Development/Plasticity/Repair

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

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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 rostrolateral (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.

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 rostrolateral and VTA neurons from a caudomedial mdDA precursor subpopulation (Smits et al., 2006). Several secreted and transcription factors are involved in the specification of the mdDA fate in neuroepithelial progenitors and in their differentiation into mature mdDA neurons, as well as in their survival throughout development and adulthood (Prakash and Wurst, 2006; Smidt and Burbach, 2007). The transcription factor Pitx3 has gained particular interest, as Pitx3 expression in the brain is restricted to mdDA neurons and *Pitx3* mutant mice display a progressive and preferential loss of the SNc mdDA neurons during embryonic and postnatal development (van den Munckhof et al., 2003; Smidt et al., 2004). Pitx3 cooperates with the nuclear receptor Nurr1 to activate transcription of several genes encoding DA biosynthetic enzymes, transporters, and receptors (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

Received Feb. 18, 2011; revised April 26, 2011; accepted July 13, 2011.

Author contributions: C.P., N.P., and W.L. designed research; C.P. and L.A. performed research; R.K. and M.L. contributed unpublished reagents/analytic tools; R.K., M.L., and W.W. analyzed data; C.P., W.W., N.P., and W.L. wrote the paper.

This work was supported by research grants from National Natural Science Foundation of China (NSFC30730096 and NSFC39070925) and National Basic Research Program of China (2011CB945200 and 2010CB510003) and research grants from Bayerischer Forschungsverbund 'ForNeuroCell II' (F2-F2412.18/10 086) to W.W. and N.P., by the Initiative and Networking Fund in the framework of the Helmholtz Alliance of Systems Biology (CoReNe), Federal Ministry of Education and Research (BMBF) NGFNPlus DiGtoP (FKZ 01GS0858), and European Union (mdDANEURODEV FP7-Health-2007-B-222999, EuTRACC LSHG-CT-2006-037445) to W.W. We thank X. Li for helpful discussions and T. Wen and S. Laass for excellent technical assistance.

The authors declare no conflicts of interest or relevant financial interests.

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DOI:10.1523/JNEUROSCI.0898-11.2011

 0270-6474/11/3112802-14$15.00/0

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

Gene	Forward primer/reverse primer	Length of product (bp)	T_m (°C)[a]	Cycles
Pitx3 RT-PCR (NM_019247)	5′-GAGCACAGTGACTCGGAGAAGG-3′/5′-AAGGCGAACGGGAAGGTC-3′	413	60	28
BDNF RT-PCR (NM_012513)	5′-AGCGTGAATGGGCCCAGGGCA-3′/5′-TGTGACCGTCCCACCGGACA-3′	369	60	28
GAPDH RT-PCR (NM_017008)	5′-CCATGTTTGTGATGGGTGTGAACCA-3′/5′-GCCAGTGGATGCAGGGATGATGTTC-3′	251	58	22
Pitx3 qPCR (NM_019247)	5′-GAGTTTGGGCTGCTTGGTGAGGC-3′/5′-CCATGTTCTGGAAGCGGAGGGTGT-3′	92	60	35
BDNF qPCR (NM_012513)	5′-GATGCCGCAAACATGTCTATGA-3′/5′-TAATACTGTCACACACGCTCAGCTC-3	82	60	35
Beta-actin qPCR (NM_031144)	5′-CCCGAGGCTCTCTTCCAGCC-3′/5′-TAGAGGTCTTTACGGATGTCAACGT-3′	110	60	35

[a] T_m, Melting temperature.

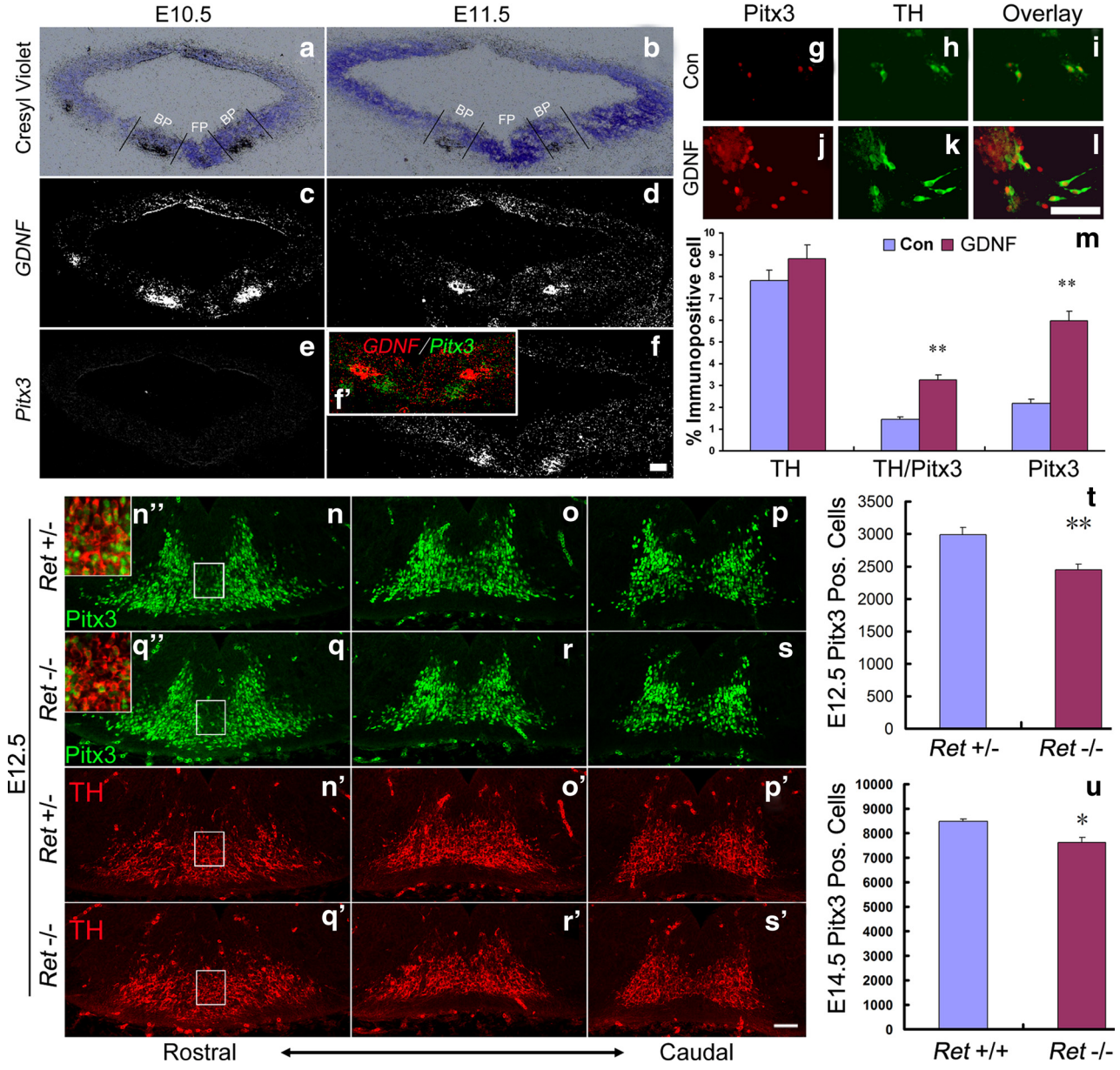

Figure 1. GDNF/*Ret* signaling is required for activation of Pitx3 expression in an mdDA neuronal subset. ***a–f***, Representative midbrain coronal sections of *wild-type* (C57BL/6) mouse embryos at E10.5 (***a***, ***c***, ***e***) and E11.5 (***b***, ***d***, ***f***), hybridized with riboprobes for *GDNF* (***c***, ***d***) and *Pitx3* (***e***, ***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–l***, 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***). ***m***, 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, 1.43 ± 0.13%; GDNF-treated, 3.25 ± 0.23%; mean ± SEM, $^{**}p < 0.01$ in the independent *t* test; Pitx3$^+$ cells: control, 2.19 ± 0.19%; GDNF-treated, 5.95 ± 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***, ***n′–p′***) and *Ret*$^{-/-}$ (***q–s***, ***q′–s′***) mouse embryos at E12.5, immunostained for Pitx3 (***n–s***) and TH (***n′–s′***). Insets (***n″***, ***q″***) are merged images and higher magnifications of the boxed areas in ***n***, ***n′***, ***q***, and ***q′***. ***t***, ***u***, Quantification on coronal or sagittal midbrain sections revealed a significant decrease of Pitx3$^+$ cells by 17.7% in E12.5 *Ret*$^{-/-}$ embryos (***t***) and 10.4% in E14.5 *Ret*$^{-/-}$ embryos (***u***) as compared to their wild-type (*Ret*$^{+/-}$ and *Ret*$^{+/+}$) littermates (Pitx3 Pos. (Pitx3$^+$) cells: E12.5 *Ret*$^{+/-}$, 2981 ± 125, $n = 3$; E12.5 *Ret*$^{-/-}$, 2455 ± 85, $n = 3$; E14.5 *Ret*$^{+/+}$, 8491 ± 105, $n = 4$; 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: (in ***f***, ***l***, ***s′***) 50 μm.

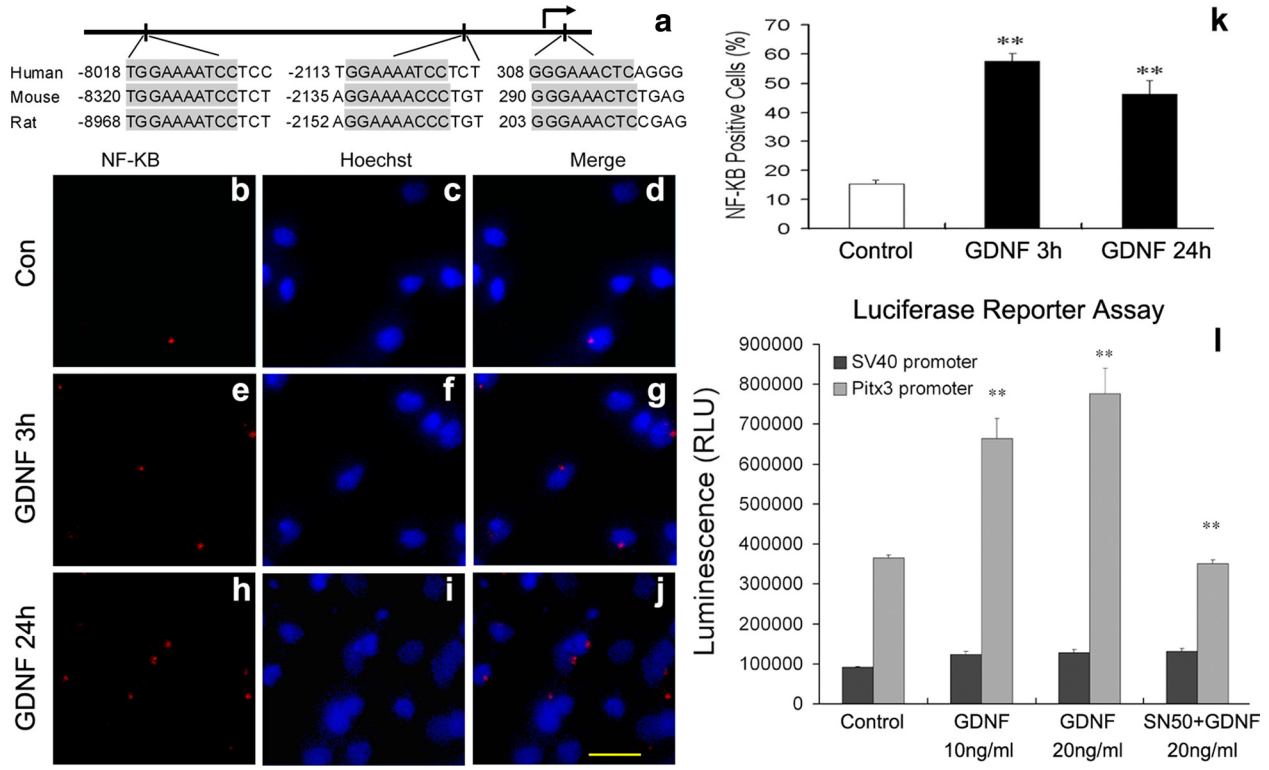

Figure 2. NF-κB-mediated GDNF-signaling activates murine *Pitx3* transcription. ***a***, The human, mouse, and rat *Pitx3* promoter region contains three highly conserved NF-κB BS (consensus sequence: 5′-GGGRNTYYCC-3′, where R = A or G, Y = C or T, and N = any nucleotide). Position of the first nucleotide of each BS is relative to the transcription start site (arrow). ***b–j***, Immunostaining for NF-κB (***b***, ***e***, ***h***) and nuclear staining (Hoechst; ***c***, ***f***, ***i***) (merged images in ***d***, ***g***, ***j***) of untreated (***b–d***) E14 rat primary VM cultures or after treatment with GDNF for 3 h (***e–g***) or 24 h (***h–j***). ***k***, Quantification of NF-κB^+ cells relative to the total number of cells in these cell cultures with or without GDNF-treatment (control: 15.3 ± 1.3%; 3 h GDNF: 57.6 ± 2.5%; 24 h GDNF: 46.1 ± 4.8%, mean ± SEM; $^{**}p < 0.005$ in the independent *t* test; $n = 3$). ***l***, Transfection of SH-SY5Y cells with *pGL3-Pitx3* promoter vector (light gray bars) and subsequent treatment of these cells with 10 or 20 ng/ml GDNF resulted in a dose-dependent activation of the *Pitx3* promoter as compared to untreated controls. Dark gray bars represent values from p*RL-SV40* vector (internal control). GDNF-mediated activation of the *Pitx3* promoter was blocked by the simultaneous application of SN50, an inhibitor of NF-κB nuclear translocation (control: *SV40* promoter, 96954 ± 2863; *Pitx3* promoter, 365722 ± 7460; GDNF at 10 ng/ml: *SV40* promoter, 123,900 ± 6980; *Pitx3* promoter, 663,831 ± 51,615; GDNF at 20 ng/ml: *SV40* promoter, 128,212 ± 7174; *Pitx3* promoter, 776,831 ± 62,941; SN50 + GDNF at 20 ng/ml: *SV40* promoter, 131,242 ± 7724; *Pitx3* promoter, 350,674 ± 9528, mean ± SEM; $^{**}p < 0.005$ in one-way ANOVA for repeated measurements). Data were derived from three independent experiments. RLU, Relative luciferase unit. Scale bar: (in ***j***), 20 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 (Airaksinen and Saarma, 2002; Zuccato and Cattaneo, 2009). 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; Oo et al., 2005). The *Ret* tyrosine kinase receptor and its GDNF family coreceptors (*GFR*) α1 and α2, however, are expressed strongly in the VM throughout the mdDA neurogenic period (Golden et al., 1999). Furthermore, conditional deletion of the *BDNF* gene during midbrain development results in a selective reduction of these neurons at birth (Baquet et al., 2005).

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 rostrolateral 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-^{35}S]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 545 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

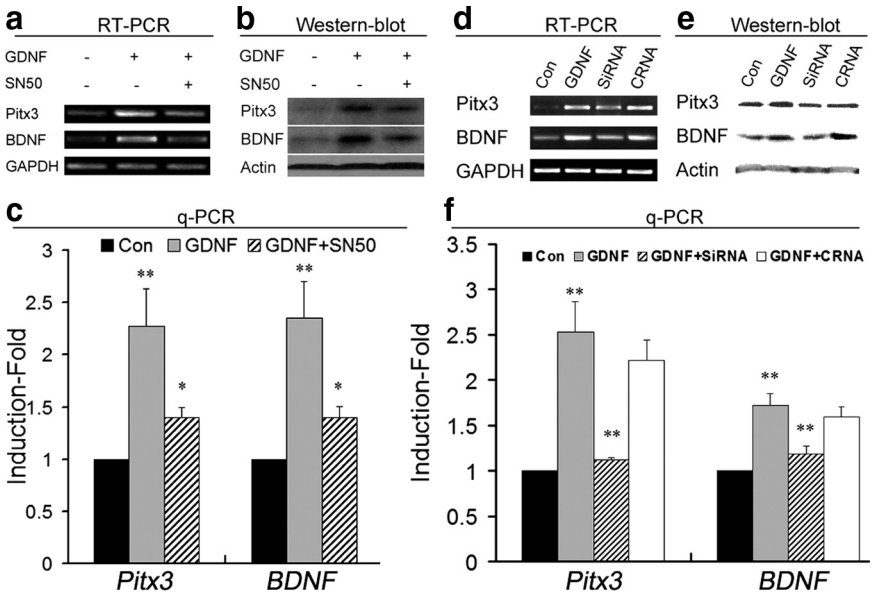

Figure 3. GDNF-mediated induction of Pitx3 expression activates *BDNF* transcription. ***a***, ***b***, RT-PCR (***a***) and Western blot (***b***) analyses of untreated (first lane), GDNF-treated (second lane), and SN50 + GDNF-treated (third lane) E14 rat primary VM cultures showed increased Pitx3 and 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 ± 0.36; GDNF + SN50, 1.40 ± 0.09, mean ± SEM.; BDNF: GDNF, 2.35 ± 0.35; GDNF + SN50, 1.40 ± 0.11, mean ± SEM; $*p < 0.05$; $**p < 0.005$ in the one-way ANOVA for repeated measurements; $n = 4$). Con, Control. ***d***, ***e***, 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.

tested by using the corresponding sense riboprobes in some experiments. Images were taken using bright- and dark-field optics on an Axioplan2 microscope or StemiSV6 stereomicroscope, AxioCam MRc camera, and Axiovision 4.6 software (Zeiss) and processed with Adobe Photoshop 7.0 or CS software (Adobe Systems).

Immunostaining and cell counting. Immunostaining of cultured cells, paraffin sections and free-floating cryosections (40 μm) was performed as described previously (Brodski et al., 2003; Peng et al., 2007). Polyclonal rabbit antisera were directed against NF-κB (1:100; Cell Signaling Technology), BDNF (1:500 (Sc-546); Santa Cruz Biotechnology), Pitx3 (1:300; Zymed), and cleaved (activated) Caspase-3 (cCasp3, 1:100; Cell Signaling Technology). The immunostaining for cCasp3 on cultured cells and tissue sections was amplified using the Tyramide Signal Amplification kit (PerkinElmer) according to the manufacturer's instructions. Monoclonal mouse antibodies were directed against tyrosine hydroxylase (TH) at 1:6000 (Sigma) and 1:600 (MAB318, Millipore Bioscience Research Reagents). Polyclonal chicken antisera were directed against GFP (1:2000; Aves Labs). Fluorescent images were taken with an Axiovert 200M inverted microscope (Zeiss), and Olympus BX51 microscope (Olympus), or an LSM510 confocal microscope (Zeiss), and processed with Adobe Photoshop 7.0 or CS software.

TH^+, $Pitx3^+$, and $DAPI^+$ cells in E14 rat VM cultures were counted in 10 random fields per well for six wells, and data were collected from three independent experiments. $Pitx3^+$ cells were counted on every fourth serial coronal hemisection through the midbrain of E12.5 and on every fourth serial sagittal section through the midbrain of E14.5 $Ret^{+/+}$, $Ret^{+/-}$ and $Ret^{-/-}$ embryos. GFP^+ and $cCasp3^+$ cells were counted on every fourth serial coronal hemisection through the midbrain of E12.5 $Pitx3^{+/GFP}$ and $Pitx3^{GFP/GFP}$ embryos. GFP^+, TH^+, $cCasp3^+$, and $DAPI^+$ cells in E11.5 $Pitx3^{+/GFP}$ and $Pitx3^{GFP/GFP}$ VM cultures were 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 $Pitx3^{+/GFP}$ and $Pitx3^{GFP/GFP}$ 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 $Pitx3^{+/GFP}$ or $Pitx3^{GFP/GFP}$ 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-SY5Y cells stably expressing mouse *Pitx3* (Peng et al., 2007). Under these conditions, one *Pitx3*-specific shRNA oligonucleotide (antisense: 5′-GCACGCCUCU-UUCAGC-UAUdTdT-3′; sense: 5′-AUAGCUGAAA-GAGGCGUGCdTdT-3′; targeting sequences around position 956 to 974 in *Pitx3* exon 4, GenBank accession no.. NM_008852.4) resulted in ~80% knock-down of *Pitx3* mRNA (data not shown). A nonsilencing shRNA oligonucleotide, as indicated by the manufacturer (Shanghai GeneChem) (antisense: 5′-CGUGACACGUUCGGAGAAdTdT-3′; sense: 5′-UUCUCCGAA-CGUGUCACGUdTdT-3′) was used as control. E14 rat primary VM cells were transfected with *Pitx3* or control shRNA oligonucleotides (40 pmol) after 7 DIV using Lipofectamine 2000 (Invitrogen) right 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).

6-Hydroxydopamine *treatments.* Dissociated E11.5 $Pitx3^{+/GFP}$ or $Pitx3^{GFP/GFP}$ 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 2 h before the addition of 10 μM 6-hydroxydopamine (6-OHDA; catalog no. H116, Sigma) or vehicle alone (PBS) to the culture medium. Cells were fixed 24 h after the addition of 6-OHDA for immunostaining.

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 CONREAL (CONserved Regulatory Elements anchored ALignment; 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 2616 bp *BDNF* promoter fragment (position −1930 to +686 relative to transcript variant 4, GenBank accession no. NM_001048142.1) were amplified from C57BL/6 mouse genomic DNA using the primer pairs *Pitx3* forward, 5′-AAGCAAACCTTTTCTAAGGC-3′; *Pitx3* reverse, 5′-TGTGGCGGCGGCGTTCT-3′; *BDNF* forward, 5′-

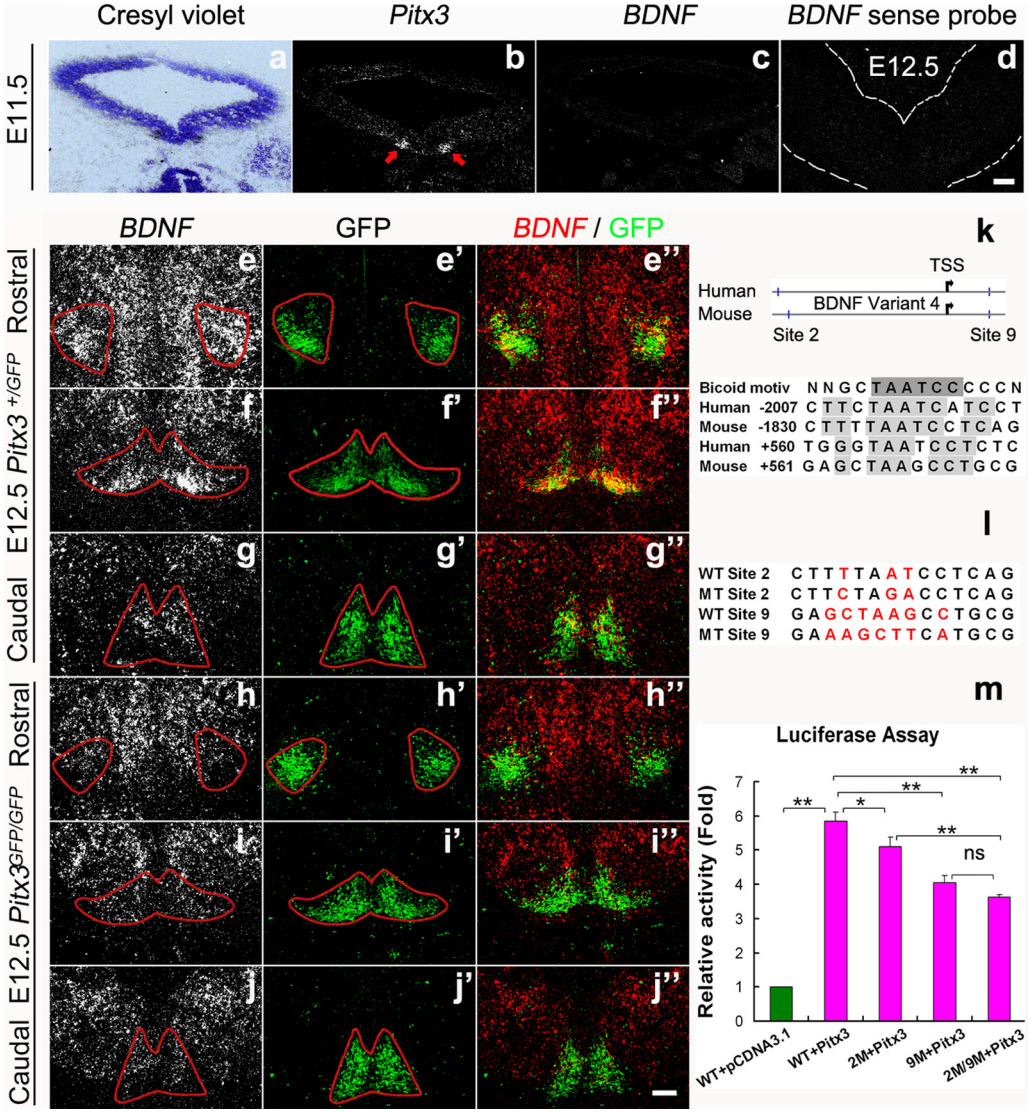

Figure 4. Pitx3 is required for the onset of *BDNF* transcription in an mdDA neuronal subpopulation at E12.5. ***a–d***, Representative midbrain coronal sections of wild-type mouse embryos at E11.5 (***a–c***, consecutive sections) and E12.5 (***d***), hybridized with *Pitx3* (***b***), *BDNF* antisense (***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–j″***, Representative coronal sections at different rostrocaudal levels of the midbrain from E12.5 $Pitx3^{+/GFP}$ (***e–g″***) and $Pitx3^{GFP/GFP}$ (***h–j″***) mice, hybridized with *BDNF* antisense riboprobe (***e–j***), or immunostained for GFP (***e′–j′***). Merged images are shown in (***e″–j″***). *BDNF* expression is not induced in a rostrolateral and medial GFP^{+} mdDA domain of the $Pitx3^{GFP/GFP}$ 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 p*GL3-BDNF* promoter vector containing these two conserved *Pitx3* BSs (WT, wild type) and p*cDNA3.1-Pitx3* vector (red bars) into HEK293 cells resulted in a 4.9-fold activation of the *BDNF* promoter relative to the control (p*cDNA3.1* only, green bar) (Pitx3: 5.86 ± 0.24, mean ± SEM; $^{**}p < 0.005$ 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) (***l***) 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 ± 0.08, mean ± SEM; $^{*}p < 0.05$; $^{**}p < 0.005$ in the one-way ANOVA for repeated measurements) (***m***). Data were derived from three independent experiments. Scale bars: (in ***d*** and ***j″***), 100 μm.

TCTCGGATACCCATTTTTGC-3′; *BDNF* reverse, 5′-CCCAACAGCT-GTCGCTCTAT-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′-CACCTGTC-TGAGGTCTAGAAGAGAGGCTCCTTCTTC-3′ (BS2) and 5′-CCAAGG-GAAGAGGACGACTTGCGCCATGGGAAAGCTTCATGCGTCCC-GGGGATCC-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 p*GL3-Pitx3* promoter vector and 8 ng/well p*RL-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 p*GL3-BDNF* wild-type or mutant promoter vector, 400 ng/well p*cDNA3.1-Pitx3* (Peng et al., 2007) or p*cDNA3.1* alone, and 8 ng/well p*RL-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,

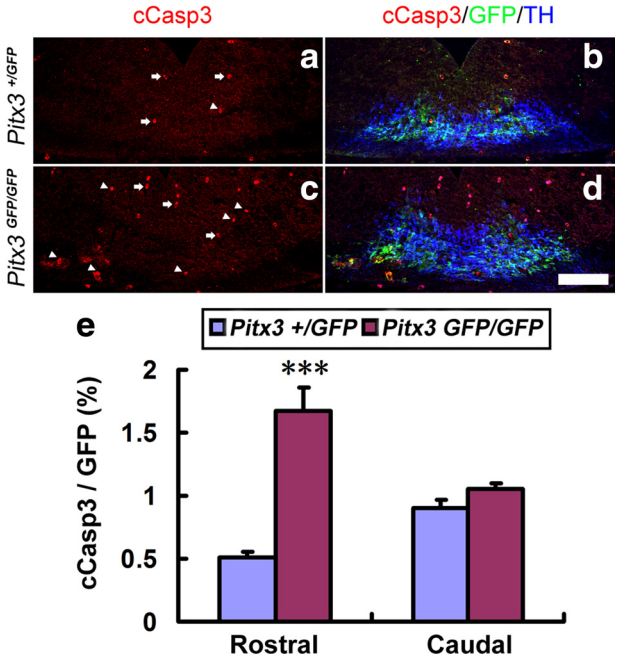

Figure 5. The loss of *BDNF* expression correlates with an increased apoptotic cell death of mdDA neurons in the E12.5 *Pitx3*$^{GFP/GFP}$ rostral VM. ***a–d***, Triple immunostaining for cCasp3, GFP, and TH on representative midbrain coronal sections of *Pitx3*$^{+/GFP}$ (***a***, ***b***) and *Pitx3*$^{GFP/GFP}$ (***c***, ***d***) embryos at E12.5 revealed an increase of apoptotic Pitx3$^+$ mdDA neurons [cCasp3$^+$ (red) and GFP$^+$ (green) double-labeled cells, white arrowheads) in the mutant VM. White arrows point at erythrocytes that were stained unspecifically for cCasp3. ***e***, Quantification of cCasp3$^+$/GFP$^+$ double-labeled cells relative to the total amount of GFP$^+$ (Pitx3$^+$) cells in these sections showed a significant increase of apoptotic mdDA neurons in the rostral (cCasp3$^+$/GFP$^+$ cells: E12.5 *Pitx3*$^{+/GFP}$, 0.506 ± 0.047%; E12.5 *Pitx3*$^{GFP/GFP}$, 1.676 ± 0.185%; $n = 3$, mean ± SEM; ***$p < 0.001$ in the independent samples *t* test), but not caudal (cCasp3$^+$/GFP$^+$ cells: E12.5 *Pitx3*$^{+/GFP}$, 0.902 ± 0.068%; *Pitx3*$^{GFP/GFP}$, 1.056 ± 0.042%, $n = 3$, mean ± SEM), VM of the *Pitx3*$^{GFP/GFP}$ 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 0.5× 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 10^2 to 10^7 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 independent experiments were analyzed in duplicate reactions for each cDNA, and all reactions were repeated more than twice.

Western blot analyses. Cells and brain tissues were processed as described 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).

Intrastriatal injections of GDNF. Adult male rats ($n = 5$) were anesthetized 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 CO_2 asphyxiation. For immunohistochemical analyses, animals were intracardially perfused with PBS followed by 4% paraformaldehyde (PFA), and brains were collected, postfixed 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 dissected 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 comparisons 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. 1*a*,*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. 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* transcription 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$^+$ cells within the VM cell population by 1.7- and 1.3-fold, respectively (Fig. 1*g–m*). Under these experimental conditions, GDNF treatment did not significantly increase the number of TH$^+$ neurons (Fig. 1*m*).

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. 1*n–u*). Pitx3 expression was particularly lost in ventromedial (Fig. 1*n*″,*q*″, 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 compensation by other GDNF receptors, such as GFRα1 and NCAM, and could explain the lack of an mdDA phenotype in postnatal *DAT-Ret*$^{flx//flx}$ 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. 2*a*), 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. 2*b–k*). To determine 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. 2*l*). 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 vitro*

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. 3*a*,*c*). These results were also confirmed at protein levels (Fig. 3*b*). 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. 3*d*,*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. 3*d*,*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. 3*e*). 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*-

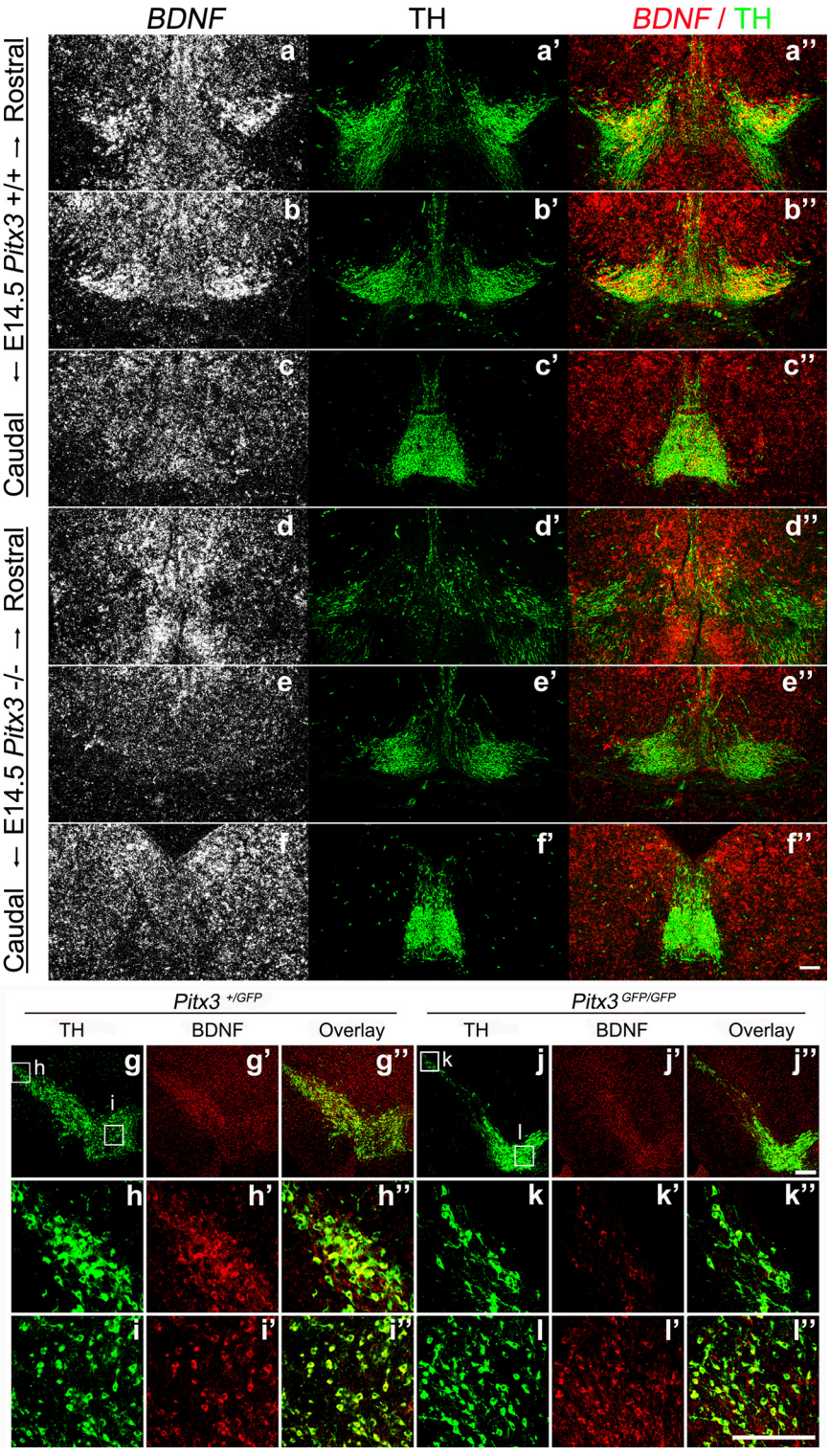

Figure 6. Lack of *BDNF* expression in an mdDA neuronal subpopulation that disappears at later embryonic/postnatal stages in the absence of *Pitx3*. (***a–l″***) Representative coronal midbrain sections from E14.5 (***a–f″***, different rostrocaudal levels) and P30 (***g–l″***) Pitx3 $^{+/GFP}$ (***a–c″***; ***g–i″***) and Pitx3 $^{GFP/GFP}$ (***d–f″***; ***j–l″***) mice hybridized with a *BDNF* riboprobe (***a–f***) or immunostained for TH (***a′–f′***, ***g–l***) and BDNF (***g′–l′***). Merged images are shown in ***a″–f″*** and ***g″–l″***. *BDNF* is expressed strongly in a rostrolateral TH $^{+}$ mdDA domain in the wild-type (*Pitx3*$^{+/GFP}$) embryos (***a–c″***; ***g–i″***), but this *BDNF* $^{+}$ domain was completely lost in the VM of *Pitx3* $^{GFP/GFP}$ embryos at E14.5 (***d***, ***d″***, ***e***, ***e″***,***f***, ***f″***), concomitant with a strong reduction at E14.5 (***d′–f′***) and followed by the almost complete loss at P30 (***j–l″***) of the rostrolateral (SNc) TH $^{+}$ mdDA neurons in these mutants. Scale bars: (in ***f″***, ***l″***, and ***l″***),100 μm.

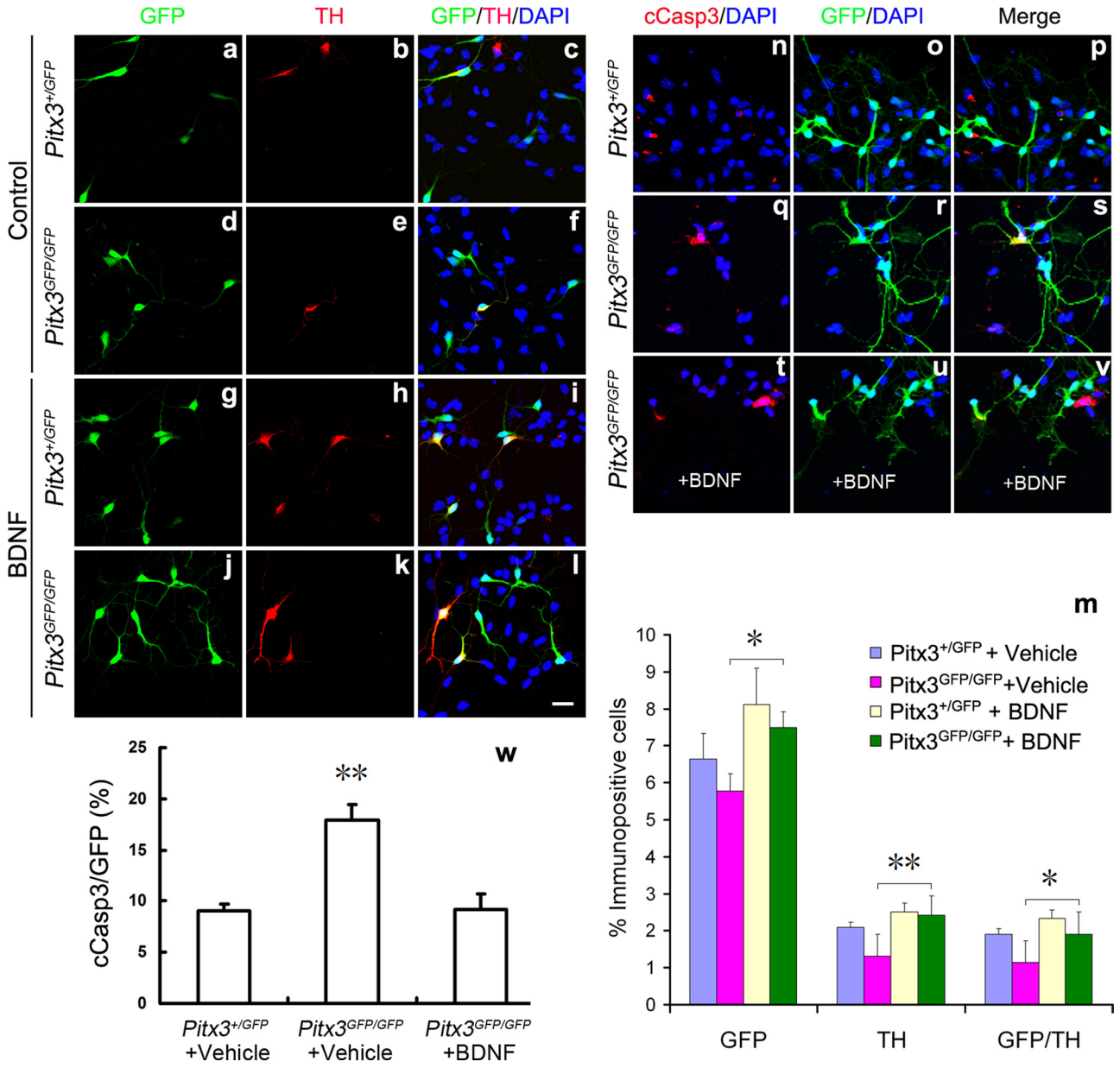

Figure 7. BDNF augments the survival of mdDA neurons in *Pitx3*$^{GFP/GFP}$ primary VM cultures. ***a–v***, Primary VM cultures derived from E11.5 *Pitx3*$^{+/GFP}$ (***a–c***, ***g–i***, ***n–p***) and *Pitx3*$^{GFP/GFP}$ (***d–f***, ***j–l***, ***q–v***) embryos were treated with vehicle (0.1% BSA) (***a–f***, ***n–s***) or 20 ng/ml BDNF (***g–l***, ***t–v***) for 4 d and immunostained for GFP (green in ***a***, ***d***, ***g***, ***j***, ***o***, ***r***, ***u***), TH (red in ***b***, ***e***, ***h***, ***k***), and cCasp3 (red in ***n***, ***q***, ***t***); merged images with DAPI stain in ***c***, ***f***, ***i***, ***l***, ***p***, ***s***, and ***v***. ***m***, Quantification of GFP and TH immunopositive cells in these four experimental groups revealed an increased number of GFP$^+$ and TH$^+$ mdDA neurons in the BDNF-treated relative to the untreated *Pitx3*$^{GFP/GFP}$ VM cultures (GFP$^+$ cells: *Pitx3*$^{+/GFP}$ untreated, 6.64 ± 0.69%; *Pitx3*$^{GFP/GFP}$ untreated, 5.78 ± 0.46%; *Pitx3*$^{+/GFP}$ BDNF-treated, 8.11 ± 0.99%; *Pitx3*$^{GFP/GFP}$ BDNF-treated, 7.49 ± 0.44%; TH$^+$ cells: *Pitx3*$^{+/GFP}$ untreated, 2.09 ± 0.14%; *Pitx3*$^{GFP/GFP}$ untreated, 1.31 ± 0.60%; *Pitx3*$^{+/GFP}$ BDNF-treated, 2.51 ± 0.24%; *Pitx3*$^{GFP/GFP}$ BDNF-treated, 2.42 ± 0.53%; TH$^+$/GFP$^+$ cells: *Pitx3*$^{+/GFP}$ untreated, 1.91 ± 0.15%; *Pitx3*$^{GFP/GFP}$ untreated, 1.14 ± 0.59%; *Pitx3*$^{+/GFP}$ BDNF-treated, 2.34 ± 0.22%; *Pitx3*$^{GFP/GFP}$ BDNF-treated, 1.90 ± 0.60%, mean ± SEM). ***w***, Quantification of the relative amount of apoptotic (cCasp3$^+$) mdDA neurons (GFP$^+$) in these cultures showed an increase of apoptotic cells in the untreated *Pitx3*$^{GFP/GFP}$ VM cultures as compared to the untreated controls that was rescued by the addition of BDNF protein to the mutant cultures (cCasp3$^+$/GFP$^+$ cells: *Pitx3*$^{+/GFP}$ untreated, 9.11 ± 0.55%; *Pitx3*$^{GFP/GFP}$ untreated, 17.92 ± 1.46%; *Pitx3*$^{GFP/GFP}$ BDNF-treated, 9.12 ± 1.63%). Paired *t* test was used for statistical analysis of treatment effects within the same genotype, and one-way ANOVA was used for statistical analysis of genotype differences for the same treatment. *$p < 0.05$; **$p < 0.005$. Data were derived from three independent experiments. Scale bar: (in ***l***), 20 μm.

null mutant (*Pitx3*$^{GFG/GFP}$) mice, which allow the fate mapping of Pitx3-expressing (GFP$^+$) 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*e*,*f*) (Smidt et al., 1997); at this or earlier stages, *BDNF* expression was not detected in the VM (Fig. 4*a–c* and data not shown). We found that *BDNF* was widely transcribed in the wild-type and *Pitx3*$^{+/GFP}$ VM at E12.5 and exhibited a particularly prominent expression domain in the rostrolateral and medial VM overlapping with GFP$^+$ (Pitx3$^+$) mdDA neurons (Fig. 4*e–g″* and data not shown). Remarkably, *BDNF* expression in this rostrolateral and medial domain was entirely lost in the *Pitx3*$^{GFP/GFP}$ embryos at E12.5, although the corresponding GFP$^+$ neurons were still present in this domain and despite the persistent expression of *BDNF* in the adjacent Pitx3-negative (GFP$^-$) VM tissue of the null mutants (Fig. 4 *h–j′*).

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. 4*k*). 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. 4*m*). 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. 4*l*,*m*), whereas mutation of both BS2 and BS9 resulted in a 47% decrease of *BDNF* promoter activity after Pitx3 cotransfection (Fig. 4*m*).

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

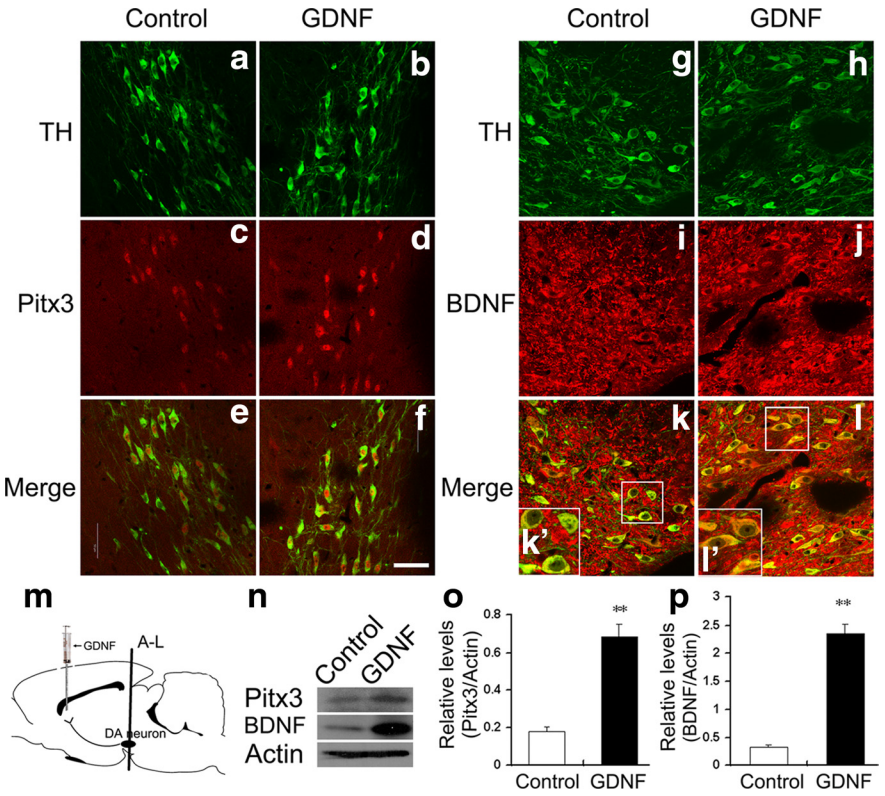

Figure 8. Intrastriatal injection of GDNF upregulates Pitx3 and BDNF expression in the adult SNc. ***a–l***, Representative coronal sections from the SNc of adult male rats who had received an unilateral injection of 0.9% saline (control, ***a***, ***c***, ***e***, ***g***, ***i***, ***k***) and 15 ng/μl GDNF into the contralateral side of the striatum (***b***, ***d***, ***f***, ***h***, ***j***, ***l***) 48 h earlier, immunostained for TH (***a***, ***b***, ***g***, ***h***), Pitx3 (***c***, ***d***) and BDNF (***i***, ***j***). Merged images are shown in ***e***, ***f***, ***k***, and ***l***. Insets in ***k′*** and ***l′*** are higher magnifications of the boxed areas in ***k*** and ***l***, respectively. ***m***, Schematic drawing of the striatal injection site and position of the VM sections shown in ***a–l***. ***n***, Western blots using protein extracts from vehicle-treated control and GDNF-treated contralateral side of the VM showed that unilateral intrastriatal injection of GDNF leads to a 2.8-fold increase of Pitx3 expression (quantification in ***o***) and 6.1-fold increase of BDNF expression (quantification in ***p***) in the ipsilateral mdDA neurons (control: Pitx3, 0.18 $\pm$ 0.02; BDNF, 0.33 $\pm$ 0.04, mean $\pm$ SEM; GDNF-treated (GDNF): Pitx3, 0.68 $\pm$ 0.07; BDNF, 2.35 $\pm$ 0.18; $^{**}p < 0.01$ in the independent samples *t* test for repeated measurements; $n = 3$). Scale bar: (in ***f***), 50 μm.

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 rostrolateral and medial VM domains of *Pitx3*$^{GFP/GFP}$ embryos at E14.5 (Fig. 6*a–f″*) and E16.5 (data not shown). Loss of *Pitx3* resulted in a notable decrease of TH$^+$ 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. 6 *g–l″*). Notably, *BDNF* transcription appeared to be very low or almost undetectable in the caudomedial mdDA domain throughout embryonic development (Figs. 4*e–g″*, 6*a–c″*), and this mdDA neuronal subset was less affected in the *Pitx3*$^{GFP/GFP}$-null mutants at embryonic and postnatal stages (Figs. 4*h–j″*, 5*e*, 6*d–f″*,*j–l″*; Maxwell et al., 2005). Collectively, our results indicate that Pitx3 directly activates the *BDNF* promoter in an mdDA neuron subpopulation located in the rostrolateral 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-null* mutant primary VM cultures

To further investigate whether the lack of *BDNF* expression contributes to the loss of mdDA neurons in *Pitx3*$^{GFP/GFP}$ 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*$^{GFP/GFP}$ embryos. We found a reduction of GFP$^+$ cell numbers (cells that would express Pitx3 in the wild type) by 13%, TH$^+$ cells by 37.5%, and GFP/TH double-positive cells by 40% in the untreated *Pitx3*$^{GFP/GFP}$ cultures as compared to untreated wild-type (*Pitx3*$^{+/GFP}$) cultures (Fig. 7*a–f*,*m*). This result is consistent with *in vivo* data from E12.5 *Pitx3*$^{GFP/GFP}$ mice showing a reduction of GFP$^+$ cells by 21%, TH$^+$ cells by 54%, and GFP/TH double-positive cells by 48% relative to the *Pitx3*$^{+/GFP}$ controls (Maxwell et al., 2005), and it confirms a selective loss of GFP$^+$ and TH$^+$ cells in the absence of *Pitx3* also in primary VM cultures. Notably, BDNF treatment of *Pitx3*$^{GFP/GFP}$ VM cultures increased the numbers of GFP$^+$ cells by 29.6%, TH$^+$ cells by 84.7%, and TH/GFP double-positive cells by 66.7% relative to the untreated *Pitx3*$^{GFP/GFP}$ cultures, thereby reaching similar numbers as in the BDNF-treated wild-type (*Pitx3*$^{+/GFP}$) cultures (Fig. 7*g–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$^+$) mdDA (GFP$^+$) neurons in the untreated and BDNF-treated VM cultures from wild-type (*Pitx3*$^{+/GFP}$) and mutant (*Pitx3*$^{GFP/GFP}$) embryos. As expected, this proportion was significantly increased in the untreated *Pitx3*$^{GFP/GFP}$ VM cultures as

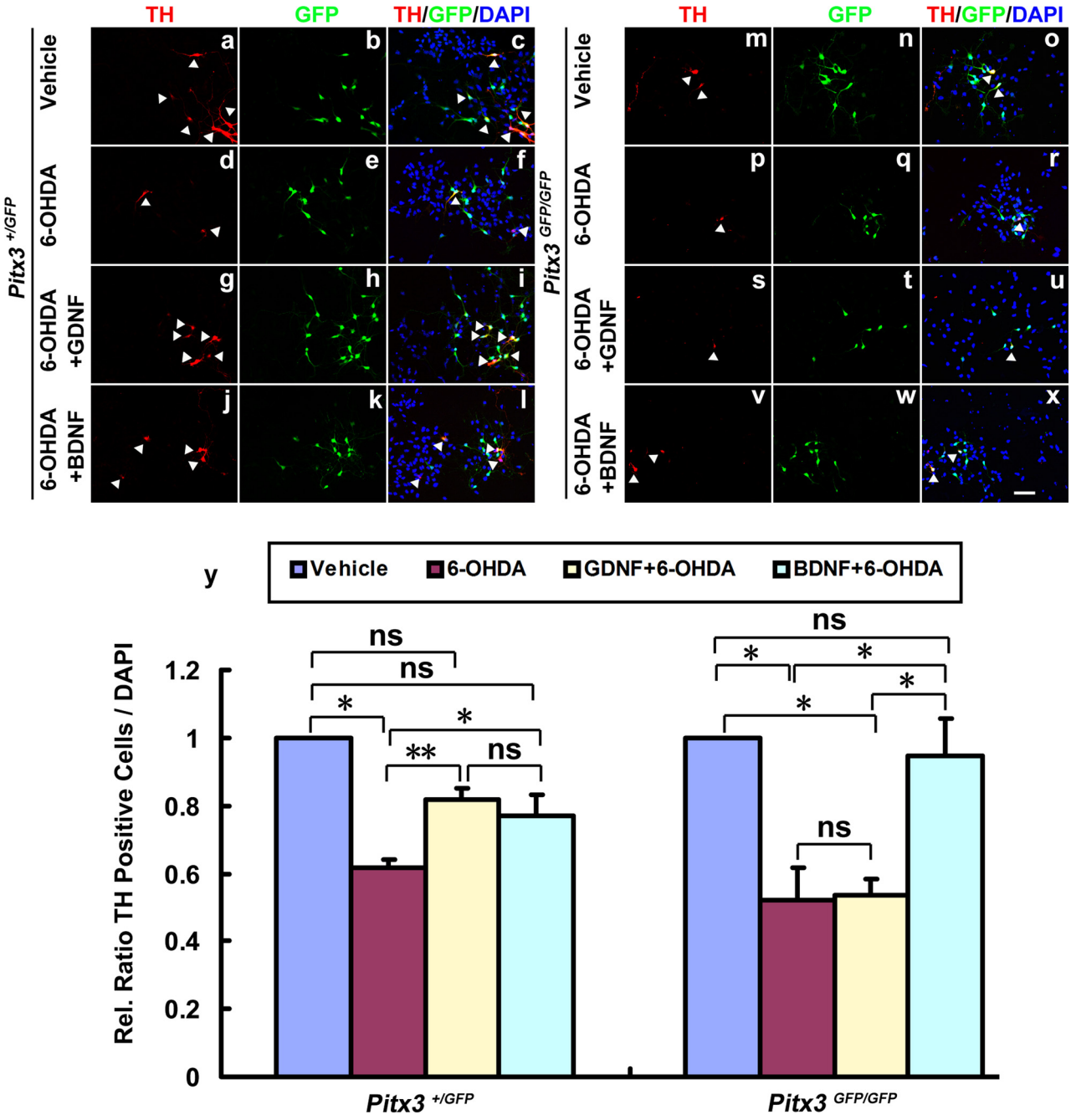

Figure 9. BDNF, but not GDNF, protects *Pitx3-null* mutant mdDA neurons against 6-OHDA neurotoxicity. ***a–x***, Primary VM cultures derived from E11.5 $Pitx3^{+/GFP}$ (***a–l***) and $Pitx3^{GFP/GFP}$ (***m–x***) embryos were treated after 3 DIV with vehicle (0.1% BSA) (***a–f***, ***m–r***), 20 ng/ml 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 TH (red in ***a***, ***d***, ***g***, ***j***, ***m***, ***p***, ***s***, and ***v***) and GFP (green in ***b***, ***e***, ***h***, ***k***, ***n***, ***q***, ***t***, and ***w***); merged images with DAPI stain are shown in ***c***, ***f***, ***i***, ***l***, ***o***, ***r***, ***u***, and ***x***. ***y***, Quantification of TH-immunopositive cells in these eight experimental groups revealed that in $Pitx3^{+/GFP}$ VM cultures both GDNF and BDNF protect TH^+ mdDA neurons against 6-OHDA toxicity, but in $Pitx3^{GFP/GFP}$ VM cultures only BDNF (and not GDNF) protects these neurons against 6-OHDA toxicity [$TH^+/DAPI^+$ cells (white arrowheads): $Pitx3^{+/GFP}$ vehicle-treated, normalized to 1; $Pitx3^{+/GFP}$ 6-OHDA-treated, 0.615 ± 0.025; $Pitx3^{+/GFP}$ 6-OHDA + GDNF-treated, 0.816 ± 0.037; $Pitx3^{+/GFP}$ 6-OHDA + BDNF-treated, 0.768 ± 0.066, mean ± SEM, $n = 3$; $Pitx3^{GFP/GFP}$ vehicle-treated, normalized to 1; $Pitx3^{GFP/GFP}$ 6-OHDA-treated, 0.521 ± 0.096; $Pitx3^{GFP/GFP}$ 6-OHDA + GDNF-treated, 0.533 ± 0.051; $Pitx3^{GFP/GFP}$ 6-OHDA + BDNF-treated, 0.946 ± 0.111, mean ± SEM, $n = 4$]. Paired *t* test was used for statistical analysis of treatment effects. *$p < 0.05$; **$p < 0.01$; ns, not significant. Data were derived from at least three independent experiments. Scale bar: (in ***x***), 50 μm.

compared to the untreated controls (Fig. 7*n–s*,*w*). Notably, the increased death of GFP^+ cells in the $Pitx3^{GFP/GFP}$ VM cultures was rescued back to control levels by the addition of BDNF to these cultures (Fig. 7*t–w*), indicating that in the absence of *Pitx3*, exogenous BDNF application prevents the cell death of cultured *Pitx3*-null ($Pitx3^{GFP/GFP}$) mdDA neurons.

Intrastriatal 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. 8*a–l*) and on Western blots (Fig. 8*n–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*$^{GFP/GFP}$) 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. 9*a–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. 9 *g–l,y*). Remarkably, the numbers of TH$^+$ mdDA neurons in the 6-OHDA-treated *Pitx3*$^{GFP/GFP}$ VM cultures were only rescued back to normal (untreated) levels after a previous incubation of these cells with BDNF, but not with GDNF (Fig. 9*s–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*$^{GFP/GFP}$) VM cultures by a reduction of neurotoxin-

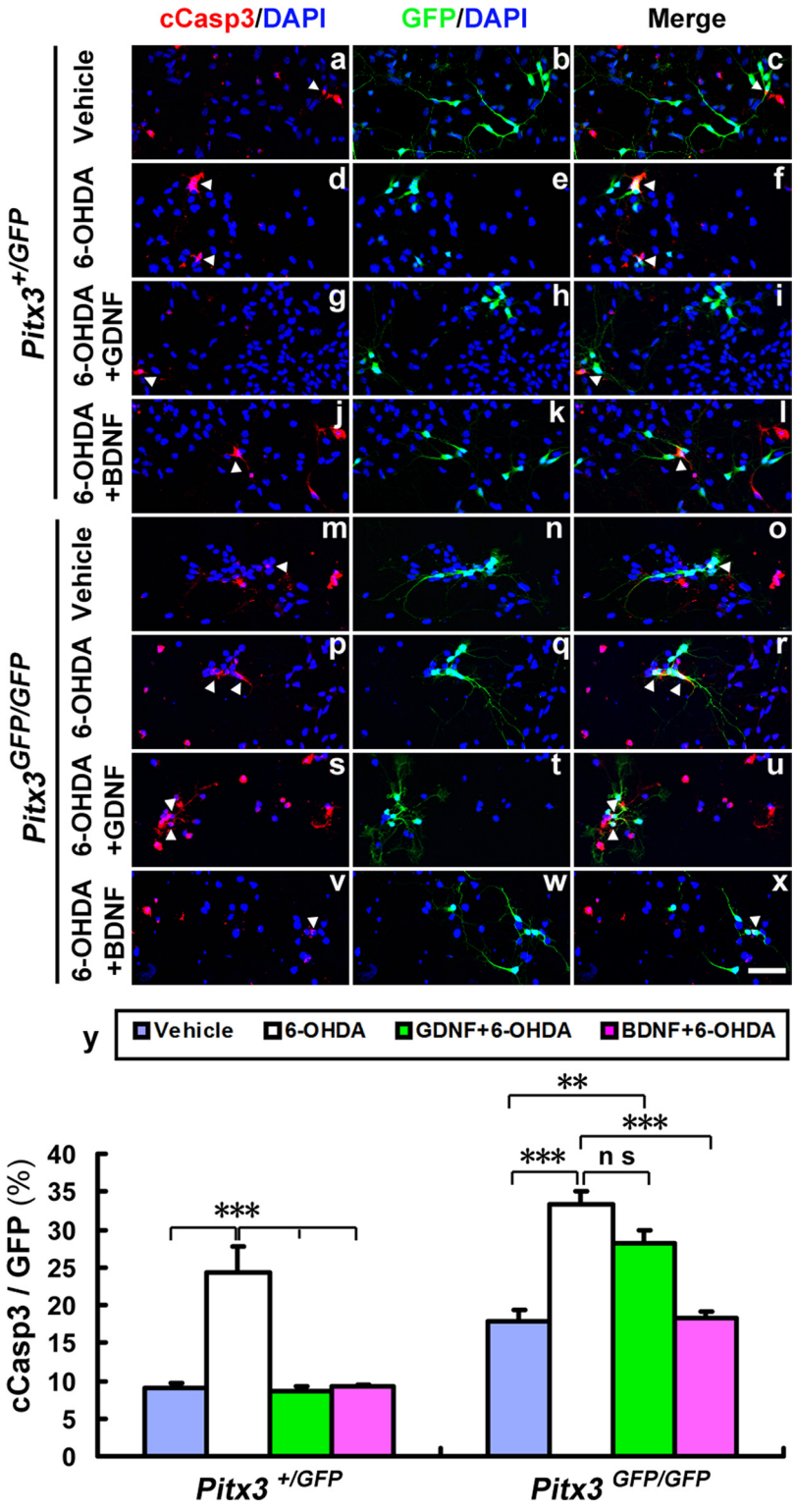

Figure 10. GDNF does not protect against 6-OHDA-induced apoptotic cell death of *Pitx3-null* mutant mdDA neurons. ***a–x***, Primary VM cultures derived from E11.5 *Pitx3*$^{+/GFP}$ (***a–l***) and *Pitx3*$^{GFP/GFP}$ (***m–x***) embryos were treated after 3 DIV with vehicle (0.1% BSA) (***a–f***, ***m–r***), 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 ***v***) 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***, ***u***, and ***x***. ***y***, Quantification of the relative amount of apoptotic (cCasp3$^+$) mdDA neurons (GFP$^+$) in these eight experimental groups revealed that both GDNF and BDNF prevent the 6-OHDA-induced cell death of *Pitx3*$^{+/GFP}$ mdDA neurons, but only BDNF (and not GDNF) prevents the 6-OHDA-induced cell death of *Pitx3*$^{GFP/GFP}$ mdDA neurons [cCasp3$^+$/GFP$^+$ cells (white arrowheads): *Pitx3*$^{+/GFP}$ vehicle-treated, 9.11 $\pm$ 0.55%; *Pitx3*$^{+/GFP}$ 6-OHDA-treated, 24.29 $\pm$ 3.49%; *Pitx3*$^{+/GFP}$ 6-OHDA + GDNF-treated, 8.70 $\pm$ 0.55%; *Pitx3*$^{+/GFP}$ 6-OHDA + BDNF-treated, 9.25 $\pm$ 0.11%; mean $\pm$ SEM, $n = 3$; *Pitx3*$^{GFP/GFP}$ vehicle-treated, 17.92 $\pm$ 1.46%; *Pitx3*$^{GFP/GFP}$ 6-OHDA-treated, 33.36 $\pm$ 1.74%; *Pitx3*$^{GFP/GFP}$ 6-OHDA + GDNF-treated, 28.11 $\pm$ 1.86%; *Pitx3*$^{GFP/GFP}$ 6-OHDA + BDNF-treated, 18.35 $\pm$ 0.78%, mean $\pm$ 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: (in ***x***), 50 μm.

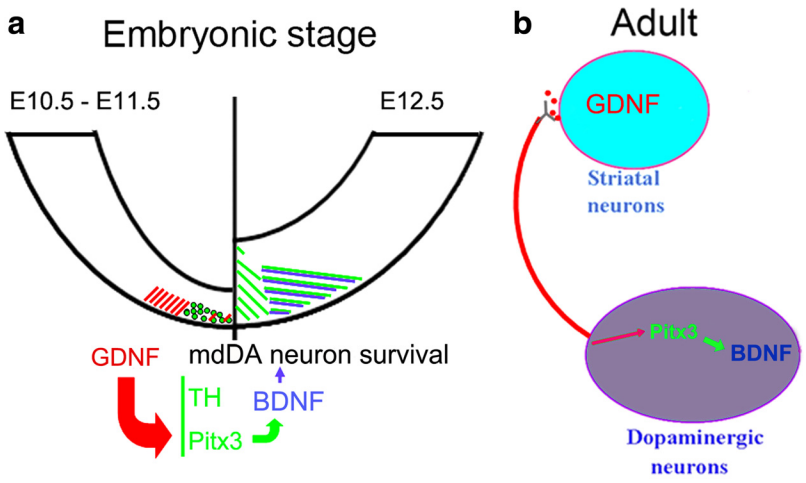

Figure 11. Feed forward regulation of GDNF, Pitx3, and BDNF expression in the embryonic and adult rodent brain. ***a***, During development, *GDNF* (red) is transiently expressed in the midbrain BP of the E10.5/E11.5 mouse embryo, where it is required for the NF-κB-mediated induction of *Pitx3* expression in an mdDA neuronal subset within the adjacent midbrain floor plate. Pitx3 (green) is in turn necessary for the activation of *BDNF* expression in a rostrolateral/medial mdDA neuronal subpopulation at E12.5. Neurotrophic support of this mdDA neuronal subset by BDNF (blue) might be required for their survival throughout development and their protection against neurotoxic insults. ***b***, In the adult brain, striatal uptake and retrograde axonal transport of GDNF (red) to the cell soma maintains the proper levels of Pitx3 (green) and BDNF (blue) expression in SNc mdDA neurons and might thereby contribute to the sustained survival and neuroprotection of these neurons throughout adulthood.

induced apoptotic cell death, we assessed the relative proportion of apoptotic (cCasp3$^+$) and GFP$^+$ 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. 10*a–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 *Pitx3*$^{+/GFP}$ and *Pitx3*$^{GFP/GFP}$ 6-OHDA-treated cultures (Fig. 10*j–l*,*v–x*,*y*), whereas GDNF only decreased these numbers significantly in *Pitx3*$^{+/GFP}$ but not in *Pitx3*$^{GFP/GFP}$ 6-OHDA-treated cultures (Fig. 10 g–*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 rostrolateral 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; Paratcha 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 intrastriatal or intranigral injections of GDNF protein during early postnatal stages, or the constitutive activation of the Ret receptor, result in increased numbers of TH$^+$ 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; Paratcha 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$^+$ 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 during subsequent prenatal and postnatal stages (Maxwell et al., 2005; Smidt and Burbach, 2007). *Aldh1a1* 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 rostrolateral and medial mdDA neuronal subpopulation that is most affected in *Pitx3*$^{-/-}$ 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*$^{GFP/GFP}$-null mutant embryos, we suggest that the failure to induce *BDNF* expression in rostrolateral and medial (SNc) mdDA precursors during development contributes to the preferential degeneration of these neurons in the *Pitx3*$^{GFP/GFP}$ mice. Interestingly, retinoid signaling via 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 *Aldh1a1* 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*$^{GFP/GFP}$-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 SNc 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*$^{GFP/GFP}$ 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 rostrolateral 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 intrastriatal 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 SNc 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 (Parsian 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$^+$ neurons in the SNc, but not VTA (Baquet et al., 2005), raise the intriguing possibility that the rostrolateral (SNc) 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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