

Regulation of α -synuclein by bFGF in cultured ventral midbrain dopaminergic neurons

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Abstract

α -Synuclein is a neuronal protein that is implicated in the control of synaptic vesicle function and in Parkinson's disease (PD). Consequently, alterations of α -synuclein levels may play a role in neurotransmission and in PD pathogenesis. However, the factors that regulate α -synuclein levels are unknown. Growth factors mediate neurotrophic and plasticity effects in CNS neurons, and may play a role in disease states. Here we examine the regulation of α -synuclein levels in primary CNS neurons, with particular emphasis on dopaminergic neurons. E18 rat cortical neurons and dopaminergic neurons of E14 rat ventral midbrain showed an induction of α -synuclein protein levels with maturation in culture. Application of basic Fibroblast growth factor (bFGF) promoted α -synuclein expression

selectively within dopaminergic, and not GABAergic or cortical neurons. This induction was blocked by actinomycin D, but not by inhibition of bFGF-induced glial proliferation. α -Synuclein levels were not altered by glial-derived neurotrophic factor (GDNF), or by apoptotic stimuli. We conclude that bFGF promotes α -synuclein expression in cultured ventral midbrain dopaminergic neurons through a direct transcriptional effect. These results suggest that distinct growth factors may thus mediate plasticity responses or influence disease states in ventral midbrain dopaminergic neurons.

Keywords: bFGF, dopaminergic, growth factor, MPP⁺, plasticity, α -synuclein.

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α -Synuclein is a neuronal protein localized primarily to presynaptic terminals of the CNS, where it closely associates with synaptic vesicles. This localization, together with its up-regulation in zebra finch during a critical period of song learning, have suggested that α -synuclein may be involved in neuronal plasticity via regulation of synaptic vesicle function (George *et al.* 1995; Clayton and George 1999). Consistent with this idea, α -synuclein knock-out mice show enhanced dopamine release in response to paired stimuli (Abeliovich *et al.* 2000). Additionally, we have shown that PC12 cells expressing α -synuclein show reduced dopamine release in response to depolarizing conditions (Stefanis *et al.* 2001a; Larsen *et al.* 2002). These findings suggest that α -synuclein acts as a negative regulator of neurotransmitter release.

The findings that mutations in α -synuclein cause autosomal dominant Parkinson's disease (PD) (Polymeropoulos *et al.* 1997; Kruger *et al.* 1998), that α -synuclein is a major component of Lewy bodies (LBs) in sporadic PD (Spillantini *et al.* 1998), and that the purified protein aggregates under certain *in vitro* conditions (Conway *et al.* 1998; Giasson *et al.* 1999) have greatly increased interest in α -synuclein biology, linking it to neurodegenerative conditions and

protein aggregation. In view of the important role of α -synuclein in control of neurotransmission and in neurodegeneration, it is critical to examine the manner in which it is regulated. α -Synuclein is up-regulated during late embryonic and early postnatal development in the rodent CNS (Hsu *et al.* 1998; Kholodilov *et al.* 1999a; Petersen *et al.* 1999). It is also induced in a variety of injury models of the rodent substantia nigra pars compacta (SNpc) (Kholodilov *et al.* 1999a; Vila *et al.* 2000; Manning-Bog *et al.* 2002). However, the role of α -synuclein up-regulation in these injury

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Abbreviations used: bFGF, basic fibroblast growth factor; DIV, day *in vitro*; GABA, γ -aminobutyric acid; GDNF, glial cell line-derived neurotrophic factor; LB, Lewy body; NGF, nerve growth factor; PBS, phosphate-buffered saline; PD, Parkinson's disease; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SN, substantia nigra; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase.

models is not understood. It is unclear whether it represents a response to maintain neuronal viability, a plastic/adaptive response to injury, or whether it is directly involved in dopaminergic neuron degeneration, perhaps through aggregate formation. It is also unclear, given the nature of these *in vivo* experiments, whether these effects are mediated directly on dopaminergic neurons by the injurious stimuli. One strategy to address these issues is to study the expression of α -synuclein in simplified cell culture systems, and to identify critical factors that regulate this expression.

Using PC12 cells and primary sympathetic neurons, we have previously found that nerve growth factor (NGF) and basic fibroblast growth factor (bFGF) induce a specific up-regulation of the rodent homolog of α -synuclein, synuclein-1. In contrast, there was no regulation with apoptotic stimuli, nor did over-expression of α -synuclein modify sensitivity to apoptosis-inducing stimuli (Stefanis *et al.* 2001b). It is known that NGF-treated PC12 cells release less dopamine in response to depolarizing stimuli compared with naive cells (Greene and Rein 1977). We concluded that α -synuclein up-regulation in neuronally differentiated PC12 cells likely represents a growth factor-mediated plasticity response that negatively controls neurotransmitter release (Stefanis *et al.* 2001b).

These data, although based only on studies in the PC12 cell line and neurons of the peripheral nervous system, raised for the first time the possibility that growth factors may be involved in regulation of α -synuclein levels. This is especially important in view of the role of growth factors, and in particular bFGF, in neuronal plasticity (Abe *et al.* 1990; Terlau and Seifert 1990; Schuman 1999; Flores and Stewart 2000; Pierce and Bari 2001) and, potentially, in disease states (Otto and Unsicker 1990; Tooyama *et al.* 1993; Unsicker *et al.* 1993). The present work was undertaken to examine the regulation of α -synuclein expression in cultured CNS neurons, with particular emphasis on midbrain dopaminergic neurons, the neurons primarily affected in PD, and on the role of growth factors.

Materials and methods

Rat ventral midbrain cultures

Dopaminergic neuron cultures were prepared from embryonic day 14 (E14) rat fetuses. Briefly, the ventral portion of the midbrain was removed in sterile ice-cold phosphate-buffered saline (PBS), cleaned free of meningeal tissue, minced and mechanically dissociated by passage through a flame-polished pasteur pipette. Dissociated cells were plated at a density of approximately 200 000 cells per cm² on poly-D-lysine coated 24-well plates or glass coverslips. The neurons were maintained in Neurobasal medium (Invitrogen) with B27 serum-free supplements, L-glutamine (0.5 mM) and penicillin/streptomycin (serum-free medium, SFM). Under these culture conditions, dopaminergic neurons comprise approximately 2–5% of the total number of cells (not shown).

Rat cortical neuronal cultures

Cultures of rat E18 cortical neurons were prepared as described previously (Stefanis *et al.* 1999; Rideout *et al.* 2001). Dissociated cells from cortical mantles were plated in SFM onto poly-D-lysine coated 35 mm plastic dishes for protein chemistry, or glass coverslips for immunocytochemistry at a density of approximately 150 000–200 000 per cm².

Addition of various reagents to the cultures

At the time of plating, bFGF (Sigma; prepared in sterile PBS, 0.5% BSA at a stock concentration of 25 μ g/mL), GDNF (Upstate Biotechnology, Lake Placid, NY, USA; prepared in sterile dH₂O at a stock concentration of 10 μ g/mL), or the antimetabolic uridine/fluorodeoxyuridine (20 and 8 μ M, respectively; Sigma, St Louis, MO, USA) were added directly to the growth medium. Whenever cultures were maintained for a period longer than 3 days, the medium was replenished with the same agents at the same concentrations. Some ventral midbrain cultures were treated with the mitochondrial complex I inhibitor MPP⁺ (Sigma/RBI), which was added either 2 h after plating, or on Day 2 *in vitro* (2 DIV) at a concentration of 1 μ M. In some cases, cortical neuron cultures were exposed to the DNA damaging agent camptothecin (Stefanis *et al.* 1999; Rideout *et al.* 2001).

Immunofluorescence

Neurons grown on glass coverslips were fixed in freshly prepared 3.7% formaldehyde for 15 min at 4°C and then incubated with 10% normal goat serum (NGS) with 0.4% Triton X-100 to block nonspecific binding, followed by incubation with the primary antibody for 1 h at room temperature (25°C). For neurons to be immunostained for GABA, coverslips were fixed in freshly prepared 3.7% formaldehyde, containing 0.0025% glutaraldehyde for 15 min at 4°C, and then incubated for 20 min in 10% heat-inactivated NGS with 0.1% Triton X-100 (Albuquerque *et al.* 1999). Specific antibodies used were rabbit anti-tyrosine hydroxylase (TH, 1 : 500; Calbiochem, San Diego, CA, USA), mouse anti-TH (1 : 200; Roche Molecular Biochemicals, Indianapolis, IN, USA), rabbit anti-GABA (1 : 3000; Sigma), mouse antisynuclein-1 (1 : 50; Transduction Laboratories, San Diego, CA, USA), or mouse anti-GFAP (1 : 500; Sigma). Following incubation with fluorescent secondary antibodies (Cy2, 1 : 100; or Cy3, 1 : 250, Jackson Immuno-Research, West Grove, PA, USA), coverslips were placed on glass slides and visualized using standard epifluorescence or confocal microscopy (Zeiss LSM410).

To determine relative changes in α -synuclein expression in dopaminergic or GABAergic neurons, cells were double immunostained for either TH or GABA and α -synuclein. The percentage of TH- or GABA-positive neurons with bright α -synuclein immunoreactivity was determined from three separate coverslips from two or more independent cultures by raters who were blinded to the experimental conditions.

Assessment of survival and death of dopaminergic neurons

Following MPP⁺ administration, we assessed surviving neurons at various time points by strip-counting TH-positive neurons in control and MPP⁺-treated wells ($n = 3$). To assess the percentage of apoptotic nuclei, we performed immunostaining for TH, as above, and then labeled the cells with the nuclear dye Hoechst 33342

(Sigma, 1 μ g/mL). Apoptotic nuclei were characterized as previously described (Stefanis *et al.* 1998, 1999; Rideout *et al.* 2001).

Western immunoblotting

Neurons were rinsed in ice-cold PBS and removed from the culture dish by scraping or trituration and then solubilized in sodium dodecyl sulfate (SDS) sample buffer containing 5% β -mercaptoethanol to generate total cell lysates. Proteins (10–25 μ g per lane) were separated by SDS–polyacrylamide gel electrophoresis (PAGE; 12%) and transferred to nitrocellulose membranes. The membranes were probed with mouse antisynuclein-1 (1 : 1000; Transduction Laboratories) or rabbit anti-TH (1 : 1000; Calbiochem). Protein bands were visualized with horseradish peroxidase conjugated secondary antibodies (Pierce, Rockford, IL, USA) and enhanced chemiluminescence (Pierce). To control for protein loading the membranes were stripped and re-probed with rabbit anti-ERK (1 : 5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Statistical analysis

Comparisons between groups were made by one-way ANOVA with Newman–Keuls *post-hoc* comparisons or Student's *t*-test unless otherwise noted. The level of significance was 0.05.

Results

The antisynuclein-1 monoclonal antibody specifically recognizes α -synuclein in cultured neurons

Throughout this study, we have used an antisynuclein-1 monoclonal antibody (Transduction Laboratories) to assess the expression of α -synuclein. This, in our experience, is the commercial antibody that gives the most specific labeling on western immunoblot analysis of neuronal rodent tissue. In order to verify that immunolabeling on western blots and fluorescent immunostaining of neuronal cultures using this antibody specifically represents α -synuclein, we have utilized cortical neuron cultures derived from synuclein null and control littermate mice (Dauer *et al.* 2002). Western immunoblots show that an 18-kDa band is specifically seen with this antibody in cortical neuron lysates derived from wild type, but not synuclein knock-out mice. Immunofluorescence images of cortical neuron cultures derived from α -synuclein heterozygotes or wild-type litter-mates show specific immunostaining in neuronal cells, which is absent in cultures derived from synuclein knock-out mice (data not shown).

α -Synuclein expression is developmentally regulated in cultured dopaminergic and cortical neurons

Up-regulation of α -synuclein mRNA and protein levels has been previously reported in the developing CNS of rodents (Hsu *et al.* 1998; Kholodilov *et al.* 1999a; Petersen *et al.* 1999), but results in neuronal cell culture systems have been conflicting (Withers *et al.* 1997; Murphy *et al.* 2000). Whether α -synuclein is regulated in cultures of ventral midbrain neurons has not, to our knowledge, been studied.

We have therefore used western immunoblotting and immunofluorescence to determine if α -synuclein expression changes in ventral midbrain GABAergic or dopaminergic neurons or cortical neurons with maturation in culture. We first used double immunofluorescence for TH or GABA and α -synuclein to assess the level of expression of α -synuclein in the ventral midbrain cultures derived from E14 rats. Following plating, most TH-positive dopaminergic neurons typically display very low, if not absent, expression of synuclein-1 by double immunofluorescence with TH (Figs 1a and b). However, with time, there was a significant increase in the percentage of TH-positive neurons that contain high levels of synuclein-1 immunoreactivity (Figs 1e and f). The pattern of α -synuclein immunoreactivity in TH-positive neurons was variable, but often staining was prominent within the cell body and punctate along cellular processes (Figs 1e and f). α -Synuclein immunostaining leveled off after 2–4 DIV and did not show any further increase during

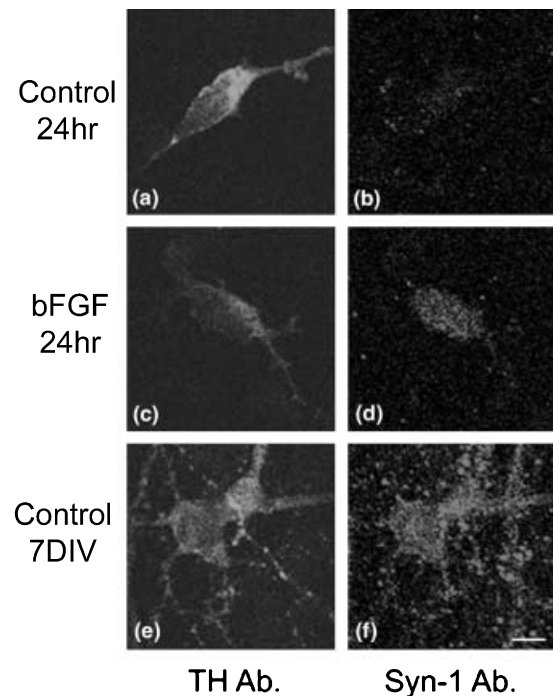


Fig. 1 Up-regulation of α -synuclein in ventral midbrain dopaminergic neurons with maturation or bFGF treatment. Cultures of ventral midbrain neurons grown on glass coverslips were untreated and fixed 24 h after plating (a and b), treated with bFGF (50 ng/mL) and fixed 24 h following plating (c and d), or left untreated and fixed 7 days following plating (e and f). Following fixation the neurons were double immunostained for TH and α -synuclein. Images were acquired using a 100 \times oil-immersion objective on a Zeiss LSM confocal microscope. Note the up-regulation in TH-positive neurons that occurred with maturation during the 7-DIV period or with bFGF treatment and the strong α -synuclein immunoreactivity present within the TH-positive neurites. Scale bar = 10 μ m.

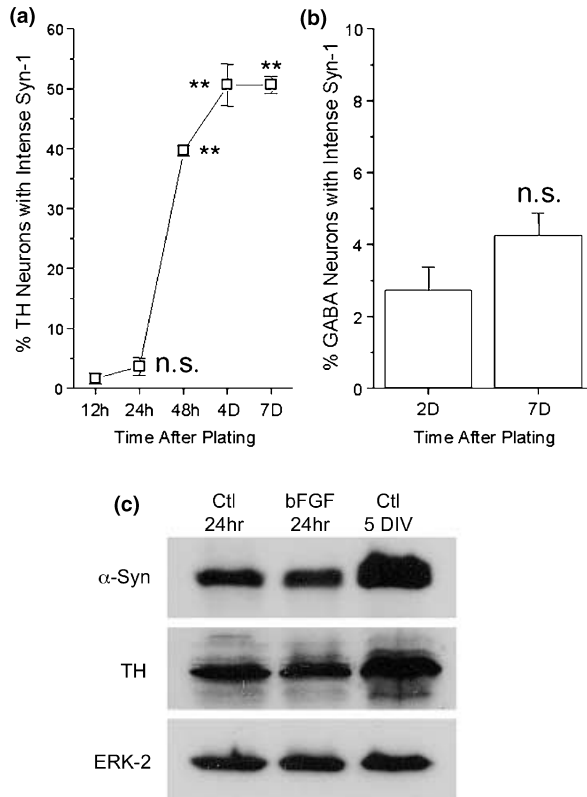


Fig. 2 Up-regulation of α -synuclein in ventral midbrain dopaminergic and cortical, but not GABAergic, neurons with maturation. (a) Neurons were fixed at the indicated times following plating and immunostained for TH and α -synuclein. In triplicate wells, the percentage of TH-positive neurons with intense α -synuclein immunoreactivity was counted. $**p < 0.001$ compared with control; n.s. not significant. (b) Neurons were fixed at the indicated times following plating and immunostained for GABA and α -synuclein. In triplicate wells, the percentage of GABA-positive neurons with intense α -synuclein immunoreactivity was counted by a rater blind to the experimental conditions. These results were verified in an additional experiment from independent cultures. (c) Total cell lysates from ventral midbrain neurons cultured in the presence or absence of bFGF (50 ng/mL) for 24 h or untreated for 5 DIV were separated by SDS-PAGE, and the membranes probed with mouse anti- α -synuclein, rabbit anti-TH, or rabbit anti-ERK2. Note the significant increase in α -synuclein levels in 5 DIV cultures.

the remaining 3 DIV of the time course examined (Fig. 2a). In contrast, GABAergic neurons, which comprise the primary neuronal subtype in these cultures, showed no up-regulation of α -synuclein over at least 7 DIV (Fig. 2b).

We then performed western immunoblotting to assess global changes in α -synuclein levels with maturation of ventral midbrain cultures and to identify the migrational pattern of α -synuclein labeling on SDS/PAGE gels. α -Synuclein in ventral midbrain lysates migrated as a broad band at the level of 18 kDa. There was a significant increase in α -synuclein levels with maturation in culture (Fig. 2c). At least in part this increase in α -synuclein levels is likely to be

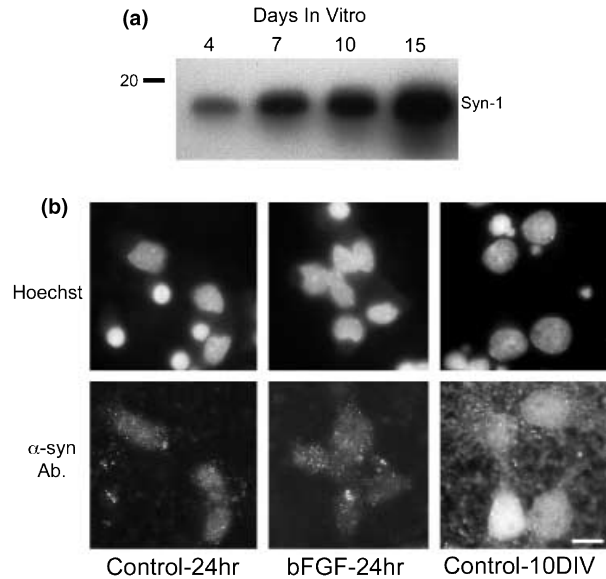


Fig. 3 Developmental up-regulation of α -synuclein in embryonic cortical neurons. (a) Embryonic cortical neurons were washed in ice-cold PBS at the indicated times following plating, and solubilized in $1 \times$ SDS sample buffer containing 5% β -mercaptoethanol and boiled for 10 min. Twenty-five μ g of protein from each sample was separated by SDS-PAGE (12%) and the membranes probed with the same monoclonal α -synuclein antibody used for immunofluorescence. (b) Cortical neurons were treated with vehicle or bFGF (50 ng/mL) for 24 h or left untreated for 10 DIV, fixed and immunostained for α -synuclein and counter-stained with the nuclear dye Hoechst. Note the dramatic increase in somal and neuritic α -synuclein in cortical neurons cultured for 10 days. Scale bar = 10 μ m.

due to the increased elaboration of processes of dopaminergic neurons with maturation, as reflected in the parallel induction of TH (Fig. 2c). Such processes are prominently immunoreactive for α -synuclein (see Figs 1e and f).

We also examined cultured embryonic cortical neurons for changes in α -synuclein immunoreactivity with maturation. Rat cortical neurons isolated from E18 embryos were allowed to mature for up to 15 DIV. There was a dramatic increase in levels of α -synuclein over time (Fig. 3a). This was confirmed also by immunofluorescence of cortical neurons, which showed a marked induction of α -synuclein immunostaining within the cell soma and along neuritic processes (Fig. 3b).

We find therefore that there is a maturational up-regulation of α -synuclein specifically within dopaminergic neurons of the ventral midbrain and within cortical neurons. In contrast, there is no maturational regulation in ventral midbrain GABAergic neurons.

bFGF induces an up-regulation of α -synuclein in dopaminergic neurons

We have previously shown that NGF and bFGF induce expression of α -synuclein in the dopaminergic PC12 cell

line, and that the presence of NGF is associated with higher α -synuclein expression in cultured rat sympathetic neurons of the peripheral nervous system (Stefanis *et al.* 2001b). There are no high affinity receptors for NGF in nigral neurons, and only a restricted population of neurons responds to NGF in the CNS (Yuen *et al.* 1996). bFGF, on the other hand, has functional effects on CNS neurons, and in particular in the substantia nigra (Ferrari *et al.* 1989; Otto and Unsicker 1990; Engele and Schilling 1996; Flores and Stewart 2000; Pierce and Bari 2001). The neurotrophic factor GDNF has specific effects on dopaminergic neurons of the CNS (Baloh *et al.* 2000). We therefore first tested whether bFGF or GDNF could modulate expression of α -synuclein in TH-positive ventral midbrain dopaminergic neurons. bFGF, applied at the time of plating at a concentration of 50 ng/mL for 24 h, significantly increased the percentage of TH-positive neurons displaying high α -synuclein immunofluorescence by a factor of 2–5, depending on the experiment (Figs 1c,d and 4a). This increase was not reflected on western immunoblotting (Fig. 2c), most likely because of the low percentage of dopaminergic neurons in our cultures, the relatively small magnitude of the effect and the lack of process extension from dopaminergic neurons at this time point. The effect of bFGF on the levels of α -synuclein was dose-dependent, and plateaued above 10 ng/mL (Fig. 4b). GDNF, at a dose previously shown to be protective for TH-positive neurons (10 ng/mL) (Burke *et al.* 1998), had no impact on α -synuclein expression (Fig. 4a). The fact that GDNF was biologically effective in the cultures was confirmed by remarkably increased arborization of TH-positive neurons after 3 days in culture, as previously reported (Burke *et al.* 1998) (data not shown).

We next determined the effects of bFGF on α -synuclein expression in dopaminergic neurons over a period of 7 days. Cultures treated with bFGF (50 ng/mL) displayed significantly greater α -synuclein expression at 12 and 24 h following plating, but subsequently showed similar increases in α -synuclein expression to untreated cultures. These data indicate that bFGF accelerates, but does not alter the final percentage of neurons expressing high levels of α -synuclein (Fig. 4c).

The bFGF-induced up-regulation of α -synuclein in nigral neurons is mediated at the transcriptional level, and does not involve glial proliferation

To investigate whether the increase of α -synuclein in bFGF-treated dopaminergic neurons was mediated at the transcriptional level, or whether it could involve post-translational mechanisms that may regulate α -synuclein levels (Bennett *et al.* 1999; Paxinou *et al.* 2001), we treated ventral midbrain cultures with the transcriptional inhibitor actinomycin D, together with bFGF. We found that this treatment abolished the induction of α -synuclein levels in dopaminergic neurons (Fig. 5a).

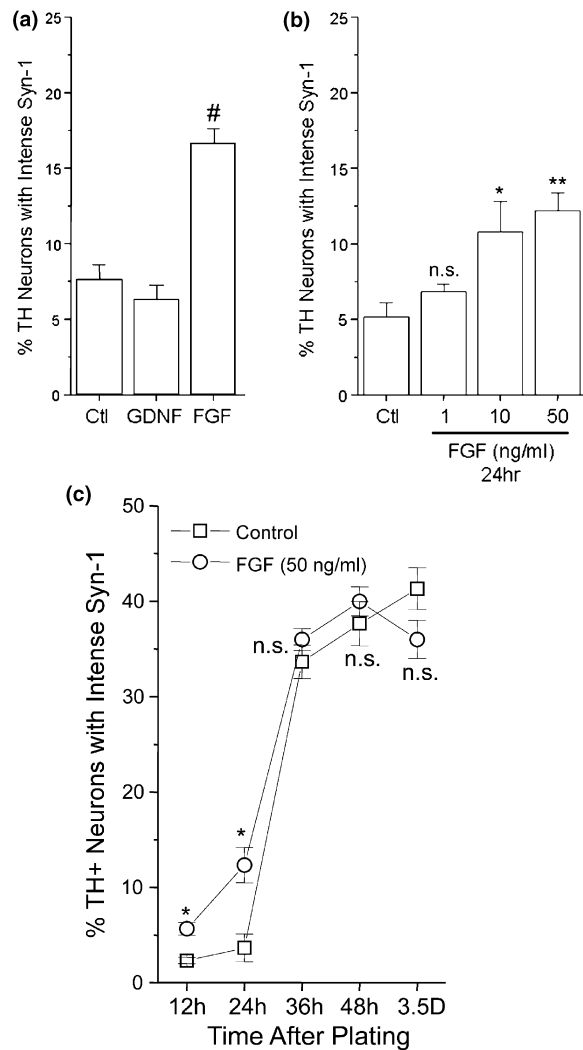


Fig. 4 Dose-dependent up-regulation of α -synuclein in ventral midbrain dopaminergic neurons with bFGF, but not GDNF, treatment. Embryonic ventral midbrain cultures were untreated, treated with GDNF (10 ng/mL), or treated with bFGF (50 ng/mL) (a) or treated with 1, 10, or 50 ng/mL bFGF (b) at the time of plating, fixed 24 h later and double immunostained for TH and α -synuclein. The percentage of TH positive neurons with intense α -synuclein immunoreactivity was determined from triplicate coverslips from two independent raters blinded to the experimental conditions. This experiment was repeated three times in independent cultures with similar results. * $p < 0.05$; ** $p < 0.01$; # $p < 0.001$ all compared with control. (c) Embryonic ventral midbrain cultures were untreated or treated with bFGF (50 ng/mL) at the time of plating, fixed at the indicated times following initial plating and double immunostained for TH and α -synuclein. The percentage of TH positive neurons with intense α -synuclein immunoreactivity was determined from triplicate coverslips from two raters blinded to the experimental conditions. * $p < 0.05$ compared with control. This experiment was repeated in independent cultures with similar results.

The survival-promoting effects of bFGF in ventral midbrain cultures are thought to occur via glial proliferation (Engele and Bohn 1991; Park and Mytilineou 1992;

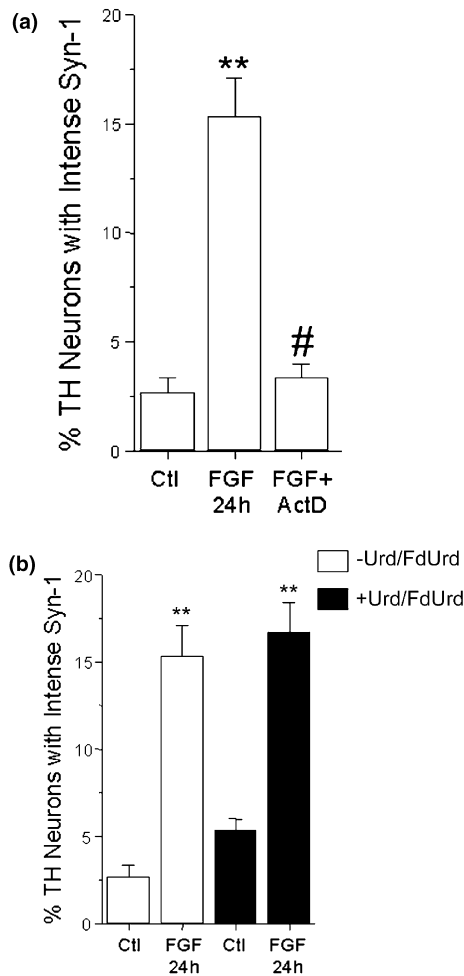


Fig. 5 The up-regulation of α -synuclein by bFGF is dependent on transcription, and is not mediated by glial proliferation. (a) Embryonic ventral midbrain cultures were untreated, treated with bFGF (50 ng/mL), or treated with bFGF plus the transcriptional inhibitor actinomycin D (10 μ M) at the time of plating, fixed at the indicated times following initial plating and double immunostained for TH and α -synuclein. (b) Embryonic ventral midbrain cultures were plated in the absence or presence of the mitotic inhibitors uridine (20 μ M) and fluorodeoxyuridine (8 μ M), denoted Urd/FdUrd, with or without bFGF (50 ng/mL), fixed 24 h later and double immunostained for TH and α -synuclein. The percentage of TH positive neurons with intense α -synuclein immunoreactivity was determined from triplicate coverslips by a rater blinded to the experimental conditions. ** $p < 0.001$ compared with control; # $p < 0.001$ compared with FGF alone. This experiment was repeated in independent cultures with similar results.

Kriegelstein *et al.* 1998). It seemed unlikely that induction of α -synuclein levels by bFGF represented such an indirect effect, because of the time course of the induction, which occurs rapidly, within 12 h (see Fig. 4c). At this time point no notable proliferative effects can be seen by bFGF administration. Nevertheless, in order to further test this possibility, we treated the cultures with a combination of

bFGF and the antimetabolic agents uridine/fluorodeoxyuridine (Park and Mytilineou 1992). We found no difference in the induction of α -synuclein by bFGF in the presence of the antimetabolic agents 24 h after plating compared with neurons treated with bFGF alone (Fig. 5b). Proliferation of GFAP-positive midbrain astrocytes induced by bFGF was inhibited by coadministration of the anti-metotics (data not shown).

We conclude that the induction of α -synuclein levels by bFGF in dopaminergic neurons is transcriptionally mediated, and that it is not dependent on glial proliferation. It therefore likely represents a direct induction of α -synuclein mRNA levels in dopaminergic neurons by bFGF.

bFGF does not modulate α -synuclein expression in GABAergic or cortical neurons

To determine if the effects of bFGF were specific for dopaminergic neurons, we next examined the ability of bFGF to modulate α -synuclein expression in cortical neurons or in the predominant neuronal subtype in ventral midbrain cultures, GABAergic neurons. bFGF, at 50 ng/mL added for 12–48 h, had no significant effect on α -synuclein levels in cortical neurons determined by western immunoblotting (Fig. 6a). This was also confirmed by immunofluorescence (see Fig. 3b). Additionally, double-immunostaining for GABA and synuclein-1 showed that bFGF had no effect on α -synuclein expression in GABAergic neurons when applied for 24 h, a time point at which the effect was maximal on dopaminergic neurons (Figs 6b and c). These data indicate that bFGF specifically modulates α -synuclein expression in dopaminergic neurons of the substantia nigra, and not in ventral midbrain GABAergic or cortical neurons.

Exposure to the dopaminergic mitochondrial toxin, MPP⁺, does not increase dopaminergic α -synuclein levels

Vila *et al.* (2000) recently showed that systemic MPTP administration in mice, at concentrations that reduce the number of TH-positive dopaminergic neurons in the SNpc, induced an up-regulation in α -synuclein levels in surviving dopaminergic neurons. This up-regulation could be mediated by a direct effect of MPP⁺ on dopaminergic neurons, or by a secondary, indirect response involving other cells within the nigrostriatal axis. Therefore we exposed ventral midbrain cultures two days after plating to the active metabolite of MPTP, MPP⁺, fixed the cells 24 h later and immunostained them for TH and α -synuclein. Counts of TH-positive neurons revealed an approximate 40% drop in MPP⁺ (1 μ M; 24 h) treated cultures compared with control (Fig. 7a, left panel). This was accompanied by morphological evidence of apoptotic death, suggesting that the decrease in TH-positive neurons did not just represent phenotypic down-regulation, but rather dopaminergic neuron degeneration (data not shown). As reported by Vila *et al.* (2000), TH-positive neurons with apoptotic nuclei, displayed very low, if not absent, levels of α -synuclein. We have had similar findings in

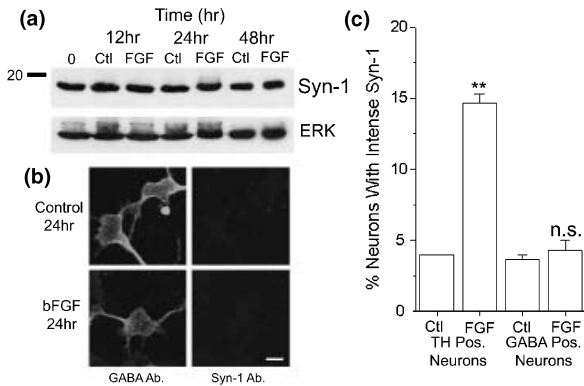


Fig. 6 bFGF does not regulate α -synuclein levels in cortical neurons or in ventral midbrain GABAergic neurons. (a) Embryonic cortical neurons were untreated or treated with bFGF (50 ng/mL) at the time of plating, and at the indicated times washed in ice-cold PBS and solubilized in $1 \times$ SDS sample buffer containing 5% β -mercaptoethanol and boiled for 10 min. Separated proteins were probed with mouse anti- α -synuclein antibody (upper panel). As a control for protein loading, the membranes were stripped and re-probed with rabbit anti-ERK2 (lower panel). Note the absence of any change in the levels of the 18-kDa α -synuclein species following treatment with bFGF. (b) Embryonic midbrain cultures were untreated or treated with bFGF (50 ng/mL) at the time of plating, fixed 24 h later and double immunostained for TH and α -synuclein or GABA and α -synuclein. Confocal images were acquired using the same exposure and contrast settings as those for TH and α -synuclein. The percentage of TH or GABA positive neurons with intense α -synuclein immunoreactivity was determined from triplicate coverslips by a rater blinded to the experimental conditions (c). ** $p < 0.001$ compared with control; n.s. not significant. This experiment was repeated in independent cultures with similar results.

cultures of trophic factor-deprived PC12 cells and sympathetic neurons (Stefanis *et al.* 2001b). However, in contrast to Vila *et al.* (2000), the percentage of remaining TH-positive dopaminergic neurons with bright α -synuclein immunoreactivity was not different from control cells (Fig. 7a, right panel). In order to ensure that the lack of α -synuclein induction by MPP⁺ was not due to a 'ceiling' effect due to maximal expression of α -synuclein by 3 DIV (see Figs 2 and 3), we also administered MPP⁺ to ventral midbrain cultures 2 h after plating, and assessed α -synuclein immunostaining 24 h later, at a time when the percentage of α -synuclein-positive dopaminergic neurons had not yet reached a plateau. Again, no induction of α -synuclein levels was seen (Fig. 7b), even though apoptotic death occurred in dopaminergic neurons (approximately 10% apoptosis). In parallel, as a positive control, we verified that in this set of cultures there was an induction of α -synuclein immunostaining with bFGF (Fig. 7b).

Therefore, we conclude that at doses that cause selective loss of cultured embryonic rat dopaminergic neurons and

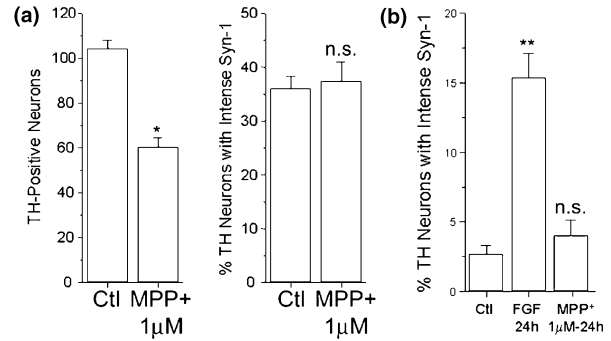


Fig. 7 Neurotoxins do not up-regulate α -synuclein levels in cultured ventral midbrain dopaminergic or cortical neurons. (a) At DIV2, embryonic ventral midbrain neurons were either untreated or treated with MPP⁺ (1 μ M), fixed 24 h later and double immunostained for TH and α -synuclein. Some parallel cultures were additionally counterstained with either Hoechst or YOYO to visualize apoptotic nuclei. The center strip from three parallel coverslips was visualized using a $40 \times$ objective and standard epifluorescence and assessed for the total number of remaining TH-positive neurons (left panel) by a rater blinded to the treatment conditions. The percentage of TH positive neurons with intense α -synuclein immunoreactivity (right panel) was then determined from triplicate coverslips. (b) Ventral midbrain neurons were either untreated or treated with MPP⁺ (1 μ M), added approximately 2 h following plating to allow for sufficient attachment of the neurons to the substrate, fixed 24 h later and double immunostained for TH and α -synuclein. Parallel cultures were additionally counterstained with either Hoechst or YOYO to visualize apoptotic nuclei. The percentage of TH positive neurons with intense α -synuclein immunoreactivity was then determined from triplicate coverslips by a rater blinded to the experimental conditions. * $p < 0.01$ compared with control; ** $p < 0.001$ compared with control; n.s., not significant. These experiments were repeated in independent cultures with similar results.

apoptotic death, MPP⁺ does not induce an up-regulation of α -synuclein levels.

Discussion

α -Synuclein is up-regulated in cultured ventral midbrain dopaminergic neurons with maturation

In this work, we have examined the regulation of α -synuclein expression in cultured primary neurons of the CNS. We find a specific up-regulation of α -synuclein expression in TH-positive neurons of ventral midbrain cultures with maturation over 7 DIV. This up-regulation, while dramatic, increasing from less than 5% to between 50 and 60% of dopaminergic neurons, levels off after 2–4 days, and there remains a considerable percentage of these neurons that never show appreciable α -synuclein immunostaining, at least within the period of 7 DIV. Within this time frame, we did not observe a complete redistribution of α -synuclein immunoreactivity to nerve terminals, in the absence of

specific cell soma staining, as was the case in a study with cultured hippocampal neurons (Withers *et al.* 1997). It is unlikely therefore that the lack of α -synuclein immunostaining of TH-positive neurons was due to such a complete redistribution of immunoreactivity away from the cell soma. These results suggest that there are two subpopulations of cultured midbrain dopaminergic neurons, one with high, and the other with low α -synuclein levels. If the existence of two such subpopulations of dopaminergic neurons is confirmed *in vivo*, this may have relevance to issues of selective vulnerability. In human SNpc, dopaminergic neurons of the ventral tier have relatively higher levels of α -synuclein mRNA (Solano *et al.* 2000). Interestingly, these neurons are selectively vulnerable in PD (Gibb and Lees 1991). In addition, subpopulations of SNpc neurons in developing (Kholodilov *et al.* 1999a, 1999b) and adult (Vila *et al.* 2000; Mori *et al.* 2002) rodents express appreciable immunoreactivity at the level of the cell soma.

The up-regulation of α -synuclein in fetal cortical and ventral midbrain dopaminergic neurons with maturation in culture correlates with what is observed *in vivo*: mRNA and protein levels of α -synuclein increase during the late embryonic/early postnatal phase in brain and in substantia nigra (Hsu *et al.* 1998; Kholodilov *et al.* 1999a; Petersen *et al.* 1999). Of note, when we derived ventral midbrain cultures from E16 rat pups, the percentage of α -synuclein-positive dopaminergic neurons approached the plateau of 40–60% within only 24 h of plating (data not shown). This further validates our approach, and suggests that the mechanisms of regulation of α -synuclein levels observed in our cell culture system are relevant to what occurs in the *in vivo* setting.

α -Synuclein is up-regulated specifically in cultured ventral midbrain dopaminergic neurons with bFGF treatment

The main finding in our study is that the developmental up-regulation of α -synuclein expression in ventral midbrain dopaminergic neurons was accelerated specifically by bFGF. This raises the possibility that maturational up-regulation of α -synuclein is, at least in part, due to the release of diffusible factors, such as bFGF. bFGF could be produced by dopaminergic midbrain neurons or by the few glia present in our cultures (Unsicker *et al.* 1993). Alternatively, maturational up-regulation of α -synuclein may occur because of diffusible signals that neurons have already received prior to tissue dissection, or because of a cell-autonomous regulation program.

In ventral midbrain cultures, both developmental and bFGF-induced up-regulation of α -synuclein appear to be restricted to dopaminergic neurons. GABAergic neurons showed no such induction of α -synuclein. The overall low percentage of GABAergic neurons that were α -synuclein-positive is consistent with studies in developing rats

(Kholodilov *et al.* 1999a,b) and humans (Solano *et al.* 2000), which show little α -synuclein expression in SN pars reticulata. Cultures of embryonic cortical neurons, which contain many neuronal subtypes, did not show an induction of α -synuclein in response to bFGF treatment, however, a marked induction was seen with maturation. It is possible that growth factors distinct from bFGF may modulate α -synuclein expression in cortical neurons.

The time course of induction of α -synuclein levels with bFGF, in conjunction with the attenuation of such induction with transcriptional inhibition and the lack of attenuation with antimetabolic agents, suggest that this effect is mediated directly in dopaminergic neurons, at the transcriptional level. Because TH-positive dopaminergic neurons comprise only 2–5% of the total cellular population under the culture conditions we employ here, it is not feasible to assess changes in levels of α -synuclein mRNA in response to bFGF treatment in these cultures. We have previously shown that such transcriptional induction occurs in PC12 cells treated with NGF or bFGF (Stefanis *et al.* 2001b). bFGF, apart from its trophic effects, which appear to be secondary to glial proliferation (Engele and Bohn 1991; Park and Mytilineou 1992; Kriegstein *et al.* 1998), exerts direct effects on cultured ventral midbrain neurons, inducing an immediate early gene response (Engele and Schilling 1996). Our findings suggest that the α -synuclein induction we observe may represent a similar direct response. There are high levels of the high affinity receptor for bFGF (FGFR1) in dopaminergic neurons of the rodent SNpc (Wanaka *et al.* 1990; Gonzalez *et al.* 1995), and functional uptake of bFGF has been demonstrated within these neurons (Ferguson and Johnson 1991). Given that the survival-promoting effects of bFGF appear to be indirect, the functional effects of signaling through FGFR1 in dopaminergic neurons are unclear. bFGF, and other growth factors, apart from their survival-promoting effects, also influence neuronal plasticity, perhaps by modulating neurotransmission (Abe *et al.* 1990; Terlau and Seifert 1990; Schuman 1999; Flores and Stewart 2000; Pierce and Bari 2001). We propose that the induction of α -synuclein by bFGF may represent part of such a direct plasticity response, mediated through FGFR1.

α -Synuclein mRNA is increased fourfold in neuronally differentiated PC12 cells (Stefanis *et al.* 2001b), and this increase is associated with a reduction of dopamine release (Greene and Rein 1977). A fourfold over-expression of wild-type α -synuclein in PC12 cells leads to a significant decrease of dopamine release, but not dopamine content (Stefanis *et al.* 2001a; Larsen *et al.* 2002). Studies in α -synuclein mice (Abeliovich *et al.* 2000) also support the idea that α -synuclein acts as a negative regulator of dopamine release. We therefore suggest that the increase of α -synuclein expression by bFGF is functionally significant, and could potentially influence dopamine release from cultured ventral midbrain neurons. Lesion models of the SNpc in rodents are consistent

with the idea that bFGF may play a role in α -synuclein regulation *in vivo*. Excitotoxin injection in the striatum of developing rats induces secondary apoptotic death of SNpc dopaminergic neurons, and up-regulation of α -synuclein in surviving SNpc neurons (Kholodilov *et al.* 1999a). In this paradigm, striatal neurons, which contain moderate levels of bFGF (Gonzalez *et al.* 1995) die, and may release bFGF, which would then be available to nigrostriatal terminals for retrograde transport. In contrast, a striatal 6-OHDA lesion in the same animals does not up-regulate α -synuclein levels in the SNpc, despite inducing apoptotic death (Kholodilov *et al.* 1999b). 6-OHDA does not damage striatal neurons, and therefore would not be expected to induce bFGF release. A chronic, but not acute, regimen of MPTP also induces α -synuclein in surviving SNpc neurons (Vila *et al.* 2000). A similar regimen has been shown to induce bFGF production in striatal glia (Leonard *et al.* 1993; Rufer *et al.* 1996), which, if released, may act on dopaminergic neurons with intact nigrostriatal projections to up-regulate α -synuclein. Further experiments are needed to test the hypothesis that bFGF may be, at least in part, responsible for α -synuclein mRNA and protein induction in such *in vivo* models.

No effect on α -synuclein levels with neurotoxin administration

It has been suggested that α -synuclein may be involved in apoptotic pathways, because it was up-regulated selectively in settings of apoptotic death (Ostrerova *et al.* 1999; Vila *et al.* 2000), and it interacted with Bad, a pro-apoptotic member of the bcl-2 family (Ostrerova *et al.* 1999). Our present and previous (Stefanis *et al.* 2001b) results, however, argue that, at least in the cell culture systems that we have tested, there is no direct relationship between apoptotic death and induction of α -synuclein levels. As mentioned, Vila *et al.* (2000) found that a systemic chronic MPTP regimen in mice induced α -synuclein levels in nigral dopaminergic neurons. Our results raise the possibility that this up-regulation is not a direct effect of the neurotoxin on dopaminergic neurons, but rather a secondary, indirect effect mediated by striatal glia. Alternatively, the discrepancy between our findings and those of Vila *et al.* (2000) may be due to species differences, developmental stage, or to other factors present in the mouse nigrostriatal axis that are not present in our isolated cell culture system.

Implications for Parkinson's disease

Given the importance of α -synuclein in PD, the regulation of its levels may be critical for disease pathogenesis. Over-expression of wild-type human α -synuclein in various cell lines has been reported to be toxic or to enhance susceptibility to certain insults (Ostrerova *et al.* 1999; Saha *et al.* 2000; Zhou *et al.* 2000; Lee *et al.* 2001). Moreover, mice and flies overexpressing wild-type α -synuclein show dopaminergic deficits and inclusion formation (Masliah *et al.*

2000; Feany and Bender 2000). On the other hand, there is also evidence that low α -synuclein levels may be associated with PD. Lymphocytes derived from patients harboring the A53T mutation showed very low or absent levels of α -synuclein mRNA compared with controls (Markopoulou *et al.* 1999). In addition, a relative reduction of α -synuclein mRNA was found in the SN of PD patients (Neystat *et al.* 1999). In certain settings, over-expression of wild-type α -synuclein has been reported to be protective against toxic stimuli, suggesting that its absence may prove deleterious (da Costa *et al.* 2000). Another indication that regulation of α -synuclein levels may be important for PD is that haplotypes in the noncoding promoter region of α -synuclein, which may influence the level of mRNA expression, are differentially represented in certain cohorts of PD patients compared with controls (Kruger *et al.* 1999; Chiba-Falek and Nussbaum 2001).

Interestingly, in view of our findings with regulation of α -synuclein levels by bFGF, loss of bFGF has been reported in PD SN (Tooyama *et al.* 1993). This could lead to a decrease of α -synuclein levels. On the other hand, a variety of injuries can lead to increased bFGF (Logan *et al.* 1992; Takami *et al.* 1992; Leonard *et al.* 1993; Rufer *et al.* 1996), and potentially to a secondary increase of α -synuclein in the SN.

In conclusion, we show here for the first time that growth factors, and in particular bFGF, regulate the expression of α -synuclein specifically in developing ventral midbrain dopaminergic neurons, the primary target of PD. Such regulation may play a role in the plasticity-mediating effects of growth factors, through control of neurotransmitter release, or in diseases, such as PD, that may critically depend on α -synuclein levels of expression.

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