Effects of noradrenergic denervation by anti-DBH-saporin on behavioral responsivity to L-DOPA in the hemi-parkinsonian rat

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Abstract

Dopamine (DA) replacement with L-DOPA remains the most effective pharmacotherapy for motor symptoms of Parkinson's disease (PD) including tremor, postural instability, akinesia, and bradykinesia. Prolonged L-DOPA use frequently leads to deleterious side effects including involuntary choreic and dystonic movements known as L-DOPA induced dyskinesias (LID). DA loss in PD is frequently accompanied by concomitant noradrenergic (NE) denervation of the locus coeruleus (LC); however, the effects of NE loss on L-DOPA efficacy and LID remain controversial and are often overlooked in traditional animal models of PD. The current investigation examined the role of NE loss in L-DOPA therapy by employing the NE specific neurotoxin anti-DA-beta hydroxylase saporin (αDBH) in a rat model of PD. Rats received unilateral 6-hydroxydopamine lesions of the medial forebrain bundle to deplete nigral DA and intraventricular injection of vehicle (DA lesioned rats) or αDBH (DANE lesioned rats) to destroy NE neurons bilaterally. Results indicated that αDBH infusion drastically reduced NE neuron markers within the LC compared to rats that received vehicle treatment. Behaviorally, this loss did not alter the development or expression of L-DOPA- or DA agonist- induced dyskinesia. However, rats with additional NE lesions were less responsive to L-DOPA's pro-motor effects. Indeed, DANE lesioned animals rotated less and showed less attenuation of parkinsonian stepping deficits following high doses of L-DOPA than DA lesioned animals. These findings suggest that severe NE loss may reduce L-DOPA treatment efficacy and demonstrate that degradation of the NE system is an important consideration when evaluating L-DOPA effects in later stage PD.

Keywords

anti-DBH saporin; norepinephrine; dyskinesia; locus coeruleus; parkinson's disease

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1. Introduction[i]

Parkinson's disease (PD) is[ii] a progressive neurodegenerative movement disorder traditionally characterized by the loss of dopamine (DA) neurons in the nigrostriatal pathway. However, over the last century it is has become increasingly apparent that PD pathology extends far beyond the DA system. Indeed, noradrenergic cell loss in the locus coeruleus (LC) precedes and is equal to, if not greater than, DA loss within the substantia nigra pars compacta (SN) in PD [1]. Noradrenergic loss has been linked to a number of cardinal motor symptoms of PD and purportedly exacerbates parkinsonian motor deficits [2–6].

Despite these findings, most pharmacological treatments for PD are aimed solely at restoring central DA function. Long-term DA replacement, especially with the DA precursor L-DOPA, is often complicated by the emergence of debilitating motor side effects, notably L-DOPA-induced dyskinesia (LID), typified by hyperkinetic involuntary movements [7]. LID has largely been attributed to pre- and post- synaptic changes in striatal DA neurotransmission leading to aberrant basal ganglia signaling [8–10]. The noradrenergic system innervates nearly all nuclei of the basal ganglia and has recently been implicated in LID by several lines of research. Recent work indicates that LID severity is positively correlated with basal firing parameters of LC neurons [11] and direct infusion of exogenous norepinephrine (NE) into the striatum induces dyskinesia in L-DOPA-primed hemiparkinsonian rats [12]. There is also evidence that the NE transporter (NET) can take up DA and may play an integral role in clearing L-DOPA-derived DA following the loss of striatal DA-transporters in PD [13]. Finally, several compounds that target the NE system have been shown to reduce LID in experimental and clinical populations [14–16]. Unfortunately, a paucity of systematic basic research exists regarding the direct impact of NE cell loss within the LC on the development and expression of LID.

Animal models of LID have focused on creating severe lesions that are specific to the nigrostriatal DA pathway. However, most do not display or account for NE cell loss typically observed in the human condition. In fact, the NET blocker desipramine is frequently given prior to 6-OHDA infusion to prevent noradrenergic cell loss in rodent models of PD. To date, only a few studies have directly examined whether the state of the noradrenergic system influences LID symptoms. These studies have thus far produced contradictory behavioral effects with some reporting that additional NE loss increased [11, 17], decreased [15], or did not change [11, 18] the severity or duration of LID expression in experimental PD models.

The selective NE neurotoxin anti-DA beta-hydroxylase saporin (αDBH) effectively destroys NE neurons [19] but has not yet been investigated in a Parkinsonian model. αDBH consists of a monoclonal antibody for DA beta- hydroxylase (DBH) conjugated to the ribosomal-

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[i]Abbreviations: 6-hydroxydopamine (6-OHDA); α2- noradrenergic receptor (α2R); anti-dopamine-beta-hydroxylase saporin (αDBH); avidin-biotin-peroxidase complex (ABC); abnormal involuntary movements (AIMs); axial, forelimb, orolingual (ALO); dopamine (DA); forepaw adjusting steps (FAS); intraventricular (ICV); locus coeruleus (LC); L-DOPA-induced dyskinesia (LID); median absolute deviation (MAD); medial forebrain bundle (MFB); norepinephrine (NE); norepinephrine transporter (NET); phosphate buffered saline (PBS); Parkinson's disease (PD); standard error of the mean (SEM); substantia nigra (SN); tyrosine hydroxylase (TH)

inactivating protein saporin [19, 20]. During neurotransmitter release, αDBH molecules bind to vesicular DBH enzymes and following endocytosis undergo retrograde transport to the cell body where saporin inactivates the ribosomes preventing protein synthesis ultimately resulting in NE cell death [21]. Therefore, the goal of the current study was to systematically address the role of NE loss on L-DOPA-mediated behaviors by characterizing differences in the development and expression of LID and motor performance in hemiparkinsonian rats using well established behavioral techniques.

2. Methods

2.1 Animals

Adult male Sprague-Dawley rats were used (N = 30; 225–250 g upon arrival; Harlan, USA). Animals were pair-housed in plastic cages (22 cm high, 45 cm deep, and 23 cm wide) and had free access to water and standard lab chow (Rodent Diet 5001; Lab Diet, Brentwood, MO, USA). The colony room was maintained on a 12/12 h light/dark cycle (lights on at 0700 hrs) at a temperature of 22–23°C. Throughout the study, animals were cared for in full accordance with the guidelines of the Institutional Animal Care and Use Committee of Binghamton University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2 Drugs

Systemically administered drugs were given at a volume of 1 ml/kg and were injected subcutaneously unless otherwise noted. All drugs were acquired from Sigma (St. Louis, MO., USA) unless specified differently. 6-hydroxydopamine (6-OHDA) and 1–3,4-dihydroxyphenylalanine methyl-ester + dl-serine 2-(2,3,4-trihydroxybenzyl) hydrazide HCL (benserazide) (L-DOPA) were dissolved in 0.9% NaCl + 0.1% ascorbic acid. Benserazide was always administered at a concentration of 15 mg/kg, regardless of L-DOPA concentration. Buprenorphine HCL (Rechitt Benckiser Pharmaceuticals Inc., Richmond, VA) was suspended in saline (0.9% NaCl). Desipramine HCL, d-amphetamine, and quinpirole were dissolved in dH$_2$O. SKF81297 was dissolved in 20% DMSO. Anti-DBH saporin (αDBH) was purchased from Millipore (Billerica, MA) and came pre-suspended in phosphate-buffered saline (PBS).

2.3 Stereotaxic surgery

One week after arrival, (day −21) rats were randomly assigned to one of two lesion groups as follows: 1) αDBH-saporin NE lesion + 6-OHDA DA lesion (DANE-lesion; n = 15); or 2) sham NE lesion + 6-OHDA DA lesion (DA-lesion; n =15). Briefly, rats were anesthetized with inhalant isoflurane (2–3%) in oxygen (1000 cc/min) and placed in a stereotaxic apparatus. All rats received the analgesic buprenorphine HCL (0.03 mg/kg, ip) prior to surgery and the day after surgery. NE- or sham-lesions were produced by infusing the NE selective neurotoxin αDBH (10 μg/10.2 μl; based on [19]) or its vehicle (10.2 μl) into the left lateral ventricle using the following coordinates relative to bregma: AP, −0.8 mm; ML 1.4 mm; DV, −3.9 mm with the interaural line at 0 (Paxinos & Watson, 1998). Immediately after, unilateral DA lesions were produced in all rats by infusing the neurotoxin 6-OHDA (12 μg/4 μl) directly into the left medial forebrain bundle (MFB) using the following

coordinates relative to bregma: AP, −1.8 mm; ML, 2.0 mm; DV, −8.6 mm. Drugs were infused at a rate of 2 μl/min and the needle was left in place for 5 min after infusion to allow for drug dispersal. All rats received Desipramine HCL (25 mg/kg, ip) 30 min prior to 6-OHDA injection to ensure that destruction of NE neurons was induced by αDBH-saporin-, not 6-OHDA. Stainless steel wound clips were used to close the surgical site and animals were returned to group housing. Animals were allowed to recover with ad lib food and water and soft chow was provided as needed to facilitate recovery during the first week after surgery.

2.4 Experimental Procedure and Design

As shown in figure 1, following recovery from surgery, rats underwent a battery of behavioral tests in order to characterize the behavioral outcome of NE lesions on L-DOPA-, and DA agonist- induced motor behaviors.

Two weeks after surgery (day −7), all rats were tested using amphetamine-induced rotations in order to assess DA lesion severity and to determine whether additional noradrenergic loss altered responsiveness to acute d-amphetamine treatment (see 2.5.1). During the next 5 d (day −6 to −1), rats were acclimated to the forepaw adjusting steps (FAS) testing procedure (see 2.5.3). On day 0, all rats were evaluated for lesion-induced stepping deficits using the FAS test. In order to determine if additional NE lesions modified the development of dyskinesia, all rats underwent 16 consecutive days of L-DOPA treatment. On days 1–8, rats received daily treatment of a low dose of L-DOPA (4 mg/kg). During this time, rats were monitored for the development of dyskinesia and rotational behavior on days 1, 5, and 8 using the rodent abnormal involuntary movements scale (AIMs; see 2.5.2) and for reversal of lesion-induced stepping deficits using the FAS test on day 6. On days 9–16, rats received the high dose of L-DOPA (12 mg/kg) which previously has been shown to induce maximal and stable LID in both DA- and DANE-lesioned animals [15, 22]. Rats were monitored for dyskinesia and rotations on days 9, 13, and 16 and for L-DOPA-induced improvements in stepping performance on day 14. Previous work has indicated that dual DANE lesioned rats demonstrating stable LID across L-DOPA (12 mg/kg) priming displayed less LID in response to a wide range of L-DOPA doses than just DA-lesioned rats [15]. Therefore, following a 4 d washout period where no drugs were administered, rats were evaluated to see if NE loss reduced sensitivity to L-DOPA's pro-rotational and dyskinesia-producing effects. Over a 2 week period (days 20–34) rats were injected with various doses of L-DOPA (0, 2, 3, 4, 6, 12 mg/kg) every 2–3 d using a counterbalanced within subjects design and were rated for AIMs and rotations. L-DOPA treatment is known to alter DA receptor sensitivity [23, 24] and subsequently, behavioral responsivity to DA-agonist treatment; however, it is unknown whether NE loss in PD further altered DA-agonist induced behavioral sensitivity. Thus, rats were tested to see if NE loss altered DA agonist-induced dyskinesia or rotations. Rats were injected with the DA D1R agonist SKF81297 (0, 0.08, 0.8 mg/kg) or the DA D2R agonist quinpirole (0, 0.05, 0.5 mg/kg) every 2–3 days using a counterbalanced within-subjects design and were again rated for AIMs and rotations (days 40–56). Following a 10 d washout period, rats were killed by injection of sodium pentobarbital (day 65), perfused with 4% paraformaldehyde suspended in PBS (PFA), and brains were removed for immunohistological analyses.

2.5 Behavioral Tests

2.5.1 Amphetamine-induced rotations—The amphetamine-induced rotation test has been extensively used to confirm DA lesion in hemiparkinsonian rats [25, 26]. Starting 5 min after injection of d-amphetamine (2.5 mg/kg; ip), DA- and DANE-lesioned rats were monitored for rotational activity ipsilateral to lesion for 1 min every 5 min for 1 h. Only full 360° turns towards the DA lesioned side of the brain were recorded. The data were expressed as the total sum of rotations observed during the 1 h period.

2.5.2 Abnormal involuntary movements—Rats were monitored for dyskinesia using a similar procedure to that described in detail previously [22]. Following L-DOPA or DA agonist injection, rats were individually placed in plastic cylinders and trained observers (interrater reliability r   .95) assessed each rat for the occurrence of axial, forelimb, and orolingual (ALO) AIMs and rotations for 1 min every 10 min for either 120 min (SKF81297 tests) or 180 min (L-DOPA and quinpirole tests). Individual dyskinesia severity scores ranging from 0 (not present) to 4 (severe and not interruptible) were given for each ALO AIMs subtype and these subtypes were summed to create a single ALO AIMs score for data analysis. Ipsilateral and contralateral rotations, defined as complete 360° turns (towards or away from the lesioned side of the brain, respectively), were tallied where contralateral rotations were expressed as positive numbers and ipsilateral rotations were expressed as negative numbers. Ipsilateral and contralateral rotations were summed for each testing day. As such, if rats displayed more ipsilateral, than contralateral, rotations the net sum rotations would be negative, and vice versa.

2.5.3 Forepaw adjusting steps—Using a procedure similar to that described previously [27], the number of adjusting steps taken by the forelimb in order to compensate for lateral movement were counted to determine the effects of lesion and drug treatment on motor performance. Rats were moved laterally across a table at a steady rate of 90 cm/10 s while being held in such a way that the rear torso, hindlimbs, and one forepaw were lifted off the table so as to bear weight on the other forepaw. Each test consisted of six trials for each forepaw, alternating between directions. Forehand steps were weight-bearing steps made towards the body and backhand steps were steps taken away from the body. Stepping data were expressed as both % intact forehand stepping (lesioned forehand steps/intact forehand steps) and % intact backhand stepping (lesioned backhand steps/intact backhand steps).

2.6 Immunohistochemistry

Rats were injected with a lethal dose of sodium pentobarbital and transcardially perfused with ice-cold PBS followed by PFA for immunohistological analysis of tyrosine hydroxylase (TH) expression in the SN and LC. After perfusion, brains were removed and immersed in PFA for 48 h, then 30% sucrose for at least 1 week, and subsequently cut with a freezing, sliding microtome into 40 μm coronal sections. Sections were stored at −20° C in cryoprotectant until staining. A "free-floating" immunohistochemistry method was used by employing standard avidin-biotin-peroxidase complex (ABC) detection methods in sections stained for TH. SN and LC sections were incubated in 0.3% H_2O_2 in 0.1M PBS for 30 min to remove endogenous peroxidases and were subsequently exposed to blocking buffer containing 0.3% Triton X-100, 1% normal goat serum, 1% bovine serum albumin, and

0.05% sodium azide in 0.01 M PBS, pH 7.4 to reduce nonspecific antibody binding (60 min for SN sections; overnight for LC sections). SN and LC sections were next incubated in blocking buffer containing primary antibody (mouse anti-TH, SN- 1:500, overnight; LC-1:1000, 4 h; Millipore) at 4° C. Sections were successively bathed in a polyclonal horse anti-mouse biotinylated secondary antibody at 1:500 (1 h) and then ABC (Elite Vectastain Kit; Vector Laboratories, Burlingame, CA, USA) diluted 1:500 in 0.01 M PBS (30 min). Chromagen was visualized with 0.005% 3,3′-diaminobenzidine (Sigma), 0.6% nickel ammonium sulfate, and 0.005% H_2O_2 in PBS.

2.7 TH positive cell counting

2.7.1 SN—Unbiased stereology was performed on every third nigral section (5 sections/animal, 120 μm apart; From Bregma: AP −5.80 through −5.20) to estimate total number of TH positive cell bodies. The SN image was captured at 5X magnification using a digital camera (Zeiss Plan-NEOFLUAR) attached to a Zeiss microscope (Axioscop 2-Plus). The region of interest was traced onto the 5X image using StereoInvestigator 8.0 software (MicroBrightField, Williston, VT, USA) based on neuroanatomical markers obtained from a rat atlas [28]. TH immunopositive cells were counted at 40X magnification (Zeiss Plan-NEOFLUAR) using a counting frame (125 μm × 125 μm) within a sampling grid (170 μm × 170 μm) by an investigator blinded to lesion status. Estimated total cells were automatically calculated according to the following equation by StereoInvestigator 8.0 software: N=ΣQ-(1/ssf)(1/asf)(1/hsf), where N is the total cell estimate, Q is the cells counted by hand in a particular animal, ssf is the sampling fraction, asf is the area sampling fraction, and hsf is a ratio between the thickness of the tissue and the height of the counting frame.

2.7.2 LC—A modified cell counting procedure was used to quantify TH positive cells in the LC. Three sections separated by approximately 120 μm were counted from each animal to evaluate the number of TH positive cells within the LC (From bregma: AP −10.04 through −9.68). Briefly, the region of interest was traced onto a 5X image as described above based on neuroanatomical markers. TH immunopositive cells were again counted at 40X magnification; however, the counting frame and sampling grid were the same size (100 μm × 100 μm) in order to sample the entire LC. Thus, LC data is expressed as the total number of cells counted throughout the three sections on the left and right hemispheres, as well as total cells counted (left and right LC hemispheres combined).

2.8 Statistics

Statistical analyses were performed using Statistica software version 7 (Statsoft Inc., Tulsa OK). Alpha was set to 0.05. Nonparametric ALO AIMs data (expressed as median ± median absolute deviation; MAD) were analyzed using nonparametric Mann-Whitney U tests for comparisons between lesion groups and Friedman tests for within-subjects comparisons. When appropriate, Wilcoxon signed rank post hoc tests were used for further evaluation of significant within-subjects effects. All parametric data were expressed as means ± standard error of the mean (SEM). Two-factor ANOVAs were used to evaluate L-DOPA- induced rotations, FAS, and immunohistochemical data. When appropriate, significant differences were determined by Fisher's LSD post-hocs. T-tests were used to compare amphetamine-induced rotations as well as some of the immunohistochemical data.

3. Results

3.1 Validation of SN and LC lesions

3.1.1 SN—Lesion induced changes in TH immunostaining within the SN were examined using a 2(DA-lesioned hemisphere) x 2(NE lesion) mixed factor ANOVA revealing a main effect of DA-lesioned hemisphere ($F_{1,28} = 400.89$, $p < 0.01$), but no effect of NE lesion or DA-lesioned hemisphere x NE lesion interaction. Unilateral infusion of 6-OHDA into the left MFB drastically reduced TH-positive cell estimates in the SN ipsilateral to 6-OHDA DA lesion (left side) in both DA- and DANE-lesioned animals (fig 2B, D, F) compared to the contralateral hemisphere (right side; fig 2C, E, F). In addition, an independent samples t-test determined there was no difference in the percentage of intact nigral TH positive cells (calculated by dividing the estimated number of TH positive cells counted in the lesioned SN by the estimated number of TH-positive cells counted in the intact SN) between DA- (M =8.15%; SD = 0.65%) and DANE- (M=7.38%; SEM = 0.60%) lesioned rats.

3.1.2 LC—The effects of αDBH on TH-positive cell counts within the LC were evaluated using a 2(DA-lesioned hemisphere) x 2(NE lesion) mixed factor ANOVA which showed a significant main effect of NE lesion ($F_{1, 28} = 186.01$, $p < 0.01$) but no effect of DA-lesioned hemisphere or DA-lesioned hemisphere x NE lesion interaction. As shown in figure 3D, intraventricular (ICV) infusion of αDBH bilaterally reduced TH immunostaining in the LC of DANE-lesioned animals by 90% compared to DA-lesioned animals alone. Since there was no effect of DA-lesioned hemisphere, the left and right LC cell counts were combined to make the "total LC cell count" (fig 3D, insert).

3.2 Amphetamine-induced rotations

As shown in figure 4, additional NE lesions did not alter rotational activity on the amphetamine induced rotations test compared to rats with just DA lesions.

3.3 NE loss does not alter the development of LID or rotations

L-DOPA 4 mg/kg: To determine whether NE loss altered the trajectory of LID development, rats were primed for 8 days with the low dose of L-DOPA (4 mg/kg) and rated for ALO AIMs and rotations on days 1, 5, and 8. Evaluation of ALO AIMs time-courses on individual treatment days revealed no lesion-induced differences in the onset or offset of LID (data not shown); therefore, ALO AIMs scores and total contralateral rotations were summed across the 3 h rating period each day. Friedman tests conducted separately on DA- ($\chi^2_{(N=15, df=2)} = 19.60$, $p < 0.01$) and DANE- ($\chi^2_{(N=25, df=2)} = 21.73$, $p < 0.01$) lesioned groups found significant differences in the progression of AIMs over time. Wilcoxon sign-rank post hoc tests revealed a dose-dependent increase in ALO AIMs expression across time for both DA- and DANE-lesioned animals ($p < 0.05$) (fig 5A). Non-parametric Mann-Whitney U tests on each day of priming demonstrate no differences in ALO AIMs expression between DA and DANE lesioned rats at any day. L-DOPA (4 mg/kg)-induced contralateral rotations were analyzed using a 2(lesion) x 3(treatment day) mixed factor ANOVA and revealed a significant main effect of treatment day ($F_{2, 56} = 17.86$, $p < 0.01$), but no effect of NE lesion or interaction. Post hoc analyses revealed that L-DOPA-induced

contralateral rotations were greater on the 5th and 8th day than the 1st day of L-DOPA treatment, where both DA- and DANE-lesioned animals displayed more ipsilateral than contralateral rotations regardless of lesion status (Fig 5B).

L-DOPA 12 mg/kg: Twenty-four hours after the final day of treatment with the low dose of L-DOPA, DA- and DANE-lesioned rats received eight consecutive days of a high dose of L-DOPA (12 mg/kg) and were rated for the expression of ALO AIMs and rotations on days 9, 13, and 16. Individual Friedman tests for the DA- and DANE-lesioned groups demonstrated that ALO AIMs expression changed following repeated L-DOPA administration in DA- ($\chi^2_{(N=15, df=2)} = 10.38$, $p < 0.01$), but not DANE-lesioned animals. Wilcoxon sign-rank post hoc tests revealed that the DA-lesioned animals displayed fewer ALO AIMs on the 16th compared to the 9th or 13th d of L-DOPA treatment ($p < 0.05$). Although these rats all displayed severe LID, results of Mann-Whitney U tests determined no difference in total ALO AIMs severity between DA- and DANE-lesioned rats on any day of treatment (fig 5C). Furthermore, analysis of individual ALO AIMs time-course days revealed no lesion-induced differences in LID onset or offset (data not shown). For L-DOPA (12 mg/kg)-induced rotations, a 3(treatment day) x 2(NE lesion) mixed factor ANOVA revealed a main effect of treatment day ($F_{2, 56} = 27.77$, $p < 0.01$), a non-significant trend for NE lesion ($F_{1, 28} = 3.47$, $p = 0.07$), and no interaction. Closer inspection revealed that both DA- and DANE-lesioned rats rotated less on the first day of L-DOPA 12 mg/kg treatment (day 9) than on the 5th or 8th day of L-DOPA (12 mg/kg) treatment (days 13 & 16) (fig 5D).

L-DOPA efficacy is diminished in hemiparkinsonian rats with NE loss: The FAS test was used to determine whether DA- and DANE-lesioned rats differed in sensitivity to L-DOPA's restorative motor benefits. Rats were observed for stepping performance at baseline (off treatment), and 60 min post L-DOPA (4 mg/kg, day 6; 12 mg/kg, day 14) treatment. Analysis of forehand stepping using a 2(NE lesion) x 3(treatment) mixed-factor ANOVA revealed significant main effects of NE lesion ($F_{1, 28} = 4.46$, $p < 0.05$) and treatment ($F_{2, 56} = 34.23$, $p < 0.01$), as well as a significant interaction ($F_{2, 56} = 6.43$, $p < 0.05$). Post hoc analyses revealed that, at baseline, NE loss in the DANE-lesioned animals did not alter forehand stepping compared to DA-lesioned rats (Fig 6A). DA- and DANE-lesioned rats did not differ in the actual number of steps taken by the left or right paws at baseline (data not shown). Forehand stepping deficits were reversed by treatment with the high dose of L-DOPA (12 mg/kg) for both DA- and DANE-lesioned animals ($p < 0.05$). However, the magnitude of this effect differed between groups where DA-lesioned animals stepped more than DANE-lesioned animals at the high dose of L-DOPA (Fig 6A) ($p < 0.05$). When evaluating % intact backhand stepping, results of a 2(NE lesion) x 3(treatment) mixed-factor ANOVA revealed a significant main effect of treatment ($F_{2, 56} = 7.63$, $p < 0.01$) but no effect of NE lesion and no interaction (both $p > 0.05$). Post hoc analyses revealed that both doses of L-DOPA (4, 12 mg/kg) increased backhand stepping compared to baseline regardless of the rats' lesion status ($p < 0.05$; Fig 6B).

NE loss reduces sensitivity to L-DOPA's pro-dyskinetic and rotational effects: To establish whether additional NE lesions altered LID liability, rats received varying doses of L-DOPA (0, 2, 3, 4, 6, 12 mg/kg) and were rated for ALO AIMs and rotations. Friedman tests

conducted separately for DA and DANE lesioned animals revealed significant effects of dose for both DA- ($\chi^2_{(N=15, df=5)}$ = 59.93, p < 0.01) and DANE- ($\chi^2_{(N=15, df=5)}$ = 60.75, p < 0.01) lesioned animals. Wilcoxon sign-rank post hoc tests were used to compare ALO AIMs induced by each dose of L-DOPA to vehicle (0 mg/kg) for DA- and DANE- lesioned animals. All doses of L-DOPA (2, 3, 4, 6, 12 mg/kg) induced significant ALO AIMs in DA lesioned rats (p < 0.05); however, a rightward shift was seen in the dose response in DANE-lesioned animals where significant ALO AIMs were not observed at the 2 mg/kg dose of L-DOPA. Mann-Whitney U tests conducted at each L-DOPA dose were used to examine potential NE-lesion-induced differences in ALO AIMs expression. DA- and DANE-lesioned animals displayed equivalent total ALO AIMs scores in response to all doses of L-DOPA, with the only exception seen at the 2 mg/kg dose, where DA-lesioned rats displayed more total ALO AIMs than DANE-lesioned animals (Fig 7A). For rotations, a 2(lesion) x 6(L-DOPA dose) mixed factor ANOVA revealed a main effect of lesion ($F_{1, 28}$ = 5.41, p < 0.05), treatment ($F_{5, 140}$ = 38.43, p < 0.01), and a significant lesion x treatment interaction ($F_{5, 140}$ = 2.67, p < .05). Post hoc analyses revealed that L-DOPA dose dependently enhanced rotations in DA- and DANE-lesioned animals; however, DANE-lesioned rats showed a blunted rotational response to L-DOPA 6 mg/kg and 12 mg/kg compared to DA-lesioned rats (p < 0.05; Fig 7B).

NE loss does not alter DA agonist-induced dyskinesia: DA agonists have repeatedly been shown to induce dyskinetic behaviors [29–31]. In order to determine if NE loss alters responsiveness to DA agonists, DA- and DANE-lesioned animals were individually administered the selective DA D1R agonist SKF81297 and the DA D2R agonist quinpirole in a counterbalanced order and were rated for ALO AIMs and rotations. An Individual Friedman test revealed significant effects of SKF81297 dose on ALO AIMs expression in both DA- ($\chi^2_{(N=15, df=2)}$ = 30.00, p < 0.01) and DANE- ($\chi^2_{(N=15, df=2)}$ = 30.00, p < 0.01) lesioned animals. Wilcoxon sign-rank post hoc analyses revealed that SKF81297 dose-dependently enhanced ALO AIMs expression in both DA- and DANE-lesioned animals (p < 0.05) (Fig 8A). An additional Friedman test also revealed significant effects of quinpirole dose on ALO AIMs expression in DA- ($\chi^2_{(N=15, df=2)}$ = 29.10, p < 0.01) and DANE- ($\chi^2_{(N=15, df=2)}$ = 27.00, p < 0.01) lesioned animals. Similar to SKF81297, post hoc analyses revealed that quinpirole dose dependently augmented ALO AIMs expression in both DA- and DANE-lesioned animals (p < 0.05). Mann-Whitney U tests at each dose revealed that additional lesion status did not alter ALO AIMs induced by either SKF81297 or quinpirole. Total sum rotational behavior was analyzed using 2(NE lesion) x 3(treatment dose) mixed factor ANOVAs for each DA agonist and revealed main effects of dose for both SKF81297 and quinpirole ($F_{2, 56}$ = 80.998, p < 0.01; $F_{2, 56}$ = 24.56, p < 0.01; respectively), but no significant effects of lesion and no interactions. As shown in figures 8B, the high dose of SKF81297 (0.8 mg/kg) induced significant contralateral rotations in both DA- and DANE-lesioned animals (p < 0.05). Quinpirole dose-dependently augmented contralateral rotations in both DA- and DANE-lesioned animals (Fig 8D; p < 0.05).

4. Discussion

The present study utilized the NE specific neurotoxin αDBH in order to clarify the role of the noradrenergic system in L-DOPA mediated behaviors in a rodent model of PD. αDBH

has previously been shown to potently and selectively destroy NE neurons by selectively inactivating ribosomal function in NE neurons [19]. In the current investigation, ICV administration of αDBH reduced LC TH immunostaining bilaterally by 90% compared to vehicle-treated animals, similar to levels often reported in PD patients [1]. Intra-MFB 6-OHDA infusions resulted in severe (>90%) unilateral loss of nigral TH-positive cells. Importantly, nigral DA loss did not differ between rats with or without this additional NE loss. This was further confirmed by showing no behavioral difference between DA- and DANE-lesioned animals following the d-amphetamine challenge. Thus, pairing αDBH lesions for NE with 6-OHDA lesions for DA allowed us to produce a late-stage model that better mimics PD neuropathology where both DA and NE cell degeneration is severe.

Using this model, our first goal was to determine whether severe NE loss altered the development and/or expression of LID. In the current investigation, the trajectory of ALO AIMs development was similar between DA- and DANE-lesioned animals. Indeed, all rats displayed stable dyskinesia following 16 consecutive days of L-DOPA treatment. In addition, we extended previous work by showing for the first time that dyskinesia induced by the DA D1R agonist SKF81297 or the DA D2R agonist quinpirole was not altered by severe NE loss.

We also assessed discrete lesion-induced differences in established LID using a wide range of L-DOPA doses (0–12 mg/kg) in L-DOPA primed rats. Using this paradigm, we demonstrated that the 2 mg/kg dose of L-DOPA was sufficient for producing significant ALO AIMs expression in DA-lesioned rats, but not in those with additional NE loss, suggesting that the integrity of the NE system influences the threshold of L-DOPA sensitivity. DA- and DANE-lesioned rats displayed equivalent levels of dyskinesia to all moderate and high doses of L-DOPA (3–12 mg/kg). This supports the previous work from our lab where hemiparkinsonian rats with mild to moderate bilateral NE loss were less dyskinetic following low to moderate doses of L-DOPA (2–6 mg/kg) than desipramine-treated animals without NE loss [15]. Along with studies reporting that bilateral NE degeneration with the neurotoxin DSP-4 did not alter the development or expression of LID [11, 18], our study provides further support for the idea that the effects of bilateral NE loss on LID may be limited.

In contrast, unilateral NE loss in rats appears to exacerbate dyskinesia symptoms [11, 17]. For example, in 6-OHDA DA-lesioned rats previously rendered dyskinetic, ibotenic acid infusion into the LC resulted in moderate (~40%) unilateral neuronal loss in the LC and prolonged LID compared to animals with DA lesions alone [11]. This lesion paradigm differs from what is observed in clinical PD where NE loss precedes DA loss and therefore precedes L-DOPA treatment. However, Fulceri et al., [17] produced simultaneous unilateral DA and NE lesions in rats prior to L-DOPA treatment, (~80% striatal NE loss) and reported an exacerbation of LID symptoms in dual lesioned rats compared to just DA-lesioned rats [17]. Remaining striatal dopamine content in the lesioned hemisphere was slightly higher in rats with both DA and NE loss compared to those with just DA lesions in the aforementioned study. This is surprising because it is generally believed that LID severity is proportional to DA loss, which would predict worse LID in the rats with more DA loss.

It is not readily clear why unilateral NE loss might enhance LID while bilateral NE loss doesn't change or may even reduce LID severity. It is well known that the CNS undergoes a number of plastic changes as a result of neurodegeneration and/or neurotoxic injury. There is precedent in the field suggesting that intrahemispheric compensation occurs following DA lesion and that the integrity of these intrahemispheric connections may influence LID susceptibility [32]. It is conceivable that the aberrant neuroplasticity associated with dyskinesia may be differentially affected by unilateral versus bilateral noradrenergic destruction. Regardless, lesion laterality seems to be an important consideration when evaluating discrepancies in the effect of NE loss in LID between models.

There is much debate surrounding the interpretation of drug-induced circling behavior (i.e. rotations) in the 6-OHDA rodent model of PD. Classically, drug-induced contralateral rotations were thought to be more indicative of antiparkinsonian efficacy than dyskinesia [33]. In support, compounds that reversed parkinsonian motor deficits frequently induced contralateral rotations [34, 35]. Additionally, compounds with low dyskinesia liability, such as bromocriptine, induced strong contralateral circling behavior [36, 37] and many compounds that effectively reduce dyskinesia often do not alter L-DOPA- or DA agonist-induced rotations [38, 39]. In fact, some antidyskinetic compounds actually enhance or extend rotational behaviors in the 6-OHDA lesioned rat model of PD when given in conjunction with L-DOPA or DA agonists [40–42]. In the current investigation, L-DOPA-induced contralateral rotations were reduced in response to the higher doses of L-DOPA (6, 12 mg/kg) with a non-significant trend towards reduced rotations across priming days for the 12 mg/kg dose of L-DOPA. This effect, supported by Barnum et al., [15], may indicate that NE loss could interfere with L-DOPA's antiparkinsonian efficacy.

Therefore, in order to discover whether NE lesions altered the effectiveness of L-DOPA's therapeutic ability, DA- and DANE-lesioned rats underwent FAS testing off treatment and during L-DOPA priming (4 mg/kg, 12 mg/kg). The FAS test is used to detect lesion-induced deficits in stepping and treatment-induced reversal of those deficits, where forehand stepping is thought to be the most sensitive measure [27, 43]. Previous research suggests that NE loss induced by DSP-4 or local 6-OHDA infusions exacerbates parkinsonian motor symptoms [5, 6, 44]. In the present study, both DA- and DANE-lesioned animals showed profound DA-lesion induced stepping deficits at baseline. Additional NE loss did not worsen stepping deficits at baseline compared to rats with just DA lesions in the current model, possibly reflecting a floor effect. L-DOPA treatment dose-dependently improved total and forehand stepping. Importantly, at the high dose of L-DOPA, rats with NE lesions showed less improvement in forehand stepping than rats with purely DA lesions. These findings complement the rotational data and suggest that severe NE loss may reduce L-DOPA's antiparkinsonian properties. This is also supported in MPTP-treated mice, whereby L-DOPA's motor stimulating effects are dramatically reduced in mice given additional NE lesions compared to those with just DA loss [44, 45].

It is not yet known whether the NE lesion-induced reduction in L-DOPA responsiveness is due to the loss of the transmitter itself or noradrenergic machinery like NET or α2 NE receptors (α2R). NE has been shown bind to DA receptors within the basal ganglia [46] and the α2R class has been implicated in DA synthesis and turnover [47, 48]. It is possible that

lesion-induced reductions in NE concentrations and NE binding at DA receptors may manifest as a dampened behavioral response to L-DOPA. However, this mechanism remains to be directly tested.

Despite the current results suggesting that NE innervation may play a more primary role in L-DOPA's antiparkinsonian effects, the NE system still remains an important mediator in the development and progression of dyskinetic behavior. Previous research has demonstrated that direct striatal infusion of exogenous NE alone can elicit dyskinesia in hemiparkinsonian rats [12] and that many noradrenergic compounds effectively reduce dyskinesia when used in concert with L-DOPA. Indeed, α2- and β-noradrenergic receptor antagonists reduce dyskinesia in experimental and clinical populations [14, 16, 38, 49–51]. Recent experimental evidence also indicates that these compounds reduce LID even when noradrenergic loss is present [15]. While presynaptic NE loss may not directly contribute to LID genesis, clearly a number of NE targets influence the neuro-circuitry involved in LID suggesting that the NE system plays a complex, and underexplored role in dyskinesia.

Despite these findings, there are a few caveats that should be considered with the current model. First, ICV αDBH administration produced bilateral loss of TH immunostaining in the LC. As mentioned above, it is unclear whether bilateral, compared to unilateral, NE loss differentially affects behavior when paired with a unilateral DA lesion. Furthermore, NE loss in clinical PD is localized to the LC and progresses over time [2, 52]. Others have demonstrated that acute ICV αDBH administration destroys NE neurons in all NE nuclei, not just within the LC [19]. While these other nuclei have not been implicated in motor performance, NE loss in these areas could influence non-motor behaviors not examined in the current investigation. Finally, the impact of αDBH-induced NE loss on L-DOPA-mediated motor behaviors was not investigated in animals with an intact DA system. Previous research has shown that genetic knock-out- or DSP-4-induced NE loss alone impairs motor performance in mice and these motor deficits are only partially restored by DA-agonist treatment [53]. Therefore, future tests with αDBH-induced NE lesions alone may further reveal a unique contribution of the NE system in L-DOPA's effects.

In closing, DA replacement therapy with L-DOPA will likely remain the mainstay treatment for PD for the foreseeable future. Noradrenergic loss is a key pathological feature of PD, however many questions remain regarding its impact on L-DOPA treatment efficacy, side effects, and function within the CNS. The lack of consistency between NE lesion models has delayed progress in understanding these issues. Clearly, a concerted effort towards systematic investigation of NE loss in PD and LID is warranted. Here we characterized a late stage model of PD that demonstrates profound DA and NE loss. Future use with this model may help unravel the mechanism(s) by which the noradrenergic system influences L-DOPA treatment in PD.

Acknowledgments

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Highlights

- αDBH treatment produced severe loss of noradrenergic neurons in the locus coeruleus

- Norepinephrine loss did not significantly influence dyskinesia in parkinsonian rats

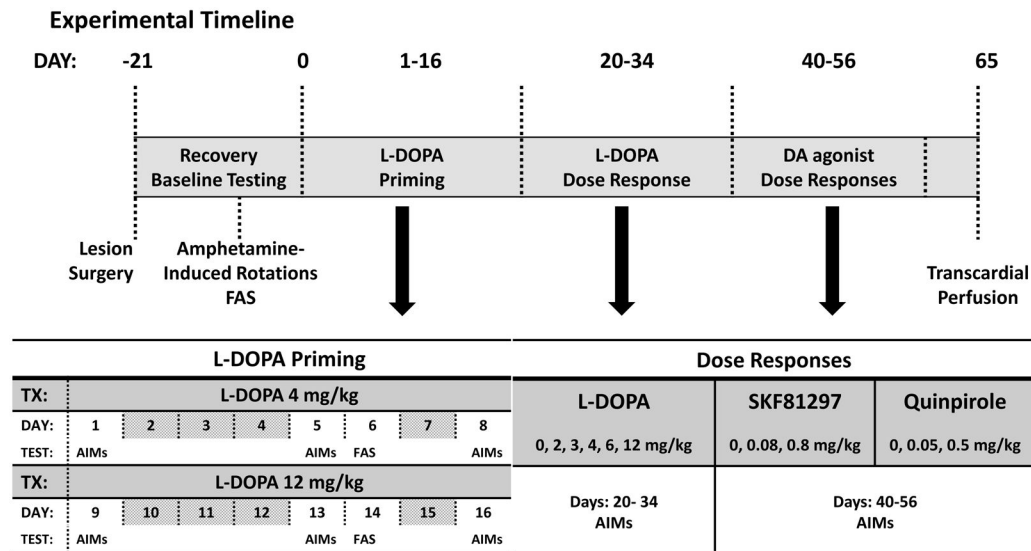- Norepinephrine lesions reduced therapeutic effects of L-DOPA in parkinsonian rats

Experimental Timeline

DAY:	-21	0	1-16	20-34	40-56	65
	Recovery Baseline Testing		L-DOPA Priming	L-DOPA Dose Response	DA agonist Dose Responses	
	Lesion Surgery	Amphetamine-Induced Rotations FAS				Transcardial Perfusion

	L-DOPA Priming					Dose Responses		
TX:	L-DOPA 4 mg/kg					**L-DOPA**	**SKF81297**	**Quinpirole**
DAY:	1	2	3	4	5	0, 2, 3, 4, 6, 12 mg/kg	0, 0.08, 0.8 mg/kg	0, 0.05, 0.5 mg/kg
					6 7 8			
TEST:	AIMs				AIMs FAS AIMs			
TX:	L-DOPA 12 mg/kg					Days: 20- 34 AIMs	Days: 40-56 AIMs	
DAY:	9	10	11	12	13 14 15 16			
TEST:	AIMs				AIMs FAS AIMs			

Figure 1. Experimental Timeline and Design

The effect of NE lesions on parkinsonian motor behaviors was assessed in DA (n = 15) and DANE (n = 15) lesioned animals. Following lesion induction surgery, DA lesion severity was evaluated in all rats using the amphetamine induced rotations test. Rats then received 16 d of L-DOPA treatment (L-DOPA priming) to evaluate whether NE loss altered the progression or development of LID. During this time, rats underwent AIMs testing for dyskinesia and FAS testing to assess L-DOPA's antiparkinsonian effects. L-DOPA primed rats underwent further AIMs testing to assess whether NE loss altered sensitivity to L-DOPA- or DA agonist-induced AIMs and contralateral rotations. Rats were killed off treatment and brains were collected for immunohistogical verification of SN DA and LC NE lesions.
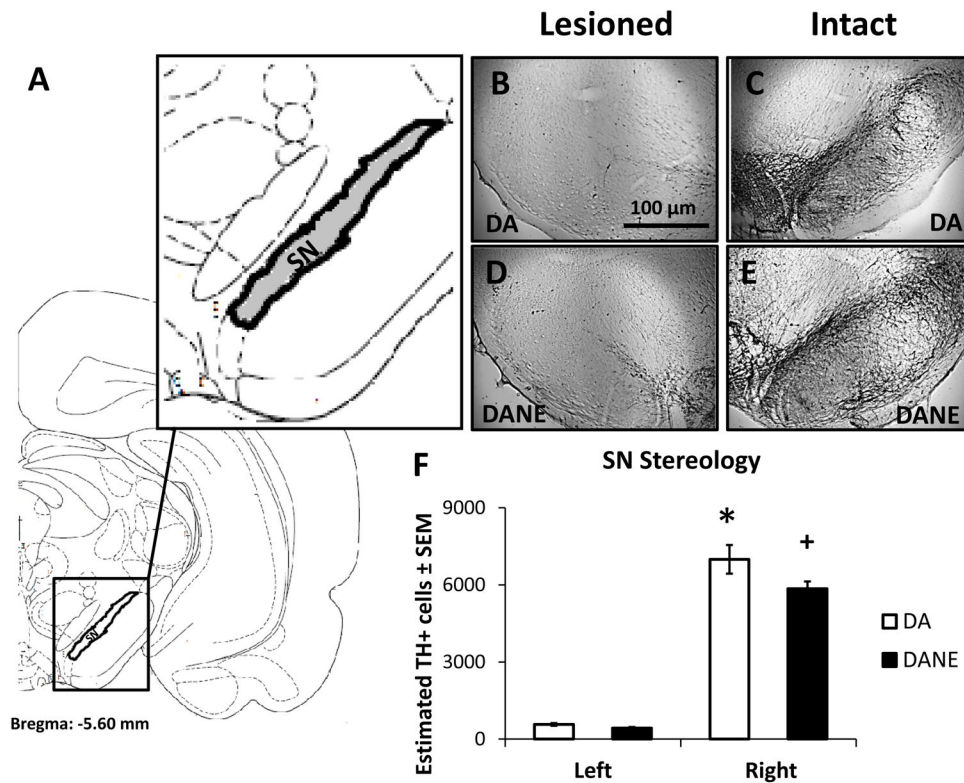
Figure 2. SN DA-lesion verification

All rats received unilateral infusion of 6-OHDA into the left MFB to produce unilateral SN DA neuronal loss and either vehicle (DA-lesioned) or αDBH (DANE-lesioned) into the left lateral ventricle. Rats were killed off treatment following the cessation of behavioral testing. (A) Schematic representation of coronal rat brain section adapted from Paxinos and Watson [26] (Bregma: −5.30mm), where the general areas of the 5 SN regions are shaded and labeled. Immunohistology and unbiased stereology were used to estimate the number of TH positive cells in the lesioned (left; B, D) and intact (right; C, E) SN of DA- (B, C) and DANE- (D, E) lesioned animals. Representative SN sections (B–E) are displayed at 5x magnification. Values, expressed as mean ± SEM, are the estimated number of cells counted across five sections per animal (F). *p < 0.05 vs. DA-lesioned- left hemisphere; +p < 0.05 vs. DANE-lesioned- left hemisphere.
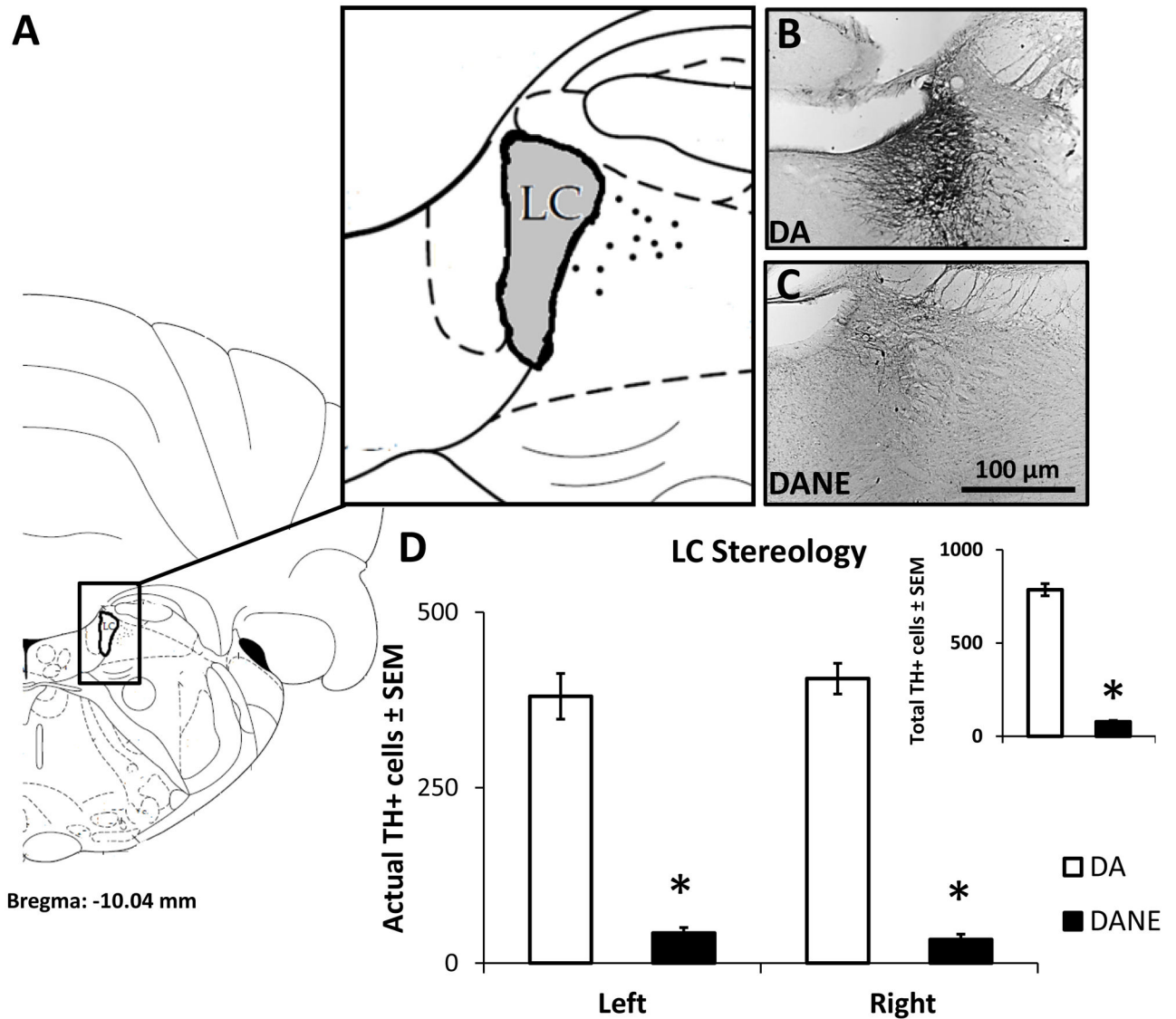
Figure 3. LC NE-lesion verification

All rats received unilateral infusion of 6-OHDA into the left MFB and either vehicle (DA-lesioned) or αDBH (DANE-lesioned) into the left lateral ventricle. Rats were killed off-treatment and three coronal LC sections from each rat were stained for lesion verification. (A) Schematic representation of coronal rat brain section containing the LC adapted from Paxinos and Watson [26] (Bregma: −10.04 mm). Representative TH-immunostained LC images from DA- (B) and DANE- (C) lesioned animals are displayed at 10x magnification. Values, expressed as mean ± SEM, are the actual number of cells counted in the left, right, and total (combined left and right) LC (D). *p < 0.05 vs. DA-lesioned.
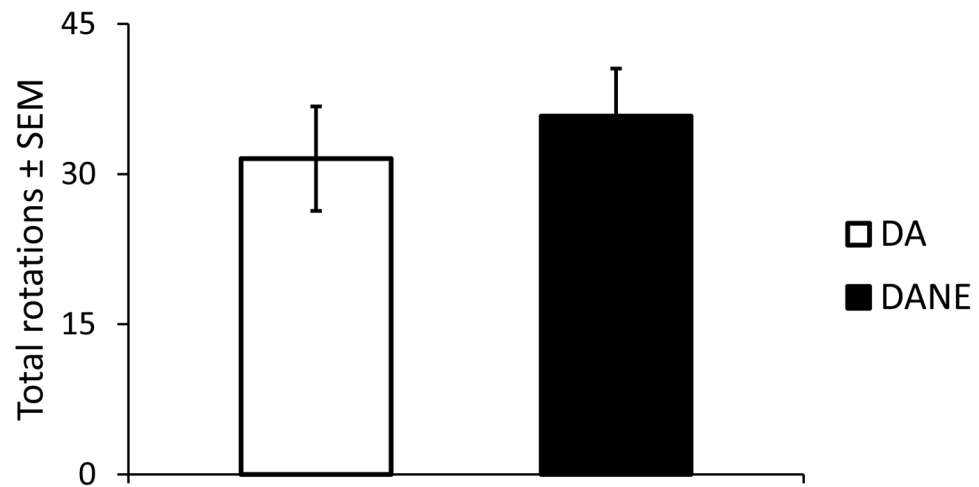
Figure 4. Effect of DA- and DANE-lesions on amphetamine responsiveness

DA- and DANE-lesioned rats were injected with d-amphetamine (2.5 mg/kg, ip) and were observed every 5 min for 1 h for rotational behavior towards the DA-lesioned hemisphere. Bars depict mean total rotations ± SEM.
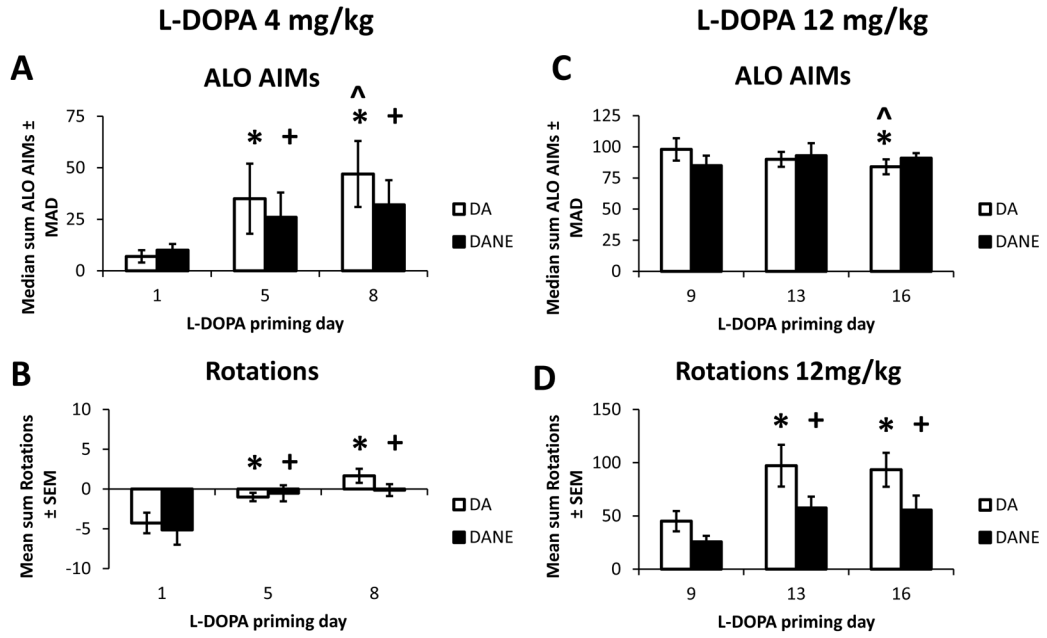
L-DOPA 4 mg/kg

A ALO AIMs

B Rotations

L-DOPA 12 mg/kg

C ALO AIMs

D Rotations 12mg/kg

Figure 5. Effect of NE loss on LID development during L-DOPA priming

DA- and DANE- lesioned rats were treated once daily with L-DOPA (4 mg/kg) for 8 d, followed by 8 d of once daily L-DOPA (12 mg/kg). During this time rats were rated for ALO AIMs and rotations on days 1, 5, 8 (L-DOPA 4 mg/kg, sc; A, B), 9, 13, and 16 (L-DOPA 12 mg/kg; C, D) of L-DOPA treatment. Bars represent total ALO AIMs (expressed as medians ± MAD) and rotations (expressed as means ± SEM) summed over the 3 h rating period each day. *p < 0.05 vs. DA-lesioned- 1st day (day 1 or 9); ^p < 0.05 vs. DA-lesioned-middle day (day 5 or 13); +p < 0.05 vs. DANE-lesioned- 1st day (day 1 or 9)
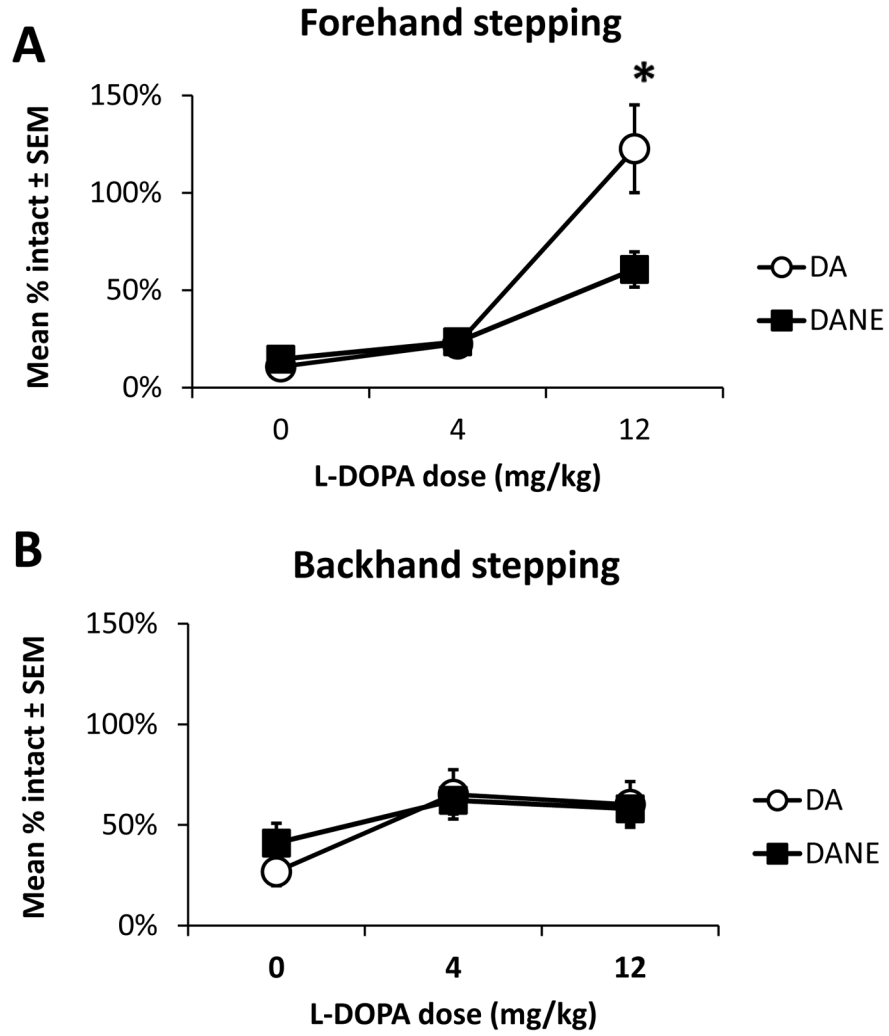
Figure 6. FAS performance in DA- and DANE-lesioned animals
DA- and DANE-lesioned rats were evaluated for lesion-induced stepping deficits at baseline (off treatment) and reversal of these deficits 60 min after L-DOPA treatment (4, 12 mg/kg) during the L-DOPA-priming period. Bars depict the effects of treatments on FAS performance expressed as mean percent ($\pm$ SEM) of intact forehand (A) and backhand (B) stepping. *$p < 0.05$ DA vs. DANE.
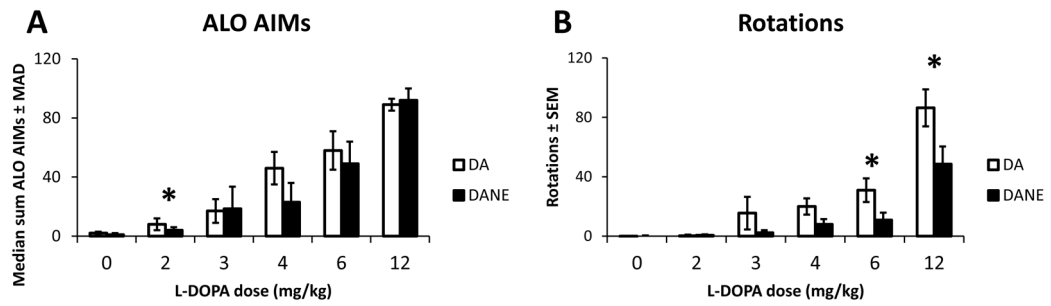
Figure 7. Effect of escalating L-DOPA doses on LID in L-DOPA-primed DA- and DANE-lesioned rats

DA- and DANE-lesioned rats received various doses of L-DOPA (0, 2, 3, 4, 6, 12 mg/kg) and were observed for ALO AIMs (A) and rotations (B) for 1 min every 10 min for 3 h. Data are presented as median sum ALO AIMs (± MAD) and mean sum rotations (± SEM). *p < 0.05 DA vs. DANE.
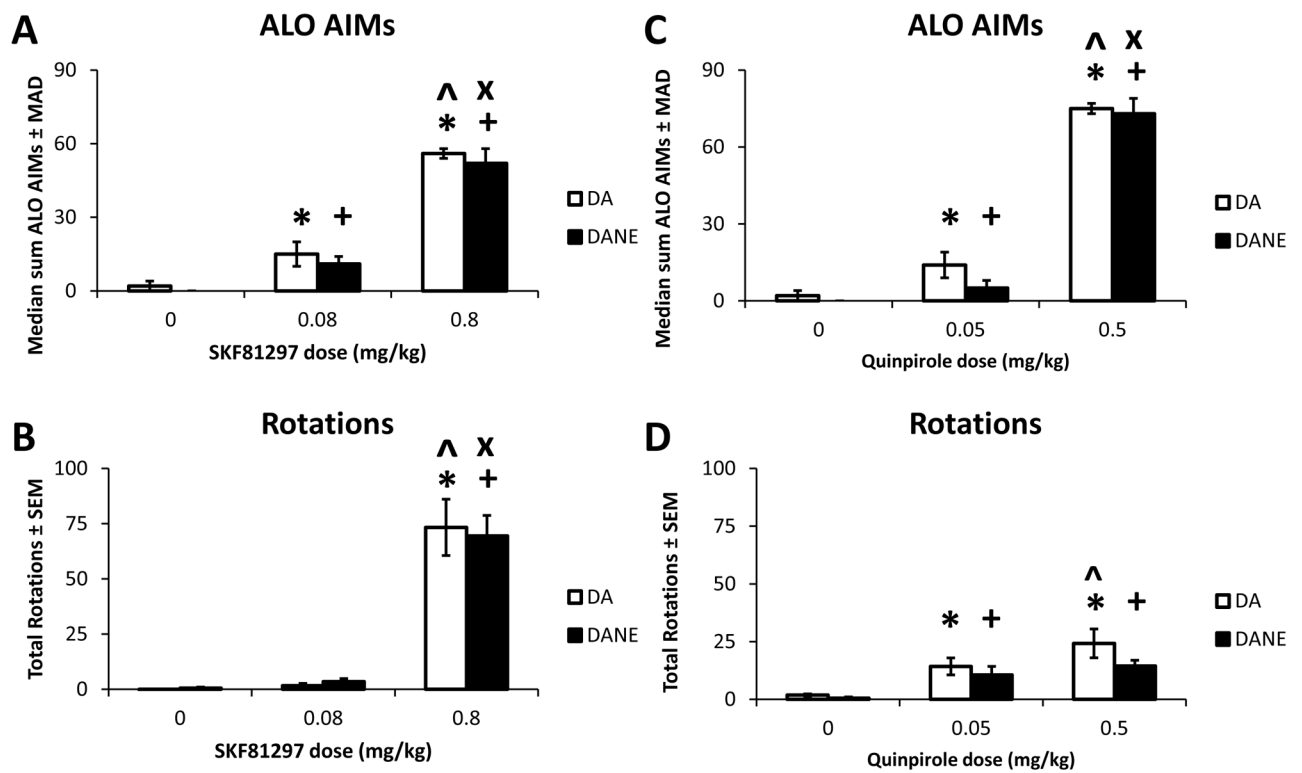
Figure 8. Expression of ALO AIMs and rotations following DA agonist treatment in L-DOPA primed DA- and DANE-lesioned rats

DA- and DANE-lesioned animals were treated with the DA D1R agonist SKF81297 or the DA D2R agonist quinpirole and were rated for ALO AIMs and rotations for 1 min every 10 min for 2 h (SKF81297) or 3 h (quinpirole), respectively. Bars represent median sum ALO AIMs (± MAD) and mean sum rotations (± SEM). *p < 0.05 vs. DA-lesioned 0 mg/kg; ^p < 0.05 vs. DA-lesioned low dose (SKF81297: 0.08 mg/kg; quinpirole: 0.05 mg/kg); +p < 0.05 vs. DANE-lesioned 0 mg/kg; xp < 0.05 vs. DANE-lesioned low dose.