prepared as described were resolved by SDS-PAGE and transferred to Immobilon-P membranes (Millipore, USA). The membranes were blocked in PBS containing 0.1% Tween-20 and 5% dry milk, and incubated

overnight with antibodies against DAT (MAB369, 1:1000) or TH (1:1000), respectively. Following incubation with HRP-conjugated anti-rat or -rabbit antibodies, respectively (both from Pierce) the blots were visualized by chemiluminescence (ECL-kit, Amersham, UK) using AlphaEase (Alpha Innotech, USA), and quantified. To verify equal protein loading, the membranes were stripped, blocked and re-probed with antibodies against β-tubulin III (Sigma) or β-actin (Sigma). In surface biotinylation experiments, band intensities of biotinylated DAT were normalized to the band intensity of DAT in the respective input lysates. Band intensities were quantified using ImageJ gel analysis software.

Dopamine uptake *in vitro*. Uptake experiments were performed on transiently transfected cells (N2A and HEK293), two days after transfection. We used two uptake regimes: competition experiments (in N2A cells), with increasing concentrations of unlabelled dopamine as described in[2], and a saturation regime (in HEK293 cells), performed as described[7], with the following modifications: 20.000 transfected cells were seeded in 96wells. We used a twofold dilution row (6.4-0.05 µM) mixture of [^{3}H]-dopamine (PerkinElmer Life Sciences, Waltham, MA) and unlabelled DA (Fluka AG, Buchs, Switzerland). Scintillation fluid (Optiphase HiSafe 3, PerkinElmer Life Sciences) was added directly to the wells before counting.

Autoradiography. DAT autoradiography was performed on coronal sections (20 µm) from striatum and midbrain. Brain sections were thawed, rinsed in binding buffer for 15 min and then incubated with 10 nM [^{3}H]-mazindol (Perkin Elmer Life Sciences, USA) for 1h at 4˚C. Desipramine (100 nM) and s-citalopram (100 nM) were used to block NET and SERT binding. After rinsing 2x5 min in binding buffer, slides were air-dried, exposed to Fuji BAS IP TR 2040 screens, and then read in a STARION FLA-9000 image scanner (both from Fujifilm Life Science, USA). Non-specific binding was determined in the presence of nomifensine (1 µM).

Calibrated [^{3}H]-polymer microscales (Amersham Bioscience, Denmark) was used for quantification of [^{3}H]-mazindol binding. For higher anatomical resolution, dried sections were exposed to tritium-sensitive films (Amersham Biosciences, Denmark), at -20˚C for 6-8 weeks and developed in Kodak GBX film developer (Sigma Aldrich, Denmark).

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