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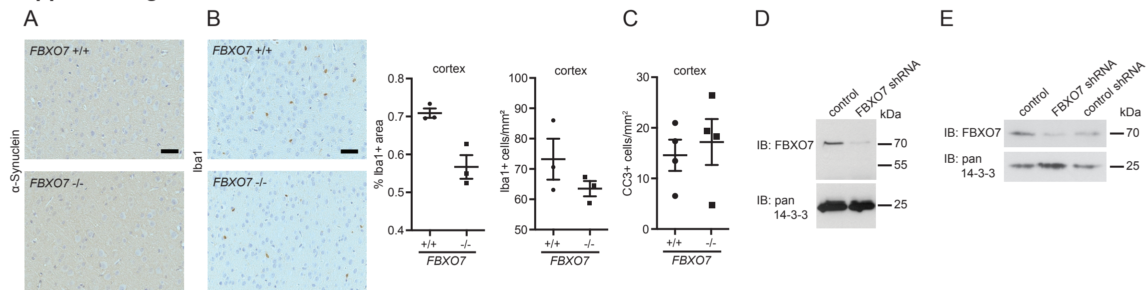
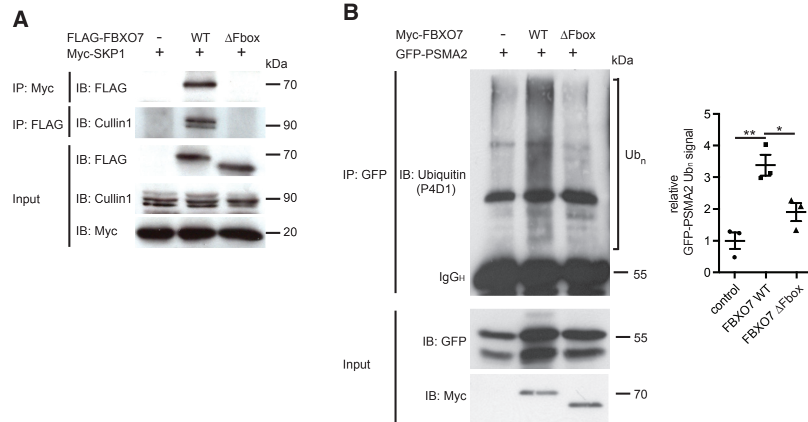
Appendix Figure S1



Appendix Fig S1: **Histological analyses of the *FBXO7-/-* mice. A.** Sagittal paraffin sections of brains from P18 *FBXO7+/+* and *FBXO7-/-* mice were subjected to immunohistochemistry using the α-synuclein antibody. Three independent litter pairs were analyzed, representative images of sections are shown. Scale bar = 40 µm. **B.** Sagittal paraffin sections of brains from *FBXO7+/+* and *FBXO7-/-* mice were subjected to immunohistochemistry using the Iba1 antibody. Three independent litter pairs were analyzed (paired t-test, mean +s.e.m.). Scale bar = 40 µm. **C.** Cortical sections from P18 WT and FBXO7 KO mice were subjected to immunohistochemistry using the cleaved caspase 3 antibody. Four independent litter pairs were analyzed (paired t-test, mean +s.e.m.). **D.** Lysates of HEK293T cells transfected with empty control vector or FBXO7 shRNA plasmid, were immunoblotted with the FBXO7 or pan 14-3-3 antibody. The latter served as loading control. **E.** Lysates of cortical neurons, nucleofected with empty control vector, FBXO7 shRNA plasmid or control shRNA plasmid, were immunoblotted with the FBXO7 or pan 14-3-3 antibody. The latter served as loading control.

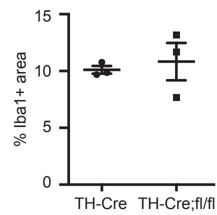
Appendix Figure S2



Appendix Fig S2: FBXO7 ubiquitinates PSMA2. A. Lysates of HEK293T cells, transfected with empty control vector, FLAG-FBXO7 or FLAG-FBXO7 ΔF-box plasmids together with the myc-SKP1 expression plasmid, were subjected to IP with the Flag or myc antibody followed by IB with the cullin-1 or FLAG antibody. **B.** Lysates of HEK293T cells, transfected with the indicated myc-FBXO7 plasmids and the GFP-PSMA2 plasmid, were subjected to boiling, followed by IP with the GFP antibody (PSMA2), followed by IB with the ubiquitin antibody (sc-P4D1) (upper panel). Inputs were immunoblotted with the GFP or myc antibody (lower panels). Three independent blots were quantified and band intensity normalized to control average. (ANOVA, *$p<0.05$, **$p<0.001$, mean + s.e.m).

Appendix Figure S3

A



Appendix Fig S3: Loss of FBXO7 in TH+ cells does not affect microglia. A. Quantitative measurements of immunohistochemistry of midbrain section from TH-Cre and TH-Cre;fl/fl mice using the Iba1 antibody. n=3 animals per genotype (t-test, mean + s.e.m.).

Methods

Yeast two-hybrid

To identify FBXO7 interaction partners we used the Matchmaker® Gold Yeast Two-Hybrid protocol with full-length *FBXO7* cloned into the pGBT9 vector (Clontech, Mountain View, CA, USA) as bait and the Mate & Plate™ Human Fetal Brain library (Clontech) as prey.

Quantitative PCR

For assessment of FBXO7 and PSMA2 mRNA expression level in *FBXO7* knock-out mice, total RNA of *FBXO7+/+*, *FBXO7+/-* and *FBXO7−/−* tissue was isolated using the TRIZOL reagent (Invitrogen). cDNA was then synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen). To amplify cDNA fragments, cDNA was mixed with the Power SYBR Green PCR Master Mix (Invitrogen). Primers for amplification were:

FBXO7 5′- tggaagtcaagtggtgtatac-3′ (forward), 5′-tactccagcagcaacgtagga-3’ (reverse);

PSMA2 5′- gtttaccaagaacccattccc-3′ (forward), 5′-gtaagctccagatggatctga-3’ (reverse);

β-actin was used as housekeeping control.

Primary neuronal cell culture

Neurons were cultured from postnatal day 0 (P0) Wistar rat cortices or P5 mouse murine cerebella as previously described (Bilimoria & Bonni, 2008, Holubowska, Mukherjee et al., 2014).

Nucleofection of primary cortical neurons

Following the primary cortical neuron culture, 8 x 10^6 rat cortical neurons were resuspended in 100 µl of 1x DMEM and 7 - 10 µg plasmid DNA added. The DNA-cell suspension was then subjected to electrical pulsing using the O-005 program of the

nucleofector (Amaxa®, Lonza) and incubated with 1 ml of prewarmed DMEM for 5 min at room temperature. Finally, neurons were plated onto polyornithine-coated 6-well plates with prewarmed nucleofection plating medium (DMEM [+] 4.5 g/L glucose [-] L-glutamine [-] pyruvate (Gibco^{TM}), 10% FCS). After 4 h, the medium was replaced by plating medium (Neurobasal^{TM} [-] L-glutamine (Gibco^{TM}), 1 % PSG, 2 % B27 supplement).

Survival assay

Rat cortical neurons were transfected with FBXO7 and control shRNA together with the transfection marker GFP at DIV3, using the modified calcium-phosphate method as previously described (Holubowska et al., 2014, Mukherjee, Holubowska et al., 2015). The neurons were fixed at DIV6 and subjected to immunocytochemistry using the GFP and cleaved-caspase-3 antibodies. In addition, the nuclei were stained with the DNA dye DAPI. Cleaved-caspase-3 was used to visualize apoptotic neurons. 100 transfected neurons from each of three independent experiments were included in the analysis.

Subcellular fractionation

Subcellular fractionation was performed as previously described (Kannan, Lee et al., 2012) by lysing cultured neurons or tissue with a 2 ml dounce homogenizer in detergent-free buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, protease inhibitors). The perinuclear fraction was separated as supernatant from the pelleted nuclei at 376 g, 4°C. The pellet was then washed thrice in 0.1% NP40-supplemented buffer A, followed by lysis in buffer B (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, protease inhibitors) and finally spun down at maximum speed (12 210 g) at 4°C. The supernatant was harvested as the nuclear fraction.

Both fractions were boiled with SDS sample buffer and subjected to immunoblot analysis.

Immortalized cell line culture and transfection

HEK293T cells purchased from ATCC were maintained and transfected as previously described (Matz, Lee et al., 2015). The cells were transfected with indicated plasmids using the modified calcium phosphate method.

Co-immunoprecipitation and immunoblot analysis

Co-immunoprecipitation analyses were carried out using transfected HEK293T cells. These were lysed in buffer containing 1% NP40, 150 mM NaCl, 20 mM Tris pH 7.5, 1 mM EDTA, 10% glycerol, protease inhibitors (3 μg/ml aprotinin, 1 μg/ml leupeptin, and 1μg/ml pepstatin) and 1 mM DTT. Lysates were incubated with indicated antibodies rotating at 4°C for 3 hours and subsequently with Protein A-Sepharose beads (GE Healthcare) for 1 hour. The protein-bound beads were washed three times with Triton X-100 buffer (1 M NaCl, 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1% Triton X-100) and once with PBS. The bound protein was eluted and separated by SDS-PAGE followed by immunoblot analysis.

Tissue samples from mice or transfected HEK293T cells were lysed in TX100 buffer (150mM NaCl, 50mM Tris-HCl pH 7.5, 1 mM EDTA, 1% Triton X-100, protease inhibitors) and appropriate amounts were subjected to SDS-PAGE followed by immunoblot analysis.

Immunoblot analysis was performed by running samples on an SDS-PAGE gel, before transfer of proteins to a nitrocellulose membrane (Amersham). After 30 minutes blocking, primary antibody diluted in 3% bovine serum albumin was applied over night

at 4°C. Secondary HRP conjugated antibodies were diluted in milk and applied to membranes for 45 minutes at room temperature. Protein bands were detected by enhanced chemiluminescence (Thermo Scientific) on high performance hyperfilms (Thermo Scientific) in an Agfa imaging station.

Densitometric analyses was performed using the ImageJ Gel Analyzer plug-in. Band intensity was normalized to loading control and compared to control sample.

Cell-based ubiquitination assay

The cell-based ubiquitination assay was carried out using an adapted version of the method described by Lu and colleagues (Lu, Pribanic et al., 2007). Transfected HEK293T cells were lysed in RIPA buffer without SDS supplemented with protease inhibitors and 10 mM NEM. Next, 1 - 2 mg of cell lysate were incubated with 1 % SDS for 5 min at 4°C on a rotator followed by boiling for 10 min at 95°C to disrupt protein-protein interactions. The SDS concentration was then reduced to 0.1 % by 10x dilution with lysis buffer (50mM HEPES pH 7.5, 150mM NaCl, 1.5 mM $MgCl_2$, 1% Triton™X-100 and 10% glycerol) and subsequently incubated with primary antibody overnight at 4°C on a rotator. For immunoprecipitation, Protein A-sepharose bead slurry was added and incubated for 45 min at 4°C on a rotator. Finally, beads were washed twice with lysis buffer, once with RIPA with SDS and once with PBS, followed by addition of 30 µl of SDS-sample buffer and boiling for 5 min at 95°C. 50 - 100 µg of the original cell lysate served as an input control.

In vitro ubiquitination assay

In vitro ubiquitination assay was carried out as described previously (Furukawa, Andrews et al., 2005). Briefly, Flag-GFP-tagged PSMA2 was expressed in HEK293T cells,

isolated with Flag-Sepharose and eluted with Flag peptide. Flag-tagged FBXO7 together with HA-tagged cullin1, HA-tagged Roc1 and myc-tagged SKP1 was expressed in HEK293T cells, immunoprecipitated with Flag antibody and Protein A-Sepharose beads. Beads were washed three times in mild co-IP buffer (0.5% NP40, 150 mM NaCl, 20 mM Tris pH 7.5, 1 mM EDTA, 10% glycerol) and once in 1X ubiquitination assay buffer (50 mM Tris-HCl pH 7.4 at 37°C, 5 mM $MgCl_2$, 0.6 mM DTT, 1 mM ATP). The ubiquitination reaction was carried out in a total volume of 50 μl. Reaction mixture containing 0.25 μM E1, 1 μM E2 (UbcH3, UbcH5c or UbcH13), 12 μg ubiquitin, 1 mM ATP and 10X ubiquitination assay buffer was assembled on ice and transferred to a tube containing the FBXO7-SCF immobilized on beads and eluted Flag-GFP-PSMA2. The mixture was incubated at 37°C for 1 hour.

Purification of GST-fusion proteins

The GST-tagged Ubl-domain of RAD23B was used to bind and precipitate 26S proteasomes with high affinity as previously shown (Schauber, Chen et al., 1998). *Ubl*^{RAD23B} cloned into pGEX-4T-1 vector was transformed into *E. coli* (strain BL21) and were grown to OD 0.5, induced with 1 mM isopropyl β-D-1-thiogalactopyranoside for 3 h and sonicated in Co-IP buffer (1% NP40, 150 mM NaCl, 20 mM Tris pH 7.5, 1 mM EDTA, 10% glycerol) with protease inhibitors (3 μg/ml aprotinin, 1 μg/ml leupeptin, 150 μM PMSF and 1μg/ml pepstatin) and 1 mM DTT. GST fusion proteins were purified from bacterial lysates by incubation with glutathione-coupled Sepharose beads (GE healthcare). A similar protocol was used to generate GST-FBXO7. GST-Flag-PSMA2 was expressed in BL21 bacteria and purified from inclusion bodies as described

previously (McGettrick & Worrall, 2004). Cleavage of the GST-tag was achieved using homemade PreScission Protease.

GST pulldown

GST pulldown of proteasomes from whole brain lysates was modified from the published protocol (Sambrook & Russell, 2006). Briefly, tissue was lysed in proteasomal activity lysis buffer (50 mM Tris-HCl pH 7,5, 250 mM sucrose, 5 mM MgCl2, 2 mM ATP, 1 mM DTT, 0.5 mM EDTA and 0.025% digitonin). As a pre-clear step 10 μg of GST protein was added to 2 mg protein lysate together with 20 μl glutathione beads (GE Healthcare) for 1 h at 4°C. 10 μg GST or GST-Ubl^{RAD23B} fusion protein was added to the supernatant for 2.5 hours followed by addition of Glutathione beads and incubation for 1.5 hours. Samples were washed four times with 0.1% NP40 in PBS, boiled in SDS sample buffer and subjected to SDS-page and immunoblot analysis as described.

Interaction assay of purified proteins

GST-FBXO7 and GST only coupled to sepharose beads were obtained after purification from bacterial lysates. Purified Flag-PSMA2 was pre-cleared with Glutathione Sepharose beads in mild co-IP buffer for 1 h at 4°C. 12 μl of GSTonly or GST-FBXO7 coupled Glutathione beads were incubated with pre-cleared Flag-PSMA2 for 45 min at 4°C, washed 3 times with mild coIP buffer and eluted with 4X sample buffer followed by boiling for 5 min at 95°C. Precipitated protein complexes were subjected to immunoblot analysis.

Proteasome affinity purification

Proteasome affinity purification was carried out using an adapted version of the method described by Besche and Goldberg (Besche & Goldberg, 2012). In this protocol, the

precipitated 26S proteasomes are eluted using the His10-tagged UIM-domain of human S5a as binding competition for the RAD23B Ubl-domain.

For 26S proteasome affinity purification, 750 mg of cortical tissue pooled from four P18 *FBXO7*+/+ and -/- mice, respectively, was lysed in proteasome lysis buffer (25 mM Tris-HCl pH 7.5, 10% glycerol, 5 mM $MgCl_2$, 1 mM ATP and 1 mM DTT) using a mechanical homogenizer (POLYTRON®, PT 1200 E). Following the centrifugation of the cortical lysates for 20 min at 4°C and 14000 rpm, 750 µg of GST-Ubl^{RAD23B} and 230 µl GST-bead slurry (Glutathione SepharoseTM 4B, GE Healthcare) were added to the lysates and incubated for 2.5 h at 4°C on a rotator. Subsequently, the beads were washed twice with 40x bed volume lysis buffer prior to the first elution by addition of 750 µg of His10-UIM^{S5a}. After mixing and followed by incubation for 15 min at 4°C, the beads were spun down. A second elution was then performed by addition of 750 µg of His10-UIMS5a. The eluates 1 and 2 were then combined and incubated with 112.5 µl Ni-NTA sepharose bead slurry for 30 min at 4°C on a rotator. Following centrifugation for 1.5 min at 4°C and 500 rpm, the supernatant was collected as the 26S proteasome elution fraction. SDS-PAGE and Coomassie blue staining determined purity of the 26S proteasome. Purified proteasomes were subjected to further analysis by native PAGE and proteasome activity assays.

Proteasome activity analysis

Proteasome activity assays were carried out as described by Kisselev and colleagues (Kisselev & Goldberg, 2005): Cells were lysed in proteasome activity lysis buffer (50 mM Tris-HCl pH 7.5, 250 mM sucrose, 5 mM MgCl2, 2 mM ATP, 1 mM DTT, 0.5 mM EDTA and 0.025% digitonin). 12 µg of cell lysate was incubated with assay buffer (50

mM Tris-HCl pH 7.5, 40 mM KCl, 5 mM MgCl2, 0.5 mM ATP, 1 mM DTT and 0.5 mg/ml BSA). 100 μM of the fluorogenic peptide substrate Suc-LLVY-AMC was used to assess chymotrypsin-like activity of the proteasome and fluorescence measured using a fluorescence plate reader (Victor) at 355/460 nm.

Glycerol density gradient centrifugation and analysis of proteasome fractions

Glycerol density gradient centrifugation was carried out using an adapted version of the method described by Koulich and colleagues (Koulich, Li et al., 2008). Transfected HEK293T cells or tissue were lysed and homogenized in proteasome cell lysis buffer (20 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 2 mM ATP and 1 mM EDTA) and then centrifuged for 10 min at 4°C and 14000 rpm. Per condition, a total amount of 0.5 - 1.5 mg of lysate was used. 10 - 40 % linear glycerol gradients were prepared in a volume of 4 ml using glycerol gradient buffer (20 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, 1 mM EDTA and 10 - 40 % glycerol) followed by loading of the sample on top. Subsequently, samples were centrifuged for 14 h at 4°C and 83000 g using a Beckman XL-90 ultracentrifuge with a SW-60 Ti rotor (Beckman Coulter). Following centrifugation, the gradients were divided equally into 20 fractions with a volume of 200 - 220 μl. 20 μl of each fraction was subjected to proteasome activity analysis and the remainder precipitated using a 4x volume of ice-cold acetone followed by incubation for 2 h at -20°C. After centrifugation for 10 min at 14000 rpm, SDS-sample buffer was added to the samples, which boiled for 5 min at 95°C. SDS-PAGE and immunoblot analysis were then carried out to confirm protein content and successful proteasome fraction.

Characterization of proteasome profiles using native PAGE

10 - 75 µg of cell or tissue lysates prepared in proteasome cell lysis buffer (50 mM Tris-HCl pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, 0.5 mM EDTA and 0.025 % digitonin) or 25 µg of purified 26S proteasomes were mixed with native PAGE sample buffer (125 mM Tris-HCl pH 6.8, 50 % glycerol, 2 % bromophenol blue) and resolved by native PAGE for 125 min at 175 V followed by immersion of the gel in assay buffer (50 mM Tris-HCl pH 7.4, 5 mM MgCl2, 1 mM ATP). Following addition of 100 µM of Suc-LLVY-AMC, the gel was incubated for 5-10 min at 37°C and the proteasome profile visualized by exposure to UV light. For stimulation of the latent 20S proteasome activity, 0.02 % SDS was added and the gel incubated for another 5-10 min at 37°C. Subsequently, the gel was immersed in pre-transfer buffer (25 mM Tris base, 192 mM glycine, 1 % SDS) and incubated for 10 min at RT. Finally, gels were transferred onto nitrocellulose membranes and subjected to immunoblot analysis.

Immunohistochemistry of NEX-Cre; fl/fl and control mice

Mice were anesthetized by intraperitoneal injection of (Ketamine/Xylazine) and transcardially perfused with PBS followed by 4% PFA. Brains were isolated and postfixed in 4 % PFA for 4 h at 4°C, washed once in PBS and then incubated in a 30 % sucrose solution overnight at 4°C for X-Gal staining. For microtome sectioning, tissues were postfixed in 4 % PFA in phosphate buffer (36% NaH_2PO_4, 3.1% Na_2HPO_4 and 1% NaCl) overnight at 4°C.

5 µm thick brain sections from FBXO7-/- and control mice as well as Nex-Cre;fl/fl and controls were cut with a microtome (Microm) and subjected to paraffin embedding using standard protocols with antigen retrieval in Citrate buffer. Sections were cooled down at room temperature for 30 min and rinsed twice in Tris buffer with 2 % milk powder.

Endogenous peroxidase activity was inhibited by treatment with 3 % H_2O_2 for 5 min followed by washing in Tris buffer with 2 % w/v milk powder. Sections were blocked in 20 % goat serum in BSA/PBS (0.04 M NaH_2PO_4, 0.16 M Na_2HPO_4, 1.8 % w/v NaCl, 1 % w/v BSA). Primary antibody was diluted in BSA/PBS and applied over night. Sections were then rinsed with Tris buffer with 2 % w/v milk powder and subjected to DAB staining using the LSAB2 kit (DAKO). Following DAB labeling, the slides were subjected to nuclear staining in 0.1 % haematoxylin (Merck) and agent Scott's solution (Thermo Fisher Scientific). Sections were rehydrated and mounted using Eukitt® (Kindler) mounting medium.

Area quantification of GFAP- or Iba1-positive areas was achieved using a custom-designed ImageJ macro provided by Dr. Miso Mitkovski. The macro functions by converting the picture into a black and white image, representing DAB-positive areas as black followed by measuring of the percentage area covered by the DAB signal as compared to the total area of interest selected.

Labeling of apoptotic cells using the TUNEL assay

Mounted 5 µm thick sections were subjected to the TUNEL assay following the DeadEnd™ Colorimetric TUNEL System kit (Promega). Samples were mounted using Aqua-Poly/Mount (Polysciences).

LacZ staining of vibratome mouse brain sections

40 µm thick sagittal whole brain sections of P18 *FBXO7* +/+ and *FBXO7* -/- mice were obtained by vibratome sectioning (VT100S, Leica), and incubated in the dark overnight at 37°C in 2 mM $MgCl_2$, 0.02% NP40, 0.01% sodium deoxycholate, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 0.5 mg/ml X-gal in PBS. Subsequently,

sections were washed in PBS and mounted onto microscope slides (Marienfeld), air-dried and mounted in 50 % glycerol. Finally, sections were imaged using a brightfield microscope (Axio Observer Z1, Zeiss) and the Zen 2011 imaging software (Zeiss, Germany).

Immunohistochemistry for TH-Cre;fl/fl mice with controls

TH-Cre;fl/fl and control mice were transcardially perfused with PBS followed by 4% PFA. Brains were harvested and post-fixed in 4% PFA o/n before cryo protection in 30% sucrose in PBS. Brains were then embedded in OCT: 30 % sucrose in PBS (1:1) and stored at -80°C. 30 μm sections from substantia nigra and striatum were then cut on a Cryostat and stored in PBS/0.1% $NaNH_3$ at 4°C. For fluorescent stainings sections were permeabilized (0.5% Triton X-100 in PBS) and blocked (10% NGS, 3% BSA, 0.5% Triton X-100 in PBS) before adding primary antibody in blocking buffer, without Trition X-100 over night. Fluorophore-conjugated secondary antibody was applied for 1 hour at RT. Sections were mounted on polysine slides (Thermo Scientific) with Mowiol mounting medium.

Image acquisition was done using a brightfield microscope (Axio Observer Z1, Zeiss) and the Zen 2011 imaging software (Zeiss, Germany).

For stereological counting every fourth section from substantia nigra was chosen and permeabilized in 40% methanol/1% H_2O_2 in TBS, blocked in 5% NGS/TBS and stained with rb α-TH (1:1000 dilution; Zytomed, 620-0336) in 2% NGS/TBS for 48 hours followed by 2 hours at RT in secondary biotinylated anti-rabbit antibody. Sections were then subjected to the Vectastain ABC Kit (Vector Laboratories) for 2 hours at RT before DAB (Vector Laboratories) staining. Nissl staining were performed on the same sections

after drying them for 5 days. Slides were hydrated in Xylol:Isopropanol:Ethanol:H_2O followed by staining in thionine acetate/0.2% acetic acid for 7 min before dehydration through a reversed alcohol row. Sections were mounted on polysine slides (Thermo Scientific) with Eukitt® mounting medium.

Stereological quantification of substantia nigra neurons

Tyrosine hydroxylase-, or Nissl-positive cells in the substantia nigra were counted using stereological methodology as previously described (Tonges, Frank et al., 2012). A Stereo Investigator software (Stereo Investigator 9.0, MicroBrightField Inc.) and a Zeiss Axioplan microscope were used to analyze every fourth section of the substantia nigra. Substantia nigra pars compacta was outlined and a point grid overlaid. Cells positive for TH or Nissl-staining were counted by the optical fractionator method (×40 objective, counting frame 50 × 50 μm). Values represent one substantia nigra per animal. Nissl cells were counted to rule out down-regulation of the tyrosine hydroxylase enzyme as opposed to loss of neurons. Experiment was performed by a blinded investigator.

Neurochemical analysis of dopamine and metabolites

HPLC analysis was conducted as previously described (Tonges et al., 2012). Mice were sacrificed and striata immediately dissected on ice. Striatal tissue was homogenized in a bead mill homogenizer (Precellys 24®, Peqlab, Erlangen, Germany) with 50 μl of 0.1 M perchloric acid per mg of tissue. Sample was subjected to centrifugation (13.4 g, 5 min), supernatant transferred to a clean tube and subjected to another centrifugation (13.4 g, 10 min, 4°C). 20 μl of supernatant was injected onto a C18 reverse-phase HR-80 catecholamine column (ESA, Bedford, MA, U.S.A.). Dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were quantified by

HPLC with electrochemical detection. The mobile phase (pH = 4.3) consisted of 6.9 g/l sodium acetate, 48 mg/l EDTA, 7.3 g/l citric acid, 105 mg/l octane sulfonic acid, and 10 % methanol. Flow rate was 0.4 ml/min. Peaks were detected by an ESA Coulochem III with a model 5010 detector (E1 = 50 mV, E2 = 400 mV). Data were collected and processed using the Chromeleon computer system (Dionex, Idstein, Germany).

Behavioral analyses

All lines were kept in a pure C57BL/6N background. TH-Cre and NEX-Cre lines were kept strictly heterozygous for Cre allele. Animals were housed under a standard 12 h light–dark cycle with food and water ad libitum in the animal facility of Max Planck Institute of Experimental Medicine, Göttingen, Germany.

For analyses of the *FBXO7* conventional KO mice, mixed gender mice of age P18 were used in all experiments. Mice were allowed to acclimatize in testing facility for 30 min before test. Due to the extreme difference in weight between *FBXO7-/-* and littermates, experimenter could not be kept blind to genotype. For animals from conditional *FBXO7*-floxed strains and conventional *FBXO7* wildtype and heterozygous mice older than P18, male mice were used. All males were allowed to acclimatize in the facility one week before testing occurred and spent at least 30 minutes in test room before every test.

Hind limb clasping

Animals were suspended by their tails for 10 seconds in 3 trials. A score was given for each trial were 0 was normal and 3 corresponded to both hind limbs being retracted towards the stomach for more than 50% of the time. Trial scores were then averaged.

Wire hang

Wire hang was adapted from a previously described method (van Putten, de Winter et al., 2010) on an 80 cm long, 1mm wide steel wire suspended 30 cm above surface. Mice were placed with front paws in the middle of the wire and hang time measured. Climbing to the end of the wire was counted as maximal time (30 sec). Each mouse was tried three times with an intra test interval of 5 min.

Open field

Open field analysis of ambulation for conventional FBXO7 KO and control mice at P18 was conducted in a small square container, as previously described (Biondi, Branchu et al., 2010). Briefly, mice were placed in the center of a plastic container measuring 28x28x20cm and observed for 5 min. The container was divided into 16 squares where the four in the middle were referred to as central and the remaining as peripheral squares. Number of peripheral and central crossings between squares was recorded manually.

Open field analysis for adult mice was conducted in a circular open field (60 cm diameter). Movement was recorded by the Viewer observation system (Noldus) and total travel length measured.

Rotarod

All rotarod experiments were conducted on a rotarod (Ugo Basile) with an accelerating protocol of 4-40 rpm in 5 min. P18 conventional KO mice were tried three times on 1 day with an intra interval time of 20 min and average latency to fall was measured. Adult mice were tried with either the same protocol on three executive days or three trials (3 and 24 hours interval) to also assess motor learning.

Balance beam

The balance beam protocol was adapted from Luong et. al (Luong, Carlisle et al., 2011). In brief, mice were placed on a beam of 12 mm width, 80 cm length, 50 cm above ground with home cage at the other end and encouraged to cross 3 times with 15 seconds rest in home cage between trials. After 10 minutes rest the procedure was repeated on a beam of 6 mm width. The training was repeated on two consecutive days before the test day where average time to cross each beam was recorded and animals scored according to number of slips on beam with 7 being given for perfect crossing and a score of 1 given to animals unable to cross the beam.

Pole test

Pole test was conducted as previously described (Ogawa, Hirose et al., 1985). Briefly, animals were placed on a 50 cm tall pole, 1 cm diameter and covered in gauze, with head facing up and encouraged to climb down for five times with 30 seconds between trials. Training was given on two consecutive days before the test day, where time to turn and total descent time was recorded manually and the animals best try used for analysis.

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