Chronic Deprivation of TrkB Signaling Leads to Selective Late-onset Nigrostriatal Dopaminergic Degeneration

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Abstract

The pathological hallmark of Parkinson's disease (PD) is a selective and progressive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc). In the vast majority of cases the appearance of PD is sporadic, and its etiology remains unknown. Several postmortem studies demonstrate reduced levels of brain-derived neurotrophic factor (BDNF) in the SNc of PD patients. Application of BDNF promotes the survival of DA neurons in PD animal models. Here we show that BDNF signaling via its TrkB receptor tyrosine kinase is important for survival of nigrostriatal DA neurons in aging brains. Immunohistochemistry revealed that the TrkB receptor was expressed in DA neurons located in the SNc and ventral tegmental area (VTA). However, a significant loss of DA neurons occurred at 12-24 months of age only in the SNc but not in the VTA of *TrkB* hypomorphic mice in which the TrkB receptor was expressed at a quarter to a third of the normal amount. The neuronal loss was accompanied by a decrease in dopaminergic axonal terminals in the striatum and by gliosis in both the SNc and striatum. Furthermore, nigrostriatal DA neurons in the *TrkB* mutant mice were hypersensitive to the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a mitochondrial complex I inhibitor that selectively kills DA neurons. These results suggest that BDNF-to-TrkB signaling plays an important role in the long-term maintenance of the nigrostriatal system and that its deficiency may contribute to the progression of PD.

Keywords

TrkB; neuronal survival; dopaminergic neurons; reactive gliosis; substantia nigra pars compacta; ventral tegmental area; MPTP

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Research Highlights

- The study created a *TrkB* hypomorphic mouse mutant with a normal lifespan

- This mutant displays selective and late nigrostriatal dopaminergic degeneration

- The degeneration accompanies reactive gliosis in substantia nigra and striatum

- Nigrostriatal dopaminergic neurons in the mutant are more vulnerable to MPTP

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by resting tremor, muscular rigidity, postural imbalance, and bradykinesia. The common pathological features of PD include a selective and progressive loss of dopaminergic (DA) neurons and deposition of cytoplasmic protein aggregates, termed Lewy bodies, in the substantia nigra pars compacta (SNc) (Dauer and Przedborski, 2003). Several genes responsible for familial forms of PD have been identified in recent years, including α-synuclein (Kruger, et al., 1998, Polymeropoulos, et al., 1997), parkin (Kitada, et al., 1998), UCH-L1 (Leroy, et al., 1998), DJ-1 (Bonifati, et al., 2003), and LRRK2 (Paisan-Ruiz, et al., 2004, Zimprich, et al., 2004). However, less than 10% of all diagnosed PD cases have a strict familial etiology (Payami and Zareparsi, 1998), and very few sporadic PD patients have mutations in any of these genes (Hu, et al., 1999, Scott, et al., 1999). Thus, the etiology of sporadic PD remains unknown.

Neurotrophins are a family of highly related, small, secreted proteins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). They exert their effects on neurons mainly through Trk receptor tyrosine kinases. NGF specifically activates TrkA; NT-3 activates TrkC; and BDNF and NT-4/5 activate TrkB. Among them, BDNF and its TrkB receptor are most abundantly and widely expressed in the brain (Conner, et al., 1997, Yan, et al., 1997). BDNF promotes neuronal survival and differentiation and modulates synaptic plasticity by activating the TrkB receptor tyrosine kinase (Huang and Reichardt, 2001). Recently, viable conditional knockouts have been employed to demonstrate that deletion of the *TrkB* gene or the *Bdnf* gene in excitatory neurons of the dorsal forebrain causes dendritic degeneration and loss of neurons (Gorski, et al., 2003, Xu, et al., 2000b). These results suggest that neurotrophin deficiencies may cause neurodegeneration in some brain regions. Several post-mortem studies show that BDNF expression is reduced in the surviving DA neurons in the SNc of PD patients (Howells, et al., 2000, Mogi, et al., 1999, Parain, et al., 1999). In addition, application of BDNF protects SNc DA neurons from the neurotoxic effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine in animals (Levivier, et al., 1995, Shults, et al., 1995, Tsukahara, et al., 1995). These observations suggest that diminished TrkB signaling in the SNc may contribute directly to the death of nigrostriatal DA neurons and the development of PD. Although ablation of the *Bdnf* gene impairs the survival and/or maturation of SNc DA neurons during development (Baquet, et al., 2005), it remains unknown if TrkB signaling is required for the long-term survival of these neurons in adults.

Here we report the development of a *TrkB* hypomorphic mutant, fB^{neo}/fB^{neo}, which expresses TrkB at 1/4-1/3 of the normal amount. Using this mouse strain we have found that a chronic reduction in TrkB signaling causes age-dependent and selective degeneration of SNc DA neurons and increases the vulnerability of these neurons to neurotoxins.

Materials and Methods

Animals

Mice were maintained on a 12 h/12 h light/dark cycle with food and water *ad libitum*. *TrkB* hypomorphic mutant mice were maintained on a mixed 129 and C57BL/6 background, and housed together with their respective control mice (either wild type or heterozygous littermates). We used polymerase chain reaction to genotype mice. The following PCR primers were used for the presence of the fB^{neo} allele: 5'-AGCCCTGAGGTGCGCACCGATATCG-3' and 5'-

CTCCAGAAGCCCAAGACCAGCAGGC-3'. All animal procedures were approved by the Georgetown University Animal Care and Use Committee.

Histology and Immunohistochemistry

Mice were anaesthetized with avertin and transcardially perfused with phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA). Brains were removed from the skull, post-fixed overnight, transferred to a 30% sucrose solution for 3-5 days, sectioned at 50 μm using a sliding microtome, and collected in PBS. Immunohistochemistry was performed as described previously (An, et al., 2008). Double-labeled immunohistochemistry was performed with the following modifications. For Nissl and tyrosine hydroxylase (TH) double-staining, sections were processed first for TH immunohistochemistry. Mounted sections were then stained with cresyl violet, dehydrated, and coverslipped with dibutyl phthalate-xylene mixture. For glial fibrillary acidic protein (GFAP) and TH double-labeling, sections were processed first for GFAP immunohistochemistry, developed in 0.04% 3,39-diaminobenzidine tetrahydrochloride (DAB; Sigma, Saint Louis, MO), 0.02% cobalt chloride and nickel sulfate (Fisher Scientific, Pittsburgh, PA), and 0.01% hydrogen peroxide dissolved in PBS for enhanced dark staining. After GFAP immunohistochemistry, sections were processed for TH immunohistochemistry, resulting in light brown staining. TH staining was used to outline boundaries of the SNc. Sources and dilutions of primary antibodies were as follows: TH (Chemicon, Temecula, CA, 1:1000; Sigma, Saint Louis, MO, 1:30000), β-galactosidase (Promega, Madison, WI, 1:300; Cappel, Aurora, OH, 1:2000), and GFAP (Sigma, Saint Louis, MO, 1:400). The same dilutions of primary antibodies were used for both immunoperoxidase and immunofluorescent detection procedures. Biotinylated and fluorescent dye-conjugated secondary antibodies were obtained from the Vector Laboratories, Inc (Burlingame, CA) or the Jackson ImmunoResearch Laboratories (West Grove, PA) and used according to the manufacturer's instructions. Sections were processed and developed simultaneously.

Analysis of TH+ terminals

Confocal images of TH immunoreactivity were obtained from the dorsal lateral striatum and nucleus accumbens using the same setting (i.e. laser intensity, gain, offset). Images were converted to gray-scale using Image J software (NIH). The threshold of the gray-scale images was adjusted, and the same parameters were used for all images. Number and size of TH$^+$ puncta larger than 0.1 μm^2 were measured using the particle analysis function of Image J. Four to six images per animal and 3-4 animals per genotype were analyzed.

Immunoblotting

Immunoblotting was performed as described previously (Gharami, et al., 2008) with the following modifications. Tissue samples were homogenized in the LW buffer (50 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, protease inhibitors, pH 7.4), and centrifuged at 13,000 rpm for 20 min at 4 °C. Protein extracts were denatured for 5 min at 100 °C, separated on SDS-PAGE gels, and transferred onto PVDF membranes. The membranes were blocked with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) for 1 hour and then incubated with primary antibodies overnight at 4 °C. Sources and dilutions of primary antibodies were as follows: TrkB (Cell Signaling, Danvers, MA, 1:500), α tubulin (Sigma, Saint Louis, MO, 1:5000). After three washes in TBST (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% Tween-20), the membranes were incubated with an appropriate secondary antibody (IR-Dye 680 and/or IR-Dye 800, 1:10,000, LI-COR Biosciences, Lincoln, NE) for 1 hour at room temperature. The membranes were then washed three times with TBST, and proteins were visualized and quantified using the Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). The amount of TrkB protein was normalized to α tubulin.

Stereology

The total number of TH-immunoreactive neurons in the SNc and VTA was determined by unbiased stereological cell counting method (West, 1993). Using Stereo Investigator software (MicroBrightField, Williston, VT), measurements were performed on every third coronal section immunostained for TH, extending from the most rostral to the most caudal parts (5-10 histological sections per brain). The two hemispheres of each brain were analyzed separately. The boundary between the SNc and the VTA was defined by the medial lemniscus. For each stereological probe (10-12 probes for each brain) TH$^+$ neurons were counted within a counting frame of 50 μm $\times$ 50 μm (a sampling site) and 10-15 sampling sites were randomly picked by the software within the outlined area. The counts were then extrapolated to estimate the total number of neurons in the SNc and the VTA. For Nissl$^+$ and GFAP$^+$ cell count, light TH staining was used to outline the SNc. For all probe runs the coefficient of error (CE Scheaffer) was less than 1.

MPTP treatment

TrkB hypomorphic mutant (*fB^{neo}/fB^{neo}*) and control mice (either wild type or heterozygous) at 8-10 weeks of age were used for MPTP injections. For 5 consecutive days, *fB^{neo}/fB^{neo}* and control mice received one i.p. injection per day of either MPTP/HCl (Sigma, Saint Louis, MO; 25 mg of free base per kg of body weight) or an equivalent volume of saline. 14 days after the last injection, mice were transcardially perfused with PBS and 4% PFA; brains were removed and processed for immunohistochemistry.

Statistical analysis

All data are expressed as mean ± SEM. All measurements were analyzed by either Student's *t* test using Exel software or two-way ANOVA using GraphPad Prism software. *Post hoc* comparisons were assessed with the Student's *t* test.

Results

The expression of the TrkB receptor in midbrain DA neurons

The TrkB receptor has been shown to be expressed in the SNc and VTA (Yan, et al., 1997), the midbrain regions that form the nigrostriatal pathway and the mesocorticolimbic pathway, respectively. To determine the extent of TrkB expression in DA neurons of these two brain regions, we took advantage of *TrkB$^{LacZ/+}$* knockin mice, where expression of β-galactosidase is under the control of the *TrkB* promoter and thus recapitulates the expression pattern of the *TrkB* gene (Xu, et al., 2000a). Fluorescent immunohistochemistry against tyrosine hydroxylase (TH) and β-galactosidase revealed that 60% (48/81) of DA neurons in the SNc (Figs. 1A-C) and 47% (57/121) in the VTA (Figs.1D-F) expressed the TrkB receptor.

Generation of a *TrkB* hypomorphic mutant

A *TrkB* hypomorphic mutant was created during the process of generating a *TrkB* lox allele. A targeting construct was created to include one *loxP* site in the 5'untranslated region of exon S that encodes the signal peptide for the TrkB receptor, a *PGKneo* expression unit flanked by two *frt* sites in the intron downstream of exon S, and the second *loxP* site immediately downstream of the *PGKneo* expression unit (Fig. 1G). Through homologous recombination in mouse embryonic stem cells, we used this targeting construct to generate an *fB^{neo}* allele (Fig. 1G). Because the *PGKneo* expression unit contains a polyadenylation signal sequence and multiple cryptic 3' splice sites, its insertion in the *TrkB* locus likely interferes with the expression of the TrkB receptor. This prediction was confirmed by immunoblotting analyses of brain protein extracts (Fig. 1H). The *TrkB* gene produces two

proteins: a full-length TrkB receptor (TrkB-F) and a truncated TrkB receptor (TrkB-T) that lacks the tyrosine kinase domain and thus is not able to activate receptor tyrosine kinase-mediated signaling cascades in neurons. Mice homozygous for the *fB^neo* allele expressed TrkB-F at approximately 1/3 of the normal amount and TrkB-T at approximately 25% of the normal amount (Figs. 1H and 1I). This result indicates that *fB^neo* is a hypomorphic *TrkB* allele. These hypomorphic mice survived well, which is different from another *TrkB* hypomorphic strain, *fBZ/fBZ* (Xu, et al., 2000b).

Selective degeneration of nigrostriatal DA neurons in aged *fB^neo^/fB^neo^* mice

To determine if DA neurons in the SNc are dependent on TrkB signaling for survival, we sectioned brains from *fB^neo^/fB^neo^* mice and littermate controls (either +/+ or *fB^neo^/+*) at 9, 12, 18, and 24 months of age, stained every third coronal section with antibodies to TH, and counted DA neurons in the SNc bilaterally using an unbiased stereological method (Fig. 2). The number of nigrostriatal DA neurons in *fB^neo^/fB^neo^* mice was similar to that in control mice at 9 months of age (Fig. 2C, $P=0.699$, $n=3$ pairs of mice). However, the number was significantly reduced by 22% at 12 months of age ($P=0.017$, $n=3$ pairs of mice), by 29% at 18 months of age ($P=0.011$, 4 control mice and 3 mutant mice), and by 26% at 24 months of age ($P=0.004$, $n=3$ pairs of mice), compared to age-matched control mice (Fig. 2C). Since TH immunoreactivity in surviving nigral DA neurons in *fB^neo^/fB^neo^* mice was similar to that in control mice at 18 months of age (Figs. 2A and 2B), the reduction in the number of TH-immunopositive (TH+) neurons in the SNc of *fB^neo^/fB^neo^* mice unlikely results from reduced TH expression. We confirmed cell loss by counting all Nissl stained neurons in the SNc at 18-24 months of age (Fig. 2D, 21% reduction, $P=0.02$, $n=6$ pairs of mice). To determine if loss of DA neurons is specific to the SNc, we counted DA neurons in the VTA bilaterally. Counts of DA neurons in the VTA were similar between *fB^neo^/fB^neo^* and control mice at 18 months of age (Fig. 2C, $P=0.593$, 4 control mice and 3 mutant mice). These results indicate that chronic reduction in TrkB signaling leads to late-onset and selective nigrostriatal degeneration.

Loss of nigrosriatal DA projections in *fB^neo^/fB^neo^* mice

To determine whether loss of DA neurons in the SNc of *fB^neo^/fB^neo^* mice was accompanied by reduced number of nigrostriatal projections, we quantified TH+ nerve terminals in the dorsal striatum (Fig. 3). Confocal images revealed reduction in TH+ fiber density in the striatum of the *fB^neo^/fB^neo^* mice (Figs. 3A and 3B). To quantify TH+ nerve terminals, we adjusted the threshold to unmask TH+ puncta representing axonal endings (Figs. 3E and 3F). The total number and average size of TH+ nerve terminals per unit of striatal area were significantly reduced in *fB^neo^/fB^neo^* mice compared to controls at 18 months of age (Figs. 3I and 3J; 23% reduction in number, $P=0.0007$; 24% reduction in size, $P=0.01$). A further analysis based on the cross area of TH+ terminals showed significant reduction in most size bins (Fig. 3K). We obtained similar results using dopamine transporter (DAT) as a selective marker for DA terminals (data not shown). To determine whether the loss of nigrostriatal projections is selective, we analyzed TH+ projections from the VTA to the nucleus accumbens and found no difference between control and *fB^neo^/fB^neo^* mice (Figs. 3C, 3D, 3G, 3H, and 3L). Thus, loss of DA neurons in the SNc of *fB^neo^/fB^neo^* mice correlates with reduction in nigrostriatal innervation.

Reactive gliosis in the striatum of *fB^neo^/fB^neo^* mice

Reactive gliosis, the cellular manifestation of neuroinflammation, is a pathological hallmark of neurodegenerative diseases including PD (Forno, et al., 1992, Hirsch, et al., 1998). To evaluate levels of gliosis, we counted reactive astrocytes that expressed glial fibrillary acidic protein (GFAP) in the striatum (Figs. 4A and 4B) and SNc (Figs. 4C and 4D) of *fB^neo^/fB^neo^* and control mice. GFAP+ cell density was significantly increased in the striatum and the

SNc of fB^{neo}/fB^{neo} mice (Figs. 4E and 4G), indicating increased inflammation that has been shown to accompany neuronal degeneration. In the striatum of fB^{neo}/fB^{neo} mice, reactive gliosis was most likely caused by axonal degeneration of nigrostriatal DA fibers, since we observed a reduction in the number and size of TH+ terminals (Fig. 3), but not in the total number of striatal neurons assessed by stereological count of Nissl+ cells (Fig. 4F).

Nigrostriatal DA neurons in fB^{neo}/fB^{neo} mice are more vulnerable to MPTP

Administration of MPTP in mice results in pathology similar to PD, with high doses of MPTP leading to massive loss of nigrostriatal DA neurons (Dauer and Przedborski, 2003). Upon systemic administration, MPTP crosses the blood brain barrier and is converted to its active metabolite (MPP+), which is selectively taken up by DA neurons within the SNc but not the VTA (Muthane, et al., 1994, Seniuk, et al., 1990). The mechanisms of MPTP induced nigrostriatal DA cell death include both apoptosis and necrosis depending on the administration regimen (Jackson-Lewis, et al., 1995). It has been shown that a sub-acute dosing of MPTP daily over five days induces cell death by apoptosis (Tatton and Kish, 1997), the mechanism of cell death that leads to nigral degeneration in PD patients (Hartmann, et al., 2000, Tatton, et al., 1998). In order to determine whether DA neurons in fB^{neo}/fB^{neo} mice at 8-10 weeks of age are hypersensitive to insults, we chose a sub-chronic, low-dose treatment that consisted of one daily i.p. injection of either MPTP (25 mg/kg) or vehicle for five consecutive days. Animals were sacrificed 14 days after the last injection to ensure that apoptosis was complete and that neuronal count of TH+ cells was not affected by decreased TH expression. Stereological cell count of TH+ neurons in the SNc showed no difference between vehicle treated control and fB^{neo}/fB^{neo} mice (Figs. 5A, 5C, and 5E). However, in fB^{neo}/fB^{neo} mice MPTP caused a significantly greater reduction in the number of TH+ neurons in the SNc compared to control mice: 43% vs 28%, respectively (Figs. 5B, 5D, and 5E; P=0.0009). Furthermore, there was a strong interaction between genotype and MPTP treatment ($F_{(1, 9)} = 11.25$, P=0.0085). These results suggest that a reduction in TrkB signaling renders nigrostriatal DA neurons more vulnerable to neurotoxin-induced cell death.

Discussion

In this study we provide evidence that TrkB signaling is important for the survival of DA neurons in the SNc during aging and neurotoxin-induced insult. Using a newly developed *TrkB* hypomorphic mutant, we demonstrate that chronic deprivation of TrkB signaling leads to late-onset DA neuronal loss that does not appear until 12 months of age. Moreover, a reduction in DA cell number is selective for the SNc, since we did not find any changes in the VTA, which is a dopaminergic area shown to be spared in PD (Dymecki, et al., 1996). In addition, we show loss of nigrostriatal projections and reactive gliosis in the striatum and SNc of *TrkB* hypomophic mutant, neuropathological changes also observed in PD (Hartmann, et al., 2003). As the majority of DA neurons in the SNc express TrkB, it is likely that the observed nigrostriatal degeneration in *TrkB* hypomorphic mice is due to the loss of an autonomous TrkB function. However, we could not exclude the possibility that the TrkB deficiency is primarily affecting a non-SNc neuronal population that secondarily causes degeneration of SNc neurons. The late onset and selective DA neuronal loss in the *TrkB* mutant resemble the pathological process of PD. Deletion of the gene for the receptor of glial cell line-derived neurotrophic factor (GDNF), Ret, has also been shown to cause late onset and selective degeneration of nigrostriatal DA neurons (Kramer, et al., 2007). Thus, a reduction in levels of neurotrophic factors such as BDNF and GDNF or their downstream signaling may contribute to the pathogenesis of PD.

Kramer and colleagues used the *DAT-Cre* transgene to delete the *TrkB* gene in dopaminergic neurons and found a negligible role for TrkB in the development and maintenance of

nigrostriatal DA neurons (Kramer, et al., 2007), which is contradictory with our observations. The discrepancy likely results from inefficient deletion of their *TrkB* lox allele in the SNc by *DAT-Cre* for unknown reasons, as evidenced by the presence of a normal amount of the TrkB receptor in the SNc of mutant mice on the basis of immunoblotting analysis (Kramer, et al., 2007).

In PD patients and neurotoxin-based animal models of PD, DA neurons in the SNc are more affected than in the VTA (Dauer and Przedborski, 2003). The mechanisms behind this selectivity are not understood. It has been suggested that possible differences in expression of cell death molecules or survival factors may contribute to hypersensitivity of the SNc to chronic and acute insults (Krieglstein, 2004). We found that the TrkB receptor was not a differentiating factor between the SNc and the VTA, since it was expressed in both brain regions at similar levels. Future studies might identify other trophic factors or their receptors responsible for the survival of DA neurons in the VTA.

Several post-mortem studies demonstrate that BDNF expression is reduced in the surviving DA neurons in the SNc of PD patients (Howells, et al., 2000, Mogi, et al., 1999, Parain, et al., 1999). These observations suggest that diminished TrkB signaling in the SNc may contribute directly to the death of nigrostriatal DA neurons and the development of PD. In fact, a recent study shows that deficiencies in TrkB signaling cause a loss of some nigrostriatal DA neurons (Zaman, et al., 2004) by using a *TrkB* hypomorphic mutant, *fBZ/fBZ*, that expresses the full-length TrkB at ~25% of the normal amount (Xu, et al., 2000a, Xu, et al., 2000b). However, as *fBZ/fBZ* mice do not survive for more than three months (Xu, et al., 2000a), they could not be used to examine age-dependent and progressive nigrostriatal degeneration.

MPTP reproduces PD pathology in both humans and mice by selectively affecting DA neurons in the SNc (Przedborski and Vila, 2003). A sub-acute chronic MPTP treatment has been shown to induce cell death by apoptosis (Tatton and Kish, 1997), a mechanism that contributes to PD neuronal loss (Vila and Przedborski, 2003). Low doses of MPTP do not cause massive neuronal loss but rather uncover if cells are hypersensitive to neurotoxic insult, as it has been shown before in other animal models (Kim, et al., 2005). We show that TrkB signaling protects DA neurons in the SNc from MPTP-induced cell death.

Currently available animal models of PD have greatly contributed to our understanding of pathogenesis of PD; however, there is still no optimal model available that recapitulates all cardinal features of PD. Although not displaying the more drastic aspects of PD, *TrkB* hypomorphic mutant still shows several important neuropathological characteristics of this disease. More detailed studies are necessary to further determine the mechanisms that protect vulnerable neurons against damaging processes of aging and insults, which may lead to the development of novel therapeutic strategies to treat neurodegenerative disorders like PD.

## Acknowledgments

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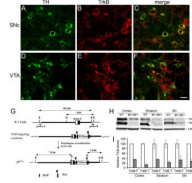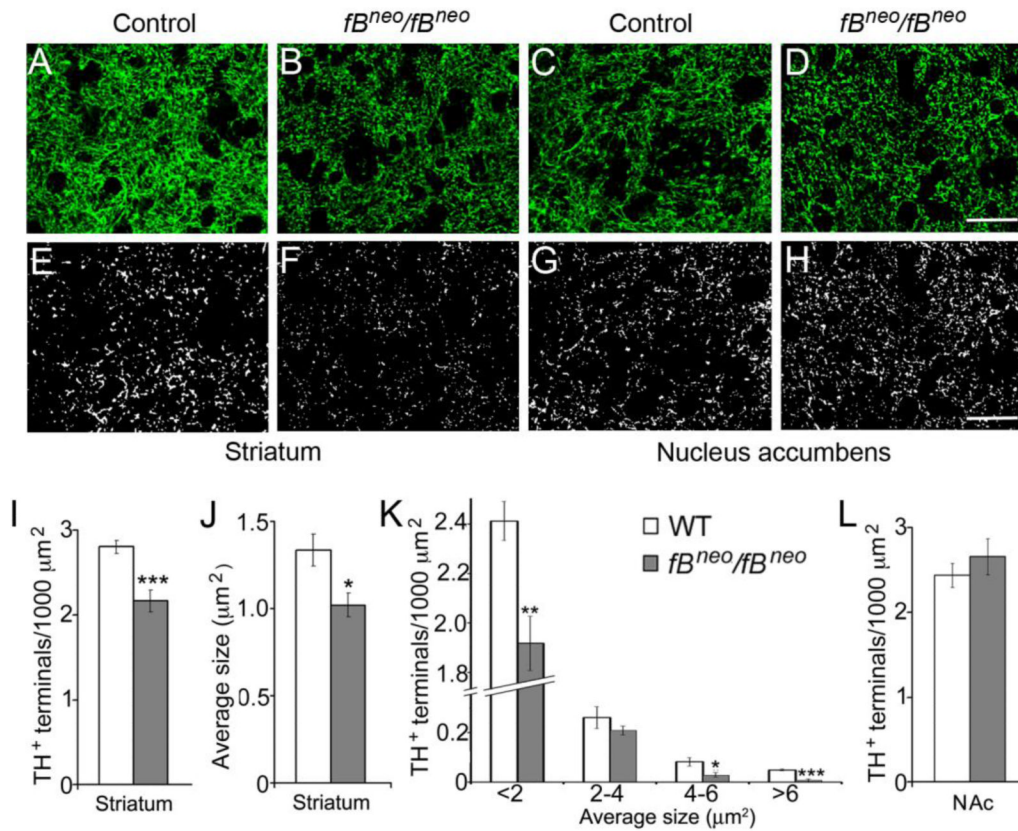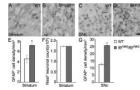
Figure 1.
TrkB expression in DA neurons and generation of a hypomorphic *TrkB* allele, *fB^{neo}*. (A-F) Expression of the TrkB receptor in DA neurons. Brain sections from *TrkB^{LacZ/+}* mice were stained with antibodies against tyrosine hydroxylase (TH) and β-galactosidase. In *TrkB^{LacZ/+}* mice β-galactosidase recapitulates the expression pattern of TrkB. Scale bar, 25 μm. (G) Schematic diagrams of the *TrkB* gene, targeting construct, and *fB^{neo}* locus. The homology arms are represented in thick lines. B, *Bam*HI; H, *Hind*III. (H) Representative immunoblots of full-length TrkB (TrkB-F) and truncated TrkB (TrkB-T). Protein extracts were prepared from the cortex, striatum, and substantia nigra of wild-type (WT) and *fB^{neo}/fB^{neo}* homozygous mice at 9 months of age, and probed with antibodies against the TrkB extracellular domain and α-tubulin. (I) Relative levels of TrkB-F and TrkB-T. TrkB levels were normalized to α-tubulin levels in the same samples (n=2 pairs of WT and mutant mice for the cortical samples and n=3 pairs of mice for other samples). The open bars represent WT mice and the filled bars *fB^{neo}/fB^{neo}* mice.
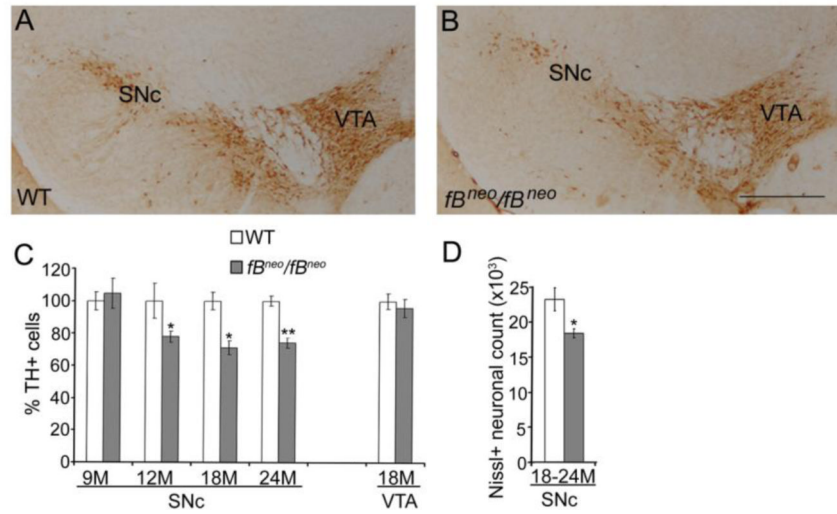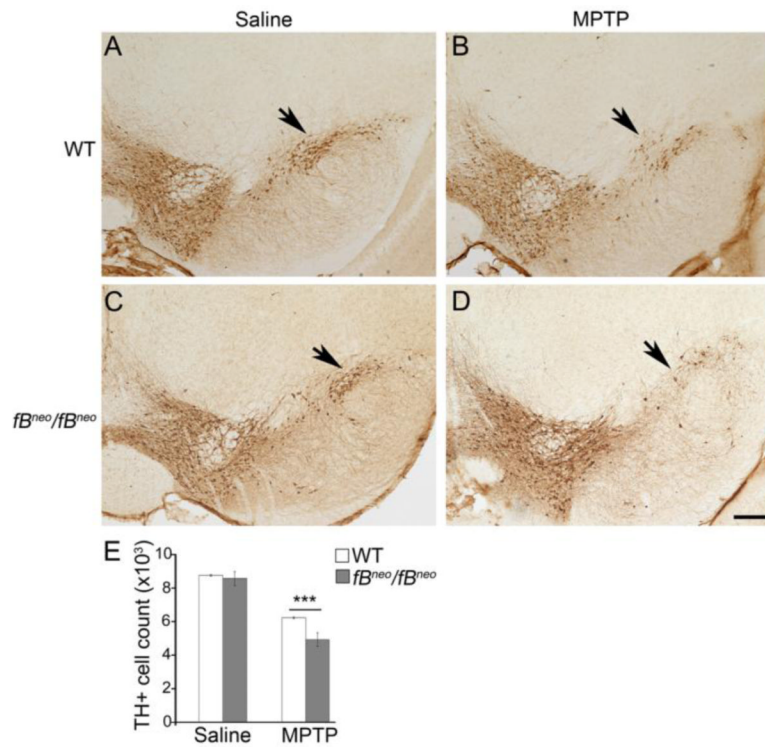
Figure 2.
Degeneration of nigrostriatal DA neurons in *fB^neo^/fB^neo^* mice. (A and B) TH immunoreactivity in the SNc and VTA of *fB^neo^/fB^neo^* and control mice at 18 months of age. Scale bar, 500 μm. (C) The number of DA neurons in the SNc and VTA. The number of DA neurons in control mice is set at 100%. At each time point 3-4 mice per genotype were used. (D) Nissl+ neuronal counts in the SNc of mice at 18-24 months of age (6 mice per genotype). Student *t* test, *, $P<0.05$; **, $P<0.01$.

Figure 3.
Loss of nigrostriatal projections in *fB^neo/fB^neo* mice. (A-D) Representative confocal images of TH⁺ nerve terminals in the dorsal striatum and nucleus accumbens of control and *fB^neo/fB^neo* mice at 18 months of age. Scale bar, 25 μm. (E-H) The same images as panels A-D with increased threshold to visualize TH⁺ puncta. Scale bar, 25 μm. (I-K) Quantification of total number, average size, and size distribution of TH⁺ nerve terminals reveals a significant reduction of nigrostriatal projections in *fB^neo/fB^neo* mice. (L) No difference was found in the TH projections from the VTA to the nucleus accumbens (NAc). Student *t* test, *, *P*<0.05; **, *P*<0.01; ***, *P*<0.001.

Figure 4.
Reactive gliosis in the striatum of fB^{neo}/fB^{neo} mice. (A-D) Representative images of GFAP immunoreactivity in the striatum and SNc of control and *fB^{neo}/fB^{neo}* mice. Scale bar, 100 μm. (E) Increased GFAP$^+$ cell density was observed in the striatum of *fB^{neo}/fB^{neo}* mice at 18months of age. (F) No changes were detected in the total number of striatal neurons. (G) Increased GFAP$^+$ cell density was observed in the SNc of *fB^{neo}/fB^{neo}* mice at 18months of age. Student *t* test, *, *P*<0.05.

Figure 5.
Downregulation of TrkB expression increases vulnerability of nigrostriatal DA neurons to MPTP. (A-D) TH immunoreactivity in the brains of mice treated with MPTP. Mice at 8-10 weeks of age were injected i.p. once a day with MPTP 25 mg/kg for 5 days. Two weeks later, mice were killed and their brains were processed for immunohistochemistry. Scale bar, 250 μm. (E) Counts of nigrostriatal DA neurons. Student t test, ***, $P<0.001$.