Chronic L-Dopa Decreases Serotonin Neurons in a Sub-region of the Dorsal Raphe Nucleus

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Abstract

L-dopa is the precursor to dopamine and has become the mainstay therapeutic treatment for Parkinson’s disease (PD). Chronic L-dopa is administered to recover motor function in PD patients. However, drug efficacy decreases over time and debilitating side effects such as dyskinesia and mood disturbances occur. The therapeutic effect and some of the side effects of L-dopa have been credited to its effect on serotonin (5-HT) neurons. Given these findings, it was hypothesized that chronic L-dopa treatment decreases 5-HT neurons in the dorsal raphe nucleus (DRN) and the content of 5-HT in forebrain regions in a manner that is mediated by oxidative stress.

Rats were treated chronically with L-dopa (6 mg/kg; twice daily) for 10 days. Results indicated that the number of 5-HT neurons was significantly decreased in the DRN after L-dopa treatment compared to vehicle. This effect was more pronounced in the caudal-extent of the dorsal DRN, a sub-region found to have a significantly higher increase in DOPAC/DA ratio in response to acute L-dopa treatment. Furthermore, pretreatment with ascorbic acid (400 mg/kg) or deprenyl (2 mg/kg), prevented the L-dopa induced decreases in 5-HT neurons. In addition, 5-HT content was decreased significantly in the DRN and prefrontal cortex by L-dopa treatment, effects that were prevented by ascorbic acid pretreatment. Taken together, these data illustrate that chronic L-dopa causes a 5-HT neuron loss and the depletion of 5-HT content in a sub-region of the DRN and as well as the frontal cortex through an oxidative-stress mechanism.
Introduction

L-dopa is the main pharmacologic treatment for Parkinson’s disease and improves motor symptoms associated with the disease by restoring striatal dopamine content (Birkmayer and Hornykiewicz, 1962; Carlsson, 2002). Unlike dopamine, L-dopa crosses the blood-brain barrier and reaches all areas of the central nervous system (Bertler et al., 1963). Acute L-dopa administration at a therapeutic doses increases dopamine concentrations in striatum as well as extrastriatal areas of the brain that contain little or no dopamine cell bodies or terminals (Lindgren et al., 2010; Navailles et al., 2010a). The increases in dopamine are due in large part to serotonin (5-HT) neurons (Navailles et al., 2011b). 5-HT neurons have the ability to synthesize dopamine in the presence of L-dopa through the enzymatic decarboxylation of L-dopa to dopamine by amino-acid decarboxylase, the enzyme which also converts 5-HTP to 5-HT (Ng et al., 1970; Arai et al., 1994). Dopamine produced from L-dopa can also be exocytosed by 5-HT neurons in an impulse-dependent manner (Miller and Abercrombie, 1999; Tanaka et al., 1999; Navailles et al., 2010b) and thus accounts for its therapeutic efficacy.

Although the ability of 5-HT neurons to synthesize and release dopamine as a “false neurotransmitter” within the striatum may explain the therapeutic efficacy of the drug, chronic treatment with high doses of L-dopa has negative side-effects such as dyskinesias and mood disturbances (Bezard et al., 2004; Carlsson et al., 2007; Carta et al., 2007; Eskow Jaunarajs et al., 2011; Porras et al., 2014). Furthermore, chronic administration of L-dopa leads to a loss of drug efficacy (Marsden, 1994; Hauser, 2009), an observation possibly attributable to decreased efficiency of L-dopa-induced
dopamine synthesis and release from 5-HT neurons (Navailles et al., 2011a). Given that the mechanisms underlying the side effect liability of L-dopa remains unclear, further investigation into the effects of L-dopa on the cellular biology of the 5-HT system is warranted.

The toxicity profile of dopamine is well known such that cytosolic, non-vesicular dopamine is subject to enzymatic degradation via monoamine-oxidase to form the by-product hydrogen peroxide. Dopamine is also susceptible to auto-oxidation with the subsequent formation of highly reactive quinone molecules that produce oxidative stress (Graham, 1978; Mena et al., 1992; Hastings et al., 1996). Previous studies have shown dopamine degradation and oxidation are toxic to serotonergic cells in culture (Stansley and Yamamoto, 2013) and to the rate limiting enzyme for 5-HT synthesis, tryptophan hydroxylase (TPH) (Kuhn and Arthur, 1998). Furthermore, rats treated chronically with L-dopa exhibit decreases in 5-HT tissue content in several brain regions (Borah and Mohanakumar, 2007; Eskow Jaunarajs et al., 2012) and decreased L-dopa-induced dopamine release by 5-HT terminals (Navailles et al., 2010a; Navailles et al., 2011a). These reports suggest that chronic L-dopa leads to the accumulation of dopamine within 5-HT neurons that is detrimental to 5-HT system physiology and function.

Despite the above findings, direct evidence for a possible loss of 5-HT neurons in vivo has not been shown. In the present study, experiments were conducted to directly test the hypothesis that chronic L-dopa at a therapeutically relevant dose, decreases 5-HT neurons in the rat dorsal raphe nucleus (DRN) and brain 5-HT tissue content through oxidative stress.
Materials and Methods

Animals. Male Sprague-Dawley rats (175-199 g, Harlan Indianapolis, IN) were used in all experiments. Rats were housed two per cage in clear plastic containers (45 x 24 x 20 cm) and allowed to acclimate for one week to the vivarium before experimentation. The rats were housed was under a 12 h light/dark cycle in a temperature (~24˚C) and humidity (~40%) controlled environment. Rats had ad libitum access to food and water. All procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the University of Toledo Institutional Animal Care and Use Committee.

6-OHDA Lesion Surgery. Unilateral 6-hydroxydopamine (6-OHDA) or sham injections were made into the medial forebrain bundle (AP: -4.3 mm, ML: +1.5mm, DV: -7.5 mm relative to bregma with the nose bar positioned -3.3 mm from horizontal). Side of lesion was counter-balanced within groups. Prior to stereotaxic surgery, desipramine HCL (25 mg/kg, i.p.) (Sigma, St. Louis, MO, USA) was administered to protect noradrenergic neurons. Rats were anesthetized with ketamine (75 mg/kg) (Hospira, INC., Lake Forest, IL) and xylazine (5 mg/kg) (Lloyd Laboratories, Shenandoah, Iowa). 6-OHDA HBr (17 µg of free base; Sigma, St. Louis, MO, USA; Cat no. H116) was prepared in 0.9% saline containing 0.01% ascorbic acid and infused at a rate of 1 µl/min for a total volume of 4 µl. After infusion, the infusion needle remained in position for 10 minutes to allow for the 6-OHDA to completely diffuse. The needle was then retracted slowly to minimize mechanical damage. Lesion severity was confirmed by tyrosine hydroxylase (TH) staining of the substantia nigra pars compacta, and only rats with TH+ loss >95% were included in the analysis.
Lesion rats were only used in experiments related to the results illustrated in Figures 1 and 2. All other subsequent experiments were carried out with non-lesioned rats due to the fact that 6-OHDA lesion itself did not affect TPH+ neuron number in the DRN.

**Pharmacological treatments.** Two weeks after surgery, rats were treated twice daily with either vehicle saline (0.9% NaCl;) or L-dopa (L-3,4-dihydroxyphenylalanine methyl ester hydrochloride; 6 mg/kg, i.p., Sigma, St. Louis, MO, USA) and benserazide (DL-serine 2-(2,3,4-trihydroxybenzyl) hydrazide hydrochloride; 12 mg/kg, i.p.; Sigma, St. Louis, MO, USA) for 10 days. This dosing regimen was chosen because it restores therapeutic concentrations of striatal dopamine to near physiologic concentrations in 6-OHDA lesioned rats (Navailles, 2010b). Four groups were evaluated: 1) Sham-lesion, Vehicle-treated, 2) Sham-lesion, L-dopa-treated, 3) 6OHDA-lesion, Vehicle-treated, 4) 6OHDA-lesion, L-dopa-treated.

To assess the roles of oxidative stress, ascorbic acid (sodium L-ascorbate; 400 mg/kg, i.p., Sigma, St. Louis, MO, USA) or deprenyl ((R)-(−)-N,α-Dimethyl-N-(2-propynyl) phenethylamine hydrochloride; 2 mg/kg, i.p., Tocris, Minneapolis, MN, USA) was administered before each L-dopa dose. These drugs were chosen because drug interactions that might prevent L-dopa accumulation in the brain are unlikely due to the fact that L-dopa is taken up into the brain by the Large-neutral amino acid transporter (Yee et al., 2001), while deprenyl readily crosses the blood-brain barrier and ascorbic acid is transported into the brain in its oxidized form, dehydroascorbic acid, via glucose transporter type-1 (Agus et al., 1997). Rats that received an acute injection of L-dopa
were killed 45 minutes after drug administration. Rats treated chronically with L-dopa were killed 48 hrs after the last drug injection.

**5-HT cell counts.** Phosphate buffered saline (1600 ml) containing 4% paraformaldehyde (PFA) was perfused intracardially at a rate of 5 mL/min under ketamine (75 mg/kg, im) + xylazine (5 mg/kg, im) anesthesia subsequent to the injection of heparin (35 USP units) (APP Pharmaceuticals, LLC, Schauburg, IL) into the left ventricle. Brains were removed from the skull and placed in 4% PFA overnight at 4˚C, followed by 10% glycerol and 20% glycerol, each for 24 hrs. Brains were then flash frozen in 2-methylbutane over dry ice.

Brains were serially sectioned through the DRN (-7.3 to -8.3 mm from bregma) (Paxinos, 1997) at a thickness of 25 µm using a cryostat microtome (Microm HM550; Thermo Scientific, Waltham, MA). Sections were mounted onto gelatin coated slides and blocked for 1 hr at room temperature with 10% normal goat serum in 0.1N PBS containing 0.25% Triton X-100. Sections were incubated with primary antibodies for tryptophan hydroxylase 2 (TPH2; Novus biological, NB100-74555 xRb, 1:1000) and NeuN (NeuN; Millipore, MAB377 xMs, 1:1000) overnight at 4˚C. Subsequently, sections were washed three times in PBS and then incubated with Alexa Fluor secondary antibodies (Alexa Fluor 688, Goat anti-Rabbit; Alexa Fluor 488 Goat anti-Mouse, 1:2000) for 30 minutes at room temperature. After three additional washes with PBS, sections were cover-slipped with fluoromount and stored in the dark before imaging.

Sections were imaged using a Leica SP5 confocal microscope and the standard Leica Applications Suite Advanced Fluorescence (LAS AF) Software. Parameters used to image included an excitation wavelength of 488 and 633 nm, and collection
wavelengths of 504-556 and 690-740 nm, respectively. Exposure, gain and offset remained constant for all images. All sections were imaged at 20x magnification to include the midline DRN structures within at least 6 slices per rat; each rat yielded an \( n \) of one. The investigator was blind to the treatment conditions during imaging and analysis.

To identify 5-HT neurons in the DRN, images were loaded into *ImageJ* (NIH) software program. The overlay of the emissions from the secondary Alexa Fluor that labeled TPH2-Red and NeuN-Green yielded a yellow co-label that signified 5-HT soma. Cell bodies were counted in serial sections to give the number of serotonin neurons in DRN per 25 \( \mu \)m section. For further analysis, the DRN was divided into rostral (-7.3 to -7.8 mm from bregma) and caudal (-7.8 to -8.3 mm from bregma) sub-regions. In addition, midline structures of the DRN were sub-divided into dorsal and ventral regions (Paxinos, 1997). These divisions formed four DRN sub-regions, 1) dorsal rostral-extent, 2) ventral rostral-extent, 3) dorsal caudal-extent, 4) ventral caudal-extent.

**High Performance Liquid Chromatography for Monoamine Tissue Content.** Brain monoamine content was measured in non-6-OHDA lesioned rats. Brain tissue was micro-punched or micro-dissected according to rat stereotactic coordinates (Paxinos, 1997). DRN sub-regions: (-7.3 to -8.3 mm from bregma) rectangular dissection with boundaries consisting of the cerebral aqueduct on top and the decussation of the superior cerebellar peduncle on bottom and 1 mm to either side of the midline; the top half was considered dorsal DRN, and bottom half ventral DRN. Hippocampus: (-3.3 to -3.8 mm from bregma; dorsal hippocampus), Striatum: (1.5 to -0.5 mm from bregma; dorsal caudate/putamen) and PFC (2.7 to 2.2 mm from bregma; prelimbic/cingulate
cortex) were micro-punched with cylindrical flat needle (14.5 gauge; 1.6 mm internal diameter). Tissue was then homogenized in cold 0.25 M perchloric acid, and centrifuged at 14000 rpm for 15 minutes. Monoamines were measured by HPLC-EC. The supernatant (20 µL) was injected onto a C18 column (100 x 2.0 mm, Phenomenex, Torrance, CA, USA). The mobile phase consisted of 21 g/L citric acid anhydrous, 10.65 g/L disodium phosphate, 380 mg/L octyl sodium sulfate, 3% acetonitrile and 15% methanol, with a pH 4.0. An LC-4B amperometric detector (BAS Bioanalytical Systems, West Lafayette, IN, USA) was used and data were analyzed with EZChrom_software (Scientific Software Inc., Pleasanton, CA, USA). The limit of detection for dopamine and 5-HT in DRN micro-dissections was 5.4 and 2.3 pg/20 µL respectively, with signal to noise ratio of 3:1. The protein content of the samples was determined using a Bradford assay (Bio-Rad, Hercules, CA, USA) after re-suspending the pellet in 1 N NaOH. Monoamine content per sample was normalized to protein and expressed as pg per µg of protein.

**Statistical analysis.** Statistical comparison of cell counts or monoamine content was performed using a Student’s t-test or a two-way analysis of variance (ANOVA) with posthoc Tukey’s multiple comparison test where appropriate. Analyses were performed using SigmaPlot 11.0 software (SigmaPlot for Windows, Systat Software). All data are presented as mean ± SEM. Significance levels in all studies is p≤0.05.
Results

Effects of L-dopa on TPH-Positive Cell Counts in the DRN

Initial experiments employed unilateral-6-hydroxydopamine (6-OHDA) or sham-lesions of the medial-forebrain bundle prior to L-dopa treatment to investigate possible combined effects of nigrostriatal dopamine degeneration with L-dopa treatment on TPH+ cell body number in the DRN. Two weeks post-surgery, L-dopa (6 mg/kg, i.p. + Benserazide, 12 mg/kg, i.p.) was injected into rats twice daily for 10 days. Rats were killed 48 hours after last injection. A two-way ANOVA revealed the lesion had no effect on the number of TPH+ cell bodies in the DRN in the presence or absence of L-dopa (F(1,18)=0.55, p=0.47). Therefore, lesioned groups were combined with unlesioned groups in subsequent analyses (Figures 1 and 2).

L-dopa treatment alone caused a significant decrease in the average number of TPH+ cell bodies per 25 µm slice in the DRN when compared with vehicle controls (t=2.33, p<0.05) (Figure 1A).

Sub-regional Effects of L-dopa on TPH-Positive Cell Counts in the DRN

Given that the DRN is known to contain distinct anatomical sub-regions which have different afferent and efferent projections and cell types, TPH+ cell counts in sub-regions were conducted to determine if the effect of L-dopa was sub-region specific. The midline DRN was sub-divided into 1) a dorsal sub-region containing both rostral and caudal aspects, and 2) a ventral sub-region containing both rostral and caudal aspects. Of the four sub-regions analyzed, L-dopa caused a 28% decrease in TPH+ cell bodies within the dorsal sub-region in the caudal-extent (t=2.62, p<0.05) (Figure 2B). In
contrast, there were no significant effects of L-dopa treatment within the rostral portion of dorsal sub-region (t=0.02, p=0.98) (Figure 2A) or throughout the entire rostral-to-caudal extent of the ventral sub-regions (rostral: t=2.01, p=0.06) (caudal: t=0.87, p=0.39) (Figures 2C, 2D).

**Effects of Ascorbic Acid and Deprenyl on L-dopa Induced Decreases in TPH-Positive Cell Counts**

To elucidate a mechanism by which L-dopa treatment decreases TPH+ neurons, an antioxidant (ascorbic acid; 400 mg/kg, i.p.) or monoamine-oxidase type B (MAO-B) specific inhibitor (deprenyl; 2 mg/kg, i.p.) was administered before each L-dopa injection. A two-way ANOVA revealed a significant interaction between L-dopa and ascorbic acid treatment ($F_{(1,16)}=4.55$, $p<0.05$) on the number of TPH+ cell bodies in the caudal-extent of the dorsal sub-region of the DRN, indicating that the effects of L-dopa treatment on TPH+ cell counts varied according to whether the rats were treated with ascorbic acid. Furthermore, Tukey’s post-hoc analysis revealed a significant effect of L-dopa treatment ($q=3.19$, $p<0.05$). Ascorbic acid treatment alone had no significant effect on TPH+ cell counts ($q=1.13$, $p=0.44$); however, ascorbic acid treatment blocked the L-dopa induced decreases in TPH+ cells ($q=3.13$, $p<0.05$) (Figure 3A).

A separate group of rats was injected with the MAO-B inhibitor deprenyl prior to each L-dopa injection. A two-way ANOVA revealed a significant interaction between L-dopa and deprenyl treatment ($F_{(1,16)}=5.18$, $p<0.05$). A Tukey’s post-hoc test revealed that deprenyl had no effect on TPH+ cells alone ($q=0.31$, $p=0.83$); however, deprenyl treatment significantly blocked the L-dopa induced decreases in TPH+ cells ($q=4.86$, $p<0.05$).
Acute L-dopa and Dopamine Tissue Content in the DRN

To investigate the effects of L-dopa on dopamine content in the DRN sub-regions, acute L-dopa was administered and the DRN was micro-dissected into raphettes, consisting of dorsal and ventral sub-regions to parallel the TPH+ cell count analyses. Dopamine content within the dorsal DRN was significantly increased by more than 2.5-fold in rats that received L-dopa (t=-10.09, p<0.05), relative to rats that received saline vehicle (Figure 4A). Similarly, acute L-dopa caused a significant increase in dopamine content within the ventral DRN (t=-9.25, p<0.05) (Figure 4B). Acute treatment also significantly increased dopamine metabolites, DOPAC and HVA in both dorsal (DOPAC: t=-14.74, p<0.05; HVA: t=-5.17, p<0.05) and ventral (DOPAC: t=-14.62, p<0.05; HVA: t=-5.04, p<0.05) sub-regions. The magnitude of dopamine elevation above control levels did not differ when dorsal and ventral sub-regions were compared (t=-2.08, p<0.06) (Figure 4C). However, there was a greater increase in the ratio of DOPAC to dopamine in the dorsal (saline: 0.19 ± 0.02; L-dopa: 0.98 ± 0.14) compared to the ventral DRN (saline: 0.47 ± 0.05; L-dopa: 1.15 ± 0.10) after acute L-dopa when normalized to saline controls (t=3.60, p<0.05) (Figure 4D).

Chronic L-dopa and 5-HT Tissue Content

To evaluate the possible consequences of L-dopa induced decreases in TPH+ neurons, the amount of 5-HT within the DRN and forebrain regions was measured. Non-lesioned rats were treated for 10 days with L-dopa and killed 48 hours after the last injection. Chronic L-dopa treatment significantly lowered 5-HT concentrations in both the DRN and the prefrontal cortex (PFC). More specifically, the dorsal sub-region of the DRN was significantly depleted of 5-HT and 5HIAA content by 23% and 29% (data not
shown) respectively, after L-dopa treatment (5-HT: t=2.55, p<0.05; 5HIAA: t=2.48, p<0.05). 5-HT content in the PFC was significantly decreased by 28% in rats treated with L-dopa (t=2.87, p<0.05) (Figure 5) whereas 5HIAA content was unaffected (t=1.61, p=0.12); however the ratio of 5HIAA to 5-HT was significantly elevated after L-dopa compared to vehicle (t=−2.36, p<0.05) (data not shown). There was no effect of L-dopa treatment on 5-HT tissue content of the ventral DRN, hippocampus or striatum, (DRV: t=1.84, p=0.09; HIPP: t=1.38, p=0.19; STR: t=1.16, p=0.27) (Figure 5).

Effects of Ascorbic Acid on L-dopa Induced Decreases in 5-HT Tissue Content

To examine the effects of an antioxidant on chronic L-dopa induced decreases in 5-HT content within the dorsal DRN and PFC, ascorbic acid was administered before each L-dopa dose, twice daily for ten days. Rats were killed 48 hours after the last injection. There was a significant interaction between L-dopa and ascorbic acid on 5-HT in the dorsal DRN sub-region (F(1,38)=5.18, p<0.05), as the effect of L-dopa on 5-HT tissue content depended on ascorbic acid co-treatment. Ascorbic acid alone had no effect on 5-HT content but blocked the decreases in 5-HT content when administered with L-dopa (q=3.06, p<0.05) (Figure 6A). In the PFC, there was a significant interaction between L-dopa and ascorbic acid (F(1,36)=7.48, p<0.05). Whereas L-dopa alone decreased 5-HT tissue content significantly (q=3.42, p<0.05), this effect was blocked by ascorbic acid co-treatment (q=3.66, p<0.05) (Figure 6B).
Discussion

The effects of chronic L-dopa on TPH+ neuron cell bodies in the dorsal raphe and 5-HT tissue content in the rat brain were investigated. Chronic L-dopa decreased TPH+ cell bodies within the caudal-extent of the dorsal DRN and decreased 5-HT tissue content in the dorsal DRN as well as the PFC. Ascorbic acid and deprenyl prevented the decreases in TPH+ cell bodies when administered prior to L-dopa. Ascorbic acid pre-treatment also blocked the decreases in 5-HT tissue content caused by L-dopa.

Chronic L-dopa decreased the number of TPH+ cell bodies throughout the entire rostral-caudal extent of the DRN (Figure 1), while a 6-OHDA lesion had no effect on cell body number in the presence or absence of L-dopa. When the DRN was analyzed sub-regionally, the caudal-extent of the dorsal DRN was specifically susceptible to L-dopa (Figure 2B). This is the first direct evidence for decreased TPH+ cell bodies after L-dopa treatment in vivo. The lack of a 6-OHDA lesion effect on 5-HT neurons is in contrast to a previous report (Eskow Jaunarajs et al., 2012) that found bilateral 6-OHDA lesions, but not chronic L-dopa, decreased 5-HT immunoreactive cell bodies throughout the entire DRN. In the current study, no effect of unilateral 6-OHDA lesion on TPH+ was noted but L-dopa alone differentially decreased TPH+ cell bodies in a DRN sub-region dependent manner. These seemingly contradictory results between Eskow Jaunarajs et al. and the current study may be explained by the differences in whether bilateral lesions (Eskow Jaunarajs et al., 2012) or unilateral lesions (current study) were made. Moreover, the current study provides a more detailed assessment of the DRN by considering possible sub-regional variations in susceptibility to L-dopa, the effects of which might be otherwise masked if the DRN was analyzed as a homogeneous structure,
Previous *in vitro* studies using 5-HT neurons in culture showed that L-dopa can be toxic to 5-HT neurons through a pro-oxidant mechanism that is dependent on the intracellular synthesis of dopamine and its metabolism (Stansley and Yamamoto, 2013). The current study extends these findings to demonstrate that chronic L-dopa administered *in vivo* to intact rats decreases TPH+ cell bodies in the DRN by similar mechanisms as that demonstrated *in vitro*. The finding that the antioxidant ascorbate blocked the decrease in TPH+ neurons within the DRN is consistent with a pro-oxidant effect of L-dopa on TPH+ neurons (Figure 3A) demonstrated *in vitro* with cultured 5-HT cells (Stansley and Yamamoto, 2013).

In addition to antioxidant protection, rats pretreated with deprenyl before each L-dopa dose also blocked the decreases in TPH+ cell number within the DRN (Figure 3A), suggesting that the dopamine formed from L-dopa is metabolized by MAO-B to a pro-oxidant species that damages TPH+ neurons. These data contrast with Camp et al., 2000, who found that L-dopa, acutely or chronically (5 days), did not elevate extracellular hydroxyl radicals in the rat striatum (Camp et al., 2000). This difference may be due to a longer duration of treatment in the present study (10 days) and the finding that a separate group showed that when L-dopa was given for an even longer duration (16 days), hydroxyl radical production was significantly increased (Ishida et al., 2000). Regardless, the current study is consistent with the finding that L-dopa can produce intracellular reactive-oxygen species and cell death to cultured 5-HT neurons in a manner that is dependent on the formation of dopamine and the degradation of dopamine by MAO (Stansley and Yamamoto, 2013).
It is well known that non-neuronal cells such as glia can decarboxylate L-dopa to dopamine (Juorio et al., 1993) but glial cells have a limited ability to exocytose dopamine formed from L-dopa due to the lack the vesicular monoamine transporter, a necessary component of L-dopa induced dopamine release (Kannari et al., 2000). Therefore, it is likely that the decreases in 5-HT are direct effects of dopamine within 5-HT neurons and not and indirect effect of glial derived dopamine.

The role of dopamine and dopamine metabolism in mediating the differential effects of L-dopa on sub-regions within the DRN was also determined. Acute L-dopa increased dopamine and its metabolites to a similar magnitude in both the dorsal and ventral sub-regions (Figures 4A-C). Although no sub-regional differences in dopamine concentration after acute L-dopa were noted, chronic treatment may result in a greater increase in dopamine, its degradation, and thus oxidative stress. Moreover, there may be a persistent conversion of L-dopa to dopamine within 5-HT neurons during chronic treatment because 5-HT neurons lack the autoregulatory mechanisms of dopamine production inherent to dopaminergic neurons such as the dopamine receptor mediated reduction in aromatic amino acid decarboxylase activity and dopamine synthesis (Zhu et al., 1994). Another factor contributing to DRN sub-regional differences after L-dopa may be a greater degradation of dopamine and thus increased oxidative stress. Indeed, there was a greater increase in the DOPAC to dopamine ratio after L-dopa in the dorsal compared to the ventral sub-region (Figure 4D), indicating that dopamine is degraded by MAO to a greater extent in the dorsal sub-region. The higher dopamine turnover in the dorsal sub-region likely results in increased hydroxyl radicals formed by the MAO dependent production of hydrogen peroxide (Spina and Cohen, 1989). This higher
turnover rate and oxidation of dopamine by MAO-B in the dorsal sub-region could account for the sub-regional effects of chronic L-dopa on 5-HT neurons within the DRN.

In addition to the decrease in TPH+ cell bodies after chronic L-dopa, there was a parallel decrease in 5-HT tissue content in the dorsal, but not ventral sub-region of the DRN (Figure 5). Interestingly, only the PFC exhibited significantly decreased 5-HT tissue content after L-dopa compared to vehicle injected rats, while no changes were observed in the striatum or hippocampus. These forebrain region specific decreases may be explained by the fact that TPH+ cells are decreased only in the dorsal DRN sub-region. In fact, the PFC not only receives dense 5-HT innervations from the DRN, the majority of those innervations emanate from 5-HT neurons in the caudal extent of the dorsal DRN (Van Bockstaele et al., 1993). Thus, decreases in 5-HT tissue content within the PFC and dorsal DRN may be a consequence of TPH+ cell body loss within the caudal extent of the dorsal DRN. Furthermore, pretreatment with ascorbic acid, prevented TPH+ cell body loss (Figure 3) and decreases in 5-HT tissue content in the dorsal DRN and PFC (Figure 6A, 6B), and supports the interpretation that decreased 5-HT tissue content is a consequence of TPH+ cell body loss through oxidative mechanisms.

The chronic L-dopa induced 5-HT decreases in the present study were restricted to dorsal DRN and PFC, in contrast to the study by Navailles et al. (2011) which demonstrated a more homogenous decrease in 5-HT tissue content and release throughout the rat brain after chronic L-dopa (Navailles et al., 2011a). These differences may be attributable to when 5-HT was measured. Indeed, in the present study 5-HT was measured at 48 hours after the last injection when exogenous L-dopa is no longer
elevated, suggesting that these longer lasting decreases are likely due to 5-HT cell loss or terminal damage as opposed to competition between L-dopa and 5-HTP at the amino acid decarboxylase enzyme. While it is possible that a higher L-dopa dose may have produced a 5-HT deficit in other brain regions, it is noteworthy that chronic low dose L-dopa results in persistent decreases in concentrations of 5-HT.

The current findings have implications for the non-motor symptoms of Parkinson’s disease. Chronic L-dopa specifically affected the caudal extent of the dorsal DRN and the PFC, two brain regions involved in affective behaviors (Lowry et al., 2005; Lowry et al., 2008; Warden et al., 2012; Albert et al., 2014). The cross-talk between these two regions is involved in fear and anxiety responses in rats (Hashimoto et al., 1999; Bishop et al., 2004; Amat et al., 2005; Forster et al., 2006) and several preclinical and clinical studies propose that chronic L-dopa therapy for Parkinson’s disease could result in mood disturbances such as anxiety and depression (Damasio et al., 1971; Choi et al., 2000; Borah and Mohanakumar, 2007; Eskow Jaunarajs et al., 2010; Eskow Jaunarajs et al., 2012). These mood disturbances may be attributable to an underlying dysregulation of the monoaminergic systems within limbic regions as demonstrated in rats (Eskow Jaunarajs et al., 2012) and more recently in monkeys (Engeln et al., 2014). Additionally, the current findings support the use of selective MAO-B inhibitors in the treatment of Parkinson’s disease, as deprenyl prevented the toxicity of L-dopa to 5-HT neurons. Furthermore, MAO-B inhibitors have been clinically proven to be an effective adjunct therapy to L-dopa for the treatment of motor-symptoms associated with Parkinson’s disease and the depression associated with the disease (Mendlewicz and Youdim, 1983; Youdim and Bakhle, 2006; Fernandez and
Chen, 2007; Riederer and Laux, 2011). Thus, the underlying the efficacy of MAO-B inhibitors and perhaps the potential use of antioxidants in this context may be related to the amelioration of L-dopa induced dopamine and dopamine–dependent reactive oxygen species that would otherwise lead to 5HT neuron loss.
Authorship Contributions

Participated in research design: Stansley and Yamamoto.

Conducted experiments: Stansley.

Contributed new reagents or analytical tools: Yamamoto.

Performed data analysis: Stansley and Yamamoto.

Wrote or contributed to the writing of the manuscript: Stansley and Yamamoto.
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release in a region-dependent manner in a rat model of Parkinson's disease.

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Footnotes

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Figure Legends

Figure 1. Effects of L-dopa on TPH+/NeuN+ cell counts in the DRN. Rats received 10 days of L-dopa or vehicle treatment. (A) Chronic L-dopa treatment significantly decreased TPH+/NeuN+ cell bodies (*p<0.05 compared to vehicle, Student’s t-test). (B) Representative DRN images (20x) illustrating TPH+/NeuN+ co-labeling (Green = NeuN+, Red = TPH+, Yellow = TPH+/NeuN+ co-label) (n = 9-11 per group).

Figure 2. Sub-regional effects of L-dopa on TPH+ cell counts in the DRN. (A) Chronic L-dopa treatment had no effect on TPH+/NeuN+ cell bodies compared to vehicle in the rostral-extent of the dorsal sub-region. (B) L-dopa treatment significantly decreased TPH+/NeuN+ cell bodies in the caudal-extent of the dorsal sub-region (*p<0.05 compared to vehicle, Student’s t-test). (C,D) L-dopa treatment had no effect on TPH+/NeuN cell bodies compared to vehicle in the either rostral or caudal-extent of the ventral sub-region (n = 9-11 per group).

Figure 3. Effects of ascorbic acid and deprenyl on L-dopa induced decreases in TPH+ cell counts. TPH+/NeuN+ cell bodies were counted within the caudal-extent of the dorsal DRN sub-region. (A) Both ascorbic acid (400 mg/kg) and deprenyl (2 mg/kg) prevented the effects of L-dopa at decreasing TPH+/NeuN+ cell bodies (*p<0.05 compared to vehicle, two-way ANOVA and Tukey’s post-hoc test). (B) Representative DRN images (20x) illustrating TPH+/NeuN+ co-labeling (Green = NeuN+, Red = TPH+, Yellow = TPH+/NeuN+ co-label) (n = 4-6 per group).
Figure 4. Acute L-dopa and dopamine tissue content in the DRN. Dopamine, DOPAC, and HVA tissue content in the DRN were measured 45 minutes after L-dopa injection. (A, B) Acute L-dopa significantly elevated dopamine and its metabolites in both the dorsal and ventral DRN compared to vehicle. (C) The magnitude of dopamine increase after acute L-dopa did not differ between dorsal and ventral DRN. (D) The DOPAC/dopamine ratio was significantly greater in the dorsal compared to ventral DRN after acute L-dopa. (*p<0.05 compared to vehicle controls, # p<0.05 compared to ventral DRN, Student’s t-test) (n= 6 per group).

Figure 5. Chronic L-dopa and 5-HT tissue content. Rats received 10 days of L-dopa or vehicle treatment. Chronic L-dopa significantly reduced 5-HT tissue content in the dorsal DRN and the PFC (*p<0.05 compared to vehicle, Student’s t-test). L-dopa treatment did not affect the ventral DRN, striatum, or hippocampus (n= 8-12 per group).

Figure 6. Effects of ascorbic acid on L-dopa induced decreases in 5-HT tissue content. (A) Ascorbic acid prevented chronic L-dopa induced decreases in 5-HT tissue content in the dorsal DRN. (B) Ascorbic acid pretreatment also prevented the 5-HT tissue content decreases in the PFC (* p<0.05 compared to vehicle, two-way ANOVA and Tukey’s post-hoc test) (n= 9-12 per group).
Figure 1

Dorsal Raphe Nucleus

A

B

Vehicle
L-dopa

Vehicle
L-dopa

TPH+ Neurons (per 25μm section)

*
Figure 2

Dorsal DRN Sub-region

A  Rostral

B  Caudal

Ventral DRN Sub-region

C  Rostral

D  Caudal

TPH+ Neurons (per 25 µm section)

Vehicle  L-dopa

Vehicle  L-dopa

*
Figure 3

A  Dorsal DRN: Caudal-Extent

B  Vehicle  Ascorbic Acid  Deprenyl

L-dopa  Ascorbic Acid  Deprenyl

L-dopa  L-dopa  L-dopa
Figure 4

Dorsal Raphe Sub-regions

(A) Dorsal DRN

(B) Ventral DRN

(C) Dopamine levels in dorsal and ventral regions.

(D) DOPAC/Dopamine ratio in dorsal and ventral regions.
Figure 6

A. Dorsal DRN

B. Pre-frontal Cortex