Published in final edited form as:

Dev Neurobiol. 2012 June ; 72(6): 906–917. doi:10.1002/dneu.20989.

Inclusion of a portion of the native SNCA 3'UTR reduces toxicity of human S129A SNCA on striatal-projecting dopamine neurons in rat substantia nigra

Christina E. Khodr, Ph.D., Jyothi Pedapati, M.S., Ye Han, Ph.D., and Martha C. Bohn, Ph.D.*

Department of Pediatrics Neurobiology Program Children's Memorial Research Center Feinberg School of Medicine Northwestern University 2300 Children's Plaza, Box 209 Chicago, IL 60614, USA

Abstract

Experimental models of Parkinson's disease (PD) created by aberrant expression of the alpha-synuclein (SNCA) coding region have been reported. However, non-coding regions function in normal physiology and recent *in vitro* studies have shown that microRNAs-7 and -153 regulate SNCA expression by binding the 3'UTR. Here, effects of different hSNCA forms were examined *in vivo*. Adult, male rats were injected into one substantia nigra (SN) with AAV-wtSNCA, AAV-S129A hSNCA, or AAV-S129DhSNCA either with or without a portion of the native 3'UTR. DA neurons in SN that maintained striatal (ST) projections at the end of treatment were retrogradely labeled by bilateral ST fluorogold (FG) injections and FG-positive DA neurons in SN were counted. At 5wks, hSNCA coding vectors reduced numbers of FG-positive neurons in injected SN compared to uninjected SN (wtSNCA, $p = 0.05$; S129A/D hSNCA, $p = 0.01$). At 7 and 9wks, wtSNCA- and S129D hSNCA-treated rats exhibited recovery, but S129A hSNCA-injected rats did not ($p = 0.01$). In contrast, numbers of FG-positive neurons were unaffected by hSNCA expression when the 3'UTR was included. When FG-positive neurons were expressed as the ratio of numbers in injected to uninjected sides, the S129AhSNCA coding vector resulted in the highest decrease at 9wks versus wtSNCA ($p = 0.05$) or S129D hSNCA ($p = 0.01$). Inclusion of the 3'UTR resulted in no significant differences in FG-positive neuron ratios. These data suggest that inclusion of the 3'UTR protects against S129A hSNCA-induced loss of nigrostriatal-projecting DA neurons *in vivo* and that mis-regulation of hSNCA expression and function at non-coding regions contribute to PD pathogenesis.

Keywords

microRNA; phosphorylation; neurodegeneration; gene therapy; retrograde labeling

INTRODUCTION

Abnormal deposition and aggregation of human alpha-synuclein (hSNCA) are observed in a group of neurodegenerative disorders termed synucleinopathies, which include Parkinson's disease (PD), dementia with Lewy bodies and multiple systems atrophy (McCormack and Di Monte, 2009). Involvement of aberrant hSNCA expression in PD pathogenesis is suggested by the presence of cytoplasmic and neuritic inclusions termed Lewy bodies and Lewy neurites, respectively, the diagnostic hallmarks of PD (Lee and Trojanowski, 2006). SNCA is the major protein component of Lewy inclusion bodies (Spillantini et al., 1997; Spillantini

*Corresponding Author: Phone: 1-773-755-6355 Fax: 1-773-755-6344 m-bohn@northwestern.edu.

et al., 1998), which occur in the majority of PD cases (Lee and Trojanowski, 2006). Further, three mis-sense mutations in the hSNCA gene, as well as multiplication of the gene, are associated with familial forms of PD (Polymeropoulos et al., 1997; Kruger et al., 1998; Singleton et al., 2003; Chartier-Harlin et al., 2004; Zarranz et al., 2004). Roles for SNCA in dopamine (DA) neurotransmission and synaptic processes have been elucidated, although a full understanding of its physiological function remains unclear (Wood-Kaczmar et al., 2006; Auluck et al., 2010).

Post-translational processes are critical to levels of SNCA expression and action. Phosphorylation, ubiquitination and C-terminal truncation act to regulate the structure, function, aggregation and toxicity of SNCA (Oueslati et al., 2010). The majority of SNCA in Lewy bodies is phosphorylated at serine 129 (Fujiwara et al., 2002; Anderson et al., 2006). This site has been mutated to an alanine (S129A SNCA) or an aspartate (S129D SNCA) residue in order to mimic an SNCA form that is not phosphorylatable at residue 129 or a form that is constitutively phosphorylated at residue 129, respectively (Oueslati et al., 2010). In *d. Melanogaster*, which lacks endogenous SNCA, co-expression of G protein-coupled receptor kinase 2 with hSNCA or expression of S129DhSNCA results in greater hSNCA toxicity than wtSNCA or S129A hSNCA (Chen and Feany, 2005). However, findings in rat have varied. Adeno-associated viral (AAV) vector mediated delivery of S129A hSNCA into rat substantia nigra (SN) has been reported to result in a more rapid reduction of tyrosine hydroxylase (TH) immunoreactivity (IR) and DA content in the striatum (ST), as well as number of TH-IR neurons in the SN than wtSNCA or S129D hSNCA (Gorbatyuk et al., 2008; Azeredo da Silveira et al., 2009). Another study reported equivalent toxicity of all hSNCA forms on SN TH-IR neurons, but reduced ST DA content in S129A and S129D mutant hSNCA-injected rats (McFarland et al., 2009).

Post-transcriptional processes also appear critical to SNCA expression. Many SNCA-based models of PD have been developed by aberrant expression of the coding region of hSNCA. While these models have been valuable for studying the actions of SNCA in DA neurons, they may not faithfully represent the normal physiological state where the non-coding regions also appear to play a role. For example, recent *in vitro* studies suggest that SNCA expression is transcriptionally downregulated by microRNA-7 and by microRNA-153 acting through consensus binding sequences in the 3'UTR (Junn et al., 2009; Doxakis, 2010). The microRNA-7 mediated downregulation was also shown to reduce SNCA toxicity in mouse neuroblastoma NS20Y cells (Junn et al., 2009). However, an *in vivo* role for the 3'UTR has not yet been demonstrated. To begin to address this issue, this study examines the effect of post-transcriptional regulation, specifically at the 3'UTR, as well as effects of phosphorylation at serine 129 on toxicity of hSNCA *in vivo* in DA neurons in rat substantia nigra (SN).

MATERIALS AND METHODS

Generation of adeno-associated viral (AAV) shuttle plasmids

pAAV-CBA-wtSNCA was generated as previously described (Khodr et al., 2011). All hSNCA mutant vectors were generated from pAAV-CBA-wtSNCA. The coding region of hSNCA was mutated to S129D hSNCA using a mutated 3' primer (mutation underlined and in bold). The forward and reverse primers used follow: 5'-GGAAGCTTATGGATGTATTCATGAAAGGA-3'; 5'-GGCTCGAGTTAGGCTTCAGGTCGTAGTCTTGATACCCTTCCTCATCAGGCAT TTCATAAGCCTCATT-3'. The sequences for S129A hSNCA, S129D hSNCA and wtSNCA with 301 base pairs of the native hSNCA 3'UTR (+3'UTR) were synthesized by Genscript (Piscataway, NJ). The S129A hSNCA sequence was amplified from the S129A hSNCA+3'UTR sequence using the previously mentioned forward primer and the following

reverse primer: 5'-GGCTCGAGTTAGGCTTCAGGTTCG-3'. All hSNCA and hSNCA mutant sequences were inserted into pAAV-CBA plasmid to make pAAV-CBA-S129D hSNCA, pAAV-CBA-S129A hSNCA+3'UTR, pAAV-CBA-S129DhSNCA+3'UTR, pAAV-CBA-S129A hSNCA and pAAV-CBA-wtSNCA+3'UTR. Plasmids were sequenced and transgene expression was confirmed *in vitro* by immunocytochemistry.

Virus packaging

All vectors were packaged as AAV2 by the Children's Memorial Viral Vector Core, following the protocol of Zufferey *et al* (Zufferey et al., 1998; Zufferey and Trono, 2002) with minor modifications. Shuttle plasmid and the appropriate packaging plasmids were transfected into 293T cells. Cells were lysed three days after transfection by freeze-thawing in order to collect virus and cellular debris was removed by centrifugation. The supernatant was treated with octyl- β -D-glucopyranoside and benzonase and applied to a 15-60% iodixanol discontinuous gradient. The 40% layer was further purified using a Mustang Q ion exchange membrane. A Centriplus 100,000 MS cut off membrane was used to concentrate virus, which was stored in phosphate buffered saline (PBS), pH 7.4 containing 5% sorbitol and 0.001% PF-68. qRT-PCR was used to determine viral titers and electron microscopy was used to examine the purity of viral preparations. Viral titers were: AAV-CBA-wtSNCA – 1.5×10^{12} vector genomes (vg)/ml, AAV-CBA-wtSNCA+3'UTR – 5.8×10^{12} vg/ml, AAV-CBA-S129D hSNCA – 2.8×10^{12} vg/ml, AAV-CBA-S129D hSNCA+3'UTR – 1×10^{12} vg/ml, AAV-CBA-S129A hSNCA – 1.2×10^{12} vg/ml, AAV-CBA-S129A hSNCA+3'UTR – 1.8×10^{12} .

Animals

Adult male Sprague Dawley rats weighing 175-200g were purchased from Harlan laboratories (Indianapolis, IN). Rats were housed on a 12 hour light/dark cycle with food and water available *ad libitum* in the Children's Memorial Research Center animal facility. Animal care and use procedures were conducted in accordance with NIH, USDA and institutional guidelines.

Treatment groups and sample sizes

Rats were generated for morphological examination at 5, 7 and 9 weeks after injection of hSNCA or hSNCA mutants. Sample sizes for each treatment group follow: wtSNCA, 5wk (n=8), 7wk (n=9), 9wk (n=5); wtSNCA+3'UTR, 5wk (n=6), 7wk (n=5), 9wk (n=4); S129A hSNCA, 5wk (n=6), 7wk (n=6), 9wk (n=6); S129A hSNCA+3'UTR, 5wk (n=6), 7wk (n=8), 9wk (n=8); S129D hSNCA, 5wk (n=7), 7wk (n=7), 9wk (n=5) and S129D hSNCA+3'UTR, 5wk (n=8), 7wk (n=5), 9wk (n=9). Rats were also generated to assess levels of vector DNA and RNA at 2 weeks after injection of S129A hSNCA with or without the 3'UTR (n=3).

Stereotaxic surgery and post-operative animal care

Rats were anesthetized with isoflurane and the skin overlying the skull was shaven. A hole was drilled in the skull over the appropriate injection site using a Stoelting stereotaxic apparatus equipped with a Stoelting quintessential stereotaxic injector holding a 10 μ l Hamilton syringe with a 26 gauge needle. The syringe was inserted into the brain at a speed of ~1 mm/min and then allowed to sit for 2 min before injection. Injection of 1.5 μ l of each virus into one SN occurred at a rate of 0.5 μ l/min. The stereotaxic coordinates for SN injection were 5.5 mm posterior, -1.9 mm lateral and 7.4 mm ventral from Bregma. Five days before euthanasia, rats were also bilaterally injected with the retrograde tracer fluorogold (FG) into the ST. 0.3 μ l of 2% FG was injected into both ST at a rate of 0.05 μ l/min at stereotaxic coordinates of 1.0 mm anterior, +/-3.0 mm lateral and 5.0 mm ventral from Bregma. Injected fluid was allowed to settle for 5 min to prevent diffusion up the

needle track and the syringe was withdrawn at ~1 mm/min. Bleeding was controlled by plugging the drill hole in the skull with gel foam, the analgesic Marcaine was spread around the surgical site, and the skin was sutured and treated with topical antibiotic ointment. Rats were returned to the vivarium upon recovering from anesthesia. Rats were transferred to a clean cage and the bedding was autoclaved 24 hours after surgery.

Tissue preparation

Rats were anesthetized with sodium pentobarbital (75 mg/kg) and perfused with either 0.9% saline for assessment of RNA and DNA levels or with 0.9% saline containing 0.002% Na nitrite, followed by 4% phosphate buffered paraformaldehyde (pH=7.4) for morphological assessment. For molecular analysis, the left and right ventral midbrain were dissected and homogenized in 1XPBS containing 1% Triton X-100 and 5mM EDTA. After freeze-thaw cycles, samples were centrifuged at 100,000 x g for 1 hour at 4°C. The RNA/DNA-containing pellet was dissolved in TRI reagent and stored at -80°C until further processing. For histology, brains were removed and post-fixed overnight in 5% sucrose-4% paraformaldehyde and then cryoprotected by an increasing gradient of sucrose concentrations (10%-30%) in 0.1 M PBS. Tissues were cut coronally at 40 µm using a sliding microtome (Leica SM2000 R) and separated into 6 serial sets, which were placed in cryoprotectant solution until used.

Immunohistochemistry

Free-floating tissue sections were rinsed of cryoprotectant and incubated for 1 hour in normal goat serum (NGS) to block nonspecific antibody binding. Sections were then incubated overnight at room temperature in primary antibody (rabbit anti-pan TH, Millipore, 1:500, Billerica, MA; mouse anti-hSNCA, 1:50, Invitrogen, Carlsbad, CA). Following further rinses, sections were incubated in secondary antibody (Cy3-conjugated goat anti-mouse, 1:100, Jackson ImmunoResearch, West Grove, PA; Cy2-conjugated goat anti-rabbit, 1:200, Jackson ImmunoResearch) for 2.5 hours at room temperature. Sections were mounted on slides, air dried overnight, coverslipped with Fluorosave (Calbiochem, La Jolla, CA), and evaluated using a Leica DMR upright microscope equipped for epifluorescence microscopy.

Quantitative real time (qRT-) PCR

RNA and DNA were extracted using TRI Reagent™ (Ambion, Austin, TX) and RNA samples were DNase-treated. Quantitative TaqMan™ real-time PCR was carried out using an Applied Biosystems (Foster City, CA) 7500 fast real-time PCR system to measure RNA levels. Each reaction contained 25 ng of RNA or DNA, 12.5 µl of 2x TaqMan Universal PCR buffer, 6.25 U of MuLV reverse transcriptase, 1.25 U of RNase inhibitor, 0.25 µl of each primer (10 µM forward β-actin, 20 µM forward hSNCA and 20 µM reverse), and 0.5 µl of probe (5 µM) in a 25 µl volume. Primer Express 3.0 (Applied Biosystems) and BLAST (blast.ncbi.nlm.nih.gov) were used to design specific primers and probes. Probes contained a FAM reporter dye and a TAMRA quencher dye. Primers and probes used are as follows: hSNCA (fwd primer:

GACCAGTTGGGCAAGAATGAAG, rev primer:

GGTTCGTAGTCTTGATACCCTTCCT, probe:

TCTGGAAGATATGCCTGTGGATCCTGACA); rat β-actin (fwd primer:

TCACCCCTGTGCCCATCTATG, rev primer:

CATCGGAACCGCTCATTGCCGATAG, probe:

ACGCGCTCCCTCATGCCATCCTGCGT). Samples were incubated at 48 °C for 30 min, 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 59 °C for 1 min. Data are expressed as relative quantity ($2^{-\Delta\Delta C_t}$) according to the method of Livak and Schmittgen (Livak and Schmittgen, 2001), where $\Delta\Delta C_t = \Delta C_{t\text{sample}} - \Delta C_{t\text{reference sample}}$ and $\Delta C_t = C_{t\text{hSNCA}} - C_{t\beta\text{-actin}}$. The sample that was used as the reference sample was the uninjected SN of rats injected with S129A hSNCA+3' UTR. Therefore, all samples were normalized to β -actin levels and then normalized to the uninjected SN of rats injected with S129A hSNCA+3' UTR.

Fluorogold (FG) cell counts

Neurolucida™ software was used to quantify FG positive cells in the SN. Five sections centered around the injection site (~240 μm apart) were counted for each brain. Only large FG-positive neurons (greater than ~15 μm in diameter) in the SN were counted to ensure that only DA neurons were counted, not dying neurons or microglia.

Imaging

A Retiga 4000R digital camera on a Leica DMR upright microscope was used to collect images of hSNCA-IR, TH-IR, and FG fluorescence. Multi-photo plate figures were made using Adobe Photoshop CS2.

Statistical analysis

Prism™ (Graphpad Software, Inc) was used to analyze data. Mann-Whitney t-tests were used to compare cell counts in injected SN to those in uninjected SN within a single treatment group. Kruskal-Wallis one-way ANOVAs followed by Dunn's post-hoc tests were used to compare ratios of FG positive neurons (injected SN / uninjected SN) between treatment groups. For analysis of smaller sample sizes (qPCR analysis), a parametric one-way ANOVA followed by Newman-Keuls multiple comparison tests were used, as well as unpaired t-tests. Statistically significant differences were set at $p < 0.05$. Data are expressed as mean $\pm$ SEM.

RESULTS

Prevention of phosphorylation at Ser129 exacerbates toxicity of hSNCA on nigrostriatal-projecting DA neurons

In order to examine effects of phosphorylation at Ser129 on hSNCA toxicity, rats were stereotactically injected with an AAV2 harboring the coding region of wtSNCA, S129A hSNCA or S129D hSNCA into one SN and euthanized at 5 wks (Fig. 1A), 7 wks (Fig. S1A) or 9 wks (Fig. S1B). To identify DA neurons in the SN that maintained projections to a defined site in each ST, the retrograde tract tracer FG was bilaterally and stereotactically injected into the ST 5 days before perfusion. This also allowed for detection of nigrostriatal-projecting neurons without relying on IR for the DA phenotypic marker TH which could be compromised by high levels of SNCA expression (Perez et al., 2002; Baptista et al., 2003).

The different levels of toxicity for the three forms of hSNCA on nigrostriatal DA neurons were examined by counting neurons in the SN that were positive for FG (Table 1; Fig. 1B-D). To examine whether each hSNCA form induced a toxic effect on ST-projecting neurons in the SN, raw cell counts in injected and uninjected SN were compared for each treatment. Numbers of FG positive DA neurons in the injected SN were significantly reduced compared to those in the uninjected SN at 5 wks in all treatment groups (Table 1; $p < 0.05$ for wtSNCA, $p < 0.01$ for S129A hSNCA and S129DhSNCA). A reduction in FG positive DA neuron numbers in the injected SN was not evident after 7 wks in rats that received wtSNCA or S129D hSNCA, i.e. FG labeled neuron numbers were not significantly different between injected and uninjected SN at 7 or 9 wks in rats injected with wtSNCA or S129D hSNCA.

However, numbers of FG positive neurons in injected SN were reduced compared to those in uninjected SN in rats that received S129A hSNCA (Table 1, $p = 0.01$). To directly compare the effects of the hSNCA mutants on SN neurons that projected to the ST, cell count data were expressed as the ratio of FG positive neuron numbers in injected SN to uninjected SN in each rat. statistically significant difference in the effect of the hSNCA mutants was revealed at 9 wks when S129A hSNCA-treated rats exhibited a reduced ratio of FG positive neurons compared to both wtSNCA- ($p = 0.05$) and S129D hSNCA-injected rats ($p = 0.01$, Fig. 1B-D).

Inclusion of the portion of the native hSNCA 3'UTR containing a predicted microRNA-7 binding site diminishes hSNCA toxicity on striatal-projecting DA neurons in SN

To examine effects of including a 3'UTR on hSNCA toxicity, rats were stereotactically injected with an AAV2 harboring the wtSNCA, S129A hSNCA or S129DhSNCA containing a portion of the native 3'UTR into one SN and euthanized at 5 wks, 7 wks (Fig. 2) or 9 wks. Five days before euthanasia, rats received bilateral striatal stereotaxic injection of FG to label DA neurons in the SN that maintained projections to that defined ST site. DA neurons in the SN that were FG positive were counted (Table 2; Fig. S2A-C). To examine whether each hSNCA form containing the 3'UTR had a toxic effect on striatal-projecting DA neurons in the SN, raw cell counts in injected and uninjected SN were compared for each treatment. FG positive neurons in the injected SN were not significantly different from those in the uninjected SN (Table 2). Cell count data were expressed as the ratio of FG positive neuron numbers in injected SN to uninjected SN in each rat in order to directly compare the effects of the hSNCA mutants containing the 3'UTR on SN neurons that project to the ST. Ratios of FG positive neurons in injected SN to uninjected SN were not significantly different in any treatment groups (Fig. S2A-C).

To further examine whether post-transcriptional regulation might play a role in the toxicity of hSNCA, the numbers of FG-positive neurons in the SN were compared in rats that received the different hSNCA forms containing or not containing a portion of the 3'UTR (Fig. 3). Ratios of FG positive cell numbers in injected SN of rats that received wtSNCA were not significantly different from those in rats that received wtSNCA+3'UTR (Fig. 3A). In contrast, ratios of FG positive neurons were reduced in rats that received S129A hSNCA compared to those in rats that received S129AhSNCA+3'UTR (Fig. 3B; $p = 0.05$ for 5 wk and 7 wk, $p = 0.01$ for 9 wk). Reduced FG positive neuron ratios were observed at 5 wks in rats that received S129D hSNCA compared to rats that received S129D hSNCA+3'UTR ($p = 0.05$). At 7 and 9 wks, there was no significant difference between FG positive neuron ratios in rats that received S129D hSNCA with or without the 3'UTR (Fig. 3C).

***In vivo* expression levels of S129A hSNCA mRNA from vectors with or without the 3'UTR**

To examine whether the differential toxicity of the hSNCA forms observed *in vivo* resulted from differential expression, levels of hSNCA RNA and DNA in the ventral midbrain were assessed in rats injected with AAV-S129A hSNCA or AAV-S129A hSNCA+3'UTR for 2 weeks. A two week time point was chosen since expression from AAV begins to plateau around this time and at longer survival times, transduced cells may begin to die. Levels of hSNCA DNA, measured using qRT-PCR were not significantly different in the injected SN of rats that received S129A hSNCA with or without the 3'UTR (Fig. 4A) suggesting that brains were transduced with equivalent amounts of vector. In contrast, hSNCA RNA levels were significantly reduced ($p = 0.001$) in SN injected with S129A hSNCA+3'UTR compared to those in SN injected with S129A hSNCA (Fig. 4B), suggesting differential transcriptional or post-transcriptional regulation of hSNCA coding mRNAs.

DISCUSSION

A clearer understanding of the physiological function and regulation of SNCA will aid in defining mechanisms involved in SNCA toxicity in synucleinopathies, as well as in identifying novel therapeutic targets for these disorders. The current study examined the effect of post-transcriptional regulation, specifically at the 3'UTR, and post-translational modification, specifically phosphorylation at S129, on hSNCA toxicity in nigral DA neurons. In agreement with previous studies, we found that the non-phosphorylatable mimic, S129A hSNCA, was more toxic than wtSNCA or the constitutively phosphorylated mimic S129D hSNCA on nigrostriatal-projecting DA neurons. Importantly, toxicity of hSNCA was reduced when a portion of the native 3'UTR was included. This was most evident in SN injected with S129A hSNCA which induced the most severe reduction of nigrostriatal-projecting DA neurons at all time points examined, but which had little effect when the 3'UTR was included. These findings suggest that both regulation of SNCA at the 3'UTR mRNA region and phosphorylation are important to determining levels of toxicity of ectopically expressed hSNCA. However, this observation might also be explained by different levels of vector transduction. To rule out this possibility, levels of vector DNA and hSNCA mRNA levels were examined at 2 weeks in the ventral midbrain of rats injected with AAV-S129A hSNCA with or without the 3'UTR, a time point chosen so that transgene expression would be high, but death of transduced cells would not yet have progressed significantly. Levels of vector DNA were not statistically different in the injected SN of both treatment groups, suggesting that neurons in the ventral midbrain were transduced with equivalent amounts of virus. However, inclusion of the 3'UTR resulted in significantly reduced hSNCA mRNA levels in the injected SN. These data suggest that the differential effects on survival of nigrostriatal DA neurons *in vivo* in rats transduced with S129A hSNCA with or without the 3'UTR was primarily due to transcriptional or post-transcriptional regulation of hSNCA mRNA. The lower levels of hSNCA mRNA might be due to lower levels of transcription or more rapid mRNA degradation when the 3'UTR is present. Our findings suggest that while differential regulation of hSNCA expression and modification are both important in determining levels of SNCA toxicity, down-regulation of mRNA levels mediated by the 3'UTR can override toxicity due to a lack of hSNCA post-translational phosphorylation.

The effect of phosphorylation at serine 129 on toxicity of SNCA is of interest because the majority of hSNCA in Lewy bodies is phosphorylated at serine 129 (Fujiwara et al., 2002; Anderson et al., 2006). The first study to examine the phosphorylation state of serine 129 was carried out in *D. Melanogaster*, which lacks endogenous SNCA expression. This study showed that co-expression of G protein-coupled receptor kinase 2 with wtSNCA or expression of S129D hSNCA results in greater SNCA toxicity than wtSNCA or S129A hSNCA (Chen and Feany, 2005). However, findings in rodents have revealed a different picture. Three different groups have examined effects of hSNCA phosphorylation at serine 129 in rat SN using AAV-mediated delivery. These studies reported somewhat different findings, possibly due to different serotypes of AAV used or time points examined. McFarland and colleagues (2009) reported equivalent toxicity of all hSNCA forms on SN TH-IR neurons, but reduced ST DA content in S129A and S129D hSNCA-injected rats. Gorbatyuk et al (2008), as well as Azeredo da Silveira et al (2009), reported that S129A hSNCA results in a more rapid reduction of DA markers in ST, such as DA content or TH- and vesicular monoamine transporter 2-IR fibers, as well as a more rapid reduction in the number of TH-IR neurons in the SN compared to wtSNCA or S129D hSNCA. Our findings are in agreement with these latter reports and expand the evidence for greater toxicity of S129AhSNCA to effects on DA neurons that maintain projections to ST as assessed by retrograde labeling with FG.

The observed effects of S129D hSNCA and wtSNCA at 8 wks differed in the studies of Gorbatyuk et al (2008) and Azeredo da Silveira et al (2009). Gorbatyuk et al (2008) did not observe an effect of S129D hSNCA on DA markers in the SN or ST, but did observe a significant wtSNCA-induced decrease in number of TH-IR neurons in SN. Azeredo da Silveira and colleagues (2009), in contrast, did not observe a significant difference between the effect of wtSNCA and S129D hSNCA on number of TH-IR neurons in SN, but observed a greater reduction in ST TH-IR fiber density in response to wtSNCA compared to S129D hSNCA. In the current study, we also did not detect a difference between the effects of wtSNCA and S129D hSNCA on SN DA neurons that maintained projections to ST, whereas these were clearly reduced by S129A hSNCA. In contrast to previous studies, only our study specifically looked at the effects of hSNCA on the subpopulation of DA neurons in the SN that maintained axonal projections to the ST.

The findings presented in this study, as well as those from previous studies (Gorbatyuk et al., 2008; Azeredo da Silveira et al., 2009; McFarland et al., 2009), demonstrate that the phosphorylation state of SNCA at serine 129 is critical to SNCA function and toxicity. Other phosphorylation sites that may contribute to determining SNCA function and toxicity include serine 87 and tyrosine 125, which also have been correlated with the PD-related synucleinopathy, dementia with Lewy bodies in humans (Chen et al., 2009; Paleologou et al., 2010). SNCA in Lewy bodies is also characterized by other post-translational modifications, such as C-terminal truncations and ubiquitination. Targeting enzymes or pathways that regulate these modifications may provide another potential therapeutic route of action (Oueslati et al., 2010).

Aberrant expression of SNCA has been implicated in PD pathogenesis in both familial and sporadic forms of PD. There is evidence that aberrant SNCA expression can be due to SNCA gene multiplications (Singleton et al., 2003; Chartier-Harlin et al., 2004), promoter polymorphisms (Maraganore et al., 2006) or changes in SNCA protein processing as in sporadic PD cases that present with Lewy bodies (Lee and Trojanowski, 2006), review) of which SNCA is the major protein component (Spillantini et al., 1997; Spillantini et al., 1998). Based on these reports, many informative models of PD have been created by expressing the coding region of hSNCA in the brains of mice, rats and non-human primates. These models have provided insight into mechanisms of SNCA toxicity. Our findings suggest downregulation of the hSNCA gene at its 3'UTR also decreases levels of toxicity of hSNCA on DA neurons *in vivo*.

Regulation of mRNA expression at the 3'UTR is likely to involve microRNA action as recent studies have suggested. The expression of SNCA *in vitro* in HEK 293 fibroblasts, neuroblastoma and primary cortical neurons was shown to be regulated by miR-7 and miR-153 through the SNCA 3'UTR (Junn et al., 2009; Doxakis, 2010). Inclusion of the 3'UTR also was shown to reduce SNCA toxicity when miR-7 was present in H₂O₂-treated mouse neuroblastoma NS20Y cells (Junn et al., 2009). Our results are in agreement with these *in vitro* findings and expand these findings to DA neurons in the rat SN, i.e. inclusion of a portion of the native hSNCA 3'UTR that contains a predicted miR-7 binding site reduced toxicity of the hSNCA coding region. Further, endogenous rat regulatory molecules in DA neurons, such as miR-7, must have been involved in the reduction of hSNCA toxicity by acting at the 3'UTR since no exogenous miR-7 was added in any of our treatments. In this respect, expression of miR-7 has been reported in mouse midbrain and ST (Junn et al., 2009; Doxakis, 2010). At this point, we cannot attribute the effects of the 3'UTR observed in this study to miR action. Future studies will need to examine the effects of including a 3'UTR with mutated miR binding sites and/or effects of silencing specific miR's *in vivo*.

Single nucleotide polymorphisms (SNPs) in the SNCA promoter (Maraganore et al., 2006), as well as in the 3'UTR (Mueller et al., 2005; Ross et al., 2007; Winkler et al., 2007; Myhre et al., 2008; Sotiriou et al., 2009) are associated with increased risk for PD. Although increased SNCA expression levels are correlated with disease-associated SNPs in the SNCA promoter, this is not necessarily the case for SNPs in the 3'UTR (Fuchs et al., 2008; Linnertz et al., 2009). Mis-regulation of splicing machinery has been suggested by one study, which associated certain 3'UTR SNPs with increased levels of the SNCA-112 splicing variant encoding a truncated SNCA isoform (McCarthy et al., 2011). Other potential regulatory paths that act at non-coding regions of SNCA include regulation by an iron-responsive element in the 5'UTR (Rogers et al., 2011). Further, epigenetic dysregulation by reduced methylation of the SNCA gene also has been observed in PD (Jowaed et al., 2010; Matsumoto et al., 2010).

In conclusion, our study highlights the importance of non-coding regions and post-translational modifications in the regulation of hSNCA expression and toxicity in DA neurons. Our results are relevant to aberrant SNCA expression and function underlying synucleinopathies, in particular PD, and to the discovery of regulatory molecules as potential therapeutic targets for these disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by the Department of Defense Neurotoxicology Program (NO06079001) and NIH grants (NS31957 and NS054989 to MCB and T32 NS041234 to CEK), the Harry F and Elaine M Chaddick Foundation and the Medical Research Institute Council of Children's Memorial Hospital. Support from the Chicago Biomedical Consortium and the Illinois Excellence in Academic Medicine to the CMRC Viral Vector Core is acknowledged. The advice of Dr. Honglin Li and technical assistance of Jianping Xie, Thomas Mangatu, Xue Song Wang, David George and Brian Corstange are appreciated.

This paper is dedicated to my postdoctoral mentor, Dr. Ira Black. I am grateful for his friendship and insights in the field of neuroscience that forever remain with me. Marty Bohn.

REFERENCES

- Anderson JP, Walker DE, Goldstein JM, de Laat R, Banducci K, Caccavello RJ, Barbour R, Huang J, Kling K, Lee M, Diep L, Keim PS, Shen X, Chataway T, Schlossmacher MG, Seubert P, Schenk D, Sinha S, Gai WP, Chilcote TJ. Phosphorylation of Ser-129 is the dominant pathological modification of alphasynuclein in familial and sporadic Lewy body disease. *J Biol Chem.* 2006; 281:29739–29752. [PubMed: 16847063]
- Auluck PK, Caraveo G, Lindquist S. alpha-Synuclein: membrane interactions and toxicity in Parkinson's disease. *Annu Rev Cell Dev Biol.* 2010; 26:211–233. [PubMed: 20500090]
- da Silveira, S Azeredo; Schneider, BL.; Cifuentes-Diaz, C.; Sage, D.; Abbas-Terki, T.; Iwatsubo, T.; Unser, M.; Aebischer, P. Phosphorylation does not prompt, nor prevent, the formation of alpha-synuclein toxic species in a rat model of Parkinson's disease. *Hum Mol Genet.* 2009; 18:872–887. [PubMed: 19074459]
- Baptista MJ, O'Farrell C, Daya S, Ahmad R, Miller DW, Hardy J, Farrer MJ, Cookson MR. Co-ordinate transcriptional regulation of dopamine synthesis genes by alpha-synuclein in human neuroblastoma cell lines. *J Neurochem.* 2003; 85:957–968. [PubMed: 12716427]
- Chartier-Harlin MC, Kachergus J, Roumier C, Mouroux V, Douay X, Lincoln S, Levecque C, Larvor L, Andrieux J, Hulihan M, Waucquier N, Defebvre L, Amouyel P, Farrer M, Destee A. Alpha-synuclein locus duplication as a cause of familial Parkinson's disease. *Lancet.* 2004; 364:1167–1169. [PubMed: 15451224]

- Chen L, Feany MB. Alpha-synuclein phosphorylation controls neurotoxicity and inclusion formation in a *Drosophila* model of Parkinson disease. *Nat Neurosci*. 2005; 8:657–663. [PubMed: 15834418]
- Chen L, Periquet M, Wang X, Negro A, McLean PJ, Hyman BT, Feany MB. Tyrosine and serine phosphorylation of alpha-synuclein have opposing effects on neurotoxicity and soluble oligomer formation. *J Clin Invest*. 2009; 119:3257–3265. [PubMed: 19855133]
- Doxakis E. Post-transcriptional regulation of alpha-synuclein expression by mir-7 and mir-153. *J Biol Chem*. 2010; 285:12726–12734. [PubMed: 20106983]
- Fuchs J, Tichopad A, Golub Y, Munz M, Schweitzer KJ, Wolf B, Berg D, Mueller JC, Gasser T. Genetic variability in the SNCA gene influences alphasynuclein levels in the blood and brain. *FASEB J*. 2008; 22:1327–1334. [PubMed: 18162487]
- Fujiwara H, Hasegawa M, Dohmae N, Kawashima A, Masliah E, Goldberg MS, Shen J, Takio K, Iwatsubo T. alpha-Synuclein is phosphorylated in synucleinopathy lesions. *Nat Cell Biol*. 2002; 4:160–164. [PubMed: 11813001]
- Gorbatyuk OS, Li S, Sullivan LF, Chen W, Kondrikova G, Manfredsson FP, Mandel RJ, Muzyczka N. The phosphorylation state of Ser-129 in human alphasynuclein determines neurodegeneration in a rat model of Parkinson disease. *Proc Natl Acad Sci U S A*. 2008; 105:763–768. [PubMed: 18178617]
- Jowaed A, Schmitt I, Kaut O, Wullner U. Methylation regulates alpha-synuclein expression and is decreased in Parkinson's disease patients' brains. *J Neurosci*. 2010; 30:6355–6359. [PubMed: 20445061]
- Junn E, Lee KW, Jeong BS, Chan TW, Im JY, Mouradian MM. Repression of alpha-synuclein expression and toxicity by microRNA-7. *Proc Natl Acad Sci U S A*. 2009; 106:13052–13057. [PubMed: 19628698]
- Khodr CE, Sapru MK, Pedapati J, Han Y, West NC, Kells AP, Bankiewicz KS, Bohn MC. An alpha-synuclein AAV gene silencing vector ameliorates a behavioral deficit in a rat model of Parkinson's disease, but displays toxicity in dopamine neurons. *Brain Res*. 2011; 1395:94–107. [PubMed: 21565333]
- Kruger R, Kuhn W, Muller T, Woitalla D, Graeber M, Kosel S, Przuntek H, Epplen JT, Schols L, Riess O. Ala30Pro mutation in the gene encoding alphasynuclein in Parkinson's disease. *Nat Genet*. 1998; 18:106–108. [PubMed: 9462735]
- Lee VM, Trojanowski JQ. Mechanisms of Parkinson's disease linked to pathological alpha-synuclein: new targets for drug discovery. *Neuron*. 2006; 52:33–38. [PubMed: 17015225]
- Linnertz C, Saucier L, Ge D, Cronin KD, Burke JR, Browndyke JN, Hulette CM, Welsh-Bohmer KA, Chiba-Falek O. Genetic regulation of alphasynuclein mRNA expression in various human brain tissues. *PLoS One*. 2009; 4:e7480. [PubMed: 19834617]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using realtime quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001; 25:402–408. [PubMed: 11846609]
- Maraganore DM, de Andrade M, Elbaz A, Farrer MJ, Ioannidis JP, Kruger R, Rocca WA, Schneider NK, Lesnick TG, Lincoln SJ, Hulihan MM, Aasly JO, Ashizawa T, Chartier-Harlin MC, Checkoway H, Ferrarese C, Hadjigeorgiou G, Hattori N, Kawakami H, Lambert JC, Lynch T, Mellick GD, Papapetropoulos S, Parsian A, Quattrone A, Riess O, Tan EK, Van Broeckhoven C. Collaborative analysis of alpha-synuclein gene promoter variability and Parkinson disease. *Jama*. 2006; 296:661–670.
- Matsumoto L, Takuma H, Tamaoka A, Kurisaki H, Date H, Tsuji S, Iwata A. CpG demethylation enhances alpha-synuclein expression and affects the pathogenesis of Parkinson's disease. *PLoS One*. 2010; 5:e15522. [PubMed: 21124796]
- McCarthy JJ, Linnertz C, Saucier L, Burke JR, Hulette CM, Welsh-Bohmer KA, Chiba-Falek O. The effect of SNCA 3' region on the levels of SNCA-112 splicing variant. *Neurogenetics*. 2011; 12:59–64. [PubMed: 21046180]
- McCormack AL, Di Monte DA. Enhanced alpha-synuclein expression in human neurodegenerative diseases: pathogenetic and therapeutic implications. *Curr Protein Pept Sci*. 2009; 10:476–482. [PubMed: 19538156]

- McFarland NR, Fan Z, Xu K, Schwarzschild MA, Feany MB, Hyman BT, McLean PJ. alpha-Synuclein S129 Phosphorylation Mutants Do Not Alter Nigrostriatal Toxicity in a Rat Model of Parkinson Disease. *J Neuropathol Exp Neurol*. 2009; 68:515–524. [PubMed: 19525899]
- Mueller JC, Fuchs J, Hofer A, Zimprich A, Lichtner P, Illig T, Berg D, Wullner U, Meitinger T, Gasser T. Multiple regions of alpha-synuclein are associated with Parkinson's disease. *Ann Neurol*. 2005; 57:535–541. [PubMed: 15786467]
- Myhre R, Toft M, Kachergus J, Hulihan MM, Aasly JO, Klungland H, Farrer MJ. Multiple alpha-synuclein gene polymorphisms are associated with Parkinson's disease in a Norwegian population. *Acta Neurol Scand*. 2008; 118:320–327. [PubMed: 18485051]
- Oueslati A, Fournier M, Lashuel HA. Role of post-translational modifications in modulating the structure, function and toxicity of alpha-synuclein: implications for Parkinson's disease pathogenesis and therapies. *Prog Brain Res*. 2010; 183:115–145. [PubMed: 20696318]
- Paleologou KE, Oueslati A, Shakked G, Rospigliosi CC, Kim HY, Lamberto GR, Fernandez CO, Schmid A, Chagini F, Gai WP, Chiappe D, Moniatte M, Schneider BL, Aebischer P, Eliezer D, Zweckstetter M, Masliah E, Lashuel HA. Phosphorylation at S87 is enhanced in synucleinopathies, inhibits alphasynuclein oligomerization, and influences synuclein-membrane interactions. *J Neurosci*. 2010; 30:3184–3198. [PubMed: 20203178]
- Perez RG, Waymire JC, Lin E, Liu JJ, Guo F, Zigmond MJ. A role for alphasynuclein in the regulation of dopamine biosynthesis. *J Neurosci*. 2002; 22:3090–3099. [PubMed: 11943812]
- Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science*. 1997; 276:2045–2047. [PubMed: 9197268]
- Rogers JT, Mikkilineni S, Cantuti-Castelvetri I, Smith DH, Huang X, Bandyopadhyay S, Cahill CM, Maccacchini ML, Lahiri DK, Greig NH. The alphasynuclein 5' untranslated region targeted translation blockers: anti-alpha synuclein efficacy of cardiac glycosides and Posiphen. *J Neural Transm*. 2011; 118:493–507. [PubMed: 21221670]
- Ross OA, Gosal D, Stone JT, Lincoln SJ, Heckman MG, Irvine GB, Johnston JA, Gibson JM, Farrer MJ, Lynch T. Familial genes in sporadic disease: common variants of alpha-synuclein gene associate with Parkinson's disease. *Mech Ageing Dev*. 2007; 128:378–382. [PubMed: 17531291]
- Singleton AB, Farrer M, Johnson J, Singleton A, Hague S, Kachergus J, Hulihan M, Peuralinna T, Dutra A, Nussbaum R, Lincoln S, Crawley A, Hanson M, Maraganore D, Adler C, Cookson MR, Muenter M, Baptista M, Miller D, Blancato J, Hardy J, Gwinn-Hardy K. alpha-Synuclein locus triplication causes Parkinson's disease. *Science*. 2003; 302:841. [PubMed: 14593171]
- Sotiriou S, Gibney G, Baxevanis AD, Nussbaum RL. A single nucleotide polymorphism in the 3' UTR of the SNCA gene encoding alpha-synuclein is a new potential susceptibility locus for Parkinson disease. *Neurosci Lett*. 2009; 461:196–201. [PubMed: 19540308]
- Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert M. alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. *Proc Natl Acad Sci U S A*. 1998; 95:6469–6473. [PubMed: 9600990]
- Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-synuclein in Lewy bodies. *Nature*. 1997; 388:839–840. [PubMed: 9278044]
- Winkler S, Hagenah J, Lincoln S, Heckman M, Haugarvoll K, Lohmann-Hedrich K, Kostic V, Farrer M, Klein C. alpha-Synuclein and Parkinson disease susceptibility. *Neurology*. 2007; 69:1745–1750. [PubMed: 17872362]
- Wood-Kaczmar A, Gandhi S, Wood NW. Understanding the molecular causes of Parkinson's disease. *Trends Mol Med*. 2006; 12:521–528. [PubMed: 17027339]
- Zarranz JJ, Alegre J, Gomez-Esteban JC, Lezcano E, Ros R, Ampuero I, Vidal L, Hoenicka J, Rodriguez O, Atares B, Llorens V, Tortosa E Gomez, del Ser T, Munoz DG, de Yebenes JG. The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. *Ann Neurol*. 2004; 55:164–173. [PubMed: 14755719]

- Zufferey R, Dull T, Mandel RJ, Bukovsky A, Quiroz D, Naldini L, Trono D. Selfinactivating lentivirus vector for safe and efficient in vivo gene delivery. *J Virol.* 1998; 72:9873–9880. [PubMed: 9811723]
- Zufferey, R.; Trono, D. Production of high-titer lentiviral vectors. John Wiley & Sons, Inc.; New York: 2002. p. 1-12.

Figure 1. Ectopic expression of a hSNCA coding region that is non-phosphorylatable at serine 129 (S129A hSNCA) has greater toxicity on striatal-projecting DA neurons in SN than wild type (wt) hSNCA or a serine 129 phosphorylation mimic (S129D hSNCA)

Adult male rats received unilateral SN stereotaxic injection of AAV-CBA-wtSNCA, AAV-CBA-S129A hSNCA, or AAV-CBA-S129D hSNCA. 5 days before perfusion, rats received bilateral striatal injection of FG. **A)** Fluorescence immunostaining for hSNCA was performed on SN brain sections 5 wks post-injection. hSNCA-IR is in red, TH-IR is in green and FG fluorescence is in blue. A representative uninjected SN is shown in the upper row. Note the reduced FG fluorescence and TH-IR in rats that received S129A hSNCA. **B-D)** The coding region of S129A hSNCA induces a reduction in the number of DA neurons in the SN that maintain projections to striatum. FG positive neurons in the SN were counted in 5 sections surrounding the injection site. Ratios of FG positive neuron numbers (injected/uninjected SN) are shown at 5, 7 and 9 wks after injection; Kruskal-Wallis one-way ANOVA was used to compare treatments. Significant differences compared to S129A hSNCA are as follows: *= p 0.05, **= p 0.01.

Figure 2. Inclusion of a portion of the native hSNCA 3'UTR containing a predicted miR-7 binding site does not result in significant toxicity on striatal-projecting DA neurons
Adult male rats received unilateral SN stereotaxic injection of AAV-CBA-wtSNCA +3'UTR, AAV-CBA-S129A hSNCA+3'UTR, or AAV-CBA-S129DhSNCA+3'UTR. 5 days before perfusion, rats received bilateral striatal injection of FG. Fluorescence immunostaining was performed on SN brain sections at 5, 7 and 9 wks post-injection. Representative images for each treatment at all time points, which did not qualitatively differ, are shown. hSNCA-IR is in red, TH-IR is in green and FG fluorescence is in blue. A representative uninjected SN is shown in the upper row. Note that hSNCA forms do not differ in their toxic effects when the 3'UTR is included.

Figure 3. The toxicity of the coding region of S129A hSNCA on striatal-projecting DA neurons is reduced when a portion of the native hSNCA 3'UTR containing the miR-7 binding site is included

Ratios of FG positive neuron numbers (injected/uninjected SN) were compared between wtSNCA coding region and wtSNCA+3'UTR (**A**), S129A hSNCA coding region and S129A hSNCA+3'UTR (**B**), and S129D hSNCA coding region and S129D hSNCA+3'UTR (**C**). A Mann-Whitney t-test was used to compare coding region to 3'UTR. Significant differences are as follows: *= p 0.05, **= p 0.01. Note that inclusion of the 3'UTR reduces toxicity of S129AhSNCA on striatal-projecting neurons at all time points. Further, the toxicity of S129DhSNCA observed at 5 wks was reduced by inclusion of the 3'UTR. However, inclusion of the 3'UTR did not affect toxicity of wtSNCA, although toxicity of this coding region was also observed at 5 wks.

Figure 4. AAV-S129A hSNCA containing the 3'UTR expresses at lower RNA levels than an AAV harboring only the coding region of S129A hSNCA in the ventral midbrain, *in vivo*
 To examine differential expression of hSNCA as a potential cause for the differential toxicity observed *in vivo*, rats were injected with AAV-S129A hSNCA (n=3) or AAV-S129A hSNCA+3'UTR (n=3) and hSNCA DNA (A) and RNA (B) levels were assessed in ventral midbrain by qPCR two weeks after injection. hSNCA Ct was normalized to β -actin Ct within each sample (ΔCt) and then ΔCt of each sample was normalized to ΔCt of the uninjected SN of rats injected with S129A hSNCA+3'UTR ($\Delta\Delta Ct$). Data are expressed as relative quantity ($2^{-\Delta\Delta Ct}$). One-way ANOVA with Newman Keuls post-hoc tests were used to assess significant differences. Asterisks denote differences compared to the injected SN of

rats that received S129A hSNCA: $*=p \leq 0.05$, $***=p \leq 0.001$. An unpaired t-test also showed that hSNCA RNA levels were significantly higher ($p \leq 0.05$) in the injected SN of rats that received S129A hSNCA+3'UTR compared to the uninjected SN. Note that hSNCA RNA expression levels are reduced when the 3'UTR is included, which correlates with its reduced toxicity on nigrostriatal-projecting neurons.

Table 1

Numbers of nigrostriatal projecting neurons after ectopic expression of the coding region of an SNCA form.

	Time Points:					
	5 wks		7 wks		9 wks	
	Injected SN	Uninjected SN	Injected SN	Uninjected SN	Injected SN	Uninjected SN
AAV Injection:						
wtSNCA	504±44 *	616±19	513±28	579±28	466±27	541±32
S129A SNCA	332±29 **	525±11	379±26 **	539±25	323±35 **	535±38
S129D SNCA	466±21 **	590±31	381±35	460±50	388±27	428±41

* Cell numbers were compared between injected and uninjected SN within a single treatment and were analyzed using a Mann-Whitney t-test. Asterisks represent significant differences between injected and uninjected SN for each time point: =p 0.05,

** =p 0.01.

Table 2

Numbers of nigrostriatal projecting neurons after ectopic expression of an SNCA form containing a portion of the native 3'UTR.

	Time Points:					
	5 wks		7 wks		9 wks	
AAV injection:	Injected SN	Uninjected SN	Injected SN	Uninjected SN	Injected SN	Uninjected SN
wtSNCA+3'UTR	451±35	547±22	408±24	455±35	467±58	540±37
S129A SNCA+3'UTR	452±48	526±57	614±54	674±58	446±27	511±31
S129D SNCA+3'UTR	472±25	507±32	502±73	527±68	504±36	575±51

Cell numbers were compared between injected and uninjected SN within a single treatment and were analyzed using a Mann-Whitney t-test.