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Mickael Decressac *et al.*

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Editor's Summary

NURRishing Dopamine Neurons with GDNF

Glial cell line–derived neurotrophic factor (GDNF) and its close relative neurturin are currently in clinical trials for neuroprotection in patients with Parkinson disease (PD). Although effective in classic neurotoxin animal models of this disease, GDNF has failed to afford protection in PD rodent models in which dopamine neurons are killed by α-synuclein toxicity. Using a rat model of α-synuclein–mediated PD, Decressac *et al.* now show that excess cellular concentrations of α-synuclein effectively block the trophic response of dopamine neurons to GDNF. They provide evidence that blockade of GDNF signaling is caused by reduced expression of the transcription factor Nurr1 and its downstream target, the GDNF receptor Ret. Deletion of Nurr1 resulted in reduced Ret expression, accompanied by a complete failure of dopamine neurons to respond to exogenously applied GDNF. However, when the investigators induced expression of Nurr1, Ret receptor expression was restored as well as the response to GDNF in the dopamine neurons subjected to α-synuclein toxicity. These results suggest that Nurr1 is a key player in the cellular defense against α-synuclein toxicity and highlight Nurr1 as a promising new target for neuroprotective therapy.

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PARKINSON'S DISEASE

α-Synuclein–Induced Down-Regulation of Nurr1 Disrupts GDNF Signaling in Nigral Dopamine Neurons

Mickael Decressac,[1]* Banafsheh Kadkhodaei,[2] Bengt Mattsson,[1] Ariadna Laguna,[2] Thomas Perlmann,[2,3] Anders Björklund[1]*

Glial cell line–derived neurotrophic factor (GDNF) and its close relative neurturin are currently in clinical trials for neuroprotection in patients with Parkinson disease (PD). However, in animal models of PD, GDNF fails to protect nigral dopamine (DA) neurons against α-synuclein–induced neurodegeneration. Using viral vector delivery of human wild-type α-synuclein to nigral DA neurons in rats, we show that the intracellular response to GDNF is blocked in DA neurons that overexpress α-synuclein. This block is accompanied by reduced expression of the transcription factor Nurr1 and its downstream target, the GDNF receptor Ret. We found that Ret expression was also reduced in nigral DA neurons in PD patients. Conditional knockout of Nurr1 in mice resulted in reduced Ret expression and blockade of the response to GDNF, whereas overexpression of Nurr1 restored signaling, providing protection of nigral DA neurons against α-synuclein toxicity. These results suggest that Nurr1 is a regulator of neurotrophic factor signaling and a key player in the cellular defense against α-synuclein toxicity.

INTRODUCTION

The pathogenic processes leading to the progressive loss of nigral dopamine (DA) neurons and aggregation of α-synuclein in Parkinson disease (PD) are poorly understood. Current clinical interventions provide symptomatic relief, but none of the neuroprotective or neuroregenerative strategies successfully tested in preclinical models of PD have so far provided therapeutic benefits in clinical trials (*1*). Glial cell line–derived neurotrophic factor (GDNF) and its close relative neurturin, in particular, have been shown to provide substantial anatomical and functional rescue of the nigrostriatal pathway in both rodent and primate models of PD in which the nigrostriatal DA neurons were killed by the toxins 6-hydroxydopamine (6-OHDA) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (*2*–*6*). Despite promising preclinical data, however, it remains unclear whether GDNF or neurturin can provide neuroprotection or restorative efficacy in PD patients (*7*–*11*).

The development of disease-modifying drugs for PD is impeded by the lack of animal models that faithfully mimic the human disease. The standard toxin-based models of PD reproduce the canonical loss of midbrain DA neurons but fail to replicate the progressive, time-dependent nature of the degenerative process and the development of α-synucleinopathy (*12*). Over the last decade, our insights into the pathogenic mechanisms of PD have increased. Mechanisms related to free radical stress and mitochondrial damage play a role, but they constitute only part of the intricate neuropathological cascade leading to neuronal dysfunction and cell death. In particular, considerable progress has been made on the involvement of α-synuclein in PD pathogenesis (*13*, *14*). Thus, increased cellular concentrations of native α-synuclein, or formation of toxic forms of the protein, have been shown to induce progressive degenerative changes in the affected DA neurons. This is revealed at early stages as axonal damage and synaptic dysfunction and later is followed by degeneration and loss of nigral DA neurons (*15*–*17*). Because of the broad impact of α-synuclein toxicity that extends beyond free radical damage and mitochondrial dysfunction modeled in the 6-OHDA and MPTP toxin models of PD, we and others have reexamined the neuroprotective effects of GDNF in the more recently introduced α-synuclein overexpression models of PD. We have found that in these α-synuclein overexpression models, GDNF indeed fails to protect nigral DA neurons against α-synuclein–induced toxicity (*18*, *19*).

The marked discrepancy in neuroprotective potency of GDNF in neurotoxin and α-synuclein models of PD raises the possibility that increased cellular concentrations of α-synuclein may interfere with GDNF signaling, rendering nigral DA neurons desensitized to its trophic effect. Here, we have used targeted delivery of an α-synuclein expressing adeno-associated viral (AAV) vector to overexpress human wild-type α-synuclein in nigral DA neurons in adult rats. We show that the intracellular signaling response induced by GDNF is almost completely blocked in the α-synuclein–affected DA neurons. Markedly, this blockade was accompanied by reduced expression of the transcription factor Nurr1 and its downstream regulated targets, including the GDNF receptor Ret. Down-regulation of Nurr1, induced by α-synuclein, affected not only the expression of components of the DA synthesis and release machinery but also the ability of the nigral DA neurons to respond to GDNF through regulation of Ret expression. We show that this blockade of the GDNF response is effectively reversed by overexpression of Nurr1 in the affected cells and that increased expression of Nurr1 can provide near-complete protection of nigral DA neurons against α-synuclein toxicity, also in the absence of exogenously administered GDNF.

RESULTS

α-Synuclein impairs the response of rat nigral DA neurons to GDNF

We used a recombinant AAV vector to overexpress human wild-type α-synuclein in nigral DA neurons of rat brain. We injected 3 μl of the vector unilaterally into the substantia nigra of adult rats. Control rats received a similar injection of an AAV vector expressing a gene encoding green fluorescent protein (GFP) instead of the gene encoding

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human α-synuclein. α-Synuclein was expressed at a level sufficient to induce progressive degeneration of DA neurons and time-dependent DA neuron loss (*16*). First, we assessed the trophic response induced by GDNF in midbrain DA neurons overexpressing either α-synuclein or GFP. GDNF was given as a single 1-μg injection into the striatum 2 weeks after vector injection, that is, at a time when α-synuclein (and GFP) was expressed but before any cell loss had occurred (*16*); animals were sacrificed 2 days after GDNF injection. The induced trophic response was assessed by monitoring the expression of the phosphorylated ribosomal protein S6 by immunohistochemistry. Phosphorylation of ribosomal protein S6 is an indicator of the upstream activation of the neuroprotective Akt/mTOR (mammalian target of rapamycin) pathway. In the AAV-GFP–injected control rats, injection of GDNF into the striatum elicited a distinct trophic response specifically confined to the nigral DA neurons, identified by their expression of tyrosine hydroxylase (TH), an enzyme necessary for the production of DA. In Fig. 1A and fig. S1, GDNF induced intense immunoreactivity of phosphorylated ribosomal protein S6 selectively in the TH-positive DA neurons on the side ipsilateral to the injection. Consistent with the well-established neuroprotective effect of GDNF in the standard toxin-based models of PD, a similar response was observed in rats that received an injection of 6-OHDA into the medial forebrain bundle (MFB) 2 days before the striatal injection of GDNF (Fig. 1A and fig. S1). By contrast, 2 weeks after intranigral injection of AAV–α-synuclein, the transduced DA neurons failed to elicit such activation (Fig. 1A and fig. S1). We confirmed that the absence of phosphorylated ribosomal protein S6 staining did not result from loss of nigral DA neurons because 2 weeks after AAV–α-synuclein injection, most nigral neurons expressed the transgene, but no significant neuronal or terminal loss was observed at this time point [fig. S2; see also (*16*)].

Previous studies suggest that α-synucleinopathy may result in blockade of axonal transport (*15*, *20*). To circumvent this possible defect, we performed a similar experiment where GDNF (1 μg) was delivered into the substantia nigra. Whereas nigral DA neurons overexpressing GFP showed a clear response to GDNF, phosphorylated ribosomal protein S6 immunoreactivity was markedly reduced in neurons overexpressing α-synuclein (bottom row in Fig. 1A).

To further investigate this lack of response, we analyzed the activation of several proteins involved in the GDNF signaling cascade including Ret, Akt, extracellular signal–regulated kinase 1/2 (ERK1/2), S6, and Bad by Western blot. Consistent with our histological observation, we found that GDNF triggered activation of these effectors in intact, GFP-overexpressing, 6-OHDA–treated neurons, as seen by increased concentrations of phosphorylated Ret, Akt, ERK, S6, and Bad, suggesting a prosurvival effect of the neurotrophic factor (Fig. 1, B and C). By contrast, striatal injection of GDNF in α-synuclein–overexpressing rats failed to activate the Akt and mitogen-activated protein kinase (MAPK) pathways, although Ret was phosphorylated to the same magnitude as in the other conditions, suggesting a blockade of the pathway downstream of the receptor (Fig. 1C). Recent studies suggest that GDNF induces the expression of the transcription factors Nurr1 and Pitx3 (*21*, *22*). Here, using quantitative real-time polymerase chain reaction (qPCR) analysis, we show that intrastriatal injection of GDNF induced increases in the concentrations of both Nurr1 (2.3 ± 0.3–fold increase) and Pitx3 (2.4 ± 0.3–fold increase) mRNAs, as well as those of their transcriptional targets TH (2.0 ± 0.2–fold), vesicular monoamine transporter 2 (VMAT-2) (1.8 ± 0.2–fold), DA transporter (DAT) (1.7 ± 0.1–fold), aromatic L-amino acid decarboxylase (AADC) (1.4 ± 0.1–fold), and Ret (1.9 ± 0.2–fold) relative to the intact side (all $P < 0.05$) (open bar in Fig. 1D). Brain-derived neurotrophic factor (BDNF) mRNA was also increased (1.8 ± 0.2–fold), whereas expression of GFRα1 (GDNF family receptor α1), NeuN, and Girk-2 transcripts remained unchanged ($P > 0.05$). By contrast, GDNF failed to trigger changes in expression of these targets in α-synuclein–overexpressing animals (all $P < 0.05$ compared to the GFP group) (black bar in Fig. 1D). The expression of the genes is reduced below that seen on the control side, which is due to the effect of α-synuclein overexpression (see below). Similarly, histological analysis revealed that induction of phosphorylated CREB (adenosine 3′,5′-monophosphate response element–binding protein), a transcription factor involved in Nurr1-mediated neuroprotection (*23*), was absent 2 days after intrastriatal injection of GDNF in AAV–α-synuclein–injected animals compared to the GFP group (Fig. 1E).

Enzyme-linked immunosorbent assay (ELISA) measurements revealed that the expression of endogenous GDNF was significantly elevated in the substantia nigra (183 ± 18%) and striatum (161 ± 15%) (all $P < 0.01$) at 2 weeks after AAV–α-synuclein injection compared to the GFP group (Fig. 1F). Therefore, we looked at whether endogenous GDNF was able to trigger any related intracellular pathways in the nigral DA neurons. We found that Ret was not activated (119 ± 14%), and the concentrations of phosphorylated Akt (p-Akt; 61 ± 6%) and phosphorylated S6 (p-S6; 33 ± 9%) were significantly reduced ($P < 0.01$) in α-synuclein–transduced animals not challenged with exogenous GDNF. This suggested that the α-synuclein–overexpressing nigral neurons were not able to respond to either exogenous or endogenous GDNF (Fig. 1G).

To ascertain that the lack of response to GDNF was not due to a misleading observation at a single time point (48 hours after GDNF injection in Fig. 1), we performed a time-course study and examined the activation of the same markers, as above, after 6, 24, and 72 hours. The same defect in the response to GDNF was apparent in the α-synuclein–overexpressing nigral neurons at all time points, as assessed by histological and Western blot analyses (Fig. 2, A and B). The immunostaining against human GDNF showed that the injection of the recombinant peptide (1 μg) covered the entire striatal region (fig. S1). Thus, we speculated that the activation of nigral DA neurons might be dose-dependent. Therefore, we performed an additional experiment where GDNF was delivered at a 10-fold higher dose (10 μg). Likewise, at this very high dose, the response of α-synuclein–transduced neurons to GDNF was almost completely blocked compared to control AAV-GFP and 6-OHDA–injected rats (Fig. 2, C and D).

Finally, we measured the expression of human GDNF by ELISA in the rat midbrain 48 hours after injection (10 μg) and found that the amount of protein transported back to the substantia nigra was significantly reduced in the α-synuclein–transduced animals (1.0 ± 0.3 pg/mg) compared to the AAV-GFP group (3.6 ± 0.6 pg/mg) ($P < 0.01$) (Fig. 2E). Histological analysis further confirmed this finding, as abundant punctate GDNF staining was observed in the soma of nigral neurons overexpressing GFP 6 hours after injection of GDNF into the striatum, whereas very few cells contained human GDNF in the AAV–α-synuclein group (Fig. 2F).

GDNF signaling cascade is impaired in nigral DA neurons overexpressing α-synuclein

Next, we performed qPCR analysis to assess the impact of α-synuclein on components of the GDNF signaling cascade and the DA synthesis and release machinery. Overexpression of human α-synuclein induced down-regulation of genes involved in DA neurotransmission, including

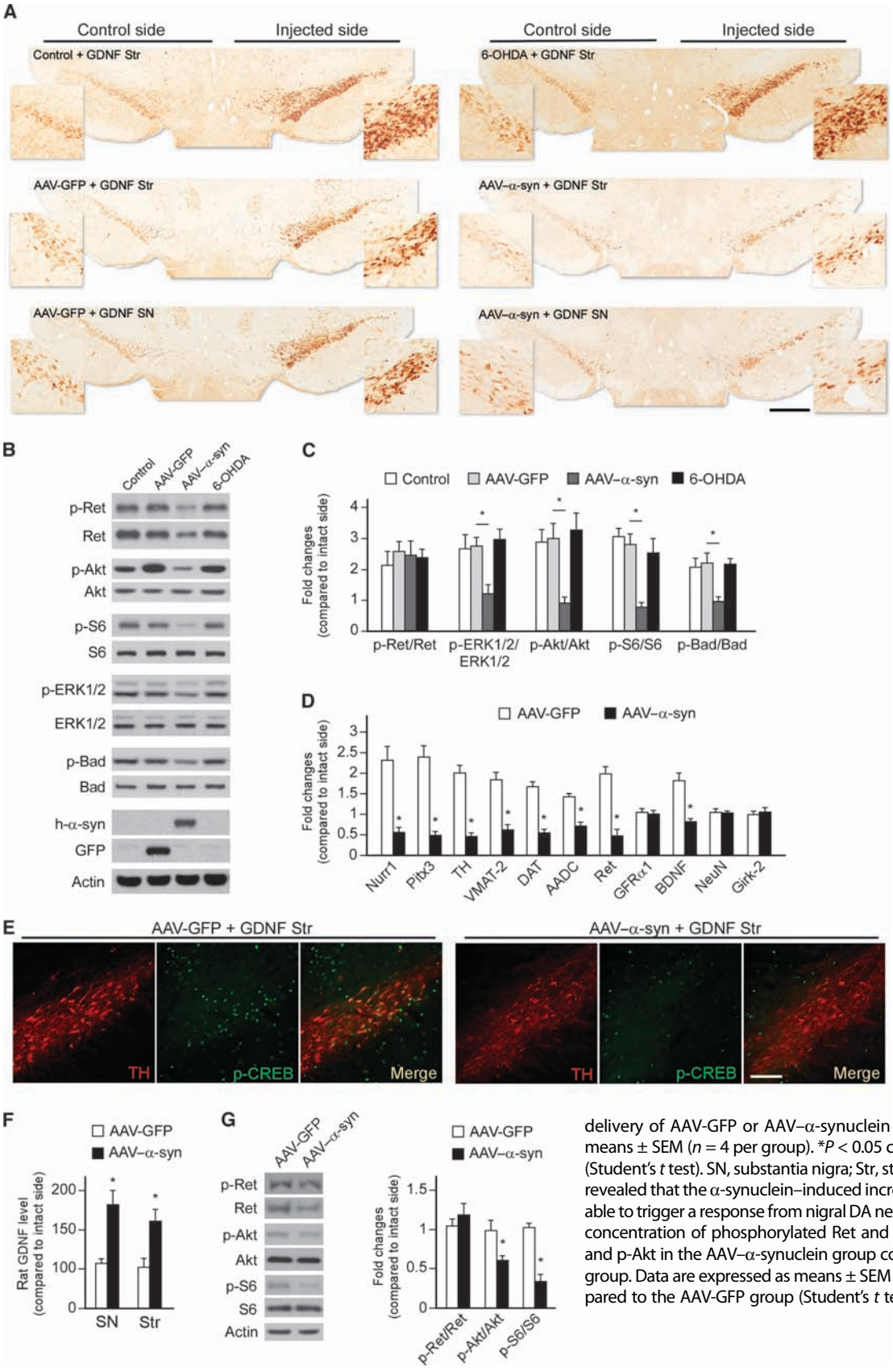

Fig. 1. α-Synuclein suppresses the response to GDNF. (**A**) Immunostaining of phosphorylated ribosomal protein S6 in the midbrain of rats 2 days after unilateral (right side) intrastriatal or intranigral injection of human recombinant GDNF (1 μg/3 μl). Control (top left), 6-OHDA–injected (top right), GFP-overexpressing (middle and bottom left), or α-synuclein (α-syn)–overexpressing (middle and bottom right) midbrain neurons. Scale bar, 600 μm. SN, injection in the substantia nigra; Str, injection in the striatum. (**B** and **C**) Western blot of Ret, ERK1/2, Akt, S6, and Bad activation in control, 6-OHDA–treated, GFP-overexpressing, or α-synuclein–overexpressing rat midbrain 48 hours after intrastriatal injection of GDNF. Data are expressed as means ± SEM ($n = 5$ per group). *$P < 0.05$ compared to the AAV-GFP group (Student's t test). (**D**) qPCR analysis showing mRNA expression of human α-synuclein, Nurr1, Pitx3, TH, VMAT-2, DAT, AADC, Ret, GFRα1, BDNF, NeuN, and Girk-2 in rat midbrain overexpressing GFP or α-synuclein 2 days after intrastriatal injection of GDNF. *$P < 0.05$ compared to the AAV-GFP group (Student's t test). (**E**) Double immunofluorescence staining showing the activation of phosphorylated CREB in nigral neurons stimulated 48 hours previously by intrastriatal injection of GDNF. By contrast, α-synuclein–overexpressing neurons failed to elicit a similar response on challenge with GDNF. Scale bar, 50 μm. (**F**) ELISA measurements of endogenous rat GDNF concentrations from midbrain and striatal tissue 2 weeks after intranigral delivery of AAV-GFP or AAV–α-synuclein vectors. Data are expressed as means ± SEM ($n = 4$ per group). *$P < 0.05$ compared to the AAV-GFP group (Student's t test). SN, substantia nigra; Str, striatum. (**G**) Western blot analysis revealed that the α-synuclein–induced increase of endogenous GDNF is unable to trigger a response from nigral DA neurons, as seen by the unchanged concentration of phosphorylated Ret and the reduced expression of p-S6 and p-Akt in the AAV–α-synuclein group compared to the AAV-GFP control group. Data are expressed as means ± SEM ($n = 5$ per group). *$P < 0.05$ compared to the AAV-GFP group (Student's t test).

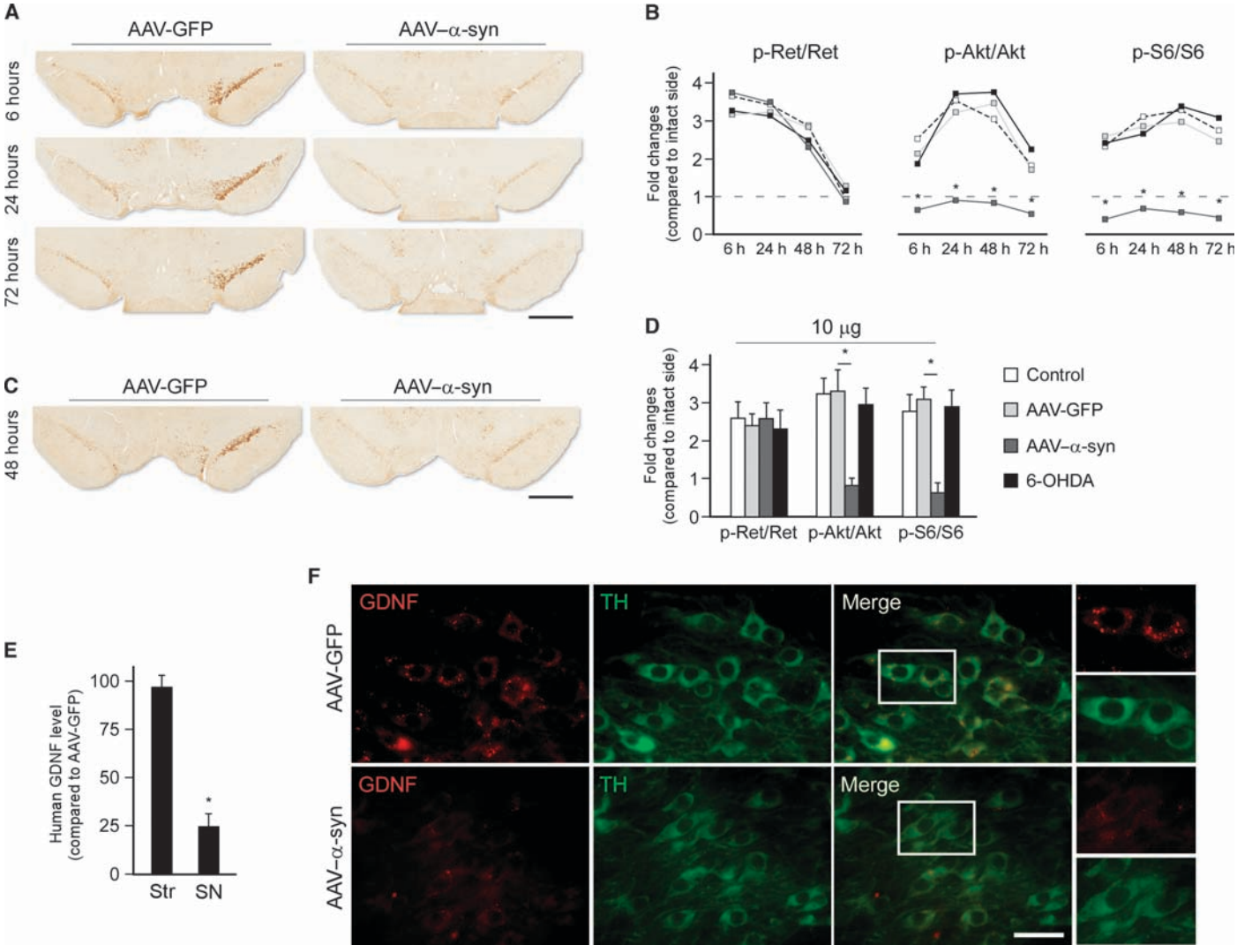

Fig. 2. Impairment of the response to GDNF is not time- or dose-dependent. (**A**) Immunostaining of p-S6 in the midbrain of rats overexpressing GFP as control or α-synuclein 6, 24, and 72 hours after unilateral (right side) intrastriatal injection of human GDNF (1 μg/3 μl). Scale bar, 800 μm. (**B**) Phosphorylated Ret, Akt, and S6 proteins in the midbrain were quantified from Western blot experiments. The plots represent mean values (n = 5 per group at each time point). *P < 0.05 compared to the AAV-GFP group (Student's t test). (**C**) Immunostaining of p-S6 in the midbrain of rats overexpressing GFP as control or α-synuclein 48 hours after intrastriatal injection (right side) of a higher dose of human GDNF (10 μg/3 μl). Scale bar, 800 μm. (**D**) Quantification of Western blots of phosphorylated Ret, Akt, and S6 in control, 6-OHDA–injected, GFP-overexpressing, or α-synuclein–overexpressing rats 48 hours after intrastriatal injection of a high dose of GDNF (10 μg). Data are expressed as means ± SEM (n = 5 per group). *P < 0.05 compared to the AAV-GFP group (Student's t test). (**E**) Human GDNF measured by ELISA in striatal and midbrain tissues from rats overexpressing GFP or α-synuclein 6 hours after intrastriatal injection of 10 μg of human GDNF. Data are expressed as percentage of GFP control group and presented as means ± SEM (n = 4 per group). *P < 0.05 compared to the AAV-GFP group (Student's t test). (**F**) Double immunofluorescence staining showing the retrograde transport of human GDNF 6 hours after intrastriatal injection in control rats overexpressing GFP (top). Reduced GDNF immunoreactivity (red) is observed in the TH-positive nigral neurons (green) of rats overexpressing α-synuclein (bottom). Scale bar, 20 μm.

the dopaminergic markers TH (43 ± 9%), VMAT-2 (40 ± 12%), DAT (58 ± 11%), and AADC (36 ± 11%) and the transcription factors Nurr1 (35 ± 10%), Pitx3 (48 ± 8%), and MEF2D (63 ± 8%; all P < 0.01) (Fig. 3A). Notably, we found a marked reduction in the expression of the GDNF receptor Ret (33 ± 6%; P < 0.001), whereas the expression of GFRα1 was unaltered (96 ± 8%) (Fig. 3A). A significant reduction in the expression of endogenous rat α-synuclein (55 ± 12% of control), BDNF (65 ± 7%), and the PD-related gene DJ-1 (64 ± 9%) was observed (all P < 0.01) (Fig. 3A). We also examined the expression of microRNAs (miRs) and found reduced expression of miR-21 (64 ± 10%) and miR-22 (47 ± 9%), regulators of phosphatase and tensin homolog (PTEN) (*24*, *25*), as well as of miR-133b (55 ± 11%), regulator of Pitx3 (*26*) (all P < 0.01) (Fig. 3A). By contrast, concentrations of miR-7 (170 ± 15%) and miR-153 (180 ± 19%), regulators of α-synuclein (*27*, *28*), were

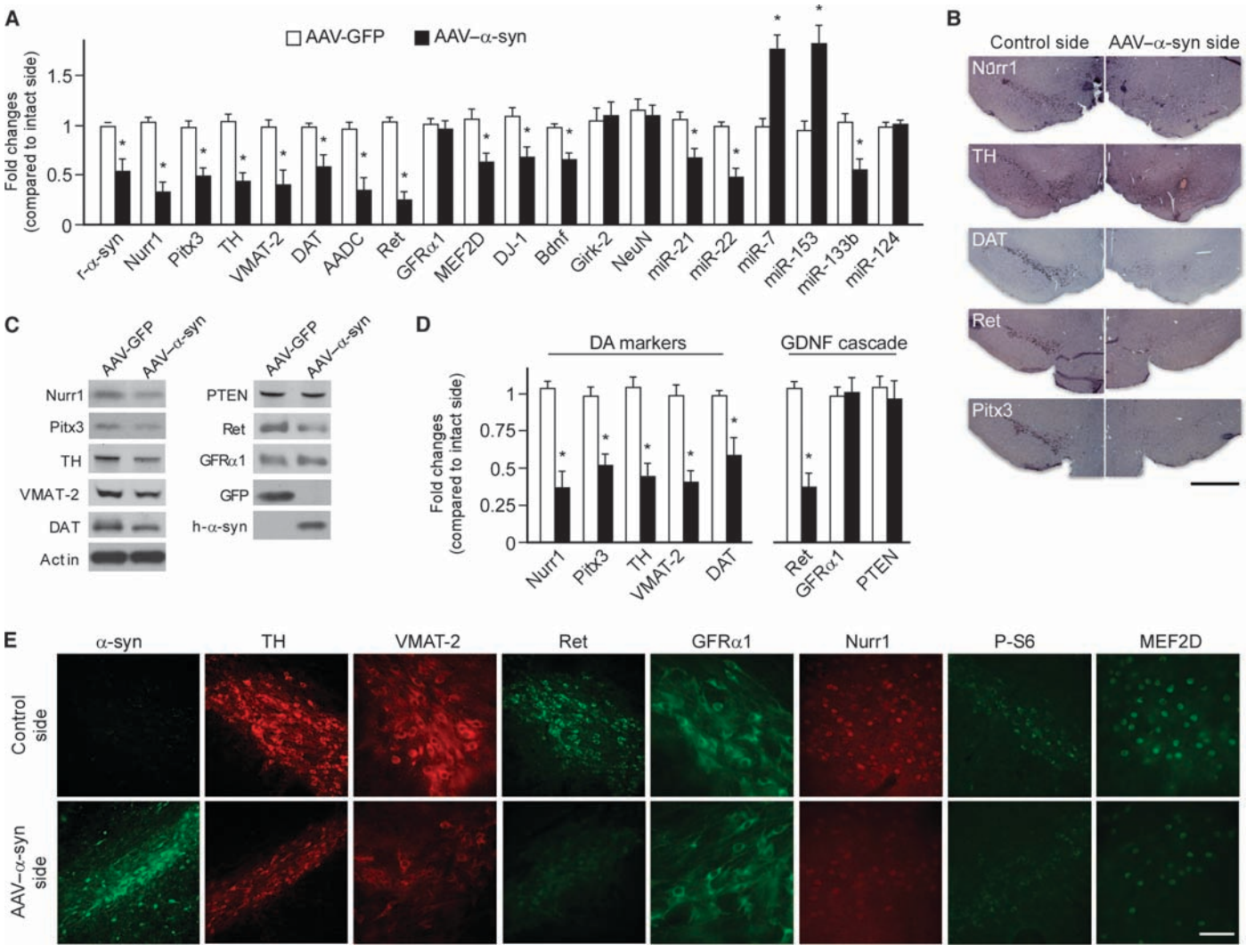

Fig. 3. α-Synuclein overexpression represses DA synthesis and release. (**A**) qPCR analysis of human and rat α-synuclein, Nurr1, Pitx3, TH, VMAT-2, DAT, AADC, Ret, GFRα1, MEF2D, DJ-1, BDNF, Girk-2, and NeuN mRNAs, and miR-21, -22, -7, -153, -133b, and -124, in the rat midbrain 2 weeks after injection of AAV-GFP or AAV–α-synuclein vectors. Data are expressed as means ± SEM ($n = 5$ per group). *$P < 0.05$ compared to the AAV-GFP group (Student's t test). (**B**) In situ hybridization of TH, Nurr1, Pitx3, DAT, and Ret mRNAs in rat midbrain sections 2 weeks after intranigral injection of AAV–α-synuclein vector (right side). Scale bar, 1 mm. (**C** and **D**) Concentrations of Nurr1, Pitx3, TH, VMAT-2, DAT, PTEN, Ret, and GFRα1 in midbrain from GFP or α-synuclein–overexpressing animals were analyzed by Western blot. Data are expressed as means ± SEM ($n = 5$ per group) *$P < 0.05$ compared to the AAV-GFP group (Student's t test). (**E**) Immunofluorescence images illustrating the reduced expression of TH, VMAT-2, Ret, Nurr1, p-S6, and MEF2D in nigral DA neurons overexpressing human α-synuclein (bottom row) compared to the noninjected control side (top row); expression of GFRα1 and NeuN were unaffected. Scale bar, 60 μm for α-synuclein, TH, Ret, Nurr1, and p-S6 and 30 μm for VMAT-2, GFRα1, and MEF2D.

significantly increased, which is consistent with the reduced expression of their target (all $P > 0.001$) (Fig. 3A). No changes in the expression of NeuN (108 ± 10%), Girk-2 (109 ± 12%), and miR-124 (102 ± 4%) were observed in the AAV–α-synuclein group compared to the AAV-GFP groups (Fig. 3A). Reduction in mRNA concentrations of Nurr1, Pitx3, TH, DAT, and Ret was confirmed by in situ hybridization 2 weeks after AAV–α-synuclein injection (Fig. 3B) and was also observed at the protein level, as assessed by Western blot analysis (Fig. 3, C and D) and immunohistochemistry (Fig. 3E).

As previously reported (*29*), examination of human postmortem midbrain sections from six PD patients revealed that TH immunoreactivity was markedly reduced in nigral DA neurons containing α-synuclein–positive inclusions compared to DA neurons without Lewy bodies or neurons from control specimens (Fig. 4, top panel). This supports the idea that this down-regulation might be a pathological event characteristic of PD and not just a restricted observation in our animal model. We also found that Ret immunoreactivity was reduced in nigral DA neurons from PD brains compared to age-matched healthy controls (Fig. 4, bottom panel).

Sections stained for α-synuclein phosphorylated on serine 129 (Ser^{129}) showed prominent nuclear localization of α-synuclein in many DA neurons (fig. S3A). Previous studies have suggested that nuclear

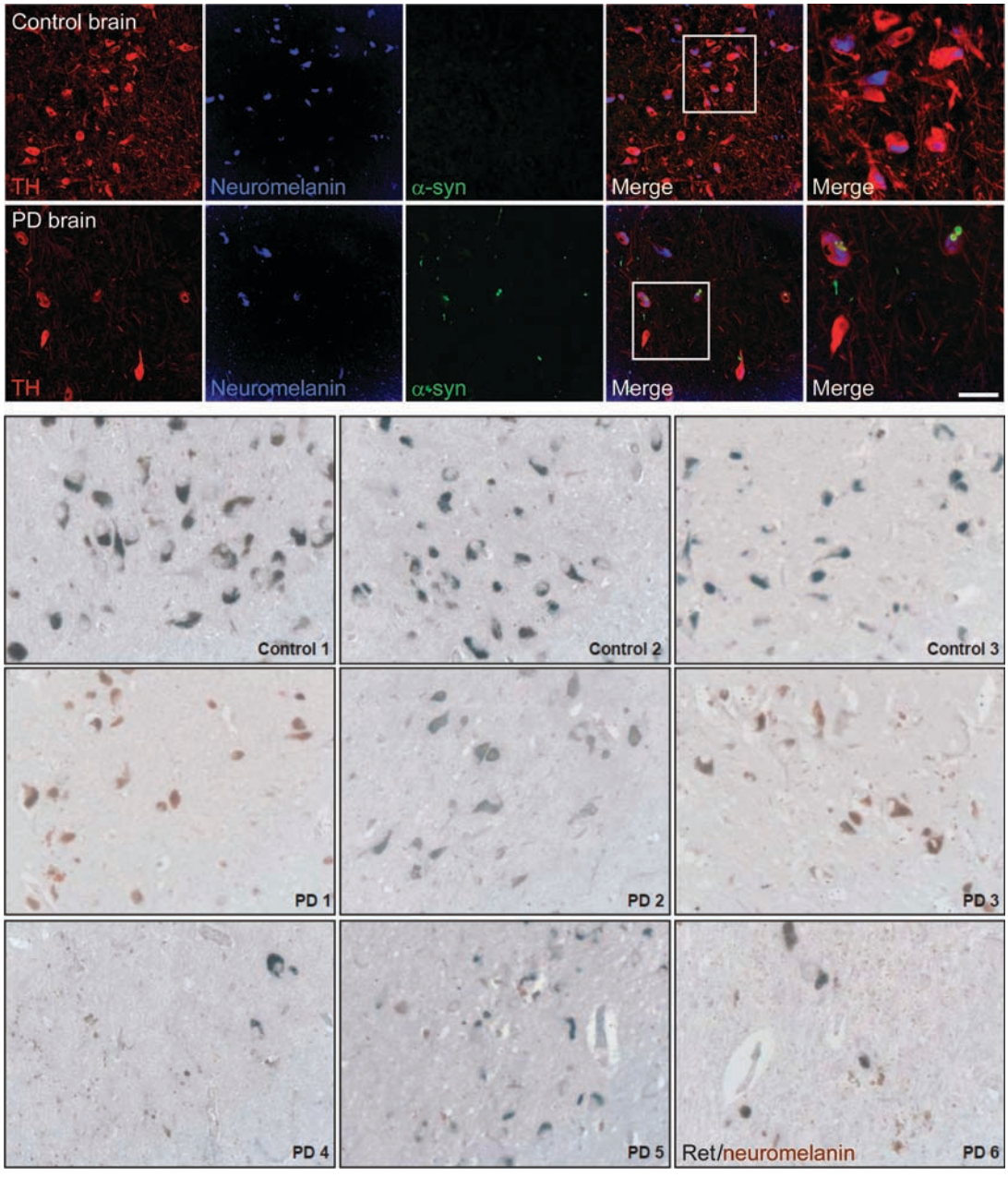

Fig. 4. Down-regulation of TH and Ret in human PD brain. (Top) Immunofluorescence staining of human control and PD postmortem midbrain tissue showing the reduced expression of TH (red) in nigral neurons containing α-synuclein–positive inclusions (green). Nigral neurons were identified by the presence of neuromelanin using light microscopy (pseudocolor, blue). (Bottom) Immunostaining of Ret (gray/blue) in three human control and six PD midbrain sections illustrating the reduced expression of the GDNF receptor Ret in nigral DA neurons identified by neuromelanin pigment (brown). Scale bar, 20 μm.

translocation of α-synuclein, which is facilitated by phosphorylation at Ser^{129}, may be detrimental for DA neurons and may have an impact on the transcriptional machinery (*30*–*34*). Marked reduction in the concentration of the dopaminergic marker VMAT-2 and the survival-related transcription factor MEF2D (*35*) was specifically observed in nigral neurons with strong immunoreactivity for phosphorylated α-synuclein in the nucleus (fig. S3, A and B). Co-immunoprecipitation experiments revealed that α-synuclein interacts with histone H3 in the nucleus (fig. S3D) and reduces its acetylation in α-synuclein–overexpressing animals (fig. S3C), suggesting a negative effect on transcriptional activity. Consistent with a previous study performed in α-synuclein transgenic mice (*36*), we found that the expression of the histone acetyltransferase p300 was lower in rats overexpressing α-synuclein compared to controls (fig. S3E). In addition, expression of sirtuin-2, a histone deacetylase known to be abundant in the aging brain and whose inhibition was shown to protect against α-synuclein–induced toxicity (*37*, *38*), was higher in the AAV–α-synuclein–transduced midbrain compared to that of control rats (fig. S3E). Given that sirtuin-2 is known to deacetylate tubulin, it may affect axonal transport. Western blot analysis showed reduced concentrations of acetyl-α-tubulin, which is in line with the defect of GDNF transport described above (fig. S3E).

Nurr1 is a key regulator of the GDNF signaling cascade

The prominent α-synuclein–induced down-regulation of Nurr1 and its target, the GDNF receptor Ret, suggested a potential role for Nurr1 in

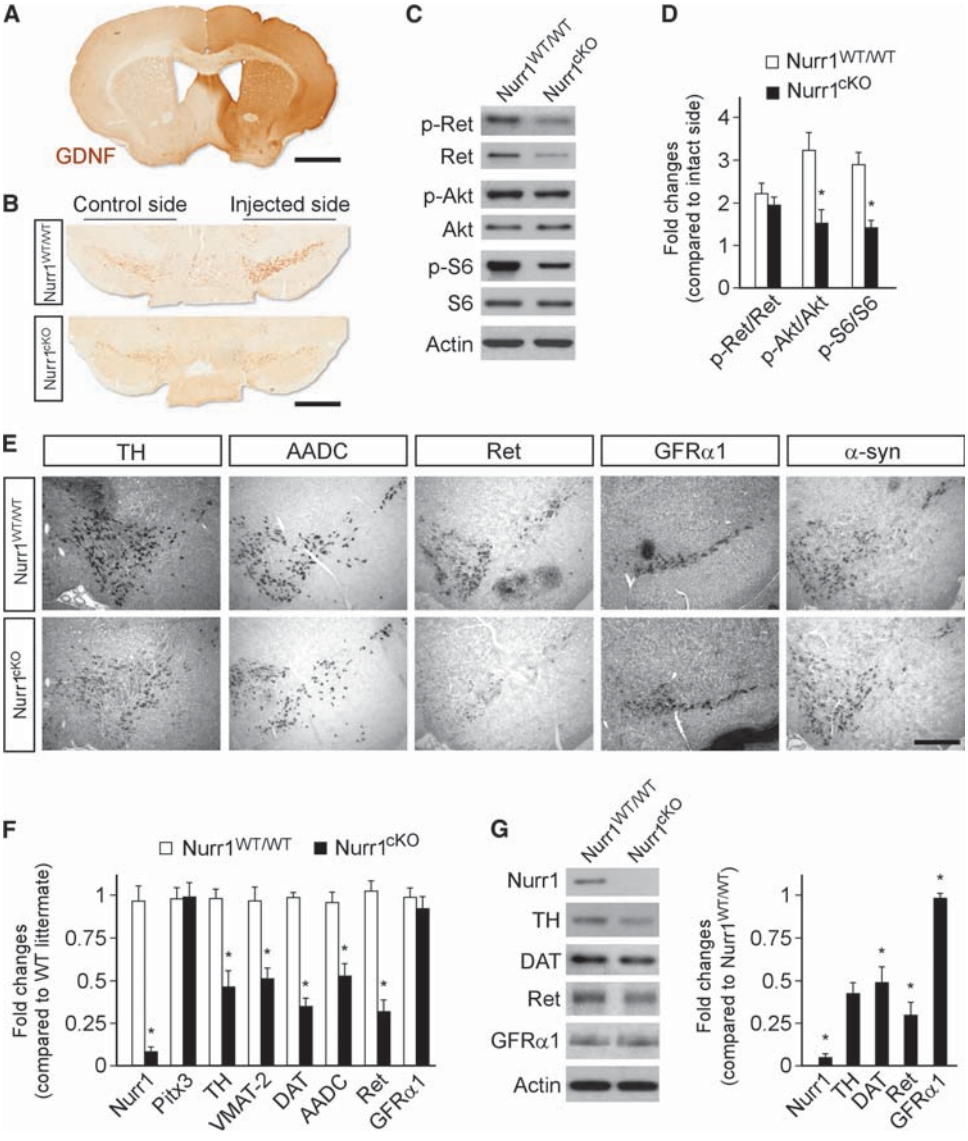

Fig. 5. Nurr1 is crucial for the maintenance of DA markers and Ret expression. (**A**) Immunostaining for human GDNF showing distribution of the protein 24 hours after unilateral injection of 1 μg of human recombinant GDNF into the mouse striatum (right side). Scale bar, 1.5 mm. (**B**) Immunostaining for p-S6 in control Nurr1$^{WT/WT}$ (top) and Nurr1$^{cKO/cKO}$ mice (bottom) 24 hours after unilateral intrastriatal injection of GDNF (1 μg/1 μl) (right side). Scale bar, 800 μm. (**C** and **D**) Western blot of phosphorylated Ret, Akt, and S6 in Nurr1$^{WT/WT}$ and Nurr1$^{cKO/cKO}$ mice 24 hours after intrastriatal injection of GDNF (1 μg/1 μl) showing the response of nigral DA neurons in transgenic mice compared to controls. Data are expressed as means ± SEM (n = 5 per group). *P < 0.05 compared to the Nurr1$^{+/+}$ group (Student's t test). (**E**) In situ hybridization of TH, AADC, Ret, GFRα1, and α-synuclein in midbrain sections 8 weeks after tamoxifen treatment of control and Nurr1 floxed mice. Scale bar, 200 μm. (**F**) qPCR analysis of Nurr1, Pitx3, TH, VMAT-2, DAT, AADC, Ret, and GFRα1 mRNAs in Nurr1-ablated and control mice 8 to 10 weeks after tamoxifen treatment. Data are expressed as means ± SEM (n = 5 per group). *P < 0.05 compared to the Nurr1$^{WT/WT}$ group (Student's t test). (**G**) Western blot analysis of Nurr1, TH, DAT, Ret, and GFRα1 in midbrain tissue from Nurr1$^{WT/WT}$ and Nurr1$^{cKO/cKO}$ mice 8 to 10 weeks after tamoxifen treatment. *P < 0.05 (Student's t test).

the regulation of the cell's responsiveness to GDNF. To investigate this further, we used a strain of gene-targeted (floxed) mice in which Nurr1 can be conditionally ablated in adult DA neurons by treatment with tamoxifen (Nurr1 conditional knockout mice) to determine the importance of this transcription factor in the maintenance of the GDNF signaling pathway. Homozygous floxed mice (1.5 months old) and control littermates were treated with tamoxifen, received an intrastriatal injection of GDNF (1 μg) 8 to 10 weeks later, and were sacrificed after 24 hours (Fig. 5A). Histological analysis showed that GDNF injection triggered phosphorylation of ribosomal protein S6 in nigral DA neurons on the injected side in control animals, whereas immunoreactivity for phosphorylated ribosomal protein S6 was similar on the two sides in the transgenic mice with conditional knockout of Nurr1 (Fig. 5B). Western blot analysis revealed that Ret activation in the striatum was similar in the control and Nurr1 conditional knockout mice (222 ± 18% increase in control mice and 194 ± 19% increase in Nurr1 conditional knockout mice, P = 0.68), whereas downstream activation of Akt and S6 was significantly impaired in the Nurr1 conditional knockout mice (156 ± 29% increase for p-Akt and 142 ± 18% increase for p-S6) compared to control littermates (323 ± 41% for p-Akt and 289 ± 29% for p-S6) (all P < 0.01) (Fig. 5, C and D).

We then examined how deletion of Nurr1 affected the expression of dopaminergic markers and GDNF receptors. Eight weeks after tamoxifen treatment, Nurr1 conditional knockout mice exhibited reduced expression of TH (47 ± 9%), VMAT-2 (51 ± 7%), DAT (35 ± 6%), AADC (53 ± 7), and Ret mRNAs (32 ± 7%) (all P < 0.05), whereas GFRα1 (92 ± 7%) and Pitx3 (98 ± 10%) concentrations were not altered compared to wild-type littermates (P > 0.05; Fig. 5F). These observations were further confirmed by in situ hybridization (Fig. 5E) and Western blot analysis (Fig. 5G). Together, our data show similar down-regulation of dopaminergic markers and disruption of GDNF signaling as a result of α-synuclein overexpression or Nurr1 ablation.

Nurr1 overexpression restores the response to GDNF

Next, we asked whether enhanced expression of Nurr1 (using AAV vector delivery) in α-synuclein–overexpressing neurons could restore their ability to respond to GDNF. First, we rescued the expression of this transcription factor in Nurr1 conditional knockout mice (Fig. 6A) and observed that it restored the responsiveness of nigral DA neurons to GDNF. This was seen as increased phosphorylated ribosomal protein S6 immunoreactivity induced by GDNF delivery to the striatum; mice receiving AAV-GFP

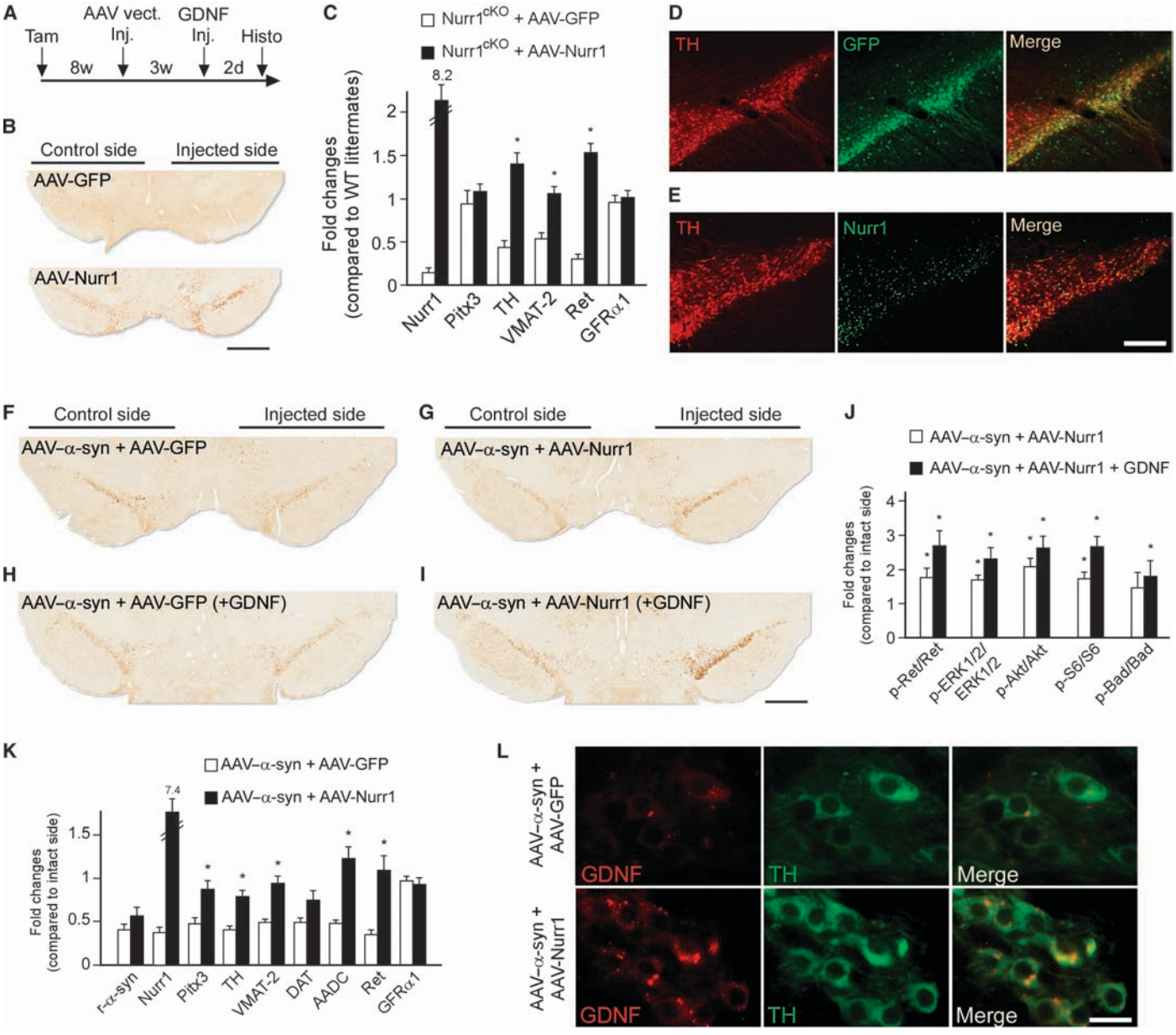

Fig. 6. Nurr1 overexpression restores DA marker expression and response to GDNF. (**A**) Schematic representation of the experimental timeline performed in the conditional knockout (cKO) Nurr1 mice. Tam, tamoxifen treatment. (**B**) p-S6 staining of midbrain section from Nurr1-ablated mice overexpressing GFP or Nurr1 2 days after unilateral intrastriatal injection of GDNF (1 μg) (right side). Scale bar, 800 μm. (**C**) qPCR analysis of Nurr1, Pitx3, TH, VMAT-2, Ret, and GFRα1 mRNAs in Nurr1$^{cKO/cKO}$ mice after AAV-mediated overexpression of GFP or Nurr1. Data are expressed as means ± SEM (n = 5 per group). *P < 0.05 compared to the AAV-GFP group (Student's t test). (**D** and **E**) Immunofluorescence images illustrating the AAV-mediated overexpression of GFP (D) or Nurr1 (E) (green) in nigral DA neurons (TH in red) when vectors were mixed with the AAV–α-synuclein vector. Scale bar, 150 μm. (**F** to **I**) p-S6 immunoreactivity in midbrain DA neurons overexpressing α-synuclein + GFP (F and H) or α-synuclein + Nurr1 (G and I) after injection of GDNF (H and I) or vehicle (F to G) into the striatum 2 days earlier (right side). Scale bar, 500 μm. (**J**) Quantification of Western blot analysis showing activation of Ret, Akt, S6, ERK1/2, and Bad in midbrain tissue after overexpression of α-synuclein + Nurr1, with or without stimulation by intrastriatal GDNF. Data are expressed as means ± SEM (n = 5 per group). *P < 0.05, Student's t test. (**K**) qPCR analysis of human and rat α-synuclein, Nurr1, Pitx3, TH, VMAT-2, DAT, AADC, Ret, and GFRα1 mRNA concentrations in rat midbrain 2 weeks after AAV vector–mediated overexpression of α-synuclein + GFP or α-synuclein + Nurr1. Data are expressed as means ± SEM (n = 5 per group). *P < 0.05 compared to the AAV–α-synuclein + AAV-GFP group (Student's t test). (**L**) Double immunofluorescence staining of human GDNF (red) and TH (green) showing that Nurr1 overexpression was efficient in restoring retrograde transport of the neurotrophic factor in nigral DA neurons affected by α-synuclein. GDNF (10 μg/3 μl) was injected 2 days earlier into the striatum. Scale bar, 20 μm.

failed to show any such activation (Fig. 6B). This functional effect was accompanied by the normalization of the expression of all dopaminergic markers and Ret expression (Fig. 6C).

We then explored the same strategy in rats overexpressing α-synuclein and found that AAV-mediated expression of Nurr1 was sufficient to partially restore the trophic response to GDNF in nigral DA neurons, as assessed histologically by staining for phosphorylated ribosomal S6 protein (Fig. 6, D to I). Notably, Western blot analysis revealed that activation of the intracellular signaling pathways was restored not only in Nurr1-overexpressing rats that had received an additional injection of GDNF but also in rats that did not receive any exogenous GDNF (Fig. 6J). This suggests that nigral DA neurons overexpressing Nurr1 were capable of responding not only to exogenous GDNF but also to the increased concentrations of endogenous GDNF induced in response to α-synuclein overexpression (Fig. 1F).

qPCR data revealed that the expression of all dopaminergic markers and Ret were significantly greater, or even fully restored, in Nurr1-overexpressing rats (all $P < 0.05$ compared to the AAV–α-synuclein group) (Fig. 6K). Finally, we found that forced Nurr1 expression in α-synuclein–transduced nigral neurons was able to restore GDNF retrograde transport compared to the GFP-treated control mice, where GDNF immunoreactivity in DA neurons was very low 6 hours after striatal delivery of GDNF (Fig. 6L).

Nurr1 protects against α-synuclein–induced toxicity

To investigate the protective role of Nurr1 in the context of PD pathology, we studied whether modulation of Nurr1 expression affects survival of α-synuclein–overexpressing nigral DA neurons. We first evaluated the impact of Nurr1 ablation on α-synuclein–induced DA neurodegeneration (see experimental design in Fig. 7A). To do so, we injected AAV–α-synuclein vector at a concentration that induces marginal damage in wild-type $Nurr1^{WT/WT}$ mice (91 ± 4% of control side) (Fig. 7, C and D). In tamoxifen-treated Nurr1 conditional knockout mice, the amphetamine-induced rotation test revealed a significantly higher number of turns in homozygous $Nurr1^{cKO/cKO}$ mice (6.4 ± 1.1 turns) compared to either wild-type (0.6 ± 0.5 turns) or heterozygous animals (2.0 ± 0.7 turns) [($F_{2,42} = 6.56$; $P < 0.01$); two-way analysis of variance (ANOVA) vector × genotype interaction] (Fig. 7B). Consistent with the behavioral data, stereological counts showed that α-synuclein caused significant loss of DA neurons in heterozygous $Nurr1^{WT/cKO}$ mice (74 ± 6% of control side) and an even more profound degeneration in homozygous $Nurr1^{cKO/cKO}$ mice (44 ± 7% of control side) [($F_{2,42} = 11.06$; $P < 0.001$); two-way ANOVA vector × genotype interaction] (Fig. 7, C and D).

We then examined whether Nurr1 overexpression can prevent dopaminergic cell loss caused by α-synuclein toxicity. Rats received intranigral injection of AAV–α-synuclein together with AAV-Nurr1 or AAV-GFP and were examined 8 weeks later (*16*, *39*). We found that Nurr1 overexpression prevented the development of motor impairment (1.2 ± 0.9 turns/min) compared to the GFP control group (5.6 ± 0.7 turns/min) ($P < 0.05$) (Fig. 7E). Stereological analysis showed that AAV-mediated Nurr1 expression afforded robust protection of VMAT-2–positive nigral neurons (81 ± 9% of control side) compared to the GFP control group (35 ± 7% of control side) ($P < 0.01$) (Fig. 7, F and G), as well as near-complete protection of the striatal TH-positive innervation on the injected side (Fig. 7, H and I). Notably, this was accompanied by a marked activation of phosphorylated ribosomal protein S6 and retained Ret expression in the rescued nigral DA neurons in the Nurr1-treated rats (Fig. 7, I and J).

Insulin-like growth factor 1 signaling is not affected in the AAV–α-synuclein model

The marked effect of α-synuclein overexpression on GDNF signaling raises the question on whether the alterations described above are selective for signaling triggered by GDNF through the Ret receptor or whether other trophic factors acting through a receptor with tyrosine kinase activity are equally affected. We selected insulin-like growth factor 1 (IGF-1) for comparison because, like GDNF, it has previously been shown to exert neuroprotection in the rat 6-OHDA lesion model and because the A9 nigral neurons express high concentrations of IGF-1 receptor (IGF-1R) (*40*, *41*). qPCR analysis showed that expression of IGF-1R was not altered by α-synuclein overexpression (fig. S4A), despite the increased expression of miR-7, reported above (Fig. 3A), which has been described as a negative regulator of this receptor in other cells (*42*). Delivery of IGF-1 into the striatum triggered an intracellular response in nigral DA neurons, as assessed by the expression of p-S6 (fig. S4B). Markedly, this response was unaffected in α-synuclein–overexpressing neurons (fig. S4B). Western blot analysis confirmed that the Akt, ERK1/2, and S6 pathways were equally stimulated by IGF-1 in all experimental groups (fig. S4C). Maintenance of IGF-1 signaling in the absence of Nurr1 was confirmed in tamoxifen-treated Nurr1 conditional knockout mice. In these mice, IGF-1R expression was unchanged, and midbrain DA neurons were stimulated by IGF-1 with the same efficiency as in control wild-type animals (fig. S4, D and E). This may possibly explain why the IGF-1 signaling system in DA neurons escapes from the repressive effects of α-synuclein.

DISCUSSION

GDNF and related neurotrophic factors have profound protective effects on DA neurons in toxin animal models of PD. However, clinical trials using exogenous GDNF have provided inconclusive results. The data presented in this study provide a mechanistic explanation for these apparently conflicting results and suggest the intriguing possibility that α-synuclein toxicity acting at the level of gene expression leads to the disruption of neurotrophic signaling. The results show that increased cellular concentrations of α-synuclein block the intracellular signaling response to GDNF, thus affecting the ability of the nigral DA neurons to respond to both exogenous and endogenous GDNF, and that Nurr1 may mediate this effect. Consistent with previous findings in PD patients (*29*), the expression of Nurr1 was reduced by as much as 70 to 80% in the α-synuclein–overexpressing DA neurons of rat brain. As a consequence, expression of several Nurr1 target genes involved in the regulation of DA neurotransmission, such as TH, DAT, and VMAT-2, was substantially reduced. This was also the case for the expression of the GDNF receptor Ret, which suggests that the blockade of the GDNF trophic response is due to reduced availability of the receptor on the affected cells. In support of this idea, we observed that Ret is down-regulated in nigral neurons in PD patients, whereas the response to another trophic factor, IGF-1, which acts through a different receptor independent of Nurr1 regulation, was unaltered in rats overexpressing α-synuclein.

Recent studies have shown that the NR4A family of transcription factors, of which Nurr1 (NR4A2) is a member, has a broad impact on

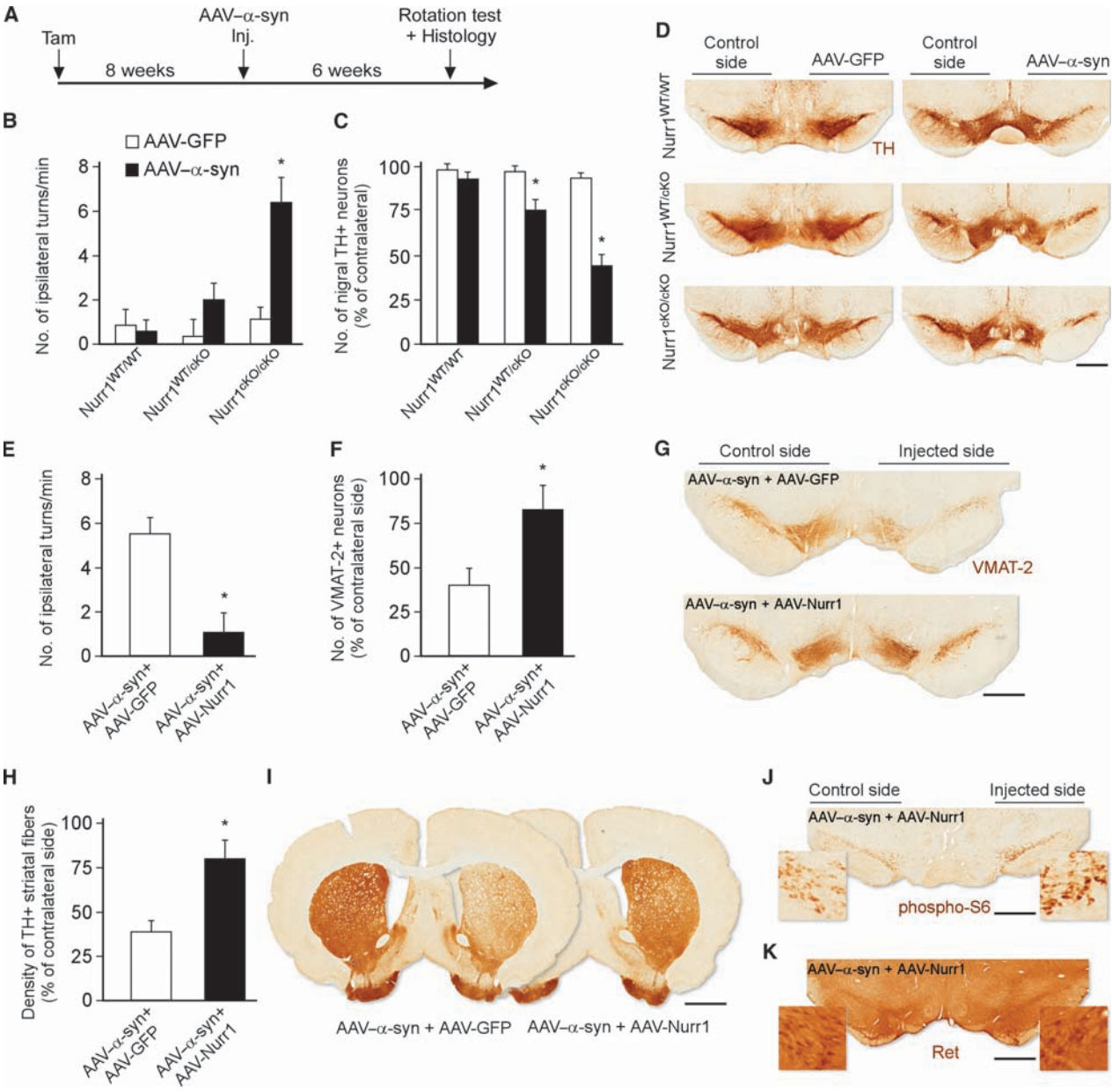

Fig. 7. Nurr1 protects nigral DA neurons against α-synuclein toxicity. (**A**) Schematic representation of the experimental timeline. (**B**) α-Synuclein toxicity studied in Nurr1 conditional knockout mice with one or both Nurr1 genes deleted. Motor impairment was assessed using amphetamine-induced rotation. Data are expressed as means ± SEM ($n = 8$ per group). *$P < 0.05$ compared to the AAV-GFP group (Student's t test). (**C**) Quantification of TH-positive nigral DA neurons 6 weeks after injection of AAV–α-synuclein or AAV-GFP in Nurr1$^{WT/WT}$, Nurr1$^{WT/cKO}$, and Nurr1$^{cKO/cKO}$ mice. *$P < 0.05$; two-way ANOVA (vector × genotype interaction) followed by Bonferroni post hoc test. (**D**) Immunostaining of TH-positive neurons in the substantia nigra 6 weeks after nigral delivery of AAV-GFP or AAV–α-synuclein vector in Nurr1$^{WT/WT}$, Nurr1$^{WT/cKO}$, and Nurr1$^{cKO/cKO}$ mice. Scale bar, 600 μm. (**E** to **I**) Protection against α-synuclein–mediated toxicity in rats treated with AAV–α-synuclein. (E) Quantification of amphetamine-induced rotation 8 weeks after intranigral injection of AAV–α-synuclein + AAV-Nurr1 or AAV–α-synuclein + AAV-GFP. *$P < 0.05$ compared to the AAV–α-synuclein + AAV-GFP group (Student's t test). (F) Stereological estimation of the number of VMAT-2–positive nigral neurons 8 weeks after intranigral injection of α-synuclein + Nurr1 or α-synuclein + GFP. *$P < 0.05$ compared to the AAV–α-synuclein + AAV-GFP group (Student's t test). (G) VMAT-2–immunostained midbrain sections from rats overexpressing α-synuclein + Nurr1 or α-synuclein + GFP (right side). Scale bar, 600 μm. (H) Density of the striatal TH-positive innervation in rats 8 weeks after intranigral injection of α-synuclein + Nurr1 or α-synuclein + GFP. *$P < 0.05$ compared to the AAV–α-synuclein + AAV-GFP group (Student's t test). (I) TH-immunostained forebrain sections from rats overexpressing α-synuclein + Nurr1 or α-synuclein + GFP (right side). Scale bar, 1.2 mm. (**J** and **K**) Immunostaining of phosphorylated S6 (J) and Ret (K) 2 days after intrastriatal injection of GDNF (1 μg/1 μl; right), showing restoration of the GDNF-induced trophic response in rats overexpressing Nurr1. Scale bar, 800 μm.

cell survival. NR4A-deficient neurons are generally more sensitive to neurodegeneration, induced, for example, by excitotoxic or oxidative stress, which is likely to result from down-regulation of an NR4A-dependent neuroprotective gene program (*23*, *43*). Nurr1 is an important factor in the development and maintenance of midbrain DA neurons (*44*–*46*). Our new data, as well as the results from a previous study in MPTP-lesioned mice (*47*), show that Nurr1-deficient adult mouse DA neurons are more sensitive to both α-synuclein and MPTP-induced toxicity. Conversely, we show that overexpression of Nurr1 can provide protection against the α-synuclein–induced toxic insult in nigral DA neurons.

These observations support the idea that Nurr1 acts as a key factor in the maintenance of DA neurons, not only as a regulator of trophic signaling but probably also as a mediator of multiple signaling pathways that are involved in the control of both survival and function of midbrain DA neurons. It has been shown that Nurr1 is activated in response to excitotoxic and oxidative stress and that this effect is mediated by CREB (*23*, *43*). This suggests that Nurr1 is a mediator of CREB-dependent neuroprotective responses that are activated by pathological stimuli or cellular stress. Moreover, as CREB is an effector in the two principal neuroprotective pathways, phosphatidylinositol 3-kinase (PI3K)/Akt and MAPK (*48*), this points to a role of Nurr1 downstream of neurotrophic factor signaling. In support of this idea, we show that Nurr1 expression in nigral DA neurons is increased 2.3-fold in response to exogenously administered GDNF and that this is accompanied by two- to threefold increases in the concentrations of p-Akt, ERK1/2, and S6, as well as induction of phosphorylated CREB. This observation is in line with a recent study reporting transcriptional activation in nigral DA neurons on striatal injection of GDNF (*22*). Markedly, these downstream changes induced by injection of GDNF were all blocked in α-synuclein–overexpressing DA neurons. In AAV–α-synuclein–injected rats not treated with GDNF, the endogenous GDNF concentration in the substantia nigra, as measured by ELISA, was increased by 60 to 80% compared to the contralateral intact side. Nevertheless, Ret was not activated and p-Akt and p-S6 concentrations were reduced below normal. This suggests that the blockade induced by increased cellular α-synuclein affected not only the response to exogenous GDNF but also endogenous GDNF signaling.

The profound effect of α-synuclein on the expression of Nurr1 and other transcription factors involved in regulation of cell survival, such as Pitx3 and MEF2D, seen in the α-synuclein–overexpressing DA neurons, suggests that the toxic effects of α-synuclein, at least in part, may be induced at the level of gene transcription. α-Synuclein is located not only in the cytoplasm but also in the nucleus (*30*, *32*–*34*, *49*), and a previous study in *Drosophila* (*31*) has shown that α-synuclein targeted to the nucleus promotes toxicity, whereas cytoplasmic sequestration is protective. Here, they also showed that α-synuclein binds directly to histones, thus inhibiting acetylation in histone acetyltransferase assays and that the toxicity of α-synuclein could be rescued by administration of histone deacetylase inhibitors in both cell culture and transgenic flies. Consistent with these observations, we found that human α-synuclein expressed by the AAV vector interacts with histone H3 in the nucleus and that the expression of histone acetyltransferase p300 was reduced in the AAV–α-synuclein–treated animals, compared to the intact or GFP-transduced controls. By confocal microscopy, we could show that DA neurons with accumulation of phosphorylated α-synuclein in the nucleus had markedly reduced concentrations of two selected factors, the DA phenotypic marker VMAT-2 and the survival-related transcription factor MEF2D. Together, these data support the view that elevated concentrations of α-synuclein have profound effects on gene expression caused by α-synuclein entering the nucleus and that the impact on transcriptional activity, including expression of Nurr1, may be mediated by interactions with histones leading to inhibition of histone acetylation as previously suggested from studies in vitro (*50*).

The central role of Nurr1 as a regulator of the GDNF-induced trophic response is further supported by the observations made in Nurr1 conditional knockout mice. The expression of genes involved in DA neurotransmission (TH, AADC, DAT, and VMAT-2) was reduced by 50 to 70% in the Nurr1 conditional knockout mice. The expression of the Ret receptor was reduced to a similar extent (−68% in the Nurr1 conditional knockout mice compared to wild-type mice) as found in α-synuclein–overexpressing rats (−74% compared to the intact side). The response to GDNF stimulation, as measured by the increase in p-Akt and S6, was impaired. We show that enhanced expression of Nurr1 using AAV vector delivery reversed these gene expression changes and restored the trophic response to GDNF, not only in Nurr1 conditional knockout mice but also in DA neurons in the AAV–α-synuclein–overexpressing rats, showing that Nurr1 is both necessary and sufficient for maintenance of GDNF signaling in DA neurons affected by increased cellular concentrations of α-synuclein.

In this analysis, we made two additional observations. First, retrograde transport of GDNF from striatum to nigra was markedly reduced (by about 75%) in the α-synuclein–overexpressing rat DA neurons, and this defect was reversed by Nurr1. Second, enhanced expression of Nurr1, in the absence of any exogenous GDNF, was able to induce a GDNF-like trophic response, seen as an activation of both the Ret receptor and its downstream signaling pathways (p-Akt, phosphorylated ERK, and p-S6). Consistent with these observations, we show that enhanced expression of Nurr1 is sufficient to protect the nigral DA neurons—both the cell bodies and the axonal projections in the striatum—against α-synuclein–induced toxicity. This suggests that Nurr1 can act on its own to activate the cell's neuroprotective machinery in a way that mimics the response induced by GDNF. This intriguing similarity suggests the possibility that Nurr1 may act by restoring the ability of the α-synuclein–affected neurons to access and respond to endogenous GDNF.

Similar to its mode of action in the peripheral nervous system, it has been proposed that GDNF can function as a retrograde trophic factor also in midbrain DA neurons, internalized by the terminals in the striatum with help from the Ret receptor, and transported to the cell bodies where it exerts its protective effects (*51*). Indeed, Bartus *et al.* (*7*) have suggested that the modest clinical response seen in PD patients who received AAV-neurturin injections in the striatum may be explained by impaired retrograde transport of the trophic factor back to the cell bodies in the substantia nigra. In AAV–α-synuclein–overexpressing rats, however, the GDNF response was equally impaired when GDNF was delivered into the striatum or into the substantia nigra. This suggests that α-synuclein–induced down-regulation of the Ret receptor is the primary defect and that reduced transport is the result of reduced availability of the Ret receptor at the axon terminals. Given that deletion of GDNF or Ret does not cause any acute DA neuron cell loss in adult mice (*52*–*54*), it is unlikely that limited access to GDNF, or reduced Ret signaling, is sufficient on its own to cause the pronounced neurodegenerative changes seen in α-synuclein–overexpressing neurons. In the AAV–α-synuclein–overexpressing rats, however, the endogenous concentration of GDNF was increased above normal, which makes it possible that the GDNF-like trophic response induced by Nurr1 is

due to restoration of GDNF signaling and transport in the α-synuclein–overexpressing cells.

In conclusion, our results reveal a new mechanism of action for α-synuclein–induced neurotoxicity that points to effects on gene expression as an important pathological mechanism. Thus, α-synuclein–mediated down-regulation of Nurr1 results in a blockade of GDNF-dependent trophic signaling in midbrain DA neurons, pointing to Nurr1 as a key player in the neuroprotective machinery mobilized in response to pathological stimuli and cellular stress. In human PD, increased cellular concentrations of α-synuclein are associated with reduced concentrations of Nurr1 (*29*, *55*), and mutations in the Nurr1 gene that lead to reduced expression of Nurr1 have been linked to the development of PD (*56*, *57*). Our data show that reduced concentrations of Nurr1 make DA neurons more susceptible to α-synuclein–induced damage and, moreover, that elevated concentrations of α-synuclein cause a down-regulation of Nurr1 that is likely to contribute to the progression of the disease. In the AAV–α-synuclein–overexpressing rat model, down-regulation of Nurr1 happens early, before any overt cell loss has occurred, indicating that impaired Nurr1 function is an early event linked to the presymptomatic stages of the neurodegenerative process. Impaired Nurr1 function, in turn, will cause down-regulation of enzymes and transporters involved in DA synthesis and release, resulting in synaptic dysfunction in DA neurons, as well as an increased vulnerability to α-synuclein. Our study shows that restoration of Nurr1 function by overexpression of Nurr1 provides almost complete protection against α-synuclein–induced toxicity, as well as restoration of GDNF signaling.

Together, these data highlight Nurr1 as a promising target for neuroprotective and restorative therapy in PD. The development of drugs designed to activate Nurr1 function may hold particular promise. Nurr1 belongs to a nuclear receptor family that can be targeted by small lipophilic ligands, and as Nurr1 can form heterodimers with RXR (retinoid X receptor), it seems possible that RXR ligands may be used to modulate Nurr1 function. Such drugs may be powerful as neuroprotective agents by themselves (*58*), but they would also be of great interest as a tool to increase the efficacy of neuroprotective therapies based on GDNF or neurturin delivery.

MATERIALS AND METHODS

Vector production

Production of the AAV–α-synuclein, AAV-GFP, and AAV-Nurr1 vectors was performed as detailed in the Supplementary Materials.

Animals

Adult female Sprague-Dawley rats (Charles River), 225 to 250 g at the time of surgery, were housed two to three per cage with ad libitum access to food and water during a 12-hour light/dark cycle. All surgical procedures and treatments were conducted in accordance with guidelines set by the Ethical Committee for the use of laboratory animals in the Lund-Malmö region and the European Ethical Committee (86/609 EEC).

Conditional Nurr1 knockout (cKO) mice and the tamoxifen-inducible Cre recombinase transgenic line ($DAT^{CreERT2}$) were generated as previously described (*44*, *59*). Crosses between these transgenic lines allow inducible Nurr1 gene ablation exclusively in DA neurons by generating mice that are homozygous for the conditional targeted Nurr1 allele and heterozygous for the $DAT^{CreERT2}$ allele ($Nurr1^{flox/flox}$; $DAT^{CreERT2/WT}$). Littermates of genotype $Nurr1^{+/+}/DAT^{CreERT2/+}$ were used as control. For the neuroprotection study, heterozygous mice $Nurr1^{+/-}/DAT^{CreERT2/+}$ were also used. Wild-type controls and heterozygous and homozygous floxed mice are referred in the manuscript as $Nurr1^{WT/WT}$, $Nurr1^{WT/cKO}$, and $Nurr1^{cKO/cKO}$ mice, respectively.

Ablation of the Nurr1 gene was induced in 1.5-month-old mice by intraperitoneal injection of tamoxifen [Sigma; 20 mg/ml in ethanol/corn oil (9:1)] twice daily (2 mg per injection) during five consecutive days. Mice were housed two to seven per cage with ad libitum access to food and water during a 12-hour light/dark cycle.

Surgical procedures

All surgical procedures in rats were performed under general anesthesia with a 20:1 mixture of fentanyl citrate (Fentanyl) and medetomidin hypochloride (Dormitor), injected intraperitoneally. Surgeries in mice were performed under gaseous anesthesia and analgesia (2% isoflurane in 2:1 oxygen/nitrous oxide) with a stereotaxic mouse frame (Stoelting).

Vector solutions were injected with a 5- or 10-μl Hamilton syringe fitted with a glass capillary (outer diameter of 250 μm). Rats received 3 μl of AAV–α-synuclein, AAV-GFP, or a 1:1 mixture of AAV–α-synuclein and AAV-Nurr1 or AAV-GFP vectors. Infusion was performed at a rate of 0.2 μl/min, and the needle was left in place for an additional 3-min period before it was slowly retracted. Injection was carried out unilaterally on the right side, above the substantia nigra, at the following coordinates (flat skull position) (*60*): anteroposterior, −5.3 mm; mediolateral, −1.7 mm; dorsoventral, −7.2 mm below dural surface, calculated relative to bregma according to the stereotaxic atlas of Paxinos and Watson (*61*).

For the neuroprotection study, rats received similar intranigral injection of or a 1:1 mixture of AAV–α-synuclein + AAV-Nurr1 or AAV-GFP vectors.

For comparison with the standard toxin model of PD, a set of animals received an injection of 3 μl of 6-OHDA (1 μg/μl free base dissolved in a solution of 0.2 mg/ml L-ascorbic acid in 0.9% w/v NaCl; Sigma; 0.2 μl/min) in the MFB at the following coordinates (flat skull, as above): anteroposterior, −4.4 mm; mediolateral, −1.1 mm; dorsoventral, −7.8 mm.

Two weeks after AAV vector injection, or 2 days after 6-OHDA injection, rats received an injection of human recombinant GDNF (1 μg in 3 μl) or IGF-1 (1 μg in 3 μl) into the substantia nigra (same coordinates as above), or into the striatum at the following coordinates (relative to bregma): anteroposterior, +0.5 mm; mediolateral, −3.1 mm; dorsoventral, −4.5 mm below dural surface.

Eight to 10 weeks after tamoxifen treatment, mice received an intrastriatal injection of GDNF (1 μg in 1 μl) or IGF-1 (1 μg in 1 μl) at the following coordinates (relative to bregma): anteroposterior, +0.5 mm; mediolateral, −1.8 mm; dorsoventral, −2.5 mm below dural surface. Nigral injection of the AAV-Nurr1 or AAV-GFP vectors (1 μl) in mice was performed at the following coordinates (relative to bregma): anteroposterior, −2.8 mm; mediolateral, −1.1 mm; dorsoventral, −4.3 mm below dural surface. For the neuroprotection study, 1.5-month-old $Nurr1^{WT/WT}$, $Nurr1^{WT/cKO}$, and $Nurr1^{cKO/cKO}$ mice were treated with tamoxifen as described above and received intranigral injection of AAV–α-synuclein or AAV-GFP 6 weeks later.

Behavioral testing

Assessment of drug-induced rotational behavior was performed in automated bowls coupled to the Rotameter software (AccuScan Instruments), as described previously (*62*). Rats and mice received an

intraperitoneal injection of D-amphetamine sulfate (2.5 mg/kg, respectively) (Apoteksbolaget). Right and left full body turns were recorded over a period of 90 min. Data are expressed as net full turns per minute, with turns ipsilateral to the injection side assigned a positive value.

Tissue processing and immunohistochemistry

Immunohistochemistry was performed as described in the Supplementary Materials.

Stereological estimation of nigral neurons and optical densitometry analysis of fiber density

Assessment of the total number of TH-positive or VMAT-2–positive neurons in the substantia nigra was made by stereology according to the optical fractionator principle with the CAST Grid System (Olympus Denmark A/S), as described (*39*). Every sixth section covering the entire extent of the substantia nigra was included in the counting procedure. A coefficient of error of <0.10 was accepted. The data are expressed as a percentage of the corresponding area from the intact side.

Density of TH-positive fibers was measured by densitometry in the striatum at four coronal levels (+1.2, 0.8, 0.00, and −0.4 mm relative to bregma) and in the nucleus accumbens at three coronal levels (+2.3, 1.7, and +0.8 mm relative to bregma) with the ImageJ software [version 1.32j; National Institutes of Health (NIH)]. The measured values were corrected for nonspecific background staining by subtracting values obtained from the cortex. The data are expressed as a percentage of the corresponding area from the intact side.

Study of gene expression by reverse transcription–qPCR

The animals (n = 5 per group) were sacrificed, the brains were rapidly removed, and the ventral midbrain, ipsilateral and contralateral to the site of injection, was dissected and snap-frozen. In an attempt to dissociate A9 and A10 neuronal populations, the lateral region comprising the nigral neurons was separated from the medial region comprising ventral tegmental area neurons under the microscope. mRNA and miRs were isolated with the miRNeasy Mini Kit (Qiagen) according to the supplier's recommendations. RNA concentration was determined with the NanoDrop (Thermo Scientific), and a 500-ng quantity of RNA was used for the reverse transcription (RT) performed with random primers (Invitrogen) and SuperScript III (Invitrogen) according to the manufacturer's recommendations. Primers were designed with Primer Blast (NIH), and primer sequences are presented in table S1. For qPCR of miR, we used the Universal cDNA (complementary DNA) Synthesis Kit and LNA™-PCR primer sets against miR-7, -21, -22, -124, and -153 (Exiqon) (*63*). SYBR Green qPCR was performed with LightCycler 480 SYBR Green I Master (Roche) using standard procedures. Data were quantified by the $\Delta\Delta C_t$ method and normalized to glyceraldehyde-3-phosphate dehydrogenase and β-actin expression.

Determination of GDNF concentrations by ELISA

The animals (n = 4 per group) were sacrificed, and nigral and striatal concentrations of endogenous GDNF were determined by a method previously described (*18*). Briefly, brains were removed, and ventral midbrains and striata were dissected and quickly frozen on dry ice. Tissue samples were sonicated in buffer [150 mM NaCl, 50 mM tris (pH 7.4), 1% Triton X-100, phenylmethylsulfonyl fluoride (1.7 μg/ml), leupeptin (1.0 μg/ml), aprotinin (10 μg/ml), and pepstatin (1.0 μg/ml)] at a concentration of 30 mg/ml. Tissue concentrations of GDNF were determined on these homogenates by ELISA according to the supplier's recommendations (IBL-America for detection of rat GDNF, and Promega G7620 for detection of human GDNF).

In situ hybridization

Animals were perfused as described above, and midbrain was cut on a cryostat (Leica). Slides were fixed for 10 min in 4% paraformaldehyde, washed in phosphate-buffered saline (PBS), permeabilized with Protease K for 5 min [Protease K (1 μg/ml) in 50 mM tris-HCl, 5 mM EDTA solution], and then acetylated for 10 min (1.3% triethanolamine, 0.05% HCl, and 0.25% acetic anhydride). After rinses in PBS, slides were incubated for 3 hours at room temperature for prehybridization [50% formamide, 5× SSC, 5× Denharts, Baker's yeast RNA (250 μg/ml), and salmon sperm DNA (500 μg/ml)]. Hybridization was performed overnight at 72°C with digoxigenin-labeled probe for TH, Nurr1, Pitx3, or DAT. The following day, slides were washed in 72°C heated solutions of 5× SSC and 0.2× SSC and then equilibrated in B1 [0.1 M tris-HCl (pH 7.5) and 0.15 M NaCl]. After preincubation for 1 hour in 10% heat-inactivated fetal calf serum (HIFCS) in B1, the sections were incubated overnight with anti–digoxigenin alkaline phosphatase Fab fragments (Roche; 1:5000 in B1 with 1% HIFCS). After washes in B1, sections were placed in a solution of 0.1 M tris-HCl (pH 9.5), 0.1 M NaCl, and 50 mM $MgCl_2$ (B2). Finally, mRNA expression was visualized after overnight incubation with 0.2 mM nitro blue tetrazolium/bromochloroindolyl phosphate (Roche) and levamisole (0.24 μg/ml) in 10% poly-(vinyl alcohol)/B2 solution. The reaction was terminated with water, and sections were mounted in Aquatex (Merck). Photographs were taken with an Eclipse E1000M microscope (Nikon) coupled to a digital camera (Spot2; Diagnostic Instruments Inc.).

Statistical analysis

All statistics were conducted with the GraphPad Prism software (version 5.0). All values are presented as means ± SEM. Comparison between experimental groups was performed with a Student's t test. In the neuroprotection study, vector × genotype interaction was analyzed with a two-way ANOVA followed by Bonferroni post hoc test. Statistical significance was set at $P < 0.05$.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. S1. Trophic response induced by intrastriatal injection of human GDNF in nigral DA neurons.

Fig. S2. Expression of GDNF and α-synuclein, GFP transgene expression, and survival of nigral DA neurons.

Fig. S3. α-Synuclein overexpression represses transcriptional activity in DA neurons.

Fig. S4. IGF-1 signaling is preserved in DA neurons overexpressing α-synuclein.

Table S1. List of RT-qPCR primers.

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