Norepinephrine transporter inhibition with desipramine exacerbates L-DOPA-induced dyskinesia: role for synaptic dopamine regulation in denervated nigrostriatal terminals

Tanya Chotibut, Victoria Fields, Michael F. Salvatore

Department of Pharmacology, Toxicology, & Neuroscience
Louisiana State University Health Sciences Center
1501 Kings Highway
Shreveport, Louisiana 71130
Running title: Norepinephrine transporter and L-DOPA dyskinesia

Corresponding Author:
Michael F. Salvatore, Ph.D.
Department of Pharmacology, Toxicology, & Neuroscience
Louisiana State University Health Sciences Center
1501 Kings Highway
Shreveport, Louisiana 71130

Email: msalva@lsuhsc.edu

Document statistics:
number of text pages: 31
figures: 8
references: 81
words in Abstract: 245
  Introduction: 801
  Discussion: 1688

Abbreviations used:
AIMS: abnormal involuntary movements
DA: dopamine
DAT: dopamine transporter
DMI: desipramine
ERK: extracellular signal-regulated protein kinase
L-DOPA: L-dihydroxyphenylalanine
LID: L-DOPA-induced dyskinesia
NE: norepinephrine
NET: norepinephrine transporter
PD: Parkinson's disease
Abstract

Pharmacological dopamine (DA) replacement with Levodopa (L-DOPA) is the gold standard treatment for Parkinson’s disease (PD). However, long term L-DOPA treatment is complicated by eventual debilitating abnormal involuntary movements termed L-DOPA induced dyskinesia (LID), a clinically significant obstacle for the majority of patients who rely on L-DOPA to alleviate PD-related motor symptoms. The manifestation of LID may in part be driven by excessive extracellular DA derived from L-DOPA, but potential involvement of DA reuptake in LID severity or expression is unknown. We recently reported that in 6-OHDA-lesioned striatum, norepinephrine transporter (NET) expression increases and may play a significant role in DA transport. Furthermore, L-DOPA preferentially inhibits DA uptake in lesioned striatum. Therefore we hypothesized that desipramine (DMI), a NET antagonist, could affect the severity of LID in an established LID model. While DMI alone elicited no dyskinetic effects in lesioned rats, DMI + L-DOPA treated rats gradually expressed more severe dyskinesia compared to L-DOPA alone over time. At the conclusion of the study, we observed reduced NET expression and norepinephrine-mediated inhibition of DA uptake in the DMI + L-DOPA group compared to L-DOPA alone group in lesioned striatum. LID severity positively correlated with striatal ERK phosphorylation among the three treatment groups, with increased ppERK1/2 in DMI + L-DOPA group compared to the L-DOPA- and DMI-alone groups. Taken together, these results indicate that the combination of chronic L-DOPA and NET-mediated DA reuptake in lesioned nigrostriatal terminals may have a role in LID severity in experimental Parkinsonism.
INTRODUCTION

Parkinson's Disease (PD) is the most common neurodegenerative movement disorder and its incidence is likely to only increase with the impending growth of the aging population (Siderowf and Stern, 2003). PD is primarily characterized by the loss of dopamine (DA) neurons in the substantia nigra pars compacta. Therapeutically, the primary loss in nigral dopamine necessitates its replacement through the exogenous administration of L-DOPA, a dopamine precursor or with DA agonists (Hornykiewicz and Kish, 1987; Steiger and Quinn, 1995). L-DOPA in particular, has remained the drug of choice for treating PD for nearly half a century (Calne and Sandler, 1970). Despite the ability of L-DOPA to significantly improve motor function, it is not without considerable side effects that severely limit its use long-term. L-DOPA-induced dyskinesia (LID) is a debilitating movement disorder brought on by chronic L-DOPA use. Approximately 90% of patients within the first 10 years of treatment develop LID (Ahlskog & Meunter 2001; Marsden, 1994; Mones et al., 1971; Olanow and Koller, 1998). Not only is the onset of dyskinesia a significant setback for the patient, its presence is often permanent, occurring with every subsequent exposure to L-DOPA, which limits the clinical efficacy of what has long been considered our gold standard in PD treatment.

Evidence suggests chronic L-DOPA leads to major adaptive molecular changes occurring within the basal ganglia that may underlie LID pathophysiology (for review see Cenci and Konradi, 2010). Loss of nigrostriatal dopaminergic neurons not only impairs pre-synaptic control of DA regulation, but also leads to large variances in extracellular levels of DA that parallel L-DOPA dosing regimens (Cenci and Lundblad, 2006). It is these supra-physiological fluctuations in extracellular DA content that are thought to underlie the induction of L-DOPA induced dyskinesia (Chase, 1998). LID has been associated with plastic changes in post-synaptic neuronal targets within the striatum, including abnormal trafficking of the DA D1 receptor (Berthet et al., 2009; Guigoni et al., 2007). This has also been shown clinically as well:
positron emission tomography (PET) imaging studies reveal an association between peak-dose dyskinesia with abnormally high levels of synaptic DA in the caudate-putamen of L-DOPA induced dyskinetic patients (de la Fuente-Fernandez et al., 2004; Pavese et al., 2006). In line with these studies, pre-clinical data show that dyskinetic rats exhibit higher levels of extracellular DA after L-DOPA administration than those seen in non-dyskinetic animals (Meissner et al., 2006; Lindgren et al., 2010). These data raise the possibility that not only does striatal extracellular DA play a pivotal role in the onset of dyskinesia, but could be the triggering element of the post-synaptic alterations identified in both clinical and pre-clinical models. However, despite considerable efforts directed at delineating the role L-DOPA plays in altering dopaminergic striatal signaling, the exact mechanism of how this occurs remains conflicting and inconclusive (Cenci & Konradi 2010).

In PD, the loss of the dopamine transporter (DAT) likely impairs its ability to maintain DA bioavailability, yet most studies indicate motor symptoms are seen only when ~70% of striatal DAT is lost (Bernheimer et al., 1973; Bezard et al., 2001), indicating an alternate mechanism through which DA may still be effective to produce normal locomotion. Indeed, many studies have concluded that serotonergic terminals may transport L-DOPA or DA, and subsequently release DA in order to maintain DA signaling, albeit in a dysregulated fashion (Arai et al., 1995; Miller and Abercrombie et al., 1999; Tanaka et al., 1999; Kannari et al. 2001, Carta et al., 2007). However, it is a comparatively neglected observation that in sparsely dopaminergic innervated regions, such as the frontal cortex, the norepinephrine transporter (NET) can also transport DA (Moron et al., 2002), and NE uptake inhibitors which can result in increased extracellular DA levels within the prefrontal cortex (Carboni et al., 1990; Di Chiara et al., 1992; Tanda et al., 1997; Gresch et al., 1995; Masana et al., 2012; Yamamoto and Novotney, 1998; Wayment et al., 2001). In line with these observations, we have recently reported that DA transport still occurs in the 6-OHDA-lesioned striatum, despite major loss of DAT, and that a potential role for...
NET in DA uptake is evident (Chotibut et al., 2012). In these experiments, L-DOPA preferentially inhibits DA reuptake when striatal DAT loss exceeds the level necessary for PD symptom appearance, and preferentially inhibits norepinephrine over DA uptake in striatal tissue. Therefore, based upon these observations, chronic L-DOPA could exacerbate the L-DOPA induced-dysregulation of synaptic DA by its influence on a NET-dominated regulation of DA uptake that evolves with the gradual loss of DA terminals.

In an effort to further explore this possibility, we examined how the blockade of NET in a chronic desipramine (DMI) paradigm would affect dyskinesia expression, NET expression, DA uptake, and an established post-translational event in striatum associated with dyskinesia, extracellular signal-regulated protein kinase phosphorylation (ppERK), in an established 6-OHDA LID rodent model.

MATERIALS and METHODS:

Animals

Male Sprague Dawley rats purchased from Charles-River were used in all experiments. A total of 21 test subjects were used at the start of the study, were 4-8 months of age, and were housed 1 per cage and under controlled lighting conditions (12:12 light:dark cycle) with food and water available ad libitum. All animals were used in compliance with federal and the institutional Animal Care and Use Committee guidelines at LSU Health Sciences Center-Shreveport. All behavioral testing was performed between 10:00 and 16:00 h. Behavioral data obtained from test subjects that did not have >70% TH loss, as discovered after completion of LID assessments and tissue analysis, was excluded from the results. We note that protein recovery was limited in some rats due to the fact we analyzed DA uptake, TH, NET, tERK, and ppERK all from the same striatal tissues and as such, the treatment group numbers are not of equal size.
In all experiments, rats were rendered briefly unconscious with isoflurane and immediately decapitated on day 40 of the experiment (1 day post last behavioral test and treatment (L-DOPA + DMI, L-DOPA or vehicle) for dissection of striatal tissues.

6-OHDA Lesions

Rats were anesthetized with 40 mg/kg Nembutal intraperitoneal (i.p.) (pentobarbital Lundbeck Inc, Deerfield, IL) with supplement of 9.0, 0.6, and 0.3 mg/kg ketamine, xylazine, and acepromazine, respectively. Each animal then underwent survival surgery to deliver the neurotoxin 6-OHDA unilaterally to the medial forebrain bundle while immobilized in a stereotaxic frame (coordinates ML +1.5, AP -3.8, DV -8.0 relative to Bregma). All stereotaxic coordinates are cited according to the stereotaxic atlas of Paxinos and Watson rat brain atlas, 4th ed. (Academic Press, 1998). A total of 16 µg of 6-OHDA in a total of 4 µl in 0.02% ascorbic acid (concentration of 4 mg/ml) was infused unilaterally at a rate of 1 µl/minute. The contralateral medial forebrain bundle was infused with vehicle (0.02% ascorbic acid) and infused at the same rate and coordinates. The syringe was left in place for 10 min before removal to allow for maximal diffusion of drug and to avoid further mechanical damage to the tissue. Body temperature was maintained at 37° during surgery using a temperature monitor with probe and heating pad (FHC, Bowdoingham, ME). Animals were kept warm after surgery and monitored closely after anesthesia. In our hands (Chotibut et al., 2012, Fig 7), we have previously shown that after 6-OHDA infusion, there was no significant difference in lesioned striatal tissue NE content compared to contralateral striatum and as such, have based these experiments from these observations. As such, we did not pre-treat these rats with DMI.

Amphetamine Testing

The extent of the lesion was evaluated 7 days post-surgery based on the net ipsilateral rotations measured over a 60 minute period following an injection of 2.5 mg/kg D-amphetamine
i.p (in 0.9% saline) (Ungerstedt and Arbuthnott, 1970). 100 net full turns on the ipsilateral side to the lesion was necessary to be included in the study. In our hands, we have established this time frame as enough to consistently catch >60% of TH loss (Chotibut et al., 2012; 2014; Salvatore, et al., 2014). Ultimately however, post-mortem verification of the lesion severity was determined by assessment of tyrosine hydroxylase protein as previously reported (Chotibut et al., 2012; 2014). And thus, any further exclusions were due to rats that did not also have >70% TH loss after western blot analysis at the end of the study. The behavioral AIMS data reflects test subjects with >70% TH protein loss.

Desipramine and L-DOPA Administration

9 days post lesion, rats were randomly divided into 3 groups. 2 groups received a treatment of DMI (Tocris, cat #3067) (12mg/kg) and 1 group received Vehicle (.9% saline 12mg/kg) i.p for 30 consecutive days. Then, at 19 days post lesion, an additional treatment of either L-DOPA (12mg/kg) and Benserazide-hydrochloride (15mg/kg) or Vehicle (.9% saline) was given once daily for 20 consecutive days (Fig 1). In summary, 3 treatment groups were created: (1) DMI Pretreatment + L-DOPA/Benserazide, (2) Vehicle Pretreatment + L-DOPA/Benserazide and (3) DMI Pretreatment + Vehicle (0.9% NaCl Saline, Hospira, Lake Forest, IL). With 6-OHDA lesion present in each group, these three groups were used to evaluate the impact of DMI on LID behavior caused by chronic L-DOPA, with the DMI alone group controlling for whether NET-blockade alone could produce LID.

Behavioral AIMS ratings

L-DOPA-induced abnormal involuntary movements (AIMs) were then rated at 6 discrete time points (days 19, 23, 27, 31, 35, 39) during the 20-day administration of L-DOPA starting on day 19-post lesion until the end of the study. AIMs were assessed by an investigator blind to treatment. Twenty minutes after L-DOPA administration, rats were placed in separate cages
and individual dyskinetic behaviors were quantified based on severity and frequency during a 1-minute observation period performed every 20 minutes over a period of 160 minutes. AIMs were classified into three subtypes as follows: axial AIMs (dystonic posturing of the upper trunk towards the side contralateral to the lesion), limb AIMs (hyperkinetic or jerky movements of the forelimb contralateral to the lesion), and orolingual AIMs (abnormal jaw movements, facial twitching and tongue protrusion). Each subtype was scored based on severity and frequency from 0 to 4, with 4 being the most severe and/or occurring continuously during the entire 1-minute observation period (for review, see Cenci et al., 1998 and Lundblad et al., 2004). Global AIMs scores were calculated by multiplying the severity score x frequency score for each observation period and combining the scores on all monitoring periods. Theoretically, the highest AIMs score achievable in one behavioral test day (with 8 observation periods) would be 384.

**Preparation of Synaptosomes**

In order to ascertain uptake properties in conjunction with protein and protein phosphorylation expression levels present in the rats immediately following the last LID assessment, synaptosomes were prepared according to the protocol previously described (Salvatore et al. 2003) with the following modifications: Tissue dissected from dorsal striatum was homogenized in 5 mL of 0.32 M sucrose solution using a Teflon/glass homogenizing wand (Glas-Col, Terre Haute, IN) then spun at 1000 x g for 10 minutes in a chilled (4° C) centrifuge. The resulting pellet was stored as the P1 fraction while the supernatant was spun further at 16,500 x g for 30 minutes at 4° C, yielding the P2 fraction. An aliquot of the P1 fraction was saved for determination of TH protein, ppERK, and total ERK protein from the 6-OHDA-lesioned and contralateral (control) striatum against a standard curve of TH protein standard (Salvatore et al., 2009). We have determined in previous experiments that this fraction is sufficient for
precise assessment of cytosolic proteins (Chotibut et al., 2012, 2014), reflecting the relative quantities recovered in fresh frozen preparations (Salvatore et al., 2009). An aliquot of P2 fraction was saved for determination of NET protein from the 6-OHDA lesioned and contralateral (control) striatum. The supernatant was aspirated and resuspended in 1 mL of Kreb’s buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 25 mM NaHCO3, 1.0 mM Na2EDTA, 1.7 mM CaCl2, 10 mM glucose, 100 μM parglyline, 100 μM ascorbic acid). Protein concentration was determined using a BCA colormetric assay (Thermo Scientific, Rockford, IL). All tissue was kept on ice or at 4 °C from the moment of brain excision until the uptake assay took place.

**[^3H]DA uptake into Synaptosomes**

Synaptosomes were distributed in ice-cold test tubes to prepare for DA uptake. The determination of[^3H]DA uptake in the crude synaptosomes from dorsal striatum harvested from the contralateral and 6-OHDA-infused hemispheres was conducted simultaneously and included assessments of DA uptake capacity in the presence of unlabeled 1 μM NE and DA. We previously determined that this concentration of NE or DA had differential impact on DA uptake (Chotibut et al., 2012) and consistent with previous observations. Each determination was done in triplicate for each assay condition and uptake was determined comparing the lesioned striatum with the contralateral control striatum. Non-specific uptake was determined by counts obtained in synaptosomes incubated with 500 nM DA (all as labeled DA) on ice during the time period of uptake. Background was determined and subtracted in the same manner as in the DA uptake studies.

Synaptosomes (30 μg protein per replicate) were added to 4 °C oxygenated Kreb’s buffer and test ligand (if indicated) to reach a total volume of 100 μL. The synaptosomes were then warmed to 35°C for 5 min, then 100 μL of pre-warmed 1μM[^3H]-dopamine, prepared from ViTrax, [7-, 8-[^3H]-DA], specific activity of 25 Ci/mmol was added to the synaptosome
preparations (giving a 500 nM final \(^{3}H\)DA concentration), allowed to incubate for uptake, and terminated after 120 seconds with an excess volume of ice-cold Kreb’s buffer and re-immersing the tubes in the ice-bath. The uptake time for DA was chosen to be as close as technically and practically possible to the approximately 2-minute uptake time of striatal dopamine observed \textit{in vivo} as seen by Sabeti \textit{et. al.} (2002) and where differences in uptake capacity between lesioned and intact striatum have been previously reported (Chotibut \textit{et. al.}, 2012). Synaptosomes were washed extensively to remove excess labeled-dopamine with equal-osmolarity PBS buffer through a Brandel M24-TI (Gaithersburg, MD) cell harvester using Brandel GF/C filter paper pretreated with a 2% polyethylenimine solution to reduce non-specific binding of label. The filter paper containing the rinsed synaptosomes was transferred into scintillation vials containing 5 mL of biodegradable scintillation cocktail (Research Products International, Mount Prospect, IL) and counted with a Beckman Coulter LS6500 scintillation counter (Brea, CA).

\textbf{Calculating DA uptake}

To determine the quantity of DA uptake, the percent of \(^{3}H\)DA recovered in the synaptosomes against the total amount of \(^{3}H\)DA added during the uptake experiment was first determined. The total pmole of recovered \(^{3}H\)DA was then determined based upon the percent \(^{3}H\)DA recovery in the synaptosomes after subtracting the non-specific binding value, and the result was normalized to synaptosome protein and expressed as pmole DA per mg protein per minute.

\textbf{Analyses of proteins and ERK phosphorylation}

Synaptosome fraction (P1) and the processed preparatory sample (P2) were sonicated in a 1% sodium dodecyl sulfate solution (pH ~8) using a Branson Sonifier 150 (Danbury, CT). Protein concentration was determined using the bicinchoninic acid colormetric assay. Following gel electrophoresis, proteins were transferred for 500 volt hours in a
Tris/glycine/methanol buffer onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA).

The nitrocellulose membrane was stained with Ponceau S to further normalize staining in each sample lane. These lanes were scanned and quantified by Image J to normalize protein in each sample. This relative total level then served as an additional normalizing value to determine the quantity of each protein assayed (Salvatore et al., 2012). To continue processing, the membranes were blocked in PVP buffer (1% polyvinylpyrrolidone and 0.05% Tween 20) for a minimum of two hours to reduce nonspecific antibody binding. The membrane was soaked in primary antibody for 1-3 hours. Specific primary antibodies were as follows: NET (Alpha Diagnostics, cat NET11-A) and TH (Millipore, cat #AB152), D1 receptor (Santa Cruz, cat #14001), ERK1/2 (Millipore cat #442704), ppERK1/2 (Sigma, cat #M8159). Protein loads for linear detection were 50ug total protein for NET, 10ug for TH and total ERK1/2, and 40ug for D1 and ppERK1/2. After primary treatment, blots were exposed to secondary antibody (swine anti-rabbit IgG for TH and NET) signal enhancement, followed by 1 hour incubation with [¹²⁵I] protein A (PerkinElmer, Waltham, MA).

**Statistical Analysis**

Data were analyzed using SPSS (Chicago, IL, USA) with correction for multiple comparisons and p-values < 0.05 considered significant. A two-way ANOVA (time x treatment) was used to analyze AIM scores over time within each session. Total AIM scores (axial + limb + oral (ALO)) summed across the 160-minute sessions were analyzed by two-way ANOVA. One-way ANOVA and t-paired student tests were used in instances with more than 3 or more groups or 2 groups, respectively. One-way ANOVA analyses was followed by Post-Hoc Tukey multi-comparison test.
Surgeries were performed in triplicates with the assumption that any irregularities in efficacy of the 6-OHDA lesion may occur in all animals lesioned with the same 6-OHDA on that day. Specifically, surgeries were performed in multiples of three each day to accommodate for the three treatments (L-DOPA, L-DOPA + DMI, DMI). Thus, any variances that may be encountered on each day that may affect lesion severity is assumed to occur to each rat on that given date of surgery. Additionally, the data was also analyzed using a paired t test when comparing contralateral and lesioned striatum or one way ANOVA between the 3 treatment groups.

Due to no dyskinesia elicited in DMI treatment group alone, area under the curve dyskinesia values (Fig 3A, B) plots only included DMI + L-DOPA and L-DOPA treatment groups. As such, a paired t-test (Fig 3A) was performed to examine for differences in dependent measures between these two treatments as well as regression analyses to determine if LID differed over the course of L-DOPA treatment (Fig 3B).

RESULTS:

LID severity increases with desipramine (DMI) pretreatment + L-DOPA compared to L-DOPA alone

L-DOPA-induced abnormal involuntary movements (AIMs) were rated at 6 discrete time points (days 19, 23, 27, 31, 35, 39 post 6-OHDA lesion, or days 1, 5, 9, 13, 17, 21 of daily L-DOPA) during the 20-day administration of L-DOPA starting on day 1 (19 days post 6-OHDA lesion). As L-DOPA treatment duration increased, the norepinephrine transporter inhibitor, desipramine (DMI), exacerbated dyskinesia expression in 6-OHDA lesioned male Sprague Dawley rats compared to L-DOPA alone beginning on day 27 and through day 39 post-6-OHDA lesion induction (Fig 2A-F). However, on day 19, when the first dose of L-DOPA was administered, DMI significantly attenuated LID severity (Fig 2A). However, this attenuation of
LID was not evident after day 5 of L-DOPA (or 23 days post-lesion) (Fig. 2B). Thereafter, DMI exacerbated LID severity (Fig. 2C-F). By the end of the study on day 39, DMI pre-treatment with L-DOPA significantly exacerbated LID at all time periods of observation compared to L-DOPA treatment alone.

Desipramine alone (no chronic L-DOPA, 6-OHDA lesion only) did not elicit dyskinesia, supporting previous findings that DA replacement (with its precursor, L-DOPA) is a necessary component of L-DOPA induced dyskinesia, despite lesion severity.

The area under the curve (AUC) reflects the accumulative LID score attained between the two groups. L-DOPA + DMI elicited greater dyskinesia than L-DOPA alone over the course of the 20 day treatment regimen (Fig 3A). Because dyskinesia was not observed with DMI alone (Fig 2A-F, average abnormal involuntary movement score = 0), a comparison of L-DOPA + DMI versus L-DOPA alone was made.

Additionally, LID severity did not change over time in the L-DOPA alone group. However, in the L-DOPA+DMI group there was a significant increase in dyskinesia over the 20-day course of treatment with L-DOPA + DMI, with the severity increasing as the study progressed (Fig. 3B).

**Tyrosine Hydroxylase loss in treatment groups**

There were no significant differences in tyrosine hydroxylase (TH) loss among the three treatment groups between lesioned and contralateral control striatum (Fig. 4A, B). Therefore, the exacerbation of LID severity in DMI + L-DOPA treated rats most likely was not due to increased dopaminergic terminal destruction or lesion severity (L-DOPA: Average TH loss 80.2% ± 4.9; DMI: 79% ± 5.3; L-DOPA + DMI: 77% ± 4.1).
Relative abundance of NET: lesioned vs. contralateral striatum

Either DMI alone or DMI + L-DOPA treatment significantly decreased NET abundance in the lesioned striatum compared to contralateral control striatum in the respective groups (Fig 5A, B, D). No difference in NET expression between control and lesioned striatum was observed in the L-DOPA group (Fig 5C, D).

ERK phosphorylation in treatment groups

Levels of phosphorylated and total ERK1/2 were assessed in tissue fractions prepared from the 6-OHDA lesioned striatum from rats from each treatment group. Both antibodies revealed two bands with the expected molecular weights of ERK1 (44kDa) and ERK2 (42kDa). Accounting for ppERK ½ in lesioned striatum, when compared to DMI alone, L-DOPA and DMI + L-DOPA treatment groups showed increased ppERK/ERK1 and ppERK/ERK2 (Fig 6A, B respectively; representative western blot Fig 6E). Interestingly, these changes were blunted in the control striatum (Fig 6C, D). ppERK/ERK1 increased compared to DMI alone in both L-DOPA and L-DOPA + DMI treatments but there were no differences between L-DOPA and L-DOPA + DMI groups (Fig 6C). Conversely, ppERK/ERK2 was increased only in L-DOPA treated rats compared to DMI (Fig 6D). No differences were observed in total ERK between treatments (data not shown).

ERK phosphorylation and dyskinesia severity

The levels of ppERK1 are increased in L-DOPA preclinical studies (Santini et al., 2007; Westin et al., 2007). We also observed a correlation between the severity of dyskinesia (as seen in the L-DOPA + DMI treatment group) and the ppERK1 signal, as measured in the lesioned striatum in each treatment group (Fig 7), indicating that NET blockade in combination with L-DOPA may increase DA signaling associated with LID manifestation as observed at the end of the study (day 21 of L-DOPA). Specifically, simple regression of phospho-ERK1 levels
on the cumulative axial, limb and orolingual global AIM scores recorded from chronically L-DOPA, DMI + L-DOPA and DMI treated rats had a positive correlation with dyskinesia severity (Fig 7).

**DA uptake and NE inhibition**

DA uptake was determined in synaptosomes from lesioned versus control striatal tissue in the presence of a concentration of DA or NE (1 µM) previously shown to differentially inhibit uptake of labeled [³H]-DA (Chotibut et al., 2012). In the L-DOPA group, we did not observe a significant difference in inhibition [³H]-DA uptake by unlabelled DA between the lesioned and control striatum (% inhibition in control ~70%) (Fig. 8A). However, NE inhibited DA uptake in lesioned striatum to a greater extent compared to that in control striatum (% inhibition in control ~50%) (Fig. 8B). In the DMI + L-DOPA group, unlabelled DA did not differentially inhibit [³H]-DA uptake between lesioned and control striatum (% inhibition in control ~70%) (Fig. 8C). However, NE was significantly less effective to inhibit DA uptake in lesioned compared to control striatum (% inhibition in control ~70%) (Fig. 8D). NE also inhibited DA uptake to a significantly greater extent in lesioned striatum in the DMI alone group (data not shown).

**DISCUSSION:**

Dysregulation of extracellular (DA) in the DA-denervated Parkinsonian striatum is associated with LID (Carta *et al.*, 2006; 2007; Cenci and Lundblad, 2006). However, the relative contribution of DA uptake in LID onset or severity has not been established. The 6-OHDA lesion may increase striatal NET expression and NET may affect DA uptake therein (Chotibut et al., 2012). In intact CNS tissue, such as prefrontal cortex, there is evidence for NET-mediated DA reuptake (Carboni *et al.*, 1990; Tanda *et al.*, 1997; Moron *et al.*, 2002). Accordingly, in lesioned striatum, the tissue content level of NE is comparable to that of DA (Chotibut et al.,...)
2012), suggesting that remaining DAT and inherent NET are potentially in comparable abundance and NET may therefore assume a greater role in DA uptake. Our results are limited to striatal analysis because of the relative abundance of DA neuropil (although lesioned) over other DA regions like substantia nigra. DA uptake in the substantia nigra could also affect LID severity (Navailles et al., 2014), given significant noradrenergic innervation in this region. However, there is comparatively less DAT protein and DA uptake in the substantia nigra, which precluded us from examining this possibility.

L-DOPA may inhibit DA uptake in lesioned striatum (Chotibut et al., 2012; Hashimoto et al., 2005), which could lead to accumulation of extracellular DA, and therefore affect LID onset or severity. Increased extracellular DA in DA-denervated striatum is observed following DMI (Arai et al., 2008). This finding is congruent with our observations that reuptake of DA, derived from L-DOPA, may be modulated by NET. Thus, reducing NET function, either by L-DOPA-blockade or reduced NET expression, could reduce DA clearance and exacerbate LID severity. Accordingly, we found that NET inhibition (with DMI) and L-DOPA, gradually exacerbated LID severity over the 20-day course of L-DOPA administration, compared to L-DOPA alone (Fig. 3). These differences in LID severity were unrelated to differences in lesion severity (Fig. 4). Our previous finding that striatal NET expression increases at a comparatively earlier time point in lesion progression (Chotibut et al., 2012), coupled with the present findings, give credence to involvement of NET in DA uptake and regulating LID expression or severity.

Our results also indicate that extracellular signal-regulated kinases 1 and 2 (ERK) phosphorylation was increased in the DMI + L-DOPA group compared to L-DOPA alone group (Fig. 6). Increased signaling through D1 receptors is implicated in the molecular and synaptic responses in striatal neurons associated with LID onset (Aubert et al. 2005; Konradi et al. 2004; Picconi et al. 2003; Westin et al., 2007) and enhanced ERK1/2 phosphorylation in DA denervated striatum occurs with selective agonists for D1 or D2 receptors (Cai et al. 2000;
Pavon et al., 2006; Zhen et al. 2002). Thus, increased ERK1/2 activity in striatal neurons is at the very least a biochemical marker of L-DOPA–induced dyskinesia through D1 or D2 over-activation. As such, phosphorylation of ERK1/2 may provide a molecular counterpart for increased D1 activity and be involved in LID induction. Reduced ERK1/2 phosphorylation dose-dependently decreases LID and other molecular correlates causally linked to LID development (Santini et al., 2007). Thus, the observation that DMI pretreatment with L-DOPA both exacerbates LID and increases ERK1/2 phosphorylation over that of L-DOPA alone supports a dopaminergic mechanism in LID severity wherein NET-mediated DA uptake and L-DOPA together modulate LID expression. Given that chronic DMI also reduced NET expression in lesioned striatum (Fig. 5), this observation suggests that increasing NET function or expression could reduce LID severity.

Compensatory changes in DA regulation do occur during the loss of DA-regulating proteins in 6-OHDA rodent models (Snyder et al., 1990; Sarre et al., 2004; Perez et al., 2008), but the potential involvement of NET function is a relatively novel concept. We have reported increased NET expression in lesioned striatum (Chotibut et al., 2012). Increased locomotion is observed in monkeys with DAT inhibitors with high NET, but low serotonin transporter, affinity in cases of severe DAT loss (80%) compared to those with moderate DAT loss (46%) (Madras et al., 2006). LID severity could also be diminished by other interactions with noradrenergic inputs to the basal ganglia (Lundblad et al., 2002; Dekundy et al., 2007; Gomez-Mancilla and Bedard 1993). Furthermore, rats with combined noradrenergic and dopaminergic lesions have greater LID severity, compared to dopaminergic lesions alone (Fulceri et al., 2007; Shin et al., 2014). Indeed, noradrenergic lesions can produce dyskinesias through DA-mediated locomotor impairment (Donaldson et al. 1976, Rommelfanger et al. 2007). As such, NET blockade via DMI may not be the only factor in worsening LID behavior. For example, in LID pathology, norepinephrine has the ability to act as a D1 dopaminergic agonist (Kubrusly et al., 2007).
Thus, it may be possible that agents which decrease NET expression could lead to increased extracellular norepinephrine, thereby increasing D1 activation and worsening LID.

Reduced NET expression in the lesioned striatum therefore appears to play one of two critical components of LID and its severity. The other component of LID relates to the impact of chronic L-DOPA. DMI alone did not produce LID, but the combination of DMI and L-DOPA gradually worsened LID compared to L-DOPA alone. The neurobiological background of nigrostriatal lesion is also an important component. Chronic DMI reduced NET expression in lesioned striatum (Fig. 5), arguably offsetting any lesion-induced increase (Chotibut et al., 2012). Chronic DMI can reduce NET expression in other CNS regions such as amygdala, striatum (Jeannotte et al., 2009) and hippocampus (Kitayama et al., 2006). We also point out that the serotonin terminals or transporter (SERT) can affect LID severity in preclinical models (Carta et al., 2007; Eskow et al., 2009; Bishop et al., 2012). However, chronic DMI is not reported to alter SERT expression or function (Hyttel, 1994; Mantovani et al., 2009), thus making it unlikely that changes in SERT expression or SERT-mediated uptake are associated with our behavioral observations. From a clinical perspective, this leads to questions as to whether an antidepressant with NET-affinity could produce, hasten the onset of, or worsen the severity of LID, given the prevalence of PD-related depression and that depression commonly precedes motor manifestations (Burn 2002; Aarsland et al., 2012; Brichta et al., 2013). DMI or other tricyclics are often prescribed for this patient population. In small-scale clinical trials (17 test subjects completing each study), methylphenidate (which also affects NET function (Pan et al., 1994)), tended to increase LID (Devos et al., 2007; Espay et al., 2011). Exacerbation of dyskinesia in a clinical setting may go unnoticed given the absence of baseline readings before DMI administration. Therefore, further study could answer to the possibility that NET-blocking drugs could exacerbate LID severity or its frequency.
Desipramine may also interfere with alternate receptors such as muscarinic, adrenergic and histamine. There is a functional interaction between the cholinergic and dopaminergic systems (Quik and Wonnacott 2011), and DA denervation can increase acetylcholine levels in striatum. Given that acetylcholine mediates its effects via muscarinic and nicotonic receptors and that DMI may affect the sensitivity of post and/or pre-synaptic muscarinic receptors (Murugaiah and Ukponmwan, 2003), it is possible DMI may be mediating its effects through muscarinic receptors, which may contribute to development of motor signs.

Behaviorally, we observe an initial delay in LID in the L-DOPA + DMI treatment group compared to L-DOPA, which may be related to increased NET expression with 6-OHDA lesion (Chotibut et al., 2012). Therefore, this initial increase in NET in the lesioned compared to the contralateral unlesioned striatum may explain why dyskinetic behavior was not worsened on the first day of L-DOPA (day 19 post lesion) and that subsequent L-DOPA, in conjunction with DMI treatments, offset this increase in NET expression, revealing increased LID severity. Our DA uptake experiments (at the end of the LID assessment) also indicated that DA uptake is more inhibited by NE in the L-DOPA alone group, but less inhibited by NE in the DMI + L-DOPA group (Fig. 8). We speculate that these differences may be related to NET expression. Whereas, NET expression is not changed relative to control striatum in the L-DOPA group, it is decreased in the groups with DMI. Thus, this decrease in NE-sensitivity in DMI groups could be due to decreased NET availability in lesioned striatum. We also speculate that the lack of difference in NET expression in rats treated with L-DOPA alone may be due to increased NET with 6-OHDA lesion that is offset by possibility that L-DOPA could reduce NET expression over time. As such, this is an important unresolved question moving forward.

The cellular sources of NET are an important point of consideration. Noradrenergic innervation of dorsal striatum is relatively sparse, although NE levels are comparable with remaining DA in lesioned striatum (Chotibut et al., 2012). In prefrontal cortex, NET transports
DA and conversion of DA to NE may be slower than DA uptake via NET. As such, DA could be co-released along with NE (Ahn and Klinman 1989). Therefore, pharmacologically-induced alteration of the noradrenergic system, such as chronic DMI, could also influence extracellular DA levels (Devoto and Flore, 2006). Thus, our results may also be related to compensatory alterations in DA release. Another possible source of NET-mediated DA transport may be glia, as glial express NET (Takeda et al., 2002). Astrocyte and microglia cell numbers may increase with DA neuron loss (Teismann and Schulz, 2004), possibly increasing NET protein abundance and DA uptake. Future studies examining alterations in DA uptake in glial preparations from dyskinetic rats may further delineate the cellular basis for NET-mediated DA uptake in LID pathophysiology.

**CONCLUSION:**

In summary, our results indicate the possibility that increasing NET expression within the striatum may be a novel therapeutic avenue to attenuate LID severity. Our previous work has shown an upregulation of NET expression in lesioned striatum compared to contralateral striatum after DA denervation in the absence of L-DOPA treatment, but further work could establish whether this increase in NET is transient or alters with L-DOPA administration. Our results also indicate that L-DOPA must be present for any development of LID following 6-OHDA lesion. Our results add to a growing body of literature that NET regulates dopamine uptake dynamics in the CNS, including pathophysiological mechanisms of L-DOPA induced dyskinesia.

**Authorship Contributions:**

*Participated in research design:* Chotibut, Salvatore  
*Conducted experiments:* Chotibut, Fields  
*Performed data analysis:* Chotibut, Salvatore  
*Wrote or contributed to writing of the manuscript:* Chotibut, Salvatore
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Footnotes:

This work was funded in part by the Ike Muslow Predoctoral Fellowship Award to TC and an award to MFS through the Edward P. Stiles Trust Fund-Louisiana State University Health Sciences Center-Shreveport and The Biomedical Research Foundation of Northwest Louisiana.

Reprint requests:

Michael F. Salvatore, Ph.D.
Department of Pharmacology, Toxicology, & Neuroscience
Louisiana State University Health Sciences Center
1501 Kings Highway
Shreveport, Louisiana 71130

Email: msalva@lsuhsc.edu
Legends for Figures

Figure 1. Timeline of treatment paradigm. Male Sprague Dawley rats were lesioned unilaterally with 6-OHDA and randomly assigned a treatment group. 7 days post lesion, rats were tested with amphetamine (2.5 mg/kg i.p) and number of turns ipsilateral to lesion turns were assessed to confirm for a successful lesion. Treatment began on day 9 with 3 treatments: (1) Desipramine + L-DOPA, (2) Desipramine + Vehicle, (3) Vehicle + L-DOPA. Beginning on day 19, behavioral AIMS were assessed at 6 discrete time points for the remainder of the study (days 19, 23, 27, 31, 35, 39). On Day 39, rats were sacrificed and dopamine uptake and biochemical markers were assessed.

Figure 2. Abnormal movements induced by the chronic treatment with L-DOPA in animals lesioned with 6-OHDA. The time course of changes in dyskinesia evaluated from the product of the frequency and amplitude behavior (oralingual, axial, forelimb) induced by a 20-day treatment with L-DOPA (6 mg/kg plus benserazide 12 mg/kg, i.p). (A) Day 19 (first day of L-DOPA) and (B) Day 23 showed minimal difference at timepoints in dyskinesia severity. (C) Day 27, (D) Day 31 and (E) Day 35, approximately 1 week after treatment initiation, shows DMI + L-DOPA treatment is worsening dyskinesia severity at latter timepoints in the observation period. (F) By the end of the study on Day 39, DMI + L-DOPA treatment worsens dyskinesia severity at all timepoints compared to L-DOPA alone. In all observation periods, DMI alone did not elicit any dyskinesia. Data analyzed by repeated ANOVA followed by Bonferroni post hoc test (time course) Significant difference from L-DOPA only treatment: * p<.05, **p<.01, ***p<.001.

Figure 3. Dyskinesia over time with L-DOPA versus L-DOPA + DMI treatment. (A) Area Under the Curve (AUC) of dyskinesia elicited between day 4 until end of 20 day treatment regimen between L-DOPA and L-DOPA + DMI (Paired Student t-Test n = 5 *p<.05) (B) Linear regression of dyskinesia and time with L-DOPA and L-DOPA + DMI treatment (L-DOPA r² = .30, p = .24; L-DOPA +DMI r² = .82, p = .0028). Percentages reflect the difference in the AUC score between the two treatment groups as a function of L-DOPA administrations.

Figure 4. (A) Tyrosine Hydroxylase (TH) loss with treatment between control and lesioned striatum. There was no significant differences between TH loss among treatments (one-way ANOVA, p = .87, n = 3-5 (DMI n = 3, DMI + L-DOPA = 4, L-DOPA = 5). DMI + L-DOPA vs L-DOPA F(2,9) = .74, DMI + L-DOPA vs DMI F(2,9) = .42; L-DOPA vs DMI F(2,9) = .24 (B) Tyrosine Hydroxylase loss representative western blot (Standards of TH shown in ng (0.5, 2, 3, 4); UL = unlesioned, L = lesioned).
Figure 5. NET Expression after treatment between lesioned and intact striatum. (A) Lesioned striatum showed less NET expression compared to intact contralateral striatum with chronic DMI + L-DOPA treatment. (B) DMI treatment also decreased NET expression in lesioned striatum compared to control. (C) Treatment with chronic L-DOPA however, did not show changes in striatal NET expression. Paired Student t-test (A) $t = 2.692$, $p = .03$, (B) $t = 12.92$, $p = .006$ (C) $t = 2.077$, $p = .06$ (D) Representative western blot (L = Lesion, UL = Unlesion).

Figure 6. ppERK1/2 in lesioned striatum after treatment. (A) ppERK/ERK1 was significantly increased in both DMI + L-DOPA and L-DOPA compared to DMI. DMI + L-DOPA treatment also increased ppERK/ERK1 compared to L-DOPA alone in lesioned striatum. One-Way ANOVA $n = 3$, $p = .0001$, $f = 58.33$. (DMI + L-DOPA vs DMI: $F_{(2, 6)} = 15$, DMI + L-DOPA vs L-DOPA $F_{(2, 6)} = 10$, L-DOPA vs DMI $F_{(2, 6)} = 5$) (# denotes significance compared to DMI, * compared to L-DOPA). (B) ppERK/ERK2 was significantly increased in both DMI + L-DOPA and L-DOPA compared to DMI. DMI + L-DOPA treatment also increased ppERK/ERK2 compared to L-DOPA alone in lesioned striatum. One-Way ANOVA $n = 3$, $p = .0007$, $f = 30.33$ (DMI + L-DOPA vs DMI: $F_{(2, 6)} = 5$, DMI + L-DOPA vs L-DOPA $F_{(2, 6)} = 11$, L-DOPA vs DMI $F_{(2, 6)} = 6$) (# denotes significance compared to DMI, * compared to L-DOPA). (C) ppERK/ERK1 was significantly increased in both L-DOPA and DMI groups compared to DMI alone in unlesioned striatum $n = 3$, $p = .02$, $f = 9.0$ $F_{(2, 6)} = 5.20$, DMI + L-DOPA vs DMI $F_{(2, 6)} = 5.19$ (* denotes significance compared to DMI alone). (D) ppERK/ERK2 was significantly increased in L-DOPA + DMI treated rats compared to DMI alone in the unlesioned striatum $n = 3$, $p = .02$, $f = 7.8$, $F_{(2, 6)} = 5.42$, L-DOPA vs DMI $F_{(2, 6)} = 3.87$, DMI + L-DOPA vs DMI $F_{(2, 6)} = 1.55$ (* denotes significance to DMI alone) (E) ppERK 1/2 representative western blot (L = Lesion, UL = Unlesion).

Figure 7. Correlation analysis of the severity of dyskinesia and the ppERK/ERK1 in all treated animals. ppERK/ERK1 values with their corresponding Global AIMS over all treatments showing a significant correlation between ppERK1 and dyskinesia severity. Correlation analysis, $n = 7$, $R = .86$, $p = .003$. 

Figure 8. Uptake of DA in presence of unlabelled DA or NE. DA uptake was measured in preparations of crude striatal synaptosomes from the contralateral control and lesioned (>70% TH loss) striatum. Results are presented as the percent inhibition of tritiated DA uptake by 1 µM DA or NE. Note, the assessments of inhibition by unlabelled monoamine were conducted simultaneously in lesioned and control synaptosome preparations, one test subject per group at a given time. **(A) DA inhibition of DA uptake in L-DOPA group.** DA uptake (2 min) was determined by the parameters described in methods (ns, Student's two-tailed t-test, t=2.36; df=4). **(B) NE inhibition of DA uptake in L-DOPA group.** DA uptake (2 min) was determined by the parameters described in methods. There was a significant increase (37%) in inhibition of DA uptake in the lesioned striatum by NE (*p<0.05, Student's two-tailed t-test, t=3.38; df=4). **(C) DA inhibition of DA uptake in DMI + L-DOPA group.** DA uptake (2 min) was determined by the parameters described in methods (ns, Student's two-tailed t-test, t=3.03; df=3). **(D) NE inhibition of DA uptake in DMI + L-DOPA group.** DA uptake (2 min) was determined by the parameters described in methods. There was a significant decrease (45%) in inhibition of DA uptake in the lesioned striatum by NE (***p<0.01, Student's two-tailed t-test, t=8.33; df=3).
Figure 1
Figure 2

A

- Desipramine + L-DOPA
- Vehicle + L-DOPA
- Desipramine + Vehicle

D19: Time (min) post-L-DOPA injection

B

- Desipramine + L-DOPA
- Vehicle + L-DOPA
- Desipramine + Vehicle

D23: Time (min) post-L-DOPA injection

C

- Desipramine + Vehicle
- Vehicle + L-DOPA
- Desipramine + L-DOPA

D27: Time (min) post-L-DOPA injection

D

- Desipramine + L-DOPA
- Vehicle + L-DOPA
- Desipramine + Vehicle

D31: Time post-L-DOPA injection

E

- Desipramine + L-DOPA
- Vehicle + L-DOPA
- Desipramine + Vehicle

D35: Time (min) post-L-DOPA injection

F

- Desipramine + L-DOPA
- Vehicle + L-DOPA
- Desipramine + Vehicle

D39: Time (min) post-L-DOPA injection

Globus AIMS (Measure of Dyskinesia)
Figure 3

A

Area Under the Curve

<table>
<thead>
<tr>
<th></th>
<th>L-DOPA</th>
<th>L-DOPA + DMI</th>
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<tbody>
<tr>
<td>0</td>
<td>2000</td>
<td>4000</td>
</tr>
<tr>
<td>1</td>
<td>3000</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4000</td>
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B

AUC LID score

days of L-DOPA

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<tr>
<td>3</td>
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<tr>
<td>6</td>
<td>52%</td>
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<tr>
<td>9</td>
<td>45%</td>
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<tr>
<td>12</td>
<td>68%</td>
<td></td>
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<tr>
<td>15</td>
<td>115%</td>
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L-DOPA + DMI
L-DOPA
Figure 4

A

TH % Loss

0 20 40 60 80 100

DMI + L-DOPA L-DOPA DMI

B

.5 ng 2ng 3ng 4ng UL / L UL / L

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Figure 5

A

NET expression in chronically treated DMI and L-DOPA: Lesioned vs Control Striatum

DMI w/DOPA Control  DMI w/L-DOPA Lesion

B

NET expression in chronically treated DMI alone: Lesioned vs Control Striatum

Contralateral Control  DMI Alone Lesion

C

NET expression in chronically treated L-DOPA: Lesioned vs Control Striatum

L-DOPA Contralateral Control  L-DOPA Lesion

D

<table>
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<tr>
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</table>

80 kDA
Figure 6

A

ppERK/ERK1 in Lesioned Striatum

B

ppERK/ERK2 in Lesioned Striatum

C

ppERK/ERK1 in Control Striatum

D

ppERK/ERK2 in Control Striatum

E

<table>
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<td>L/UL</td>
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44 kDA ERK1
42 kDA ERK2
Figure 7

The graph shows the relationship between ppERK/ERK1 and Gobal AIMS. The data points are represented as follows:

- **DMI** = ★
- **DMI + L-DOPA** = ■
- **L-DOPA** = ●

The statistical values are:

- **p = .003**
- **r = .86**