

## RESEARCH PAPER

# Exendin-4 reverts behavioural and neurochemical dysfunction in a pre-motor rodent model of Parkinson's disease with noradrenergic deficit

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## BACKGROUND AND PURPOSE

Parkinson's disease (PD) is characterized by progressive dopaminergic cell loss; however, the noradrenergic system exhibits degeneration as well. Noradrenergic deficit in PD may be responsible for certain non-motor symptoms of the pathology, including psychiatric disorders and cognitive decline. The aim of this study was to generate a pre-motor rodent model of PD with noradrenergic denervation, and to assess whether treatment with exendin-4 (EX-4), a glucagon-like peptide 1 receptor agonist, could reverse impairment exhibited by our model.

## EXPERIMENTAL APPROACH

We generated a model of PD utilizing *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine and 6-hydroxydopamine to create partial lesions of both the noradrenergic and dopaminergic systems respectively. We then assessed the validity of our model using an array of behavioural paradigms and biochemical techniques. Finally, we administered EX-4 over a 1 week period to determine therapeutic efficacy.

## KEY RESULTS

Our model exhibits anhedonia and decreased object recognition as indicated by a decrease in sucrose preference, increased immobility in the forced swim test and reduced novel object exploration. Tissue and extracellular dopamine and noradrenaline were reduced in the frontal cortex and striatum. TH<sup>+</sup> cell counts decreased in the locus coeruleus and substantia nigra. Treatment with EX-4 reversed behavioural impairment and restored extracellular/tissue levels of both dopamine and noradrenaline and TH<sup>+</sup> cell counts.

## CONCLUSION AND IMPLICATIONS

We conclude that early treatment with EX-4 may reverse certain neuropsychiatric dysfunction and restore dopamine and noradrenaline content.

## Abbreviations

6-OHDA, 6-hydroxydopamine; DSP-4, *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine; EX-4, exendin-4; FST, forced swim test; GLP-1, glucagon-peptide 1; NOR, novel object recognition; OFT, open field test; SPT, sucrose preference test

## Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder affecting the population (de Lau and

Breteler, 2006). It is characterized by the progressive loss of dopaminergic neurons in the substantia nigra (SN) which results in the hallmark motor symptoms of the pathology including resting tremor, rigidity, bradykinesia and postural

instability (Lees *et al.*, 2009). However, accumulating evidence suggests other neurotransmitter systems are severely compromised. Studies have shown deficits in the noradrenergic, serotonergic and acetylcholinergic systems (Braak *et al.*, 2003; Dauer and Przedborski, 2003). Braak and his research group have suggested a six-stage scheme in which alpha synuclein pathology migrates rostrally from the brainstem to higher cortical areas (Braak *et al.*, 2003). Regions such as the locus coeruleus (LC), the main site of noradrenergic cell bodies, and the raphe nuclei, the principal site of serotonergic cell bodies, may be affected before dopaminergic degeneration (Hornykiewicz, 1963). In fact, it has been shown that the LC experiences greater cell loss than the SN in PD patients (Zarow *et al.*, 2003). Neurodegeneration of the LC has also been implicated in Alzheimer's disease (AD) pathology (Braak *et al.*, 2003).

Loss of NA may be responsible for several non-motor symptoms experienced by PD patients including affective disorders and cognitive decline (Schrag, 2004; Chaudhuri *et al.*, 2006; Ziemssen and Reichmann, 2007; Benarroch, 2009). These symptoms have been shown to appear in the early stages of the disease and significantly affect the patient's quality of life (Tolosa *et al.*, 2007; Ziemssen and Reichmann, 2007).

Recent *in vivo* studies utilizing PET have shown a correlation between dysfunctional noradrenergic transmission and worsening of PD pathology (Marie *et al.*, 1995; Remy *et al.*, 2005). Remy *et al.* reported decreased NA transporter binding in several limbic regions of the brain in depressed PD patients (Remy *et al.*, 2005). This is an important finding as noradrenergic transmission in these regions has been shown to regulate aspects of emotional and cognitive processing (Benarroch, 2009). In addition, *post-mortem* studies have described increased cell loss in the LC of patients suffering from depression (Chan-Palay and Asan, 1989). Depression affects more than 50% of PD patients and it has been hypothesized that loss of NA may contribute to both neuropsychiatric alterations and cognitive impairment (Cummings and Masterman, 1999; Shiba *et al.*, 2000; Tolosa *et al.*, 2007).

Given the prominent role of NA in both psychiatric and cognitive processes and the suggested pathological progression of PD, we sought to explore whether partial degeneration of the LC could account for the cognitive and emotional deficits that many patients in the early stages of PD experience. We utilized an optimized dose of *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP-4, 25 mg·kg<sup>-1</sup>) to partially ablate the noradrenergic system (Sachs and Jonsson, 1975). DSP-4 has long been used to create lesions of NA afferents stemming from the LC (Ross and Reis, 1974; Srinivasan and Schmidt, 2003; Archer and Fredriksson, 2006). Four days after DSP-4 treatment, we injected 15 µg of 6-hydroxydopamine (6-OHDA) into each striatum in order to generate a pre-motor rodent model of PD simulating the partial noradrenergic and dopaminergic loss evident in early PD.

We then assessed the validity of our model to express anhedonic and depressive behaviour in two behavioural paradigms: sucrose preference and the forced swim test (FST) (Porsolt *et al.*, 1978; Bambico *et al.*, 2009). We also evaluated cognitive ability utilizing a novel object recognition (NOR) test (Ennaceur and Delacour, 1988). Tissue and extracellular dopamine (DA) and NA content in both the frontal cortex

(FC) and striatum (STR) were also analysed by HPLC with electrochemical detection (ECD). Finally, we administered exendin-4 (EX-4), a glucagon-like peptide 1 receptor agonist, to determine therapeutic efficacy. EX-4 has been shown to reverse neurodegeneration and promote neuroprotection in animal models of PD, AD, Huntington's disease, stroke and amyotrophic lateral sclerosis (Bertilsson *et al.*, 2008; Harkavyi *et al.*, 2008; Martin *et al.*, 2009; Li *et al.*, 2012). EX-4 is currently prescribed for the treatment of type II diabetes and holds promise as a non-invasive treatment for PD (Gallwitz, 2011).

## Methods

### Chemicals and reagents

All chemicals and reagents used in subsequent experiments were purchased from Sigma-Aldrich, Inc. (Gillingham, UK) unless otherwise indicated.

### Animals

Male albino Wistar rats (180–250 g, aged 56–63 days) were purchased from Harlan Laboratories, Inc., Blackthorn, UK, and group housed in the Biological Services Unit of the university, under fixed conditions of constant humidity (40–60%), temperature (18–22°C) and a 12 h light-dark cycle in accordance with Home Office regulations. Access to food (standard rodent diet) and water was *ad libitum*. All experimental procedures were conducted in strict adherence to the terms of the 1986 Animals (Scientific Procedures) Act, EU Directive 2010/63/EU and ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). Each experimental group consisted of 40 rats (6 experimental groups were used, therefore a total of 240 rats were employed). An experimental group was further subdivided into 4 groups of 10 rats. This was done to ensure that a subset of each group would undergo only one behavioural test to prevent confounding results due to multiple test exposure. For example, in the 6-OHDA only treatment group-10 rats underwent sucrose preference testing ( $n = 10$ ), 10 rats performed the FST ( $n = 10$ ), 10 rats performed the NOR test ( $n = 10$ ), and 10 rats were subjected to the open field test (OFT) ( $n = 10$ ) ( $10 \times 4 = 40$  rats per experimental group). In addition to the behavioural testing, 10 rats from each group were randomly selected for tissue content analysis, another 10 for microdialysis, and a further 10 rats for immunohistochemical analysis.

### Stereotaxic surgery and drug administration

DSP-4 was injected at a dose of 25 mg·kg<sup>-1</sup>, i.p., to induce partial degeneration of the noradrenergic nerve terminals and cell bodies 4 days before stereotaxic surgery. This methodology was selected to mimic the sequential condition of neurodegeneration present in PD patients. In other words, a noradrenergic deficit was induced before a dopaminergic insult. DSP-4 was stored away from light to prevent oxidation and a fresh batch of the toxin was prepared before administration. Stereotaxic surgery was performed to administer a bilateral 6-OHDA injection into each striatum, thereby inducing a Parkinsonian rodent model. Animals were anaesthetized using isoflurane (5% v v<sup>-1</sup> in O<sub>2</sub> for induction and

2% v v<sup>-1</sup> in O<sub>2</sub> for maintenance delivered through a fitted anaesthetic nose mask; Abbot Laboratories, Ltd., Kent, UK) and secured using blunt ear bars to a stereotaxic frame (David Kopf, Tujunga, CA, USA). Stereotaxic coordinates from the atlas of Paxinos and Watson (1982) were used to locate the ventrolateral area of the dorsal STR (from bregma in mm; AP 1.1 mm, ML 3.2 mm and DV -7.2 mm). This area is analogous to the putamen in primates, which incurs the greatest dopaminergic deficit in PD patients (Tadaiesky *et al.*, 2008). About 15 µg of 6-OHDA (containing 0.2% ascorbic acid as a stabilizer) or vehicle (saline) was injected into the left and right STR of each rat at a flow rate of 1 µl·min<sup>-1</sup> to induce partial destruction of the nigrostriatal dopaminergic system. 6-OHDA was kept in a closed container during the duration of the study and a freshly prepared vial was used every five surgeries. Prior to receiving an intracerebral injection of 6-OHDA, animals were given i.p. injections of both pargyline (a monoamine oxidase-B inhibitor) (50 mg·kg<sup>-1</sup>) and desipramine (a noradrenergic reuptake inhibitor) (25 mg·kg<sup>-1</sup>) to ensure maximal selectivity of 6-OHDA towards dopaminergic neurons. EX-4 was administered at a dose of 0.5 µg·kg<sup>-1</sup> via i.p. injections (with saline as vehicle) twice daily for a period of 7 days 1 week following 6-OHDA insult. This dose was selected based on the work of Harkavyi *et al.* (2008). We chose to commence treatment 1 week following 6-OHDA administration to more closely mimic clinical manifestation and treatment of PD. In other words, patients present to the clinic and begin a treatment protocol after a substantial amount of neurodegeneration has already taken place.

### Behavioural assessments

**Sucrose preference test (SPT).** Sucrose consumption was employed to measure anhedonia in rodents. Rats were first exposed to a 4 day habituation phase in which they were provided with two bottles, one containing water and the other containing a 1% sucrose solution. After the habituation phase, the animals were individually housed and sucrose consumption was measured over a 2 day period by weighing the bottles daily. This test took place 2 weeks after either neurotoxins or vehicle had been administered. Sucrose preference was calculated as total sucrose solution consumed divided by total fluid intake multiplied by 100.

**Forced swim test.** A FST was utilized to assess depressive behaviour (Porsolt *et al.*, 1978). Rats were individually placed in plastic rectangular containers (56 cm in height and 24 cm in diameter) filled with water (40 cm water depth and temperature was maintained at 25 ± 1°C). Total percentage of time spent immobile was recorded over a period of 15 min. Immobility was defined as stagnant floating behaviour or minimal motility serving only to keep the subject's head above the water. The FST was conducted 2 weeks after 6-OHDA lesion to assess behavioural impairment or the therapeutic value of EX-4.

**Novel object recognition.** Object-recognition memory was tested using a NOR test. The test apparatus was a circular arena (100 cm in diameter and 35 cm in height). Rats were exposed to the arena for 2 days before testing (for a period of 5 min) to habituate the animals to the testing conditions. The

NOR test consisted of three phases: a habituation phase, a familiar object phase and, finally, the novel object phase. During the habituation phase, the animal was placed in the arena with no objects for a period of 10 min. The familiar object phase followed 2 min after the habituation phase. In this portion of the test, the rat was reintroduced into the arena, which now contained two familiar objects for a period of 5 min. Finally, the novel object phase followed 1 h after the familiar object phase. In this segment of the test, the rat was reintroduced to the arena, which now contained one familiar and one novel object for a period of 5 min. All test phases were recorded and percentage of time spent exploring the novel object was determined. Object exploration was defined as sniffing the novel object or making direct contact with forepaws. Animals were subjected to the NOR test 2 weeks after 6-OHDA treatment.

**Open field test.** The OFT was employed to assess spontaneous locomotor activity. The testing apparatus consisted of a circular arena (same from NOR test) with a grid drawn on the floor. The grid was composed of 14 cm × 14 cm squares. Rats were exposed to the arena for 2 days (for 5 min each day) before testing to induce habituation. Rats were placed in the arena for a period of 5 min and the total number of squares crossed during that time period was determined. The animals were subjected to the OFT 2 weeks after 6-OHDA lesion.

### In vivo microdialysis and tissue assay

Animals were anaesthetized with isoflurane (5% induction v v<sup>-1</sup> in O<sub>2</sub> and 2% maintenance) and then secured on a stereotaxic frame. Depth of anaesthesia was monitored by observing the rate of respiration (expansion of the chest cavity), palpating the sides of the chest to give an indication of heart rate, monitoring of body temperature (heating pad utilized to prevent hypothermia), assessment of reflexes (pedal and tail), and observation of muscle relaxation.

CMA 11 guide cannulae were purchased from CMA microdialysis (CMA/microdialysis, Stockholm, Sweden) and inserted stereotaxically into either the STR (from bregma in mm; AP +0.2, ML 3.0, DV 4.2) or FC (AP +3.2, ML 1.5, DV 3.0), then secured with dental cement (DuraLay; Reliance Dental Mfg. Co., Worth, IL, USA) and anchor screws. The animals were then placed in individual microdialysis cages and allowed to recover for 24 h before dialysis commenced. Cuprophane CMA/11 microdialysis probes (4 mm membrane length, 0.24 mm o.d.) were utilized during the dialysis procedure (CMA/microdialysis). During dialysis, the rats were perfused with artificial cerebrospinal fluid (2.5 mM KCl, 125 mM NaCl, 1.18 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 1.26 mM CaCl<sub>2</sub>) pH 7.4 at a rate of 1 µl·min<sup>-1</sup> using Harvard Apparatus model 22 syringe infusion pump. Samples were collected every 30 min in tubes containing 5 µl ascorbic acid (0.2 µM), which served as an antioxidant. In total, eight samples were collected per experiment. Collected samples were frozen at once at -80°C and analysed for DA and NA within 1 week using HPLC with ECD. HPLC injection volume for microdialysis samples was always 30 µl. Amount of DA and NA was averaged and expressed as fmol 10 µl<sup>-1</sup>.

Striatal and frontal cortical tissue homogenates were treated with 0.1 M perchloric acid containing ascorbic acid

(0.2  $\mu\text{M}$ ) and EDTA (0.2  $\mu\text{M}$ ) to precipitate the cell debris (40  $\mu\text{L}$  aliquot of tissue sample + 40  $\mu\text{L}$  of perchloric acid mixture). These were then centrifuged at 13 000 $\times g$  for 10 min at 4°C, afterwards supernatant was passed through a syringe filter and DA and NA tissue levels were estimated using HPLC with ECD (25  $\mu\text{L}$  HPLC injection volume) (Biggs *et al.*, 1992). DA and NA chromatogram peaks were converted to amount values using an external standard method and expressed as ng g<sup>-1</sup> tissue homogenate. Both DA and NA tissue and extracellular levels were evaluated 2 weeks after 6-OHDA insult.

### Transcardial perfusion fixation and immunohistochemistry

Perfuse fixation of rats was carried out when the only assessment intended was immunohistochemistry. In this procedure, animals were administered a lethal dose of pentobarbital (60 mg·kg<sup>-1</sup>; Euthatal, Merial, UK), and once animals were under deep anaesthesia, the heart was surgically exposed and a butterfly cannula (Butterfly-21; Hospira Venisystems, Sligo, Ireland) was inserted into the left ventricle while the right atrium was incised. At first, animals were perfused transcardially with 100 mL of heparin-PBS solution 10 U·mL<sup>-1</sup> (Multiparin, Wockhardt, UK) to prevent coagulation of blood, followed by another 100 mL of 4% paraformaldehyde (PFA) solution in 0.1 M PBS to fix the tissue, after 30 min rats were decapitated and the brains carefully removed and post-fixed again in 4% PFA in 0.1% PBS for 24 h at 4°C. Brains were then cryoprotected in a 30% sucrose solution for 24 h at 4°C then flash frozen on dry ice and stored at -80°C until use.

Slide-mounted 12  $\mu\text{m}$  cryostat sections (depicting LC, and SN) from frozen brain blocks were removed from the freezer and allowed to equilibrate to room temperature for 30 min before post-fixation in 4% w v<sup>-1</sup> PFA, containing 1% w v<sup>-1</sup> glutaraldehyde in de-ionized water for 5 min at 0°C. Following rinsing in 0.01 M PBS for 5 min, sections were dehydrated through graded alcohols (70%–90%–100%) and endogenous peroxidase activity was blocked by incubation in 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 10 min. The sections were then rehydrated and non-specific immunoreactivity was blocked using 10% mouse serum in 0.01 M PBS for 10 min. Sections were then incubated in primary antibody [anti-TH IgG raised in rabbit at 1:1000 in 0.01 M PBS (Merck Chemicals, Ltd., Beeston, Nottingham, UK; product number 657012-100 $\mu\text{L}$  reference) (Du *et al.*, 2001)] for 15 h at 4°C. After being rinsed, the sections were incubated sequentially in biotinylated mouse anti-rabbit antibody 1:500 in 0.01 M PBS (Millipore Corporation, Billerica, MA, USA; catalogue number MAB201B), and ABC (Vector Labs, Ltd., Burlingame, CA, USA) complex was applied for 30 min at room temperature following the manufacturer's instructions. Immunoreactivity was visualized through incubation in 0.5 mg·mL<sup>-1</sup> 3-diaminobenzidine, containing 0.009% H<sub>2</sub>O<sub>2</sub> for 2 min at room temperature. The sections were counterstained in Harris haematoxylin, dehydrated, cleared and mounted for microscopic examination. TH+ cell counting was performed at the level of the third cranial nerve (bregma - 5.6 mm; Paxinos and Watson, 1982) in the SN and in the LC (bregma - 10.3 mm; Paxinos and Watson, 1982) based on the protocol of Iravani *et al.* (2005). Cells across the entire region of the SN and LC as defined by

Paxinos and Watson (1982) at the relevant bregma level were counted. Cell counts were made in a blinded manner in three adjacent tissue sections per rat in both the left SN and left LC using a Leica DC500 system ( $\times 40$  and  $\times 100$ ) and the manufacturer's software (Leica Microsystems, Ltd., Bucks, UK). A countable element was defined as a distinctly independent densely stained cell body. Cell counts were an average of stained neuronal bodies across three sections. Care was taken not to count the same cell twice by observing cell morphology and superimposing adjacent images using CorelDRAW 5 (Corel Corporation, Ontario, Canada).

### Statistics

The data obtained from the behavioural OFT, SPT, NOR test, FST, and tissue DA and NA assays were all subjected to one-way ANOVA and *post hoc* Bonferroni's multiple comparison tests to compare the difference between selected treatments. Microdialysis data were subjected to a two-way ANOVA with *post hoc* Bonferroni's multiple comparison tests. In both cases, data are expressed as mean  $\pm$  SEM. GraphPad Prism 5.03 software was used for all of the statistical manipulations.

## Results

### Behavioural assessments

**Sucrose preference test.** Our results show that rats treated with 6-OHDA alone had a significant decrease in sucrose preference compared with shams ( $P < 0.001$ ) (Figure 1). The DSP-4 + 6-OHDA group showed a significant decrease in sucrose preference compared with both shams ( $P < 0.001$ ) and the 6-OHDA only group ( $P < 0.05$ ). This decrease in sucrose preference was reversed in DSP-4 + 6-OHDA groups treated with EX-4. Groups treated with only DSP-4 or EX-4 did not show a significant decrease in sucrose preference compared with sham measurements.

**Forced swim test.** Our results showed that rats treated with 6-OHDA alone had a significant increase in immobility time compared with the shams ( $P < 0.001$ ) (Figure 1). DSP-4 + 6-OHDA group showed a significant increase in immobility time compared with both shams ( $P < 0.001$ ) and the 6-OHDA only group ( $P < 0.05$ ). This increase in immobility time was reversed in DSP-4 + 6-OHDA groups receiving EX-4. Groups treated with EX-4 alone showed a significant decrease in immobility time compared with the sham group ( $P < 0.05$ ).

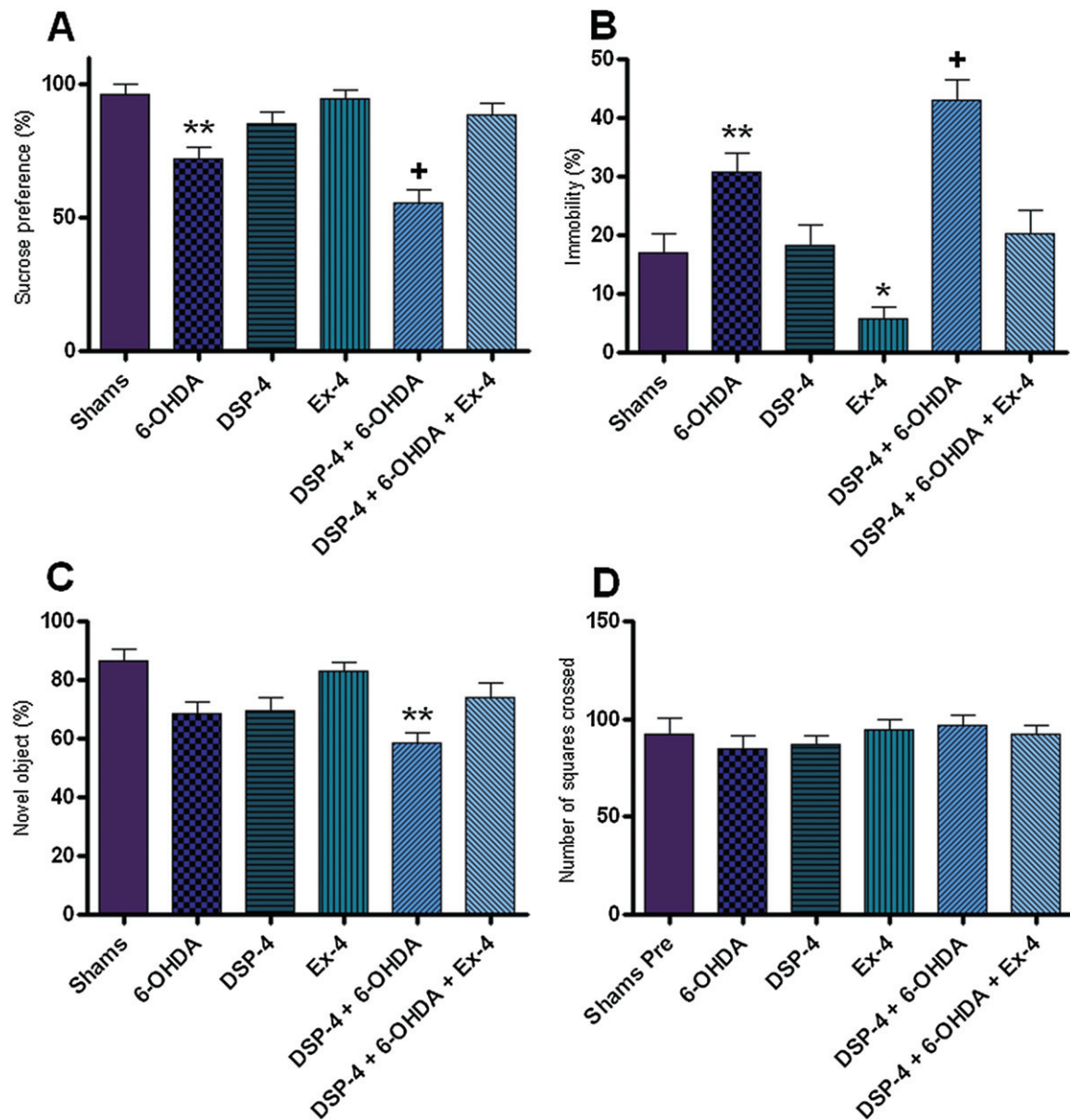
**Novel object recognition.** DSP-4 + 6-OHDA treated rats showed a significant decrease in novel object exploration compared with shams ( $P < 0.001$ ) (Figure 1). This decrease in novel object exploration was reversed in groups treated with EX-4.

**Open field test.** None of the experimental groups displayed a significant decrease in locomotor activity in the OFT (Figure 1).

### In vivo microdialysis and tissue levels

**Extracellular and tissue levels of DA in the FC and STR.** DA tissue levels in the FC and STR of 6-OHDA and DSP-4 +





**Figure 1**

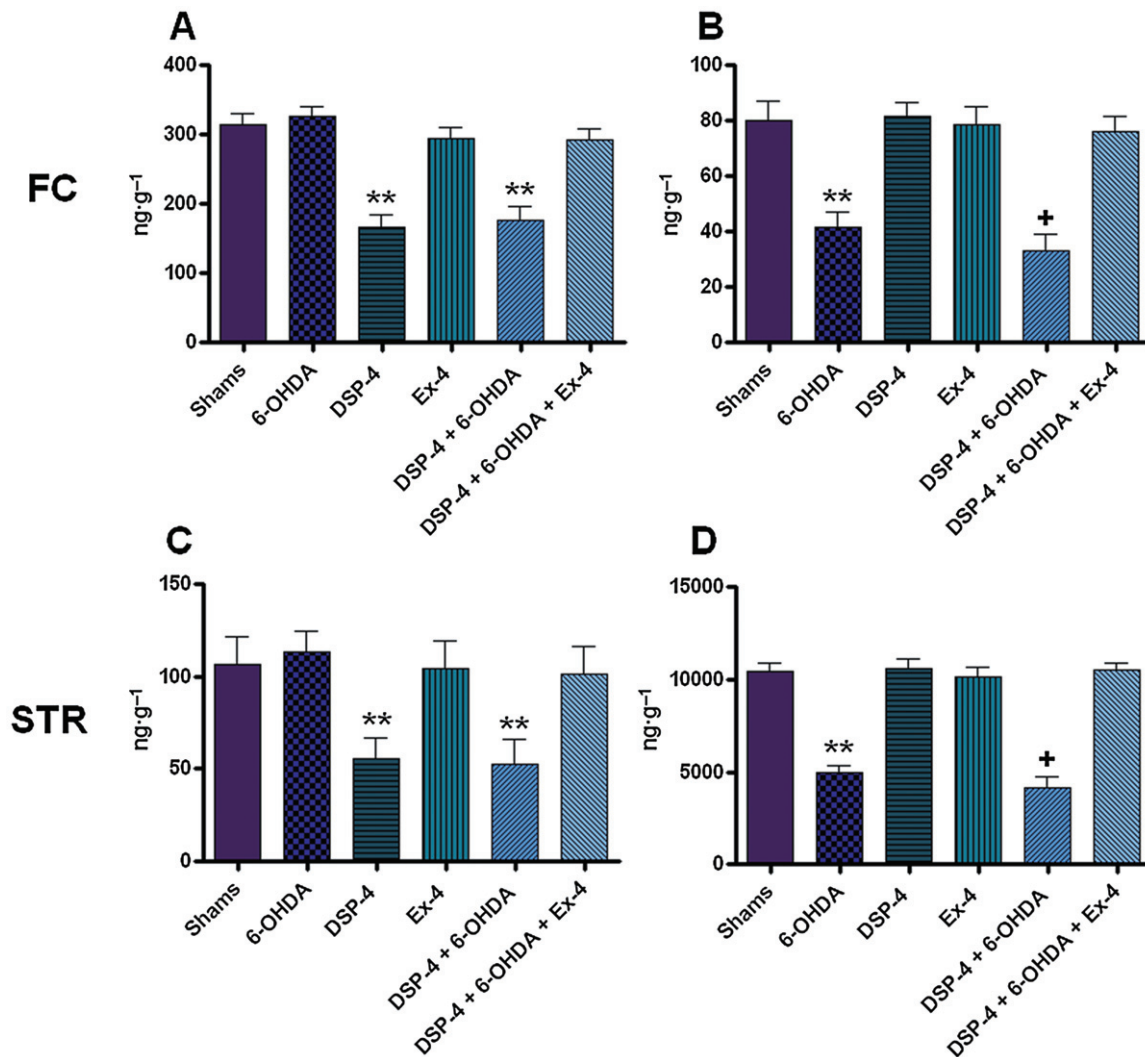
Behavioural assessments. This figure illustrates the various behavioural paradigms utilized in this study. (A) Sucrose preference test, expressed as preference percentage. (B) Forced swim test, measured as percentage of time spent immobile. (C) Novel object recognition, shown as percentage of time exploring the novel object. (D) Open field test, depicted as number of squares crossed. All results were analysed using one-way ANOVA and a *post hoc* Bonferroni's test to compare differences between groups ( $n = 10$ ). \*Indicates different from sham,  $P < 0.05$ . \*\*Indicates different from sham  $P < 0.001$ . +Indicates different from 6-OHDA only,  $P < 0.05$ .

6-OHDA groups were significantly lower than the sham group ( $P < 0.001$ ) (Figure 2, Table 1). The DSP-4 + 6-OHDA group had a significantly lower level of DA compared with the 6-OHDA only group ( $P < 0.05$ ). The decrease in DA levels present in groups treated with 6-OHDA was recovered in DSP-4 + 6-OHDA groups treated with EX-4.

Basal extracellular DA levels in the FC and STR of 6-OHDA and DSP-4 + 6-OHDA were significantly lower than the sham group ( $P < 0.001$ ) (Figure 3, Table 2). The DSP-4 + 6-OHDA group had a significantly lower level of DA compared with

the 6-OHDA only group ( $P < 0.05$ ). This decrease in extracellular DA levels was also reversed in groups given EX-4 treatment.

*Extracellular and tissue levels of NA in the FC and STR.* NA tissue levels in the FC and STR of DSP-4 only and DSP-4 + 6-OHDA groups were significantly lower than the sham group ( $P < 0.001$ ) (Figure 2, Table 1). This decrease in NA levels was restored in DSP-4 + 6-OHDA groups with EX-4 administration.



**Figure 2**

Noradrenaline (NA) and dopamine (DA) tissue levels in the frontal cortex and striatum. All values are expressed as mean  $\pm$  SEM denoted in ng·g<sup>-1</sup> tissue. All results were analysed using one-way ANOVA and a *post hoc* Bonferroni's test to compare differences between groups ( $n = 10$ ). (A) NA in the FC; (B) DA in the FC; (C) NA in the FC; and (D) DA in the STR. \*\*Indicates different from sham  $P < 0.001$ ; +Indicates different from 6-OHDA only,  $P < 0.05$ . FC, frontal cortex; STR, striatum.

Basal extracellular NA levels in the FC and STR of DSP-4 and DSP-4 + 6-OHDA groups were significantly lower than the sham group ( $P < 0.001$ ) (Figure 3, Table 2). This decrease in extracellular NA levels was also reversed in groups receiving EX-4.

### Immunohistochemistry

**Locus coeruleus.** TH+ cell counts were reduced in DSP-4 ( $28.50 \pm 1.60$ ) and DSP-4 + 6-OHDA ( $26.20 \pm 2.67$ ) groups compared with shams ( $41.70 \pm 1.43$ ) ( $P < 0.001$ ) (Figure 4). TH+ cell count values were no longer significantly less than those from shams in the groups treated with the toxins and EX-4.

**Substantia nigra.** TH+ cell counts in the SN were reduced in the 6-OHDA ( $54.55 \pm 1.30$ ) and DSP-4 + 6-OHDA ( $48.44 \pm$

$1.65$ ) groups compared with shams ( $82.28 \pm 2.54$ ) ( $P < 0.001$ ) (Figure 5). TH+ cell counts in the DSP-4 + 6-OHDA were lower than those in the 6-OHDA group; however, it did not reach a level of statistical significance. The decrease in TH+ staining and cell bodies was restored to sham levels in groups treated with EX-4.

### Discussion and conclusions

In this study, we generated a completely novel rodent model of early stage PD. Our toxin administration protocol adheres to the principal of a progressive clinical presentation of the disorder observed in a majority of patients (Braak *et al.*, 2003). It is also important to note that our paradigm mimics several behavioural and neurochemical deficiencies also observed in

**Table 1**

Numerical noradrenaline and dopamine tissue levels in the frontal cortex and striatum

	FC NA	DA	STR NA	DA
Shams	313.80 ± 16.76	79.77 ± 7.32	106.32 ± 14.84	10455.33 ± 401.73
6-OHDA	325.63 ± 14.65	41.68 ± 5.22**	113.09 ± 11.54	4927.92 ± 427.47**
DSP-4	165.67 ± 18.67**	81.31 ± 5.33	55.63 ± 10.92**	10606.27 ± 471.55
EX-4	294.74 ± 15.76	78.50 ± 6.74	104.10 ± 15.02	10144.70 ± 502.81
6-OHDA + DSP-4	176.64 ± 19.52**	33.11 ± 5.84+	52.76 ± 12.88**	4142.00 ± 566.33+
6-OHDA + DSP-4 + EX-4	291.50 ± 16.88	75.95 ± 5.34	101.14 ± 15.11	10467.07 ± 407.29

All values are expressed as mean ± SEM denoted in ng·g<sup>-1</sup> tissue. All results were analysed using one-way ANOVA and a *post hoc* Bonferroni's test to compare differences between groups (*n* = 10).

\*\*Indicates different from sham *P* < 0.001; +indicates different from 6-OHDA only, *P* < 0.05.

FC, frontal cortex; STR, striatum.

**Table 2**

Extracellular noradrenaline and dopamine numerical concentrations in the frontal cortex and striatum

	FC NA	DA	STR NA	DA
Shams	3.51 ± 0.34	4.55 ± 0.41	5.73 ± 0.26	22.83 ± 0.44
6-OHDA	3.65 ± 0.25	2.14 ± 0.35**	5.91 ± 0.33	12.33 ± 0.47**
DSP-4	1.16 ± 0.27**	4.73 ± 0.38	2.40 ± 0.48**	23.89 ± 0.52
EX-4	3.75 ± 0.19	4.79 ± 0.50	5.87 ± 0.23	23.11 ± 0.39
6-OHDA + DSP-4	1.19 ± 0.22**	0.98 ± 0.47+	2.96 ± 0.28**	8.95 ± 0.48+
6-OHDA + DSP-4 + EX-4	3.83 ± 0.30	5.01 ± 0.55	5.75 ± 0.31	22.80 ± 0.50

All values are expressed as mean ± SEM denoted in fmol 10µl<sup>-1</sup>. All results were analysed using two-way ANOVA and a *post hoc* Bonferroni's test to compare differences between groups (*n* = 10).

\*Indicates different from sham, *P* < 0.05; \*\*indicates different from sham *P* < 0.001; +indicates different from 6-OHDA only, *P* < 0.05.

FC, frontal cortex; STR, striatum.

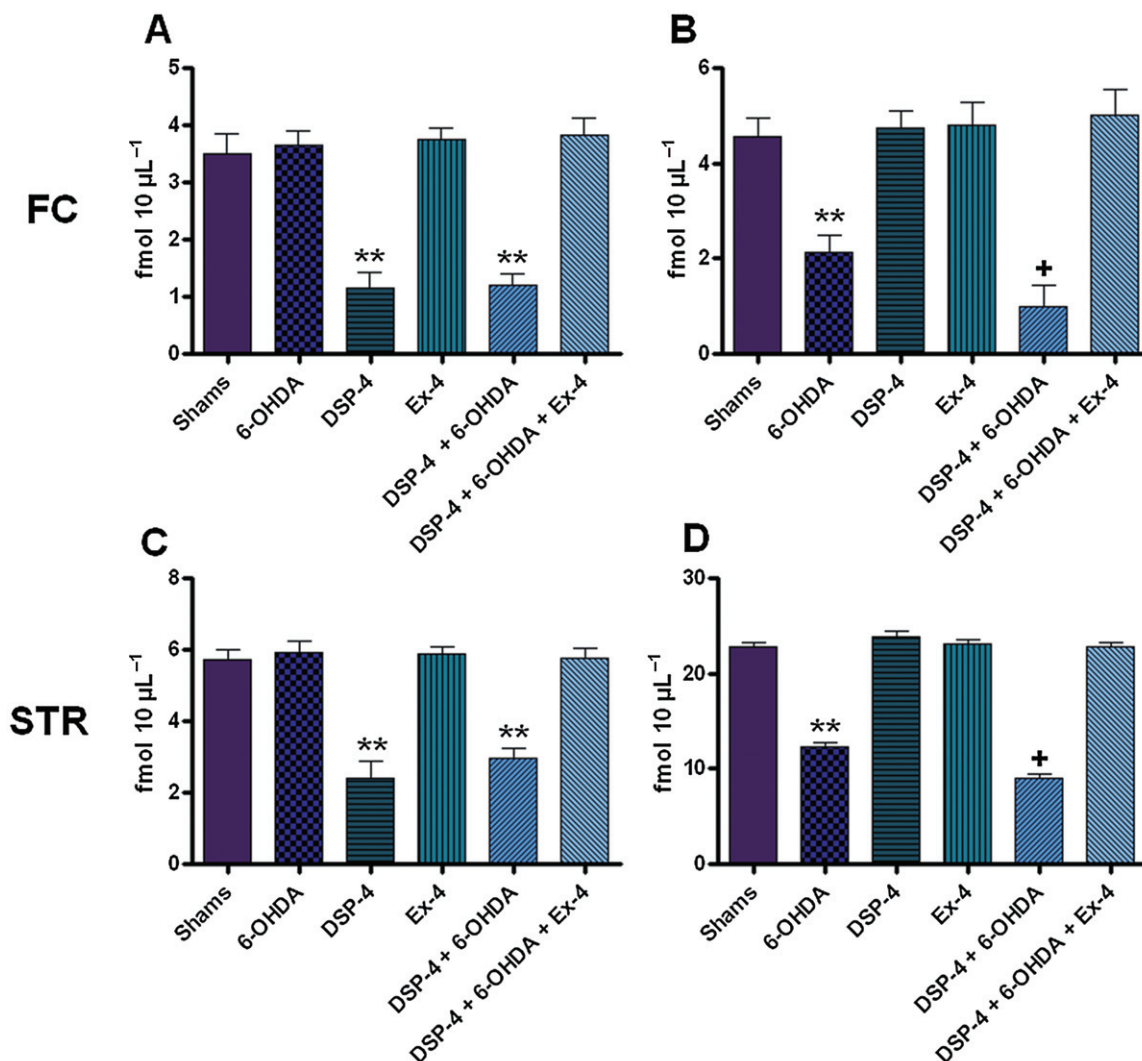
the patient population (Remy *et al.*, 2005; Chaudhuri *et al.*, 2006).

The results of this study suggest that EX-4 may be an effective therapy for certain psychiatric and cognitive deficits experienced in the early stages of PD. In addition, EX-4 treatment was able to restore neurochemical changes in both the dopaminergic and noradrenergic systems. This is an important finding as neurodegeneration of the LC and subsequent loss of NA have been implicated in PD pathology (Chan-Palay and Asan, 1989). Deficits in NA transmission have been correlated with increased severity of both motor and non-motor symptoms of the condition. In this study, we generated a pre-motor model of PD and attempted to validate depressive behaviour and cognitive dysfunction in several behavioural paradigms.

The SPT and the FST were employed to measure anhedonia and the depressive condition (Bambico *et al.*, 2009; Wang *et al.*, 2009). In the SPT, healthy rodents demonstrate a distinct preference for sweetened solution. However, depressed animals will no longer exhibit that preference (Bambico *et al.*, 2009; Wang *et al.*, 2009). In the FST, immobility signifies

a depressive disposition. The FST has been extensively employed to test the therapeutic value of antidepressants (Kim *et al.*, 2010). The 6-OHDA only group showed a decrease in sucrose preference (~28% reduction) compared with shams. The DSP-4 + 6-OHDA group also demonstrated a reduction in sucrose preference (~44% reduction) that was significantly lower than the 6-OHDA only group. The findings of the FST mimicked the SPT results. The 6-OHDA only group demonstrated an increase in percentage of immobility (~108% increase) compared with sham values. The DSP-4 + 6-OHDA group showed an increase in percentage of immobility (~206% increase) that was significantly higher than the 6-OHDA only group.

The results of the SPT and FST suggest a purely dopaminergic deficit is able to produce a depressive phenotype. This observation has been previously made by our lab group (unpublished results) and others utilizing a pre-motor bilateral 6-OHDA or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD (Branchi *et al.*, 2008; Tadaiesky *et al.*, 2008). Delaville *et al.* also observed decreased sucrose consumption and heightened immobility in the FST



**Figure 3**

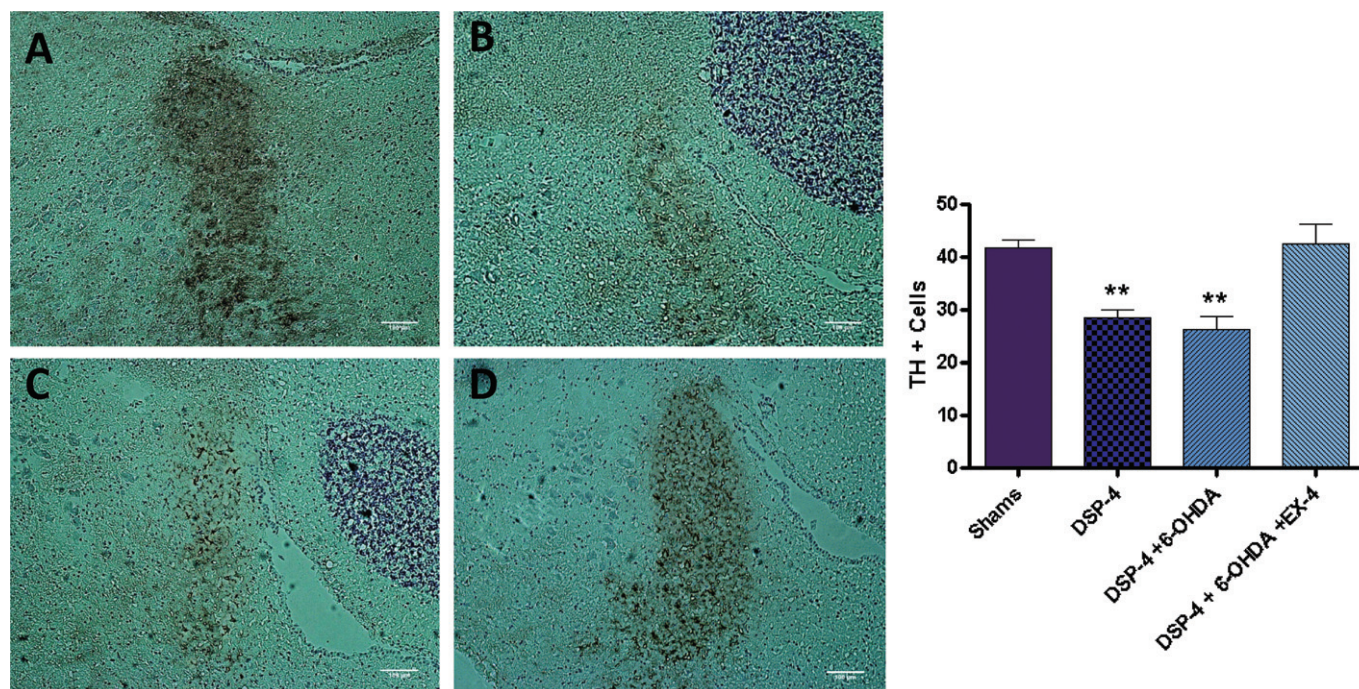
Extracellular noradrenaline (NA) and dopamine (DA) concentrations in the frontal cortex and striatum. All values are expressed as mean  $\pm$  SEM denoted in fmol  $10 \mu\text{L}^{-1}$ . All results were analysed using two-way ANOVA and a *post hoc* Bonferroni's test to compare differences between groups ( $n = 10$ ). (A) NA in the FC; (B) DA in the FC; (C) NA in the STR; and (D) DA in the STR. \* Indicates different from sham,  $P < 0.05$ . \*\* Indicates different from sham  $P < 0.001$ . + Indicates different from 6-OHDA only,  $P < 0.05$ . FC, frontal cortex; STR, striatum.

in a hemiparkinsonian model of PD treated with DSP-4 and parachlorophenylalanine (Delaville *et al.*, 2011). Increasing evidence suggests dopaminergic deficit in the nigrostriatal pathway influences limbic function (Mayberg and Solomon, 1995; Frisina *et al.*, 2009). In addition, it has been shown that independent lesioning of SN and ventral tegmental area in animal models resulted in depressive behaviour (Winter *et al.*, 2007). Our results also indicate that additional noradrenergic lesions further potentiated anhedonic/depressive behaviour. This observation supports the work of several groups that have shown there is decreased NA transporter binding in depressed patients with PD (Remy *et al.*, 2005). This lack of transporter binding occurs in LC and several limbic regions that receive dense noradrenergic innervation including the thalamus, amygdala, anterior cingulate and ventral STR. These regions have been implicated in various depressive

disorders and anxiety (Ressler and Nemeroff, 1999; LeDoux, 2000). A reduction in LC pigmentation and NA transporter binding has also been observed in suicidal patients and patients with major depression (Arango *et al.*, 1996; Klimek *et al.*, 1997).

In addition to evaluating depressive behaviour, we assessed level of interest in a novel object. Cognitive impairment is experienced by a significant number of early PD patients. Our results indicate that dopaminergic insult alone does not produce memory deficits; however, when combined with a NA lesion, a reduction in novel object exploration is observed. The DSP-4 + 6-OHDA treatment group showed a decrease in percentage of time exploring the new object (~34% reduction) compared with shams. Object recognition has never been assessed in a 6-OHDA pre-motor model of PD. Several groups have demonstrated spatial memory impair-





**Figure 4**

TH immunostaining in the locus coeruleus – each slice is 12  $\mu$ m in thickness and the scale bar represents a length of 100  $\mu$ m. Noradrenergic cell bodies appear dark brown in colour visualized using DAB staining. (A) Shams; (B) DSP-4; (C) DSP-4 + 6-OHDA; and (D) DSP-4 + 6-OHDA + EX-4. Bar graph on the right-hand side shows TH+ cell counts. \*\* Indicates  $P < 0.001$  compared with sham value. Cell counts were an average of stained neuronal bodies across three adjacent tissue sections.

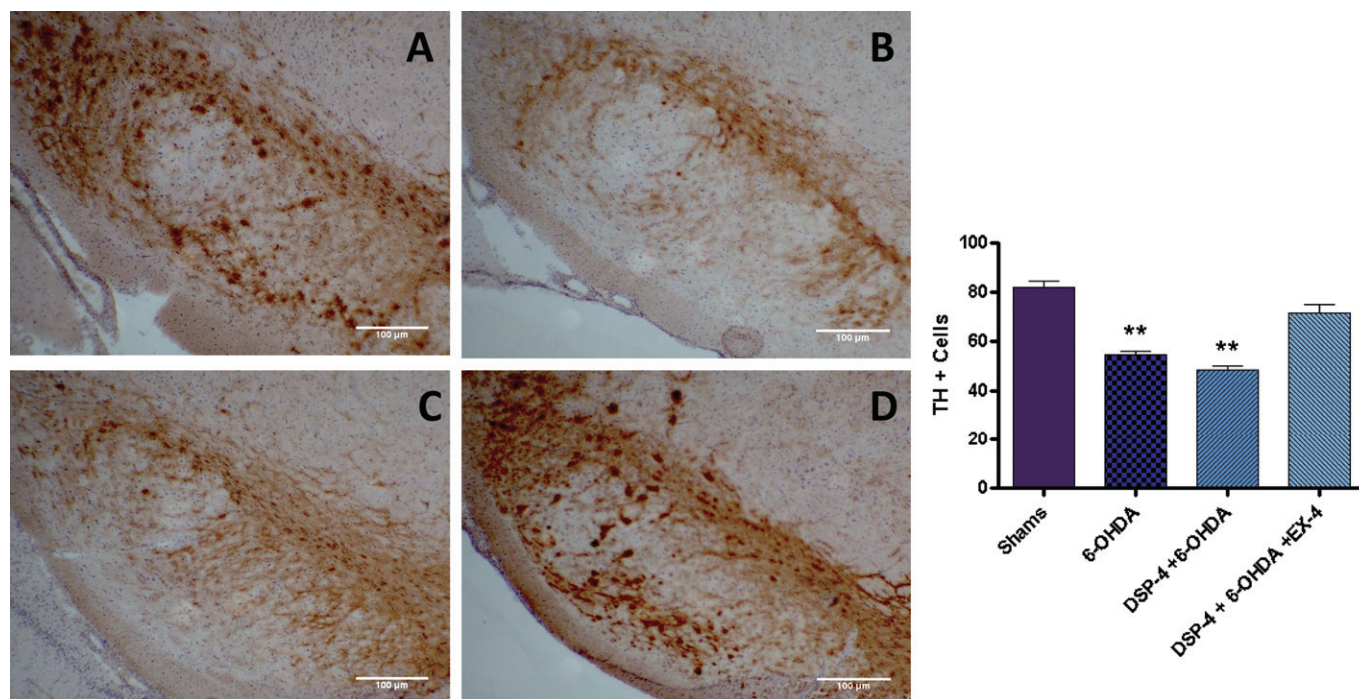
ment in the Morris water maze and elevated plus maze in pre-motor rodent models of PD (Branchi *et al.*, 2008; Tadaiesky *et al.*, 2008; Delaville *et al.*, 2011). These deficits may be the result of DA loss in the FC and STR, regions that partially govern procedural and working memory. NA has also been implicated in memory consolidation in rats exposed to the object recognition task (Rooszendaal *et al.*, 2008). LC degeneration has been shown to be an early indicator of AD pathology and increases both neuroinflammatory response and amyloid deposition (Jardanhazi-Kurutz *et al.*, 2010). In addition, the possibility that inflammation due to our 6-OHDA administration may be affecting various brain regions implicated in object recognition processing, including the FC, STR and hippocampus, cannot be ruled out (Owen, 2004). Our results suggest that both NA and DA have a role in object memory formation.

We conducted the OFT to determine spontaneous locomotor activity. This test was performed to ensure that our DA lesion was not producing motor dysfunction that would confound our results. All treatment groups failed to differ significantly from shams.

NA and DA tissue levels were assessed in the FC and STR to determine the reduction of monoamines produced by our toxin protocol. NA levels were only reduced in groups treated with DSP-4. In the FC and STR, there was an approximately 47–51% reduction in NA levels. DA levels were reduced in the FC and STR of all groups treated with 6-OHDA. DA levels were further significantly reduced in 6-OHDA groups co-treated with DSP-4. These results indicate DSP-4 treatment potenti-

ated DA loss in groups treated with 6-OHDA. The potentiation of dopaminergic deficit in PD models as a result of additional NA lesioning has been observed by several other groups (Lategan *et al.*, 1990; Mavridis *et al.*, 1991; Srinivasan and Schmidt, 2003). Both Mavridis *et al.* and Fornai *et al.* noticed that the MPTP-induced damage to nigrostriatal DA neurons was potentiated by pretreatment with DSP-4 (Mavridis *et al.*, 1991; Fornai *et al.*, 1995). Recently, Wang *et al.* observed increased apomorphine-induced circling behaviour after dual 6-OHDA lesions into the LC and SN respectively (Wang *et al.*, 2010). Stimulation of the LC facilitates burst firing of SNc neurons (Rommelfanger and Weinshenker, 2007). NA appears to act as a compensatory mechanism for dopaminergic loss through its ability to modify the firing rate of SNc neurons and thus the release of DA. In addition, it has been shown that extracellular NA exhibits antioxidant and anti-inflammatory properties. Release of cofactors such as galanin and brain-derived neurotrophic factor may also aid in the neuroprotection of surviving DA neurons (Rommelfanger and Weinshenker, 2007).

Our microdialysis results largely reflected the data obtained from the tissue content assays. Basal NA levels were only reduced in groups treated with DSP-4. In the FC and STR, there was an approximately 53–57% reduction in NA levels. Basal DA levels were reduced in the FC and STR of all groups treated with 6-OHDA. DA levels were further significantly reduced in 6-OHDA groups treated with DSP-4. These results again indicate DSP-4 treatment potentiated DA loss in groups treated with 6-OHDA. The decreased levels of NA and



**Figure 5**

TH immunostaining in the substantia nigra – each slice is 12  $\mu$ m in thickness and the scale bar represents a length of 100  $\mu$ m. Dopaminergic cell bodies appear dark brown in colour visualized using DAB staining. (A) Shams; (B) 6-OHDA; (C) DSP-4 + 6-OHDA; and (D) DSP-4 + 6-OHDA + EX-4. Bar graph on the right side shows TH+ cell counts. \*\* Indicates  $P < 0.001$  compared with sham value. Cell counts were an average of stained neuronal bodies across three adjacent tissue sections.

DA that we observed mimic clinical data obtained from PD patients experiencing non-motor symptomology.

TH immunohistochemistry was performed in the SN and LC to assess damage to both noradrenergic and dopaminergic cell bodies. The results showed decreased TH+ cell number (~31–37% reduction) in the LC of groups treated with DSP-4. DSP-4 treatment has previously been shown to reduce dopamine  $\beta$  hydroxylase staining in the LC indicating loss of functional neuronal phenotype (Ross and Reis, 1974). Reduced TH staining was also observed in the SN of all groups treated with 6-OHDA (~31–45% reduction). This result was expected since 6-OHDA treatment has long been known to cause degeneration of dopaminergic neurons in the SN. However, it may be that the decreases in TH+ staining observed in both the LC and SN represent a phenotypical shift in protein expression rather than cell loss. Other groups have observed a loss of TH+ expression without the concomitant loss of neurons in rodents (Bowenkamp *et al.*, 1996; Cohen *et al.*, 2011). One interesting finding that we observed was that DSP-4 treatment along with 6-OHDA administration did not significantly reduce TH + staining in the SNc compared with 6-OHDA only groups, although cell numbers appeared reduced. We hypothesize that DSP-4 may be altering the firing rate of SNc neurons and thus reducing the release of DA at this particular time point (Rommelfanger and Weinshenker, 2007). However, the possibility that DSP-4 can promote the degeneration of SN neurons over a longer time course or higher dosage cannot be ruled out.

Treatment with EX-4 was able to promote both behavioural and biochemical recovery in our pre-motor model.

Deficits in sucrose preference, immobility time in the FST and novel object exploration in the NOR were all restored to sham levels. We also confirmed an anti-depressant effect of EX-4 observed previously by our lab (unpublished results) and Isacson *et al.* (2010). EX-4 also restored both extracellular and tissue DA and NA content. TH+ cell counts also reverted to sham levels.

Although the exact mechanism of action of EX-4 has yet to be elucidated, we hypothesize that EX-4 is exerting an anti-apoptotic, neurotrophic and anti-inflammatory effect via activation of glucagon-like peptide 1 receptors (GLP-1 receptors) in the SN, LC, STR and FC (Merchenthaler *et al.*, 1999; Perry *et al.*, 2002). We have previously observed that the beneficial effects of EX-4 are reversed by co-administration of the GLP-1 receptor antagonist EX-9-39 in motor models of PD (Harkavyi and Whitton, 2010). GLP-1 receptor activation increases the expression of anti-apoptotic genes such as *Bcl2* and *Bclxl* and hinders caspase-3 activation and Bax expression (Li *et al.*, 2012). It exerts anti-inflammatory effects by reducing the expression of IL-1 $\beta$ , TNF $\alpha$  and IL-6. GLP-1 receptor stimulation has previously been shown to promote neuronal restoration/protection in two *in vivo* rodent models of PD (Bertilsson *et al.*, 2008; Harkavyi *et al.*, 2008; Kim *et al.*, 2009; Li *et al.*, 2009). GLP-1 receptor stimulation can also initiate activation of the PKA/cAMP pathway (DeCastro *et al.*, 2005). This pathway has been shown to promote axonal sprouting and regulate both TH and TPH gene expression (Piech-Dumas *et al.*, 2001). Increased stimulation of the PKA/cAMP pathway could lead to enhanced TH/TPH expression and subsequent restoration



of DA and NA storage and release (Chen *et al.*, 2008). Recent studies suggest activation of GLP-1 receptors in the subventricular zone promotes neurogenesis and could possibly be a contributor to neurorestorative effect of EX-4 (Bertilsson *et al.*, 2008). EX-4 may also contribute to enhanced mitochondrial functioning, as suggested by the increased mitochondrial number and biogenesis in human islet amyloid polypeptide-treated clonal insulinoma (INS-1E) cells (Fan *et al.*, 2010). These authors suggested this effect may be indirectly related to restoration of Akt activity (Fan *et al.*, 2010).

The results of this study suggest that EX-4 is able to preserve the functional integrity of both the noradrenergic and dopaminergic systems. The use of EX-4 as a non-invasive therapeutic treatment in the early stages of PD would be highly advantageous in addressing not only motor dysfunction but also the emotional and cognitive deficits that patients experience.

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## Conflict of interest

The authors have no conflict of interest to disclose.

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