

Monoamine transporter contributions to L-DOPA effects in hemi-parkinsonian rats



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ABSTRACT

L-DOPA is the standard treatment for Parkinson's disease (PD), but chronic treatment typically leads to abnormal involuntary movement or dyskinesia (LID) development. Although poorly understood, dyskinetic mechanisms involve a complex interaction between the remaining dopamine system and the semi-homologous serotonin and norepinephrine systems. Serotonin and norepinephrine transporters (SERT and NET, respectively) have affinity for dopamine uptake especially when dopamine transporters (DAT) are scant. Monoamine reuptake inhibitors have been reported to modulate L-DOPA's anti-parkinsonian effects, but DAT, SERT, and NET's contribution to dyskinesia has not been well delineated. The current investigation sought to uncover the differential expression and function of DAT, SERT, and NET in the L-DOPA-treated hemi-parkinsonian rat. Protein analysis of striatal monoamine transporters in unilateral sham or 6-hydroxydopamine-lesioned rats treated with L-DOPA (0 or 6 mg/kg) showed lesion-induced DAT loss and L-DOPA-induced gain in SERT:DAT and NET:DAT ratios in lesioned rats which positively correlated with dyskinesia expression, suggesting functional shifts among monoamine transporters in the dyskinetic state. SERT blockade with citalopram (3, 5 mg/kg) reduced LID while DAT and NET blockade with GBR-12909 (5, 10 mg/kg) and nisoxetine (5, 10 mg/kg), respectively, mildly exacerbated dyskinesia expression. Transporter inhibition did not significantly alter L-DOPA's ability to reverse motor deficit. Overall, DA and DAT loss with L-DOPA treatment appear to precipitate gain in SERT and NET function. Strong correlations with LID and direct behavioral comparisons of selective transporter blockade reveal novel implications for SERT, DAT, and NET as potential biomarkers and therapeutic targets in the hemi-parkinsonian model and dyskinetic PD patients.

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1. Introduction

Due to the growing elderly population in the United States, the prevalence rate of Parkinson's disease (PD) diagnosis is expected to drastically climb (Kowal et al., 2013). Since the progressive deterioration of voluntary movement is typically due to nigrostriatal dopamine (DA) loss, the gold standard treatment has been DA replacement therapy with the precursor L-DOPA (Smith et al., 2012). However, chronic treatment precipitates motor fluctuations in the majority of PD patients chief of which are disabling abnormal involuntary movements (AIMs), referred to as L-DOPA-induced dyskinesia (LID; Ahlskog and Muenter, 2001). As novel treatment strategies are sought to improve L-DOPA therapy, there

remains an urgent need to better understand the mechanisms contributing to L-DOPA's efficacy and dyskinetic liability.

Several new mechanisms and thus potential targets have emerged as research has identified compensatory neuroplasticity in response to DA loss, including monoamine transporters capable of extracellular DA uptake like the serotonin (5-HT) and norepinephrine (NE) transporters (SERT and NET, respectively; Arai et al., 2008; Kannari et al., 2006; Nirenberg et al., 1996). Reduced DA transporter (DAT) levels predominantly resulting from nigrostriatal degeneration and subsequent L-DOPA treatment may exacerbate LID severity by prolonging receptor exposure to DA (Adams et al., 2005; Cai et al., 2012; Kraemmer et al., 2014; Sossi et al., 2009; Troiano et al., 2009). Changes in striatal SERT levels in PD are less consistent but likely reflect 5-HT cell degeneration in some patients. Interestingly, compensatory SERT increases have been reported with severe DAT loss (Politis et al., 2010; Stansley and

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Nomenclature

PD	Parkinson's disease
DA	dopamine
AIMs	abnormal involuntary movements
LID	L-DOPA-induced dyskinesia
DAT	dopamine transporter
5-HT	serotonin
SERT	serotonin transporter
NE	norepinephrine
NET	norepinephrine transporter
6-OHDA	6-hydroxydopamine
FAS	forepaw adjustment steps
L-DOPA	L-DOPA methyl ester
Benserazide	DL-serine 2-(2,3,4-trihydroxybenzyl) hydrazine hydrochloride
ALO	axial limb orolingual
HPLC	high performance liquid chromatography
GBR	GBR 12909
NIS	nisoxetine
CIT	citalopram
M.A.D.	median absolute deviation
S.E.M.	standard error mean

Yamamoto, 2014; Strecker et al., 2011). More uniform are the effects of L-DOPA treatment, which increases SERT in dyskinetic PD patients and animal models (Politis et al., 2014; Roussakis et al., 2016; Rylander et al., 2010). Despite pronounced NE neuronal loss in PD, changes in NET expression are inconsistent where striatal NET is increased but locus coeruleus NET is reduced (Chotibut et al., 2014, 2012; Marien et al., 2004; McMillan et al., 2011; Zarow et al., 2003). That said, NET has the ability to regulate synaptic L-DOPA-derived DA and therefore could modify LID (Arai et al., 2008; Moron et al., 2002). Although changes in striatal DAT, SERT, and NET expression have been reported, coincident assessment of how DA-depletion and L-DOPA treatment impacts these transporters provides a unique view of their shared role in DA uptake and its impact on behavior.

In recent years there has been renewed interest in targeting monoamine transporters for parkinsonian motor symptoms and LID (Chotibut et al., 2014; Conti et al., 2016; Devos et al., 2008; Paumier et al., 2015). Pharmacological DAT blockade has been shown to increase L-DOPA's hyperlocomotive effects (Dekundy et al., 2015; Sossi et al., 2009; Troiano et al., 2009). Growing evidence has implicated SERT in LID where SERT inhibitors have been associated with reduced LID development and expression while chronic SERT blockade does not appear to modify anti-parkinsonian benefit (Conti et al., 2016, 2014; Fidalgo et al., 2015; Mazzucchi et al., 2015). Although NET is capable of DA uptake, whether NET inhibitors provide anti-parkinsonian benefit and/or exacerbate LID remains an important question (Arai et al., 2008; Chotibut et al., 2014; Conti et al., 2016; Hansard et al., 2002). Although the effects of SERT inhibition on LID have been best characterized, the relative degree to which selective DAT, SERT, and NET blockade modifies L-DOPA's motor effects is generally unknown.

To better understand the unique roles of DAT, SERT, and NET during L-DOPA's dyskinetic and anti-parkinsonian outcomes, the current investigation sought to provide a direct comparison of striatal transporter expression and behavioral measures following selective transporter inhibition. First, DA depletion- and L-DOPA-

induced changes on striatal DAT, SERT, NET, SERT:DAT, and NET:DAT were characterized and correlated with LID. Next, the relative functional contribution of SERT, DAT, and NET in LID and motor performance was determined using selective monoamine transporter blockers in L-DOPA-primed, hemi-parkinsonian rats.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats were used (N = 47; approximately 2 months old and 225–250 g upon arrival; Harlan Farms, USA). Rats were housed in plastic cages (22 cm high, 45 cm deep, and 23 cm wide) and given free access to standard lab chow (Rodent Diet 5001; Lab Diet, Brentwood, MO, USA) and water. The colony room was kept on a 12 h light/dark cycle (light on at 0700 h) and maintained at 22–23 °C. Rats were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of Binghamton University and the "Guide for the Care and Use of Laboratory Animals" (Institute for Laboratory Animal Research, Crossgrove and Fletcher, 2011).

2.2. Medial forebrain bundle 6-hydroxydopamine surgery

One week after arrival, rats received either unilateral sham (n = 12) or 6-hydroxydopamine (6-OHDA; n = 35) lesions of the left medial forebrain bundle to destroy DA neurons. All rats received injections of Buprenex (buprenorphine HCl; 0.03 mg/kg, i.p.; Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA) as analgesic treatment 5 min pre-surgery. Desipramine HCl (25 mg/kg, i.p.; Sigma, St. Louis, MO, USA) was given to each rat 30 min prior to 6-OHDA injection to protect NE neurons. Rats were anesthetized with inhalant isoflurane (2–3%; Sigma) in oxygen (1000 cc/min) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). The coordinates for 6-OHDA injections were AP: –1.8 mm, ML: +2.0 mm, DV: –8.6 mm relative to bregma, with the incisor bar positioned 5.0 mm below the interaural line (Paxinos and Watson, 1998). After drilling a small hole in the skull above the injection site, a 10 µL Hamilton syringe attached to a 26 gauge needle was used to deliver 4 µL of 6-OHDA (3 µg/µL; Sigma; (Conti et al., 2014)) dissolved in 0.9% NaCl + 0.1% ascorbic acid at a rate of 2 µL/min. The needle was withdrawn 5 min later to allow the drug to disperse. Post-surgery, rats were pair-housed in clean cages and provided with soft chow, fruit, and saline as needed to facilitate recovery.

2.3. Pharmacological treatments and experimental procedure

2.3.1. Experiment 1: effects of lesion and L-DOPA treatment on striatal transporter expression

As shown in Figs. 1A and 3 weeks post-surgery, 6-OHDA-lesioned rats' (n = 16) forepaw adjustment step (FAS) habituation performance was established to counterbalance treatment groups based on parkinsonian motor deficit. Then lesioned and sham (n = 12) rats were given 1 week of daily vehicle or L-DOPA methyl ester (L-DOPA; 12 mg/kg, s.c.; Sigma) + DL-serine 2-(2,3,4-trihydroxybenzyl) hydrazine hydrochloride (benserazide; 15 mg/kg, s.c.; Sigma) dissolved in 0.9% NaCl + 0.1% ascorbic acid to prime for maximal LID (Dupre et al., 2011). Rats were rated for axial, limb, and orolingual (ALO) AIMs (see description below) on day 7 with inclusion criteria set at ALO AIMs scores ≥ 50. During a 1 week wash out period, rats were tested on FAS to establish baseline motor performance. Rats were then treated with either vehicle or L-DOPA (6 mg/kg + benserazide 15 mg/kg, s.c.) daily for 2 weeks. Rats were tested on LID expression on days 1, 8, and 14 and on FAS (off

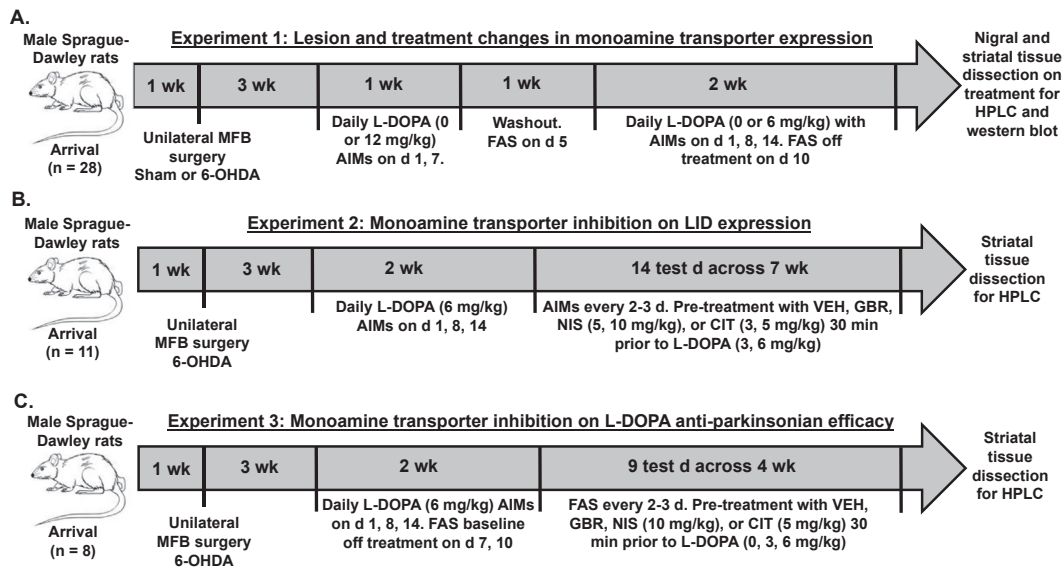


Fig. 1. Experimental timeline and design. In experiment 1(A), rats were either given unilateral sham or 6-OHDA lesions then, after 3 weeks, primed for 1 week with daily ι -DOPA (12 mg/kg) and rated for abnormal involuntary movements (AIMs) on day 1 and 7. After a 1 week washout period where rats were tested on the forepaw adjustment steps (FAS) test on day 5, rats were given daily vehicle or ι -DOPA (6 mg/kg) for 2 weeks then tested for AIMs on day 1, 8, and 14. FAS testing on day 10 occurred off treatment. After testing, rats were sacrificed 1 h post-treatment then nigral and striatal tissue was dissected for monoamine analysis via HPLC and SERT, DAT, and NET quantification via western blotting, respectively. In experiment 2 (B) rats were given unilateral 6-OHDA lesions. Three weeks later, rats were primed with daily ι -DOPA (6 mg/kg) for 2 weeks with AIMs ratings on days 1, 8, and 14. Across 14 test days and using a within-subjects counterbalanced design, rats were pretreated with VEH, GBR, NIS (5, 10 mg/kg), or CIT (3, 5 mg/kg) 30 min later. AIMs and rotations were rated for the following 3 h. Each test day was separated by at least 2 days to allow for drug washout. After the last test day, striatal tissue was dissected for monoamine analysis via HPLC. In experiment 3 (C) rats were lesioned and ι -DOPA primed with AIMs on day 1, 8, and 14 and FAS baseline off treatment on day 7 and 10. Across 9 test days and using a within-subjects counterbalanced design, rats were pretreated with VEH, GBR, NIS (10 mg/kg), or CIT (5 mg/kg) 30 min prior to ι -DOPA (0, 3, 6 mg/kg). One hour later, rats were rated on motor performance with the FAS test. Test days were separated by at least 2 days and striatal tissue dissected for HPLC after last test day.

treatment) on day 10. On day 15, rats were sacrificed 1 h post-treatment and bilateral nigral and striatal tissue was dissected and frozen for high performance liquid chromatography (HPLC) analysis of monoamine levels and western blot analysis of SERT, DAT, and NET expression, respectively.

2.3.2. Experiment 2: effects of selective transporter inhibitors on LID in severely DA-lesioned rats

As Fig. 1B describes, 3 weeks post-surgery, rats ($n = 11$) were primed with ι -DOPA (6 mg/kg) daily for 2 weeks to produce stable dyskinesia (Conti et al., 2014). Inclusion criteria was set at ALO AIMs scores ≥ 25 on priming day 14. After priming, rats were pre-treated with either vehicle (20% dimethyl sulfoxide (DMSO) + 80% distilled water, s.c.) or the respective DAT, NET, or SERT blocker: GBR 12909 (GBR; 5, 10 mg/kg, s.c.; Sigma; Lane et al., 2005), nisoxetine (NIS; 5, 10 mg/kg, s.c.; Sigma; Davids et al., 2002), or citalopram (CIT; 3, 5 mg/kg, s.c.; LKT Laboratories Inc, St. Paul, MN; Bishop et al., 2012) using a within-subjects counterbalanced design where every pre-treatment was given on each test day. Thirty min following pre-treatment, rats were injected with ι -DOPA (3, 6 mg/kg) and AIMs were rated. Test days were separated by 2–3 days to account for active transporter blocker metabolites. Therefore, rats were tested on each pre-treatment in a randomized order before ι -DOPA across 14 separate test days that spanned 7 weeks. Forty-eight hours after the last test day, rats were killed off treatment and bilateral striatal was dissected and frozen for HPLC analysis of DA depletion.

2.3.3. Experiment 3: effects of selective transporter inhibitors on ι -DOPA motor benefit in severely DA-lesioned rats

As shown in Fig. 1C, 3 weeks post-surgery, rats' ($n = 8$) baseline FAS performance was established after which ι -DOPA (6 mg/kg) was given daily for 2 weeks. Using the same within-subjects counterbalanced design as experiment 2, rats were pre-treated with vehicle, GBR (10 mg/kg), NIS (10 mg/kg), or CIT (5 mg/kg)

30 min prior to ι -DOPA (0, 3, 6 mg/kg). An hour after ι -DOPA treatment, rats were tested on FAS to evaluate motor performance. Test days were separated by 2–3 days. Therefore, rats were tested on each pre-treatment before ι -DOPA across 9 test days that spanned 4 weeks. At the end of the study rats were sacrificed and bilateral striata were dissected for HPLC analysis of DA depletion.

2.4. General methods

2.4.1. Abnormal involuntary movements (AIMs)

Rats were monitored for rodent dyskinesia using a procedure previously described (Bishop et al., 2012; Lundblad et al., 2002). During testing (0900–1700 h), rats were placed in clear plastic cylinders (22.2 cm diameter, 25.4 cm height; Thermo Fisher Scientific, Rochester, NY) immediately after ι -DOPA injection. After injections, a trained observer blind to treatment conditions recorded AIMs involving axial, limb, and orolingual (ALO) regions. “Axial” AIMs include dystonic twisting of the neck and torso directed toward the side of the body that is contralateral to lesion. “Limb” AIMs refer to excessive and purposeless movements of the forelimb contralateral to lesion. “Orolingual” AIMs are repetitive openings and closings of the jaw as well as multiple tongue protrusions that are not associated with eating or grooming. Each ALO AIM was rated and given a severity score (0–4) for 1 min every 10 min for 3 h: 0, not present; 1, present for less than 30 s or half the time; 2, present for 30–59 s or majority of the time; 3, present for the whole 60 s but interrupted by stimulus (tap on the cylinder); or 4, present for the whole 60 s and was not interrupted by stimulus. A maximum ALO score at a given time point was 12 and ALO scores were summed to create a single ALO AIMs score for data analysis.

2.4.2. Forepaw adjustment steps test (FAS)

The FAS test was used to measure rodent forelimb akinesia since motor deficit due to DA loss and therapeutic reversal has been

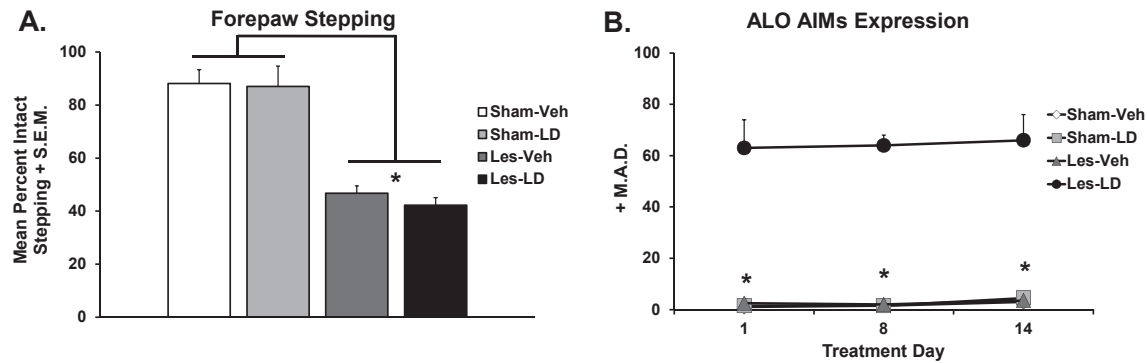


Fig. 2. Parkinsonian motor deficit (A) and α -DOPA-induced abnormal involuntary movements (AIMs) expression (B) in sham or 6-OHDA lesioned rats. Rats with either unilateral sham or 6-OHDA lesions were treated daily with either vehicle or α -DOPA (LD; 6 mg/kg; s.c.) for 14 days. On days 1, 8, and 14, axial, limb, and orolingual (ALO) AIMs were recorded for 3 h following LD. Total ALO values are expressed as medians (AIMs \pm median absolute difference; M.A.D.). On day 10, the forepaw adjustment steps (FAS) test was used to evaluate motor deficit. Values (as means \pm standard mean error; S.E.M.) are expressed as percent intact. ALO AIMs significant differences were determined by non-parametric Kruskal-Wallis ANOVAs within day and Mann-Whitney U post-hoc tests. * $p < 0.05$ vs Les-LD. FAS significant differences were determined by a 2×2 (lesion \times treatment) between-subjects ANOVA. When appropriate, motor performance differences were analyzed with Tukey HSD post-hocs. * $p < 0.05$ vs Sham.

demonstrated in hemi-parkinsonian rats (Chang et al., 1999; Olsson et al., 1995). An experimenter blind to treatment held the rat's hindlimbs and one forepaw so that weight would be imposed on the other forepaw. Rats were moved laterally across a table at a rate of 90 cm/10 s. Each test consisted of 6 trials for each forepaw: 3 trials each for forehand (adjusting for movement toward the body) and backhand (adjusting for movement away from the body) directions. Data is presented as "percent intact" (sum of lesioned forepaw steps divided by sum of intact forepaw steps multiplied by 100) indicating the degree of disability seen in the lesioned paw. Lower percent intact scores indicate greater parkinsonian motor impairment.

2.4.3. Neurochemical analyses with high performance liquid chromatography (HPLC)

Upon experimental completion, rats were rapidly decapitated and tissue was dissected and frozen at -80°C for later analysis for monoamine levels via HPLC with electrochemical detection. Reverse-phase HPLC was performed on bilateral nigral tissue from rats in experiment 1 and bilateral striatal tissue obtained from rats in experiments 2 and 3, according to a previous protocol (Eskow Jaunarajs et al., 2011; Kilpatrick et al., 1986). The limit of detection was 10^{-10} M for monoamines. The final oxidation current values were plotted on a standard curve of known concentrations from 10^{-6} M to 10^{-9} M, adjusted to respective tissue weights and expressed as pg of monoamine per mg tissue.

2.4.4. Sample preparations and western blot analysis

Using striatal tissue obtained from rats in experiment 1, lesion and treatment effects on DAT, SERT, and NET expression were investigated using methods previously described (Santerre et al., 2014; Werner et al., 2011). For whole-cell lysates (total expression), following dissection, striatal samples were homogenized in a mixture of 1% sodium dodecyl sulfate, 1 mM ethylenediaminetetraacetic acid, and 10 mM of Tris (Grosshans et al., 2002). Protein concentrations of all samples were quantified using a bicinchoninic acid method. Samples were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using Novex Tris-Glycine gels (8–16%) and transferred to polyvinylidene difluoride membranes (Invitrogen, Carlsbad, CA, USA). Membranes were probed with antibodies for the DAT (Millipore, Lake Temecula, CA, USA), NET, and SERT (Santa Cruz Biotechnology Inc., Dallas, TX, USA) proteins. Blots were then exposed to an antibody directed against β -actin (Millipore) for normalization. Secondary antibodies

were obtained from Thermo Scientific (Waltham, MA). Samples were run in duplicate or triplicate and averaged. All bands were detected by enhanced chemiluminescence under non-saturating conditions (GE Healthcare, Piscataway, NJ, USA) and exposed to x-ray films and analyzed using NIH Image J.

2.5. Data analyses

All AIMs data (expressed as medians + median absolute difference; M.A.D.) were analyzed by non-parametric statistics. For within-subjects designs, the Friedman test was employed with Wilcoxon post-hoc tests. For between-subjects designs, the Kruskal-Wallis ANOVA with Mann-Whitney U post-hoc test was used to determine significant differences. All FAS data (presented as mean percent intact + standard mean error; S.E.M.) were measured by ANOVAs with Tukey HSD post-hoc analyses. All HPLC monoamine and metabolite data (mean pg/mg of tissue \pm S.E.M.) as well as western blot data (expressed as mean percent control + S.E.M.) were analyzed using T-tests or ANOVAs with Tukey HSD post hoc tests. Pearson correlations were used for comparing SERT:DAT and NET:DAT ratios with day 14 AIMs data from experiment 1. Analyses for all experiments were performed with 'Statistica software '98 (Statsoft Inc., Tulsa, OK, USA) and alpha was set at $p < 0.05$.

3. Results

3.1. Experiment 1: DA lesion and α -DOPA treatment differentially modulate striatal monoamine transporter expression

Changes in striatal SERT, DAT, and NET expression as well as SERT:DAT and NET:DAT ratios were quantified via western blot using tissue from unilateral sham or DA-lesioned rats that received either daily vehicle or α -DOPA (6 mg/kg) for 2 weeks. On day 10 prior to receiving treatment, rats were examined for motor deficit with FAS. A main effect of lesion was found where DA-depleted rats exhibited ~60% deficit in stepping compared to sham rats ($F_{1,23} = 84.39$, $p < 0.05$; Fig. 2A). As expected, α -DOPA-treated, DA-lesioned rats demonstrated significant ALO AIMs on days 1, 8, and 14 (median = 66; $H_{3,27} = 87.15$, $p < 0.05$; Fig. 2B).

After the final test day, rats were sacrificed an hour after treatment and striatal tissue was dissected for whole cell SERT, DAT, and NET total protein levels via western blot. β -actin levels were equivalent across groups within blots, therefore it was used as a housekeeper for SERT, DAT, and NET levels. To determine SERT:DAT

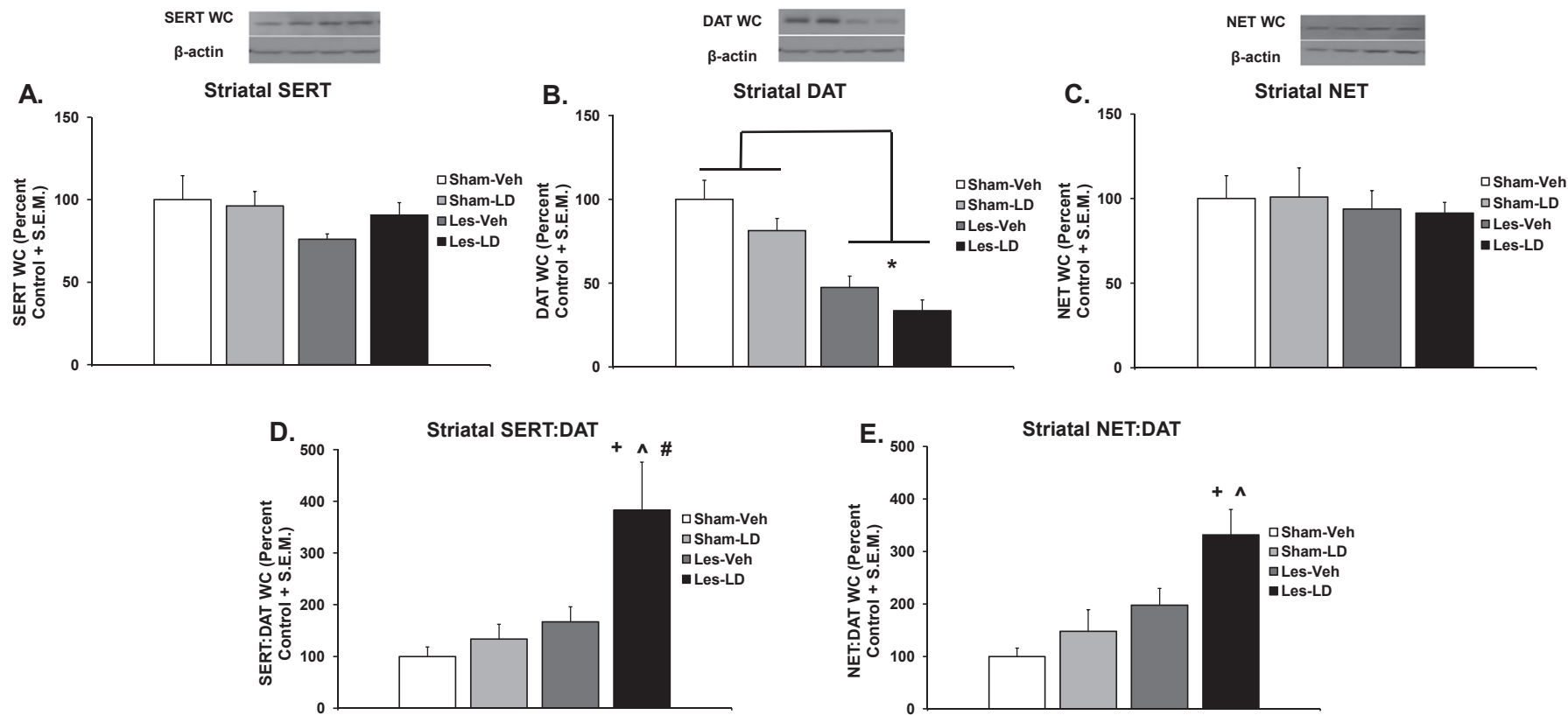


Fig. 3. Total monoamine transporter expression in intact or 6-OHDA lesioned striatum of vehicle- or l-DOPA-treated rats. Rats with either unilateral sham or 6-OHDA lesions were treated daily with either vehicle or l-DOPA (LD; 6 mg/kg; s.c.) for two weeks. Rats were then sacrificed 1 h following respective treatment and bilateral striatal tissue dissected for whole cell SERT (A), DAT (B), and NET (C) expression as well as SERT:DAT (D) and NET:DAT ratios (E). Representative blots are shown for SERT, DAT, and NET. Values (as means \pm standard mean error; S.E.M.) are expressed as percent control. Significant differences were determined by 2×2 (lesion \times treatment) between-subjects ANOVAs within each transporter. When appropriate, expression differences were analyzed with Tukey HSD post-hocs. * $p < 0.05$ vs Sham; + $p < 0.05$ vs Sham-Veh; $\wedge p < 0.05$ vs Sham-LD; # $p < 0.05$ vs Les-Veh.

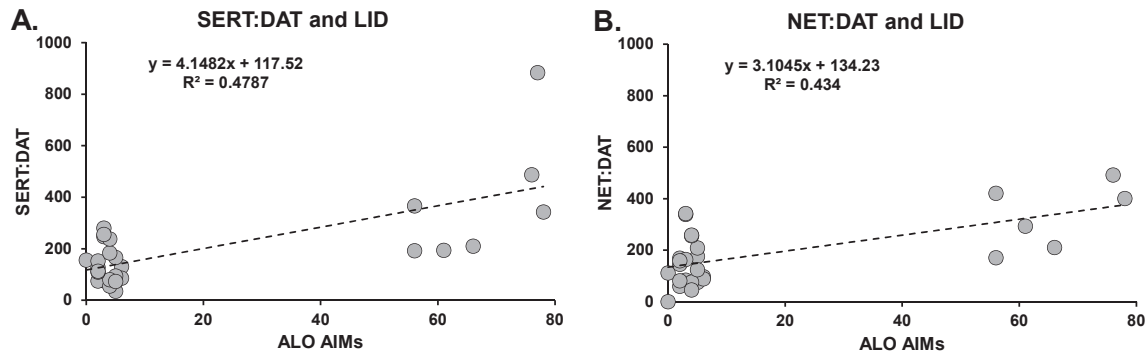


Fig. 4. Striatal SERT:DAT (A) and NET:DAT (B) ratios correlate with *l*-DOPA-induced abnormal involuntary movements (AIMs) expression. Rats with either unilateral sham or 6-OHDA lesions were treated daily with either vehicle or *l*-DOPA (LD; 6 mg/kg; s.c.) for two weeks. Rats were then sacrificed 1 h following respective treatment and bilateral striatal tissue dissected for whole cell SERT:DAT and NET:DAT ratios. Ratios were correlated with axial, limb, and orolingual (ALO) AIMs scores from day 14 of priming (see Fig. 2B).

and NET:DAT, optical densities of SERT and NET were divided by DAT and then normalized to the sham and vehicle-treated group which served as the control. Fig. 3 shows representative blots for SERT (A), DAT (B), and NET (C). A main effect of lesion was seen for DAT expression where ~50% loss was seen compared to intact striatal tissue ($F_{1,23} = 38.39$, $p < 0.05$; Fig. 3B). While neither lesion nor *l*-DOPA treatment significantly affected striatal SERT or NET alone, although ~25% lesion-induced reduction of SERT was observed in vehicle-treated rats, main effects of lesion and treatment were seen for SERT:DAT and NET:DAT ratios. Planned comparisons demonstrated that DA lesion with *l*-DOPA increased SERT:DAT compared to sham-vehicle, *l*-DOPA, and lesion-vehicle treated rats ($F_{1,23} > 5.31$, $p < 0.05$; Fig. 3D) while NET:DAT was greater than sham rats ($F_{1,22} > 5.95$, $p < 0.05$; Fig. 3E). In fact, higher SERT:DAT ($r = 0.69$; Fig. 4A) and NET:DAT ($r = 0.66$; Fig. 4B) ratios significantly correlated with more severe ALO AIMs expression (both $p < 0.05$).

To verify DA depletion for experiment 1, lesioned nigral tissue was collected and DA levels were compared between lesion and treatment groups. As expected, lesion-vehicle treated rats had less DA (93.14 pg/mg \pm 28.8) than sham-vehicle (365.52 pg/mg \pm 24.7) and sham-*l*-DOPA treated rats (478.37 pg/mg \pm 68.9). *l*-DOPA treatment slightly elevated nigral DA levels in lesioned rats (220.82 pg/mg \pm 33.2) though this remained significantly less than sham-*l*-DOPA treated rats ($F_{1,23} > 8.746$, all $p < 0.05$).

3.2. Experiment 2: SERT blockade uniquely attenuates *l*-DOPA-induced AIMs

In order to determine the effect of systemic DAT, SERT, and NET inhibitors on established LID, rats rendered dyskinetic by chronic *l*-DOPA began counterbalanced treatments of vehicle, GBR (5, 10 mg/kg), CIT (3, 5 mg/kg), or NIS (5, 10 mg/kg) 30 min prior to low or moderate doses of *l*-DOPA (3, 6 mg/kg). Statistical analyses revealed that both doses of CIT attenuated AIMs induced by both doses of *l*-DOPA, with a floor effect occurring in 3 mg/kg *l*-DOPA and a dose-dependent effect observed with 6 mg/kg *l*-DOPA (Fig. 5A, D). GBR had no effect with 3 mg/kg *l*-DOPA but GBR (10 mg/kg) prompted an earlier onset of peak ALO AIMs induced by 6 mg/kg *l*-DOPA (Fig. 5B, E). NIS (10 mg/kg) extended LID expression with 3 mg/kg *l*-DOPA and NIS (5 mg/kg) did so with 6 mg/kg *l*-DOPA (Fig. 5C, F). Interestingly, NIS (10 mg/kg) did not exacerbate total ALO AIMs induced by moderate *l*-DOPA doses (all $\chi^2 > 7.1$; all $p < 0.05$).

3.3. Experiment 3: selective transporter blockade minimally affects *l*-DOPA's anti-parkinsonian benefits

To examine whether acute selective monoamine transporter

blockade modifies *l*-DOPA's improvement on lesion-induced motor deficit, dyskinetic rats received counterbalanced treatment of vehicle or high doses of GBR (10 mg/kg), CIT (5 mg/kg), or NIS (10 mg/kg) 30 min prior to *l*-DOPA (0, 3, 6 mg/kg). Motor performance was measured using the FAS test 60 min post *l*-DOPA, a time when *l*-DOPA's effects are often pronounced. As shown in Fig. 6, a significant improvement was seen with 6 mg/kg *l*-DOPA ($F_{2,14} = 6.25$, $p < 0.05$). Though there were some trends toward improved stepping, no significant effects were seen with any transporter blocker and 3 mg/kg *l*-DOPA. While GBR and NIS did not affect 6 mg/kg *l*-DOPA anti-parkinsonian efficacy ($F_{2,14} > 5.45$, all $p < 0.05$; Fig. 6E, F) pre-treatment with acute CIT (Fig. 6D) produced an intermediate response that did not differ from vehicle-vehicle or vehicle-*l*-DOPA.

Upon completion of experiments 2 and 3, bilateral striatal tissue was collected and analyzed using HPLC to verify DA lesion. DA levels in the lesioned striatum were significantly reduced by ~98–99% when compared to the intact striatum. For experiment 2, intact DA levels were an average of 6253.28 pg/mg and lesioned DA levels were an average of 105.17 pg/mg ($t_{10} = 28.33$, $p < 0.05$). For experiment 3, intact DA levels were an average of 7623.9 pg/mg and lesioned DA levels were an average of 94.2 pg/mg ($t_7 = 22.58$, $p < 0.05$).

4. Discussion

Increasingly, research has suggested that DAT, SERT, and NET expression levels are distinctly affected by severe parkinsonian DA loss and subsequent *l*-DOPA treatment; however, a direct comparison of these transporter actions in *l*-DOPA's motor effects has been lacking. This work systematically investigated DAT, SERT, and NET expression and function in order to gain insight into their respective contributions in *l*-DOPA-induced behaviors. For the first time, our findings showed that DA loss and *l*-DOPA treatment promoted a functional shift from DAT to both SERT and NET and that this shift was related to LID expression. Moreover, SERT inhibition was confirmed to have the greatest anti-dyskinetic benefit while NET and DAT inhibition mildly promoted *l*-DOPA-induced locomotive behavior. Collectively, these findings provide significant support for potential biomarkers for *l*-DOPA's therapeutic response and FDA-approved treatment strategies targeting monoamine transporters with various affinities to improve *l*-DOPA therapy for dyskinetic PD patients.

Although DAT naturally declines in aging, in PD, precipitous DAT loss is strongly associated with DAergic cell loss, disease duration, and motor symptom severity (Buddhala et al., 2015; Cai et al., 2012; Kerényi et al., 2003; Liu et al., 2015). In the current investigation, the 6-OHDA lesion reduced striatal DAT by ~50% corresponding

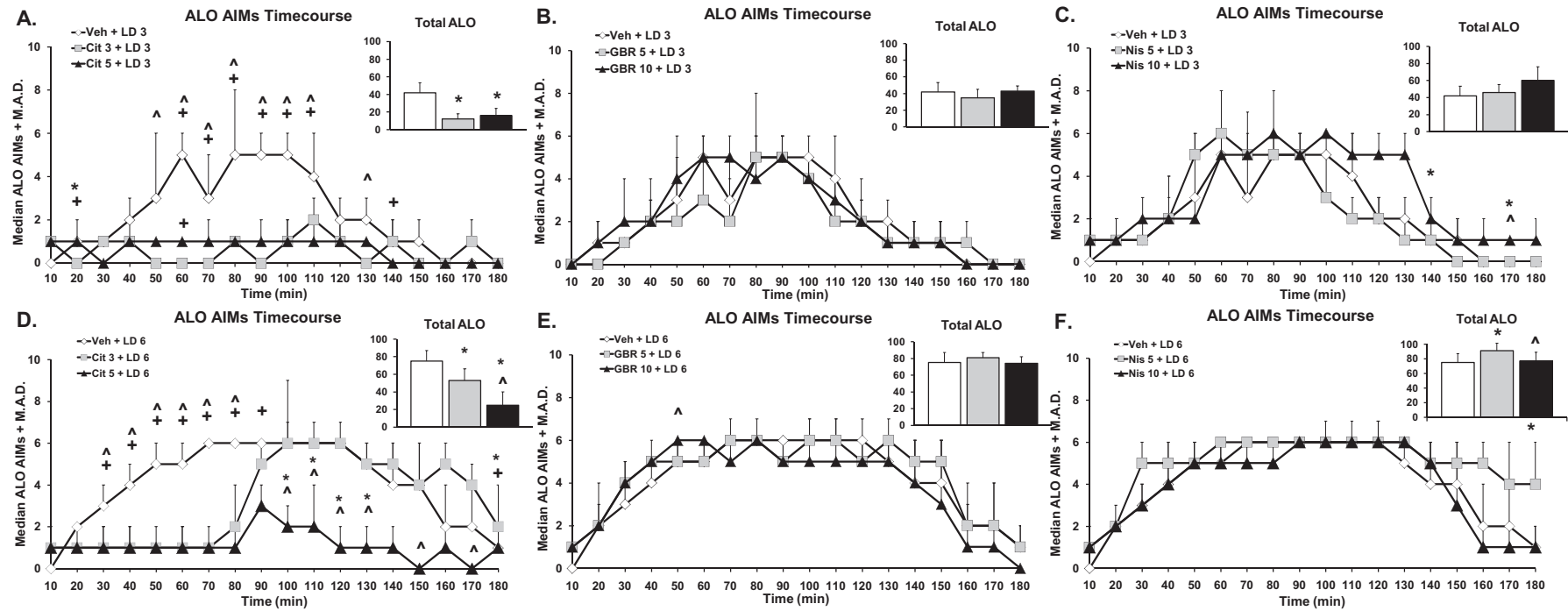


Fig. 5. Selective SERT (A, D), DAT (B, E), and NET (C, F) blockade modifies established *l*-DOPA-induced abnormal involuntary movements (AIMs) expression. Rats were *l*-DOPA-primed for 14 days and axial, limb, and orolingual (ALO) AIMs were recorded. Using a within-subjects, counterbalanced design, rats were treated with vehicle, citalopram (3 or 5 mg/kg, s.c.), GBR 12909 (5 or 10 mg/kg, s.c.), and nisoxetine (5 or 10 mg/kg, s.c.) 30 min before *l*-DOPA (3 or 6 mg/kg, s.c.) + benserazide (15 mg/kg, s.c.). ALO AIMs were evaluated for 3 h after *l*-DOPA. Timecourse and total ALO values are expressed as medians (AIMs \pm median absolute difference; M.A.D.). Significant differences were determined by non-parametric Friedman ANOVAs with Wilcoxon Matched Pairs post-hoc tests. * $p < 0.05$ vs Veh + LD, ^ $p < 0.05$ vs low dose + LD, + $p < 0.05$ vs high dose + LD.

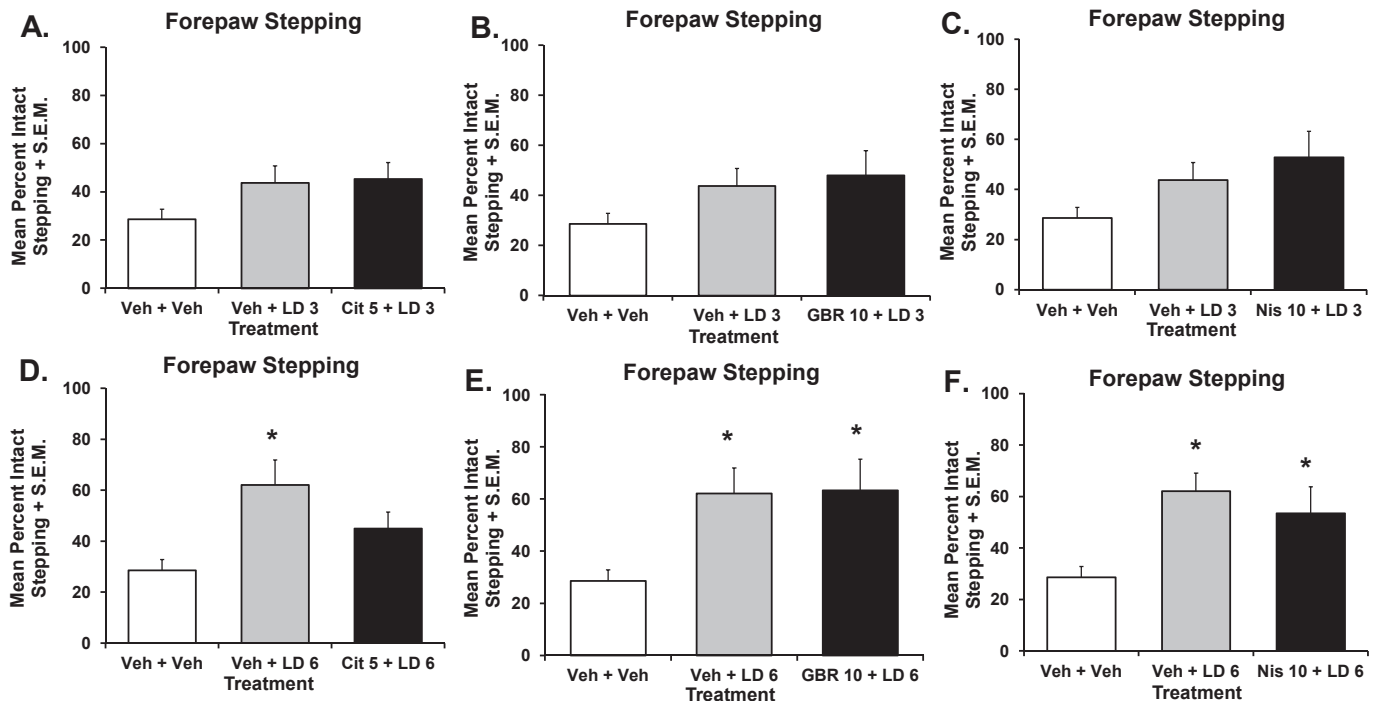


Fig. 6. Effect of selective SERT (A, D), DAT (B, E), and NET (C, F) inhibition on parkinsonian motor deficit on the forepaw adjustment steps (FAS) test. In a counterbalanced within-subjects design, L-DOPA-primed rats were treated with vehicle, citalopram (5 mg/kg, s.c.), GBR 12909 (10 mg/kg, s.c.), and nisooxetine (10 mg/kg, s.c.) 30 min prior to L-DOPA (0, 3, or 6 mg/kg, s.c.) + benserazide (15 mg/kg, s.c.). FAS was evaluated 60 min after L-DOPA. Values (as means \pm standard error; S.E.M.) are expressed as overall percent intact. Significant differences were determined by repeated measure ANOVAs within each treatment condition. When appropriate, motor performance differences were analyzed with Tukey HSD post-hocs. * $p < 0.05$ vs Veh + Veh.

with motor deficit when compared with sham-lesioned rats, regardless of prior L-DOPA treatment. Some have suggested that lower DAT levels independent of DA cell loss may reflect beneficial compensatory processes that prolong synaptic DA signaling (Hansard et al., 2002; Lee et al., 2000; Madras et al., 2006; Sossi et al., 2009). Interestingly however, there is evidence that patients displaying dyskinesia have even less DAT than non-dyskinetic patients (Cai et al., 2012; Hong et al., 2014). In such cases DAT loss may lead to reduced DA uptake linked to wearing-off, LID development, and even non-motor complications in PD patients (Kiferle et al., 2014; Lohle et al., 2016; Santangelo et al., 2015). When we tested the effects of the DAT inhibitor in L-DOPA-treated rats, it mildly shifted peak LID onset to an earlier time-point without affecting L-DOPA's anti-parkinsonian efficacy (Figs. 5E and 6E). Other DAT blockers have also been shown to accentuate L-DOPA-induced locomotive behaviors (Dekundy et al., 2015). Addiction liability is a potential concern regarding DAT inhibitors, however this is only at high doses (De Vries et al., 1999; Glowa et al., 1995; Schenk, 2002). Research regarding DAT blockers as an adjunct to L-DOPA therapy is limited though future studies focusing on sub-threshold doses of combined DAT blockers and L-DOPA may delay LID development while alleviating akinetic motor symptoms.

SERT levels are often cited as a proxy for 5-HT neuronal integrity and can be affected by severe experimental-induced DA loss and contribute to PD pathophysiology (Ballanger et al., 2016; Hahn et al., 2014; Qamhawi et al., 2015). Although present results showed no significant effect of the 6-OHDA lesion on striatal SERT expression, past findings are inconsistent where no difference (Cheshire et al., 2015; Suwijn et al., 2013), progressive loss (Buddhala et al., 2015; Kerényi et al., 2003; Politis et al., 2010; Roussakis et al., 2016), or increases are found in PD patients or associated models (Strecker et al., 2011). While it is unclear how DA

loss influences SERT expression, increases in SERT have been shown in dyskinetic rat and primate models as well as in PD patients (Politis et al., 2014; Rylander et al., 2010). Even though prior L-DOPA treatment did not alter striatal SERT expression in the current study, a treatment-induced increase in SERT:DAT ratio that strongly correlated with LID was found in dyskinetic PD rats. This increase was not simply due to lesion-induced DAT reduction but depended upon DA depletion and L-DOPA treatment since the increase was not found in vehicle-treated, lesioned rats. Such a shift suggests compensatory mechanisms due to severe DAergic cell loss that are contingent upon L-DOPA-induced elevations in DA signaling. Elevated SERT:DAT in L-DOPA-treated, lesioned rats is remarkably consistent with recent clinical work showing increased putaminal SERT:DAT in dyskinetic versus non-dyskinetic PD patients (Lee et al., 2015; Roussakis et al., 2016). This consistency across species, from preclinical rodent model to PD patient, implicates SERT:DAT as a potential biomarker for dyskinesia.

Selectively blocking SERT conveyed dose-dependent anti-dyskinetic effects without observable hypo-locomotor side effects similar to other SERT inhibitor and tricyclic antidepressant intervention of LID in similar preclinical models (Bishop et al., 2012; Conti et al., 2016; Inden et al., 2012; Lindenbach et al., 2015). A possible therapeutic mechanism of SERT blockade on LID expression may be the coincident activation of inhibitory 5-HT_{1A} and 5-HT_{1B} receptors via increased extracellular 5-HT since stimulation of these receptors has led to LID relief while antagonizing 5-HT_{1A} partially reverses SERT inhibitor effects (Conti et al., 2014; Munoz et al., 2008; Politis et al., 2014). While SERT inhibition has minimal side effects and has not been shown to compromise L-DOPA's anti-parkinsonian efficacy when given acutely or chronically (Conti et al., 2014; Lindenbach et al., 2015), in the current experiment, the highest dose of CIT partially attenuated 6 mg/kg L-DOPA's

improvement on motor performance. Some evidence suggests that high acute doses of selective SERT inhibitors may initially interfere with L-DOPA benefit, however, with prolonged use, these effects dissipate suggesting dependency on treatment-induced plasticity (Benmansour et al., 1999; Conti et al., 2014; Fidalgo et al., 2015). Recent research has supported SERT blockade as a viable strategy for regulating LID expression though future studies investigating SERT and DAT interactions may lead to more optimal pharmacological co-therapies with L-DOPA (Huot et al., 2014, 2012).

While NET is found on cell bodies and terminals of locus coeruleus projection neurons to the basal ganglia, idiopathic or lesion-induced DA loss affects NET expression in a region dependent manner. The present results show no effect of 6-OHDA lesion on striatal NET expression but others have reported increased striatal NET in the PD rat model (Chotibut et al., 2012) and reduced locus coeruleus NET in PD patients (McMillan et al., 2011). Similar to SERT, L-DOPA treatment did not alter NET expression, but it did increase the NET:DAT ratio in the DA-depleted striatum which also correlated with LID suggesting compensatory shifts that are dependent upon DA lesion and L-DOPA treatment and not simply loss of DAT (Chotibut et al., 2014). To our knowledge, this is the first presentation of an increased NET:DAT that relates with dyskinesia, which suggests another potential biomarker for LID susceptibility. Selective NET inhibition mildly exacerbated LID with no effects seen in L-DOPA-induced motor improvement. Similarly, other NET blockers, such as Desipramine, can significantly increase dyskinesia severity in part by elevating extracellular L-DOPA-derived DA (Arai et al., 2008; Conti et al., 2016). The effect of NET inhibition on motor performance may depend on dose, where high doses may worsen parkinsonian deficit (Hansard et al., 2002). However, NE neurons were explicitly protected from 6-OHDA-induced loss with Desipramine which may account for present changes in NET expression and NET blockade.

Overall, the present findings demonstrate the differential roles that DAT, SERT, and NET play in L-DOPA-induced motor behavior. For the first time, we revealed that lesion and L-DOPA treatment elicited compensatory shifts towards both SERT and NET, but SERT appeared to be the optimal target for alleviating LID expression while NET and DAT may be targeted for pro-motor benefits. Clinically, investigating NET:DAT changes in dyskinetic PD patients may yield additional biomarkers for LID that may also implicate novel NE-related strategies. While the current results show functional shifts in transporters through behavioral measures, future work will need to identify the longevity and translatability of these effects for use in PD patients.

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