Pitx3 Is a Critical Mediator of GDNF-Induced BDNF Expression in Nigrostriatal Dopaminergic Neurons

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Introduction

The majority of the brain’s dopamine (DA) is synthesized by neurons located in two nuclei of the mammalian ventral midbrain (VM): the substantia nigra pars compacta (SNC), innervating predominantly the dorsolateral striatum, and the ventral tegmental area (VTA) projecting mainly to limbic and cortical areas of the brain ( Björklund and Dunnett, 2007 ). The SNC neurons control the execution of voluntary movements, and their selective degeneration in Parkinson’s disease (PD) is responsible for the characteristic motor symptoms of this disease (Dauer and Przedborski, 2003 ).

During development, mesodiencephalic DA (mdDA) precursors arise from progenitors located in the midbrain floor plate (Smidt and Burbach, 2007 ). It is assumed that SNC neurons derive from a rostromedial and VTA neurons from a caudomedial mdDA precursor subpopulation (Smits et al., 2006 ). Pitx3 cooperates with the nuclear receptor Nurr1 to activate transcription of several genes encoding DA biosynthetic enzymes, transporters, and receptors (Jiao et al., 2008 ; Jacobs et al., 2009 ). The gene encoding the retinoic acid (RA)-synthesizing enzyme Aldh1a1 is a target of Pitx3, but maternal RA complementation is only able to partially rescue SNC neurodegeneration in Pitx3 mutant mice (Jacobs et al., 2007 ). Pitx3 might

Pitx3 is a critical homeodomain transcription factor for the proper development and survival of mesodiencephalic dopaminergic (mdDA) neurons in mammals. Several variants of this gene have been associated with human Parkinson’s disease (PD), and lack of Pitx3 in mice causes the preferential loss of substantia nigra pars compacta (SNC) mdDA neurons that are most affected in PD. It is currently unclear how Pitx3 activity promotes the survival of SNC mdDA neurons and which factors act upstream and downstream of Pitx3 in this context. Here we show that a transient expression of glial cell line-derived neurotrophic factor (GDNF) in the murine ventral midbrain (VM) induces transcription of Pitx3 via NF-κB-mediated signaling, and that Pitx3 is in turn required for activating the expression of brain-derived neurotrophic factor (BDNF) in a rostromedial (SNC) mdDA neuron subpopulation during embryogenesis. The loss of BDNF expression correlates with the increased apoptotic cell death of this mdDA neuronal subpopulation in Pitx3−/− mice, whereas treatment of VM cell cultures with BDNF augments the survival of the Pitx3−/− mdDA neurons. Most importantly, only BDNF but not GDNF protects mdDA neurons against 6-hydroxydopamine-induced cell death in the absence of Pitx3. As the feedforward regulation of GDNF, Pitx3, and BDNF expression also persists in the adult rodent brain, our data suggest that the disruption of the regulatory interaction between these three factors contributes to the loss of mdDA neurons in Pitx3−/− mutant mice and perhaps also in human PD.

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Table 1. Primers used for RT-PCR and qPCR experiments

| Gene                  | Forward primer/reverse primer                          | Length of product (bp) | Tm (°C) | Cycles |
|-----------------------|--------------------------------------------------------|------------------------|---------|--------|
| Pitx3 RT-PCR (NM_019247) | 5′-GAGCACTAGTCTGGAGAGG-3′/5′-AAAGCGACGGAAAGTC-3′       | 413                    | 60      | 28     |
| BDNF RT-PCR (NM_012513) | 5′-AGGTGACTGGGCGGAGCG-3′/5′-TGTCCTCCACGGACA-3′        | 369                    | 60      | 28     |
| GADPH RT-PCR (NM_017008) | 5′-CCCAATAAGACCTTGAGACG-3′/5′-GCGAGTCGCTGACATGC-3′    | 251                    | 58      | 22     |
| Pitx3 qPCR (NM_012947)  | 5′-GTTCAAGGCTTCGAGGCTG-3′/5′-TGGTAAAATTGCGACG-3′       | 92                     | 60      | 35     |
| BDNF qPCR (NM_012513)  | 5′-GATCGCCTACATGTTCAAG-3′/5′-TATATGTCACAGCCTCAGC-3′    | 82                     | 60      | 35     |
| Beta-actin qPCR (NM_031144) | 5′-CCCCAGGCTCTCTCCAGCCC-3′/5′-TAGAGCTTTACGCTGACGT-3′   | 110                    | 60      | 35     |

\* Tm, Melting temperature.

Figure 1. GDNF/Ret signaling is required for activation of Pitx3 expression in an midDA neuronal subset. (a–f) Representative midbrain coronal sections of wild-type (C57BL/6) mouse embryos at E10.5 (a, c, e) and E11.5 (b, d, f), hybridized with riboprobes for GDNF (c, d) and Pitx3 (a, f). a and b are Nissl-stained bright-field views of the dark-field pictures shown in c and d. Inset (f′) is a pseudo-colored overlay and magnification of the VM from the consecutive sections shown in d and f (red, GDNF; green, Pitx3). GDNF is expressed in the midbrain basal plate (BP) at E10.5 and E11.5 adjacent to the midbrain floor plate (FP), where Pitx3 starts to be transcribed at E11.5. (g–i) Immunostaining of untreated (control [Con], g–i) and GDNF-treated (GDNF, j–l) E14 rat primary VM cultures with antibodies for Pitx3 (g, j) and TH (h, k) (merged images in i, l). Quantification of TH−/−, TH+/−, Pitx3−/−, and Pitx3−+/− cells relative to the total number of cells in untreated (Con, blue bars) and GDNF-treated (GDNF, red bars) primary rat VM cultures revealed a significant increase of Pitx3−/− and Pitx3−+/−/TH−/− cells, but not of TH−/− cells, in the GDNF-treated cultures as compared to untreated controls (TH−/− cells: control, 7.82 ± 0.47%; GDNF-treated, 8.83 ± 0.61%; mean ± SEM, not significant, p = 0.26; TH+/−/Pitx3−/− cells: control, 1.45 ± 0.13%; GDNF-treated, 3.25 ± 0.23%; mean ± SEM, **p < 0.01 in the independent t test; TH−/− cells: control, 2.19 ± 0.19%; GDNF-treated, 3.59 ± 0.46%; mean ± SEM; **p < 0.01 in the independent t test). Data were derived from three independent experiments. n–s, Representative VM close-up views on coronal sections of Ret−/− (n–p, q–p′) and Ret+/− (q–s, q′–s′) mouse embryos at E12.5, immunostained for Pitx3 (a–s) and TH (a′–s′). Insets (a′–s′) are merged images and higher magnifications of the boxed areas in n–s, q–s, and q′–s′. Quantification on coronal or sagittal midbrain sections revealed a significant decrease of Pitx3−/− cells by 77.7% in E12.5 Ret−/− embryos (d) and 10.4% in E14.5 Ret−/− embryos (a) as compared to their wild-type (Ret+/− and Ret+/+) littermates (Pitx3 Pos. (Pitx3−/−) cells: E12.5 Ret−/−, 2983 ± 125, n = 3; E12.5 Ret+/−, 2455 ± 85, n = 3; E14.5 Ret−/−, 8491 ± 105, n = 6; E14.5 Ret+/−, 7609 ± 224, n = 3 mean ± SEM; **p < 0.01 in the paired t test; *p < 0.05 in the independent t test). Scale bars: (a, f, s′) 50 μm.
therefore activate additional target genes to promote the survival of SNC mdDA neurons.

Glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) were the first potent survival factors identified for mdDA neurons (Golden et al., 1999; Oo et al., 2005). Previous reports indicated that GDNF is highly transcribed in the striatum when nigrostriatal innervation takes place, suggesting that GDNF is a target-derived neurotrophic factor for mdDA neurons (Golden et al., 1999; Saarma, 2002; Zuccato and Cattaneo, 2009). Previous reports therefore activate additional target genes to promote the survival of SNC mdDA neurons.

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We have previously shown that Pitx3 activates BDNF expression in primary cell cultures (Li et al., 2009). In the present study, we demonstrate that GDNF, Pitx3, and BDNF are engaged in a feedforward regulatory pathway in the rodent VM during development and in adulthood. This feedforward interaction promotes the survival of a rostral lateral mdDA neuronal subset during embryogenesis and possibly also of adult and aging SNC neurons and protects mdDA neurons against neurotoxic insults in vitro, suggesting that it might also be relevant for the pathogenesis of PD.

Materials and Methods

Animals. C57BL/6 mice were purchased from Charles River. Adult male and pregnant female Sprague Dawley rats were purchased from the Experimental Animal Center (Shanghai, China). Generation and genotyping of Pitx3+/-GFP knock-in mice is described by Zhao et al. (2004). Mice carrying the floxed Ret allele and a transgene encoding the Cre recombinase driven by the dopamine transporter (Dat) promoter (Kramer et al., 2007) often undergo Cre-mediated excision in the germline; their progeny thus carries one Ret-null allele in all cells (Ret−/− mice). Ret−/− mice were intercrossed and their offspring was genotyped by PCR. Newborn Ret−/− pups lacked kidneys and died shortly after birth, thus confirming that these mice were Ret-null mutants. Collection of embryonic stages was done from timed pregnant females; noon of the day of vaginal plug detection was designated as embryonic day 0.5 (E0.5). Animal treatment was conducted in accordance with the Laboratory Animal Care Guidelines approved by Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China) or under federal guidelines as approved by the HMGU Institutional Animal Care and Use Committee (Munich, Germany).

Radioactive in situ hybridization. Paraffin sections (8 μm) were processed for radioactive ([α-35S]UTP, GE Healthcare) in situ hybridization as described by Fischer et al. (2007). Riboprobes used were Pitx3 (Brodski et al., 2003), an 813 base pair (bp) fragment of mouse GDNF (bp 29–841; GenBank accession no. NM_010275.2), and a 543 bp fragment of mouse BDNF (bp 548–1092, GenBank accession no. NM_001048139.1) that detects all murine BDNF isoforms. Specificity of the GDNF and BDNF antisense riboprobes was
counted in 12 random fields per well, and data were collected from at least three independent experiments.

Primary VM cultures and neurotrophin treatments. Primary VM cultures were prepared from E14 rat embryos or E11.5 Pitx3GFP/GFP and Pitx3GFP/GFP mouse embryos as described by Du et al. (2005). For GDNF treatments, E14 rat primary VM cells were treated after 7 days in vitro (DIV) with 10 ng/ml recombinant rat GDNF (Sigma) in 0.9% saline or with 0.9% saline alone (control) for 3, 24 or 48 h. In some cases, 10 μg/ml NF-κB inhibitor SN50 (Calbiochem) were added for 1 h to the culture medium before the GDNF treatment. Cells were harvested for immunostaining and RT-PCR assays after 3 or 24 h and for Western blot analyses after 2 d of GDNF treatment. For BDNF treatments, dissociated E11.5 Pitx3GFP/GFP or Pitx3GFP/GFP mouse VM cells were plated at a density of 1.4 × 10^5 cells/well and cultured in DMEM/F12 with 2% B27 supplement (Invitrogen) containing 20 ng/ml recombinant human BDNF (R & D Systems) in 0.1% bovine serum albumin (BSA) or 0.1% BSA alone. BDNF or vehicle (0.1% BSA) was added with each medium change every second day. Cells were fixed for immunostaining after 4 d.

siRNA treatments. Three different mouse Pitx3-specific short hairpin RNA (shRNA) oligonucleotide pairs were designed and synthesized by Shanghai GeneChem. Downregulation of Pitx3 mRNA by these shRNAs was tested in SH-SYSY cells stably expressing mouse Pitx3 (Peng et al., 2007). Under these conditions, one Pitx3-specific shRNA oligonucleotide (antisense: 5'-GGAGCAGCUCU-UUCAGC-UAUdTdT-3'; sense: 5' -AUAAGGUGAA-GAGGGUGGATdFdT-3') targeting sequences around position 956 to 974 in Pitx3 exon 4, GenBank accession no. NM_000852.4) resulted in ~80% knock-down of Pitx3 mRNA (data not shown). A nonsilencing shRNA oligonucleotide, as indicated by the manufacturer (Shanghai GeneChem) (antisense: 5'-GGUGACAGCUGUCGGAAdTdT-3'; sense: 5' -UUCCCGGAA-CGGUCUGCAUdTdT-3') was used as control. E14 rat primary VM cells were transfected with Pitx3 control siRNA or vehicle (0.1% BSA) alone or with each medium change every second day. Cells were fixed for immunostaining after 4 d.

6-Hydroxypseudoephedrine treatments. Dissociated E11.5 Pitx3GFP/GFP or Pitx3GFP/GFP mouse VM cells were plated at a density of 1.4 × 10^5 cells/well and cultured in DMEM/F12 with 2% B27 supplement for 3 DIV. Cells were treated after 3 DIV with 20 ng/ml BDNF or 20 ng/ml recombinant human GDNF (R & D Systems) or with vehicle (0.1% BSA) alone. Cells were fixed 2 h after the addition of GDNF to the culture medium. Cells were harvested after 24 h (for RT-PCR assays) or 2 d (for Western blot analyses).

Luciferase reporter assays. A search for conserved NF-κB binding sites (BSs) within the Pitx3 promoter (~10kb to +1kb) and conserved Pitx3 Bicoid-like transcription factor BSs within the BDNF promoter region (~2.5kb to +0.5kb) was done using CONNEX (CONserved Regulatory Elements anchored AListgment; http://conreal.niob.knaw.nl/) and Gene2promoter (Genomatix) software, respectively. A 2514 bp Pitx3 promoter fragment (position –2425 to +89, GenBank accession no. NC_000085) and a 2661 bp BDNF promoter fragment (position –1930 to +686 relative to transcription start) were cloned into pCR-Blunt II-TOPO (Invitrogen). The vectors were digested with each of the BSs and then ligated into the corresponding sites of the pGL3-Basic vector (Promega). The inserts were amplified from C57BL/6 mouse genomic DNA using the primer pairs Pitx3 forward, 5’-AACGAAAACCTTTCTTAAAGG-3’; Pitx3 reverse, 5’-TGTGGCGCCCGCGTCTT-3’; BDNF forward, 5’-

Figure 3. GDNF-mediated induction of Pitx3 expression activates BDNF transcription. a, b, RT-PCR (a) and Western blot (b) analyses of untreated (first lane), GDNF-treated (second lane), and SN50+GDNF-treated (third lane) E14 rat primary VM cultures showed increased Pitx3 and BDNF expression after GDNF treatment, which was blocked by simultaneous treatment with NF-κB inhibitor SN50. c, qPCR analysis indicated an 1.3-fold increase of Pitx3 and 1.4-fold increase of BDNF mRNA levels after GDNF treatment and 70% reduction in both cases after SN50 application (Pitx3: GDNF, 2.27 ± 0.56; GDNF + SN50, 1.40 ± 0.09, mean ± SEM; BDNF: GDNF, 2.35 ± 0.53; GDNF + SN50, 1.40 ± 0.25, mean ± SEM; *p < 0.05; **p < 0.005 in the one-way ANOVA for repeated measurements; n = 4). Con, Control. d, RT-PCR (d) and Western blot (e) analyses of untreated (first lane), GDNF-treated (second lane), Pitx3 siRNA + GDNF-treated (third lane), and control siRNA + GDNF-treated (fourth lane) E14 rat primary VM cultures revealed that siRNA-mediated knockdown of Pitx3 (siRNA), but not a control siRNA (CRNA) abolished the increased expression of BDNF in these cultures after GDNF treatment. f, qPCR analysis indicated that Pitx3 siRNA + GDNF treatment resulted in a 56 and 32% reduction of Pitx3 and BDNF mRNA levels, respectively, as compared to GDNF-only treated cultures and a control siRNA (Pitx3: GDNF, 2.53 ± 0.33; GDNF + Pitx3 siRNA, 1.12 ± 0.02; GDNF + control siRNA, 2.21 ± 0.22, mean ± SEM; BDNF: GDNF, 1.73 ± 0.12; GDNF + Pitx3 siRNA, 1.18 ± 0.09; GDNF + control siRNA, 1.59 ± 0.11, mean ± SEM; **p < 0.005 in the one-way ANOVA for repeated measurements). Data were derived from at least three independent experiments in each case.
 Pitx3 is required for the onset of BDNF transcription in an mdDA neuronal subpopulation at E12.5. a–d, Representative midbrain coronal sections of wild-type mouse embryos at E11.5 (a–c, consecutive sections) and E12.5 (d), hybridized with Pitx3 (b), BDNF antisense (c) and BDNF sense (d) riboprobes. a, Nissl-stained bright-field view of the dark-field picture shown in b. BDNF is not expressed in the murine VM at E11.5, although Pitx3 starts to be expressed in a bilateral VM domain at this stage (red arrows in b). e–f', Representative coronal sections at different rostrocaudal levels of the midbrain from E12.5 Pitx3+/GFP (e–g') and Pitx3−/−/GFP (h–j') mice, hybridized with BDNF antisense riboprobe (e–f'), orimmunostained for GFP (e'–f''). Merged images are shown in (e'–f''). BDNF expression is not induced in a rostrocaudal and medial GFP + mdDA domain of the Pitx3+/GFP embryos at E12.5 (h–j'). Note that the BDNF sense probe does not give a signal at this stage in wild-type embryos (section shown in d), demonstrating the specificity of the BDNF antisense riboprobe. k–m, Two highly conserved Pitx3/Bicoid-like transcription factor BSs in the human and mouse BDNF promoter regions (Site 2 and Site 9, positions relative to the transcription start site (TSS, arrow)) were tested for BDNF promoter activation by Pitx3 (k). Cotransfection of the pGL3-BDNF promoter vector containing these two conserved Pitx3 BSs (WT, wild type) and pcDNA3.1-Pitx3 vector (red bars) into HEK293 cells resulted in a 4.9-fold activation of the BDNF promoter relative to the control (pcDNA3.1 only, green bar) (Pitx3: 5.86 ± 0.24, mean ± S.E.M.; **p < 0.005 in the one-way ANOVA for repeated measurements) (m). Site-directed mutagenesis (MT) of Pitx3 BS 2 (MT Site 2) and/or 9 (MT Site 9) (j) significantly attenuated BDNF promoter activation by Pitx3 (BDNF WT promoter: 5.86 ± 0.24; BDNF promoter with MT Site 2 (2M): 5.11 ± 0.27; BDNF promoter with MT Site 9 (9M): 4.04 ± 0.22; BDNF promoter with MT Site 2 and MT Site 9 (2M/9M): 3.6 ± 0.06, mean ± S.E.M.; *p < 0.05; **p < 0.005 in the one-way ANOVA for repeated measurements) (m). Data were derived from three independent experiments. Scale bars: (in d and f'), 100 μm.

Figure 4. Pitx3 Mediates GDNF-Induced BDNF Expression

TCTCGGATACCCATTTTTGC-3'; BDNF reverse, 5'-CCCAACAGCT-GTCGCTATAT-3'; and cloned into a pGL3 basic vector (Promega). Site-directed mutagenesis of two conserved Pitx3/Bicoid BSs within the BDNF promoter region was done using the primer 5'-CACCTCTCTGTGGGTCTAGAGAGAGGCTTTCC-3' (BS2) and 5'-CCAAGG-GAAGAGGAGCAGCTTGCCATGGAAAGCTTACATGCCGGGATCC-3' (BS9), and the QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. SH-SY5Y cells were transfected with 400 ng/well pGL3–Pitx3 promoter vector and 8 ng/well pRL–SV40 vector using Lipofectamine 2000 (Invitrogen). SN50 (10 μg/ml) was added 30 min before 10 ng/ml or 20 ng/ml GDNF was given to the medium. HEK293 cells were cotransfected with 200 ng/well pGL3–BDNF wild-type or mutant promoter vector, 400 ng/well pcDNA3.1–Pitx3 (Peng et al., 2007) or pcDNA3.1 alone, and 8 ng/well pRL–SV40 using Lipofectamine LTX and Plus Reagent (Invitrogen). Cells were lysed in Passive Lysis Buffer 24 h (BDNF promoter assays) or 36 h (Pitx3 promoter assays) post-transfection, and Firefly and Renilla Luciferase luminescence were measured in a Centro LB 960 luminometer (Berthold Technologies) using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturers' instructions. Firefly luminescence was normalized against Renilla luminescence for each well, and relative values (fold induction) were calculated by setting the normalized value of the control transfection as 1.

Semiquantitative RT-PCR and real-time PCR assays. Semiquantitative RT-PCR was performed as reported in (Peng et al., 2007). PCR primers and conditions are provided in Table 1. For quantitative (q) PCR assays,
GFP, and TH on representative midbrain coronal sections of between groups was assessed by paired cDNA, and all reactions were repeated more than twice. Setting the normalized value of controls as 1. Samples from three inde-

48 h, animals were sacrificed by CO₂ asphyxiation. For immunohistochem-

the cannula was left in place for another 10 min before it was retracted. After

Pitx3GFP/GFP

Pitx3

One-4.2.0; Bio-Rad).

The loss of

Pitx3GFP/GFP

and GFP

and GFP

0.068%; E12.5

Pitx3GFP/GFP

0.047%; E12.5

Pitx3GFP/GFP

0.3, mean ± SEM), VM of the

Pitx3GFP/GFP

embryos (Fig. 1

b, d, f), and becomes undetectable in the mouse VM at E12.5 (data not shown).

Given the spatiotemporal correlation of GDNF expression and the initiation of Pitx3 expression in the embryonic VM, we next asked whether GDNF can promote the expression of Pitx3 in primary VM cells in vitro. Treatment of E14 rat primary VM cell cultures (grown in the absence of neurotrophins for 7 DIV) with 10 ng/ml GDNF for 24 h significantly increased the number of Pitx3

and Pitx3

/TH

3, mean

SEM), VM of the

Pitx3GFP/GFP

embryos. Scale bar: (in d), 100 µm.

1 µl of a 1:3 diluted cDNA was amplified in a DNA Engine Opticon (MJ Research) together with 0.5X SYBR Green I (Roche Applied Science) and 5 pmol of each primer (Table 1). A standard curve was constructed using plasmid DNA containing the Pitx3 cDNA (from 10⁵ to 10 molecules) (Peng et al., 2007). Signals from specific Pitx3 and BDNF PCR products were normalized against β-actin, and relative values were calculated by setting the normalized value of controls as 1. Samples from three inde-

Western blot analyses. Cells and brain tissues were processed as de-

scribed by Peng et al. (2007). Antibodies used were rabbit anti-Pitx3 (1:500, Zymed), anti-BDNF (1:500, catalog no. sc-546, Santa Cruz Biotechnology), and anti-β-actin (1:2000, Sigma). Optical densities of the specific protein bands were quantified with an Image Analyzer (Quantity One-4.2.0; Bio-Rad).

Intrasтратial injections of GDNF. Adult male rats (n = 5) were anesthe-

tized with chloral hydrate (400 mg/kg, i.p.). Twenty microliters of a 15 ng/µl GDNF solution in 0.9% saline were injected into the right striatum (according to bregma: anterior-posterior, +0.7; medial-lateral, ±3.5; dorsal-ventral, −4.0) with a Hamilton syringe. Vehicle (0.9% saline) was injected into the contralateral striatum. The injection rate was 1 µl/min, and the cannula was left in place for another 10 min before it was retracted. After 48 h, animals were sacrificed by CO₂ asphyxiation. For immunohistochem-

analyses, animals were intracardially perfused with PBS followed by 4% paraformaldehyde (PFA), and brains were collected, postfixied in 4% PFA for 24 h, cryosectioned, and processed as described above. For Western blot analyses, brains were immediately removed and ventral midbrain tissue was dissection and processed as described before.

Statistics. All values shown are mean ± SEM. Statistical significance between groups was assessed by paired t tests, independent samples t tests, or one-way ANOVA followed by post hoc S-N-K multiple compar-

isons using the SPSS 10.0 software (SPSS Inc.). A value of p < 0.05 was considered significant.

Results

Induction of Pitx3 expression by NF-κB-mediated GDNF/Ret signaling

To determine whether GDNF-mediated signaling is involved in the transcriptional activation of the Pitx3 gene during embryogenesis, we first compared the spatiotemporal expression patterns of GDNF and Pitx3 in the embryonic mouse VM. GDNF is expressed in the mantle zone (MZ) of the midbrain basal plate (ventrolateral midbrain) at E10.5, one day before the onset of Pitx3 transcription in this brain region (Fig. 1a,c,e). Pitx3 expression is detected at E11.5 in the MZ of the adjacent floor plate. At this stage, GDNF expression is restricted to a smaller domain in the midbrain basal plate (Fig. 1b,d,f), and becomes undetectable in the mouse VM at E12.5 (data not shown).

To explore whether GDNF signaling is required for induction of Pitx3 expression in vivo, we counted the number of Pitx3

+ cells in GDNF receptor Ret knock-out embryos. We found that the number of Pitx3

+ cells in Ret−/− embryos was reduced by 17.7% at E12.5 and by 10.4% at E14.5 compared to their heterozygous or wild-type littermates (Fig. 1n–u). Pitx3 expression was particularly lost in ventromedial (Fig. 1n′–u′, insets) and lateral (data not shown) TH

+ cells in the Ret−/− embryos, suggesting that GDNF signaling might specifically induce Pitx3 expression in a TH

+ mdDA neuron subpopulation. The partial recovery of Pitx3

+ cell numbers in the E14.5 Ret−/− embryos might be due to compensa-

tion by other GDNF receptors, such as GFRα1 and NCAM, and could explain the lack of an mdDA phenotype in postnatal DAT−/−Ret−/− mice (Kramer et al., 2007; Paratcha and Ledda, 2008).

We next tested whether GDNF signaling directly activates the Pitx3 promoter in rodent VM cells. GDNF-mediated signaling enhances the nuclear translocation of NF-κB protein complexes and subsequent activation of NF-κB target genes in mdDA neurons (Cao et al., 2008; Wang et al., 2008). Analysis of the human, mouse, and rat Pitx3 promoter regions using bioinformatics prediction tools revealed the existence of conserved NF-κB BSs in these regions (Fig. 2a), and treatment of E14 rat primary VM cultures after 7 DIV with GDNF for 3 or 24 h increased the nuclear translocation of NF-κB, as expected (Fig. 2b–k). To de-

etermine whether this treatment resulted in increased activation of the Pitx3 promoter, we made use of a Pitx3 promoter/reporter construct containing a conserved NF-κB BS. GDNF treatment of SH-SY5Y cells transfected with this Pitx3 reporter construct resulted in a dose-dependent activation (80–110% increase) of the Pitx3 promoter (Fig. 2l). This activation was prevented when we treated the cells with SN50, an inhibitor of NF-κB nuclear translocation (Lin et al., 1995). We thus concluded that GDNF/Ret signaling is necessary and sufficient for the activation of the Pitx3 promoter in the embryonic rodent VM, and we identified NF-κB as a mediator of this process.
GDNF-mediated activation of Pitx3 expression induces BDNF transcription in vivo

We have previously shown that overexpression of Pitx3 upregulates the transcription and secretion of BDNF protein from neuroblastoma cells and primary VM as well as astrocyte cultures (Peng et al., 2007; Yang et al., 2008). We therefore hypothesized that GDNF-mediated activation of Pitx3 expression might subsequently induce the transcription of BDNF in these cells. To test this hypothesis, we treated E14 rat primary VM cultures after 7 DIV with GDNF and evaluated the transcription of Pitx3 and BDNF under these conditions. As expected, GDNF treatment increased the transcription of Pitx3 and BDNF in these cultures by 1.3- and 1.4-fold, respectively, and this effect of GDNF on Pitx3 and BDNF transcription was blocked by the application of the NF-κB inhibitor SN50 (Fig. 3a,c). These results were also confirmed at protein levels (Fig. 3b). To further establish whether the GDNF-induced activation of BDNF transcription was mediated by Pitx3 and not by other Pitx3-independent pathways, we depleted Pitx3 in these cultures by siRNA-mediated knock-down. Quantitative RT-PCR indicated that transfection of a Pitx3 siRNA after 1 h of GDNF treatment-reduced Pitx3 mRNA levels by 56%, concomitant with a 32% reduction of BDNF mRNA levels in these cultures (Fig. 3d,f). A slight but not significant decrease in Pitx3 and BDNF mRNA levels was also observed after transfection of a control (nonsilencing) siRNA oligonucleotide (Fig. 3d,f). This reduction was most likely unspecific and due to the lipofection of the oligonucleotides, as a similar decrease in mRNA levels was observed in sham-lipofected GDNF-treated cultures as compared to untransfected controls (data not shown). We also used Western blotting to confirm that Pitx3 knock-down leads to a reduction of Pitx3 and BDNF protein levels in GDNF-treated primary VM cultures (Fig. 3e). These results therefore suggest that Pitx3 mediates the transcriptional activation of the BDNF gene following treatment of primary VM cells with GDNF.

Pitx3 is required for BDNF transcription in an mdDA neuronal subset in vivo

Our results raised the possibility that Pitx3 is an essential regulator of BDNF gene expression in the rodent VM. To address this possibility, we analyzed the time course of BDNF expression in wild-type (Pitx3+/+ and Pitx3^{GFP/} mice) and Pitx3-
null mutant (Pitx3<sup>GFP/GFP</sup>) mice, which allow the fate mapping of Pitx3-expressing (GFP<sup>/H11001</sup>) cells (Maxwell et al., 2005). In wild-type mouse embryos, Pitx3 expression in the VM was first detected around E11.0 – E11.5 (Fig. 1<sup>e, f</sup>) (Smidt et al., 1997); at this or earlier stages, BDNF expression was not detected in the VM (Fig. 4<sup>a</sup>–<sup>c</sup> and data not shown). We found that BDNF was widely transcribed in the wild-type and Pitx3<sup>/H11001</sup>/GFP VM at E12.5 and exhibited a particularly prominent expression domain in the rostrolateral and medial VM overlapping with GFP<sup>/H11001</sup> (Pitx3<sup>/H11001</sup>) mdDA neurons (Fig. 4<sup>e–g</sup>/H11033 and data not shown). Remarkably, BDNF expression in this rostrolateral and medial domain was entirely lost in the Pitx3GFP/GFP embryos at E12.5, although the corresponding GFP<sup>/H11001</sup> neurons were still present in this domain and despite the persistent expression of BDNF in the adjacent Pitx3-negative (GFP<sup>/H11002</sup>) VM tissue of the null mutants (Fig. 4<sup>h–j</sup>).

To determine whether Pitx3 can directly activate the transcription of the BDNF gene, we searched for conserved Pitx3/Bicoid-like
BSS within the mouse and human BDNF promoters. We found two highly conserved Pitx3/Bicoid-like BSs in these two species, one located ~1.8–2.0 kb upstream (referred to as BS2) and the other one located ~560 bp downstream (referred to as BS9) of the putative transcription start site for the mouse BDNF variant 4 (Fig. 4k). Cotransfection of a luciferase reporter construct containing 2.6 kb of mouse BDNF promoter sequences (including these two highly conserved Pitx3 BSs and seven other putative Pitx3 BSs) and a Pitx3 expression vector resulted in a 4.9-fold increase in luciferase (promoter) activity relative to the control (Fig. 4m). Moreover, site-directed mutagenesis of either one of these two conserved Pitx3 BSs decreased the Pitx3-induced activation of the BDNF promoter by 15% (BS2) and 37% (BS9) (Fig. 4l,k), whereas mutation of both BS2 and BS9 resulted in a 47% decrease of BDNF promoter activity after Pitx3 cotransfection (Fig. 4m).

To establish whether the loss of BDNF expression in the rostralateral and medial VM of the Pitx3<sup>gfp/gfp</sup> embryos is rapidly followed by the death of GFP<sup>+</sup> (Pitx3<sup>+</sup>) mdDA neurons in these embryos, we assessed the proportion of apoptotic (cCasp3<sup>+</sup>) and GFP<sup>+</sup> cells relative to the total number of GFP<sup>+</sup> mdDA neurons in wild-type (Pitx3<sup>+/gfp</sup>) and mutant (Pitx3<sup>gfp/gfp</sup>) embryos at E12.5 (Fig. 5a–c). This proportion was significantly increased by ~3-fold in the rostral but not caudal VM of the Pitx3<sup>gfp/gfp</sup> embryos as compared to their wild-type littersmates (Fig. 5e), indicating that the lack of BDNF expression indeed correlates with a reduced survival of mdDA neurons in the rostral VM of the Pitx3<sup>gfp/gfp</sup> embryos.

We next investigated the effect of Pitx3 inactivation on BDNF expression in late gestational and postnatal mdDA neurons. BDNF expression was still not detected in the rostralateral and medial VM domains of Pitx3<sup>gfp/gfp</sup> embryos at E14.5 (Fig. 6a–f) and E16.5 (data not shown). Loss of Pitx3 resulted in a notable decrease of TH<sup>+</sup> SNC mdDA neurons in the null mutant VM at postnatal day 30 (P30), concomitant with a strong reduction of BDNF-expressing cells in the same region (Fig. 6g–l). Notably, BDNF transcription appeared to be very low or almost undetectable in the caudomedial mdDA domain throughout embryonic development (Figs. 4e–g', 6a–c'), and this mdDA neuronal subset was less affected in the Pitx3<sup>gfp/gfp</sup>-null mutants at embryonic and postnatal stages (Figs. 4h–j', 5e, 6d–f', j–l'; Maxwell et al., 2005). Collectively, our results indicate that Pitx3 directly activates the BDNF promoter in an mdDA neuron subgroup located in the rostralateral and medial VM of the mouse embryo and suggest that the Pitx3-mediated activation of BDNF expression is necessary for the survival of this mdDA neuronal subset at embryonic and postnatal stages.

BDNF augments the survival of mdDA neurons in Pitx3<sup>+</sup> mutant primary VM cultures

To further investigate whether the lack of BDNF expression contributes to the loss of mdDA neurons in Pitx3<sup>gfp/gfp</sup> mice (Fig. 5 and Maxwell et al., 2005), we tested whether BDNF treatment is sufficient to rescue the numbers of mdDA neurons in primary VM cultures derived from E11.5 Pitx3<sup>gfp/gfp</sup> embryos. We found a reduction of GFP<sup>+</sup> cell numbers (cells that would express Pitx3 in the wild type) by 13%, TH<sup>+</sup> cells by 37.5%, and GFP/TH double-positive cells by 40% in the untreated Pitx3<sup>gfp/gfp</sup> cultures as compared to untreated wild-type (Pitx3<sup>+/+</sup>) cultures (Fig. 7a–f,m). This result is consistent with in vivo data from E12.5 Pitx3<sup>gfp/gfp</sup> mice showing a reduction of GFP<sup>+</sup> cells by 21%, TH<sup>+</sup> cells by 54%, and GFP/TH double-positive cells by 48% relative to the Pitx3<sup>+/+</sup> controls (Maxwell et al., 2005), and it confirms a selective loss of GFP<sup>+</sup> and TH<sup>+</sup> cells in the absence of Pitx3 also in primary VM cultures. Notably, BDNF treatment of Pitx3<sup>gfp/gfp</sup> VM cultures increased the numbers of GFP<sup>+</sup> cells by 29.6%, TH<sup>+</sup> cells by 84.7%, and TH/GFP double-positive cells by 66.7% relative to the untreated Pitx3<sup>gfp/gfp</sup> cultures, thereby reaching similar numbers as in the BDNF-treated wild-type (Pitx3<sup>+/+</sup>) cultures (Fig. 7g–m). To determine whether this was due to a survival-promoting effect of the BDNF treatment on the cultured mdDA neurons, we assessed the proportion of apoptotic (cCasp3<sup>+</sup>) mdDA (GFP<sup>+</sup>) neurons in the untreated and BDNF-treated VM cultures from wild-type (Pitx3<sup>+/+</sup>) and mutant (Pitx3<sup>gfp/gfp</sup>) embryos. As expected, this proportion was significantly increased in the untreated Pitx3<sup>gfp/gfp</sup> VM cultures as compared to untreated wild-type (Pitx3<sup>+/+</sup>) cultures.
compared to the untreated controls (Fig. 7n–s,w). Notably, the increased death of GFP+/H11001 cells in the Pitx3+/H11001 VM cultures was rescued back to control levels by the addition of BDNF to these cultures (Fig. 7t–w), indicating that in the absence of Pitx3, exogenous BDNF application prevents the cell death of cultured Pitx3-null (Pitx3+/GFP/GFP) mdDA neurons.

Intrastrital injection of GDNF upregulates Pitx3 and BDNF expression in the adult SNc
Our previous results strongly suggested the existence of a feed-forward mechanism for the initiation and/or maintenance of GDNF, Pitx3, and BDNF expression in the murine VM during embryonic development. We therefore hypothesized that this...
feedforward mechanism might also persist in the adult brain, as it is known that GDNF released from striatal target cells is taken up and retrogradely transported to the soma of mdDA neurons, where it promotes their survival in the early postnatal and adult rodent brain (Tomac et al., 1995; Oo et al., 2003; Kholodilov et al., 2004). To test this hypothesis, we injected GDNF protein unilaterally into the striatum of adult rats, and 48 h later we analyzed the endogenous expression levels of Pitx3 and BDNF proteins in the ipsilateral (GDNF-treated) SNc relative to the contralateral (vehicle-treated) control side. We found that Pitx3 and BDNF protein levels were increased by 2.8- and 6.1-fold, respectively, in the ipsilateral (GDNF-treated) SNc, as determined by immunohistochemistry (Fig. 8a–l) and on Western blots (Fig. 8n–p). These data provide direct evidence that retrograde GDNF signaling stimulates Pitx3 and BDNF expression in mature SNc mdDA neurons.

BDNF, but not GDNF, protects Pitx3+/− mdDA neurons against 6-OHDA neurotoxicity

Given the previous result, we wanted to know whether the neuroprotective effect of GDNF against 6-OHDA toxicity on mdDA neurons might be due to Pitx3-mediated activation of BDNF expression in these neurons. Therefore, we treated wild-type (Pitx3+/−/GFP) and Pitx3-null mutant (Pitx3−/−/GFP) VM cultures after 3 DIV with 6-OHDA and assessed the effect of a previous 2 h incubation with GDNF or BDNF on these 6-OHDA-treated cultures. Treatment with 6-OHDA significantly reduced the numbers of TH+ cells in both wild-type and mutant cultures (Fig. 9a–f, m–r, y) as expected. Prior incubation with GDNF or BDNF significantly rescued the numbers of TH+ mdDA neurons in the wild-type (Pitx3+/−/GFP) VM cultures, reaching a similar level as that under normal (untreated) conditions (Fig. 9a–f, m–r, y). Remarkably, the numbers of TH+ mdDA neurons in the 6-OHDA-treated Pitx3−/−/GFP VM cultures were only rescued back to normal (untreated) levels after a previous incubation of these cells with BDNF, but not with GDNF (Fig. 9s–y), indicating that GDNF cannot protect TH+ mdDA neurons against 6-OHDA-induced neurotoxicity in the absence of Pitx3.

To determine whether these two neurotrophic factors exerted their protective effect on the 6-OHDA-treated wild-type (Pitx3+/−/GFP) and mutant (Pitx3−/−/GFP) VM cultures by a reduction of neurotoxin-induced cell death of Pitx3−/−/GFP mdDA neurons. a–x: Primary VM cultures derived from E11.5 Pitx3+/GFP (a–l) and Pitx3−/−/GFP (m–x) embryos were treated after 3 DIV with vehicle (0.1% BSA) (a–f, m–r), 20 ng/ml GDNF (g–i, s–u), or 20 ng/ml BDNF (j–l, v–x); after 2 h, cells were incubated with 10 μM 6-OHDA (d–l, p–x) or vehicle (PBS; a–c, m–o) for 24 h and immunostained for cCasp3 (red in a, d, g, j, m, p, s, and u) and GFP (green in b, e, h, k, n, q, t, and w); blue, DAPI stain; merged images in c, f, i, l, o, r, and y. Quantification of the relative amount of apoptotic (cCasp3+; red) mdDA neurons (GFP+; green) in these eight experimental groups revealed that both GDNF and BDNF prevent the 6-OHDA-induced cell death of Pitx3+/+ mdDA neurons, but only BDNF (and not GDNF) prevents the 6-OHDA-induced cell death of Pitx3−/− mdDA neurons. cCasp3+/GFP cells (white arrowheads); Pitx3+/−/GFP vehicle-treated, 17.92 ± 1.46%; Pitx3−/−/GFP 6-OHDA-treated, 33.28 ± 1.74%; Pitx3−/−/GFP 6-OHDA + GDNF-treated, 28.11 ± 1.86%; Pitx3+/−/GFP 6-OHDA + BDNF-treated, 18.35 ± 0.78%; mean ± SEM; n = 3. One-way ANOVA was used for statistical analysis of treatment effects. ***p < 0.005; ***p < 0.001. Data were derived from three independent experiments. Scale bar: (a,x), 50 μm.
induced apoptotic cell death, we assessed the relative proportion of apoptotic (cCasp3ˢ⁺) and GFP ᵇ mdDA neurons in the 6-OHDA-treated VM cultures alone and after previous incubation with GDNF or BDNF. As expected, treatment with 6-OHDA alone significantly increased the numbers of apoptotic mdDA neurons in these cultures, regardless of their genotype (Fig. 10a–f, m–r, y). In line with our previous findings, BDNF significantly decreased the numbers of apoptotic mdDA neurons down to normal (untreated) levels in both Pitx3ˢ⁻/GFP and Pitx3ˢGP⁴/GFP 6-OHDA-treated cultures (Fig. 10j–l, v–x, y), whereas GDNF only decreased these numbers significantly in Pitx3ˢ⁻/GFP but not in Pitx3ˢGP⁴/GFP 6-OHDA-treated cultures (Fig. 10g–i, s–u, y). Altogether, these results strongly suggest that GDNF exerts its neuroprotective and survival-promoting effect on at least a subpopulation of mdDA neurons through the activation of Pitx3-mediated BDNF expression in these cells. In the absence of Pitx3, GDNF cannot protect the mutant mdDA neurons against 6-OHDA-induced neurotoxicity and apoptotic cell death, and these cells are only rescued by the exogenous application of BDNF acting downstream of GDNF and Pitx3, as suggested by our previous results.

Discussion

The secreted factors GDNF and BDNF and the transcription factor Pitx3 are individually required for the proper development of mdDA neurons or for their survival during postnatal and adult stages (Baquet et al., 2005; Smidt and Burbach, 2007; Pascual et al., 2008; Oo et al., 2009), but it remained unclear whether a regulatory interaction exists between these three factors during embryonic development or in the adult brain. Here we show that NF-κB-mediated GDNF/Ret signaling is, at least in part, sufficient for the activation of Pitx3 expression, which is in turn required for the transcription of BDNF in a rostralateral and medial mdDA neuronal subpopulation, thereby promoting the survival of these neurons during mouse embryonic development (Fig. 11). We also show that this feedforward regulation of GDNF, Pitx3, and BDNF expression is still active in the adult rodent brain and that, in the absence of Pitx3, BDNF but not GDNF protects mdDA neurons against 6-OHDA toxicity in vitro. Altogether, our data indicate that the regulatory interaction between GDNF, Pitx3, and BDNF is necessary for the survival and protection of mdDA neurons against neurotoxic insults during embryogenesis, and that this regulatory interaction might also be relevant for the survival and neuroprotection of adult mdDA neurons (Fig. 11).

GDNF/Ret signaling activates Pitx3 expression in an mdDA neuronal subpopulation

The relevance of GDNF signaling for the normal development of mdDA neurons in vivo has remained controversial until now. Inactivation of the murine GDNF, Ret, and Gfra1 genes apparently does not interfere with prenatal mdDA neuron differentiation or survival (Airaksinen and Saarma, 2002; Kramer et al., 2007; Paratsuma and Ledda, 2008). Moreover, persistent overexpression of GDNF driven by the TH promoter leads to a reduction of SNc DA neurons (Chun et al., 2002), whereas intrastratal or intranigral injections of GDNF protein during early postnatal stages, or the constitutive activation of the Ret receptor, result in increased numbers of TH ᵇ SNc neurons and an enhanced DA metabolism (Beck et al., 1996; Mijatovic et al., 2007). The transient but detectable expression of GDNF in the midgestational VM shown here might have been missed by previous in situ hybridization studies due to lower sensitivity or slight differences in the staging of the embryos (Hellmich et al., 1996; Golden et al., 1999), but GDNF expression in the embryonic rat VM was also shown by Choi-Lundberg and Bohn (1995) using a more sensitive RT-PCR-based detection method. Our and previous findings therefore suggest that the transient expression of GDNF in the rodent VM might have two different functions during development: on the one hand, GDNF signaling might activate Pitx3 transcription in an mdDA precursor subpopulation; on the other hand, GDNF might provide transient trophic support to developing progenitors and postmitotic neurons in this region of the brain. The requirement of GDNF signaling for these two processes, however, might be compensated by other factors during embryonic development, leading to the absence of an mdDA phenotype in the GDNF, Ret, and Gfra1-null mutants at birth (Airaksinen and Saarma, 2002; Paratsuma and Ledda, 2008). Our and previous results also suggest that the tight regulation of GDNF protein levels is critical for proper mdDA neuron development. The notion that a transient (rather than persistent) expression of GDNF during embryonic development might have an important permissive role is supported by the observation that sustained GDNF expression in differentiating TH ᵇ SNc neurons results in a marked reduction of these neurons shortly after birth (Chun et al., 2002).

Pitx3 is necessary for the activation of BDNF transcription in an mdDA neuronal subset

Pitx3 is required for the initiation of TH expression in an mdDA neuronal subset during development and for the survival of these neurons...
neurons during subsequent prenatal and postnatal stages (Maxwell et al., 2005; Smidt and Burbach, 2007). Aldh1al1 was identified as a target gene that could mediate the prosurvival activity of Pitx3, as maternal RA complementation partially rescues the loss of mdDA neurons in Pitx3 mutants (Jacobs et al., 2007). Here we identify BDNF as another target gene of Pitx3 in the rostralateral and medial mdDA neuronal subpopulation that is most affected in Pitx3 <sup>−/−</sup> mice (Smidt et al., 2004; Maxwell et al., 2005). Because BDNF treatment augmented the survival of mdDA neurons in primary VM cultures prepared from E11.5 Pitx3<sup>GFP/GFP</sup>-null mutant embryos, we suggest that the failure to induce BDNF expression in rostralateral and medial (SNC) mdDA precursors during development contributes to the preferential degeneration of these neurons in the Pitx3<sup>GFP/GFP</sup> mice. Interestingly, retinoic acid receptors was recently shown to induce BDNF transcription and to prevent the inflammatory degeneration of mdDA neurons in vitro and in vivo (Katsuki et al., 2009). While our data strongly suggest a direct activation of the BDNF gene by Pitx3, we cannot exclude that Pitx3 might indirectly maintain BDNF expression through the induction of Aldh1al1 and subsequent local production of RA in an mdDA neuronal subset.

GDNF protects mdDA neurons against 6-OHDA toxicity only in the presence of Pitx3

Treatment of Pitx3<sup>GFP/GFP</sup>-null mutant VM cultures with 6-OHDA resulted in an increased survival of mdDA neurons in these cultures only after preincubation with BDNF, but not with GDNF, suggesting that GDNF acts upstream of Pitx3 and BDNF to protect mdDA neurons against cytotoxic insults and to increase their survival under these conditions. BDNF is in fact more potent than GDNF in promoting the survival of mdDA neurons after unilateral 6-OHDA lesion of the SNCs in organotypic cultures of the rat VM (Stahl et al., 2011). Moreover, GDNF appears to promote primarily the survival of calbindin-expressing VTA neurons under basal conditions (Meyer et al., 1999) and of VTA and rostromedial SNC neurons after 6-OHDA lesion (Barroso-Chinea et al., 2005), which are the cell groups that are not affected by the loss of BDNF expression in our Pitx3<sup>GFP/GFP</sup> mice. Together, our and previous findings therefore suggest that GDNF acts as a potent survival factor for those medial mdDA neuronal subpopulations projecting to the GDNF-rich ventral striatum (Barroso-Chinea et al., 2005). However, the rostralateral mdDA neurons projecting to the dorsolateral striatum (where lower levels of GDNF are expressed; Barroso-Chinea et al., 2005) depend on Pitx3-mediated local BDNF synthesis for their survival and neuroprotection.

The feedforward regulation of GDNF, Pitx3, and BDNF expression in the adult SNC might be relevant for the pathogenesis of PD

We also found that the intrastral delivery of GDNF protein in the adult rat brain increased the expression of Pitx3 and BDNF in the ipsilateral SNC. This finding raises the possibility that retrograde transport of GDNF or local signaling at axon terminals in the striatum controls the levels of Pitx3 and BDNF proteins in adult mdDA neurons (Fig. 11) and might have implications for the pathogenesis and treatment of PD. Among all neurotrophic factors, GDNF exhibits the most severe decrease in the SNCs of PD patients (Chauhan et al., 2001). Reduced intranigral GDNF levels might lead to reduced Pitx3 expression and thus to decreased expression of BDNF in SNC mdDA neurons. This might render these neurons more susceptible to death in the diseased brain. A reduction of BDNF mRNA and protein levels in the SNC of PD patients has been consistently reported by several groups (Mogi et al., 1999; Parain et al., 1999; Howells et al., 2000; Chauhan et al., 2001). Interestingly, some of these studies also noted in the patients the preferential loss of a subset of SNC neurons that normally express high levels of BDNF (Parain et al., 1999; Howells et al., 2000). Moreover, several polymorphisms in noncoding regions of the PITX3 gene (which might alter PITX3 expression) are associated with idiopathic or early onset PD (Fuchs et al., 2009; Bergman et al., 2010; Haubenberger et al., 2011; Le et al., 2011), and one polymorphism in the promoter region of the human BDNF gene was associated with familial PD (Persiani et al., 2004). These findings, together with our results and the observation that conditional ablation of the BDNF gene during mouse development leads to the selective loss of a subset of TH<sup>+</sup> neurons in the SNCs, but not VTA (Baquet et al., 2005), raise the intriguing possibility that the rostralateral (SNCs) mdDA neuron subpopulation expressing high levels of BDNF is most affected by the loss of PITX3 in human PD patients and cannot be protected by the exogenous application of GDNF. We suggest that a developmental failure to induce normal expression of Pitx3 coupled to age-related decreases in GDNF levels (Miyazaki et al., 2003) might lead to a depletion of BDNF in SNC neurons and predispose them to neurodegeneration (Porritt et al., 2005). According to this model, alterations in this neurotrophic factor reinforcement loop might lead to reduced neurotrophic support of SNC mdDA neurons, thus contributing to PD.

Further dissection of the molecular underpinnings of this regulatory network might therefore facilitate the development of novel therapeutic approaches for the treatment of PD.

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