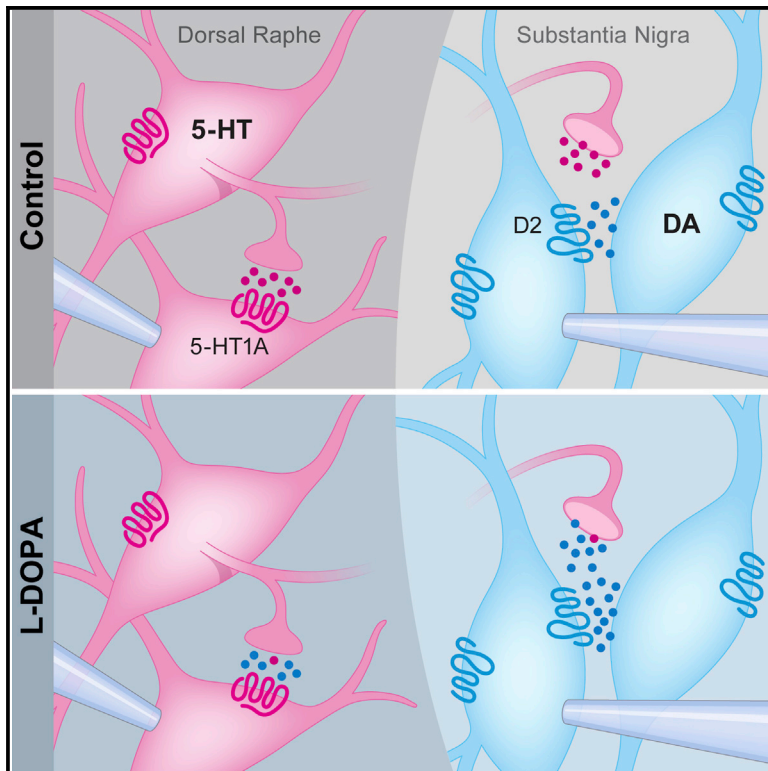


Depression of Serotonin Synaptic Transmission by the Dopamine Precursor L-DOPA

Graphical Abstract



Authors

Stephanie C. Gantz, Erica S. Levitt, Nerea Llamosas, Kim A. Neve, John T. Williams

Correspondence

williamj@ohsu.edu

In Brief

Gantz et al. demonstrate that after exposure to L-DOPA, serotonin neurons release dopamine in the midbrain and within the dorsal raphe. The production and vesicular release of dopamine produces a long-lasting depression of 5-HT_{1A}-receptor-mediated synaptic transmission.

Highlights

- L-DOPA augments D₂-receptor-mediated synaptic transmission in the midbrain
- Serotonin terminals contribute to dopamine synaptic transmission after L-DOPA
- L-DOPA depresses 5-HT-mediated synaptic transmission in the dorsal raphe
- In the dorsal raphe, serotonin neurons release dopamine from vesicles after L-DOPA



Depression of Serotonin Synaptic Transmission by the Dopamine Precursor L-DOPA

Stephanie C. Gantz,¹ Erica S. Levitt,¹ Nerea Llamosas,² Kim A. Neve,³ and John T. Williams^{1,*}

¹Vollum Institute, Oregon Health & Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239, USA

²Department of Pharmacology, Faculty of Medicine and Dentistry, University of the Basque Country UPV/EHU, Leioa 48940, Spain

³Research Service, VA Portland Health Care System and Department of Behavioral Neuroscience, Oregon Health & Science University, Portland, OR 97239, USA

*Correspondence: williamj@ohsu.edu

<http://dx.doi.org/10.1016/j.celrep.2015.07.005>

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SUMMARY

Imbalance between the dopamine and serotonin (5-HT) neurotransmitter systems has been implicated in the comorbidity of Parkinson's disease (PD) and psychiatric disorders. L-DOPA, the leading treatment of PD, facilitates the production and release of dopamine. This study assessed the action of L-DOPA on monoamine synaptic transmission in mouse brain slices. Application of L-DOPA augmented the D2-receptor-mediated inhibitory postsynaptic current (IPSC) in dopamine neurons of the substantia nigra. This augmentation was largely due to dopamine release from 5-HT terminals. Selective optogenetic stimulation of 5-HT terminals evoked dopamine release, producing D2-receptor-mediated IPSCs following treatment with L-DOPA. In the dorsal raphe, L-DOPA produced a long-lasting depression of the 5-HT_{1A}-receptor-mediated IPSC in 5-HT neurons. When D2 receptors were expressed in the dorsal raphe, application of L-DOPA resulted in a D2-receptor-mediated IPSC. Thus, treatment with L-DOPA caused ectopic dopamine release from 5-HT terminals and a loss of 5-HT-mediated synaptic transmission.

INTRODUCTION

The monoamine neurotransmitters, noradrenaline, dopamine, and serotonin, modulate basic physiological functions including sleep, motor control, food intake, sexual arousal, as well as influence mood, temperament, and behavioral reinforcement. Dysfunction of the dopamine system has been associated with substance abuse, schizophrenia, attention-deficit hyperactivity disorder, and Parkinson's disease (PD). Imbalance in the serotonin (5-hydroxytryptamine [5-HT]) system has been linked to the etiology of complex emotional disorders including anxiety, bipolar, impulsivity, and depression. The prevalence of comorbidity in these disorders implicates significant interaction between the dopamine and 5-HT systems. Understanding interactions between the monoamine systems is necessary to reduce side effects of pharmaceuticals that target these systems.

The focus of this study is the immediate dopamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA), the leading therapy to treat the motor symptoms of PD. Chronic L-DOPA treatment produces undesirable side effects, including the development of dyskinesias and fluctuations in mood that exacerbate psychiatric symptoms such as anxiety, impulsivity, and depression in humans (Cools et al., 2003; Damásio et al., 1971) and rodent models (Borah and Mohanakumar, 2007; Eskow Jaunarajs et al., 2012). Many studies have implicated an imbalance between dopamine and 5-HT as the cause of these detrimental side effects (Borah and Mohanakumar, 2007; Carta et al., 2007; Eskow Jaunarajs et al., 2012; Hornykiewicz, 1975; Navailles et al., 2010). There is also significant recent interest in using L-DOPA to treat a myriad of health conditions, including Angelman syndrome, Tourette syndrome, restless legs syndrome, subacute back pain, and cocaine dependence. Because these conditions are often comorbid with 5-HT-linked psychiatric symptoms, a greater understanding of the action of L-DOPA on the serotonergic system is necessary.

5-HT neurons can convert L-DOPA to dopamine through the L-amino acid decarboxylase (AADC; Arai et al., 1994; Ng et al., 1970). Exposure of 5-HT neurons to L-DOPA results in vesicular packaging of dopamine via the vesicular monoamine transporter (VMAT2) and activity-dependent release of dopamine (Kannari et al., 2000; Tanaka et al., 1999). Since VMAT2 preferentially transports dopamine (Finn and Edwards, 1997), production of dopamine in 5-HT neurons could reduce the content of vesicular 5-HT. But, the consequence of L-DOPA on 5-HT-dependent synaptic transmission has not been reported. Furthermore, evidence that 5-HT terminal-derived dopamine activates postsynaptic dopamine receptors has not been described. The present study describes a depression in serotonin synaptic transmission after L-DOPA. The results reveal that following treatment with L-DOPA serotonin terminals participate in D2-receptor-dependent dopamine signaling in the substantia nigra (SN) and reduce 5-HT_{1A}-receptor-dependent signaling in the dorsal raphe (DR).

RESULTS

Acute L-DOPA Enhances Dopamine Transmission in the Substantia Nigra

In the SN, vesicular dopamine release elicits D2-receptor-mediated inhibitory postsynaptic currents (IPSCs) through the

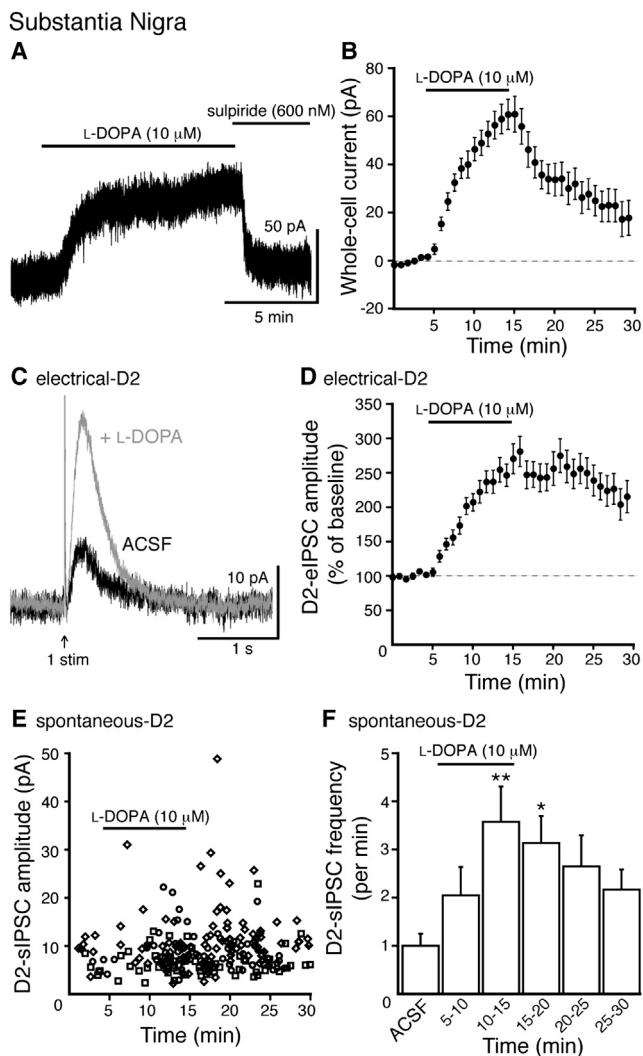


Figure 1. Acute L-DOPA in the SN Enhances Evoked and Spontaneous D2-IPSCs

(A) Representative trace of a whole-cell voltage-clamp recording of the outward current induced by bath application of L-DOPA (10 μ M), which was reversed by the D2 receptor antagonist sulpiride (600 nM). (B) Outward current to L-DOPA application, mean \pm SEM (n = 23). (C) Representative traces of D2-eIPSCs from a single experiment in ACSF and after L-DOPA. (D) D2-eIPSCs were evoked once every 50 s. L-DOPA increased the D2-eIPSC amplitude, mean \pm SEM, baseline: mean amplitude of six D2-eIPSCs preceding L-DOPA application (n = 22). (E) Plot of spontaneous D2-sIPSC amplitude versus time from three experiments (circle, square, and diamond) showing the increase in frequency by L-DOPA. (F) The frequency of D2-sIPSCs (per min, 5-min bins) increased during and after L-DOPA application, mean \pm SEM (n = 8–17, one-way ANOVA). *p < 0.05 and **p < 0.01.

activation of G protein-coupled inwardly rectifying potassium (GIRK) channels (Beckstead et al., 2004). Whole-cell voltage-clamp recordings were made from SN dopamine neurons in horizontal midbrain slices from wild-type mice. A single electrical

stimulus was used to evoke D2-IPSCs (D2-eIPSC) once every 50 s in the presence of NMDA, AMPA, GABA_A, GABA_B, and nACh receptor antagonists. As previously reported, application of L-DOPA (10 μ M, 10 min) had three actions (Beckstead et al., 2004; Gantz et al., 2013; Mercuri et al., 1990). First, L-DOPA (10 μ M) produced an outward current (62 \pm 6 pA, p < 0.001, paired t test, Figures 1A, 1B, and 2A), which returned to baseline upon wash out or was reversed by the D2 receptor antagonist, sulpiride (600 nM). Pretreatment with sulpiride prevented the outward current induced by L-DOPA (5 \pm 3 pA, p = 0.22, n = 6). Second, L-DOPA produced a robust augmentation of the D2-eIPSC amplitude (252% \pm 19% of baseline, p < 0.001, paired t test, Figures 1C, 1D, 2B, and 3C). Last, L-DOPA increased the amplitude (p < 0.001, Mann-Whitney U test) and frequency of spontaneous D2-sIPSCs (p = 0.02, one-way ANOVA, Figures 1E and 1F) that result from vesicular release of dopamine in the absence of stimulation (Gantz et al., 2013). The transient outward current produced by iontophoretically applied dopamine (I-DA) was not increased by L-DOPA. In fact, there was a small decrease in I-DA that correlated with the amplitude of the outward current induced by L-DOPA (reduction in I-DA: 34 \pm 11 pA; outward current: 45 \pm 9 pA, p < 0.05, n = 9, Pearson correlation). The results indicate that acute application of L-DOPA enhanced evoked and spontaneous dopamine transmission in the SN by a presynaptic mechanism.

The Conversion of L-DOPA to Dopamine Is Required

L-DOPA is converted to dopamine by the L-amino acid decarboxylase (AADC). To determine whether the conversion to dopamine was required, brain slices were treated with the AADC inhibitor, NSD-1015 (20 μ M, >15 min). NSD-1015 blocked the outward current induced by L-DOPA (NSD: 7 \pm 3 pA, NSD and L-DOPA: 0 \pm 4 pA, p = 0.37, Figure 2A). In addition, NSD-1015 prevented the L-DOPA-induced enhancement in D2-eIPSC amplitude (NSD: 18 \pm 2 pA, NSD and L-DOPA: 16 \pm 2 pA, p = 0.07, paired t test, Figure 2B), and the L-DOPA-induced increase in spontaneous D2-sIPSC frequency (ACSF: 0.9 per min, NSD: 1.2 per min, NSD and L-DOPA: 0.8 per min, p = 0.27, Figure 2C). Thus, the metabolism of L-DOPA to dopamine was required to affect dopamine-dependent transmission.

5-HT Terminals Release Dopamine in the Substantia Nigra after Acute or In Vivo L-DOPA

Dopamine neurons in the SN receive a prominent projection from 5-HT neurons in the raphe nuclei (Dray et al., 1976; Moukhes et al., 1997). Given that 5-HT neurons can convert L-DOPA to dopamine through the AADC (Arai et al., 1994), the ability of 5-HT terminals in the SN to participate in dopamine signaling after L-DOPA was assessed. 5-HT terminals, but not dopamine neurons, have 5-HT1B/D receptors that strongly inhibit 5-HT release (Cameron and Williams, 1994; Morikawa et al., 2000). Application of the selective 5-HT1B/D receptor agonist, sumatriptan (1 μ M), alone had no effect on the D2-eIPSC (p = 0.77, Figures 3A and 3B), consistent with a previous report that used the 5-HT1 receptor agonist, 5-CT (Ford et al., 2006). However, sumatriptan reduced and reversed the L-DOPA-induced enhancement of the D2-eIPSC. Sumatriptan, when applied before L-DOPA, significantly reduced the enhancement of the

Substantia Nigra

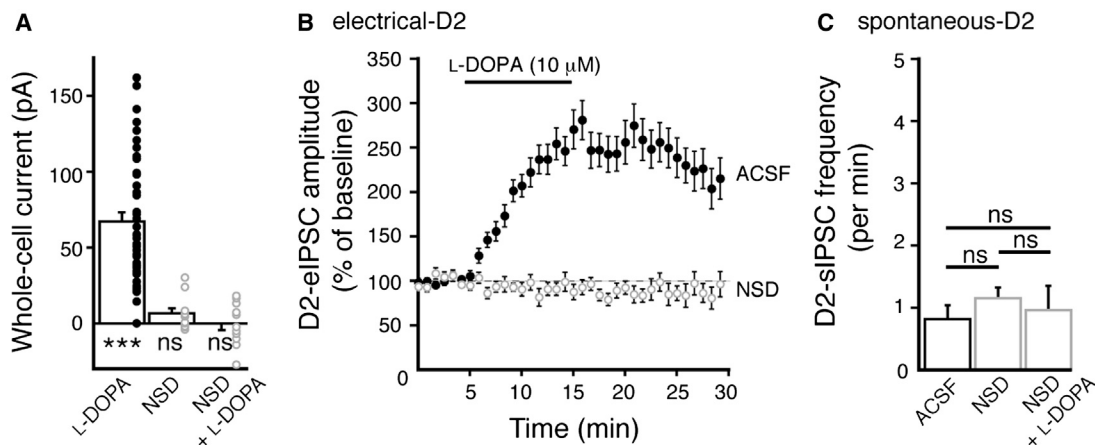


Figure 2. In the SN, the Conversion of L-DOPA to Dopamine Is Required

(A) Peak outward current to L-DOPA (10 μ M) application, mean \pm SEM (black circles, $n = 23$, paired t test). The AADC inhibitor, NSD-1015 (NSD, 20 μ M, 15 min), prevented the outward current induced by L-DOPA (open circles, $n = 9$, RM one-way ANOVA).

(B) D2-eIPSCs were evoked once every 50 s. NSD prevented the augmentation of D2-eIPSC by L-DOPA (black circles in ACSF, as shown in Figure 1D, open circles in NSD, $n = 13$), mean \pm SEM, baseline: mean amplitude of six D2-eIPSCs preceding L-DOPA application.

(C) NSD prevented the L-DOPA-induced increase in spontaneous D2-sIPSC frequency, mean \pm SEM (per min, $n = 11$, RM one-way ANOVA). ns, not significant and *** $p < 0.001$.

D2-eIPSC by L-DOPA ($136\% \pm 11\%$ of baseline, $p < 0.001$, Figures 3C and 3D). When applied following L-DOPA, sumatriptan decreased the D2-eIPSC to $142\% \pm 13\%$ of its original amplitude, which was not statistically different from the enhancement by L-DOPA following pretreatment of sumatriptan ($p = 0.79$, $n = 9$, unpaired t test). While sumatriptan markedly reduced the L-DOPA-induced enhancement of the D2-eIPSC, the L-DOPA-induced increase in the presence of sumatriptan was still significant ($p = 0.001$, paired t test, Figures 3A, 3C, and 3D). This indicates that L-DOPA also increased dopamine release from dopamine neurons. Furthermore, in the presence of sumatriptan, L-DOPA increased the frequency of spontaneous D2-sIPSCs ($p < 0.001$, Figure 3E), to the same extent and with a similar time course as in control ($p = 0.997$, two-way ANOVA, Figures 1F and 3E, Gantz et al., 2013). Pretreatment with sumatriptan also had no effect on the outward current produced by L-DOPA (82.5 ± 10.5 pA, $p = 0.53$, $n = 21$, Mann-Whitney U test). These results suggest that 5-HT terminals released dopamine after acute exposure to L-DOPA.

To determine whether this phenomenon persists after chronic L-DOPA treatment, mice were treated with L-DOPA (100 mg/kg, subcutaneous) and a peripheral AADC inhibitor, benserazide (200 mg/kg, subcutaneous), or with benserazide alone once daily for 6 days. Brain slices were prepared 1 hr after the final injection. D2-eIPSCs were evoked with a single stimulus (one stim) or a train of five stimuli (five stims), to obtain better signal-to-noise ratio (Figure 3F). In slices from mice treated with benserazide alone, sumatriptan had no effect on the D2-eIPSC amplitude (one stim: $94\% \pm 5\%$; five stims: $100\% \pm 5\%$ of baseline, Figure 3G). In slices from mice treated with L-DOPA, however, sumatriptan significantly decreased the D2-eIPSC amplitude, whether evoked by one stim ($67\% \pm 5\%$ of baseline, $p < 0.001$) or five stims ($72\% \pm 5\%$ of baseline, $p < 0.001$, Figures 3F and

3G). Taken together, these results demonstrate that dopamine release in the SN, after acute or chronic in vivo L-DOPA exposure, was attenuated by activation of 5-HT_{1B/D} receptors, suggesting that dopamine was released from 5-HT terminals.

An optogenetic strategy was employed to selectively activate 5-HT terminals in the SN, using the ePet-Cre transgenic mouse line that expresses Cre recombinase in serotonin neurons (Scott et al., 2005). The selective expression of Cre recombinase in 5-HT neurons in the ePet-Cre line was validated by immunohistochemistry. ePet-Cre^{+/−} mice were crossed with the Ai9 Cre reporter mouse line that produces cytosolic tdTomato in Cre⁺ cells. Mice were transcardially perfused and the brains were sectioned for immunohistochemistry (Figures S1A–S1C and 4A). No tdTomato⁺ cells were detected in ePet-Cre^{−/−} sections. In ePet-Cre^{+/−} sections, cell counts ($n = 4,330$) were made in the DR (Figure S1), which contains the majority of 5-HT neurons in the rodent brain (Dahlstrom and Fuxe, 1964). Sections were stained for tyrosine hydroxylase (TH) and tryptophan hydroxylase (TpH), the rate-limiting enzymes in the production of dopamine and 5-HT, respectively. In the DR, 82% of the Cre⁺ cells were TpH⁺, whereas 7% of the Cre⁺ cells were TH⁺ and not TpH⁺. Generally, the TH⁺ cells had smaller somas and were located close to the cerebral aqueduct. The remaining Cre⁺ cells (11%) did not co-stain with TpH or TH (Figure S1B). Last, 24% of TpH⁺ neurons were not Cre⁺ (Figure S1C). In midbrain sections, TpH⁺/Cre⁺ fibers were found throughout the SN, as well as Cre[−] only and TpH⁺-only fibers. Importantly, no SN dopamine neurons (TH⁺) were Cre⁺ (Figure 4A).

The ePet-Cre^{+/−} mice were crossed with the Ai32 mouse line to express the light-activated cation channel, channelrhodopsin-2 (ChR2) in Cre⁺ cells. In slices from these mice, L-DOPA augmented the electrically evoked D2-eIPSC to the same extent as in slices from wild-type mice ($244\% \pm 28\%$, $p = 0.81$, $n = 10$,

Substantia Nigra acute *in vitro* L-DOPA

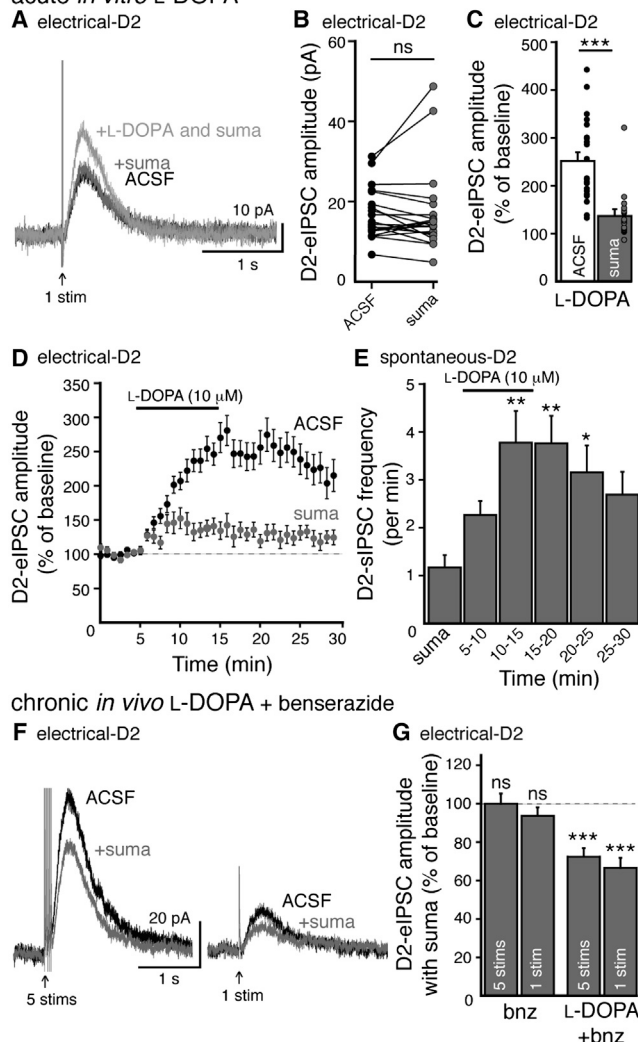


Figure 3. Activation of 5-HT Autoreceptors Attenuates the L-DOPA-Induced Increase in Evoked D2-IPSCs in the SN

(A) Representative traces of D2-eIPSCs from a single experiment in ACSF, after the addition of the 5-HT_{1B/D} receptor agonist, sumatriptan (1 μ M), and after bath application of L-DOPA (10 μ M) with sumatriptan.

(B) Sumatriptan alone did not change the D2-eIPSC amplitude ($n = 21$, paired t test).

(C and D) D2-eIPSCs were evoked once every 50 s. Pretreatment with sumatriptan significantly blunted the L-DOPA-induced increase in the D2-eIPSC amplitude, compared to the increase in ACSF (ACSF: black circles, sumatriptan: gray circles, $n = 20$ – 22 , unpaired t test), mean \pm SEM, baseline: mean amplitude of six D2-eIPSCs preceding L-DOPA application.

(E) In the presence of sumatriptan, L-DOPA increased the frequency of spontaneous D2-sIPSCs (per min, 5 min bins), mean \pm SEM ($n = 14$ – 17 , one-way ANOVA).

(F and G) Sumatriptan decreased the D2-eIPSC amplitude following chronic *in vivo* treatment with L-DOPA and a peripheral AADC inhibitor, benserazide (bnz). (F) Representative traces of D2-eIPSCs evoked by a single (one stim) or train (five stims) of electrical stimuli, in ACSF and after application of sumatriptan. (G) Sumatriptan decreased the D2-eIPSC amplitude following chronic *in vivo* L-DOPA and bnz treatment, but had no effect after bnz treatment alone, mean \pm SEM, baseline: mean amplitude of six D2-eIPSCs

unpaired t test). ChR2-expressing axon terminals were activated with a light pulse (LED, 470 nm, 5–15 ms) every 50 s, in the presence of NMDA, AMPA, GABA_A, GABA_B, and nACh receptor antagonists, and 4-aminopyridine (100 μ M, see [Supplemental Experimental Procedures](#) and [Figure S2](#)). In 16/18 recordings, the activation of ChR2 had no effect prior to the application of L-DOPA. In the other two recordings, activation of ChR2 evoked an IPSC that was blocked by the 5-HT_{1A} receptor antagonist, WAY 100635 (100 nM). This observation is similar to previous work done in slices from guinea pig, demonstrating a small population of dopamine neurons with 5-HT_{1A}-receptor-mediated inhibitory postsynaptic potentials ([Cameron et al., 1997](#)). After treatment with L-DOPA (10 μ M, 10 min), in every case, activation of ChR2 induced a D2-receptor-mediated oIPSC (10 ± 1 pA, [Figures 4B and 4C](#)). The oIPSCs were completely eliminated by the D2 receptor antagonist, sulpiride (600 nM, 1 ± 0.4 pA, $p = 0.004$, $n = 5$, paired t test). The time-to-peak and duration of the D2-oIPSC were longer than the D2-eIPSC, recorded in the same conditions (time-to-peak: oIPSC: 389 ± 11 ms; eIPSC: 305 ± 14 ms; $p < 0.001$, [Figure 4D](#); duration at 20%: oIPSC: $1,071 \pm 83$ ms, eIPSC: 795 ± 37 ms, $p = 0.007$, Mann-Whitney U test). Activation of 5-HT_{1B/D} receptors with sumatriptan or 5-CT (100 nM) inhibited the D2-oIPSCs ($80\% \pm 3\%$ inhibition, $p < 0.001$, [Figures 4B and 4C](#)). Thus, after treatment with L-DOPA, selective activation of 5-HT terminals in the SN evoked dopamine release and produced a D2-receptor-mediated synaptic current from 5-HT terminal-derived dopamine.

Acute L-DOPA Reduces Serotonin Transmission in the Dorsal Raphe

To determine whether L-DOPA-induced synaptic release of dopamine from 5-HT terminals affected vesicular 5-HT release, recordings were made from 5-HT neurons in the DR. In the DR, 5-HT release produces 5-HT_{1A}-receptor-mediated IPSCs through the activation of GIRK channels ([Williams et al., 1988](#)). Whole-cell recordings were made from 5-HT neurons in coronal DR slices from wild-type mice. 5-HT neurons were identified by slow, broad, and regular action potential firing upon current injection. 5-HT_{1A}-eIPSCs were evoked by a single electrical stimulus once every 50 s, in the presence of NMDA, AMPA, GABA_A, and α -1 adrenergic receptor antagonists. The 5-HT_{1A}-eIPSC was blocked by the 5-HT_{1A} antagonist, WAY 100635 (100 nM), or inhibited by activation of 5-HT_{1B/D} receptors with sumatriptan (1 μ M). Application of L-DOPA (10 μ M, 10 min) attenuated the 5-HT_{1A}-eIPSC amplitude ($80\% \pm 6\%$ of baseline, $p = 0.002$, [Figure 5A](#)), without changing the holding current (-9 ± 4 pA, $p = 0.06$, $n = 11$, paired t test). Higher concentrations of L-DOPA (30 and 50 μ M) caused larger reductions in the 5-HT_{1A}-eIPSC that did not recover upon L-DOPA wash out (30 μ M: $33\% \pm 7\%$ of baseline, $p < 0.001$; 50 μ M: $10\% \pm 2\%$ of baseline, $p < 0.001$, [Figures 5A–5C and 5F](#)).

In recordings from 5-HT neurons from the ePet-Cre^{+/-}/Ai32^{+/-} mice, light stimulation (10 ms, LED, 470 nm) caused a depolarization that elicited one to two action potentials ([Figure S1D](#)).

preceding sumatriptan application. ($n = 9$ – 11 , two-way RM ANOVAs). ns, not significant.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

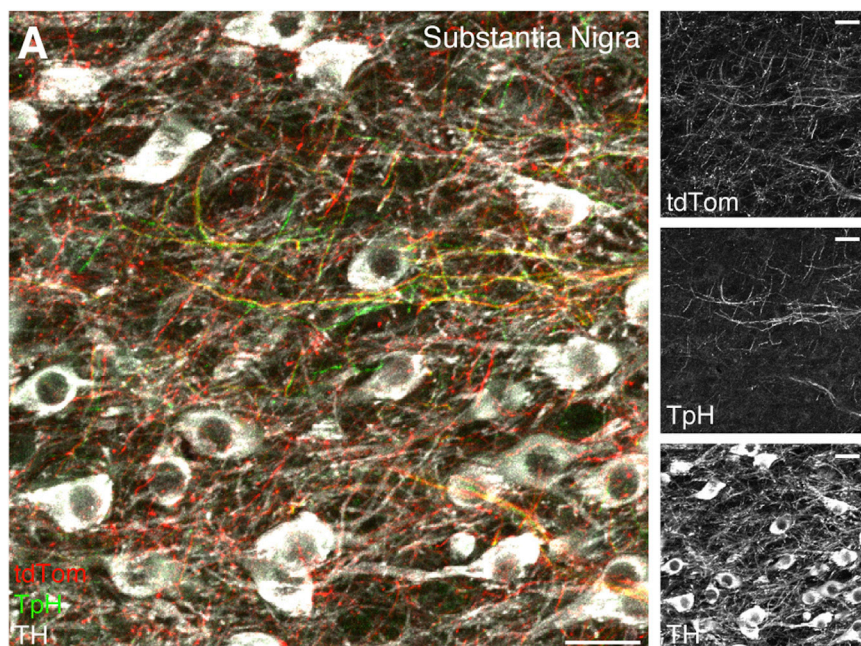
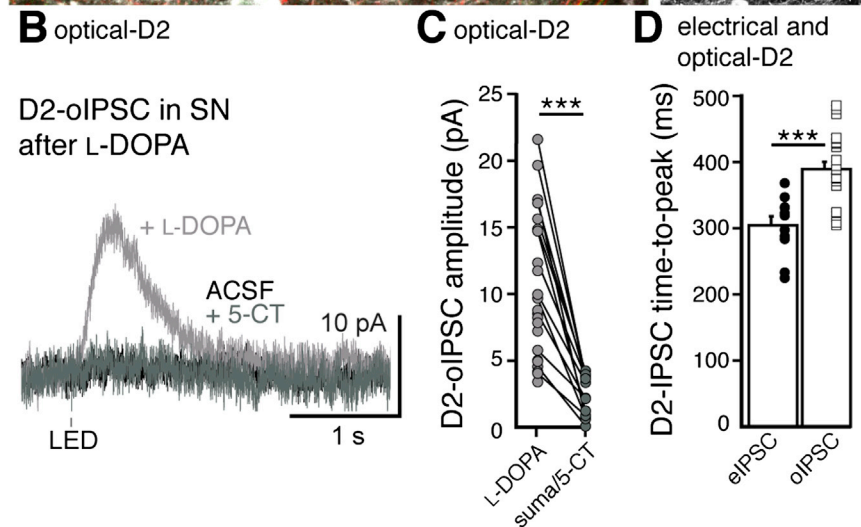


Figure 4. Selective Activation of 5-HT Terminals in the SN Elicits a D2-IPSC after L-DOPA

(A) Representative maximum intensity projection confocal image of dopamine neurons in the SN of ePet-Cre^{+/+}/Ai9^{+/+} brain slices. Cre⁺ cells express cytosolic tdTomato (tdTom, red). Sections were immunostained for tyrosine hydroxylase (TH, gray), labeling dopamine neurons, and tryptophan hydroxylase (TpH, green), labeling 5-HT fibers. Cre⁺ fibers colocalized with 5-HT fibers, and no dopamine neurons were Cre⁺; scale bars, 25 μ m. (B and C) Chr2 activation in ePet-Cre^{+/+}/Ai32^{+/+} midbrain slices (5–15 ms, 470 nm LED) did not produce a current before application of L-DOPA. With L-DOPA (10 μ M), Chr2 activation evoked a D2-oIPSC (n = 24) that was inhibited by 5-CT, shown in (B) representative traces and (C) the decrease in amplitude with 5-CT (100 nM) or sumatriptan (1 μ M) (n = 14, paired t test). (D) The time-to-peak of the D2-eIPSC (electrically evoked) was significantly faster than that of the D2-oIPSC (optically evoked), mean \pm SEM (n = 11–24, unpaired t test). ***p < 0.001. See also Figures S1 and S2.



Chr2 activation evoked a 5-HT1A-oIPSC (Figure 5D) that followed the Chr2-induced inward current. 5-HT1A-IPSCs were evoked once every 25 s, alternating between electrical and optical stimulation. Application of L-DOPA (30 μ M, 10 min) significantly attenuated the 5-HT1A-oIPSC (14% \pm 6% of baseline, p = 0.004, paired t test, Figures 5D and 5E), which did not recover after a 20-min wash. The L-DOPA-induced attenuation in amplitude and limited recovery of electrically or optically evoked synaptic currents was indistinguishable (p = 0.73, two-way ANOVA, Figure 5E). These results indicate a selective action of L-DOPA on 5-HT terminals.

In PD patients, the level of L-DOPA in the cerebral spinal fluid (CSF) fluctuates and varies considerably from patient to patient. The concentration in the CSF varies from \sim 130 nM up to 8 μ M and remains elevated for >1 hr following a single dose (Nyholm et al., 2002; Olanow et al., 1991; Stocchi et al., 2005; Tohgi

et al., 1995). In the present study, prolonged application of a low concentration of L-DOPA (1 μ M, 50–65 min) resulted in a significant reduction of the 5-HT1A-eIPSC (48% \pm 8% of baseline, p = 0.03, paired t test, Figure S3). Thus, a clinically relevant concentration of L-DOPA depressed 5-HT1A-receptor-mediated synaptic currents.

L-DOPA Reduced Serotonin Transmission through the Production of Dopamine

The AADC inhibitor, NSD-1015 (20 μ M, >15 min) was used to determine whether the conversion of L-DOPA to dopamine was required. In slices exposed to NSD-1015, L-DOPA (30 μ M) had no effect on the 5-HT1A-eIPSC (NSD: 90 \pm 17 pA, NSD and L-DOPA: 90 \pm 17 pA, p > 0.05, Figure 5F). Therefore, the production of dopamine from L-DOPA was required to affect 5-HT-dependent transmission. When NSD-1015 was applied after L-DOPA (30 μ M, 10 min), the amplitude of the 5-HT1A-eIPSC remained depressed (recovery following 10- to 15-min wash: ACSF: 36% \pm 7% of baseline, n = 7; NSD: 33% \pm 3% of baseline, n = 4, p = 0.76, unpaired t test). Thus, ongoing dopamine synthesis was not required after L-DOPA wash out.

When 5-HT currents were evoked by photolysis of caged-serotonin (l-5-HT, 5–25 ms, 365 nm), application of L-DOPA (50 μ M, 10 min) caused a modest reduction (81% \pm 5% of baseline, p < 0.05, Figures 6A and 6B) and a small outward current (16 \pm 3 pA, p < 0.001, Figures 6A and 6C). This postsynaptic effect of L-DOPA was readily reversed with wash out, or the

Dorsal Raphe

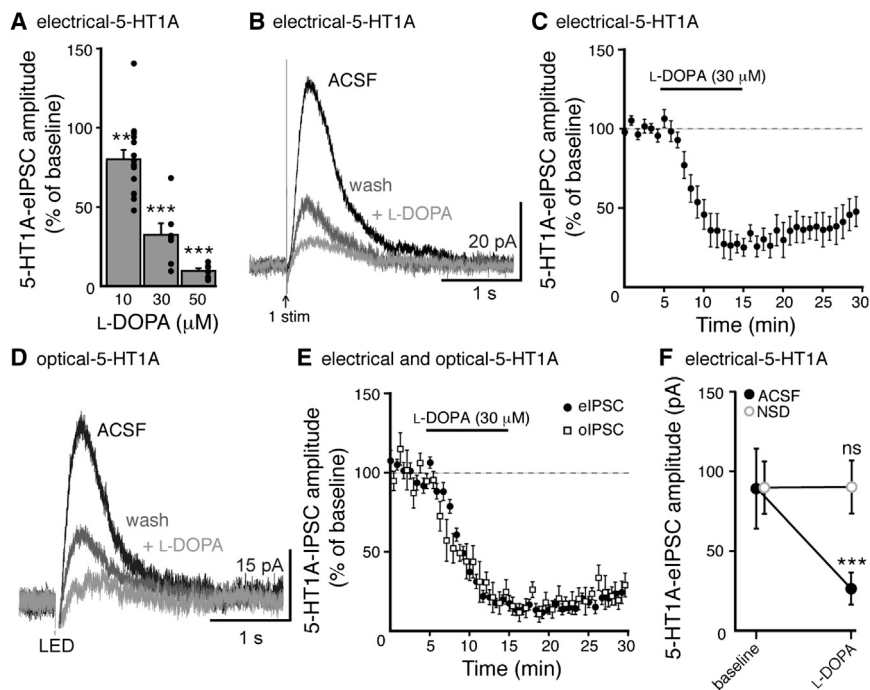


Figure 5. Acute L-DOPA Reduces 5-HT1A-IPSCs in the DR

(A) Concentration-dependent depression induced by L-DOPA (10 min) in 5-HT1A-eIPSCs, mean \pm SEM, baseline: mean amplitude of six 5-HT1A-eIPSCs preceding L-DOPA application ($n = 15, 7,$ and 10 for $10, 30,$ and $50 \mu\text{M}$, respectively, paired t tests).

(B) Representative traces of 5-HT1A-eIPSCs from a single experiment in ACSF, after bath application of L-DOPA ($30 \mu\text{M}$), and after a 20-min wash.

(C) 5-HT1A-eIPSCs were evoked once every 50 s. L-DOPA significantly depressed the 5-HT1A-eIPSC with little recovery after wash, mean \pm SEM ($n = 7$).

(D and E) In ePet-Cre^{+/-}/Ai32^{+/-} DR slices, 5-HT1A-IPSCs were evoked once every 25 s, alternating between electrical and optical stimulation. (D) Representative traces of 5-HT1A-IPSC, evoked by ChR2 activation (oIPSC). L-DOPA depressed the 5-HT1A-oIPSC with little recovery in wash. (E) L-DOPA-induced depression in the 5-HT1A-IPSC, whether electrically evoked (eIPSC, black circles) or optically evoked (oIPSC, open squares) was indistinguishable, mean \pm SEM ($n = 7-8$).

(F) Application of the AADC inhibitor, NSD-1015 (NSD, $20 \mu\text{M}$, 15 min), prevented the depression of the 5-HT1A-eIPSC by L-DOPA, mean \pm SEM (ACSF: black circles, $n = 7$; NSD: open circles, $n = 7$, two-way RM ANOVA).

Baseline: mean amplitude of six 5-HT1A-IPSCs preceding L-DOPA application; ns, not significant; ** $p < 0.01$ and *** $p < 0.001$. See also Figures S1 and S3.

addition of NSD-1015 ($20 \mu\text{M}$, 1-5-HT: $98\% \pm 6\%$ of baseline, $p < 0.001$; outward current: $-9 \pm 4 \text{ pA}$, $p < 0.001$, Figures 6A–6C). The results suggest that dopamine produced from L-DOPA exerted a small, transient postsynaptic effect. Therefore, the long-lasting L-DOPA-induced depression of 5-HT-mediated synaptic transmission occurred primarily through a presynaptic dopamine-dependent mechanism.

In the DR, 5-HT activates presynaptic 5-HT1B receptors that inhibit further 5-HT release (Morikawa et al., 2000). To determine whether L-DOPA or dopamine activates 5-HT1B receptors, the effect of dopamine on the 5-HT1A-eIPSC was tested in 5-HT1B receptor knockout mice (5-HT1B KO) and their wild-type (WT) littermates. Sumatriptan ($1 \mu\text{M}$) caused a marked inhibition of the 5-HT1A-eIPSC in WTs ($85\% \pm 5\%$ inhibition) and only a small inhibition in 5-HT1B KOs ($34\% \pm 5\%$ inhibition, $p < 0.001$, Figure 6D). In slices from WT mice, dopamine ($10 \mu\text{M}$, 10 min) inhibited the 5-HT1A-eIPSC ($41\% \pm 5\%$ of baseline, $p < 0.001$, Figure 6E). The inhibition induced by dopamine reversed after wash out (after 10- to 15-min wash: $98\% \pm 14\%$ of baseline, repeated-measures (RM) one-way ANOVA, Figure 6E). The transient action of dopamine was unlike the prolonged depression induced by L-DOPA. In slices from 5-HT1B KO mice, dopamine inhibited the 5-HT1A-eIPSC ($60\% \pm 6\%$ of baseline, $p < 0.001$) and recovered after wash out (after 10- to 15-min wash: $105\% \pm 7\%$ of baseline, RM one-way ANOVA), though the inhibition was slightly less than in WT slices ($p = 0.02$, unpaired t test, Figure 6E). The dopamine-induced reduction in the 5-HT1A-eIPSC also persisted in the presence of su-

matriptan ($1 \mu\text{M}$, $58\% \pm 7\%$ of baseline, $n = 5$) in 5-HT1B KO mice, indicating 5-HT1D receptors were not involved. In addition, dopamine ($10 \mu\text{M}$) decreased the 5-HT1A-oIPSC in slices from ePet-Cre^{+/-}/Ai32^{+/-} mice ($41\% \pm 5\%$ of baseline, $p = 0.009$, $n = 8$, paired t test). L-DOPA ($30 \mu\text{M}$, 10 min) also produced similar inhibitions of the 5-HT1A-eIPSC in both WT and 5-HT1B KO mice that did not recover upon wash out (WT: $24\% \pm 3\%$ of baseline; 5-HT1B KO mice: $28\% \pm 2\%$ of baseline, $p = 0.31$, unpaired t test, Figure 6F). Thus, the L-DOPA-induced depression of the 5-HT1A-IPSC cannot be explained by action on the 5-HT1B autoreceptor.

5-HT Neurons Release Dopamine in the Dorsal Raphe after Acute L-DOPA

The ability of DR 5-HT neurons to package and release dopamine after treatment with L-DOPA was examined through the activation of virally expressed D2 receptors in the DR. Wild-type mice received a midline injection of an adeno-associated virus vector generating D2 receptor and GFP expression, as previously described (Neve et al., 2013). Infected neurons were identified by visualization of GFP (Figure 7A). Electrical stimulation (one or five stims) evoked IPSCs, in the presence of NMDA, AMPA, GABA_A, and α -1 adrenergic receptor antagonists. The resulting eIPSCs were inhibited by the 5-HT1A receptor antagonist, WAY 100635 (100 nM , $88\% \pm 3\%$ inhibition, $p < 0.001$, Figures 7B and 7C). In 7/12 recordings, the eIPSC was not completely blocked by WAY 100635 ($9 \pm 2 \text{ pA}$). It is unclear whether this WAY-resistant D2 component originates from

Dorsal Raphe

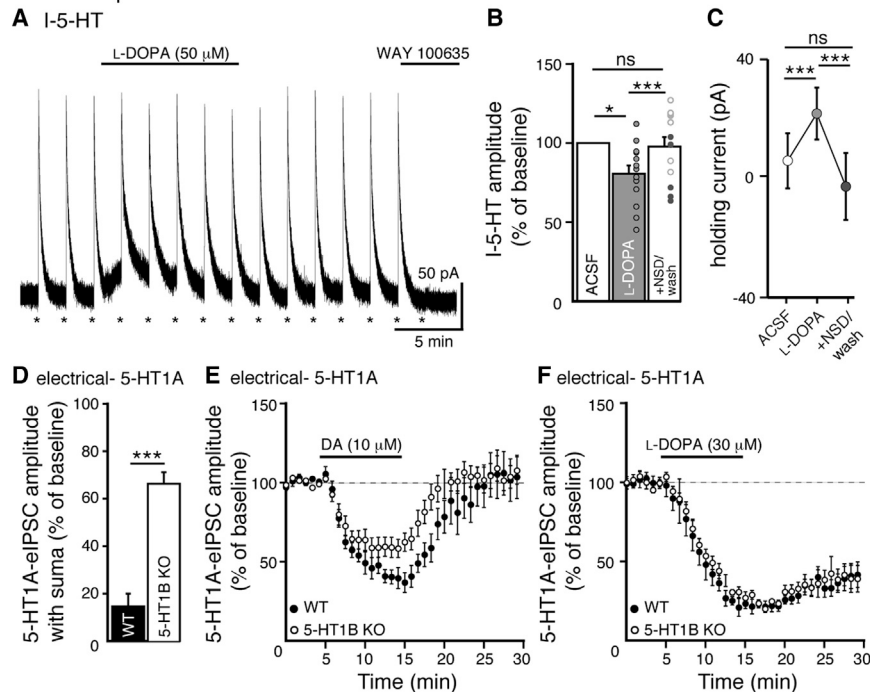


Figure 6. Dopamine Poorly Activates 5-HT1A and 5-HT1B Receptors

(A) Whole-cell voltage-clamp recording of the 5-HT1A-receptor-mediated currents induced by photolysis of caged-serotonin once every 2 min (I-5-HT, denoted by *) during bath application of L-DOPA (50 μ M). I-5-HT was blocked by WAY 100635 (10 μ M). (B) L-DOPA reduced I-5-HT and the reduction was reversed with the addition of NSD-1015 (20 μ M, open circles) or wash out (filled circles), mean \pm SEM (n = 13, RM one-way ANOVA). (C) L-DOPA induced an outward current that was reversed with the addition of NSD-1015 or wash out, mean \pm SEM (n = 14, RM one-way ANOVA). (D) Inhibition of the 5-HT1A-eIPSC by sumatriptan (1 μ M) was less in slices from 5-HT1B receptor KO mice (5-HT1B KO) than from their wild-type littermates (WT), mean \pm SEM (n = 7–8, unpaired t test). (E) Dopamine (10 μ M) transiently inhibited the 5-HT1A-eIPSC in slices from WT (black circles, n = 10) and 5-HT1B KO mice (open circles, n = 8), mean \pm SEM. (F) L-DOPA (30 μ M)-induced depression in the 5-HT1A-eIPSC was indistinguishable in slices from 5-HT1B KO (open circles, n = 7) and WT mice (black circles, n = 6), mean \pm SEM. Baseline: mean amplitude of three I-5-HTs or six 5-HT1A-IPSCs preceding L-DOPA application; ns, not significant; *p < 0.05 and ***p < 0.001.

dopamine release from local dopamine (TH⁺) neurons (Figures S1A and S1B) or dopamine terminal projections (Pollak Dorocic et al., 2014; Kitahama et al., 2000) or D2 receptor activation from the release of noradrenaline (Onali et al., 1985; Yoshimura et al., 1985). In the presence of WAY 100635, L-DOPA (10 μ M, 10 min) produced a transient outward current (69 \pm 11 pA, p < 0.001, n = 12, paired t test, Figure 7B), and D2-receptor-mediated eIPSCs that persisted long after the outward current recovered upon wash out (29 \pm 6 pA, p = 0.01, Figures 7B and 7D). 5-CT (100 nM) significantly inhibited the L-DOPA-induced D2-eIPSCs (65% \pm 6% inhibition, p = 0.002, Figure 7E), indicating that most of the dopamine was from 5-HT terminals. The D2 receptor antagonist, sulpiride (600 nM) completely abolished the D2-eIPSC (1 \pm 0.3 pA, p < 0.001, Figure 7F). Disruption of the vesicular monoamine transporter with reserpine (1 μ M, 1 h) abolished the 5-HT1A-eIPSC (4% \pm 0.3% of baseline, p = 0.005, n = 3, paired t test, see also Pan et al., 1989). After reserpine, L-DOPA still produced an outward current (61 \pm 22 pA, n = 4). However, pretreatment with reserpine completely prevented the L-DOPA-induced D2-eIPSC (in WAY and reserpine: 2 \pm 0.6 pA; in WAY, reserpine, and L-DOPA: 1 \pm 0.5 pA, p = 0.15, n = 6, paired t test). Thus, viral-mediated expression of D2 receptors in 5-HT neurons revealed that treatment with L-DOPA resulted in dopamine release by a vesicular mechanism. The time course of dopamine release mirrored that of the depression of the 5-HT1A-IPSC.

DISCUSSION

The ability of 5-HT terminals to convert and release dopamine after L-DOPA was first described 45 years ago (Ng et al., 1970).

Despite this, the ability of 5-HT terminal-derived dopamine to activate postsynaptic dopamine receptors or the inhibition of serotonin transmission by displacement with dopamine has not been reported. This study assessed the action of L-DOPA on monoamine synaptic transmission on dopamine neurons in the SN and 5-HT neurons in the DR. The results reveal that treatment with L-DOPA causes ectopic dopamine release from 5-HT terminals and a simultaneous loss of 5-HT-mediated synaptic transmission.

Ectopic Release of Dopamine from 5-HT Terminals

Numerous studies have demonstrated that 5-HT terminals can convert L-DOPA to dopamine that is then packaged into vesicles and released in an activity-dependent manner (Arai et al., 1994; Kannari et al., 2000; Ng et al., 1970; Tanaka et al., 1999). However, the majority of studies isolated 5-HT terminals by first ablating nigrostriatal dopamine neurons. By preserving the nigrostriatal dopamine neurons, this study assessed the ability of 5-HT terminals to release dopamine, activate postsynaptic D2 receptors, and otherwise mimic dopamine-dependent synaptic transmission.

In the SN, dopamine is released from the somatodendritic compartment spontaneously and after electrical stimulation to produce D2-IPSCs (Beckstead et al., 2004; Gantz et al., 2013). This study found that L-DOPA increased dopamine release from dopamine neurons, augmenting D2-IPSCs. These dopamine neurons also receive projections from the 5-HT neurons in the raphe nuclei (Dray et al., 1976; Moukhles et al., 1997). The release of dopamine from 5-HT terminals after L-DOPA was demonstrated using pharmacological and optogenetic experiments. The terminals of 5-HT neurons have 5-HT1B/D

Dorsal Raphe viral-mediated expression of D2 receptors

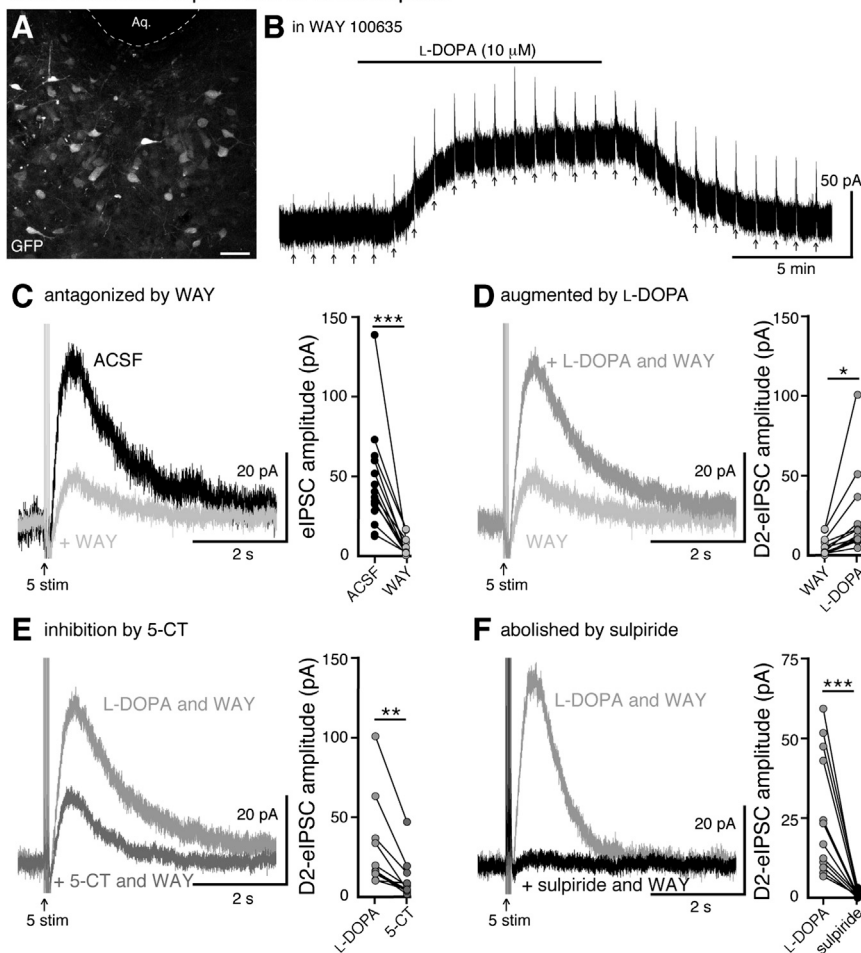


Figure 7. Virally Expressed D2 Receptors in the DR Mediate D2-IPSCs after L-DOPA

(A) Representative maximum intensity projection confocal image of GFP⁺ neurons in the DR (ventral to the cerebral aqueduct, aq.) infected with AAV-D2; scale bar, 50 μm.

(B–F) Representative traces from a single experiment.

(B) Whole-cell voltage-clamp recording of the outward current and D2-eIPSCs induced by bath application of L-DOPA (10 μM), in the presence of WAY 100635 (100 nM). eIPSCs were evoked by electrical stimulation (arrow, one or five stims) once every 50 s, stimulation artifacts were blanked for clarity.

(C) eIPSCs in ACSF and after WAY 100635. 5-HT_{1A}-eIPSCs were inhibited by WAY 100635 (n = 12, paired t test).

(D) D2-eIPSCs in WAY 100635 and after L-DOPA in the continued presence of WAY 100635. L-DOPA augmented the D2-eIPSC (n = 18, paired t test).

(E) L-DOPA-induced D2-eIPSCs in WAY 100635 and after 5-CT (100 nM). D2-eIPSCs were inhibited by 5-CT (n = 11, paired t test).

(F) L-DOPA-induced D2-eIPSCs in WAY 100635 and after sulpiride (600 nM). Sulpiride completely eliminated the D2-eIPSCs (n = 12, paired t test).

*p < 0.05, **p < 0.01, and ***p < 0.001.

receptors, which inhibit transmitter release (Cameron and Williams, 1994; Morikawa et al., 2000). Under control conditions, the activation of these receptors did not alter the amplitude of the D2-eIPSC. However, after acute L-DOPA application, activating 5-HT_{1B/D} receptors decreased the D2-eIPSC. In addition, following 6 days of in vivo L-DOPA treatment, activation of 5-HT_{1B/D} receptors inhibited the D2-eIPSC. Last, following L-DOPA, selective stimulation of ChR2-expressing 5-HT axon terminals found throughout the SN evoked dopamine release resulting in a D2-receptor-mediated IPSC. Thus, acute and in vivo treatment with L-DOPA produced dopamine release from 5-HT terminals in the SN.

Taken together, this study made three observations related to the ability of 5-HT terminals to mimic dopamine synaptic transmission after treatment with L-DOPA: (1) 5-HT terminals in the SN are positioned such that the release of dopamine activates D2 receptors on dopamine neurons; (2) 5-HT terminal-derived dopamine does not likely contribute to spontaneous D2-receptor-mediated synaptic transmission; and (3) the release of newly synthesized dopamine from 5-HT terminals is greater and persists for longer than the release from dopamine neurons.

these regions following an intraperitoneal injection of L-DOPA, and destruction of 5-HT neurons attenuated the L-DOPA-induced increase in dopamine (Navailles et al., 2010). As found in the present study in the SN, dopamine receptors in each of these projection areas could be activated by dopamine from 5-HT terminals.

Depression of 5-HT-Mediated Synaptic Currents by L-DOPA

Reduced 5-HT levels and altered 5-HT metabolism are often reported in PD patients (reviewed in Eskow Jaunarajs et al., 2011) and rodent models of PD treated with L-DOPA (Borah and Mohanakumar, 2007; Navailles et al., 2011; Ng et al., 1970; Stansley and Yamamoto, 2014). However, the impact of L-DOPA on 5-HT-receptor-mediated signaling had yet to be examined.

The present study describes a long-lasting L-DOPA-dependent depression of 5-HT_{1A}-receptor-mediated IPSCs in 5-HT neurons in the DR. The decrease in 5-HT transmission resulted from the production and packaging of dopamine into vesicles. This most likely resulted from displacement of vesicular 5-HT, since VMAT2 transports dopamine more efficiently than

The projections of 5-HT neurons are widespread, including the prefrontal cortex, hippocampus, and nucleus accumbens, and form somatic, dendritic, and axonal contacts in these areas (Milner and Veznedaroglu, 1993; Van Bockstaele et al., 1993). An in vivo microdialysis study, using a rodent model of PD, found that dopamine levels were increased in

serotonin (Finn and Edwards, 1997). Inhibition of the AADC prevented the L-DOPA-induced depression in the 5-HT1A-eIPSC, demonstrating the synthesis of dopamine was required. Finally, with the expression of D2 receptors in the DR, L-DOPA resulted in D2-receptor-mediated IPSCs. Inhibition of VMAT2 disrupted the loading of dopamine into vesicles and completely prevented the D2-eIPSCs. Therefore, the D2-eIPSCs were due to vesicular release of dopamine from 5-HT terminals. The ectopic release of dopamine from 5-HT terminals is likely dysregulated, since exogenous application of dopamine had little effect on 5-HT1B autoreceptors. However, dopamine caused a transient inhibition of the 5-HT1A-IPSC that was not explained fully by action on the 5-HT1A and 5-HT1B/D receptors, suggesting another mechanism by which dopamine may inhibit release from 5-HT terminals. It is possible that 5-HT terminal-derived dopamine contributes to the depression of the 5-HT1A-IPSC through this dopamine-mediated inhibition. Taken together, this study demonstrates that after L-DOPA, dopamine is released from 5-HT neurons impairing 5-HT-mediated synaptic transmission.

The significance of these results likely extends beyond the depression of the 5-HT1A-eIPSC in the DR. The projections of 5-HT neurons will be affected similarly (Törk, 1990). 5-HT can activate as many as 14 distinct 5-HT receptors, influencing multiple effectors and modulating the release of glutamate, GABA, acetylcholine, in addition to monoamine neurotransmitters (reviewed in Fink and Göthert, 2007). Thus, the reduction in 5-HT release will have widespread actions throughout the CNS.

Implications for the Experimental and Clinical Use of L-DOPA

This study demonstrates that after treatment with L-DOPA, 5-HT terminals participate in dopamine signaling at the expense of 5-HT synaptic transmission. These actions were observed with clinically relevant concentrations of L-DOPA and without ablation of dopamine neurons. Therefore, the participation of 5-HT terminals in dopamine release must be considered during experimental and clinical use of L-DOPA.

In advanced PD with substantial dopamine denervation, the release of dopamine from 5-HT terminals in the forebrain is thought to be beneficial, but the ectopic release has been suggested to be more detrimental than therapeutic, particularly to affective symptoms (Borah and Mohanakumar, 2007; Carta et al., 2007; Eskow Jaunarajs et al., 2012; Hornykiewicz, 1975). Thus, in the context of PD treatment, this study provides a cellular and synaptic basis for testing pharmaceuticals that recruit 5-HT neurons to synthesize and release dopamine while sparing 5-HT-receptor-mediated signaling. In addition to PD, there is substantial recent interest in L-DOPA treatment for other health conditions, including Angelman syndrome, Tourette syndrome, and cocaine dependence. These conditions are often comorbid with affective disorders, notably impulsivity (Belin et al., 2008; Ersche et al., 2010; Palumbo and Kurlan, 2007; Pelc et al., 2008), as seen in PD patients treated with L-DOPA (Cools et al., 2003). Impulse control is influenced by 5-HT neurons in the dorsal raphe, where reduced 5-HT levels promote impulsive behavior (Fonseca et al., 2015; Miyazaki et al., 2014). Thus, a greater understanding of the action of L-DOPA on the serotonin system is necessary when considering its use when certain af-

fective symptoms are also present. Indeed, in PD patients and animal models, combination therapies with L-DOPA, 5-HT1A, and 5-HT1B receptor agonists have proved effective in treating dyskinesias and psychiatric symptoms, such as depression and anxiety (Muñoz et al., 2008 and reviewed in Shimizu and Ohno, 2013).

Concluding Remarks

Taken together, this study demonstrates L-DOPA is driving ectopic dopamine release from 5-HT neurons. Dopamine derived from 5-HT terminals can activate D2 receptors in the SN and mediate inhibitory postsynaptic currents. However, in 5-HT neurons, newly synthesized dopamine is transported into vesicles, where it ultimately impairs 5-HT-mediated synaptic transmission in the DR. Thus, L-DOPA generates an imbalance between the dopamine and 5-HT neurotransmitter systems. Given the extensive projections of each system, such an imbalance will have widespread consequences throughout the CNS.

EXPERIMENTAL PROCEDURES

Animals

All studies were conducted in accordance with the Institutional Animal Care and Use Committee at Oregon Health and Science University. All mice were group-housed in standard plastic containers on a 12-hr-light/dark cycle, with food and water available ad libitum. Male and female wild-type (C57BL/6) mice (>30 days old) were used, and principal results (Figures 1, 2, 3, and 5) were repeated in DBA/2J mice. Treated mice received a once-daily subcutaneous injection of L-DOPA methyl ester (100 mg/kg) and benserazide (200 mg/kg) dissolved in saline, or an equal volume of benserazide only (200 mg/kg), for 6 days and were killed 1 hr after the last injection. ePet-Cre^{+/−} mice on a C57BL/6 background were obtained from The Jackson Lab (stock no. 012712). Ai9 and Ai32 mice (stock no. 007905 and 012569, respectively), backcrossed onto C57BL/6 background, were kindly provided by Dr. Tianyi Mao (Vollum Institute). 5-HT1B receptor knockout mice, maintained on a BALB/c background, were kindly provided Dr. Mark Pennesi (Casey Eye Institute).

Stereotaxic Virus Injection

D2 receptors were ubiquitously expressed in the DR using an adeno-associated virus (AAV) vector (AAV9 D2-IRES-GFP; Virovek) encoding a mixture of short and long splice variants of the rat D2 receptor (Neve et al., 2013). Mice were injected when 5–7 weeks old. Mice were immobilized in a stereotaxic alignment system under isoflurane anesthesia and received one midline injection, 500 nl delivered over 5 min. The coordinates for injection were (with respect to bregma) AP −3.9 mm, ML 0.0 mm, DV −2.8 mm. Animals recovered for 4–5 weeks to allow for expression. Infected neurons were identified in the slice by visualization of GFP.

Slice Preparation and Electrophysiology

Detailed procedures and recipes for slice preparation and electrophysiology can be found in Supplemental Experimental Procedures. Briefly, horizontal mouse midbrain or coronal dorsal raphe (DR) slices were obtained using a vibrating microtome (Leica) and incubated in modified Krebs' buffer solution, at 30°C in vials with 95%/5% O₂/CO₂ saline with MK-801 (10 μM) for >30 min prior to recording. Dopamine neurons of the substantia nigra pars compacta (SN), and 5-HT neurons in the DR were identified by their location and physiological characteristics. Whole-cell voltage-clamp recordings were made with a K-methanesulfonate-based internal solution containing BAPTA (10 mM). Transmitter release was evoked electrically or in ePet-Cre^{+/−}/Ai32^{+/−} slices with a 470-nm LED pulse. Currents were isolated pharmacologically, with AMPA, GABA_A, α-1 adrenergic (DR only), GABA_B (SN only), nACh (SN only) receptor antagonists. Currents peaks were determined by averaging the current ±20 ms from the greatest upward deflection. For each cell,

three to 12 consecutive currents were averaged to determine amplitude, time-to-peak, and duration at 20% of the peak. sIPSCs were detected as previously described (Gantz et al., 2013). Data were acquired using AxoGraph software (AxographX) and LabChart 7 (AD Instruments) and were post hoc filtered.

Immunohistochemistry

Mice were anesthetized with Avertin (i.p.) and transcardially perfused with 5% sucrose in H₂O followed by ice-cold 4% paraformaldehyde in PBS (pH 7.4). Brains were fixed overnight at 4°C and then sliced coronally (DR) and horizontally (SN) in 60- μ m sections. Free-floating slices were permeabilized in PBS with 0.3% Triton-X (PBS-T) then blocked in PBS-T with 0.5% fish skin gelatin for 1 hr. Slices from ePet-Cre^{+/+}/Ai9^{+/+} mice were incubated 24 hr at 24°C in mouse anti-TH (1:1,000) primary antibody. Slices were washed then incubated in Alexa-Fluor-647-conjugated goat anti-mouse secondary antibody (1:1,000) for 3 hr. Slices were washed then incubated for 48 hr at 24°C in rabbit anti-TpH primary antibody (1:500). Following washing, slices were incubated in Alexa-Fluor-488-conjugated goat anti-rabbit secondary antibody (1:1,000) for 3 hr. Slices from AAV-D2-infected wild-type mice were incubated in rabbit anti-GFP conjugated to Alexa Fluor 488 (1:1,000) 24 hr at 4°C. Washed slices were mounted with Fluoromount (Sigma) aqueous mounting medium. Confocal images were collected on a Zeiss LSM 780 microscope with a 20 \times (0.8 NA) or 40 \times water-immersion lens (1.2 NA) and processed using Fiji.

Materials

CGP 55845, 5-CT, WAY 100635, and NPEC-caged-serotonin were obtained from Tocris Bioscience. MK-801 was obtained from Abcam. Sumatriptan was from Glaxo Wellcome Research and Development. All other drugs were obtained from Sigma-Aldrich.

Data Analysis

Values are given as means \pm SEM. Unless otherwise noted, n = number of cells. All data sets with n > 10 were tested for normality with Shapiro-Wilk normality test. Nonparametric statistics were used if any data set failed a normality test and was skewed. Significant differences in within-group comparisons were determined for two group comparisons by paired two-tailed t tests or two-tailed Mann Whitney U tests, and in more than two group comparisons by one- or two-way repeated-measures (RM) ANOVA. Significant mean differences in between-group comparisons were determined by unpaired two-tailed t tests, or by one- or two-way ANOVAs. ANOVAs were followed, when appropriate (p < 0.05) by Bonferroni's multiple comparisons post hoc tests. Correlations were determined by two-tailed Pearson Correlation test. Statistical analysis was performed using GraphPad Prism 6 (GraphPad).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.07.005>.

AUTHOR CONTRIBUTIONS

S.C.G., E.S.L., and J.T.W. designed research. S.C.G., E.S.L., N.L., and J.T.W. performed research and analyzed the data. K.A.N. provided reagents. E.S.L., N.L., and K.A.N. assisted with the preparation of the manuscript. S.C.G. and J.T.W. prepared the figures and wrote the manuscript.

ACKNOWLEDGMENTS

This work was supported by NIH DA04523 and DA034388 (J.T.W.) and by Merit Review Award BX000810 from the US Department of Veterans Affairs Biomedical Laboratory Research and Development (K.A.N.). We thank Drs. Tianyi Mao and Mark Pennesi for supplying Ai9 and Ai32 mice, and 5-HT1B receptor KO mice, respectively, and Maozhen Qin for help with mouse husbandry.

Received: May 14, 2015

Revised: June 16, 2015

Accepted: July 1, 2015

Published: July 30, 2015

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