JPET Fast Forward. Published on March 14, 2011 as DOI: 10.1124/jpet.111.179416

Behavioral and Cellular Modulation of L-DOPA-Induced Dyskinesia by β-Adrenoceptor Blockade in the 6-OHDA-Lesioned Rat[¶]

David Lindenbach, Corinne Y. Ostock, Karen L. Eskow Jaunarajs, Kristin B. Dupre, Christopher J. Barnum, Nirmal Bhide, Christopher Bishop

Behavioral Neuroscience Program, Department of Psychology, Binghamton University, Binghamton, NY, USA

JPET Fast Forward. Published on March 14, 2011 as DOI: 10.1124/jpet.111.179416
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β-Adrenoceptor Blockade in L-DOPA-Induced Dyskinesia

Corresponding Author:
Christopher Bishop
Behavioral Neuroscience Program
Department of Psychology
Binghamton University
PO Box 6000
Binghamton, NY 13902-6000
Telephone: (607) 777-3410
Fax: (607) 777-4890
Email: cbishop@binghamton.edu

29 Text Pages
0 Tables
6 Figures
41 References
Abstract: 238 words
Introduction: 445 words
Methods: 2643 words
Results: 991 words
Discussion: 1501 words

Abbreviations: 6-hydroxydopamine (6-OHDA); Abnormal Involuntary Movements (AIMs); Axial Limb and Orolingual (ALO); β-adrenergic receptor (βAR); Dopamine (DA); Forepaw Adusting Steps (FAS); Glyceraldehyde 3-phosphate dehydrogenase (GAPDH); High Performance Liquid Chromatography with Electrochemical Detection (HPLC-ED); L-3,4-dihydroxyphenylalanine (L-DOPA); L-DOPA-Induced Dyskinesia (LID); Medial Forebrain Bundle (MFB); Norepinephrine (NE); Parkinson's disease (PD); Preprodynorphin (PPD); Preproenkephalin (PPE); Preprotachykinin (PPT); Serotonin (5-HT)

Recommended Section: Behavioral Pharmacology

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Abstract

Chronic dopamine (DA) replacement therapy in Parkinson's disease (PD) leads to deleterious motor sequelae known as L-3,4,-dihydroxyphenylalanine (L-DOPA)-induced dysinesias (LID). No known therapeutic can eliminate LID, but preliminary evidence suggests that dl-1-Isopropylamino-3-(1-naphthyloxy)-2-propanol ((±)propranolol), a non-selective β-adrenergic receptor (βAR) antagonist, may reduce LID. The present study used the rat unilateral 6-hydroxydopamine (6-OHDA) model of PD to characterize and localize the efficacy of (±)propranolol as an adjunct to therapy with L-DOPA. We first determined if (±)propranolol was capable of reducing the development and expression of LID without impairing motor performance ON and OFF L-DOPA. Coincident to this investigation, we used RT-PCR techniques to analyze the effects of chronic (±)propranolol on markers of striatal activity known to be involved in LID. In order to determine whether (±)propranolol reduces LID through βAR blockade, we subsequently examined each enantiomer separately since only the (-) enantiomer has significant βAR affinity. Next, we investigated the effects of a localized striatal βAR blockade on LID by cannulating the region and microinfusing (±)propranolol prior to systemic L-DOPA injections. Results showed that a dose range of (±)propranolol reduced LID without deleteriously affecting motor activity. Pharmacologically, only (-)propranolol had anti-LID properties indicating βAR-specific effects. Aberrant striatal signaling associated with LID was normalized with (±)propranolol co-treatment and intrastriatal (±)propranolol was acutely able to reduce LID. This research confirms previous work suggesting that (±)propranolol reduces LID through βAR antagonism, and presents novel evidence indicating a potential striatal locus of pharmacological action.

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Introduction

For the last half century, the DA precursor L-DOPA has been the treatment of choice for PD even though its chronic use induces side effects that can be as debilitating as PD itself (Jankovic, 2008). These side effects, typified by LID are commonly slow to develop, occurring in less than 10% of patients during the first year of L-DOPA therapy, but in as many as 90% of patients after 9 years of use (Ahlskog and Muenter, 2001).

The primary mechanism(s) underlying LID are thought to include extraphysiological DA release and aberrant receptor signaling in the striatum, which dysregulates subsequent striatal output (Cenci, 2007; Winkler et al., 2002). Following DA depletion, serotonin (5-HT) neurons synthesize DA from exogenous L-DOPA and release it in a pulsatile manner, which is a putative source of LID (Carta et al., 2007). However, recent evidence demonstrates that noradrenergic (NE) terminals also synthesize and release L-DOPA-derived DA (Arai et al., 2008). Thus, in recent years, there has been increased interest in the use of adrenergic compounds to prevent or reduce LID (Brotchie, 2005; Colosimo and Craus, 2003). While most research has focused on α-adrenergic compounds (Buck et al., 2010; Rommelfanger and Weinshenker, 2007; Savola et al., 2003), the striatum contains a high density of βARs (Rainbow et al., 1984), which are preserved in PD patients (Waeber et al., 1991). These receptors represent a unique therapeutic target for LID since βAR blockade prevents drug-induced facilitation of DA release in intact and DA-depleted animals, which may blunt downstream signaling abnormalities associated with LID (Goshima et al., 1991; Reisine et al., 1982). In line with these findings (±)propranolol, a non-selective-βAR antagonist, reduces the expression of LID in humans (Carpentier et al., 1996), and in animal models of LID

(Buck and Ferger, 2010; Dekundy et al., 2007; Gomez-Mancilla and Bedard, 1993). However, several key pragmatic and mechanistic questions remain regarding the anti-dyskinetic efficacy of βAR blockade.

Therefore, the following studies were undertaken to determine the cellular and behavioral effects of βAR blockade in L-DOPA-treated hemiparkinsonian rats. The first experiment examined the effects of (±)propranolol on the development of abnormal involuntary movements (AIMs) and on the expression of genes routinely used as markers of pathway-specific striatal DA output: preproenkephalin (PPE), preprodynorphin (PPD) and preprotachykinin (PPT) (Cenci et al., 1998; Gerfen et al., 1990). The second experiment determined whether anti-dyskinetic doses of (±)propranolol modify L-DOPA efficacy or basal motor activity. By employing active and inactive enantiomers, the third experiment examined (±)propranolol's βAR specific effects. Lastly, to determine if the striatum is a pharmacological site of action, striatal microinfusions of (±)propranolol were performed. Results suggest that (±)propranolol dose-dependently suppresses AIMs through a normalization of signaling in the dorsal striatum.

Methods

Animals

These studies used male Sprague-Dawley rats (N=98; Taconic Farms, Hudson, NY, USA) which were 8 weeks old and 225-250g upon arrival. Rats were kept in plastic cages (45cm long, 23cm wide, 22cm high) and given free access to water and food (Rodent Diet 5001; Lab Diet, Brentwood, MO, USA). The colony room was maintained at 22-23°C on a 12h light/dark cycle with lights on at 0700. The guidelines of the

Institutional Animal Care and Use Committee of Binghamton University and the "Guide for the Care and Use of Laboratory Animals" were maintained throughout the study (Institute of Laboratory Animal Resources, National Academic Press 1996; NIH publication number 85-23, revised 1996).

Drugs

Systemic drug administrations were done at a volume of 1ml/kg, with the injection given intraperitoneally, except for L-DOPA, which was given subcutaneously. 3-(5,6-dihydrobenzo[b][1]benzazepin-11-yl)-N-methylpropan-1-amine hydrochloride (Despiramine hydrochloride, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dH_2O. 21-cyclopropyl-7-a-[(*S*)-1-hydroxy-1,2,2-trimethylpropyl]-6,14-*endo*-ethano-6,7,8,14-tetrahydrooripavine hydrochloride (Buprenorphine hydrochloride, Hospira Inc., Lake Forest, IL, USA) was dissolved in dH_20 containing 0.9% NaCl (saline). All enantiomers of propranolol hydrochloride (Sigma-Aldrich) were dissolved in 90% saline and 10% dimethylsulfoxide. L-DOPA methyl ester hydrochloride (Sigma-Aldrich) and 6-OHDA hydrobromide (Sigma-Aldrich) were dissolved in saline with 0.1% ascorbic acid. Doses of 4, 6 and 12mg/kg L-DOPA were administered during this experiment, but the peripheral decarboxylase inhibitor 2-amino-3-hydroxy-N'-[(2,3,4-trihydroxyphenyl)methyl]propanehydrazide (Benserazide, Sigma-Aldrich) was always co-administered with L-DOPA in the same vehicle at a dose of 15mg/kg. Overall, we believe this to be more beneficial than testing at a single dose of L-DOPA since PD patients differ greatly in L-DOPA dose and dyskinesia severity. In experiment 1, we used a dose of 6mg/kg L-DOPA to study the development of dyskinesia because animals gradually manifest AIMs of increasing severity for several weeks. We tested 4 and

12mg/kg L-DOPA in fully primed animals in order to test whether propranolol was capable of reducing mild or severe AIMs, respectively.

Surgeries

One week after arrival, rats were given a unilateral DA lesion to the left medial forebrain bundle (MFB). Prior to surgery, rats were given injections of desipramine (25mg/kg) to protect NE neurons, and buprenorphine (0.03mg/kg) as pre-emptive analgesia. Animals were then anaesthetized with 1-2% 2-chloro-2-(difluoromethoxy)-1,1,1-trifluoroethane

(Isoflurane, Baxter Healthcare Corp., Deerfield, IL, USA) mixed with oxygen (2.5L/min). The following coordinates relative to bregma were used to target the MFB according to the rat brain atlas of Paxinos and Watson (1998): AP -1.8mm; ML +2.0mm; DV -8.6mm, with the incisor bar 5mm below the interaural line. A small hole was drilled into the skull, and a 10µl syringe with 26 gauge needle (Hamilton Company, Reno, NV, USA) was lowered into the target. 6-OHDA (12µg in 4µl) was injected at a constant flow rate of 2µl/min for 2 min and timed to begin 30 min after desipramine injection. The needle was withdrawn 5 min later.

A sham lesion in the right hemisphere was not performed, which is consistent with current protocols in the field (e.g. Cenci and Lundblad, 2007; Buck and Ferger, 2010). The rationale is that a sham lesion in the right hemisphere would partially deplete DA due to mechanical damage to the MFB.

Rats in experiment 4 had a 15mm guide cannula (22 gauge, C313/G/SPC; Plastics One Inc., Roanoke, VA, USA) inserted into the dorsal striatum coincident with lesion surgery, using a procedure from Dupre et al. (2008). The following coordinates were used

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relative to bregma: AP +0.4mm; ML +2.9mm; DV -3.6mm. Cannulae were fixed in place with Jet Denture Repair Acrylic (Lang Dental, Wheeling, IL, USA). The guide cannula was then fitted with a 28 gauge inner stylet (Plastics One) to maintain guide cannula patency.

Abnormal Involuntary Movements Test

The AIMs test is a metric of dyskinesia, a primary side effect of L-DOPA therapy. Rats were monitored for AIMs using a procedure modified from Lundblad et al. (2002) and described in Eskow et al. (2009). Following treatment with L-DOPA, rats were placed in plastic cylinders (22.2cm diameter, 25.4cm height; Thermo Fisher Scientific, Rochester, NY, USA) and rated by trained observers blind to the experimental condition for 1 min every 10 min over a 180-min period. Axial AIMs were defined as dystonic twisting of the neck and torso contralateral to the lesioned hemisphere. A limb AIM was operationalized as a rapid, purposeless movement of the forelimb controlled by the lesioned hemisphere. Orolingual AIMs were coded as repetitive mastication or tongue protrusions when the rat's mouth was empty and not in contact with any object. A severity score was assigned to each of these AIMs: 0, not present; 1, present for <50% of the observation period; 2, present for >50% but <100% of the observation period; 3, present for the entire observation period and interrupted by a loud stimulus (a pencil tap on the side of the cylinder); or 4, present for the entire observation period and not interrupted by the stimulus. Scores for axial, limb and orolingual (ALO) were combined to create a single ALO AIMs score for data analysis.

Forepaw Adjusting Steps Test

The forepaw adjusting steps (FAS) test is a measure of akinesia, a cardinal symptom of PD. Rats with >80% unilateral DA depletion perform poorly on the test with the lesioned side of the body (Chang et al., 1999). L-DOPA reduces this deficit so the test can be used to determine if an L-DOPA adjunct is interfering with the relief of PD symptoms provided by L-DOPA (Eskow et al., 2007). To perform the test, an experimenter blind to treatment condition held the rat's hindlimbs and one forelimb such that the free forelimb was forced to bear the rat's body weight. Rats were then moved laterally for 90cm over 10 seconds across a marked surface while another experimenter counted the number of steps taken in the forehand direction (defined as movement toward the rat's midline) and backhand direction (movement away from the rat's midline). Each FAS test consisted of 3 backhand and 3 forehand trials with each limb, for a total of 12 trials per rat. The score for percent intact stepping was derived by summing the total steps with the lesioned forepaw, dividing by the number of steps with the unlesioned forepaw, and multiplying this number by 100. Lower percent intact scores indicate greater forelimb akinesia.

Experiment 1: Chronic (±)propranolol administration and the development of dyskinesia

Thirty-four rats received unilateral 6-OHDA lesions of the MFB. Two weeks later, DA-lesion severity was assessed using the FAS test. Rats were included in the study if they averaged <5 forehand adjusting steps with the lesioned forelimb per trial, since this score is associated with >80% striatal DA-depletion (Chang et al., 1999). Thirty-three rats were included in the final analysis and assigned to 3 groups of 11 rats each, with each group having equivalent average disability on the FAS test. These FAS scores,

measured off L-DOPA, were used as a baseline against which scores on L-DOPA would be compared.

Three weeks after surgery, treatment with (±)propranolol and L-DOPA began. Rats received (±)propranolol (VEH, 5 or 20mg/kg) 5 min prior to L-DOPA (6mg/kg) for 16 consecutive days. AIMs were assessed on days 1, 5, 8, 12 and 15. FAS data were collected 1 hour after L-DOPA treatment on days 2, 9 and 14. On day 16, rats were killed by decapitation 2 hours after L-DOPA injection. The dorsal striatum was removed for analysis using real-time reverse-transcription polymerase chain reaction (RT-PCR).

Real-Time Reverse Transcription Polymerase Chain Reaction. Two hours after receiving their last VEH or (±)propranolol injections coincident with L-DOPA, rats in experiment 1 were killed and the left and right striata were dissected individually and placed in RNAlater (Qiagen, Valencia, CA, USA) for subsequent analyses of PPE, PPD, and PPT mRNA expression in addition to two housekeeper genes, β-Actin, and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Tissue was processed with Qiagen's RNeasy mini protocol, as detailed in Barnum et al. (2008). cDNA was amplified with the IQ SYBR® Green Supermix kit (BioRad, Hercules, CA, USA). A reaction master mix of volume 40µl was created consisting of 20µl SYBR® Green, 17.6µl RNase-free water, 0.4µl cDNA template, and 2µl of sample. 10µl of each master mix was pipetted in triplicate into a 384-well plate (BioRad) and analyzed using BioRad CFX1000 Thermal Cycler. Relative gene expression was quantified using the $2^{-\Delta CT}$ method, with expression levels normalized to 100% of ultimate control values. Gene sequences were obtained from GenBank at the National Center for Biotechnology Information and primer specificity was verified by the Basic Local Alignment Search Tool

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(http://www.ncbi.nlm.nih.gov/). The primer sequences used were for β-actin (5'-AGCATCACCCCATTTGATGT-3'/5'-GTCGTACCACTGGCATTGTG-3') GAPDH (5'-GCCATCTCTTGCTCGAAGTC-3'/5'-ATGACTCTACCCACGGCAAG-3') PPD (5'-GGGTTCGCTGGATTCAAATA-3'/5'-TGTGTGGAGAGGGACACTCA-3') PPT (5'-AGCCTCAGCAGTTCTTTGGA-3'/5'-CGGACACAGATGGAGATGAA-3') and PPE (5'-AAAATCTGGGAGACCTGCAA-3'/5'-CATGAAACCGCCATACCTCT-3').

Experiment 2: Impact of (±)propranolol on spontaneous motor activity

Fourteen rats received unilateral 6-OHDA lesions in the aforementioned manner. Three weeks later, rats were acclimated to motion chambers 4 times in 1 week for a period of 90 min each, since this length corresponds to the duration of antidyskinetic efficacy of 20mg/kg (±)propranolol. Rats were kept L-DOPA naïve and tested 4 times in a within-subjects design. Each test session was separated by a 2-3 day washout period, which insured full clearance of the drug given that the half-life of (±)propranolol in rats is approximately 1 hour (Bianchetti et al., 1980). Rats were injected with (±)propranolol (VEH, 5, 20 or 40mg/kg) and immediately placed in motion chambers for analysis. The order of treatments was counterbalanced across test days. Testing was done between 1000h and 1600h with each rat placed in the same chamber for all acclimation and testing sessions.

Locomotor activity was assessed in 6 identical acrylic chambers measuring 41cm in length and width and 30.5cm in height (Accuscan Instruments, Columbus, OH, USA). Each chamber was surrounded by infrared photocell arrays synched with a program running Versamax and Versadat software (Accuscan Instruments). The software analyzes

patterns of photo beam breaks to measure horizontal and vertical movements. Data were analyzed over a period of 90 min, with data grouped into 6 blocks of 15 min. We include data on the total distance traveled (in cm), the number starts and stops separated by at least 1 second ("movement number"), and the number of beam breaks in the vertical plane ("vertical activity"). These behaviors have been previously used to measure drug-induced locomotor changes in hemiparkinsonian rats (Bishop et al., 2004).

Experiment 3: Enantiomers of (±)propranolol in the expression of established dyskinesia

Twenty rats received unilateral 6-OHDA lesions to the MFB. After a 3-week recovery period, all rats were "primed" with daily injections of L-DOPA (12mg/kg) for one week. This process is referred to as priming since subsequent doses of L-DOPA will cause rats to manifest reliably similar levels of dyskinesia in response to a given dose of L-DOPA (Bishop et al., 2009). On day 7 of L-DOPA priming, ALO AIMs were monitored and rats with >25 ALO AIMs (n=17) were included in the study.

The following week, rats were given (±)propranolol (VEH, 5 or 20mg/kg) in a within-subjects design 5 min prior to L-DOPA (12mg/kg) and monitored for ALO AIMs. Rats were tested 3 days per week with each test day separated by a 2-day washout period and the order of treatments was counterbalanced. The individual enantiomers of propranolol (+ and -) were tested in the same manner in weeks 5 and 6 respectively. To address potential changes in dyskinesia levels across treatment, each rat's VEH+L-DOPA ALO AIMs were compared across weeks and did not differ between weeks 4, 5 and 6 ($p>.05$).

Experiment 4: Striatal (±)propranolol infusion and dyskinesia expression

Two cohorts each consisting of 15 rats received cannulae in the left dorsal striatum in addition to unilateral 6-OHDA lesion to the MFB. Three weeks later, all rats were primed with L-DOPA (12mg/kg) for 1 week. On the last day of priming, AIMs were assessed and only rats with >25 ALO AIMs score were included in the study. Four times during the priming week, rats were wrapped in a small towel for several min to habituate them to gentle restraint during microinfusions. Two days after the final priming dose of L-DOPA, the first cohort of rats was given a striatal microinfusion of (±)propranolol (VEH, 1, or 10μg in 2μl) at a constant flow rate of 0.5μl/min over 4 min in a counterbalanced within-subjects manner. Previous research indicated that this flow rate and volume of fluid at the coordinates used would cause the liquid vehicle to permeate only the dorsal striatum (Dupre et al. 2008). For micro-infusions, the dummy cannula was removed and a 16mm injector was placed inside the 15mm guide cannula, making the injector extend 1mm further into the striatum than the guide. The injector was removed 4 min after the flow was stopped and rats were immediately injected with L-DOPA (4mg/kg) and assessed for ALO AIMs. The second cohort was tested in the same manner, except the L-DOPA dose was increased to 12mg/kg. Rats were removed from the study if they did not become dyskinetic after priming, or the cannula placement was incorrect, leaving 13 rats in the first cohort and 12 in the second.

Tissue dissection and cresyl violet staining. Following decapitation, the brain was removed and the striatum was bisected coronally with a razor blade. The posterior striatum was dissected and frozen at -80°C for analysis of DA levels using high performance liquid chromatography with electrochemical detection (HPLC-ED). The anterior section of the brain (containing the cannula site) was placed in 4% formaldehyde

(Fisher Scientific, Hanover Park, IL, USA). Coronal slices (30μm) showing the cannula placements were sectioned using a microtome (Model SM2000R; Leica Microsystems Inc., Bannockburn, IL, USA). Slices were mounted on slides and stained with cresyl violet (FD NeuroTechnologies, Inc. Baltimore, MD, USA). Light microscopy was then used to determine injection sites.

High-performance liquid chromatography. Reverse-phase HPLC-ED was performed on striatal tissue as outlined by Bishop et al. (2009), based on a protocol for catecholamine analysis by Kilpatrick and colleagues (1986). The system included an autoinjector (ESA, Model 542, Chelmsford, MA, USA), a solvent delivery system (ESA, Model 582), an external pulse dampener (ESA), and a MD-150X3.2 column (150x3.2 mm, 3μm particle size, ESA). Samples were homogenized in 0°C perchloric acid (0.1M), 1% ethanol, and 0.02% EDTA (Sigma-Aldrich). The homogenates were spun for 45 min at 14,000g with the temperature maintained at 4°C. Aliquots of supernatant were then analyzed for abundance of DA. Samples were separated using a mobile phase composed of 90mM sodium dihydrogen phosphate, 50mM citric acid, 50μM EDTA, 1.7mM octane sulfonic acid, and 10% acetonitrile, adjusted to pH 3.0 with orthophosphoric acid (Sigma-Aldrich). A coulometric detector configured with 3 electrodes (Coulochem III, ESA) measured the content of DA. An ESA model 5020 guard cell (+500mV) was positioned prior to the autosampler. The analytical cell (ESA model 5011A; first electrode at -100mV, second electrode at +250mV) was located immediately after the column. EZChrom Elite software (ESA) recorded and analyzed the electrochemical reaction that occurred on the second analytical electrode. The final oxidation current values were plotted on a standard curve of known concentrations from 10^{-6}M to 10^{-9}M and values

were adjusted to striatal tissue weights.

Statistical Analysis

ALO AIMs data for between-subjects comparisons was analyzed using the non-parametric Kruskal-Wallis test. If the omnibus comparison for a given test day was significant, Mann-Whitney contrasts were used to distinguish significant differences between vehicle and treatment groups. For within-subjects designs, the non-parametric Friedman test was used followed by the Wilcoxon signed-rank post-hoc. All non-ALO AIMs data were analyzed with standard parametric statistics using between-subjects, repeated measures and mixed model ANOVAs when appropriate. Fisher's least significant difference (LSD) contrasts on post hoc comparisons were used throughout the study.

For analysis where the primary comparisons of interest were between-subjects, (Exp. 1 ALO AIMs and RT-PCR data) scores more than 2 standard deviations from the group mean were discarded and replaced with the new group mean; degrees of freedom for comparisons were adjusted accordingly. No data were discarded in other analyses. Statistical analysis was done using SPSS v18.0 (IBM, Chicago, IL, USA) with alpha set at .05.

Results

Experiment 1

(±)Propranolol dose-dependently reduced the development of ALO AIMs. As shown in figure 1A, co-administration of (±)propranolol at 5mg/kg "(±)PRO5" or (±)propranolol at 20mg/kg "(±)PRO20" along with L-DOPA reduced ALO AIMs when

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compared to L-DOPA alone "VEH". Axial, limb and orolingual AIMs were examined individually and it was found that (±)propranolol dose-dependently suppressed all three subtypes (data not shown). Therefore, all further analyses were conducted using a composite ALO AIMs score. Kruskal-Wallis test revealed that this difference was statistically significant on all test days: Day 1 (χ^2=12.74, p<.01), Day 5 (χ^2=19.45, p<.001), Day 8 (χ^2=7.53, p<.05), Day 12 (χ^2=9.40, p<.01), and Day 15 (χ^2=12.07, p<.01). Mann-Whitney comparisons indicated that compared to VEH, (±)PRO5 reduced ALO AIMs on days 5 and 15 (p<.05) and (±)PRO20 reduced ALO AIMs on all test days (p<.05).

(±)Propranolol did not affect the efficacy of L-DOPA on the FAS test. The FAS test was employed to determine whether L-DOPA improved stepping in lesioned rats, and if (±)propranolol co-treatment impacted stepping (Fig. 1B). A 3x4 (treatment by day) mixed model ANOVA was used for analysis. Omnibus ANOVA revealed a main effect of treatment ($F_{2,30}$=4.92, p<.05) where (±)PRO20 rats had a higher percent intact score than (±)PRO5 or VEH rats (p<.05). A main effect of day ($F_{3,90}$=17.62, p<.001) demonstrated that L-DOPA improved stepping on test days relative to baseline (p<.05).

Since there was a main effect of day, a 1-way repeated measures ANOVA was run on each treatment group to test for differences between baseline and treatment day stepping. ANOVA revealed an effect of treatment day for VEH ($F_{3,30}$=4.39, p<.05), (±)PRO5 ($F_{3,30}$=5.28, p<.01) , and (±)PRO20 ($F_{3,30}$=9.26, p<.001). Post hoc comparisons between baseline and treatment days revealed that L-DOPA administration improved stepping in VEH rats on days 2 and 9 (p<.05) and in (±)PRO5 and (±)PRO20 rats on all test days.

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(±)Propranolol modified gene expression of LID-related striatal prepropeptides. Tissue from the dorsal striatum was analyzed via RT-PCR to examine changes in opioid prepropeptide mRNA for PPE, PPD and PPT. Expression changes were determined with a 2x3 (lesion by treatment) mixed model ANOVA. No significant main effects or interactions were found for the housekeeper genes, β-actin and GAPDH ($p>.05$), thereby allowing for comparison of lesion- and treatment-induced effects. Subsequent opioid mRNA analysis with a 2x3 (lesion by treatment) mixed model ANOVA revealed a main effect of lesion, which increased transcription of PPE ($F_{1,26}=12.97$, $p<.01$; Fig. 2A) and PPD ($F_{1,28}=9.26$, $p<.01$; Fig. 2B) while reducing PPT transcription ($F_{1,29}=46.92$, $p<.001$; Fig. 2C). There was also a significant lesion by treatment interaction for PPE ($F_{2,26}=3.79$, $p<.05$) and PPD ($F_{2,28}=3.72$, $p<.05$). Post hoc comparisons of VEH to (±)PRO5 and (±)PRO20 on the lesioned side indicated that transcription of both PPE and PPD on the lesioned side was reduced by (±)PRO5 compared to lesioned striatum treated with VEH ($p<.05$).

Experiment 2

(±)Propranolol reduced motor activity only at high doses. The motor activity of rats given injections of (±)propranolol alone was assayed in motion chambers in order to determine if (±)propranolol's anti-LID efficacy is due to a general suppression of motor behavior. The 4 treatment groups were analyzed in 6 time blocks of 15 min using a 4x6 (treatment by time) repeated measures ANOVA. Analysis of total movement (Fig. 3A) revealed a significant effect of treatment ($F_{3,39}=10.92$, $p<.001$), time ($F_{5,65}=25.52$, $p<.001$) and a treatment by time interaction ($F_{15,195}=6.52$, $p<.001$). Compared to VEH, both (±)PRO20 and 40mg/kg (±)propranolol "(±)PRO40" suppressed total distance traveled.

(±)PRO20 suppressed distance traveled for the first 30 minutes, but subsequently increased distance traveled between 46 and 60 min ($p<.05$). (±)PRO40 reduced distance traveled for 45 min ($p<.05$). Movement number (Fig. 3B) was also affected by treatment ($F_{3,39}=5.91$, $p<.01$) and time ($F_{5,65}=25.76$, $p<.001$) and there was an interaction ($F_{15,195}=5.03$, $p<.001$). Collapsing across time, only (±)PRO40 reduced movement number, with significant reductions in the first 30 min ($p<.05$). (±)PRO20 had no overall effect on movement number, and actually increased movement at 46-60 and 76-90 min after injection ($p<.05$). Similarly, a main effect of treatment ($F_{3,39}=12.75$, $p<.001$) and time ($F_{5,65}=14.15$, $p<.001$) and a significant treatment by time interaction ($F_{15,195}=5.19$, $p<.001$) were found for vertical activity. (±)PRO5 reduced vertical activity compared to VEH from 16-30 min, but not overall ($p>.05$). Both (±)PRO20 and (±)PRO40 decreased total vertical activity compared to VEH. (±)PRO20 reduced vertical activity compared to VEH for 30 min, while (±)PRO40 reduced vertical activity for 45 min ($p<.05$).

Experiment 3

The (-)propranolol enantiomer conveyed anti-dyskinetic properties. To determine if (±)propranolol's efficacy is due to βAR binding, the enantiomers of (±)propranolol were examined individually, since (-)propranolol has 100x greater affinity for these receptors than (+)propranolol (Mehvar and Brocks, 2001). Friedman tests showed an ALO AIMs reduction by (±)propranolol ($\chi^2=22.91$, $p<.001$; Fig. 4A). (+)propranolol did not reduce ALO AIMs ($\chi^2=.82$, $p>.05$; "(+)PRO": Fig. 4B) but (-)propranolol was effective ($\chi^2=23.06$, $p<.001$; "(-)PRO": Fig. 4C). Mann-Whitney post hocs demonstrated that anti-LID efficacy began at 20 min post L-DOPA and lasted until 50 min for (±)PRO5

and until 90 min for (±)PRO20 ($p<.05$). (-)PRO5 reduced LID at min 20-60 and 80 after L-DOPA, while (-)PRO20 reduced LID from time points 20-100 and 120-160 ($p<.05$).

Experiment 4

Dopamine depletion and cannula placements. HPLC-ED analysis of posterior striatal tissue revealed that average DA depletion in the lesioned striata compared to intact striata was 97%. Cannula placements were verified using cresyl violet staining (Fig. 5A) and injection sites are schematically represented in Fig. 5B.

Striatal microinfusion of (±)propranolol reduced established ALO AIMs. Microinjections targeting the dorsal striatum were employed to determined the role of this structure in the anti-dyskinetic efficacy of (±)propranolol. (±)Propranolol was effective in reducing ALO AIMs induced by 4mg/kg of L-DOPA ($\chi^2=7.54$, $p<.05$; Fig. 6A), but not those induced by 12mg/kg L-DOPA ($\chi^2=4.67$, $p>.05$; Fig. 6B). Post hocs run on rats receiving 4mg/kg indicated that 1µg of (±)propranolol "(±)PRO1µg" reduced ALO AIMs from at 50, 60, 70, 120 and 140 min post-L-DOPA, while 10µg of (±)propranolol "(±)PRO10µg" reduced ALO AIMs compared to VEH at the 90, 100 and 130 min time points ($p<.05$).

Discussion

Previous preclinical and clinical research has indicated that (±)propranolol reduced the expression of established LID (Buck and Ferger, 2010; Carpentier et al., 1996). In the current investigation, we expand upon these findings, reporting that behaviorally (±)propranolol also reduced the development of LID without impairing motor ability. Pharmacologically, the (-) optical isomer is the only enantiomer that had

anti-dyskinetic efficacy, implicating βAR-specific effects. Neuroanatomically, at least some of (±)propranolol's therapeutic effects occured in the dorsal striatum. Collectively, these findings suggest that βAR antagonists may be useful L-DOPA adjuncts in PD and may expand the therapeutic window of L-DOPA to allow greater relief of primary PD symptoms while reducing LID.

An ideal L-DOPA adjunct would not only reduce established LID, but would also be capable of preventing the development of LID. We therefore examined chronic (±)propranolol co-treatment in rats with no prior exposure to L-DOPA. Daily L-DOPA treatment led to a gradual increase in the severity of ALO AIMs, while pretreatment with 5 and 20mg/kg (±)propranolol reduced these dyskinesias by an average of 37% and 70% respectively across test days (Fig. 1A). Importantly, both doses of (±)propranolol significantly lowered ALO AIMs on the final test day. While previous studies have suggested acute LID suppression by (±)propranolol (Buck and Ferger, 2010; Dekundy et al., 2007), and Carpentier et al. (1996) reported long-term anti-dyskinetic efficacy, this is the first study to suggest a pronounced anti-LID prophylaxis due to chronic βAR blockade.

Coincident with the ALO AIMs investigation, we examined if (±)propranolol was reducing LID via a general reduction in motor performance, since such a drug would be of limited clinical relevance. Throughout chronic L-DOPA treatment, rats were monitored for motor performance using the FAS test, since DA-lesioned rats exhibit profound stepping deficits that improve with L-DOPA (Chang et al., 1999; Winkler et al., 2002). At baseline, lesioned paw stepping averaged 18% of intact paw stepping (Fig. 1B). While L-DOPA initially improved stepping, it failed to provide a significant

improvement on the last day of testing. Meanwhile, pretreatment with (±)PRO20 improved stepping on all test days relative to baseline, with rats performing at more than 70% intact on the last day of testing. Previous studies on the motor effects of (±)propranolol have yielded equivocal results. Dekundy et al. (2007) used the same drug doses as the present investigators and found that (±)propranolol did not affect motor performance in a rotorod test. However, Gomez-Mancilla and Bedard (1993) found that 10mg/kg (±)propranolol diminished the length of L-DOPA efficacy in MPTP-treated macaques.

Therefore, in order to more fully characterize the motor effects of (±)propranolol, we employed a second test of motor behavior without L-DOPA. In locomotor chambers, 5mg/kg had no effect on behavior, whereas 20mg/kg (±)propranolol reduced some types of motor activity and 40mg/kg reduced scores on all metrics of mobility (Fig. 3). A similar study in rats found no significant reduction in motor behavior with either 10 or 20mg/kg (±)propranolol (Buck and Ferger, 2010). Collectively, these findings indicate that a range of doses of (±)propranolol can reduce LID without suppressing general motor activity or diminishing the anti-PD effects of DA replacement.

Several adrenergic receptor compounds have been investigated for potential anti-LID properties, but most target α-adrenoceptors (Brotchie, 2005; Colosimo and Craus, 2003). Far fewer studies have investigated βAR drugs despite consistent beneficial effects (Carpentier et al., 1996; Dekundy et al., 2007). However, the specificity of these effects for βARs remained untested prior to the present study. Data from figure 4 show that (±) and (-)propranolol reduced ALO AIMs, indicating that anti-LID efficacy is mediated through βAR blockade. While both enantiomers have membrane effects including sodium

channel blockade (Matthews and Baker, 1982), (-)propranolol has 100x greater affinity for $\beta_{1/2}$AR than the (+) enantiomer (Mehvar and Brocks, 2001).

Qualitatively, 20mg/kg (±)propranolol lowered ALO AIMs for 90 min (Fig. 4A), while 20mg/kg (-)propranolol was effective for almost the entire 180 min rating period (Fig. 4C). (+)Propranolol showed no significant reduction in ALO AIMs even at 20mg/kg, but a trend was observed for 50 minutes following l-DOPA injection (Fig. 4B). This time interval coincided with a reduction in motion chamber activity by 20mg/kg (±)propranolol (Fig. 3A, 3C), which may have accounted for the observed ALO AIMs reduction.

Since (-)propranolol binds to a multitude of receptors, off-target effects are possible but unlikely. For example, (-)propranolol binds to the β_3AR, but has 100x lower affinity for this receptor as for $\beta_{1/2}$AR (Mehvar and Brocks, 2001) and β_3AR have not been reported in the striatum. (-)Propranolol also has affinity for multiple 5-HT_1 receptors, but since it acts as an antagonist (Middlemiss, 1984) the 5-HT binding properties alone would actually be expected to increase dyskinesia (Bishop et al., 2009; Eskow et al., 2009). The results of this study strongly suggest that (±)propranolol reduces LID through $\beta_{1/2}$AR antagonism, although future studies should test this theory directly by using antagonists with greater receptor specificity.

The $\beta_{1/2}$AR are widely distributed throughout the CNS, but are found in particularly high concentrations in the striatum (Rainbow et al., 1984). Ample evidence exists showing that βAR activity modulates DA release in the striatum, suggesting a pre-synaptic mechanism for (±)propranolol in LID (Goshima et al., 1991; Reisine et al.,

1982). Recent research has suggested an additional post-synaptic mechanism: antagonism of β_1AR was shown to reduce downstream phosphorylation of DARPP-32 at Thr^{34} (Hara et al., 2010), which is clinically important since high levels of $pThr^{34}$-DARPP-32 are implicated in the expression of LID (Bateup et al., 2010; Santini et al., 2007).

The striatum was hypothesized to be the anatomical locus of (±)propranolol's therapeutic action, given the evidence that LID is caused by aberrant striatal DA signaling (Cenci, 2007; Winkler et al., 2002) and that (±)propranolol directly effects striatal DA signaling (Goshima et al., 1991; Reisine et al., 1982). Microinjection of (±)propranolol (1 or 10μg) into the dorsal striatum reduced ALO AIMs when L-DOPA was given at a therapeutic dose (4mg/kg; Fig. 6A), but not when given at a higher dose (12mg/kg; Fig. 6B). The reduction in ALO AIMs did not occur until 50 min after 4mg/kg L-DOPA injection, which corresponded to peak dyskinesia, so it is possible that intrastriatal (±)propranolol is only capable of blunting maximal DA spikes. Striatal (±)propranolol microinjection had no significant effect on ALO AIMs with 12mg/kg L-DOPA even though systemic (±)propranolol did. It is unlikely that increasing the dose of (±)propranolol would have provided efficacy with 12mg/kg L-DOPA since we did not observe a dose-response between 1 and 10μg with 4mg/kg L-DOPA. Thus results in figure 6 simultaneously confirm that the dorsal striatum is a key site of anti-LID action for (±)propranolol and suggest the existence of additional loci of action, such as the ventral striatum, globus pallidus and substantia nigra pars reticulata (Rainbow et al., 1984).

Regardless of the site(s) of anti-LID action of (±)propranolol, the drug appears to be affecting striatal signaling since gene expression changes were observed in striatal

mRNA following (±)propranolol adjunct therapy (Fig. 2). PPE mRNA expression, an index of D_2-mediated indirect pathway activity (Gerfen et al., 1990), was increased by L-DOPA in the lesioned hemisphere relative to the intact hemisphere (Fig. 2A). Importantly, pretreatment with (±)PRO5 prevented this increase. Surprisingly, we did not observe a significant change in PPE or PPD expression among (±)PRO20-treated rats on the lesioned side compared to L-DOPA alone. PPD and PPT expression (Fig. 2B, 2C) were also measured since they are markers of D1-mediated direct pathway activity (Gerfen et al., 1990). In corroboration with previous research, L-DOPA was found to preferentially increase PPD expression in the lesioned striata (Cenci et al., 1998). Figure 2B highlights the novel finding that this increase was blocked with (±)PRO5 pretreatment. We confirm previous reports of a lesion-induced decrease in PPT mRNA (Cenci et al., 1998); however, treatment group did not effect PPT expression.

Clinically, the use of (±)propranolol may be warranted as it is currently FDA approved for a variety of conditions including essential tremor, but potential side effects should be considered. For example, PD is associated with a reduction in sympathetic nervous system activity and patients often display basal hypotension and bradycardia (Oka et al., 2007). It is feasible that (±)propranolol might exacerbate these symptoms. However, Carpentier et al. (1996) administered (±)propranolol to PD patients for 5-6 weeks and did not observe any side effects that precluded patients from taking the drug. Systolic blood pressure and heart rate were unaffected by the maximum dose tested (60mg/day), but this dose was able to reduce LID scores by an average of 40%.

The present data suggest that there is a dose range of (±)propranolol which will reduce dyskinesia without impairing motor activity or exacerbating PD symptoms.

JPET Fast Forward. Published on March 14, 2011 as DOI: 10.1124/jpet.111.179416
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Mechanistically, we provide evidence that the dorsal striatum is one site of action for (±)propranolol in the reduction of LID. We show that aberrant striatal signaling associated with dyskinesia is normalized with doses of (±)propranolol that have anti-dyskinetic efficacy. When combined with previous data in humans and in animal models, the present research provides convergent preclinical evidence for the efficacy of βAR blockade for the treatment of LID.

JPET Fast Forward. Published on March 14, 2011 as DOI: 10.1124/jpet.111.179416
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgements

The authors thank Dr. Terrence Deak for providing expertise with RT-PCR research design and analysis. Thomas Button and Jess George analyzed HPLC-ED data. Margaret Surrena, Melanie Salamon, Evan Feinberg, Adam Goldenberg, Hannah Mitchell, Yuchen Liu and Sando O. Dickinson helped with behavioral testing. Molly M. Deak, Hollin Buck, Cara Hueston, and Nancy Monteith assisted with RT-PCR analysis. Dr. Lisa M. Savage and Dr. Ryan P. Vetreno aided with histology.

Authorship Contributions

Participated in research design: Lindenbach, Ostock, Eskow Jaunarajs, Dupre, Barnum, Bhide, Bishop

Conducted Experiments: Lindenbach, Ostock, Eskow Jaunarajs, Dupre, Barnum, Bhide

Contributed new reagents or analytic tools: Bishop

Performed Data Analysis: Lindenbach, Bishop

Wrote or Contributed to Manuscript: Lindenbach, Bishop

Provided Funding: Bishop

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Footnotes

¶This research was supported by the Center for Development and Behavioral Neuroscience at Binghamton University, and the National Institute of Health National Institute of Neurological Disorders and Stroke [Grant NS059600]

JPET Fast Forward. Published on March 14, 2011 as DOI: 10.1124/jpet.111.179416
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Figure Legends

Figure 1. (±)Propranolol dose-dependently attenuated the development of L-DOPA induced dyskinesia without interfering with L-DOPA efficacy. Unilaterally lesioned rats (n=11 per group) were given (±)propranolol (VEH, 5mg/kg, or 20mg/kg) 5 min prior to L-DOPA (6mg/kg) every day for 16 days in a between-subjects design. ALO AIMs were tested on days 1, 5, 8, 12, and 15. The FAS test was performed at baseline several days prior to the beginning of L-DOPA treatment and subsequently 1 hour after L-DOPA injection on days 2, 9 and 14. Symbols of charts denote treatment group mean ±S.E.M. for **(A)** ALO AIMs and **(B)** FAS. ALO AIMs were analyzed using the Kruskal-Wallis test with Mann-Whitney post hocs. FAS data were analyzed using a 1-way repeated measures ANOVA and LSD contrasts comparing treatment days to baseline. AIMs: *p<.05 VEH vs. (±)PRO5; ††p<.01, †††p<.001 VEH vs. (±)PRO20. FAS: +p<.05, VEH vs. baseline; ^p<.05, ^^p<.01, ^^^p<.001 (±)PRO5 vs. baseline; #p<.05, ##p<.01 (±)PRO20 vs. baseline.

Figure 2. DA lesion, L-DOPA and (±)propranolol alter the transcription of PPE, PPD and PPT mRNA in the striatum. Hemiparkinsonian rats (n=11 per group) were given (±)propranolol (VEH, 5 or 20mg/kg) 5 min prior to L-DOPA (6mg/kg) every day for 16 days in a between-subjects design. Rats were killed by decapitation 2 hours after final L-DOPA injection on day 16 and the dorsal striata from the lesioned and non-lesioned sides were dissected for subsequent RT-PCR analysis. Bars express percent change in expression compared to control (unlesioned striatum treated with L-DOPA) for **(A)** PPE **(B)** PPD and **(C)** PPT mRNA expression. Effects were determined using a 2x3 (lesion by

treatment) mixed model ANOVA followed by post hocs. *p<.05, **p<.01 VEH+Intact vs. VEH+Lesion; †p<.05, ††p<.01 VEH+Lesion vs. (±)PRO5+Lesion.

Figure 3. High doses of (±)propranolol reduce motor activity. Unilaterally lesioned rats (n=14) were injected with (±)propranolol (VEH, 5, 20 or 40mg/kg) and immediately placed in motion chambers for analysis of spontaneous motor activity over the next 90 min. Symbols represent mean for 15-min time points ± S.E.M. for **(A)** distance traveled **(B)** movement number and **(C)** vertical activity. Data were analyzed using a 4x6 (treatment by time) repeated-measures ANOVA and contrasts of vehicle to treatments. *p<.05 VEH vs. (±)PRO5; †p<.05, ††p<.01 VEH vs. (±)PRO20; +p<.05, ++p<.01 VEH vs. (±)PRO40.

Figure 4. Propranolol dose-dependently and stereo-specifically reduced the expression of ALO AIMs induced by L-DOPA. L-DOPA-primed hemiparkinsonian rats (n=17) were given injections of racemic (±), levo (-), and dextro (+) propranolol (VEH, 5 or 20mg/kg) 5 min prior to L-DOPA (12mg/kg) in a within-subjects design. ALO AIMs were subsequently monitored. Symbols on main chart represent the treatment group time point mean ALO AIMs ± S.E.M for **(A)** (±)propranolol **(B)** (+)propranolol and **(C)** (-)propranolol. Bars on inlaid figure denote total ALO AIMs ± S.E.M of treatment groups. Data were analyzed using the Friedman test with Wilcoxon signed-rank post hocs. *p<.05, **p<.01, ***p<.001 VEH vs. PRO5; †p<.05, ††p<.01, †††p<.001 VEH vs. PRO20.

Figure 5. The cannula placements from rats in experiment 4 were examined post-mortom. **(A)** Cresyl violet staining of a successful cannula placement in dorsal striatum.

Injector tract is darkly stained relative to striatum. **(B)** Schematic representation of injector tracts. Adapted from a coronal slice at +.48mm relative to bregma from the rat brain atlas of Paxinos and Watson (1998). CC: Corpus Collosum, LV: Lateral Ventricle, CPu: Caudate Putamen.

Figure 6. Intrastriatal microinfusion of (±)propranolol reduced ALO AIMs induced by low doses of L-DOPA. Unilaterally lesioned rats (n=12-13) primed with L-DOPA (12mg/kg) received microinfusions of (±)propranolol (VEH, 1 or 10μg) into the dorsomedial striatum. Rats were subsequently injected with L-DOPA and monitored for ALO AIMs. Symbols on graph represent treatment group time point mean ALO AIMs ± S.E.M. for **(A)** 4mg/kg L-DOPA or **(B)** 12mg/kg L-DOPA. Bars on inlaid figure represent total ALO AIMs ± S.E.M of treatment groups. Data were analyzed using the Friedman test and Wilcoxon signed-rank post-hocs. *$p<.05$ VEH vs. (±)PRO1μg; †$p<.05$ VEH vs. (±)PRO10μg.

JPET Fast Forward. Published on March 14, 2011 as DOI: 10.1124/jpet.111.179416
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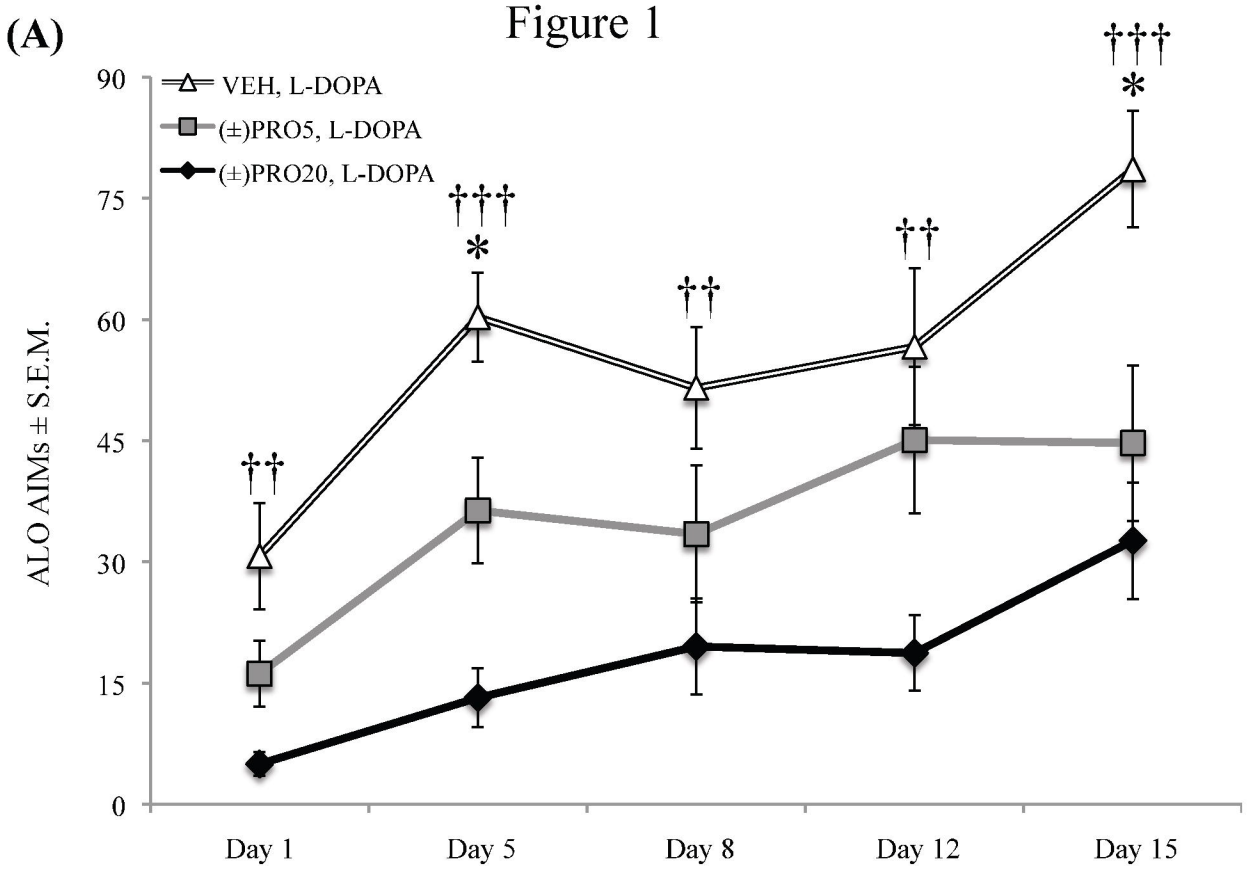
Figure 1



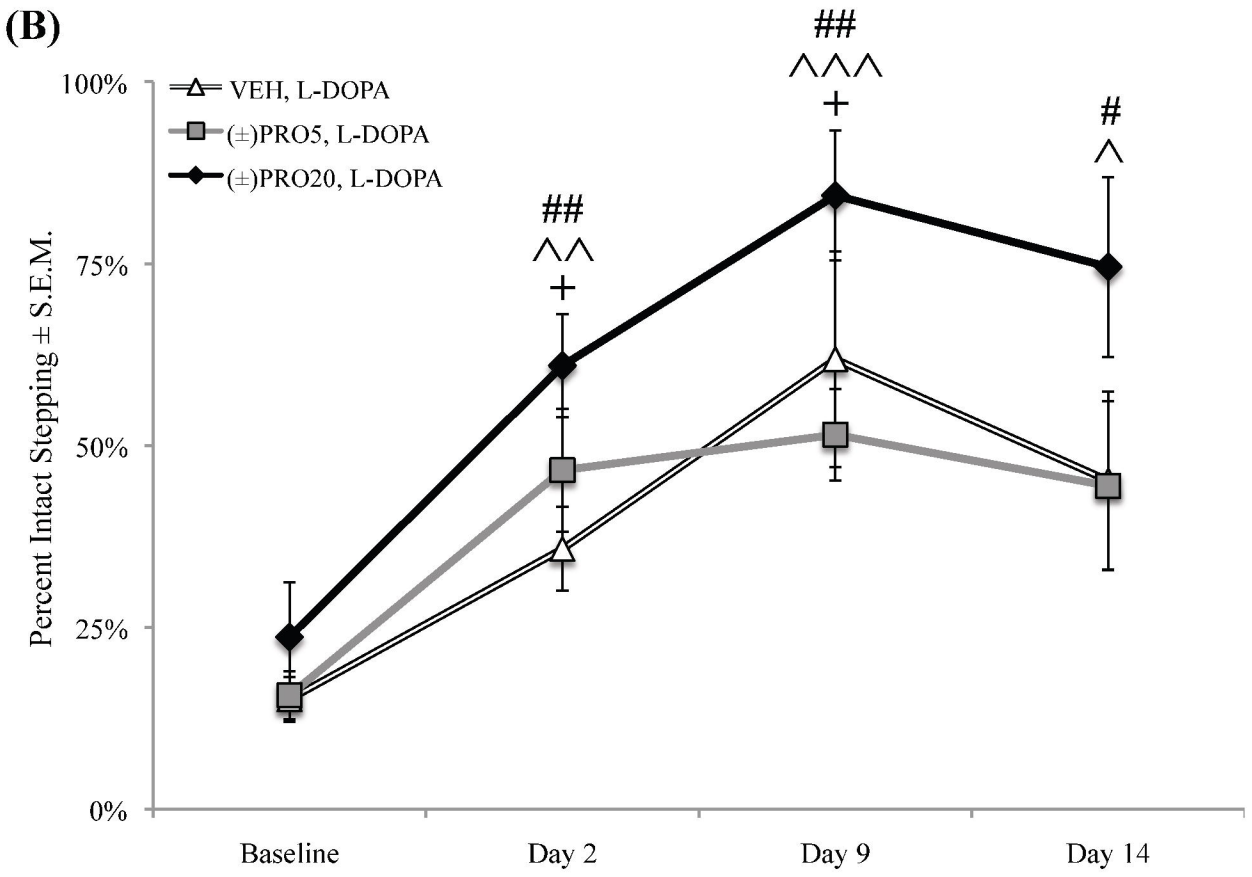


Figure 2

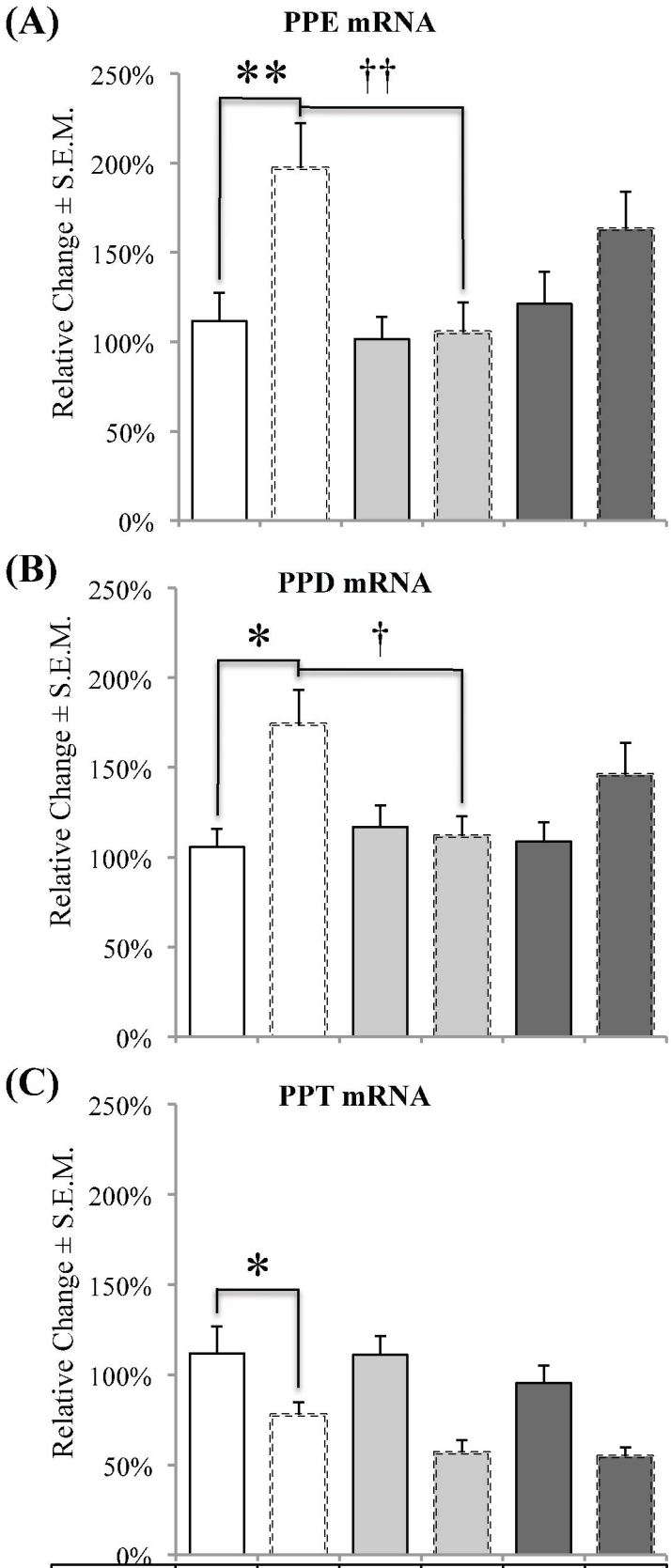

(A)
PPE mRNA
Relative Change ± S.E.M.
250%
200%
150%
100%
50%
0%
**
††
(B)
PPD mRNA
Relative Change ± S.E.M.
250%
200%
150%
100%
50%
0%
*
†
(C)
PPT mRNA
Relative Change ± S.E.M.
250%
200%
150%
100%
50%
0%
*
Lesion - + - + - +
(±)PRO - - 5 5 20 20
L-DOPA + + + + + +

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Figure 3

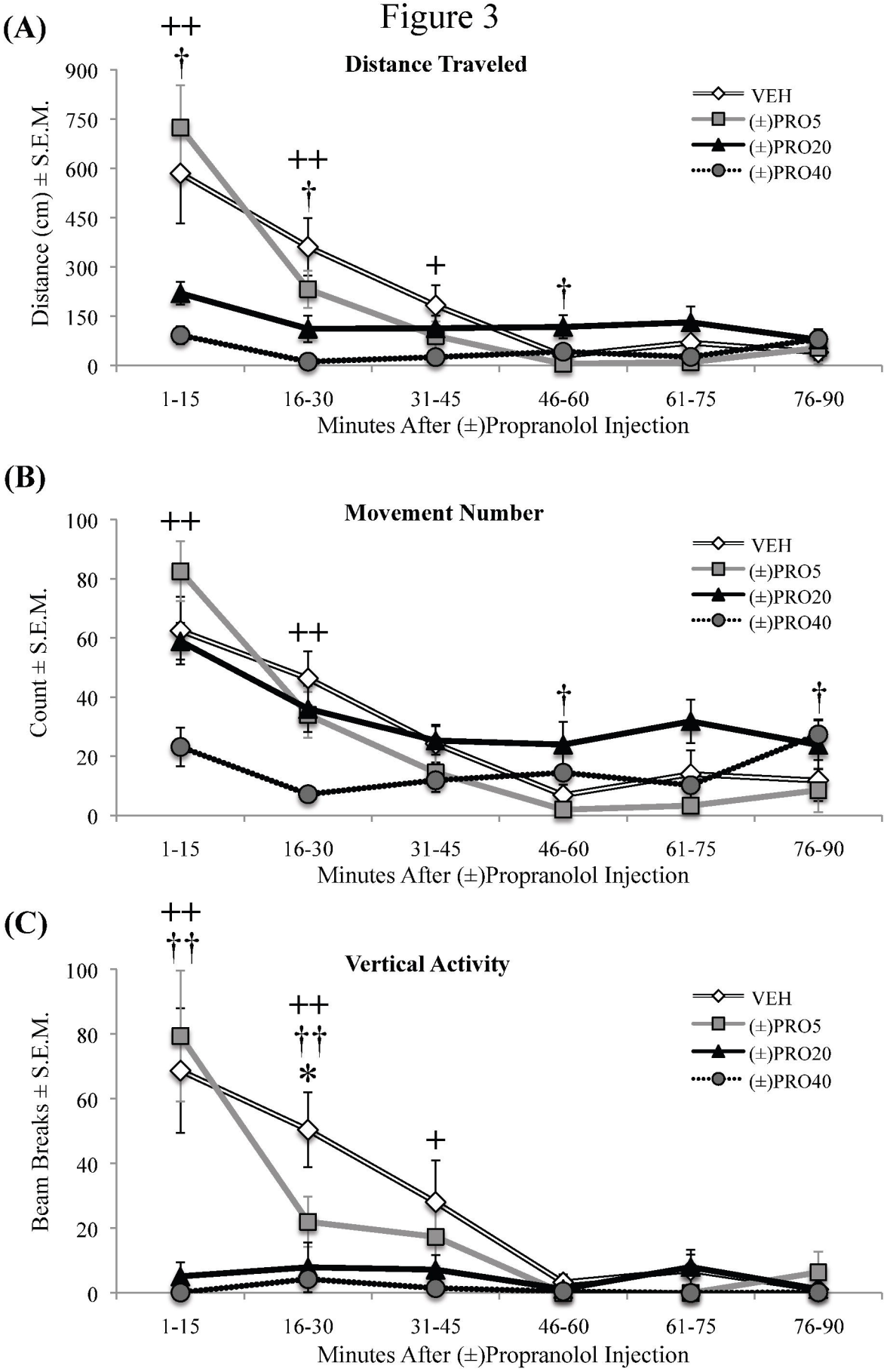

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Figure 4

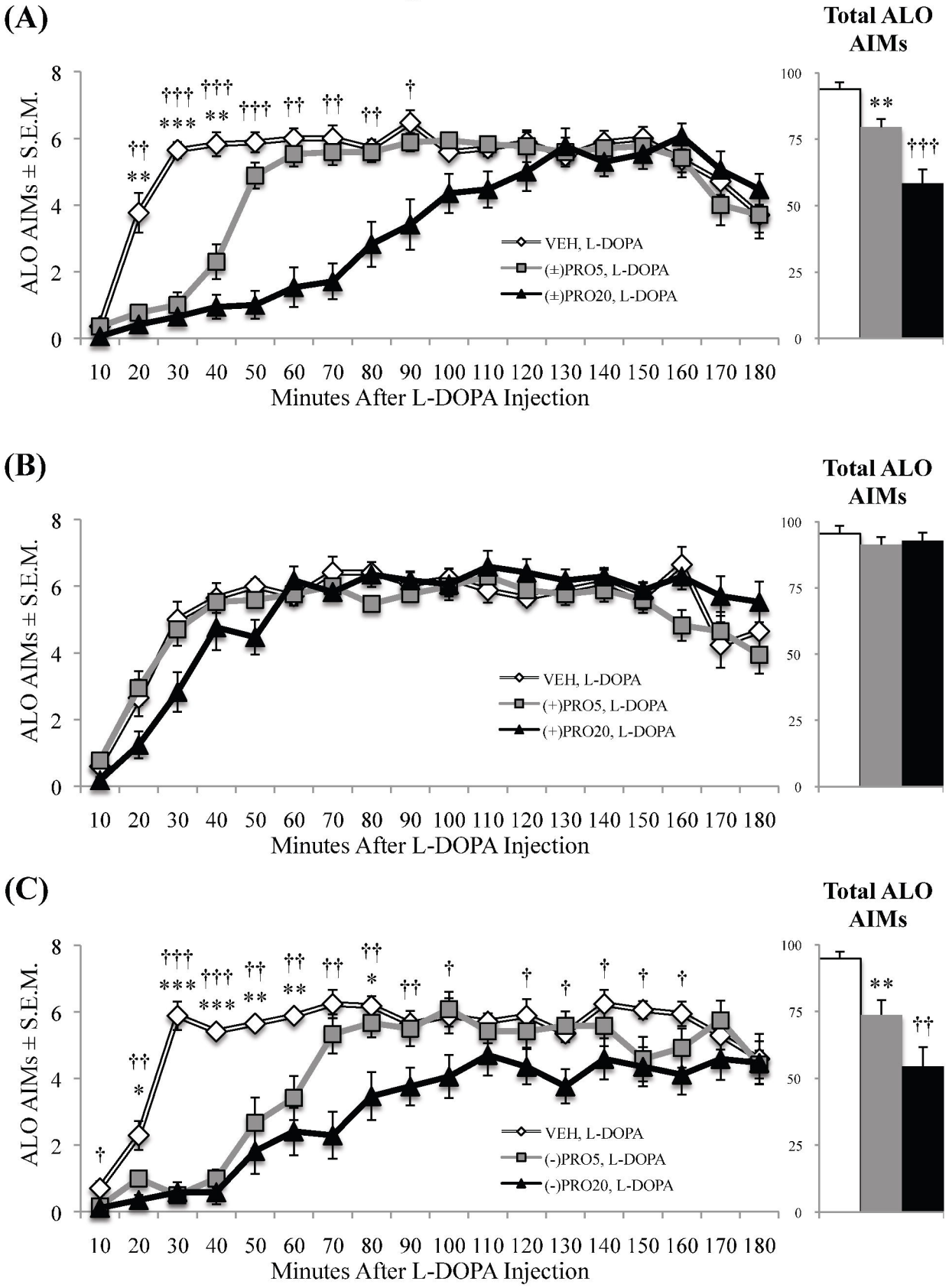

Figure 5

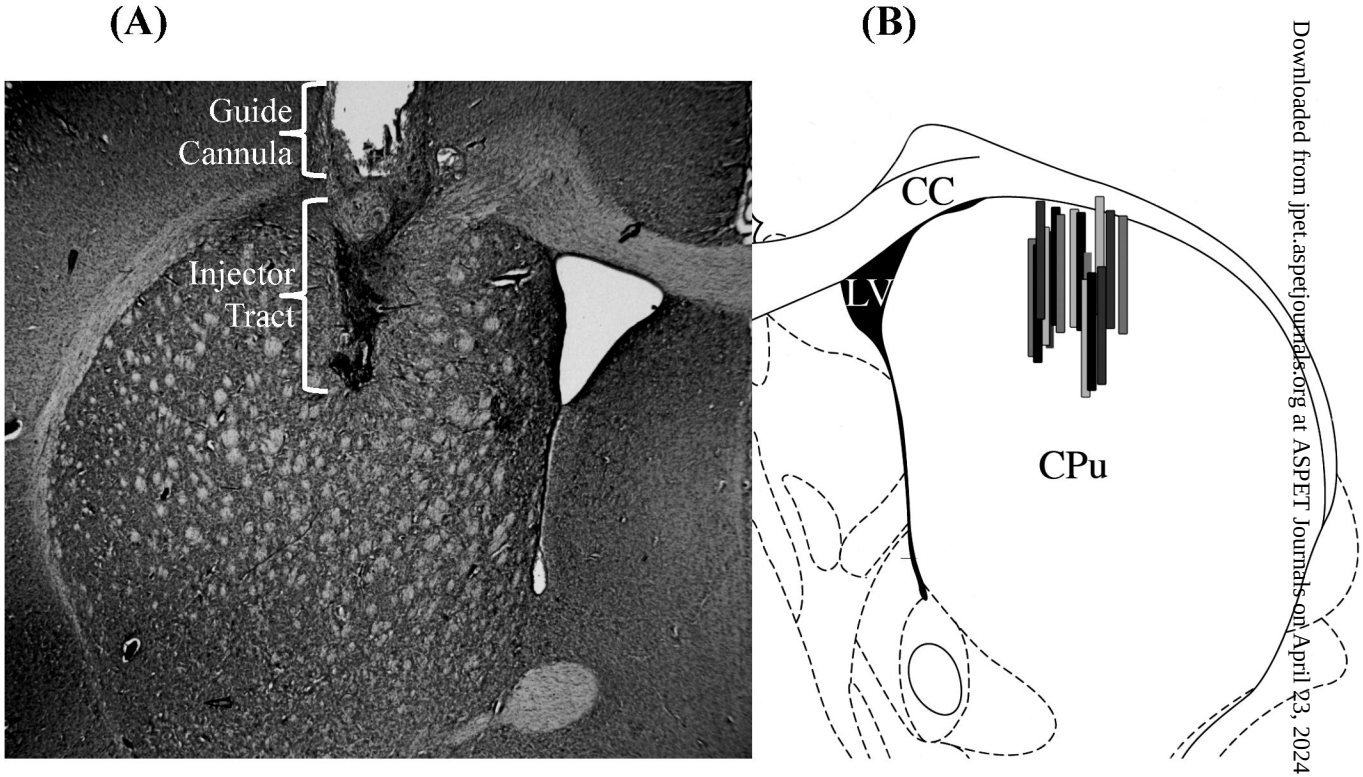

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Figure 6