

SHORT COMMUNICATION

Lack of α -synuclein does not alter apoptosis of neonatal catecholaminergic neurons

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Abstract

α -Synuclein is an abundant neuronal protein of uncertain function linked to Parkinson's disease. Numerous studies have proposed an antiapoptotic function for α -synuclein, based on overexpression experiments in cell lines. To explore whether α -synuclein has such a physiological function, we assessed the response of wild type or α -synuclein null neonatal mouse sympathetic neurons to nerve growth factor deprivation, a well-characterized stimulus of neuronal apoptosis. There was no difference in the rate of neuronal loss, neuronal apoptosis, or c-jun phosphorylation. Furthermore, the absence of α -synuclein did not alter the magnitude of naturally occurring cell death *in vivo* in substantia nigra pars compacta. Therefore, α -synuclein is unlikely to play a significant role in apoptotic signalling in catecholaminergic neurons of the neonatal nervous system.

Introduction

α -Synuclein is a protein implicated in the pathogenesis of Parkinson's disease (PD). Point mutations or gene triplication of the α -synuclein locus lead to autosomal dominant PD, presumably through a gain of function. In addition, α -synuclein is a prominent component of Lewy bodies (LBs) and has a propensity to fibrillize *in vitro*, suggesting that it may be the protein responsible for LB formation (reviewed in Vekrellis *et al.*, 2004).

The physiological function of α -synuclein is unclear, but it may be involved in control of synaptic release, through actions at the level of presynaptic vesicles (reviewed in Vekrellis *et al.*, 2004). A number of studies suggest that α -synuclein may also participate in apoptotic pathways: α -synuclein is up-regulated in certain *in vivo* models of substantia nigra pars compacta (SNpc) injury (Kholodilov *et al.*, 1999; Vila *et al.*, 2000; Manning-Bog *et al.*, 2002). It is unclear however, whether this up-regulation represents participation of α -synuclein in SNpc apoptotic pathways, an unrelated response to injury or an attempt to up-regulate antiapoptotic responses. Consistent with the latter possibility, a number of studies have shown that overexpression of wild-type (WT) α -synuclein protects from apoptotic death in a variety of cell cultures and *in vivo* settings (da Costa *et al.*, 2000; Lee *et al.*, 2001; da Costa *et al.*, 2002; da Costa *et al.*, 2003; Hashimoto *et al.*, 2002; Seo *et al.*, 2002; Manning-Bog *et al.*, 2003). In many cases, the mutant forms lack such protective effects, suggesting that, at

least in part, they may confer a loss of the antiapoptotic function of α -synuclein.

Opposite effects of α -synuclein on apoptotic pathways have also been reported. Antisense-induced down-regulation of α -synuclein was associated with reduced sensitivity to serum deprivation-induced apoptosis of 293 HEK cells; these authors argued that α -synuclein mediated apoptosis, possibly through interactions with the pro-apoptotic protein Bad (Ostrerova *et al.*, 1999).

To explore further the relationship between α -synuclein and apoptosis, in the current work we have studied the susceptibility of WT and α -synuclein null catecholaminergic neurons to well-characterized paradigms of apoptotic cell death. Importantly, we have assessed, both in cell culture and *in vivo*, neuronal populations that display selective vulnerability in PD. Accordingly, we have assessed nerve growth factor (NGF) deprivation of cultured mouse sympathetic neurons and developmental cell death in the mouse SNpc, and we have asked the question whether lack of α -synuclein alters these classical neuronal apoptotic pathways.

Materials and methods

Mice and genotyping

The generation of α -synuclein null mice has been previously described (Dauer *et al.*, 2002). Genotyping was performed by PCR on DNA derived from mouse tails, using the following primers: SYN UPPER (TCACACTTACACCAGGACTTGG), SYN LOWER (GTCCCTGTTGTTTCTGAGAGC), NEO LOWER (ATGGAAGGATTGGAG

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CTACGGG). The PCR products were a 450-base pair band from WT mice, and a 600-base pair band from the knock out (KO) mice. All procedures were approved by the Institutional Animal Care Committee of Columbia University.

Culture and assessment of mouse sympathetic neurons

Cultures were performed essentially as described for rat sympathetic neurons (Stefanis *et al.*, 2001). Sympathetic ganglia were dissected from postnatal day (PND) 0–1 mouse pups after decapitation. Ganglia from each pup were cultured separately in six wells of a 24-well collagen-coated plate and cultured as reported (Stefanis *et al.*, 2001). One day after plating, uridine and 5-fluorodeoxyuridine (20 μ M each) were added to minimize proliferation of glial cells. NGF deprivation was performed as previously described (Stefanis *et al.*, 2001). To assess survival, an examiner blinded to the genotype and the experimental conditions performed strip-counting of phase-bright sympathetic neurons in a central strip of the cultures 0, 24 and 48 h after NGF deprivation. Phase-bright round-shaped sympathetic neurons were easily distinguished from the darker heterogeneously shaped glial cells, which represented less than 5% of the cell population. Results at 24 and 48 h are reported relative to the number of neurons present at time 0, and are the mean \pm SEM.

Parallel sets of cultures were fixed with 3.7% paraformaldehyde at 4 $^{\circ}$ C for 20 min, and then immunostained with mouse antisynuclein 1 (Transduction Laboratories, 1 : 50) or rabbit antiphospho-c-Jun (targeted to phosphorylated c-jun at serine 63, Cell Signalling, 1 : 500) and counterstained with Hoechst 33342 (1 μ g/mL, Sigma), using previously described procedures (Stefanis *et al.*, 2001). At least 100 neurons in each well were assessed for nuclear apoptosis or phospho-c-Jun positivity. Nuclear apoptosis was defined as nuclear compaction, with chromatin clumping or margination. The results are reported as mean \pm SEM.

Assessment of apoptosis in SNpc

The magnitude of apoptosis due to natural cell death in dopaminergic neurons of the SNpc was assessed in littermate PND2 pups of all genotypes by first performing immunohistochemistry for tyrosine hydroxylase (TH) and then performing a thionin counterstain to identify characteristic apoptotic nuclear clumps. PND2 mice were perfused through the left cardiac ventricle with 4% paraformaldehyde/0.1 M PB. The brains were removed, postfixed and cryoprotected as previously described (Kholodilov *et al.*, 2004). Frozen sections were cut at 20 μ m and processed free-floating. They were treated with a rabbit anti-TH (Calbiochem) at 1 : 1000, then with biotinylated Protein A, and then with avidin-biotinylated-horseradish peroxidase complexes (ABC, Vector Laboratories) at 1 : 600 for one hour as described (Kholodilov *et al.*, 2004). After incubation with diaminobenzidine, sections were mounted onto subbed slides and counterstained with thionin. An assessment of the number of apoptotic profiles in each brain was performed by scanning the entire SN in each of three sections representative of the anterior, central and posterior regions of the SN at 600 \times . The number of apoptotic profiles in each of the three sections for a given region were averaged, to provide a measure for that region, and then these values for each of the three regions were added to provide an index of the number of apoptotic profiles for each brain. The criteria used to identify apoptotic profiles among dopaminergic neurons were as previously described (Oo & Burke, 1997; Oo *et al.*, 2003).

Statistics

Sympathetic neuron cultures

Statistical differences between genotypes were assessed by Student's *t*-test.

SNpc in vivo

Differences between genotypes were analysed by the use of one way ANOVA (SigmaStat).

Results

Lack of α -synuclein does not lead to alterations in NGF deprivation-induced apoptosis of mouse sympathetic neurons

We have previously reported that α -synuclein is robustly expressed in cultured neonatal rat sympathetic neurons (Stefanis *et al.*, 2001). Prominent α -synuclein immunoreactivity also occurred in the cell soma and the neurites of neonatal mouse sympathetic neurons. Immunoreactivity for α -synuclein was present in the nucleus, consistent with our previous results in cultured neuronal PC12 cells, rat sympathetic neurons and rat cortical neurons (Stefanis *et al.*, 2001; Rideout *et al.*, 2003), and those of others in *in vivo* settings (Mori *et al.*, 2002; Goers *et al.*, 2003). No immunoreactivity was seen in α -synuclein null cultures, confirming the specificity of the immunostaining (Fig. 1).

Having determined that α -synuclein was expressed in neonatal mouse sympathetic neurons, we assessed whether the lack of α -synuclein in these neurons would influence the magnitude of death following NGF deprivation. NGF deprivation of rat or mouse sympathetic neurons is a classical paradigm of neuronal cell death that mimics the developmental programmed cell death that occurs *in vivo* due to a limited supply of trophic support. It has therefore been used extensively as a paradigm of neuronal programmed cell death (Deshmukh & Johnson, 1997; Stefanis *et al.*, 2001). We found that WT and α -synuclein null neurons display a similar degree of NGF deprivation-induced survival (Fig. 2A) or apoptotic death, as assessed by nuclear morphological criteria (Figs 2B and 3).

Activation of c-Jun kinase (JNK) and subsequent phosphorylation and activation of the transcription factor c-Jun are required features of NGF deprivation-induced death of sympathetic neurons (Maroney *et al.*, 1999; Eilers *et al.*, 2001). One report suggests that the putative

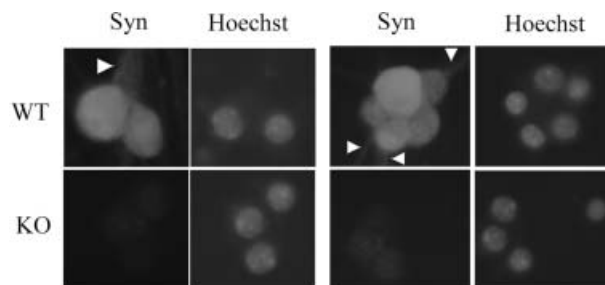


FIG. 1. α -Synuclein is prominently expressed in the cell soma and neurites of cultured mouse sympathetic neurons. Neonatal mouse sympathetic neurons derived from α -synuclein null (knock-out, KO) or WT mice were cultured for 3 days, and then immunostained with a synuclein-1 antibody (Transduction Laboratories), and counterstained with Hoechst 33342. Fluorescent images were obtained with an inverted Leica DM IRB microscope equipped with a digital SPOT camera. Representative images are shown. The arrow heads point to neuritic processes that are labelled with the Syn-1 antibody.

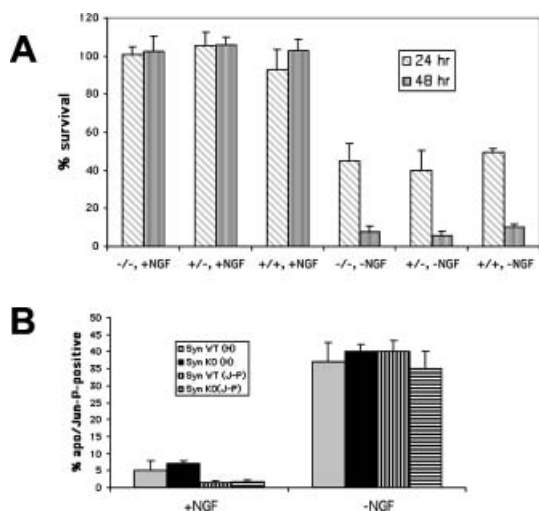


FIG. 2. Lack of α-synuclein does not lead to alterations in survival, apoptosis, or c-Jun phosphorylation following NGF deprivation. (A) Neonatal mouse sympathetic neurons derived from α-synuclein KO (-/-), heterozygote (+/-) or WT (+/+) mice were cultured for 3 days, and then deprived of NGF (-NGF), or maintained in NGF (+NGF) for 24–48 h. Survival was assessed by strip-counting phase-bright neurons in a defined area of the dish in each condition and is reported as the mean ± SEM (n = 9 independent wells for -/-, n = 6 for +/-, and n = 3 for +/+, derived, respectively, from three, two and one mouse). (B) Twenty-four hours after NGF deprivation, cultures were fixed and immunostained with phospho-c-Jun antibody and counterstained with Hoechst. The percentage of apoptotic (by nuclear criteria) and phospho-c-Jun-positive neurons was assessed in each condition, and is reported as the mean ± SEM (n = 6 independent wells for KO and n = 4 for WT, derived, respectively, from three and two mice). There were no statistical differences amongst genotypes for any of the parameters studied (Student's *t*-test).

TABLE 1. Natural cell death in dopamine neurons of the substantia nigra in wild-type and α-synuclein null mice

Genotype	Mice (n)	Apoptotic profiles (n)
WT	4	5.7 ± 1.8
Heterozygous	4	4.4 ± 1.4
α-synuclein null	6	3.9 ± 0.7

Data are presented as mean ± SEM.

survival-promoting effects of α-synuclein are due to an inhibition of JNK, possibly through activation of JIP-1b/IB1 (Hashimoto *et al.*, 2002). We therefore also assessed c-Jun phosphorylation following NGF deprivation in cultures derived from wild-type or knock-out mice. Again, we found no significant difference in this measure between the WT and α-synuclein null cultures (Fig. 2B). Representative fluorescence images of the cultures are shown in Fig. 3.

Lack of α-synuclein does not lead to significant changes in programmed cell death of SNpc

To confirm the relevance of our cell culture findings in an *in vivo* setting, we assessed the effect of the absence of α-synuclein on the apoptosis of developing midbrain dopaminergic neurons. These neurons undergo apoptotic natural cell death, which peaks at PND2 (Oo & Burke, 1997). There was no significant difference in apoptosis of SNpc dopaminergic neurons between WT, heterozygous and null α-synuclein mice (Table 1, ANOVA *P*-value = 0.6).

Discussion

We have explored whether α-synuclein participates directly in apoptotic pathways, using two classical models of neuronal programmed cell death, NGF deprivation of cultured neonatal sympathetic neurons and naturally occurring cell death in the SNpc. α-Synuclein null neurons did not show altered susceptibility to these apoptotic stimuli, compared to wild-type or heterozygote mice. Our results therefore suggest that α-synuclein is neither a proapoptotic nor an antiapoptotic mediator in the nervous system.

Our results are consistent with the lack of difference in the morphology or cell number of SNpc neurons in the α-synuclein null mice, when compared to the WT (Abeliovich *et al.*, 2000; Cabin *et al.*, 2002; Dauer *et al.*, 2002). They are also consistent with our previous observation that overexpression of human WT α-synuclein in PC12 cells did not lead to any change in serum deprivation-induced apoptosis, which shares common elements with the NGF deprivation paradigm shown here (Stefanis *et al.*, 2001).

Our results, although clear, cannot be construed as definitive. It is possible that compensatory responses are triggered with the germline lack of α-synuclein that lead to alterations in other modulators of neuronal apoptotic pathways, thus obscuring potential differences. However, we believe that this possibility is unlikely, given that no compensatory changes occur in the highly homologous β- and γ-synucleins in the null mice (Abeliovich *et al.*, 2000). Another possibility is that human α-synuclein has a role in apoptotic pathways, but its mouse homologue does not. We believe that this possibility is also unlikely, given the high degree of homology across these species (Clayton & George, 1998). It should be noted that minor differences do exist between homologues, for example

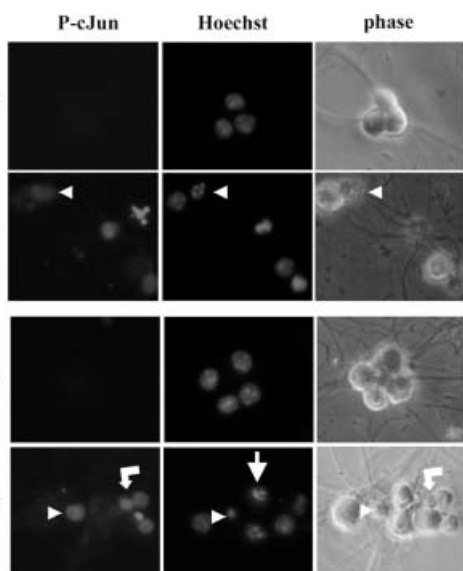


FIG. 3. Representative images of NGF-maintained or NGF-deprived, WT or α-synuclein KO sympathetic neurons. Neonatal mouse sympathetic neurons derived from α-synuclein KO or WT mice were cultured for 3 days, and then deprived of NGF (-NGF), or maintained in NGF (+NGF) for 24 h. Cultures were then fixed and immunostained with phospho-c-Jun antibody and counterstained with Hoechst. Representative fluorescence and phase photomicrographs are shown. Arrow heads depict representative phospho-c-Jun-positive neurons that are also apoptotic. Note that some neurons are apoptotic without phospho-c-Jun positivity (arrows), whereas others are phospho-c-Jun positive, but have lost nuclear staining (angled arrows).

mouse α -synuclein exhibits faster fibrillization than its human homologue at high concentrations (Rochet *et al.*, 2000).

How can we then explain the previously reported studies, which, for the most part, support an antiapoptotic function for wild-type α -synuclein? It is noteworthy, that all these studies are based on overexpression of α -synuclein, which may lead to nonphysiological levels that have little relevance to its physiological function. In addition, most studies have been performed in cell lines, with a variety of apoptotic stimuli, and thus may not be applicable to the function of α -synuclein in the developing postnatal nervous system. Importantly, our study has examined the possible role of α -synuclein in apoptotic pathways in catecholaminergic neurons that are lost in PD. Therefore, while overexpressed α -synuclein may modulate the response to apoptotic stimuli in select cell lines, this does not appear to occur in the neuronal cell types most relevant to PD.

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Abbreviations

KO, knock out; NGF, nerve growth factor, PD, Parkinson's disease; PND, postnatal day; SNpc, substantia nigra pars compacta; WT, wild-type.

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