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Alpha-synuclein ferrireductase activity is detectible *in vivo,* is altered in Parkinson's Disease and increases the neurotoxicity of DOPAL

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ABSTRACT

The normal cellular role of α-synuclein is of potential importance in understanding diseases in which an aggregated form of the protein has been implicated. A potential loss or change in the normal function of α-synuclein could play a role in the aetiology of diseases such as Parkinson's disease. Recently, it has been suggested that α-synuclein could cause the enzymatic reduction of iron and a cellular increase in Fe(II) levels. Experiments were carried out to determine if such activity could be measured *in vivo.* Experiments with rats overexpressing human α-synuclein in nigral dopaminergic neurons demonstrated a correlation between α-synuclein expression and ferrireductase activity. Furthermore, studies on tissue from Parkinson's disease patient brains showed a significant decrease in ferrireductase activity, possibly due to deposition of large amounts of inactive protein. Cellular studies suggest that increase ferrireductase activity results in increased levels of dopamine metabolites and increased sensitivity to the toxicity of DOPAL. These findings demonstrate that α-synuclein ferrireductase activity is present *in vivo* and its alteration may play a role in neuron loss in disease.

INTRODUCTION

Alpha-synuclein (α-syn) is a small protein (140 amino acid residues) predominantly found in the pre-synaptic cytosol of neurons (Maroteaux et al., 1988; Totterdell et al., 2004) that can also associate with the plasma membrane (Jao et al., 2004; Jao et al., 2008; Maroteaux and Scheller, 1991). It is one of a family of similar proteins which include beta-synuclein which is co-expressed with α-syn in neurons (Kahle et al., 2000). It is a monomeric protein, but may form tetramers under specific conditions (Bartels et al., 2011; Dettmer et al., 2013). It has been shown to bind metals such as copper and iron (Binolfi et al., 2006; Davies et al., 2010; Rasia et al., 2005), but its normal cellular activity remains to be fully determined. There is some evidence to suggest that its expression in dopaminergic neurons influences the packaging and trafficking of dopamine (Burre et al., 2010; Sidhu et al., 2004). There has also been the suggestion that it can alter the redox state of iron by reducing Fe(III) to Fe(II) (Davies et al., 2011). However, its most noted role is its association with a number of diseases that have been termed synucleinopathies (Goedert, 2001).

The synucleinopathies include Multiple System Atrophy and Dementia with Lewy Bodies. However, the most well-known member of this family of diseases is Parkinson's disease (PD) (Goedert, 2001). The nature of the possible causative role of α-syn in these diseases is unclear. However, deposits of α-syn in an aggregated, amyloidogenic form are present in Lewy Bodies which are characteristic of synucleinopathies (Spillantini et al., 1997). α-syn has also been found to be toxic both *in vitro* and *in vivo* (Winner et al., 2011; Wright et al., 2009).

PD is characterized by extensive loss of dopaminergic neurons in the *Substantia nigra* in patient brains. It is unclear if aggregated α-syn plays a direct role in this neuronal loss, especially when α-syn-rich Lewy bodies are found in surviving neurons. Some inherited forms of PD are associated with point mutations in the α-syn gene SNCA (Mullin and Schapira, 2015).

The ferrireductase (FR) activity of α-syn was first demonstrated using recombinant human α-syn generated from bacteria (Davies et al., 2011). This was verified using human cells overexpressing α-syn which showed higher FR activity. The same cells showed elevated levels of Fe(II). As a potential enzyme α-syn showed simple kinetics with a K_m and V_{max} similar to other ferrireductases. The activity was found to be dependent on the presence of an electron donor such as NADH. A more recent studies has provided a fuller picture of the kinetics of α-syn FR activity characterizing NADH and substrate dependence (McDowall et al., 2017). The study also identified the active isoform to be a membrane associated tetramer. The relation of this activity to synucleinopathy or dopamine metabolism was not determined.

α-syn has been suggested to impact dopamine metabolism (Abeliovich et al., 2000; Perez et al., 2002; Yu et al., 2005; Yu et al., 2004). There is evidence to suggest that level of expression of α-syn alters dopamine synthesis, but also dopamine entry into vesicles and its release (Gaugler et al., 2012; Lotharius and Brundin, 2002; Nemani et al., 2010). Additionally, dopamine and its metabolites have been suggested to interact with α-syn and alter its potential to aggregate (Follmer et al., 2015). A bi-product of dopamine breakdown in cells is 3,4-

Dihydroxyphenylacetaldehyde (DOPAL). DOPAL has been shown to be toxic to neurons and also able to increase the formation of α-syn aggregates (Goldstein et al., 2012; Jinsmaa et al., 2016). The level of DOPAL is increased in PD patients (Burke, 2003) and it is toxic *in vivo* (Burke et al., 2003).

In the current investigation we sought to further our investigations of α-syn FR activity and its relevance to *in vivo* models and Parkinson's disease. We also sought to determine if increase α-syn FR activity would impact dopamine metabolism and the toxicity of DOPAL. Our results confirm that α-syn is a ferrireductase *in vivo* and that levels of FR activity are altered in the striatum of PD patients. Ferrireductase activity rather than simply α-syn expression alters dopamine synthesis and breakdown and increase cellular sensitivity to DOPAL toxicity.

MATERIALS AND METHODS

Reagents were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated.

Viral vector production and titration

Serotype 6 adeno-associated viral (AAV6) vectors were produced and titrated as previously described (Gaugler et al., 2012). The number of transducing units (TU) was determined by infecting HEK293T cells. The number of S1-nuclease resistant vector genome copies was measured by real-time PCR at 48 hrs post-infection. The AAV6-α-syn vector encodes expression of full-length wild-type human α-syn under the control of the constitutive mouse pgk-1 promoter. The control non-coding AAV6 vector contains a similar empty expression cassette. The titres of the vector suspensions were 7.9E10 TU/ml for the AAV6-α-syn vector and 2.1E10 TU/ml for the non-coding AAV6 vector.

Animal experiments

All procedures were performed in accordance with Swiss legislation and the European Community Council directive (86/609/EEC) for the care and use of laboratory animals. Female adult Sprague-Dawley rats (Charles River Laboratories, France), weighing 180-200g, were housed in a 12h light-dark cycle, with *ad libitum* access to water and food. Rats were injected with AAV6 vectors in the right *substantia nigra*. For both AAV6-α-syn and non-coding AAV6 vectors, the

total injected dose for each animal was 2.8E7 TU in a volume of 2 µl. The general procedure for vector injection was as previously described (Gaugler et al., 2012). We used the following stereotaxic coordinates: 5.2 mm anterior and 1.9 mm lateral to bregma point, 7.9 mm ventral from the skull surface. The injected rats were culled at both one month and three months after injection. For biochemical analysis, fresh tissue was obtained for both *substantia nigra* and striatum from each brain hemisphere separately, after one and three months. For immunohistochemistry, the animals were perfused three months after vector injection with 4% paraformaldehyde, as described previously (Gaugler et al., 2012).

Immunohistochemistry and quantitative analysis

The primary antibodies were anti-tyrosine hydroxylase (TH) (AB152 Chemicon, CA, 1:500) and anti-α-syn antibody (AB5334P Chemicon, 1:800). For bright-field microscopy analysis, TH immunohistochemistry and 3,3'-diaminobenzidine solution (DAB) revelation were performed on nigral and striatal slices, as previously described (Gaugler et al, 2012). The percentage loss of TH-positive neuronal cell bodies was determined by stereological counting as described in (Gaugler et al., 2012). Striatal dopaminergic innervation was quantified by measuring the optical density (OD) of TH-immunoreactive terminals in one in six sections throughout the entire striatum. Sections were scanned using Nikon Super Coolscan 4000 and the OD was analyzed using the free ImageJ software. The OD

was measured as the integrated density of grey values of pixels with correction for non-specific background. Results normalised for surface area were expressed as a percentage of the non-injected side. All analyses were performed in a blinded fashion.

For immunofluorescence, slices were blocked in 10% normal donkey serum (Jackson) and 0.1% Triton X-100 in PBS. Anti-α-syn antibody was incubated overnight at 4°C. For revelation, we used a secondary antibody conjugated to Alexa Fluor 546 dye (Molecular Probes), incubated for 2 hrs at room temperature. Sections were mounted on glass slides using MOWIOL.

Brain Tissue Samples

Patient tissue samples were obtained from the London Neurodegenerative Diseases Brain Bank. The ethical permission to obtain the tissues was supplied by the London City and East NRES Committee (08/H0704/128). 10-20 samples of PD patient *substantia nigra* and striatum were obtained along with 20 samples of non-PD controls that were matched for both age of the patient and post-mortem delay in processing. Statistical analysis was carried out using Analysis of Variance (ANCOVA) and F and p values determined.

Cell culture

SH-SY5Y (human neuroblastoma) cells were cultured in DMEM (Lonza) supplemented with 10% FBS, and pen/strep. Cells were maintained at 37°C and 5% CO_2 in a humidified incubator. The neuronal status of SH-SY5Y cells was monitored by RT-PCR with primers for tyrosine hydroxylase (TH), dopamine transporter (DAT) and vesicle mono-amine transporter 2 (VMAT2). Cells were used within 20 passages of the original vial.

Stable cell lines overexpressing either wild-type or mutant human α-syn were as previously described (Wang et al., 2010). The cell lines were established from SH-SY5Y cells via transfection using Fugene (Promega). All proteins were expressed from pCDNA3.1(+). The resulting cells were then selected by growth in medium contain G418 at 800 μg/ml for at least two weeks. The lines were screened by western blot to confirm expression of the protein of interest. The mutants included in this study were a deletion of amino acid residues 2-9 of the α-syn sequence (Δ2-9) and a deletion of amino acid residues 71-82 of the α-syn sequence (ΔNAC). Additionally, cell lines for human beta-synuclein (β-syn) (Wang et al., 2010) and the human ferrireductase, Steap-3 (six-transmembrane epithelial antigen of the prostate 3) metalloreductase (Sendamarai et al., 2008) were also included. Steap-3 was cloned by isolation of mRNA from SH-SY5Y and reverse transcription of the open reading frame using specific primers before cloning into pCDNA3.1(+).

Western Blotting

Cells were lysed in 0.5% Igepal CA-630 and 'complete' protease inhibitor cocktail (Roche), sonicated 3 x 3 seconds on ice, and centrifuged 10 000 xg for 3 minutes to remove insoluble membranes. Brain samples were prepared as

described for the ferrireductase assay. Protein concentration was determined with a Bradford protein assay (Bio-Rad), according to the manufacturer's instructions. Protein concentrations were normalized and boiled for 5 minutes with 1 x Laemmli SDS-PAGE buffer. Samples were loaded into a 12% acrylamide SDS-PAGE gel, with a buffer of Tris (250 mM) + Glycine (1.92 M) + SDS (0.1% w/v), run at 250V for 45 minutes. Separated proteins were transferred to a PVDF membrane by a semi-dry transfer apparatus, run at 25 V for 1.3 hours. Membranes were blocked in 5% w/v non-fat milk powder in TBS-T for 30 minutes, incubated with primary antibody for 1-2 hours, and washed 3 x 5 minutes in TBS-T. Membranes were blocked again and incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour. A further 3 x 10 minute washes were performed, and the membranes developed with Luminata Crescendo or Luminata Forte ECL substrate (Thermo Scientific), and imaged with a Fusion SL CCD imaging system (Vilber Lourmat).

Rabbit monoclonal anti-α-synuclein (MJFR1, Abcam, immunogen human α-synuclein 1-150) was used for human α-synuclein detection at a dilution of 1:4000. Mouse monoclonal anti-α-synuclein (610787, BD Biosciences, immunogen rat α-synuclein 15-123) was used for rat α-synuclein detection at a dilution of 1:2000. Mouse monoclonal anti-α-tubulin (T5186, Sigma, immunogen acetylated tubulin from *Strongylocentrotus purpuratus* sperm axonemes) was used at a dilution of 1:10,000. Mouse monoclonal, anti-dopamine transporter (AB2231, Millipore) was used at 1:1000 dilution.

Ferrireductase Assay

The ferrireductase assay was based upon the reaction of ferrozine with Fe(II) in the presence of NADH and was similar to the previous method(Davies et al., 2011). The reaction contained 20 mM MOPS pH 7.0 as the buffer and included 500 µM NADH, 500 µM ferrozine and varying concentration of protein extract depending upon the material to be assayed. The reaction was initiated by the addition of Ferric ammonium citrate prepared as a stock concentration of 25 mM. The amount added depended on the concentration required for the reaction. The final volume of the reaction was either 250 µL or 1.0 mL depending of the instrument used to measure the kinetics of the reaction. The assay was carried out by monitoring absorbance at 562 nm for at least 30 min. Experiments were performed in 1 mL quartz cuvettes in a Libra S22 spectrophotometer (Biochrom) or in 250 µL in 96 well plates using a Fluorostar Omega plate reader (BMG). Brain samples were prepared in 0.5% Igepal in distilled water and sonicated, Samples from cultured cells were prepared by scraping a confluent T75 flask of cells into 0.5 mL of distilled water. The membranous fraction was collected by centrifugation and washed several times with PBS. The resulting pellet was then resuspended with PBS containing 0.5% Igepal. After 30 minutes on ice the protein extract was cleared of debris by centrifugation and the supernatant collected. Protein content was determined using the Biorad protein assay kit using a BSA standard curve. Brain samples were prepared by sonicating the tissue into PBS with 0.5 % Igepal.

Dopamine Assay

Dopamine was detected in the extracts of SH-SY5Y cells overexpressing α-syn or various mutants of α-syn, Steap-3 and β-syn using a commercial ELISA kit (Abnova) following the manufacturer's instruction.

Electrochemical Detection

Lysates from different SH-SY5Y cell lines were prepared by scraping of confluent T75 flasks into a mobile phase (see below). The samples were sonicated and cleared debris by centrifugation before being stored at -20°C prior to analysis. Once defrosted the samples were vortexed for 30 s to ensure homogeneity. A 100 μL aliquot of the resulting cell lysates was diluted with an additional 100 μL of mobile phase and vortexed for 30 s. A 20 μL aliquot was then examined by liquid chromatography with electrochemical detection (LC-EC). Samples were examined using C18 reverse-phase column (Symmetry Shield, RP18, 5 μm, 250 mm x 4.6 mm, Waters) at room temperature. A mobile phase of 10% acetonitrile, 90% 50 mM phosphate buffer pH 3.0 containing 20 mM octane sulfonic acid was used at a flow rate of 1.0 mL/min. Amperometric detection was carried out using an applied potential of +0.5 V (ESA Coulochem II). Quantification was undertaken by external calibration for dopamine, 3,4-Dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA).

MTT assay

For toxicity experiments, cells were plated in 24 well plates (Gibco) and grown overnight. They were then treated for 48 hours with varying concentrations of DOPAL (Cayman Chemical Company) in 10 µl of methanol. Control wells were treated with 10 µl of methanol alone. After 48 hours the viability of the cultures was assessed with an MTT assay. The MTT (3, [4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) reagent was resuspended to 2.5 mg/ml. Treatments were removed and 50 µl of MTT per well was incubated for 30 minutes. After removal of MTT, deposits were solubilised in DMSO. Readings were taken at 570 nm using FLUOstar Omega (BMG Labtech). Each treatment was conducted in triplicate, averaged, and then represented as percentage of the control (methanol alone). Each experiment was repeated 4 times. Statistical analyses were conducted using a two-tailed Student's t test, statistical significance at *p*-value of < 0.05. Data are expressed as the mean ± standard error (S.E.).

Recombinant Protein and Aggregates

Human recombinant α-syn was generated as previously described (Davies et al., 2010). Briefly, human α-syn was overexpressed in bacteria and purified from the lysed bacteria. A 15% ammonium sulphate precipitation was performed to separate α-syn from the majority of bacterial proteins. Further purification was received using anion exchange using sepharose Q hi-trap column (GE Healthcare) on an ACTA purification system (GE Healthcare). The purified protein was filtered

through a 30 kD cut off membrane (Millipore) before dialysis and further concentration as needed. Protein aggregation was achieved by rapid shaking at 1200 r.p.m. for seven days at 37°C as previously described (Wright et al., 2009). Success of the aggregation process was checked using a ThT (thioflavin T) assay as previously described (Wright et al., 2009).

RESULTS

α-syn FR activity in the rodent *substantia nigra* and striatum

Previous work has demonstrated that α-syn can act as a FR in both cell models and with purified recombinant protein (Davies et al., 2011). Therefore, it is important to establish that α-syn also has FR activity *in vivo*. A previously established model of α-syn overexpression was utilised to confirm α-syn FR activity *in vivo* (Gaugler et al., 2012). In this model, rats were unilaterally injected with an AAV6 vector to induce overexpression of human α-syn within the nigrostriatal system. Figure 1 shows analysis of rats that were injected either with the AAV6-α-syn vector or with non-coding AAV6 control vector. Analysis was carried out three months after the injection. Coronal sections through the midbrain and striatum were taken and stained to detect tyrosine hydroxylase (TH) and α-syn. After three months, there was a marked reduction (-46.3±7.4%) in the number of TH-positive neurons for the AAV6-α-syn rats when comparing the *substantia nigra pars compacta* in the injected and non-injected sides (Figure 1A & B). No such difference was observed for the rats injected with the control non-coding AAV6 vector (-1.5±6.3%). In the *striatum*, there was only a 22.5±4.9% loss of TH immunoreactivity in the AAV6-α-syn rats, which was not statistically different when compared to the rats injected with the non-coding AAV6 (Figure 1C & D). It is therefore possible that compensatory changes reduced the loss of striatal TH-positive innervation. To verify transgene expression, similar sections were stained to detect α-syn in both the *substantia nigra* and striatum. As expected, fluorescent

immunostainings showed dramatically higher levels of α-syn expression when compared to the non-injected hemisphere (Figure 1E).

Tissues from rats that had also been injected with the same AAV6 virus were analysed for both FR activity and levels of α-syn and TH protein. Samples were collected from rats both one month and three months after injection with the AAV6-α-syn vector. Samples were collected from both the *substantia nigra* and the *striatum* of both the injected and non-injected sides. Additionally, similar tissue was collected at a one month time point from rats injected with the non-coding AAV6 control. The initial rate of the FR reaction was determined for each sample using 0.7 mg/ml of protein extract and 500 μM Fe(III). Six samples for each condition were analysed and the averaged data are shown in Figure 2A. The samples from the AAV6-α-syn injected rats showed a significantly higher initial rate in the FR assay (both striatum and *substantia nigra*). No other condition showed a significant increase in FR activity. The expression of α-syn relative to the levels of dopamine transporter (DAT) were used to verify that only the AAV6-α-syn injected samples showed increased α-syn levels (Figure 2B). These results demonstrate that increased α-syn levels result in increased FR activity *in vivo*.

Cell loss in the *substantia nigra* was demonstrated three months after injection of AAV6-α-syn (see above). We therefore compared the FR activity levels in AAV6-α-syn injected rat brain regions from three months after injection to those observed at 1 month, (Figure 3A). At one month, both striatum and *substantia nigra* samples showed elevated FR activity in the AAV6-α-syn injected side compared to the control side. However at three months the elevation was lost in the *substantia*

nigra. There was no significant change in the striatum at three months compared to one month. The same samples were analysed for the expression of both α-syn and DAT (Figure 3B &C). There was a clear decline in the expression of both α-syn and DAT for *substantia nigra* samples between one month and three months and marked reduction in the ratio of the two indicating a loss of α-syn relative to the amount of DAT. There was no observed change in the levels of α-syn and DAT in the striatum. Overall, these results suggest that the level of FR activity observed in the rat brain tissue samples was proportional to the amount of α-syn expressed. This supports the suggestion that α-syn acts as a ferrireductase *in vivo*.

α-syn FR activity in PD

We obtained samples of *substantia nigra* and striatum from patients with PD and aged matched controls. We analysed the samples for FR activity, α-syn protein levels and DAT protein levels (Figure 4). The initial rate of FR activity was determined using the ferrozine assay. DAT and α-syn levels were assayed by western blot. To ensure the western blot data was equivalent a standard was included on all blots so that direct comparison could be made between all samples. Between 10 and 20 samples were analysed per group. The FR activity for the samples was compared to the level of α-syn expression and any correlation observed. In general, control samples showed increased activity in proportion to the level of α-syn expression. However, PD samples did not show the same trend and higher expression was more closely related to a decreased or unaltered activity. When comparing the PD and control samples, the striatum samples

showed a significant decrease in FR activity relative to α-syn expression when analysed by ANOVA ($F=4.94$, $p=0.033$). However, there was no significant difference between the *substantia nigra* samples when comparing PD and control ($F=1.32$, $p=0.262$). We also looked at the level of α-syn expression relative to the level of DAT for each sample (Figure 4). The distribution was fairly equivalent for PD and control striatum samples. However, PD *substantia nigra* samples were more clustered with low α-syn and low FR activity relative to the controls. These findings demonstrate that there is a decrease in FR activity in PD patient striatum.

α-syn aggregates have no FR activity

We previously demonstrated that recombinant α-syn has FR activity (Davies et al., 2011). The changes in activity identified in PD brains striatum increased levels of α-syn expression does not lead to increased activity. We therefore sought to determine if aggregated α-syn has altered FR activity. We generated aggregates of α-syn as previously described (Wright et al., 2009) using a rapid shaking method over seven days. Aggregation was assessed using the standard ThT method. Using the ferrireductase assay we could measure FR activity in non-aggregated α-syn but not in aggregated protein (Figure 5). These results suggest that when α-syn is aggregated it loses its FR activity. Therefore the decreased levels of FR activity measured in samples with high α-syn content could be due to aggregation.

FR activity and the levels of dopamine and it metabolites in cells

There has been significant discussion of the potential of α-syn to alter the secretion and possible formation of dopamine and it metabolic break down products. We were therefore keen to do determine if FR activity of α-syn could influence dopamine levels and its metabolism. We previously generated a number of cell lines using SH-SY5Y cells that express either wild-type α-syn, β-syn or mutants of α-syn. We included in this study two mutants of α-syn Δ2-9 (missing the N-terminus) and ΔNAC (missing the NAC region, amino acid residues 71-82). Lastly, we included another known ferrireductase, Steap-3. Protein extracts were prepared from the cells and used to assess FR activity (Figure 6A). Clear differences in activity were seen with both α-syn and Steap-3 showing the highest activity. The two mutants lacking specific domains showed greatly reduced activity indicating the mutations allowed expression of α-syn with diminished FR activity. In comparison extracts from cells expressing β-syn showed no FR activity. We used a commercial kit to measure the levels of dopamine in extracts from these cell lines. Despite the expression of these different proteins at high levels, none of the cell lines showed any significant difference to a cell line transfected with the empty vector (Figure 6B). These results suggest that neither α-syn nor increased FR activity alters dopamine levels.

We then used HPLC with electrochemical detection to assess if the same cell lines showed alterations in the breakdown products of dopamine. Within the cell dopamine that is free in the cytosol is rapidly metabolized to DOPAL (3,4-Dihydroxyphenylacetaldehyde) by mono-amine oxidase and then further metabolized to DOPAC (3,4-Dihydroxyphenylacetic acid) by aldehyde

dehydrogenase. Further degradation to HVA (homovanilic acid) is dependent upon encountering catechol-O-methyl transferase which has low cell expression. Therefore, we assessed DOPAC and HVA levels for the cell lines (Table 1). As can be seen, the levels of the two metabolites were similarly increased in both α-syn and Steap-3. However, neither β-syn nor the mutants of α-syn with reduced activity showed any change in these metabolites. We also measured dopamine with HPLC and the result confirmed the findings from the commercial assay that α-syn overexpression does not alter it levels in cells (data not shown). These data suggests that FR activity of α-syn may cause an increase in the rate of degradation of dopamine.

α-syn FR activity increases cellular sensitivity to DOPAL toxicity

Given that α-syn expression altered the levels of dopamine breakdown products we wished to know if this could have an impact on cellular survival. DOPAL is believed to be toxic to cells if it accumulates at high levels. Therefore we used cell survival assays to assess DOPAL was differentially toxic to cells overexpress α-syn. Cells overexpressing either α-syn or the known FR Steap-3 were compared to cells transfected with the empty vector. After 48 hours of treatment, the cells were assessed with an MTT assay to determine relative viability. A significant difference in viability was seen for both α-syn and Steap-3 expressing cell lines for 2 μM DOPAL and above when compared to the control cell line (Figure 7A). This suggests that FR activity makes cells more vulnerable to the toxicity of DOPAL.

We also tested the toxicity of DOPAL on other cell lines. Cell lines expressing β-syn or the two α-syn mutant Δ2-9 and ΔNAC showed significantly reduced levels of DOPAL toxicity when compared the α-syn cell line (Figure 7B). As these cell lines have much less FR activity it is likely that the impact of α-syn on DOPAL toxicity is due to its FR activity.

DISCUSSION

The identification of a potential enzymatic function for α-syn necessitates verification that this activity is present *in vivo*. The first aim of the research present here was to determine if increased FR activity can be detected with increased α-syn expression in a rodent model. Rats unilaterally injected with the AAV6-α-syn vector in the *substantia nigra* showed increased expression of human α-syn. Both in the *substantia nigra* and in the striatum, FR activity was increased only when there was increased α-syn expression. Secondly, neuronal death in the *substantia nigra* where there was initially increased expression resulted in loss of both α-syn expression and FR activity. Additionally, in human brain tissue (non PD) the level of FR activity correlated with increased α-syn expression. This evidence strongly supports that α-syn is an FR *in vivo*. These findings need to be assessed along-side previous studies where purified, recombinant α-syn showed FR activity in isolation from other cellular components (Davies et al., 2011; McDowall et al., 2017). Without this evidence it could be argued that α-syn expression could alter the behaviour of other iron- reducing systems without being directly involved. Additionally, we have shown previously that the FR activity is heat sensitive, membrane associated and can be selectively immunoprecipitated from the membranes of dopaminergic cells, fully accounting for any increase caused by overexpression of the protein (McDowall et al., 2017). This implies that the increase FR activity we observe in these studies is entirely due to α-syn.

Rodent models have been particularly important in assessing the impact of α-syn expression on neuronal survival. High α-syn expression has been shown to lead

to cell death (Gaugler et al., 2012). The cell death occurred in the *substantia nigra*. In this study we looked at the striatum in parallel as this is the principal area of innervation by dopaminergic neurons of the *substantia nigra*. Our analysis did not observe any changes in FR activity in the striatum following neuronal loss in the *substantia nigra*. Levels of DAT, α-syn and FR activity all remained similar between the one and three month time points in this brain region. This is likely to be due to the remaining neuronal population of the *substantia nigra* expanding axonal branching to encompass the additional targets.

The potential impact of FR activity from α-syn on the disease state is clearly very important. However, assessment of an enzymatic activity within post mortem tissue is subject to potential loss of activity due to the age and storage conditions of the material assessed. The impact of this on our results is difficult to assess. However, controls samples showed a linear relation between α-syn levels and FR activity for both the *substantia nigra* and striatum samples which is supportive that this approach has validity. More importantly, there was a significant difference in the striatum in regards of FR. Samples with higher α-syn levels showed lower activity. This trend was evident in the *substantia nigra* samples as well but did not reach significance. α-syn is an amyloidogenic protein and its aggregation in disease is a hallmark of PD. We verified that aggregated α-syn has no FR activity. It is likely that PD samples with high α-syn content probably have significant amounts of aggregated α-syn. Therefore the difference observed between control and PD samples is likely to be due to α-syn aggregation and subsequent loss of activity.

A loss of activity relative to the amount of α-syn present does not indicate a possible mechanism of dopaminergic neuron death. Lewy bodies (which contain aggregates of α-syn) are usually intracellular and therefore present in viable cells or their axons. Also, the significant change in FR/ α-syn was in the striatum which is more likely to be activity associated with axonal or terminal α-syn content. In the rodent model there was a loss of expression and a loss of FR activity in the *substantia nigra* which is more likely to be due to neuron loss. What is unclear is whether the neuronal loss is related in any way to the increased FR activity. In the rodent model, which involved viral delivery of an α-syn expression system, there is no evidence for the extent of aggregation of the protein, nor the nature of the cells overexpressing α-syn. However, there is a distinct possibility that elevated FR activity (and increased Fe(II)) would constitute an additional toxic load that may result in cell death.

It is important to consider the difference between the observations in the rat model and the changes seen in PD. Both models show a change in the levels of α-syn in the *substantia nigra*, but an actual significant reduction in FR activity was seen in the rat model only. In the case of the rat model the α-syn present is a result of transgenic overexpression and may not be expressed solely in dopaminergic neurons (which is not the case in the striatum, where it would have been transported along axons). Also, assessment in PD brain tissue is fundamentally more challenging because of the age and state of the material being not ideal for the preservation of enzymatic activity. Thus variability unrelated to changes in α-syn aggregation and FR activity might mask a result that would have been

equivalent to that observed in the striatum (i.e. reduced activity relative to the amount of protein present). It should be noted that aggregation of α-syn is often relative to its concentration, thus in the rat model significant amounts of aggregation may have been present (due to overexpression) at both one and three months and that change in the FR activity is not related to a change in the nature of the protein but simply as a result of the loss of cells expressing α-syn.

We turned to a cell model to investigate whether increased FR activity has consequences for neuronal viability. One of the most obvious sources of toxicity for dopaminergic cells would be accumulation of a dopamine metabolite that is toxic. There is good evidence that DOPAL can be toxic and it is a product of dopamine breakdown by mono-amine oxidase. We assessed the levels of dopamine and various metabolites in SH-SY5Y cells and found an increase in DOPAC, a further breakdown product of DOPAL (via aldehyde dehydrogenase) where there was increased FR activity. This change suggests that FR activity (or increased Fe(II)) accelerates dopamine breakdown or causes accumulation of metabolites in cells. Unlike other reports have suggested (Lotharius et al., 2002; Perez et al., 2002), we did not see any change in dopamine itself, as a result of α-syn overexpression, which indicates the changes in DOPAC are a change in the rate of breakdown and not just a result of their being more dopamine in cells. Also, unlike other reports, we were not able to detect DOPAL due to its significant insolubility in the HPLC mobile phase. This is in contrast to other who have report DOPAL measurements with HPLC-ECD (Wey et al., 2012). However, given the

increase in both DOPAC and HVA, it is reasonable to conclude that all breakdown species are increased.

Cell lines facilitate molecular studies but their continued division masks any potential changes in viability due to toxic molecules. We therefore treated SH-SY5Y cells with exogenous DOPAL to determine if FR activity altered the cellular response to its toxicity. FR activity increased DOPAL toxicity significantly. These findings provide a potential mechanism by which high levels of α-syn FR activity could specifically induce cell death in dopaminergic neurons. If such a mechanism was active in the *substantia nigra* it would imply that altered FR activity would contribute to cell death. Alternatively, increased Fe(II) levels could be toxic without any involvement of DOPAL. While either is possible the potential role of DOPAL is more convenient as it would be a more unique target for therapeutic intervention than the ubiquitous Fe(II). Also, it has been suggested that Fe(II) can increase the rate of DOPAL mediated α-syn aggregation (Jinsmaa et al., 2014).

The fundamental requirement for α-syn FR activity to play a role in altering neuronal viability in PD is that its expression, and subsequently FR activity, is increased during disease progression. The formation of aggregated α-syn is believe to be largely a result of increased expression and subsequent molecular crowding (Munishkina et al., 2008). However, it is unclear if there is genuinely an increase in α-syn expression during early PD. In our study, there was clearly a distribution of expression levels in human tissue. PD *substantia nigra* showed a trend to lower expression. However, such material is clearly from after significant cell loss has occurred and in general higher activity in PD patients correlated with

lower FR activity. There is strong support for the idea from inherited PD particularly in regards the PARK4 locus which is a duplication of the SNCA gene and results in significantly higher expression of α-syn (Itokawa et al., 2013). There is other evidence to support that PD patients have elevated levels of α-syn which includes studies of fibroblasts (Hoepken et al., 2008) and also from micro-analysis of mRNA levels in surviving PD patient dopaminergic neurons (Grundemann et al., 2008) or generally in the mid-brain (Chiba-Falek et al., 2006). However, there are other reports that suggest there might be no increase, but these are largely based on looking at CSF or other tissues (Tokuda et al., 2006), (Wirdefeldt et al., 2001).

FR activity is clearly an essential activity for cells that require replenishment of Fe(II) for cellular metabolism. Clearly, there is a consequence for cells of excess Fe(II) because of its potential to initiate damage through radical generation (Fenton chemistry) but whether overexpression of a ferrireductase could result in toxic levels of Fe(II) has had little consideration. We could find no cases in the literature of mutant ferrireductases associated with cell loss. However, changes in iron are well known to be associated with PD (Dexter et al., 1991; Dexter et al., 1990; Dexter et al., 1987; Dexter et al., 1989) and until this point there has been no clear explanation for this. Interestingly, a recent study of inherited PD associated with mutations in RAB39B resulted in reduced α-syn levels but increased iron accumulation (Wilson et al., 2014). The study was carried out in post-mortem tissue in which high α-syn neurons may have been lost. However, the correlation with PD and altered iron metabolism remains.

In summary, our findings verify that α-syn has FR activity *in vivo* and that FR activity correlated with α-syn expression is altered in PD. We also provide a possible mechanism by which increase FR activity in the presence of DOPAL could cause neuronal loss. Targeting excess generation of Fe(II) in PD patients could be a potential therapeutic option for the development of novel PD treatments.

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FIGURE LEGENDS

Figure 1 AAV6-α-syn injection in the rat *substantia nigra* leads to neurodegeneration

Rats were unilaterally injected in the *substantia nigra* either with a control non-coding AAV6 vector or with the AAV6-α-syn vector for overexpression of human α-syn. Degeneration of the nigrostriatal system was analysed at three months post-injection. **A** Representative sections of the ventral midbrain showing TH-positive neurons in the *substantia nigra*, comparing the injected and non-injected hemispheres near the site of injection. Note the loss of TH-positive neurons (*) in the *substantia nigra* injected with the AAV6-α-syn vector. **B** Stereological analysis of the percentage loss of TH-positive nigral cell bodies, comparing the injected with the non-injected *substantia pars compacta* in each animal. Statistical analysis: Student's t test, n=6 in each group; ***$p<0.001$. **C** Representative sections of the *striatum* showing TH-positive innervation in rats injected either with the non-coding or with the AAV6-α-syn vector. * indicates the injected side. **D** Quantification of the percentage loss of dopaminergic axons in the *striatum* by comparing optical density of TH immunostaining in the injected and non-injected hemispheres. Statistical analysis: Student's t test, n=6 (α-syn) and n=7 (non-coding); ns: not significant. **E** Representative pictures of α-syn fluorescent immunostainings showing human α-syn overexpression in the rat *substantia nigra* and *striatum* in the injected hemisphere. Scale bars: 100 µm.

Figure 2 α-syn FR activity in AAV6-α-syn injected rats at one month

Protein extracts were prepared from the *substantia nigra* (SN) and the striatum (STR) from rats that had either been injected with the AAV6-α-syn vector (Exp) or a non-coding sham vector without the α-syn ORF (Sham). Samples analysed were from both the injected side and the contralateral non-injected side (control). The extracts were used to measure FR activity using the ferrozine assay (A) or western blots to detect α-syn and DAT (B). The data in A is the average of six different animals per treatment with s.e.m. * Indicates values significantly different to control and sham groups ($p < 0.05$, Student's t test).

Figure 3 α-syn FR activity in AAV6-α-syn injected rats after 3 months

A Protein extract were prepared from the *substantia nigra* (SN) and the striatum (STR) from rats that had either been injected with the AAV6-α-syn virus. Extracts were also prepared from the *substantia nigra* (CSN) and striatum (CSTR). The samples had been collected either one month or three months after injection. The samples were analysed for FR activity using the ferrozine assay. There were nine animals used the one month time point and five for the three month time point. Shown are mean and s.e.m. of the measured initial rate for the different treatments. * Indicates a significant difference between injected and the non-injected side. # Indicates a significant difference between one month and three months.

B & C. Western blot was used to analyse the expression of α-syn and DAT in the samples of rat brain. The level of expression was compared using densitormetry

and the ratio of α-syn to DAT determine for each sample. At the one month time period the ratio of α-syn to DAT was highest for the *substantia nigra* (SN). When comparing the ratio at three months to one month there was a significant reduction in the value ($p < 0.05$, Student's t test) indicating a loss of α-syn relative to the levels of DAT. The ratio of α-syn to DAT for the striatum (STR) showed no significant change between one month and three months.

Figure 4 FR Activity in PD brain samples

Samples from both striatum (STR) and the *substantia nigra* (SN) from both PD patients and controls were obtained and protein extracts prepared. The FR activity was measured using the ferrozine assay. Levels of DAT and α-syn were also determined using western blot. A standard was used across all western blots to allow comparison between samples. Densitometric analysis was carried out on western blots and normalised to the internal standard. An example of a western blot for both α-syn and DAT is shown at the bottom. The levels of FR activity were compared to levels of α-syn for each sample and plotted (upper two panels). A trend-line was fitted to the four sets and statistical analysis was carried out by ANCOVA. The data for the STR showed a significant difference between the control and PD groups but this was not observed in the SN data. In addition, the levels of α-syn to DAT was also plotted for each sample (middle panels). The relation of α-syn to DAT is uniform when comparing the STR groups but in the SN

groups the PD samples are largely grouped showing low FR activity and α-syn levels when compared to controls.

Figure 5 FR activity of aggregated recombinant α-syn

Recombinant human α-syn was generated by bacterial expression and purified using ion affinity chromatography. Aggregation was induced by shaking at 1200 r.p.m. at 37°C for seven days. **A** The extent of aggregation was determined using a thioflavin T (ThT) assay. Fluorescence was measured at 482 nm with 440 nm excitation. The values were plotted on log scale. Aggregated samples were significantly more fluorescent than controls (n=6). **B** Samples of the aggregated protein were added to FR ferrozine assay along-side non aggregated controls. The assay was run standardly for 30 minutes but an example is shown for a much longer assay. The aggregated sample was mixed every 5 minutes. Measurements were recorded every 40 seconds.

Figure 6 FR and dopamine levels in SH-SY5Y cells

A The level of FR activity was determined in several SH-SY5Y cell lines that had been stably transfected to overexpress α-syn, two mutants of α-syn (Δ2-9 and ΔNAC), β-syn or Steap-3. A cell line transfected with the empty vector (pCDNA) was included as a control. Protein extracts were prepared from the cells and the protein content determined. The initial rate for each cell line in the FR ferrozine

assay using 500 μM Fe(III) and equivalent amounts of protein was determined. Shown are the mean and s.e.m. for n=4 experiments. α-syn, Δ2-9 and Steap-3 show significantly more activity than the control (*, $p < 0.05$, Student's t test) and both Δ2-9 and ΔNAC showed significantly less activity than wild-type α-syn.

B The levels of dopamine were assessed in the same cell lines. The levels of dopamine were measured using an ELISA based commercial assay. Shown are the mean and s.e.m. for four experiments. There were no significant differences in dopamine levels for any of the cell lines.

Figure 7 FR activity and DOPAL toxicity.

The levels of toxicity of DOPAL to SH-SY5Y cell lines were determined using an MTT assay. Cell lines were plated at equivalent density in to 24 well plates and exposed to increasing concentrations of DOPAL in 10 μL of methanol for 48 hours. The survival relative to a methanol treated control (10 μL) was determined and graphed as a percentage. Shown are the mean and s.e.m. for 4-6 experiments were shown. **A** DOPAL was significantly more toxic to cell lines overexpressing α-syn or Steap-3 than the pCDNA control at concentrations of 2 μM and above ($p < 0.05$, Student's t test). **B** When compared to wild-type α-syn the mutants of α-syn (Δ2-9 and ΔNAC) show significantly less susceptibility to DOPAL toxicity at 2 μM and above while β-syn showed less susceptibility at 10 μM DOPAL and above.

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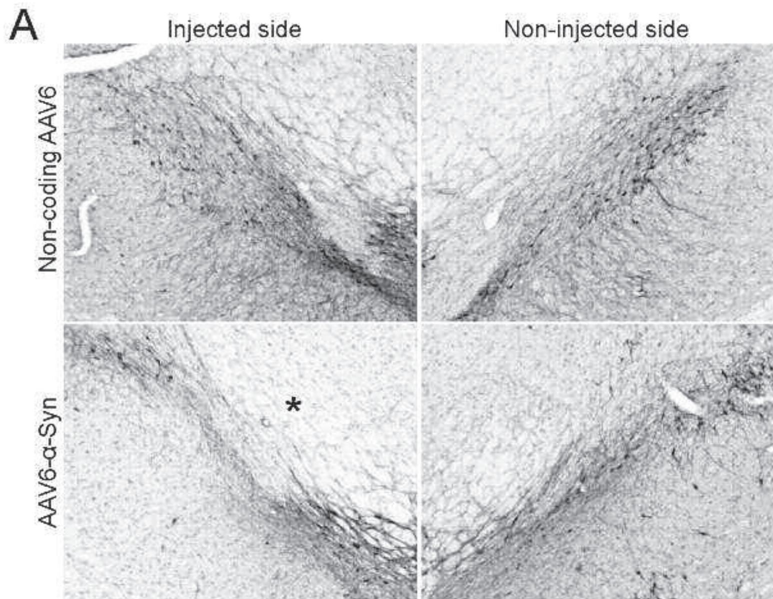
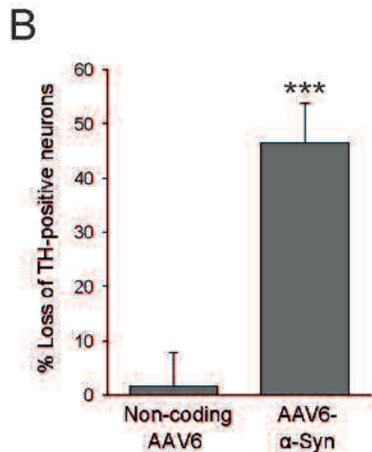
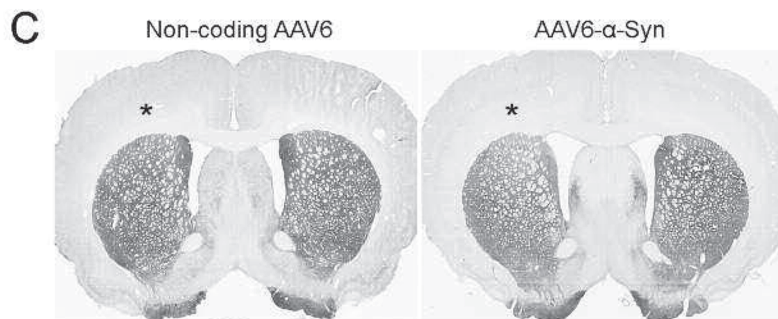
Table 1 Dopamine metabolites in SH-SY5Y cell line

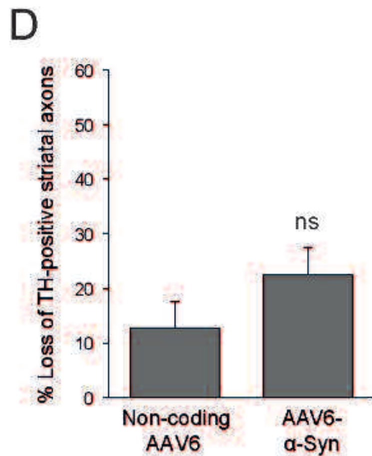
Cell Line	DOPAC (ng/mg protein)	HVA (ng/mg protein)
pCDNA	130 ± 46	640 ± 87
α-syn	320 ± 67*	876 ± 77*
β-syn	116 ± 68	527 ± 81
Δ2-9	197 ± 45	712 ± 67
ΔNAC	142 ± 44	595 ± 54
Steap-3	387 ± 81*	923 ± 102*

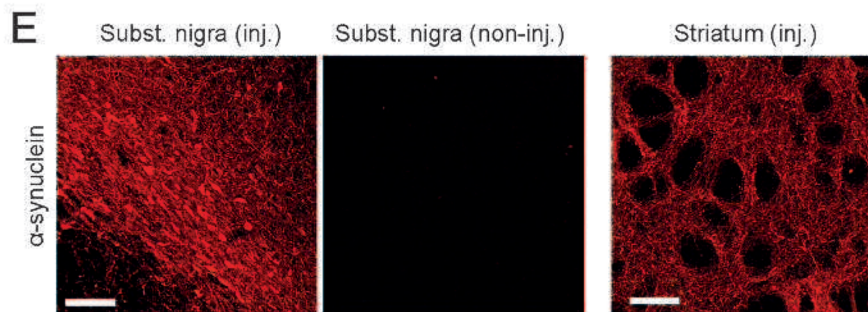
SH-SY5Y cells stably transfected with the protein indicated. Level of DOPAC and HVA were detected by HPLC with electrochemical detection. Values obtained were related to the initial protein concentration of the starting sample. Shown are the mean and s.e. for 3-4 samples each. * indicates significant difference to the to the pCDNA.3.1 transfected control.

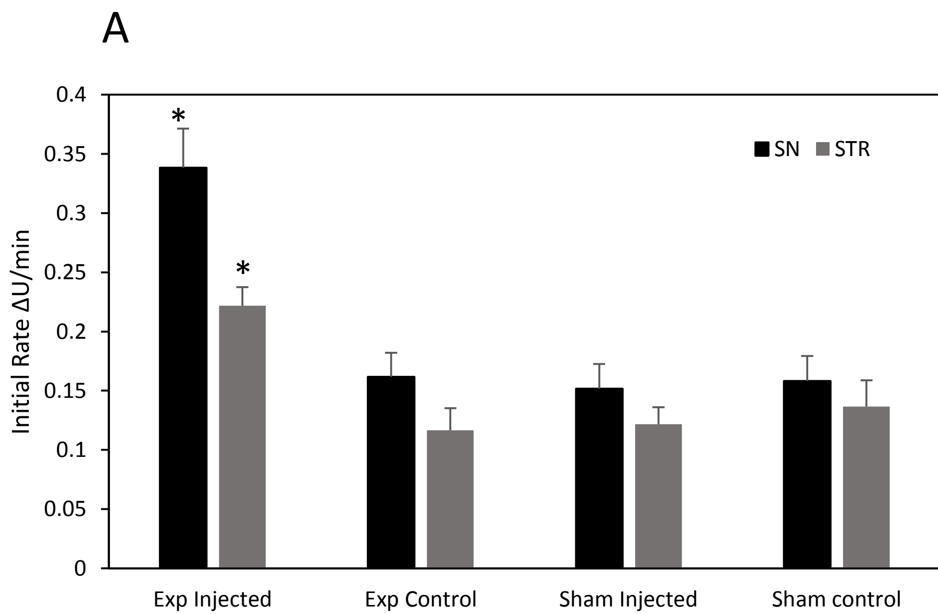

A
Injected side
Non-injected side
Non-coding AAV6
AAV6-α-Syn
*


B
% Loss of TH-positive neurons
60
50
40
30
20
10
0
***
Non-coding AAV6
AAV6-α-Syn


C
Non-coding AAV6
AAV6-α-Syn
*
*


D
% Loss of TH-positive striatal axons
60
50
40
30
20
10
0
ns
Non-coding AAV6
AAV6-α-Syn


E
Subst. nigra (inj.)
Subst. nigra (non-inj.)
Striatum (inj.)
α-synuclein



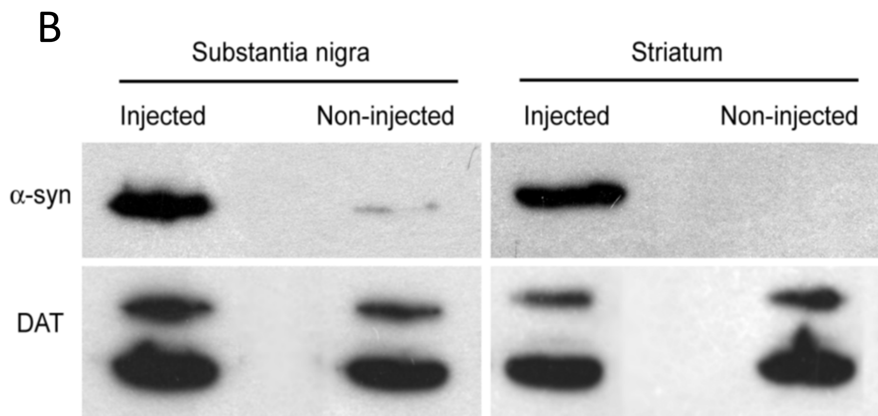
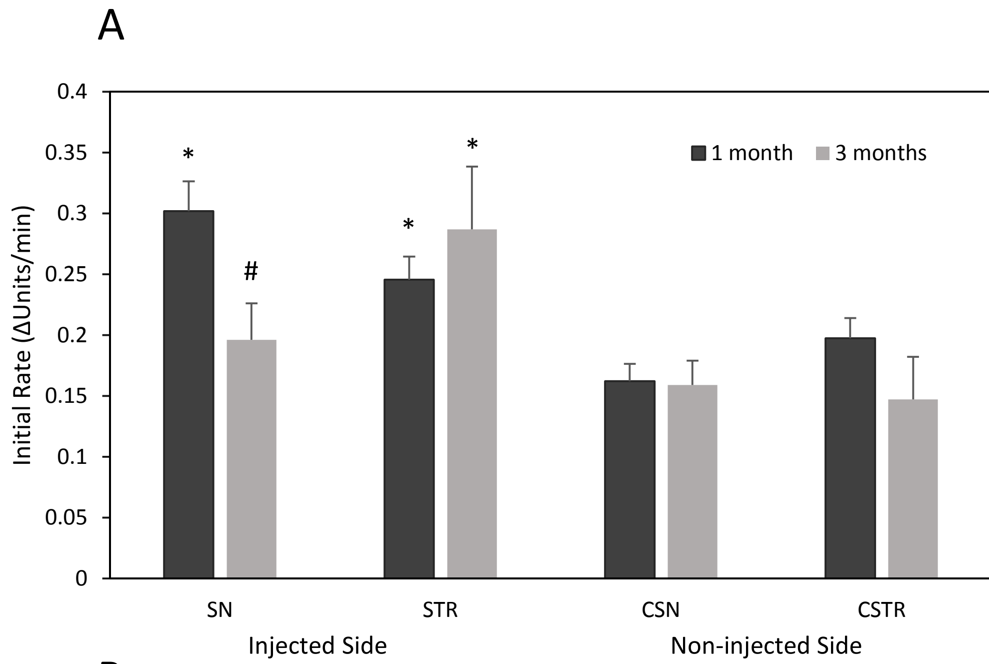
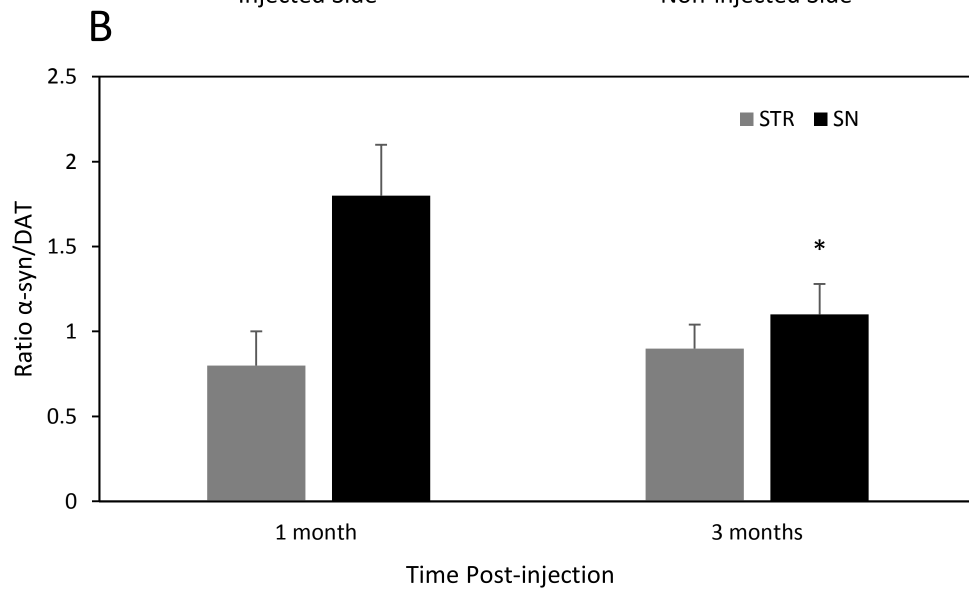


Figure 2





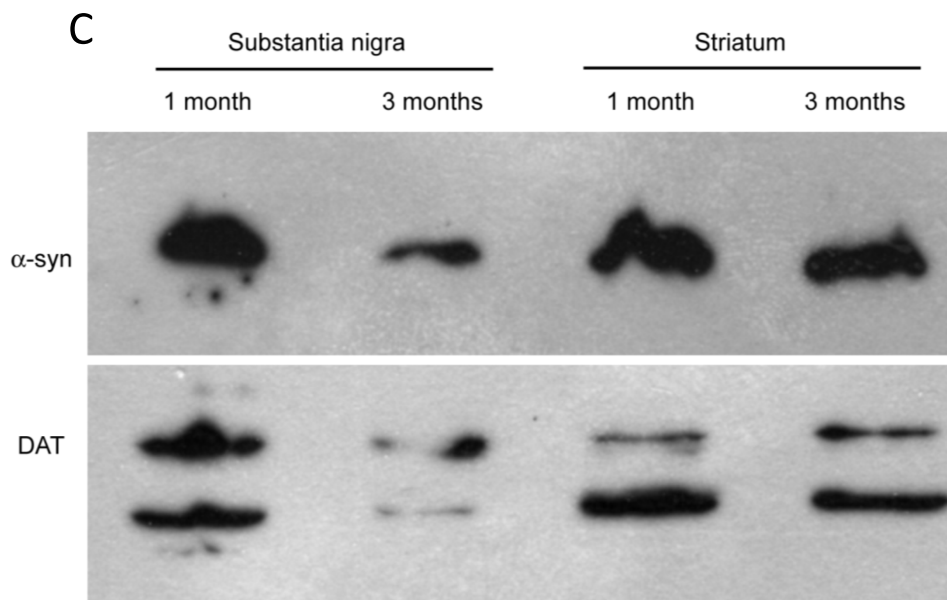


Figure 3

STR-Activity

Control
PD

Initial Rate μM Fe(III)/min

Relative α-syn

SN-Activity

Initial Rate μM Fe(III)/min

Relative α-syn

STR-Protein

Relative α-syn

Relative DAT

SN-Protein

Relative α-syn

Relative DAT

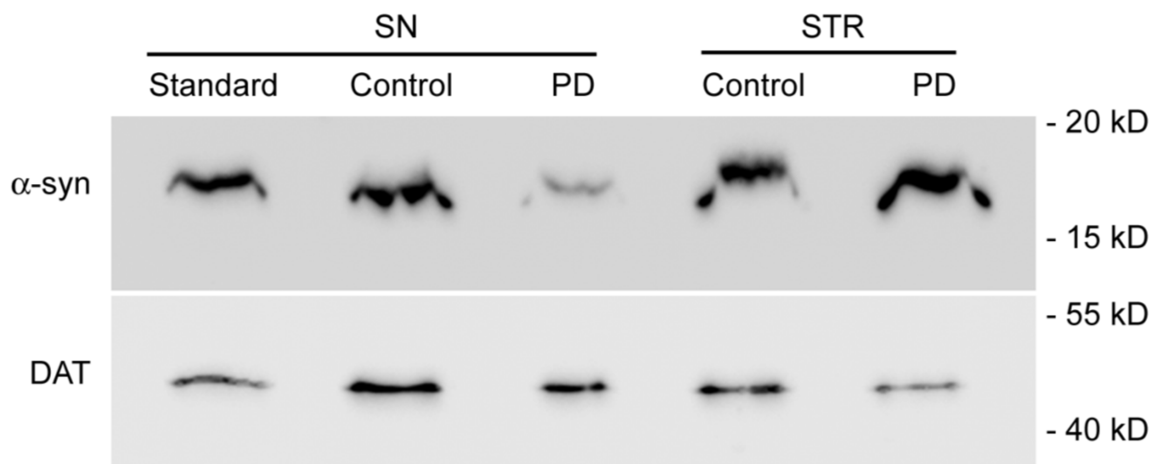


Figure 4

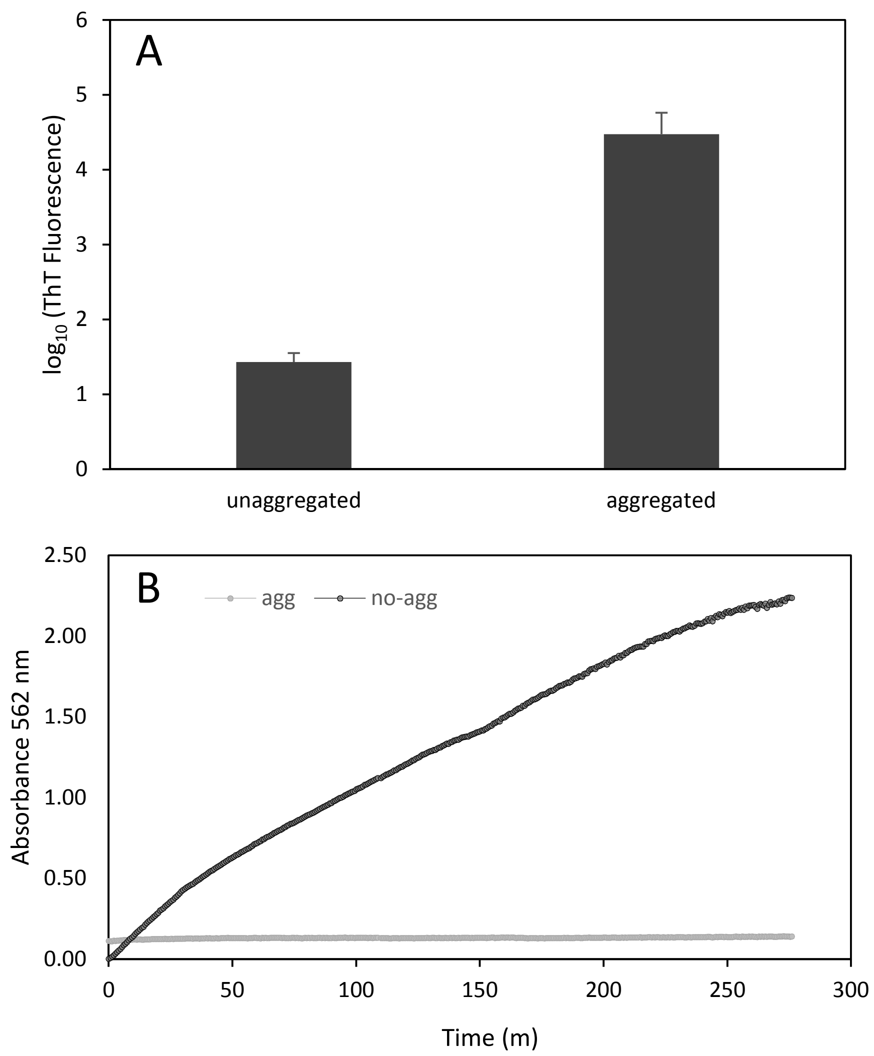


Figure 5

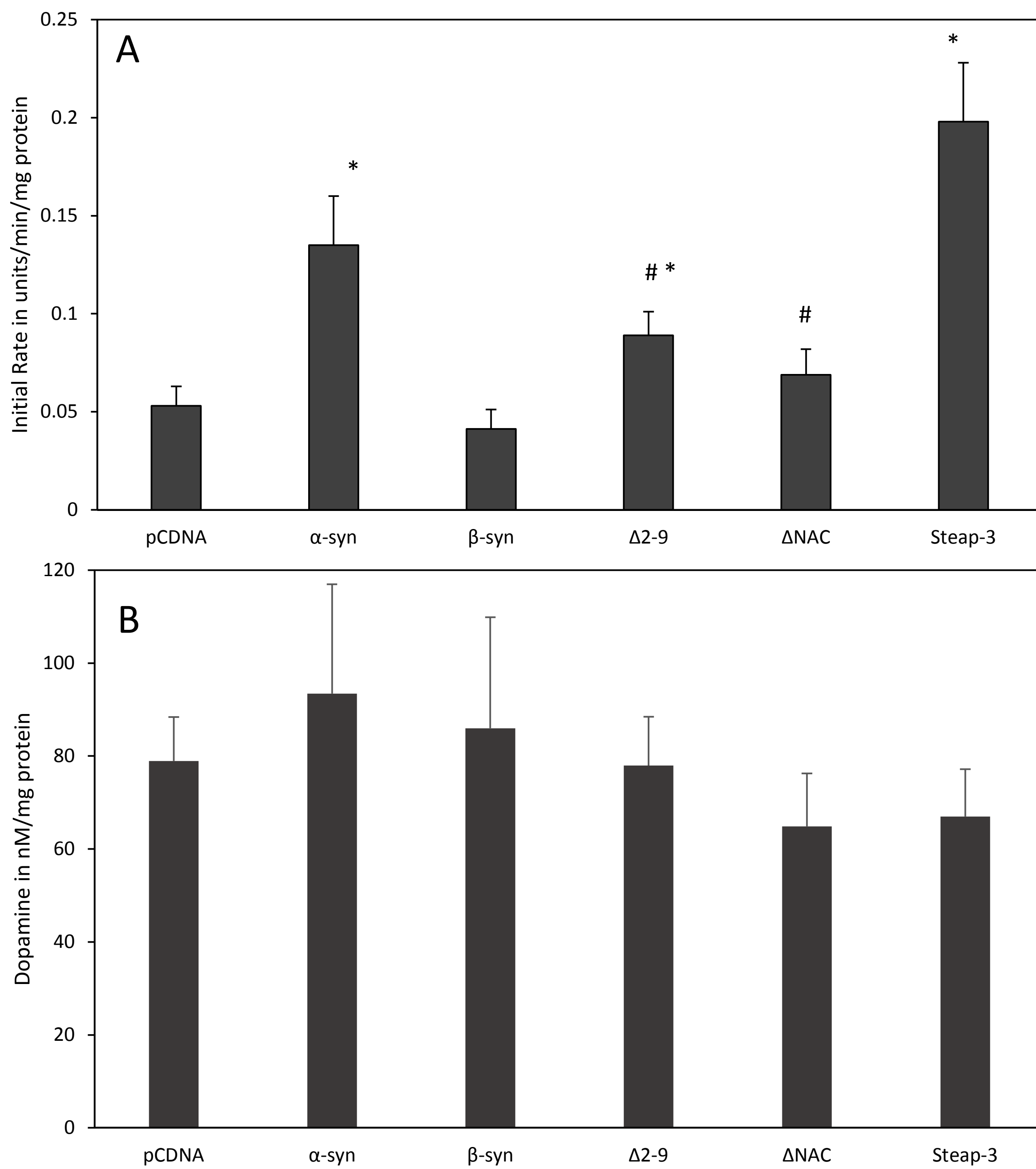
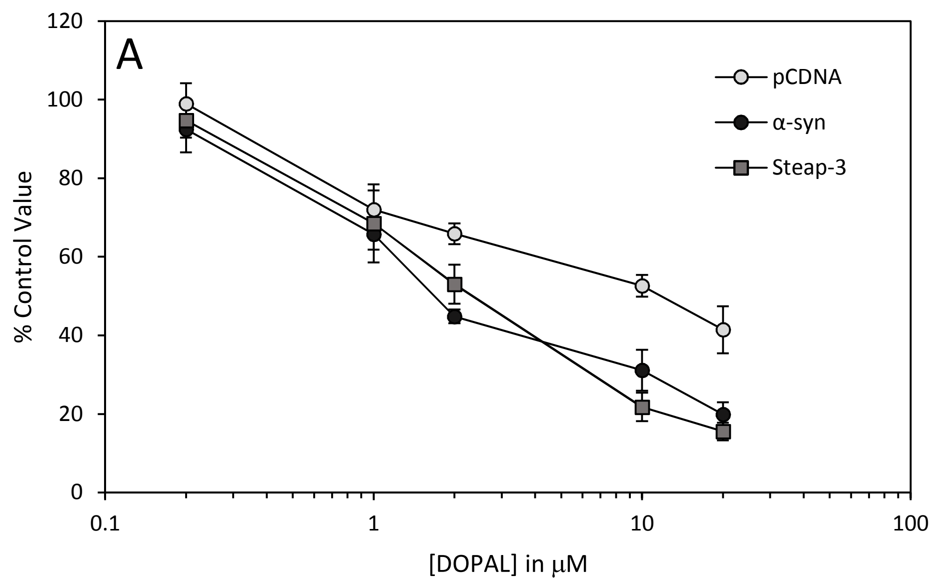


Figure 6



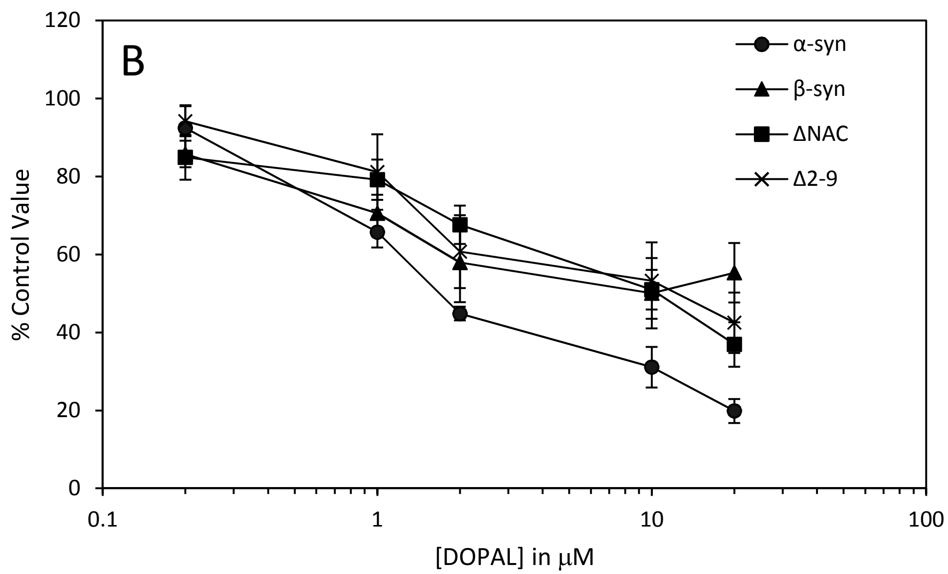


Figure 7