Supplemental Information

TGF-β signaling in dopaminergic neurons regulates dendritic growth, excitatory/inhibitory synaptic balance and reversal learning

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Immunogold Electron Microscopy

P14 *TbRII*$^{fl/fl}$ and *DAT-iCre;TbRII*$^{fl/fl}$ mice (N = 3) were transcardially perfused using 2% PFA/0.2% glutaraldehyde in 0.1 M phosphate buffer solution (PBS). Brains were removed, fixed in 2% PFA overnight and sectioned at 60 mm using a vibratome. Sections were cryoprotected in 25% sucrose/10% glycerol in PBS for 2 hours, freeze-thawed, incubated for an hour in 4% normal goat serum (NGS)/PBS, and then with anti-TH antibody (1:400, Milipore) in 1% NGS. After washing in PBS, sections were incubated for 2 hours with Ultra small gold conjugated secondary goat anti-rabbit IgG (1:100, Aurion) in 0.2% acetylated BSA solution (BSA-c™, Aurion)/0.2% fish gelatin in PBS. Silver enhancement was performed using the R-Gent SE-EM Silver Enhancement kit (Aurion) according to manufacturer's protocol. Sections were post-fixed in 0.5% OsO_4, dehydrated through increasing concentrations of ethanol and propylene oxide, and impregnated with resin overnight. They were then mounted on Sigmacote-coated glass slides with resin and heated at 60°C overnight. Areas of interest were cut out from the sections, embedded in resin and heated again at 60°C. Ultra thin sections (1 mm) were cut with a microtome (Reichert-Jung, Reichert) and collected on copper grids. Sections were examined with a JEOL 1200EX electron microscope.

Bioinformatics and Luciferase Assays for Transcriptional Regulation of *Tgfb1*

To identify potential Smad and AP-1 binding motifs that control the expression of TGF-β1, we isolated a 5 kb mouse *Tgfb1* genomic sequence upstream of the first coding exon. The mouse *Tgfb1* locus contained two transcriptional start sites (TSS)(+1 and +290), with the first ATG located at position +868. This 5 kb *Tgfb1* genomic sequence was analyzed using the "Cluster Buster" software (Frith et al., 2003) (http://zlab.bu.edu/cluster-buster/), which identified 4 Smad-binding elements (SBE)(+369, +63, -3,673 and -3,847) and 4 AP1 binding sites (+685, -1,405, -2,189 and -3,354). Given the distribution of the SBE and AP-1 sites, this 5 kb sequence was divided into 4 regions that contained one or two binding sites, amplified by polymerase chain reaction, and subcloned into the pGL4.10-Luc promoterless vector (Promega). All *Tgfb1*-Luciferase constructs were confirmed by sequencing analysis, and luciferase assays performed by transfecting N2A neuroblastoma cells with the reporter plasmids together with the control *Renilla* luciferase plasmid pRL-null (Promega) using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's recommendations. Luciferase activity was measured with the Dual-Luciferase Reporter assay system (Promega) 24 hours after transfection, and was normalized by measuring the *Renilla* luciferase activity. Each luciferase assay was done in triplicate, and repeated three times (N = 3).

Whole-cell Patch Clamp Recording of DA Neurons

Whole-cell voltage clamp recordings were made with borosilicate glass pipettes (3~4 MΩ) filled with an internal solution containing: 126 mM $CsMeSO_3$, 10 mM HEPES, 1 mM EGTA, 2 mM QX-314 chloride, 0.1 mM $CaCl_2$, 4 mM MgATP, 0.3 mM Na_3GTP, 8 mM Na_2-phosphocreatine (280~290 mOsm, pH 7.3 with CsOH). 0.2% Biocytin was added to internal solution for morphological reconstruction of DA neurons. To measure both EPSC and IPSC from the same neurons, dopamine neurons were held at –70 mV (the reversal potential of chloride) to measure EPSC and at 0 mV (the reversal potential of ionotropic glutamate receptors) to measure IPSC, respectively. For mEPSC/mIPSC experiment, TTX (500 nM) and R-CPP (10 μM) was always present to block sodium channel and NMDA receptors, respectively. At lease 5 min

stable recordings were obtained for mEPSCs and mIPSCs. For input-output curve (I/O) of evoked EPSCs and IPSCs, local electrical stimulation was delivered at 0.05 Hz (stimulus pulse duration, 0.1 ms; stimulus intensity, 6~18 mV) using a glass pipette filled with ACSF and three successive responses were averaged, representing the averaged response from one stimulus intensity. E/I ratio was calculated by dividing the amplitude of EPSC by the amplitude of IPSC. R-CPP (10 μM) was present to block NMDAR. To examine the burst firing properties of SNc dopaminergic neurons, current-clamp mode was used to measure membrane potentials with potassium-based internal solution containing: 135 mM $KMeSO_3$, 3 mM KCl, 10 mM HEPES, 1 mM EGTA, 0.1 mM $CaCl_2$, 4 mM MgATP, 0.3 mM Na_3GTP, 8 mM Na_2-phosphocreatine (280~290 mOsm, pH 7.3 with KOH). To induce burst firing, local electrical stimulation was delivered at 100 Hz (stimulus duration, 1 sec; pulse duration, 0.1 ms; intensity, 20 mV). Access resistance was 15~25 MΩ and only cells with a change in access resistance < 20% were included in the analysis. Whole-cell patch recordings were performed using Multiclamp 700B (Molecular Devices), monitored (WinWCP, Strathclyde Electrophysiology Software) and analyzed offline using Clampfit 10.0 (Molecular Devices). Signals were filtered at 2 kHz and digitized at 10 kHz (NI PCIe-6259, National Instruments). Reagents were purchased from Sigma except R-CPP, QX-314 and TTX (Tocris).

Behavioral Paradigms

Open field and elevated plus maze. Open field recordings were performed using Kinder Scientific SmartFrame Open Field apparatus (16" x 16") and MotorMonitor tracking and analysis software. Naïve control and *DAT-iCre/+;TbRII*$^{fl/fl}$ male mice between 8 and 10 weeks of age (n=12 for both genotype) were acclimated to the testing room for at least 30 min before being placed in arena. The first 10 minutes of activity was recorded for each mouse. For 60-minute activity testing, ENV-510 Open Field Arena (10.75" x 10.75" x 8" H) from Med Associates was used and analyzed using Activity Monitor software. The center zone was defined as a square in (X, Y) coordinates as (4, 4) to (12, 12). Periphery was defined as the residual space. Elevated Plus Maze recordings were performed using Kinder Scientific Elevated Plus maze apparatus with arms 2" wide x 15" long and closed wall 6" high (n=14 for control and n=8 for *DAT-iCre;TbRII*$^{fl/fl}$). MotorMonitor software was used for tracking and analysis.

Rotarod. 8-10 week old male mice (n=12 for control and *DAT-iCre;TbRII*$^{fl/fl}$) were tested for motor coordination and learning by placing them on a rotating rod that incrementally accelerated from 4 to 40 rpm over 5 min. Each mouse underwent 3 sessions per day and performance was assessed on three consecutive days as latency to fall from the rod.

Reversal Learning. The 4-choice odor discrimination and reversal task followed the protocols by Johnson and Wilbrecht (Johnson and Wilbrecht, 2011). The test arena is composed of four chambers of clear acrylic with outer dimensions 9" height x 12" x 12" and internal walls 2 ⅞" x 8 ¾" height. A central removable cylinder is 6" in diameter and 8 ¾" tall. Bowls containing cheerios and wood shavings are 2 ⅞" wide and 1 ¾" deep and made of opaque white ceramic. Two cohorts of 12-week-old male control and *DAT-iCre;TbRII*$^{fl/fl}$ mice (cohort #1: control: n=8 and *DAT-iCre;TbRII*$^{fl/fl}$: n=8; cohort #2: control: n=12 and *DAT-iCre;TbRII*$^{fl/fl}$: n=8) were food-deprived over two days to 90% of their original body weight and fed to maintain 90% body weight during training. For the habituation phase, a mouse was placed in the center of the arena with the cylinder lowered around it. A bowl containing $\frac{1}{8}^{th}$ of a Honey Nut Cheerio (General Mills, Minneapolis, MN) was placed in each of the four compartments. The cylinder was then raised and the animal was allowed to explore freely and eat from the bowls. Bowls were rebaited every 10 minutes and the total time for habituation is 30 minutes.

The first day of training was a habituation phase that allowed mice to become familiar with the arena and bowls containing Cheerio. In the shaping phase, one bowl and increasing amounts of unscented wood shavings (over 16 trials) were used to train the mouse to dig for cheerio pieces. The bowl changed compartments each trial and the time taken to complete each trial was recorded. The third day consisted of an odor discrimination phase and a reversal phase. In the odor discrimination phase, shavings scented with odors (O1-O4: anise, clove, litsea and thyme respectively) were placed in separate sham-baited bowls. Only O1 was rewarded, with $\frac{1}{8}^{th}$ of a Cheerio buried at the bottom of the bowl. The stimulus presentation was alternated so that an odor was never in the same location two trials in a row. Each trial was timed and criterion was met when a mouse completed 8 out of 10 consecutive trials successfully. Once criterion was met for discrimination, the reversal phase was initiated. In this phase, O2 (clove) became the rewarded odor and O4 (thyme) was switched out for a novel odor, eucalyptus. Again, each mouse ran this behavior to criterion.

To quantify the reversal learning phenotype, mice were recorded for the frequency of their entry to each quadrant, the latency to start digging and their digging choice (O1-O4). Errors during the reversal learning paradigm were defined as follows: (1) Reversal – mouse dig in previously rewarded odor (anise); (2) Perseverative – number of reversal errors made *before* one correct; (3) Regressive – reversal errors made *after* one correct; (4) Novel – mouse digs in novel odor (eucalyptus); (5) Irrelevant – mouse digs in never rewarded odor (litsea). Cumulative latency to start digging was quantified for the first ten trials in each phase. Data from both cohort #1 and cohort #2 were combined and analyzed using two-way ANOVA.

REFERENCES

Frith, M.C., Li, M.C., and Weng, Z. (2003). Cluster-Buster: Finding dense clusters of motifs in DNA sequences. Nucleic acids research *31*, 3666-3668.

Johnson, C., and Wilbrecht, L. (2011). Juvenile mice show greater flexibility in multiple choice reversal learning than adults. Developmental cognitive neuroscience *1*, 540-551.

SUPPLEMENTAL FIGURES

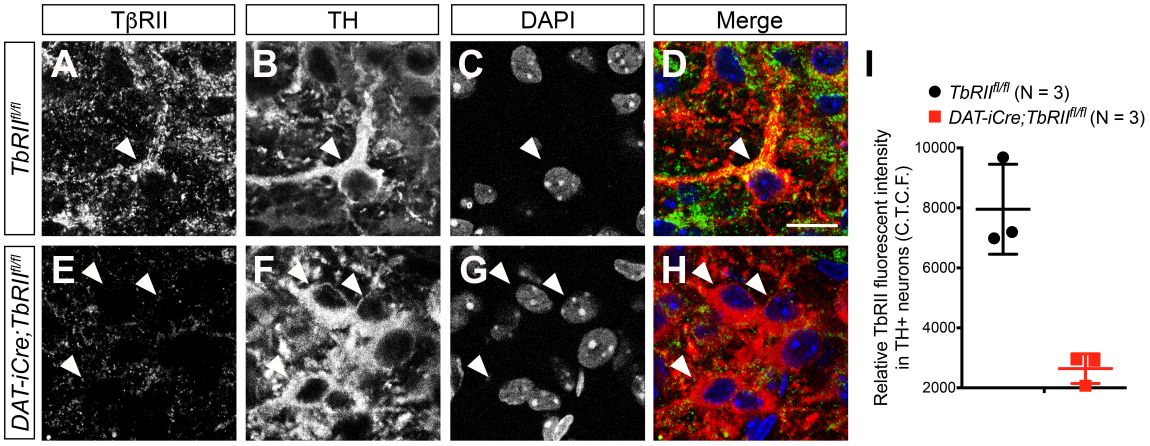

Figure S1. *DAT-iCre* removes TβRII from DA neurons in P0 *DAT-iCre;TbRII*$^{fl/fl}$ mutants, Relates to Figure 1

(A-D) Individual channel of confocal images showing abundant TβRII signal in TH+ DA neurons in the substantia nigra pars compacta (SNpc) in P0 control *TbRII*$^{fl/fl}$ mice.

(E-H) Indivdual channel of confocal images showing a marked reduction in TβRII signal in TH+ DA neurons in P0 *DAT-iCre;TbRII*$^{fl/fl}$ mutants. Scale bar, 20 μm.

(I) Quantification of TβRII immunofluorescent signal in P0 control and *DAT-iCre;TbRII*$^{fl/fl}$ mutants (N = 3, each). The TβRII immunofluorescent signal in confocal images from 202 DA neurons from control *TbRII*$^{fl/fl}$ mice and 205 DA neurons from *DAT-iCre;TbRII*$^{fl/fl}$ mutants are quantified using NIH ImageJ. Data presented as mean ± S.D., Student's *t* test, ** $p = 0.0043$. The trace amount of TβRII signal in *DAT-iCre;TbRII*$^{fl/fl}$ mutants represents background signal.

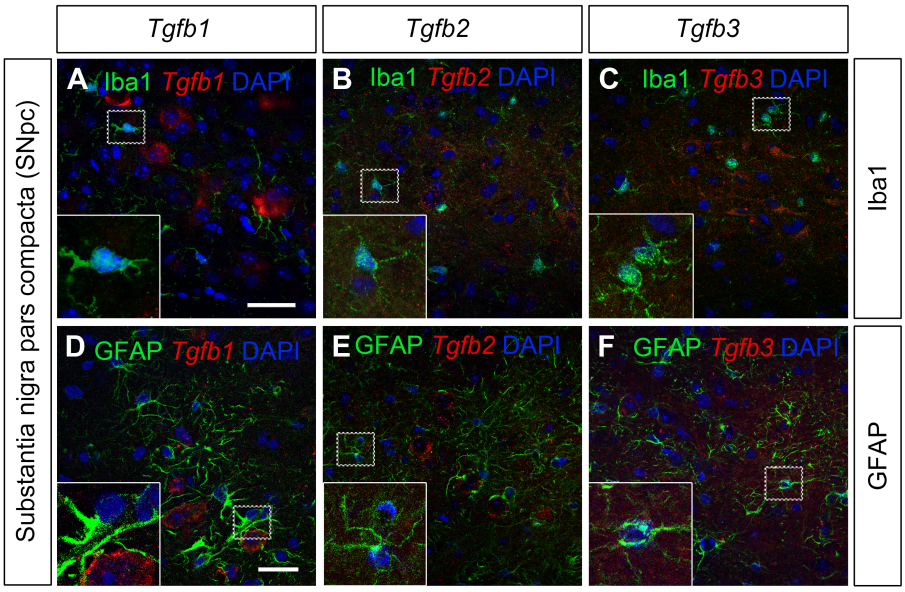

Figure S2. Lack of *Tgfb1*, *Tgfb2* and *Tgfb3* mRNA expression in microglia and astrocytes in the ventral midbrain, Related to Figure 4
(A-F) Fluorescent *in situ* hybridization for *Tgfb1* mRNA (red) combined with immunostaining for Iba1 or GFAP (both in green) in P28 control mouse brain. Note that *Tgfb1* mRNA is below detectable level in Iba1+ microglia and GFAP+ astrocytes in the substantia nigra pars compacta. Scale bars represent 50 μm in (A, D).

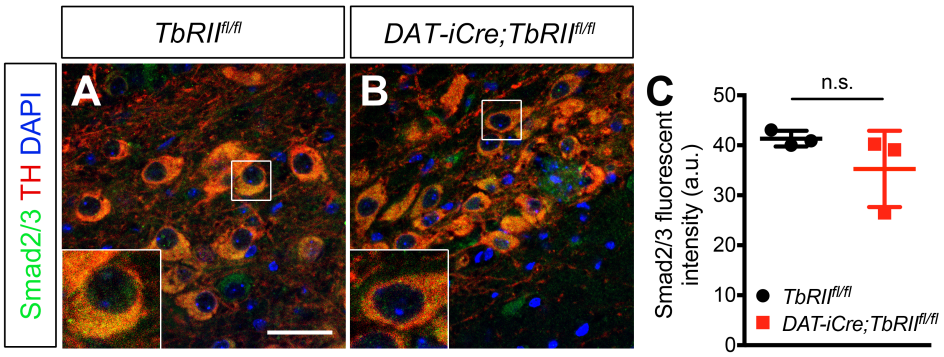

Figure S3. No detectable reduction in total Smad2/3 level in the ventral midbrain dopaminergic neurons in *DAT-iCre;TbRII*$^{fl/fl}$ mice, Related to Figure 4

(A-B) Confocal immunofluorescent microscopy showing no detectable reduction of total Smad2/3 proteins in the DA neurons of control *TbRII*$^{fl/fl}$ and *DAT-iCre;TbRII*$^{fl/fl}$ mutants. Scale bar, 50 μm.

(C) Quantification of total Smad2/3 level in DA neurons in control *TbRII*$^{fl/fl}$ and *DAT-iCre;TbRII*$^{fl/fl}$ mutants using NIH ImageJ shows no significant reduction. Data presented as mean ± S.D., n.s., not significant, Student's *t* test.

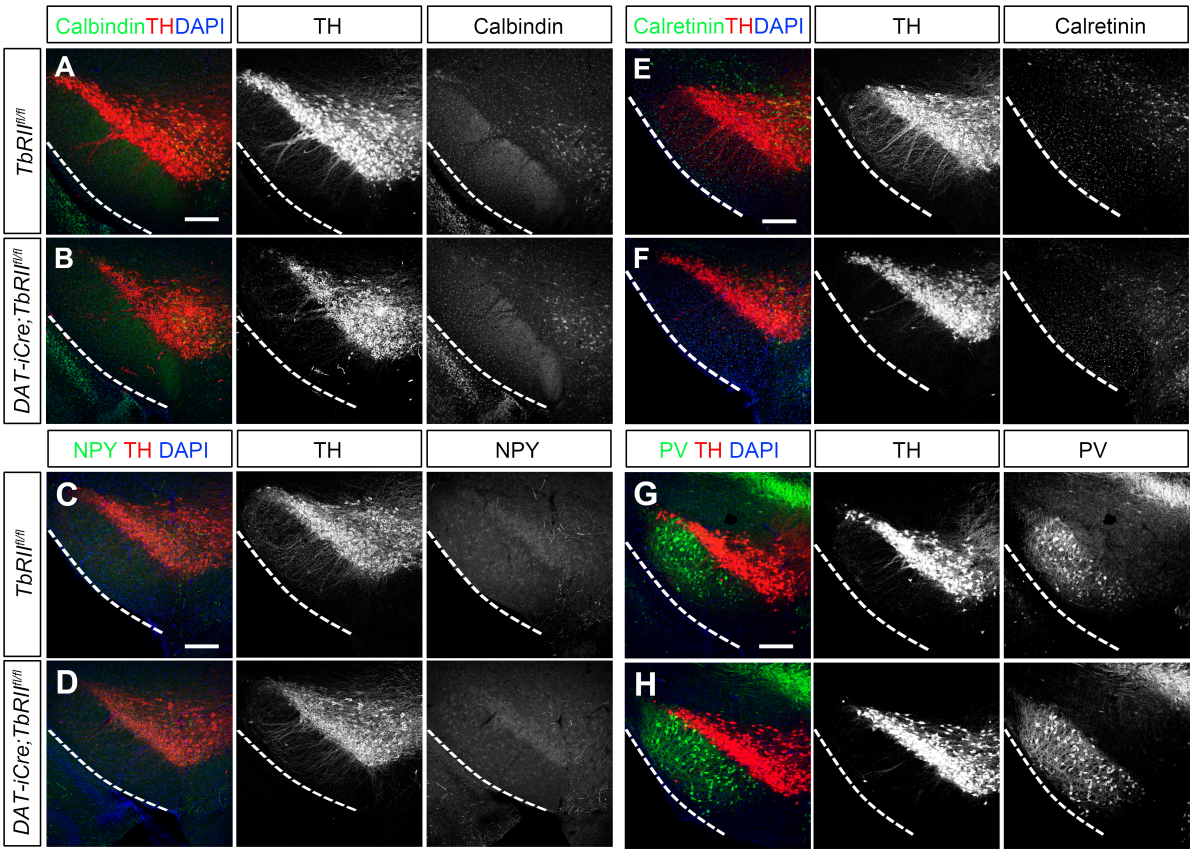

Figure S4. Parvalbumin is the most prominently expressed inhibitory neuron marker in the ventral midbrain, Related to Figure 5

(A-B) Immunostaining of calbindin (green) and TH (red) in P14 *TbRII*$^{fl/fl}$ (A) and *DAT-iCre;TbRII*$^{fl/fl}$ (B) ventral midbrain.

(C-D) Immunostaining of neuropeptide Y (NPY, green) and TH (red) in P14 *TbRII*$^{fl/fl}$ (C) and *DAT-iCre;TbRII*$^{fl/fl}$ (D) ventral midbrain.

(E-F) Immunostaining of calretinin (green) and TH (red) in P14 *TbRII*$^{fl/fl}$ (E) and *DAT-iCre;TbRII*$^{fl/fl}$ (F) ventral midbrain.

(G-H) Immunostaining of parvalbumin (PV, green) and TH (red) in P14 *TbRII*$^{fl/fl}$ (G) and *DAT-iCre;TbRII*$^{fl/fl}$ (H) ventral midbrain. Individual channels are shown on the right. Scale bars represent 200 μm. Dotted line denotes pial surface.

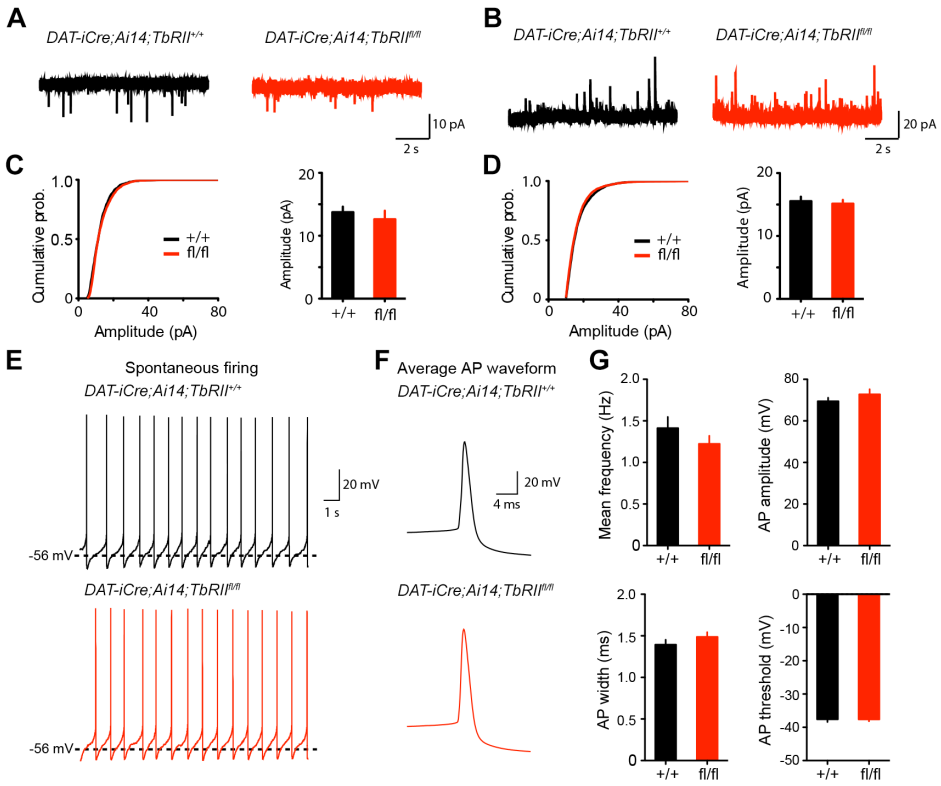

Figure S5. Loss of TβRII in DA neurons do not show changes in intrinsic firing properties, Related to Figure 6

(A) Sample recording traces showing mEPSC recorded from dopaminergic (DA) neurons from littermate control and *DAT-iCre/+;Ai14/+;TbRII*$^{fl/fl}$ conditional mutant mice.

(B) Sample recording traces showing mIPSC recorded from DA neurons from littermate control and *DAT-iCre/+;Ai14/+;TbRII*$^{fl/fl}$ conditional mutant mice.

(C) Cumulative probability plots of mEPSC amplitudes (left, mEPSC amplitude, $p > 0.51$, K.S. test) and summary of mean mEPSC amplitude (right).

(D) Cumulative probability plots of mIPSC amplitudes (left, mIPSC amplitude, $p > 0.14$, K.S. test) and summary of mean mIPSC amplitude (right, $p > 0.68$, Mann-Whitney).

(E-F) Sample recording traces showing spontaneous burst firing (E) and average action potential (AP) waveform (F) recorded from DA neurons from littermate control (top) and *DAT-iCre;Ai14;TbRII*$^{fl/fl}$ (bottom) mice.

(G) Mean frequency ($p > 0.23$), AP amplitude ($p > 0.20$), AP width ($p > 0.40$) and AP threshold of spontaneous firing in SNpc dopaminergic neurons ($p > 0.68$). Number of neuron recorded: N = 16 from 3 *DAT-iCre;Ai14;TbRII*$^{+/+}$ mice and N = 17 from 4 *DAT-iCre;Ai14;TbRII*$^{fl/fl}$ mice. Error bar represents mean ± s.e.m. Statistical significance is determined using Mann-Whitney test. Kolmogorov-Smirnov test is used for cumulative distribution of mEPSC and mIPSC.

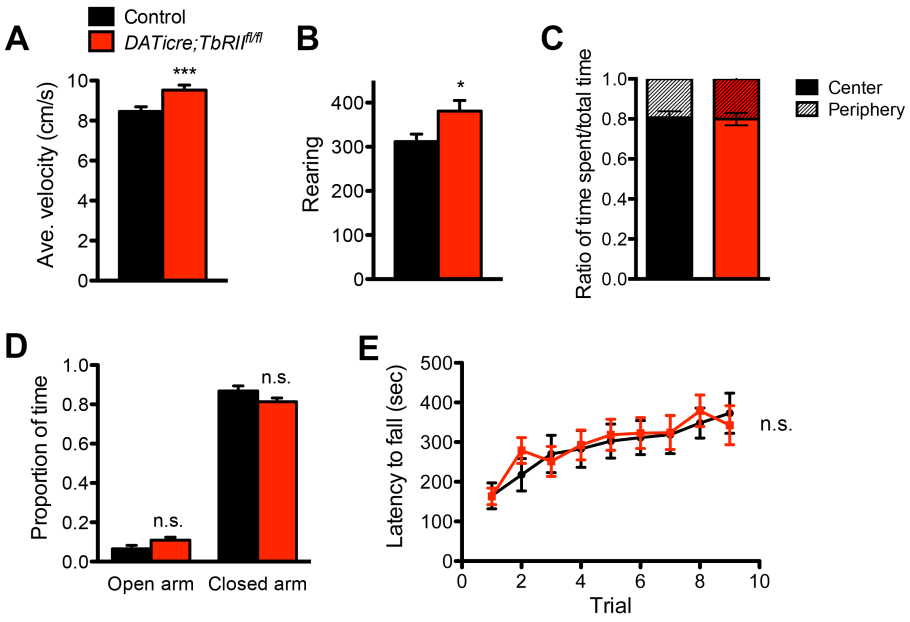

Figure S6. *DAT-iCre;TbRII*$^{fl/fl}$ mice are hyperactive in the Open Field test, but do not show deficits in the Elevated Plus Maze or rotarod, Related to Figure 7

(A-B) Motor performance by two-month old control and *DAT-iCre;TbRII*$^{fl/fl}$ mice in the Open Field test, analyzed for average velocity (A) and rearing (B) using MotorMonitor software over ten minutes of continuous monitoring (N = 12 mice per genotype, basic movement: two-way ANOVA, *** $p < 0.005$, * $p < 0.05$).

(C) Ratio of time spent in the center and periphery of open field apparatus during 60 minutes of monitoring. Control and *DAT-iCre;TbRII*$^{fl/fl}$ mice do not show any significant difference (N = 12 per genotype).

(D) Proportion of time spent in open and closed arms of the elevated plus maze by two-month old male control (N = 13) and *DAT-iCre;TbRII*$^{fl/fl}$ (N = 12) over ten minutes continuous monitoring. n.s., not significant, Student's *t* test.

(E) Latency to fall in the rotarod test over nine trials in two-month old control and *DAT-iCre;TbRII*$^{fl/fl}$ mice (N = 12 mice per genotype). Data presented as mean ± s.e.m. and analyzed using Student's *t* test, n.s. not significant.