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Noradrenaline neuron degeneration contributes to motor impairments and development of L-DOPA-induced dyskinesia in a rat model of Parkinson's disease

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Abstract

Parkinson's disease (PD) is characterized by progressive loss of dopaminergic (DA) neurons in the substantia nigra. However, studies of post-mortem PD brains have shown that not only DA neurons but also the noradrenergic (NA) neurons in the locus coeruleus degenerate, and that the NA neurodegeneration may be as profound, and also precede degeneration of the midbrain DA neurons. Previous studies in animal models of PD have suggested that loss of forebrain NA will add to the development of motor symptoms in animals with lesions of the nigrostriatal DA neurons, but the results obtained in rodents have been inconclusive due to the shortcomings of the toxin, DSP-4, used to lesion the NA projections. Here, we have developed an alternative double-lesion paradigm using injections of 6-OHDA into striatum in combination with intraventricular injections of a powerful NA immunotoxin, anti-DBH-Saporin, to eliminate the NA neurons in the locus coeruleus, and associated pontine nuclei. Animals with combined DA and NA lesions were more prone to develop L-DOPA-induced dyskinesia, even at low L-DOPA doses, and they performed significantly worse in tests of reflexive and skilled paw use, the stepping and staircase tests, compared to DA-only lesioned rats. Post-mortem analysis revealed that NA depletion did not affect the degree of DA depletion, or the loss of tyrosine hydroxylase-positive innervation in the striatum. Cell loss in the substantia nigra was similar in both single and double lesioned animals, showing that the worsening effect was not due to increased loss of nigral DA neurons. The results show that damage to brainstem NA neurons, contributes to the development of motor impairments and the appearance of L-DOPA-induced dyskinesia in 6-OHDA lesioned rats, and provide support for the view that the development of motor symptoms and dyskinetic side effects in PD patients reflects the combined loss of midbrain DA neurons and NA neurons.

Introduction

Parkinson's disease (PD) is a neurodegenerative disease associated with a profound loss of the dopaminergic (DA) neurons in the substantia nigra (SN). Current therapeutic strategies are mostly focused on pharmacological restoration of DA neurotransmission. However, studies of post-mortem PD brains have shown that not only DA neurons in the SN but also noradrenaline (NA) neurons in the locus coeruleus (LC) degenerate, and that NA neurodegeneration may be as profound, and also precede, degeneration of midbrain DA neurons (German et al., 1992; Patt and Gerhard, 1993; Zarow et al., 2003). The early involvement of the NA system is also in line with the caudal-to-rostral disease progression predicted by the model proposed by Braak et al. (Braak et al., 2003; Hawkes et al., 2007, 2009).

Studies in non-human primates have shown that NA neurodegeneration may contribute to the extent and severity of symptoms seen in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD. Mavridis and colleagues compared MPTP treated squirrel monkeys with monkeys

treated with a combination of MPTP and 6-hydroxydopamine (6-OHDA) injected into the LC (Mavridis et al., 1991). Monkeys treated with MPTP alone showed motor deficits that recovered over time, while the double lesioned monkeys developed more severe and persistent parkinsonian symptoms. In line with these observations, Pifl and colleagues (Pifl et al., 2013) recently reported that in MPTP treated monkeys with near-complete (N95%) loss of striatal DA the severity and persistence of motor deficits was correlated, not with the magnitude of DA depletion, but with the loss of NA in the motor thalamus, similar to the reductions seen in advanced PD patients (Pifl et al., 2012). These observations suggest that severe long-lasting motor impairments in MPTP treated monkeys will develop only when the NA neurons are affected, in addition to the DA neuron cell loss.

Studies on the effects of NA neuron loss on the development of motor impairments in rodent models of PD have used systemic injections of the toxin DSP-4 to lesion the NA neurons and their projections. In MPTP-treated mice Archer and Fredriksson (Archer and Fredriksson, 2006) have shown that the MPTP-induced motor deficits are further aggravated in mice when MPTP was given in combination with the DSP-4. Similar effects have so far not been obtained using DSP-4 in rats. In a study using bilateral injections of 6-OHDA into the MFB, alone or in combination with DSP-4 (Srinivasan and Schmidt, 2004) the effect of the combined lesion on motor behavior was limited to a reduction in open-field locomotor activity. In two other studies the effect of DSP-4 on L-DOPA-induced dyskinesia have been explored in rats injected with 6-OHDA into the MFB, but found no effect on dyskinesia (Migueluez et al., 2011; Perez et al., 2009). Thus, it remains unclear to what extent damage to the NA projections may contribute to the development of motor impairments and dyskinesia in rodent models of PD.

A shortcoming of these rodent studies is the use of DSP-4 to lesion the NA projections. In mice, this toxin is relatively specific for NA neurons at low to moderate doses, but when used in rats it has proved difficult to obtain more substantial and long-lasting depletions of fore-brain NA without additional damage to the serotonin system (Archer and Fredriksson, 2006; Booze et al., 1988; Cassano et al., 2009; Dailly et al., 2006; Hughes and Stanford, 1998; Kask et al., 1997; Szot et al., 2010; Thomas et al., 2007; Wolfman et al., 1994). Anti-dopamine- β -hydroxylase (DBH)-Saporin (here called DBH-Saporin) is an interesting alternative. This immunotoxin, which consists of a monoclonal antibody to DBH coupled by a disulfide bond to saporin (a ribosome inactivating protein), has been shown to be selectively toxic to NA neurons, and when injected into the ventricular space in rats it has been shown to be effective in killing the NA neurons in the LC, as well as the associated A5 and A7 pontine cell groups, eliminating the cell bodies without any detectable effect on other monoaminergic neurons in the brain (Coradazzi et al., 2010; Wrenn et al., 1996). The high selectivity and efficiency of the DBH-Saporin lesion makes it an ideal tool for studies involving lesions of the pontine NA neuron cell groups, but it has so far not been used to explore the role of NA neurons in the development of motor impairments and dyskinesia in the rat PD model. In this study we have used DBH-Saporin to obtain a near-complete lesion of the LC NA neurons bilaterally in 6-OHDA-lesioned rats, and monitored the development of L-DOPA-induced dyskinesia in rats subjected to a single unilateral 6-OHDA lesion with that obtained in rats with combined 6-OHDA and DBH-Saporin lesions. In a second experiment we have compared the motor impairments induced by DA and combined DA + NA lesions in rats treated with 6-OHDA alone or with a combination of 6-OHDA and DBH-Saporin. The results show that damage to the NA neurons promotes the development of L-DOPA-induced dyskinesia in rats with severe 6-OHDA lesions, and in addition has a worsening effect on motor impairments in rats with partial lesions of the nigrostriatal DA system.

Material and methods

Animals

Adult female Sprague–Dawley rats (225–250 g at the start of the experiment, Charles River, Sweden) were used in the present study and housed on a 12 h light/dark cycle (light on 7:00–19:00) with free access to food and water. All animal works were performed in accordance with regulations set by Swedish legislation 1988:543 and EU-directive 2010/ 63.

Drugs

All the drugs were diluted in 0.9% sterile saline and injected s.c. unless otherwise stated. Apomorphine (0.1 mg/kg in 0.002% L-ascorbic acid); benserazide (10 mg/kg); desipramine (20 mg/kg, i.p.); and 6-OHDA (3.5 µg/µl free base in 0.02% L-ascorbic acid in 0.9% saline, into the striatum) were purchased from Sigma-Aldrich, Sweden. L-DOPA (6, 12, and 24 mg/kg); amphetamine (2.5 mg/kg, i.p.) and DBH-Saporin (2 × 2.5 µg in phosphate buffered saline (PBS) into lateral ventricles, bilaterally) were purchased from Research Organics, Cleveland, OH; Apoteksbolaget, Sweden; and Advanced Targeting Systems, San Diego, CA, USA, respectively. DSP-4 (50, 75, and 100 mg/kg, i.p. was tested and 50 mg/kg was chosen due to mortality.) and reboxetine (3 and 10 mg/kg, 20 min before L-DOPA injection, i.p.) were purchased from Tocris Bioscience, Bristol, UK.

Experimental design

Before the two main experiments, the efficacy of two NA toxins was compared. DSP-4 (50 mg/kg in saline, i.p.) and DBH-Saporin (2 × 2.5 µg per rat, bilaterally into the lateral ventricles) were injected into intact rats (n = 5 each). Four weeks after the injection, rats were sacrificed and sections throughout the brain (1 in 6 series) were stained with DBH antibody (Millipore AB, Sweden). To confirm the NA depletion in DBH-Saporin rats, separate rats (n = 5) were injected with DBH-Saporin and 4 weeks later cortex and striatum were dissected for high performance liquid chromatography (HPLC) measurement of NA levels.

Experiment 1

Three groups of rats were included in this project (Fig. 1A). One group of rats (n = 8) received NA toxin DBH-Saporin into the ventricles bilaterally (for details, see below). After two weeks, two groups of rats (n = 8 each) received 6-OHDA into striatum (for details, see Lesion surgery). Three weeks after the 6-OHDA surgery, the degree of motor deficits was evaluated using four motor tests (stepping, cylinder, corridor, and amphetamine-induced rotation, each test was performed on different days). Then, rats were challenged with different doses of L-DOPA, apomorphine, and the NA transporter blocker, reboxetine. Three days after the last LID test, cortex and striatum were dissected for high performance liquid chromatography (HPLC) to measure the level of NA, DA, and DOPAC.

Experiment 2

Two groups of rats (n = 18 in total) received DBH-Saporin into the ventricles bilaterally (Fig. 1B). After two weeks, two groups of rats (n = 30 in total) received 6-OHDA into striatum (for details, see Lesion surgery). Six weeks after the 6-OHDA injection, the degree of motor deficits was evaluated using four motor tests (amphetamine-induced rotation, cylinder, corridor, and stepping test; each test was performed on different days). The rats that had received 6-OHDA only (n = 28) were divided into two groups evenly based on the four motor test results. One of the two groups (n = 13) was injected with DBH-Saporin into the lateral ventricles, as above. DBH-Saporin was injected before (n = 12) or after (n = 13) 6-OHDA injection to investigate whether the effect of the toxin was dependent on the state of DA innervation at the time of injection. Four weeks after the injection, the rats went through a battery of motor tests. At the end of the motor tests, they were perfused and sections

throughout the brain (1 in 6 series for each antibody) were stained with tyrosine hydroxylase (TH) and vesicular monoamine transporter (VMAT) antibodies for histological analysis.

Lesion surgery

Stereotaxic surgery was performed under general anesthesia, induced by i.p. injection (1.4–1.6 ml) of a 20:1 mixture of Fentanyl and Dormitor® (Apoteksbolaget, Sweden). For DBH-Saporin, 5 µg (in 14 µl of PBS) was injected bilaterally into the lateral ventricles, 2.5 µg on each side (AP = -0.6 mm from bregma; ML = ±1.5 from bregma; DV = -4.5 from the skull surface; tooth bar = 0.0; injection speed 2 µl/min) using a stereotaxic frame (Stoelting, Wood Dale, IL). In Experiment 1, a total of 28 µg 6-OHDA (3.5 µg/µl free base in 0.02% L-ascorbic acid in 0.9% sterile saline) were injected into the striatum (AP/ML = +1.3/-2.6; +0.4/-3.2; -0.4/-4.2; and -1.3/-4.5 mm from bregma; DV = -5.0 mm from the dura surface; Tooth bar = 0.0; injection speed 1 µl/min). In Experiment 2, a total of 14 µg 6-OHDA (3.5 µg/µl free base in 0.02% L-ascorbic acid in 0.9% sterile saline) were injected into the striatum (AP = ±0.5 mm from bregma; ML = 3.2/-4.2 mm from bregma; DV = -5.0 mm from the dura surface; Tooth bar = 0.0; injection speed 1 µl/min). To give less damage to the brain, a glass capillary (outer diameter 60–80 µm) was fitted onto the needle of a Hamilton syringe (Nikkhah et al., 1994). In order to protect NA fibers, the NA transporter blocker, desipramine (20 mg/kg, i.p.) was given 30 min before the 6-OHDA injection. After the 6-OHDA injection, the needle was kept in place for an additional 3 min before retracted slowly. Antisedan® (0.28 mg/kg, s.c., Apoteksbolaget, Sweden) was injected to reverse sedative effects of anesthetics and Temgesic® (0.04 mg/kg, s.c., Apoteksbolaget, Sweden) to relieve pain after the surgery.

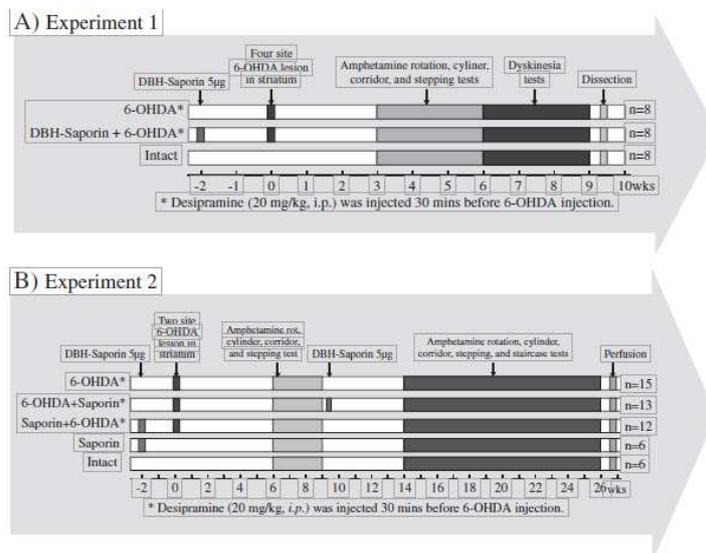


Fig. 1. Experimental design. (A) Experiment 1. Eight rats received DBH-Saporin into lateral ventricles. 2 weeks later, 16 rats received 6-OHDA (28 µg) into striatum. 3 weeks after the 6-OHDA surgery, rats were tested with four motor tests and dyskinesia test with escalating doses of L-DOPA, apomorphine, and L-DOPA plus reboxetine. 3 days after the last dyskinesia test, tissues were collected for HPLC analysis. (B) Experiment 2. Eighteen rats received DBH-Saporin into lateral ventricles. 2 weeks later, 40 rats received 6-OHDA (14 µg) into striatum. 4 weeks after the 6-OHDA surgery rats were tested with four motor tests. The group received 6-OHDA only divided into two groups where one of them received DBH-Saporin. 4 weeks after the saporin surgery, rats were tested with 5 different behavioural tests. At the end of the test, rats were perfused with 4% PFA and sliced sections went through immunostaining.

Amphetamine-induced rotation

Rats were administered 2.5 mg/kg of amphetamine sulphate (Apoteksbolaget, Sweden, i.p.) and left and right full body turns were recorded over 90 min using automated rotometer bowls (AccuScan Instrument Inc., Columbus, OH), as described previously (Ungerstedt and Arbuthnott, 1970). The data are expressed as net full body turns per min where rotation towards the lesion side (i.e. right hand side) was given a positive value.

Cylinder test

Spontaneous forelimb use was measured in the cylinder test (Schallert and Tillerson, 1999), as reported previously Kirik et al.(2001). Individual rats were placed in a glass cylinder (diameter 20 cm) and a total of 20 weight-bearing forepaw touches to the glass were recorded. The data are presented as percentage of left (impaired) forepaw contacts out of the total contacts. Symmetric use of the two forepaws would thus give a score of 50% (10 out of 20).

Corridor task

Lateralized sensorimotor response was evaluated using the corridor task, as first described by Dowd et al. (2005). The rats were food restricted (6–8 g per day, food administered only after the completion of the daily test) and placed in a 150 cm long and 7 cm wide corridor, with 10 white plastic beakers equally spaced along each wall, each beaker containing 5–7 sugar pellets. The rats were given 5 min to investigate the corridor and make decisions to investigate beakers on either side of their body. The ratio of contralateral (left) investigations to the total is used for comparison. Rats were habituated in the corridor for the first two days and the test was carried out for the next three days. Results were displayed as the average of the two most similar days.

Stepping test

Forelimb akinesia was assessed by the stepping test as described by Olsson et al. (1995). Rats were trained for 2 days and tested for the next 3 days (3 trials per day). The hind legs and one forepaw were held so that the animal has its weight only on one paw. Rats were encouraged to make reflexive adjusting steps when moved sideways along a 1 m table. Both limbs were tested in the forward and backward direction and the number of left paw (contralateral) forward adjusting steps was compared between groups. For statistical analysis the values from the last two days of testing were averaged together.

Staircase test

The staircase test, as first described by Montoya et al. (1991), is specifically designed to test skilled forelimb motor movements. First, rats were food restricted throughout the test days (same regimen as in the corridor test, above) to encourage participation in the experiment, and put into a plastic staircase box loaded with sugar pellets for 20 minutes once a day for 13 days. Each box has a platform for the rat to lie on and a removable bilateral staircase filled with sugar pellets that are in reaching distance for the rat (for detailed information on the design of the test box, see ref). This test used 10 pellets on each step, with four total steps of increasing difficulty on each side of rat. At the end of the test session the remaining pellets were counted to see how many were retrieved, as well as how many pellets were dropped. The total amount of taken, eaten and dropped on the affected side was recorded as a measure of skilled forelimb use. For statistical analysis, the 3 most similar values of the final 4 days were averaged together.

L-DOPA-induced dyskinesia

Abnormal involuntary movements (AIMs) were assessed by injecting escalating doses of L-DOPA (6, 12, and 24 mg/kg) together with the peripheral DOPA-decarboxylase inhibitor, benserazide (10 mg/kg). AIMs were evaluated according to the rat dyskinesia scale described in detail previously (Lee et al., 2000; Winkler et al., 2002) by an experimenter who was unaware of the identity of the animals. Briefly, the animals were placed individually in transparent plastic cages without bedding material and scored every 20 min following the injection of L-DOPA until no AIMs were observed. The severity of the dyskinetic movements were scored as follows: 0, absent; 1, occasional (i.e. present less than 50% of the observation time); 2, frequent (i.e. present more than 50% of the observation time); 3, continuous but interrupted by strong sensory stimuli; 4, continuous and not interrupted by strong sensory stimuli. Scores were given in three sub-types of AIMs according to their topographic distribution as forelimb, orolingual, and axial together with locomotor behaviors (contralateral circular movement). Forelimb dyskinesia was defined as movement of contralateral forelimb/paw with jerky, choreic, or dystonic character. Orolingual dyskinesia is predominantly seen as hyperkinesia with jaw movement, facial grimace, and tongue protrusion towards contralateral side. The axial dyskinesia is often of a dystonic type with torsion of the head, neck, and trunk towards contralateral side. Enhanced manifestations of normal behaviors such as grooming, gnawing, rearing and sniffing were not included in the rating. Total AIMs score was calculated by combining forelimb, orolingual and axial dyskinesia scores.

Apomorphine-induced dyskinesia

In order to evaluate DA receptor super sensitivity in striatum, apomorphine (0.1 mg/kg in 0.002% ascorbic acid) was administered s.c. and AIMs were monitored every 10 min following the drug injection, until the signs of AIMs had subsided, using the same rating scale as for LID.

HPLC measurement

To test the effect of DBH-Saporin on tissue neurotransmitter levels, rats were decapitated 4 weeks after toxin injection and cortex and striata were rapidly dissected out bilaterally, frozen on dry ice and stored in 80 °C freezer until analysis. For Experiment 1, rats were sacrificed 3 days after the last dyskinesia test and cortex and striata were rapidly dissected out, frozen and stored, same as the above. At the time of the analysis, tissues were homogenized by sonication in 0.1 N HClO₄ (1:20, weight/volume), centrifuged at 10,000 ×g, the supernatant filtered on micro spin centrifuge tubes (0.22 µm nylon filter) and analyzed by HPLC with electrochemical detection. The HPLC system was equipped with 3.0 × 150 mm C18 (3.5 µ) Symmetry columns (Waters, Milan, Italy), kept at 35 °C by Series 1100 thermostat (Agilent Technologies, Waldbronn, Germany), and ESA Coulochem II detector (Chelmsford, MA, USA). The mobile phase consisted of 80 mM Na₂HPO₄, 0.27 mM EDTA, 0.6 mM sodium octyl sulfate, 8% methanol, 3% acetonitrile, pH 2.8 with H₃PO₄, delivered at 0.35 ml/min by a 1260 Infinity pump (Agilent Technologies, Waldbronn, Germany). The Coulochem analytical cell first electrode was set at +200 mV, the second one at -300 mV. Only the second electrode signal was recorded and analyzed. In these conditions, the detection limit (signal to noise ratio 3:1) was 0.3 pg on column. Data are expressed as pg/mg tissue.

Perfusion

At the end of the experiment animals received i.p. injection of sodium pentobarbitone (60 mg/kg, Apoteksbolaget, Sweden) and were transcardially perfused with 100 ml of 0.9% saline followed by 250 ml of ice-cold 4% paraformaldehyde (PFA) in PBS. The brains were removed and post-fixed for 2 hr in the same fixative before cryoprotection in 25% sucrose in PBS overnight.

Immunohistochemistry

Cryoprotected brains were cut coronally at 35 μm thickness in 6 series using a freezing slide-microtome (Leica) and free-floating sections were quenched for 15 min with 3% H₂O₂ and 10% methanol in potassium-PBS (KPBS). Sections were incubated with blocking solution (5% normal goat/horse serum and 0.25% tritonX-100 in KPBS) for 1 hr followed by primary antibodies (mouse anti-DBH, MAB308, Millipore, 1:1000; mouse anti-NeuN, MAB377, Millipore, 1:100; rabbit anti-TH, AB152, Millipore, 1:1000; rabbit anti-VMAT, AB81855, AbCam, 1:1000) in blocking solution overnight at room temperature. Next day, incubation in blocking solution with secondary antibodies (horse anti-mouse, BA 2000 or goat-anti rabbit, BA 1000, Vector Laboratories, 1:200) for 1 h at room temperature was followed by streptavidin-biotin complex solution (ABC Elite, Vector Laboratories) for 1 h. The visualization of the primary and secondary antibodies was carried out by peroxidase (0.01% H₂O₂) driven precipitation of di-amino-benzidine. The sections were mounted onto subbed slides and air-dried overnight before being dehydrated and cover slipped using DePeX mountant (BDH Chemicals, UK).

Estimation of striatal DA fibers using optical densitometry

For densitometry TH⁺ stained striatal tissue sections were captured using a high-resolution scanner (Scanscope CS, Aperio Technologies, Oxford, UK). Using Image J software (National Institutes of Health, USA), the average optical density over the entire area of the striatum for three striatal sections in each animal was quantified: Rostral (+1.0 mm from Bregma), Medial (−0.26 mm from Bregma) and Caudal (−0.92 mm from Bregma) in each animal. To compensate for differences in background coloring between the slides, the optical density was subtracted from values obtained in the cortex of each sample. The data is expressed as a percentage of the fiber density on the lesioned side to the non-lesioned side.

Estimation of the amount of DA neurons in SN

The total number of VMAT⁺ (DA) neurons in SN was assessed by stereology. Perfused brains were cut in a 1:6 series, which gave approximately 9 successive SN sections to analyze for each animal. Briefly, using the Stereo Investigator software, the SN of each tissue section was highlighted bilaterally based on anatomical morphology (using histological references) as the sampling area. Next, it was decided how many sampling sites to visit for each SN section. To have confidence that the sample population was representative of the true population, 100–150 cells were counted in each SN to reach a variance level of 0.10 or less, except in some cases where animals had too severe lesions to reach this number. The results are expressed as a total number of cells in the lesioned (right) SN, expressed as percentage of the number on the non-lesioned (left) side.

Statistical analysis

All data are expressed as mean \pm the standard error of the mean. Statistical significance was set at $p < 0.05$. Unpaired t test has been used for the HPLC data analysis of the pilot study. One-way analysis of variance (ANOVA) with Newman Keuls multiple comparisons test was used for amphetamine-induced rotation, cylinder test, corridor task, stepping test, staircase test, L-DOPA-induced dyskinesia for total AIMs, apomorphine induced dyskinesia, TH densitometry, and VMAT⁺ cell counts to find statistical differences in three or more groups. Two-way ANOVA with Bonferroni's multiple comparisons test was used for HPLC data from experiment 1, L-DOPA-induced dyskinesia (each components data), and reboxetine data. For dyskinesia data, non-parametric test (L-DOPA-induced dyskinesia for total AIMs and apomorphine-induced dyskinesia: Kruskal-Wallis test with Dunn's multiple comparisons test; L-DOPA-induced dyskinesia (each components data): Mann-Whitney test for 6-OHDA and double lesioned pair; reboxetine data: Friedman test for each group) was applied to

con-firm the statistical significance. All statistics in this study were performed using Prism 6 for Mac OS X, version 6.0a (GraphPad Software, Inc.).

Results

Efficacy of the two NA toxins

Prior to the main experiments, DSP-4 and DBH-Saporin were compared for their ability to destroy NA neurons in the LC. DSP-4 has been widely used in order to deplete central NA levels in rodents and non-human primates. However, when used in rats, Szot and colleagues have reported that the effect of DSP-4 is transient and also not fully specific for the projections from the LC (Szot et al., 2010). In this study the authors observed reduced NA levels in prefrontal cortex, hippocampus, amygdala, septum/bed nucleus of the stria terminalis, and cerebellum between 3 days and 2 weeks after the injection, followed by progressive recovery at later time points. By contrast, DBH-Saporin, given intraventricularly, has been reported to be a powerful toxin, able to completely wipe out the LC NA system in rats (Wrenn et al., 1996). This toxin, however, has never been used in the context of 6-OHDA lesioned, parkinsonian animals. Hence, we were set to compare the efficacy of these two toxins in order to assess whether DBH-Saporin could, indeed, yield more severe and long-lasting LC NA neuron degeneration in parkinsonian rats.

In this test DSP-4 was administered i.p. at a dose of 50 mg/kg, and DBH-Saporin ($2 \times 2.5 \mu\text{g}$) was injected bilaterally in the lateral ventricles. These doses were selected based on previous reports (Fritschy and Grzanna, 1989; Wrenn et al., 1996). In a pilot experiment we found that higher doses of DSP-4 (75 and 100 mg/kg) affected the survival of the animals (not shown). Four weeks later animals were sacrificed and the tissue was stained with DBH antibody. DBH immuno-histochemistry showed a striking difference between the two toxins: DBH-Saporin treatment removed all NA cell bodies in LC (Figs. 2B,D), while the LC remained virtually intact in the DSP-4 treated animals (Figs. 2A,C). The complete loss of LC neurons, bilaterally, in the DBH-Saporin treated rats was confirmed in sections stained for the pan-neuronal marker NeuN (not shown). Consistent with these data, HPLC measurements of tissue NA levels, performed in a separate group of DBH-Saporin treated rats, revealed a near-complete loss of NA in both cortex and striatum (Figs. 2E,F).

After confirming that DBH-Saporin given intraventricularly is an efficient tool to obtain a near-complete lesion of the NA neurons in the LC, two experiments were designed. In Experiment 1 we focused on the effect of NA neuron degeneration on L-DOPA-induced dyskinesia. This study was performed in rats with a severe 6-OHDA lesion; a total of 28 μg was distributed over four sites in the striatum, sufficient to induce more prominent motor impairments and allow induction of L-DOPA-induced dyskinesia in most of the treated animals (see Winkler et al., 2002). In Experiment 2 we used a lower dose of toxin; a total of 14 μg was distributed over two sites, in order to induce more moderate behavioural impairments. This would allow us to detect worsening of motor impairments in a situation more similar to that seen in early Parkinson patients.

Experiment 1

Effect of NA neuron degeneration on 6-OHDA-induced loss of striatal DA

In Experiment 1 DBH-Saporin was administered 2 weeks before the 6-OHDA lesion and the extent of damage to the DA and NA neuronal projections were assessed by HPLC measurements of tissue levels of DA, DOPAC and NA, performed at the end of the 10 week experimental study. As shown in Figs. 3A,B, the level of DA in cortex and striatum was decreased by about 70–80% in both 6-OHDA lesioned groups, similar in magnitude in both single and double lesioned animals. This pattern was

same for the DOPAC level in cortex (Fig. 3C) and striatum (Fig. 3D). Unexpectedly, there we observed an increased level of DA and DOPAC in contralateral side of the cortex in double lesioned animals. In the double lesioned group NA was reduced bilaterally by more than 99% in cortex and by more than 80% in striatum, (Figs. 3E, F). In the 6-OHDA treated rats we used desipramine, a NA transporter blocker, to protect the NA neurons against the toxin. Nevertheless, the NA level was reduced by about 20% in the ipsilateral cortex in the animals treated with 6-OHDA alone (Fig. 3E). A minor reduction was seen also in striatum, but this did not reach significance (Fig. 3F).

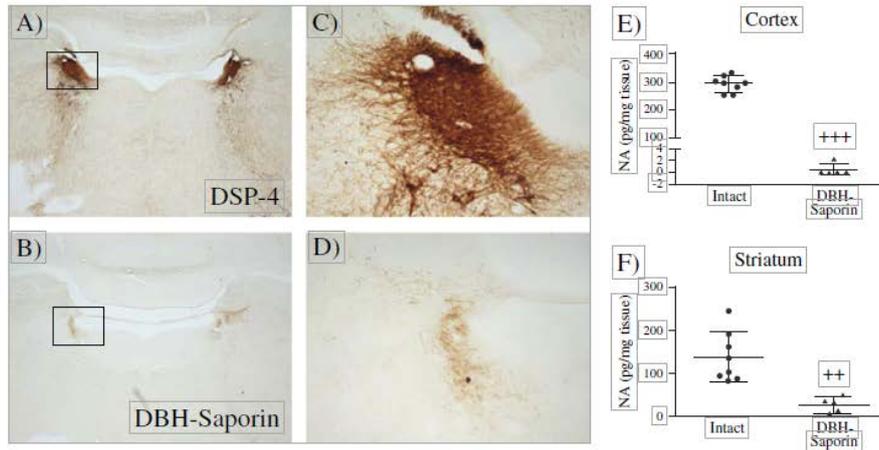
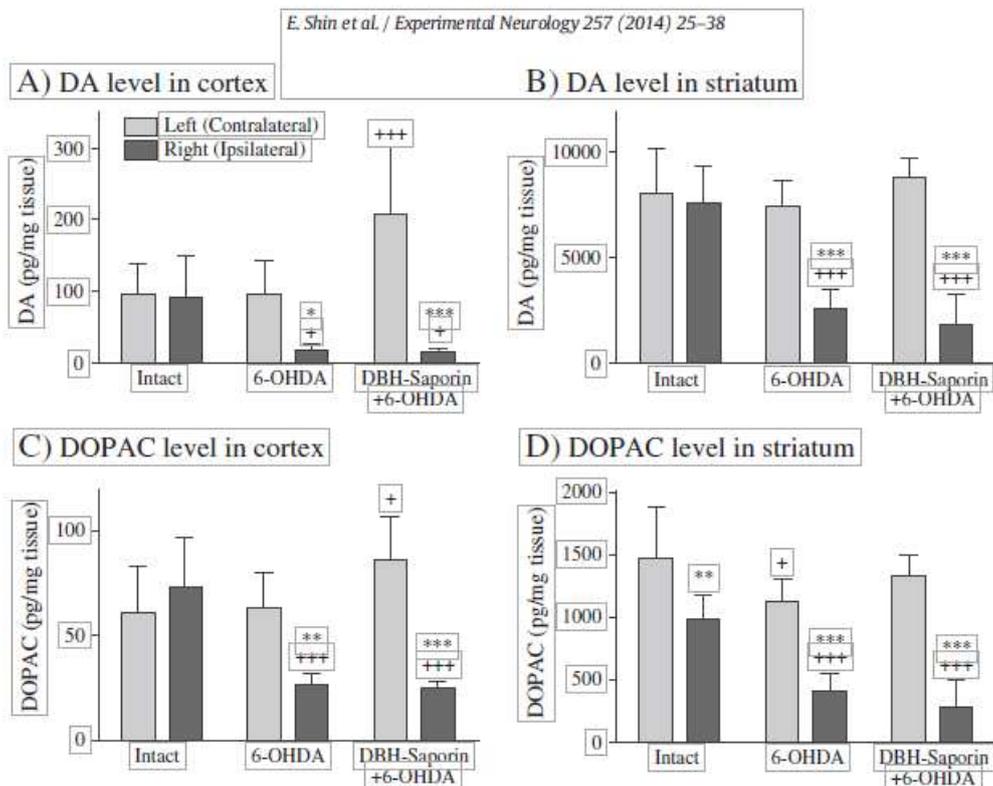


Fig. 2. Efficacy of NA toxins. The potency of DSP-4 and DBH-Saporin was evaluated with DBH immunostaining on LC (A, C for DSP-4 injected rats and B, D for DBH-Saporin injected). The tissue content level of NA from DBH-Saporin rats was analyzed by HPLC in cortex (E) and striatum (F), respectively. DBH-Saporin clearly removed NA neurons in LC and significantly lowered NA tissue level. Unpaired t-test was used for (E) and (F). +++, $p < 0.0001$, ++, $p < 0.01$.



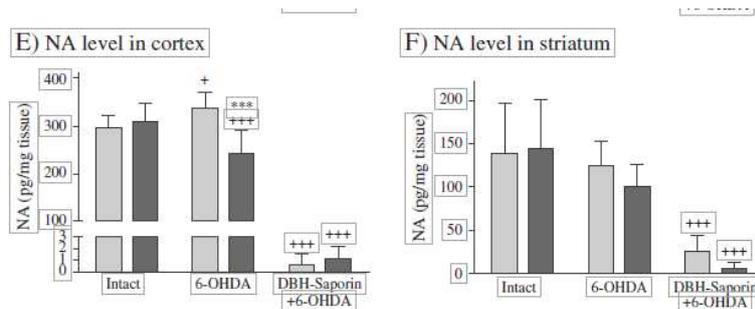


Fig. 3. HPLC analysis of NA, DA, and DOPAC levels in cortex and striatum. (A) The level of DA in cortex has dramatically decreased in the right side (6-OHDA injected side) of single and double lesioned rats. Left side of cortex in double lesioned rats showed higher level of DA compared to the same side of the intact rats. (B) The level of DA in striatum was significantly lower in the right side of the brain from both single and double lesioned rats. (C) The level of DOPAC had a same trend as the level of DA in cortex. Right side of the cortex had lower level of DOPAC in both single and double lesioned rats compared to that in intact rats. Also, the level of DOPAC in left side of the cortex of double lesioned rats was slightly higher than that of intact rats. (D) The level of DOPAC was lower in the right side of the striatum in all groups compared to the left side of the same group. Left side of the striatum in 6-OHDA rats had slightly lower level of DOPAC compared to the same side from intact rats. (E) The level of NA in both sides of the cortex from double lesioned rats was significantly decreased compared to those from intact and 6-OHDA rats. In addition, 6-OHDA rats had lower amount of NA in right cortex. Left side of cortex from 6-OHDA rats also had slightly increased NA level compared to those from intact rats. (F) The level of NA in both sides of striatum in double lesioned rats was significantly lower than those from the intact rats. Statistics: Two-way ANOVA and Bonferroni's multiple comparisons test were used for all the data in Fig. 5. (A) Lesion, $F_{2,34} = 3.930$, $p < 0.05$; Side of the brain, $F_{1,34} = 31.76$, $p < 0.001$; Interaction, $F_{2,34} = 11.00$, $p < 0.001$ (B) Lesion, $F_{2,34} = 15.28$, $p < 0.001$; Side of the brain, $F_{1,34} = 70.23$, $p < 0.001$; Interaction, $F_{2,34} = 15.77$, $p < 0.001$ (C) Lesion, $F_{2,34} = 5.782$, $p < 0.01$; Side of the brain, $F_{1,34} = 25.05$, $p < 0.001$; Interaction, $F_{2,34} = 14.53$, $p < 0.001$ (D) Lesion, $F_{2,34} = 16.04$, $p < 0.001$; Side of the brain, $F_{1,34} = 90.77$, $p < 0.001$; Interaction, $F_{2,34} = 4.209$, $p < 0.05$ (E) Lesion, $F_{2,34} = 294.7$, $p < 0.001$; Side of the brain, $F_{1,34} = 5.969$, $p < 0.05$; Interaction, $F_{2,34} = 11.32$, $p < 0.001$ (F) Lesion, $F_{2,34} = 30.31$, $p < 0.001$; Side of the brain, $F_{1,34} = 0.9130$, $p > 0.05$; Interaction, $F_{2,34} = 0.5737$, $p > 0.05$. +, $p < 0.05$; + + +, $p < 0.001$ compared to the same side of the intact rats. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to the left side within the same group.

Effect of NA neuron degeneration on 6-OHDA-induced motor impairments Three weeks after the 6-OHDA injection (5 weeks after DBH-Saporin injection), rats were subjected to four different behavioural tests. As shown in Fig. 4, the 6-OHDA lesioned rats were impaired in all four tests, but there was no significant difference between single and double lesioned rats in any of the tests. In the amphetamine-induced rotation test (Fig. 4A) both 6-OHDA lesioned groups displayed a strong ipsilateral rotation, more than 11 turns/min, thus confirming the severity of the lesion induced by the high dose of the toxin. In the cylinder test (Fig. 4B), the use of the affected (left) paw was reduced by about 60% compared to the intact rats, similar in both single and double lesioned rats. In the corridor task (Fig. 4C), intact rats explored the two sides to a similar degree, i.e. 50% left side investigation, whereas the single and double lesioned rats had significantly reduced left side investigation (15–17% left side investigation). In the stepping test (Fig. 4D), the intact rats made around 12 steps whereas the single and double lesioned rats made 1–2 steps.

Effect of NA neuronal degeneration on L-DOPA-induced dyskinesia

After completion of the motor tests the rats were subjected to daily L-DOPA treatment and the development of dyskinesia was monitored over 3 weeks (see Fig. 1A). 6, 12, and 24 mg/kg of L-DOPA (together with 10 mg/kg benserazide) was administered for 7 days, 10 days, and 7 days, respectively (Fig. 5J the dose was escalated once the AIMs score reached a plateau). At the lowest dose, 6 mg/kg, L-DOPA evoked mild-moderate dyskinesia in the double lesioned group, while the rats given 6-OHDA alone showed no or only mild dyskinetic response (Figs. 5A,D). At higher doses, 12 and 24 mg/kg (plus benserazide), L-DOPA evoked more severe dyskinesia in the double lesioned rats but only a low-to-moderate response in the single lesioned animals (Figs. 5B,E and C,F). The level of dyskinesia seen in the double lesioned rats was significantly higher than that seen in 6-OHDA only and intact rats at the two lower doses. As seen in Figs. 5A–C the higher AIMs score in the double-lesioned group was due to an overall increase in the magnitude of dyskinesia at each time point, while the duration of the response was not affected.

To investigate whether individual AIMs components were more or less affected by NA degeneration, scores were divided into three components (axial, limb, and orolingual dyskinesia). At 6 mg/kg of L-DOPA, the limb and orolingual components were most prominently increased in the double lesioned animals (Fig. 5G), whereas in 12 mg/kg of L-DOPA, axial and orolingual dyskinesia were significantly

increased (Fig. 5H). The changes seen at the highest dose, 24 mg/kg, did not reach significance (Fig. 5I).

To investigate to what extent this difference was due to a difference in sensitivity of postsynaptic DA receptors we administered the DA agonist apomorphine at a dose, 0.1 mg/kg, known to be effective in inducing dyskinesia in L-DOPA-primed 6-OHDA lesioned rats (Carta et al., 2007; Munoz et al., 2009). Unlike L-DOPA, the level of dyskinesia induced by apomorphine was similar in the single and double lesioned groups (Fig. 5K), suggesting similar sensitization of post-synaptic DA receptors, which is in agreement with HPLC data showing equal degree of striatal DA depletion in both groups.

It has been suggested that NA terminals might be involved in the elimination of extracellular DA, formed from L-DOPA, via the NA trans-porter. Indeed, there is a microdialysis study in 6-OHDA lesioned rats showing increased extracellular DA levels in the striatum when rats were pretreated with a NA transporter blocker, prior to L-DOPA administration (Arai et al., 2008). If this is the case, the L-DOPA-derived extra-cellular levels would be increased in the double lesioned rats, which in turn could lead to more severe dyskinesia. To investigate this possibility, we pretreated the rats with a NA transporter blocker, reboxetine at two doses, 3 and 10 mg/kg, shown to be effective in previous studies (Masana et al., 2011; Ortega et al., 2010; Sacchetti et al., 1999), and the dyskinesia induced by L-DOPA at 12 mg/kg was monitored. Contrary to the hypothesis, reboxetine did not worsen dyskinesia in the 6-OHDA rats (Fig. 5L), suggesting that clearance of DA by uptake into NA terminals does not account for the difference in the level of dyskinesia seen in the single and double lesioned rats.

Experiment 2

Effect of NA deficiency on DA-dependent motor behavior

In this experiment we used a less severe 6-OHDA lesion ($2 \times 7 \mu\text{g}$ unilaterally in the striatum) in order to induce more moderate behavioural deficits than those obtained in Experiment 1. DBH-Saporin was injected either 2 weeks before or 9 weeks after 6-OHDA to study whether the effect of the toxin was dependent on the state of DA innervation at the time of injection (see Fig. 1B). The rats were perfused for immunohistochemical analysis 26 weeks after the 6-OHDA lesion. The integrity of the DA system was investigated by assessment of TH+ fiber density in striatum and by stereological counting of VMAT+ cell numbers in SN (Figs. 6H–K). Figs. 6A–E illustrate the extent of TH+ fiber loss in the striatum from representative TH-immunostained sections and the loss of TH+ cell bodies in the SN from each group, while

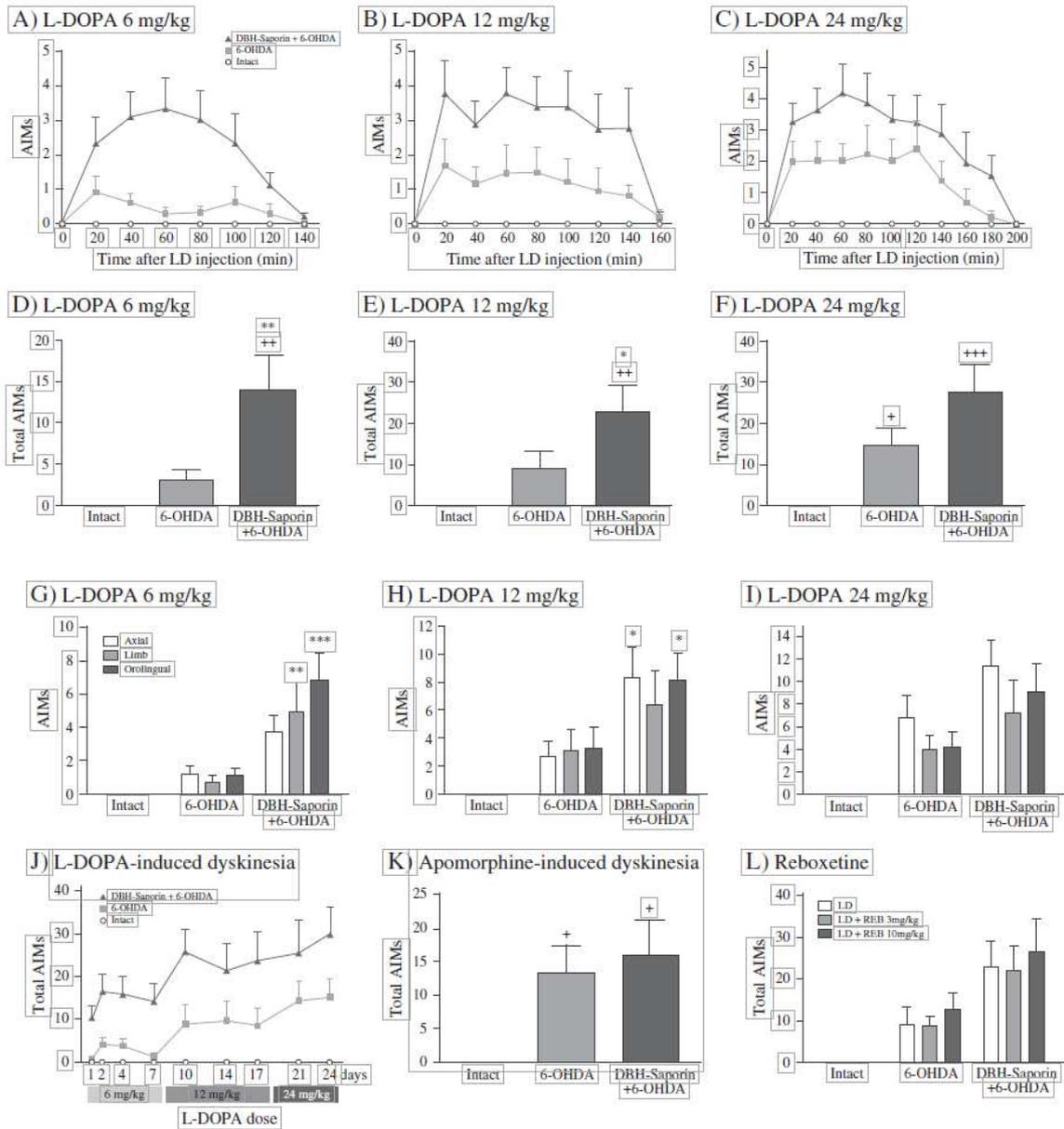


Fig. 5. Effect of NA depletion on L-DOPA-induced dyskinesia. Dyskinesia time course by 6 mg/kg (A), 12 mg/kg (B), and 24 mg/kg (C) of L-DOPA is shown. There was no prolongation of dyskinesia in double lesioned rats but was higher score in every time point. In the total score, lower doses (6 and 12 mg/kg, D and E respectively) of L-DOPA evoked significantly more severe dyskinesia in double lesioned group than in single lesioned rats. 24 mg/kg of L-DOPA (F) showed a trend of higher AIMS score in double lesioned rats but it did not reach statistical significance. Data in A, D, G give the AIMS average of the 2nd, 4th, and 7th injection; data in B, E, H from the 10th, 14th, and 17th injection; data in C, F, I give the average of the 21st and 24th injections. Each component of dyskinesia is shown in G-I. In general all three components were affected by NA removal. Development of AIMS were plotted in J. Apomorphine induced the same amount of dyskinesia in single and double lesioned groups (K). NA transporter blocker reboxetine did not affect L-DOPA-induced dyskinesia (L). *Statistics:* One-way ANOVA with Newman-Keuls multiple comparison test was used for D-F and J. (D) $F_{2,19} = 8.556, p < 0.01$ (E) $F_{2,19} = 8.163, p < 0.01$ (F) $F_{2,19} = 9.807, p < 0.01$ (K) $F_{2,19} = 5.736, p < 0.05$. Two-way ANOVA with Bonferroni's multiple comparison test was used for G-H. (G) Lesion, $F_{2,60} = 25.37, p < 0.001$; LD components, $F_{2,60} = 0.9360, p > 0.05$; Interaction, $F_{4,60} = 0.9675, p > 0.05$ (H) Lesion, $F_{2,57} = 21.73, p < 0.001$; LD components, $F_{2,57} = 0.1671, p > 0.05$; Interaction, $F_{4,57} = 0.2193, p > 0.05$ (I) Lesion, $F_{2,57} = 24.68, p < 0.001$; LD components, $F_{2,57} = 1.627, p > 0.05$; Interaction, $F_{4,57} = 0.4987, p > 0.05$ (L) Two-way repeated measure ANOVA: Lesion, $F_{2,19} = 9.191, p < 0.01$; Drug, $F_{2,38} = 2.915, p > 0.05$; Interaction, $F_{4,38} = 0.7763, p > 0.05$. Due to the nature of the data, non-parametric test was also carried out. For that, Kruskal-Wallis test with Dunn's multiple comparison test was used for D-F and K. $p < 0.001$ from Kruskal-Wallis test. For multiple comparison, (D) $p < 0.001$, Intact vs DBH-Saporin + 6-OHDA (E) $p < 0.05$, Intact vs 6-OHDA; $p < 0.001$, Intact vs DBH-Saporin + 6-OHDA (F) $p < 0.01$, Intact vs 6-OHDA; $p < 0.001$, Intact vs DBH-Saporin + 6-OHDA (K) $p < 0.01$, Intact vs 6-OHDA and vs DBH-Saporin + 6-OHDA. Mann-Whitney test was employed for C-L. Each component was compared between 6-OHDA vs DBH-Saporin + 6-OHDA group (G) $p < 0.05$ in Orolingual (H) $p < 0.05$ in Orolingual (I) all pairs were $p > 0.05$. Friedman test was used for each group in L. For both groups $p > 0.05$, +, $p < 0.05$; ++, $p < 0.01$; +++, $p < 0.001$ compared to Intact group. *, $p < 0.05$; **, $p < 0.05$; ***, $p < 0.001$ compared to 6-OHDA group.

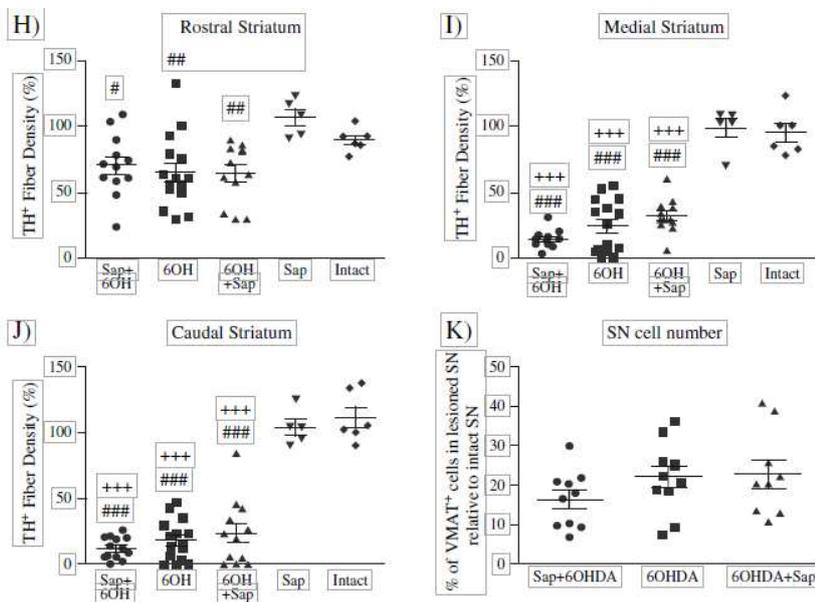
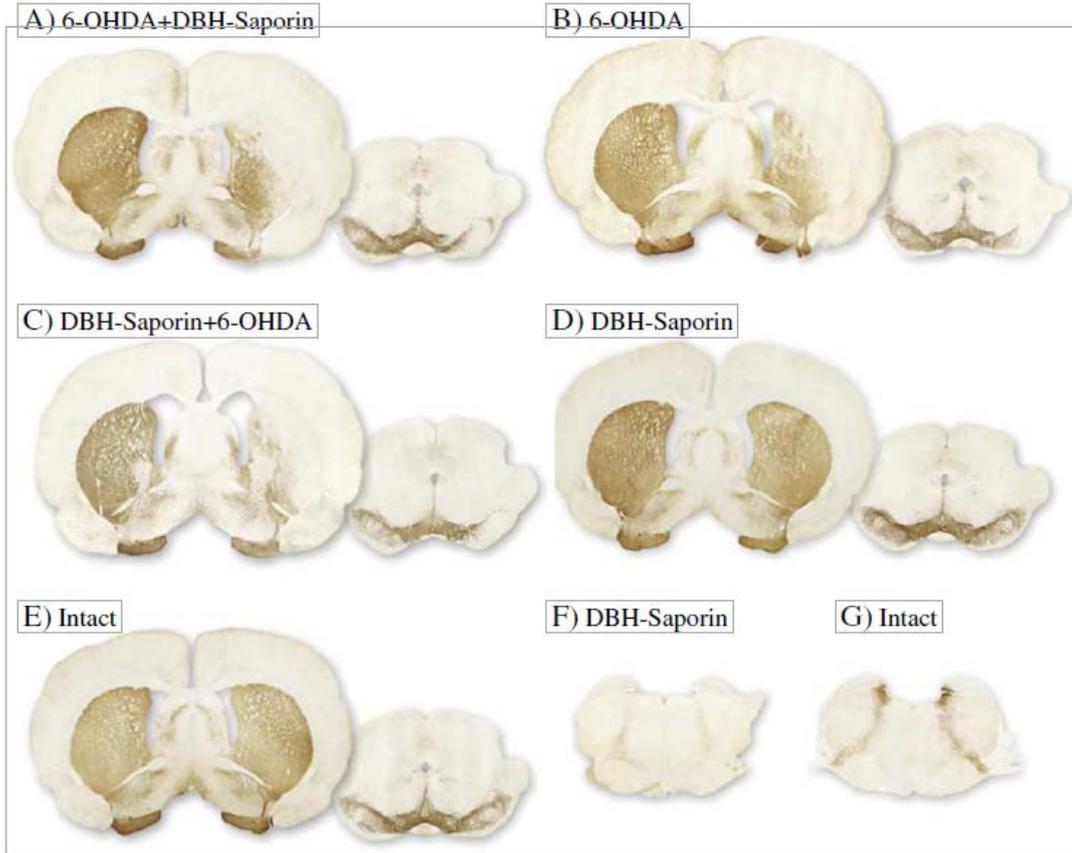


Fig. 6. Histological analysis of the DA system. (A-E) shows the representative TH stained striatum and SN. (F-G) shows the representative TH stained LC. TH⁺ fiber density (relative to the intact side of striatum) in rostral (H), medial (I), and caudal (J) striatum was not different between single (DA) and double lesioned groups, but was significantly less than Saporin and Intact rats. % of VMAT⁺ cells in SN was similar between the three groups (K). *Statistics:* one-way ANOVA was carried out with Newman-Keuls multiple comparison test. (H) $F_{4,45} = 4.467, p < 0.01$ (I) $F_{4,45} = 51.61, p < 0.001$ (J) $F_{4,45} = 58.39, p < 0.001$ (K) $F_{2,27} = 1.535, p > 0.05$. +, +, +, $p < 0.001$ compared to intact. #, #, #, $p < 0.05$; ##, ##, ##, $p < 0.01$; ###, ###, ###, $p < 0.001$ compared to Saporin group.

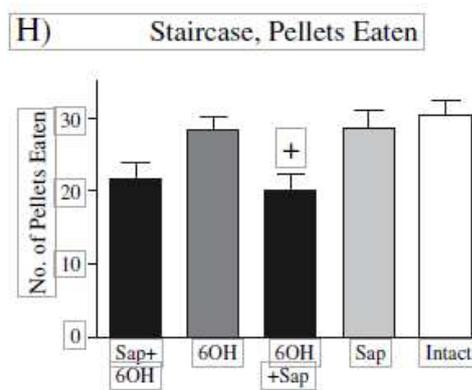
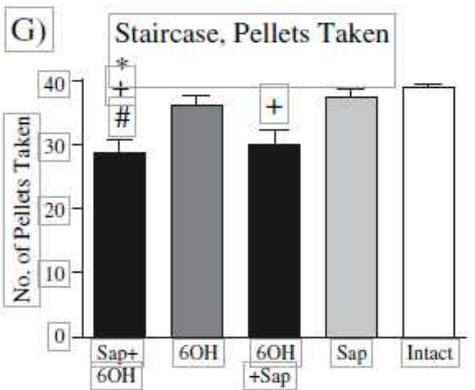
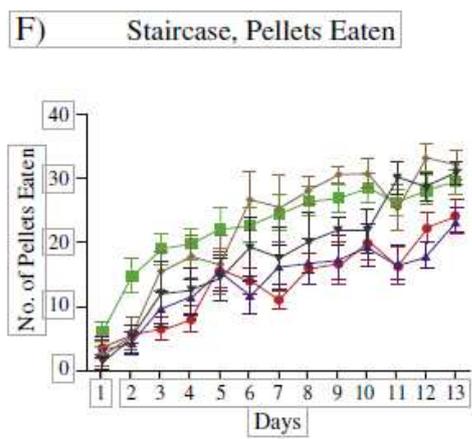
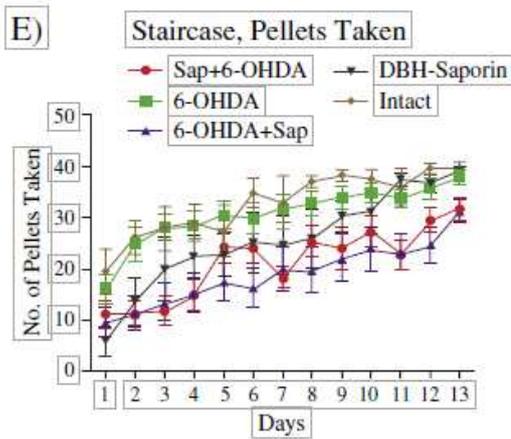
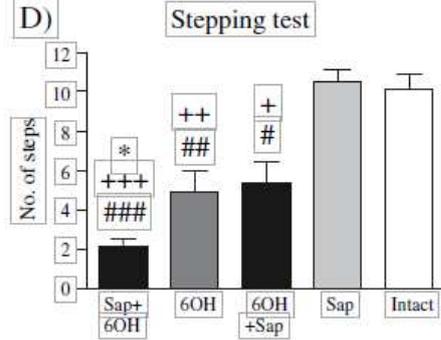
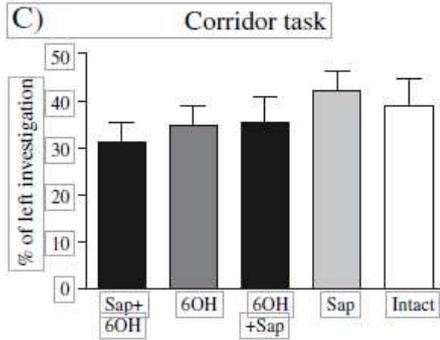
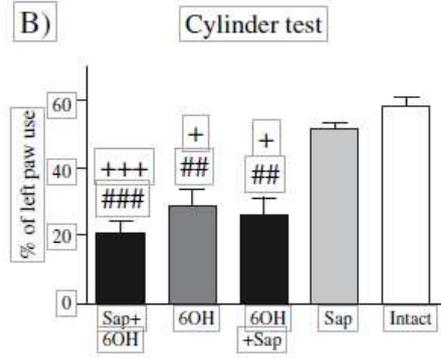
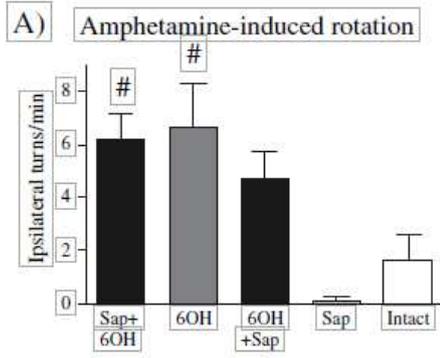


Fig. 7. Effect of NA depletion on performance in motor tests. (A) Amphetamine induced more ipsilateral rotation in Saporin + 6-OHDA and 6-OHDA group compared to Saporin group. (B) In cylinder test, three groups (Saporin + 6-OHDA, 6-OHDA, and 6-OHDA + Saporin) had impaired left paw use compared to Saporin and Intact group. (C) Corridor task was not sensitive to pick the motor differences between groups. (D) In stepping test, Saporin + 6-OHDA was significantly impaired compared to 6-OHDA. All 6-OHDA injected groups (the first three groups) were significantly impaired compared to Saporin and Intact group. The number of pellets taken (E) and eaten (F) from the affected (left) paw during 13 consecutive days was plotted. Most similar 3 days of data among the last 4 days' results were averaged and compared in G and H. Saporin + 6-OHDA group took significantly less amount of pellets compared to 6-OHDA, Saporin and Intact rats (G). 6OHDA + Saporin rats also showed lower amount of pellets taken. For pellets eaten, 6-OHDA + Saporin group had the least number of eaten pellets which were significantly less than those from Intact group (H). Statistics: one-way ANOVA with Newman-Keuls multiple comparison test was used. (A) $F_{4,47} = 3.408$, $p < 0.05$ (B) $F_{4,46} = 11.03$, $p < 0.001$ (C) $F_{4,47} = 0.5582$, $p > 0.05$ (D) $F_{4,47} = 9.74$, $p < 0.001$ (G) $F_{4,45} = 4.691$, $p < 0.01$ (H) $F_{4,45} = 4.125$, $p < 0.01$. +, $p < 0.05$; ++, $p < 0.01$; +++, $p < 0.001$ compared to intact. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ compared to Saporin group. *, $p < 0.05$ compared to 6-OHDA group.

Figs. 6F,G show the loss of TH+ cell bodies in the LC, bilaterally, in DBH-Saporin treated and intact rats. All DBH-Saporin treated rats had maximum 1–2 NA cells in each stained section.

The loss of VMAT+ cells in the SN was similar in all three groups, ranging from 40–95%, with an average of about 80% (Fig. 6K). The loss of TH+ innervation in the striatum was greatest in the caudal and medial parts, ranging from about 60–100%, and less pronounced in the rostral striatum, limited to less than 50% in most cases (Figs. 6H–J). The loss of striatal innervation was similar in all three DA-lesioned groups.

The effect of DBH-Saporin-induced loss of LC NA neurons on motor performance, on the side contralateral to the 6-OHDA lesion, was studied in 5 different behavioural tests. Four of the tests (Figs. 7A–D) –amphetamine rotation, cylinder test, corridor task and stepping test –were the same as in Experiment 1. In addition, we added a test of more complex motor performance, the staircase test, which involves skilled paw use for retrieval of pellets and is acquired through training (Figs. 7E–H). The three DA-lesioned groups (6-OHDA-injected groups with or without DBH-Saporin) showed impaired performance in the amphetamine rotation, cylinder, and stepping test but not in the corridor task. The turning response seen in the amphetamine rotation test (Fig. 7A) was less (around 6–7 turns/min) than in the more severely lesioned rats used in Experiment 1 (Fig. 4A, around 10–15 turns/min). Compared to the pronounced effects seen in Experiment 1, the impairment was overall less severe in these rats, which had received a lower dose of 6-OHDA. No significant difference was found in the motor performance between single lesioned (6-OHDA) and double lesioned (Saporin + 6-OHDA and 6-OHDA + Saporin) groups in the amphetamine-induced rotation (Fig. 7A), cylinder (Fig. 7B), and corridor (Fig. 7C) tests. By contrast, double lesioned rats (either Saporin+ 6-OHDA, or 6-OHDA + Saporin, or both) showed more severe motor deficits compared to the 6-OHDA only injected rats in the stepping (Fig. 7D) and staircase (Figs. 7E–H) tests. Overall, with the exception of the stepping test, DBH-Saporin injection appeared to induce equal deficits whether it was injected before or after the 6-OHDA lesion.

Daily performance in the staircase test (Figs. 7E,F) showed similar acquisition of the task between the 6-OHDA and the Intact group over the 13 days of training. The Saporin group performed worse in the beginning but reached a similar level at later days of testing. The two double lesioned groups learned slowly and never reached the same level of performance as the other groups. Hence, when we compared the number of pellets taken during the last 4 days, both the Saporin + 6-OHDA and the 6-OHDA + Saporin group performed significantly worse than other groups (Fig. 7G). Daily performance in the number of pellets eaten showed a similar trend as pellets taken (Fig. 7H), although the data from the last 4 days did not show statistical significance between the Saporin + 6-OHDA and the 6-OHDA group.

The scatter plots in Fig. 8 show the individual scores of the rats in the staircase test (last four days) plotted against the loss of DA neurons in the SN (Figs. 8A,B) and against the loss of TH+ innervation in the striatum (Figs. 8C,D). The performance of the 6-OHDA only lesioned rats (red circles) was unaffected by the lesion. Their scores, above 30 pellets taken, and above 20 pellets eaten, were within the range seen in the Intact control group (see dashed lines) regardless of the magnitude of

cell loss or striatal denervation, which varied from about 60 to 90% in the individual cases. By contrast, most of the double-lesioned rats (blue triangles, both groups combined) showed a markedly impaired performance already at moderate levels of damage to the nigrostriatal neurons, i.e. at 75–90% loss of DA neurons in the SN and 50–80% loss of striatal TH+ innervation, suggesting that loss of NA neurons in the LC affects the ability of the 6-OHDA lesioned rats to acquire and perform this more complex motor task.

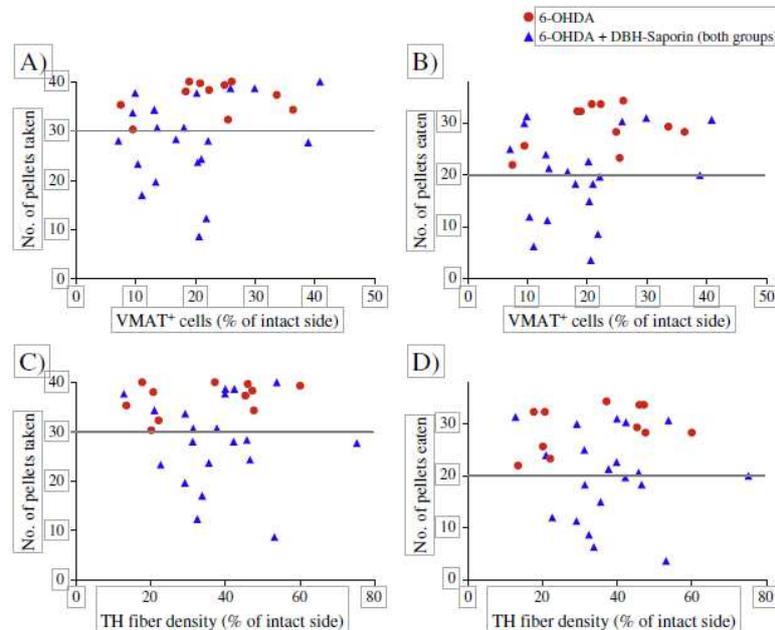


Fig. 8. Scatter plots of DA cell loss vs staircase test (A, pellets taken; B, pellets eaten) shows intact level (dashed line and above) of performance in 6-OHDA group regardless of DA cell loss in SN whereas NA + DA double lesioned rats start to show impairment when DA cell loss is above 75% (i.e. 25% VMAT⁺ cells). Similar scatter plot was made between striatal DA loss vs pellets taken (C) and eaten (D). Regardless of the striatal DA loss, 6-OHDA rats performed perfectly whereas DA + NA double lesioned rats were impaired when loss of striatal TH innervation was between 50–80%.

Discussion

In this study we demonstrate for the first time that the selective NA toxin DBH-Saporin, contrary to DSP-4, is highly effective in removing NA neurons in 6-OHDA-lesioned rats, and that the profound NA neuron loss induced by this toxin has significant impact on the development of L-DOPA-induced dyskinesia and motor impairments, as observed in tests of reflexive and skilled paw use. The worsening of L-DOPA-induced dyskinesia and motor impairments observed in parkinsonian rats after DBH-Saporin treatment does not appear to be due to decreased survival of nigral DA neurons, neither when DBH-Saporin was given before 6-OHDA, nor when given after the DA toxin. Accordingly, neither the striatal DA fibre density nor DA tissue content in cortex and striatum was changed.

Previous studies of the effect of NA depletion on the development of L-DOPA-induced dyskinesia in 6-OHDA lesioned animals have been contradictory. Both Fulceri et al. (2007) and Barnum et al. (2012) have used 6-OHDA injected into the MFB, with or without systemic injection of the uptake blocker desipramine (to protect NA terminals) in order to achieve DA depletion with or without loss of forebrain NA. The former study reported more severe dyskinesia in double lesioned group while the latter study saw the opposite result: decreased dyskinesia in double lesioned group. In a third study, Perez et al. (2009) reported that L-DOPA-induced dyskinesia remained unchanged after DSP-4 injection in 6-OHDA lesioned rats. In a different study, when DSP-4 was administered into L-DOPA-primed, 6-OHDA-lesioned dyskinetic rats, the degree of dyskinesia was unchanged; however, when

LC NA neurons were re-moved by ibotenic acid in already dyskinetic rats, there was an increase of the overall AIMs score due to the prolongation of dyskinesia (Migueluez et al., 2011).

These contradictory results are most probably due to the variable magnitude of NA depletion, and LC NA neuron damage in particular, obtained with the different types of lesion used. From the recent study of Szot et al. (2010) it seems likely that the long-term reduction of NA in the target regions of LC (cortex and hippocampus) induced by DSP-4 at the dose used in these studies (50 mg/kg) was less than 50%. In the present study we used the DBH-Saporin immunotoxin to obtain a near complete destruction of the NA neurons, resulting in a $\geq 90\%$ reduction of NA in both cortex and striatum. The worsening of L-DOPA-induced dyskinesia induced by severe damage to the NA projections from the LC neurons, as seen here, is consistent with the observations of Fulceri et al. (2007) in MFB-6-OHDA lesioned rats. In their study a loss of around 80% of striatal NA (as seen in the rats that did not receive the protective desipramine treatment) was associated with an increased level of dyskinesia.

The mechanism underlying this effect is not clear. Our results show, for the first time, that increased dyskinesia in NA + DA double lesioned rats is not due to increased sensitization of postsynaptic DA receptors, or to reduced elimination of L-DOPA-derived DA. Thus, the dyskinesia induced by a low dose of the DA receptor agonist apomorphine was similar in both single and double lesioned rats, and blockade of the NA transporter by reboxetine did not induce any worsening of L-DOPA-induced dyskinesia in single lesioned animals. This argues against the hypothesis that L-DOPA-derived DA, in part, is eliminated by uptake into NA terminals, and that removal of the NA innervation will result in an increased level of L-DOPA-derived DA acting on the supersensitive DA receptors.

As DA appears to bind to NA receptors (Cornil and Ball, 2008), it could also be hypothesized that stimulation of supersensitive NA receptors by L-DOPA-derived DA may be involved in the increased LID in double lesioned rats. However, this seems unlikely under our experimental conditions, as apomorphine has been shown to have similar affinity to NA receptors as DA itself (Cornil and Ball, 2008), but no difference was found between the two lesioned groups after its administration, in contrast to L-DOPA. As discussed below, available data speak in favor of an alternative mechanism, i.e., that the effect on the development of dyskinesia induced by the loss of NA neurons is an indirect one, mediated by changes in the activity of either the DA neurons themselves, or of neurons involved in the regulation of basal ganglia function, such as the raphe serotonergic neurons, the SN pars reticulata neurons, or the corticostriatal neurons.

In our second experiment, the effect of NA neuron degeneration on motor impairment was investigated. Double lesioned rats performed significantly worse in reflexive and skilled paw use tasks. The effect was especially clear in staircase test. Regardless of the magnitude of DA degeneration in nigra and striatum, single lesioned rats did not show any impairment in retrieving and eating pellets, whereas most of the double lesioned rats showed impairments in both parameters. In previous studies in rats, using 6-OHDA lesions in combination with DSP-4, the behavioural deficits induced by the 6-OHDA lesion was largely unaffected by the DSP-4 treatment (Delaville et al., 2012; Srinivasan and Schmidt, 2003, 2004), although one of them reported reduced open field activity in 6-OHDA + DSP-4 double lesioned rats (Srinivasan and Schmidt, 2004). In mouse and monkey models the effects of NA degeneration, induced by DSP-4 or 6-OHDA into LC, have been more consistent and pronounced, especially when MPTP has been employed as a DA toxin. In both models (monkey and mouse), NA degeneration prevented spontaneous recovery of DA after MPTP and induced more pronounced motor deficits (Archer and Fredriksson, 2006; Bing et al., 1994; Fornai et al., 1997; Mavridis et al., 1991). In our study, increased motor impairments did not seem to be caused by increased DA neuron cell loss, which is in agreement with some studies (Delaville et al.,

2012; Pifl et al., 2013; Rommelfanger et al., 2007). Other studies have reported increased DA neurodegeneration, or increased loss of striatal DA, in double lesioned animals (Archer and Fredriksson, 2006; Bing et al., 1994; Fornai et al., 1997; Mavridis et al., 1991; Srinivasan and Schmidt, 2003, 2004). It remains unclear, however, to what extent this effect on DA neurodegeneration is mediated via damage to the NA projections, or due to a direct, non-specific insult of the DSP-4 toxin on DA neurons made vulnerable by the damage induced by the 6-OHDA treatment.

The worsening of 6-OHDA-induced motor impairments induced by the loss of NA neurons is most likely mediated by changes in the activity of either the DA neurons themselves, or of neurons involved in the regulation of basal ganglia function, such as the serotonergic raphe neurons, the neurons in SN pars reticulata, or the cortical projection neurons innervating the striatum. LC NA neurons are known to innervate the SN pars compacta (Berridge and Waterhouse, 2003; Jones and Moore, 1977). Accordingly, NA has been shown to facilitate DA transmission in rats (Antelman and Caggiula, 1977; Donaldson et al., 1976; Ponzio et al., 1981; Reisine et al., 1982), and lesions of NA neurons and their terminals have been shown to decrease striatal DA release in rats (Lategan et al., 1990, 1992; Marien et al., 1994). In addition, NA seems to be involved also in modulating the activity of other types of neurons, such as SN pars reticulata neurons and cortical pyramidal neurons. For instance, bilateral lesions of LC NA neurons with 6-OHDA in DA lesioned rats increased the firing activity of SN pars reticulata neurons (Wang et al., 2010a) and medial prefrontal cortex pyramidal neurons (Wang et al., 2010b). In addition, firing rate of serotonin neurons (Haddjeri et al., 1996; Svensson et al., 1975; Vandermaelen and Aghajanian, 1983) and serotonin synthesis and release (Adell and Artigas, 1999; Amargos-Bosch et al., 2003; Mongeau et al., 1997; Pudovkina et al., 2002, 2003; Yoshioka et al., 1992) has been shown to be modified by NA input. All of these types of neurons are known to be involved in the expression of both L-DOPA-induced dyskinesia and motor performance. Thus, the loss of NA neurons, as seen in the double lesioned rats as well as in PD patients, is likely to induce functional changes not only in the surviving nigral DA neurons, but also in other subsets of neurons involved in the regulation of basal ganglia function.

In conclusion, the potent and selective effect of DBH-Saporin on NA neurons provides a new efficient tool to study the motor and non-motor implications of NA neuron degeneration in both intact and parkinsonian animals. Our results add to the increasing body of evidence suggesting that NA neuron loss, which is known to affect the PD brain already at a relatively early stage of the disease, may contribute to the development of L-DOPA-induced dyskinesia and the motor manifestations of the disease. Our data do not support a direct effect of NA on DA cell survival but may suggest an effect mediated by functional changes in neurons involved in basal ganglia function, including the surviving nigral DA neurons themselves.

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