Supporting Information

Avale *et al*. 10.1073/pnas.0807635105

SI Text

Lentiviral Vectors. The lentiviral vectors used in this work were derived from the pHR' expression vectors first described by Naldini and colleagues (1, 2), with several subsequent modifications. Vectors are based on the previously described pTRIPΔU3 (3) in which the U3 region of the 3′ long terminal repeat (LTR) was deleted (ΔU3), rendering the integrated viral DNA replication-incompetent. The central polypurine tract (cPPT) (4) and the central termination sequence (CTS) (5) of the wild-type HIV-1 were added, creating the 99-bp central DNA "flap" (6) that enhances infection of non-dividing cells by facilitating transport of the preintegration complex through the nuclear membrane pores. The woodchuck hepatitis B virus post-transcriptional regulatory element (WPRE) was added to increase RNA stability and transgene expression (7).

The re-expression lentivirus vector [PGK-Beta2-Ires2-eGFP] is a bicistronic β2-IRES2-eGFP construct that has been previously described (8). Briefly, the mouse phosphoglycerate kinase (PGK) promoter was amplified from a PGK-nls lacZ expression vector, M48, by PCR and ligated into pTRIPΔU3. To generate the β2-IRES2-eGFP construct a cloning site was created in the pIRES2-EGFP expression plasmid (Clontech) by mutagenesis (QuikChange, Stratagene), and the wild-type mouse β2 subunit, containing a consensus Kozak translation initiation site, was then ligated into plasmid pIRES-EGFP. The β2-IRES2-eGFP cassette was then ligated into the pTRIPΔU3-PGK vector using *Xho*I-*Sal*I sites. Finally, the WPRE sequence was added. The PGK-eGFP control lentivirus vector is identical to the bicistronic version but lacks the β2-IRES2 portion.

The shRNA lentivirus vectors [U6-shRNA-Ubiq-EGFP] were produced in a two-step cloning strategy. First, the shRNA oligonucleotides (see below) were cloned into expression plasmid pCMV-U6 (9) (kindly provided by Dr. Pavel Osten) via *Bbs*I (5′) and *Bst*BI (3′) restriction sites. Oligonuclotides containing the shRNA sequences were annealed following the protocol described by Yu *et al.* (10). Briefly, oligonucleotides (Invitrogen) were resuspended at 0.25 nmol/μl in light TE, mixed in equal amounts (10 μl each) and put in a bath at 100°C that was allowed to cool down to room temperature over 3 h. One μl of the annealed oligo mix was then ligated into the pCMV-U6 vector previously digested with *Bbs*I and *Bst*BI. The ligation product was transformed into *E. coli* XL-1 Blue and selected with zeocin (Invitrogen). In a second cloning step, the U6-shRNA cassette was released from pCMV-U6 via *Nhe*I and *Bst*BI digestion. This "silencing cassette" was cloned into the *Nhe*I and *Bst*BI sites introduced into the FUGW lentiviral vector (9, 11), which co-expresses eGFP from the ubiquitin promoter ("reporter cassette").

shRNA Design. Three sequences of short hairpin RNA were designed to target the mouse β2 subunit gene, following the rules suggested at http://www.rockefeller.edu/labheads/tuschl/sirna.html and using the software available at http://jura.wi.mit.edu/bioc/siRNAext/ following the guidelines provided (12). Hairpins were cloned into lentiviral vectors and tested *in vivo* to choose the one with the most efficient silencing effect over the β2 subunit. The three initial sequences targeted regions between 150 and 1,300 bp downstream from the ATG in the cDNA sequence, with an overall 45–50% GC content, and followed the rules for siRNA design as set out in (12). A BLAST analysis was performed with the candidate sequences against the mouse genome to avoid non-specific gene silencing. Each sequence was named by the position of the first base targeted in the β2 subunit cDNA from the ATG:

sh285: 5′ GCCTGAGGATTTCGACAATAT 3′;
sh11156:5′GCCATGTACCTGCTTTGTCAA 3′;
sh1300:5′ GCGGACCATATGCGAAGTGAA 3′.

The control sequence (*shScr*) was designed by a 'scrambled' analysis, obtaining a sequence with similar thermodynamic values and GC content, but with no silencing effects: *shScr*: 5′ GCCAGATTTCTCAGGTGATAA 3′.

For each of the RNA hairpins, we designed an oligonucleotide with 21 bp of the chosen sense target sequence followed by a loop sequence derived from an endogenous miRNA (5′- GTGAAGCCACAGATG-3′) and 21 bp of the antisense sequence. For cloning procedures, a 5′ TTTG overhang was added to the sense sequence, which includes the transcriptional start at G (+1). The target sequences thus always started with G.

The three sequences were tested *in vivo* in preliminary assays (see supporting information (SI) Fig. S3), and the *sh285* sequence was chosen as having the best silencing effect.

Histology. To establish the accurate injection site and to determine the virally transduced area, brains from mice injected either with the re-expression vector or the silencing vector (both containing eGFP as a reporter gene) were removed at 4 weeks post-injection and used for histological analysis. Anesthetized mice were transcardiacally perfused with 4% PFA, brains were post-fixed overnight in 4% PFA and cut on a vibratome in 50-μm thick coronal sections. Sections were analyzed by imaging eGFP autofluorescence and by immuno-histochemistry against TH or NeuN to label either DA neurons or all neurons, respectively. A typical injection location of the viral vector in the SNpc is shown in Fig. 1*C* of the main text. In this case, TH labeling of DA neurons of the VTA and SNpc allows to distinguish the specific location in the SNpc, completely avoiding the VTA.

In the virally infected area, DA neurons co-express TH and eGFP (Fig. 1*C*). eGFP-positive non-TH cells are also seen, and correspond to non-DA neurons and glial cells. The eGFP signal is almost exclusively restricted to the SNpc injected zone. However, in several cases some eGFP fluorescence was observed in the *substantia Nigra pars reticulata* (SNpr; Fig. S1*A*), although no β2*-nAChRs were detected in this area by [^{125}I]epibatidine autoradiography (Fig. 1*D*–*F* in the main text). Given the morphology of the cells (arrows in Fig. S1*B*), this off-site signal is due to glial cells migrating from the infected SNpc area. Labeling with NeuN was used to verify that eGFP was absent from neuronal cell bodies in SNpr (Fig. S1*B*). In addition, some eGFP labeling could also be observed in TH-positive dendrites and axons projecting from SNpc to SNpr (Fig. S1*C* and *D*). The few DA neurons in proximity to SNpr did not express eGFP, but they were surrounded by eGFP positive glial cells (Fig. S1*E*).

Response to Nicotine of Re-expressed *β2*-nAChRs*. To further characterize the lentivirus-re-expressed β2*-nAChRs, we tested their ability to respond to nicotine. To this end, nicotine-elicited DA release was determined by intra-cerebral microdialysis in the dorsal striatum of awake, freely-moving SNpc-RESC mice (Fig. S2). Briefly, mice were anesthetized with chloral hydrate (400 mg/kg, i.p.), placed on a stereotaxic frame and implanted unilaterally into the dorsal striatum with concentric dialysis probes made of cuprophan fibers (active length 1.0 mm, outer diameter 0.30 mm). Twenty hours after surgery, the probe was

continuously perfused with an artificial CSF (composition in mmol/L: NaCl 147, KCl 3.5, $CaCl_2$ 1.26, $MgCl_2$ 1.2, NaH_2PO_4 1.0, pH7.4) at a flow rate of 1.5 μl/min using a CMA/100 pump (Carnegie Medicin). Dialysates were collected at 15-min intervals. Measurements of DA content were made using a High Performance Liquid Chromatography (HPLC) system (XL-ODS, 4.6 $\times$ 75 mm, particle size 3 μm; Beckman) coupled to an amperometric detector (1049A, Hewlett-Packard). Eight samples were collected to determine basal values (means$\pm$ SEM) before the systemic administration of nicotine. The limit of sensitivity for [DA]ext was 0.5 fmol per sample (signal-to-noise ratio = 2). Mice were injected i.p. with 1 mg/kg of nicotine (salt) dissolved in 0.9% NaCl and control animals received an injection of 0.9% NaCl (10 ml/kg by the same route). Responses to nicotine administration were determined over a 120-min period.

In WT and *SNpc-RESC* mice, nicotine caused a significant increase in DA release. In contrast, no nicotine-elicited DA release was observed in β2KO mice, as previously reported (8, 13). This result confirms the presence of fully functional, nicotine-sensitive, nigrostriatal β2*-nAChRs in *SNpc-RESC* mice.

Knock-Down of β2*-nAChRs by shRNA Lentivirus: *Preliminary* in vivo *test of shRNA target sequences.* The three sequences designed for targeting the β2 mRNA (*sh285, sh1156 and sh1300*) were tested *in vivo* in a preliminary assay (Fig. S3*A*), with the aim to choose the best sequence for β2 gene silencing. A mismatched sequence with no silencing effect (*shScr*) was used as a control. Four mice were unilaterally injected into the SNpc with lentivirus carrying each of these sequences. Injections were performed with equal amounts of virus (120 ng p24, 1 $\times$ 10^6 transfecting units), and the only difference between vectors was the sequence targeted in the β2 mRNA. The contralateral side was not injected to serve as control. At the indicated time post injection, brains were removed, frozen and cut for [^{125}I]-epibatidine autoradiography experiments. Adjacent sections were used for OD quantification comparing the injected and the non-injected side for each level.

The only shRNA sequence that efficiently reduced the expression of β2*-nAChRs was *sh285*; *sh1300* had no effects on ^{125}I-epibatidine binding sites, as no differences were observed between injected and non-injected sides (Fig. S3*A*). Similar results were obtained with *sh1156* (data not shown). These results are consistent with reports proposing that the most efficient targets for siRNAs are placed between 150 and 500 bp downstream of the ATG codon (*12*). This experiment also demonstrates that the *shScr* (control) virus did not affect the [^{125}I]-epibatidine binding, validating the specificity of the shRNA chosen for silencing. The time course of silencing effects of *sh285* (Fig. S3*B*) revealed that 3 months were necessary to obtain a significant reduction in β2*-nAChR protein levels. This does not imply that the RNAi machinery is inactive before that time point, as it could be acting at the mRNA level all along. But by detecting [^{125}I]-epibatidine binding sites we were able to assay the most critical variable, the knock-down of functional receptor protein.

After these preliminary tests *sh285* was selected as the *shβ2* sequence for further experiments, while *shScr* was the control sequence (see the main text). All of the following injections for behavioral analysis were carried out with these two lentiviral vectors, and the experiments were performed 3 months post injection, after validating the behavioral onset of *shβ2* (see next section).

To validate the data obtained with shRNA injected mice, all shRNA bilaterally injected mice used in behavioral experiments were analyzed by quantitative [^{125}I]-epibatidine autoradiography after the behavioral tests. The radioactive signal was quantified to determine relative levels of binding in the injected area (SNpc) *vs.* adjacent non-injected areas. Four consecutive coronal (20 μm thick) sections of each mouse, containing the SNpc (-2.9 to -3.3 mm from Bregma) were analyzed using ImageJ (U.S. NIH). Pixel densities of the scanned films were converted to nCi/mg tissue using ^{125}I standards (Amersham). The average decrease of [^{125}I]-epibatidine binding was about 70% (Fig. 4*C* in the main text) in the injected area (representing 50% of the total volume of SNpc).

Some 10% of shβ2 injected mice did not show any significant reduction in [^{125}I]-epibatidine autoradiography, and they were discarded from the shβ2 group for the statistical analysis. [^{125}I]-epibatidine autoradiography of the shβ2 mice in the behavioral analysis performed in the open field (Fig. 4 *D–F*, main text) is shown in Fig. S4. Two *shScr* sections are shown for comparison. All *sh285* mice shown here developed the hyperactive behavioral phenotypes in the open field.

Behavioral analysis of shRNA injected mice. To analyze the time course of the appearance of locomotor phenotypes due to shRNA silencing, wild-type mice were injected in the SNpc with *shβ2* or *shScr* lentiviral vectors and tested in the open field at two different periods (1 month and 3 months after injection). Distance traveled and the time spent in fast and slow movements were analyzed (Fig. S5). One month post-injection no significant behavioral changes were observed between groups. However, 3 months after injection, mice injected with *shβ2* displayed an enhanced 'distance traveled' and ''fast movements' phenotype similar to the β2KO mice.

This delay after injection to observe changes in open field activity is consistent with the time course of β2*-nAChR down-regulation observed with [^{125}I]-epibatidine binding in preliminary assays (see Fig. S3), suggesting that both events are directly related. Differences observed within the same group at different times postinjection are likely due to the age of mice, as 1 month after injection mice are 3 months old, while at 3 months post injection they are 5 months old. Older mice show reduced activity scores compared to younger mice (14).

For the gene rescue experiments 4 weeks were sufficient to obtain functional re-expression of β2*-nAChRs concomitant with its behavioral effects (Fig. 3 in the main text). It is unlikely that these differences between re-expression and repression were due to the time course of expression of lentiviral vectors, as both constructs contain similar regulatory sequences (see above). Indeed, eGFP expression from the reporter cassette contained in the lentiviral silencing vector was observed shortly after injection (data not shown). Our hypothesis is that the effects of shRNA injection are not detectable until the down-regulation (turnover) of β2*-nAChRs already present at the time of injection, that may take 2 - 3 months. Further experiments are underway to characterize the time course of gene silencing by shRNA methods.

We also tested the behavioral outcome of local β2*-nAChR knock-down in the 24-h activity test, as shown for the re-expression approach (see Fig. 6 in the main text). In this case no significant effect was observed in night time activity between groups, after 3 (Fig. S6) or even 4 months post-injection (data not shown). In addition to these behavioral data, [^{125}I]-epibatidine autoradiography revealed that no changes were observed in the striatal content of β2*-nAChRs, even 3 months after the shRNA injection (data not shown). This suggests that β2*-nAChRs present at the DA striatal terminals would be less affected by the RNA interference mechanism than those present in the somato-dendritic compartment. This difference at the molecular level might be the reason for differences in the behavioral outcome after nigral β2*-nAChR knock-down. Thus, *shβ2* injection in the SNpc would alter the balance between somatodendritic *vs.* terminal receptors, giving rise to an altered behavioral pattern in the open field, but without significant effect in 24 h activity in a familiar environment. Under this hypothesis, the 24-h activity pattern would be mainly controlled by local cholinergic action in the dorsal striatum, through

$\beta 2^*$-nAChRs in the DA terminals (see Discussion in the main text). This idea is consistent with the nocturnal ACh increase at the striatal level previously described in the rat (15).

1. Naldini L, Blomer U, Gage FH, Trono D, Verma IM (1996) Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc Natl Acad Sci USA* 93:11382–11388.
2. Naldini L, *et al.* (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 272:263–267.
3. Sirven A, *et al.* (2001) Enhanced transgene expression in cord blood CD34(+)-derived hematopoietic cells, including developing T cells and NOD/SCID mouse repopulating cells, following transduction with modified trip lentiviral vectors. *Mol Ther* 3:438–448.
4. Charneau P, Alizon M, Clavel F (1992) A second origin of DNA plus-strand synthesis is required for optimal human immunodeficiency virus replication. *J Virol* 66:2814–2820.
5. Charneau P, *et al.* (1994) HIV-1 reverse transcription. A termination step at the center of the genome. *J Mol Biol* 241:651–662.
6. Zennou V, *et al.* (2000) HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell* 101:173–185.
7. Zufferey R, Donello JE, Trono D, Hope TJ (1999) Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J Virol* 73:2886–2892.
8. Maskos U, *et al.* (2005) Nicotine reinforcement and cognition restored by targeted expression of nicotinic receptors. *Nature* 436:103–107.
9. Dittgen T, *et al.* (2004) Lentivirus-based genetic manipulations of cortical neurons and their optical and electrophysiological monitoring in vivo. *Proc Natl Acad Sci USA* 101:18206–18211.
10. Yu JY, DeRuiter SL, Turner DL (2002) RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc Natl Acad Sci USA* 99:6047–6052.
11. Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D (2002) Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* 295:868–872.
12. Reynolds A, *et al.* (2004) Rational siRNA design for RNA interference. *Nat Biotechnol* 22:326–330.
13. Bolam JP, Francis CM, Henderson Z (1991) Cholinergic input to dopaminergic neurons in the substantia nigra: A double immunocytochemical study. *Neuroscience* 41:483–494.
14. Picciotto MR, *et al.* (1998) Acetylcholine receptors containing the beta2 subunit are involved in the reinforcing properties of nicotine. *Nature* 391:173–177.
15. Araghi-Niknam M, Lane L, Watson RR (1999) Modification of physical movement in old C57BL/6 mice by DHEA and melatonin supplementation. *Proc Soc Exp Biol Med* 221:193–197.
16. Day J, Damsma G, Fibiger HC (1991) Cholinergic activity in the rat hippocampus, cortex and striatum correlates with locomotor activity: An in vivo microdialysis study. *Pharmacol Biochem Behav* 38:723–729.

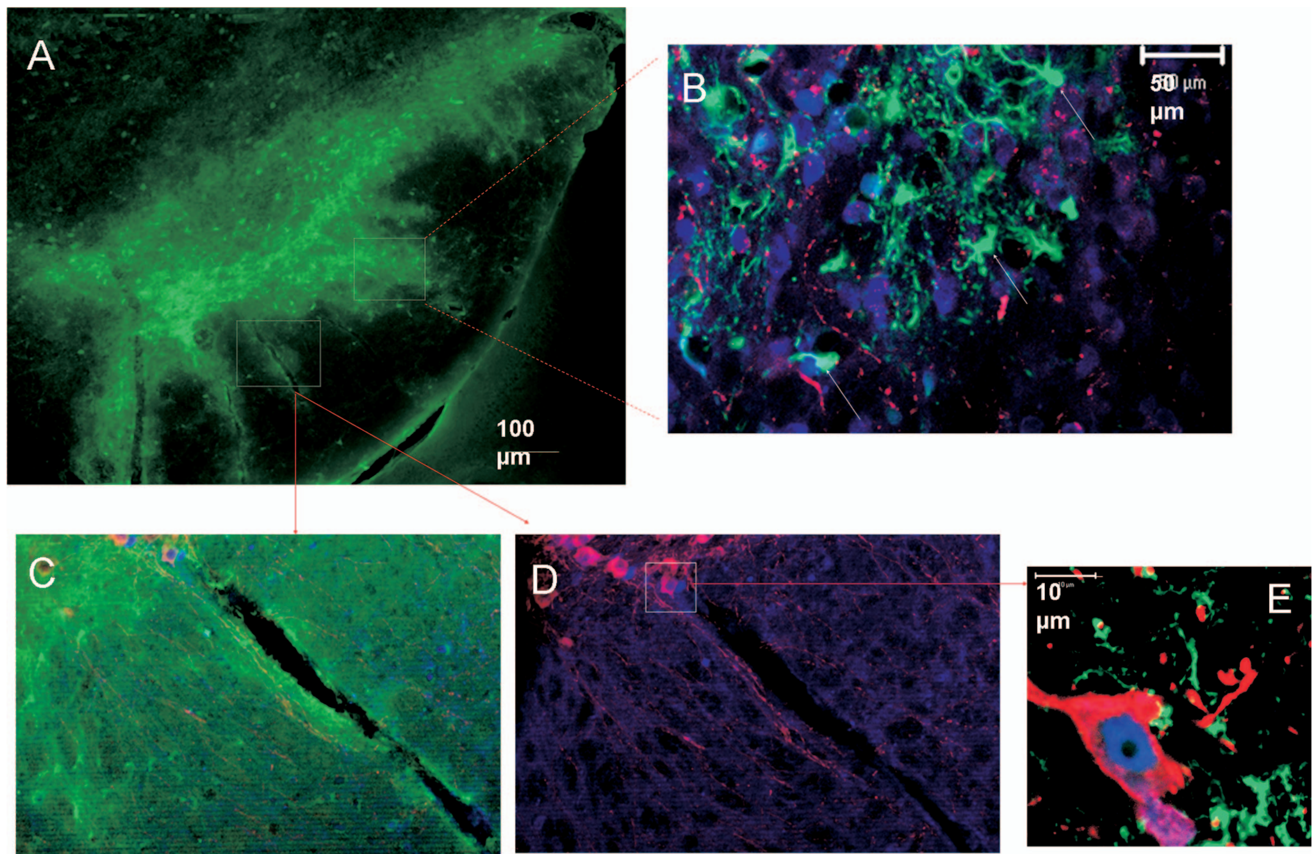

Fig. S1. Histological analysis of transduced SNpc. (*A*) Coronal sections (−3 mm from bregma) showing eGFP (green) expression in the virally transduced area in SNpc. In this case, some eGFP staining is also seen at the level of substantia nigra pars reticulata (SNpr, insets). (*B*) Absence of colocalization with neuron-specific marker NeuN (blue) at SNpr demonstrates that the eGFP-positive cells are not neurons, but glial cells (arrows). (*C* and *D*) eGFP-positive fibers projecting from SNpc to SNpr, coexpressing TH (red in *D*). Dendrites of TH-positive DA neurons in the SNpc are known to extend into the SNpr (16). (*E*) Detail of a DA neuron in the ventral part of SNpc close to the SNpr, showing TH and NeuN colocalization, but no eGFP labeling. The eGFP-positive signal in this area derives from glial cells.

Fig. S2. *In vivo* intracerebral microdialysis to determine nicotine-elicited DA release. (*A*) Time course of extracellular dopamine levels in the dorsal striatum after the injection of saline or nicotine over 2 h, measured in WT, *β2KO*, and SNpc-*RESC* mice. Saline or nicotine (1 mg/kg salt; i.p.) were administered at t_0. Each point is the mean ± SEM in fmol/20 μl. (*B*) Mean ± SEM (fmol/sample) values across the experiment were pooled for statistical analysis (Fisher's test: WT, $n = 9$; KO, $n = 10$; SNpc-RESC, $n = 11$; *, $P < 0.05$, **, $P < 0.01$.).

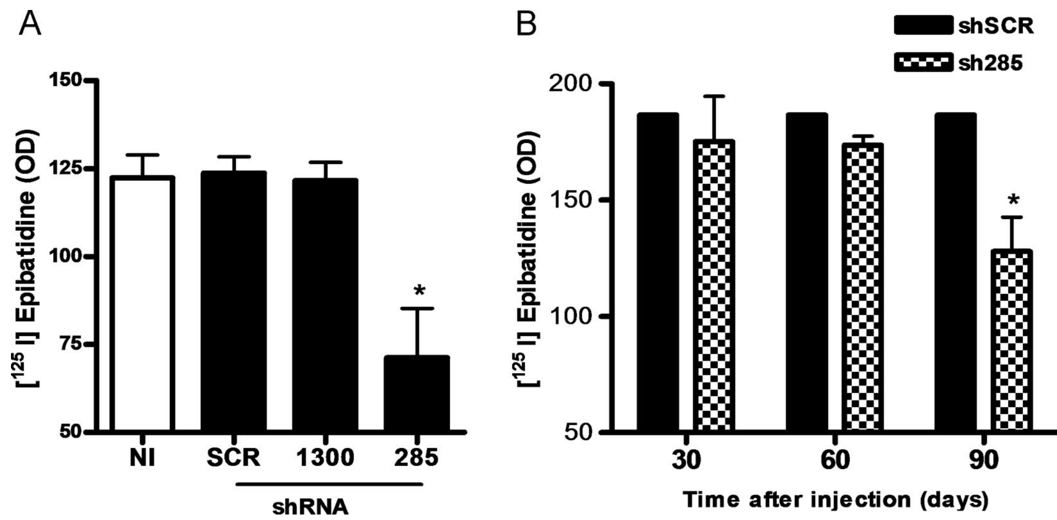

Fig. S3. Preliminary test of the *in vivo* silencing effect of siRNA sequences in lentiviral vectors. (*A*) ^{125}I-epibatidine autoradiography in coronal sections of mice injected with lentiviruses carrying different shRNA analyzed 3 months after injection. *Scr*, scrambled (control) sequence; *sh1300* or *sh285*, numbers indicate that shRNA targets the $\beta 2$ mRNA at 285 or 1,300 bp downstream of the ATG. NI: noninjected side. (One-way ANOVA; $n = 4$ per injection; *, $P < 0.05$) (*B*) Time course of *sh285* silencing effect in the SNpc ($n = 2$) compared with WT (noninjected) OD in the SNpc.

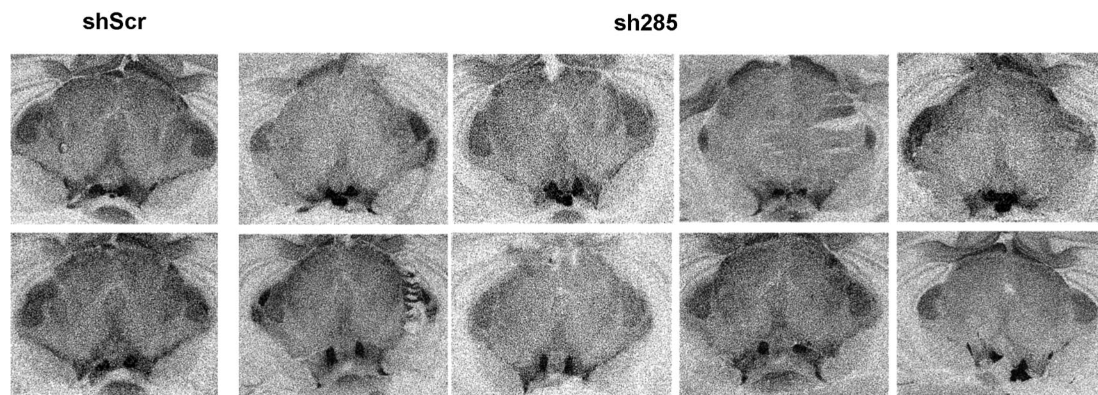

Fig. S4. [^{125}I]-epibatidine autoradiography of SNpc from *shβ2*-injected mice. Representative coronal brain sections at −3 mm from bregma of *shβ2*-injected mice used for behavioral studies described in the main text. Two control (*shScr*-injected) coronal sections are shown on the left. Mice were bilaterally injected with lentivirus *shβ2* (*sh285*) or *shScr* and [^{125}I]-epibatidine autoradiography performed 3 months after injection after behavioral analysis.

Fig. S5. Open field test at 1 and 3 months after shRNA injection in the SNpc. Mice were injected and tested at the indicated post-injection time. Despite a tendency at 1 month for distance traveled and time in fast movement, statistically significant behavioral differences were only observed 3 months after injection. Data were analyzed by two-way ANOVA followed by Bonferroni's test, *, $P < 0.05$ ($n = 6$ per group).

Fig. S6. Twenty-four hour activity in a familiar environment 3 months after shRNA injection in the SNpc. Total distance traveled in activity boxes during 24 h, starting at 3:00 p.m. (dark phase, 8:00 p.m. to 8:00 a.m.). Groups did not show differences in motor activity during the dark phase ($P > 0.05$, repeated measures ANOVA, $n = 6$ per group).