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# Functional Fast Scan Cyclic Voltammetry Assay to Characterize Dopamine D2 and D3 Autoreceptors in the Mouse Striatum

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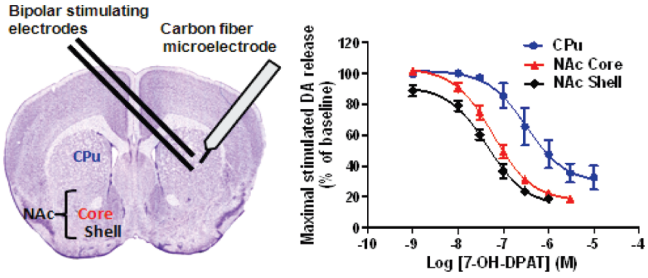
## Abstract




Dopamine D2 and D3 autoreceptors are located on presynaptic terminals and are known to control the release and synthesis of dopamine. Dopamine D3 receptors have a fairly restricted pattern of expression in the mammalian brain. Their localization in the nucleus accumbens core and shell is of particular interest because of their association with the rewarding properties of drugs of abuse. Using background subtracted fast scan cyclic voltammetry, we investigated the effects of dopamine D2 and D3 agonists on electrically stimulated dopamine release and uptake rates in the mouse caudate putamen and nucleus accumbens core and shell. The dopamine D2 agonists (−)-quinpirole hydrochloride and 5,6,7,8-tetrahydro-6-(2-propen-1-yl)-4*H*-thiazolo[4,5-*d*]azepin-2-amine dihydrochloride (B-HT 920) had the same dopamine release inhibition effects on caudate putamen and nucleus accumbens (core and shell) on the basis of their $EC_{50}$ values and efficacies. This suggests that the dopamine D2 autoreceptor functionality is comparable in all three striatal regions investigated. The dopamine D3 agonists (4*aR*,10*bR*)-3,4*a*,4,10*b*-tetrahydro-4-propyl-2*H*,5*H*-[1]benzopyrano-[4,3-*b*]-1,4-oxazin-9-ol hydrochloride ((+)-PD 128907) and (±)-7-Hydroxy-2-dipropylaminotetralin hydrobromide (7-OH-DPAT) had a significantly greater effect on dopamine release inhibition in the nucleus accumbens shell than in the caudate putamen. This study confirms that, the dopamine D3 autoreceptor functionality is greater in the nucleus accumbens shell followed by the nucleus accumbens core, with the caudate putamen having the least. Neither dopamine D2 nor D3 agonists affected the uptake rates in nucleus accumbens but concentrations greater than 0.1 $\mu$M lowered the uptake rate in caudate putamen. To validate our method of evaluating dopamine D2 and D3 autoreceptors, sulpiride (D2 antagonist) and nafadotride (D3 antagonist) were used to reverse the effects of the dopamine agonists to approximately 100% of the preagonist dopamine release concentration. Finally, these results demonstrate a functional voltammetric assay that characterizes dopamine D2-like agonists as either D2- or D3-preferring agonists by taking advantage of the unique receptor density within the striatum.

**Keywords:** Caudate putamen, nucleus accumbens core, nucleus accumbens shell, autoreceptor, dopamine transporter, dopamine uptake, quinpirole, B-HT 920, 7-OH-DPAT, (+)-PD 128907, nafadotride, sulpiride

Dopamine is a widely investigated neurotransmitter due to its involvement in the brain reward pathway and in several neuropathological conditions such as Parkinson's disease, schizophrenia, attention deficit hyperactivity disorder (ADHD), and drug abuse (*1*, *2*). Under normal physiological conditions, dopamine is known to regulate locomotor activity, cognition, learning, emotional effects, and neuroendocrine hormone secretion (*3*). Dopaminergic histological and neurochemical studies demonstrate regional differences within the striatum, which can be further subdivided into three distinct anatomical regions: the caudate putamen (CPu), the nucleus accumbens (NAc) core, and the NAc shell (*4*−*6*). Dopamine cell bodies, which innervate the NAc and the CPu, arise from two distinct areas in the midbrain, the ventral tegmental area and the substantia nigra, respectively (*7*). A variety of neurochemical studies have demonstrated differences in dopamine levels between these regions. For example, the CPu is known to have higher extracellular dopamine levels and greater dopamine uptake compared to those of the NAc (*8*−*11*). Within the NAc, the core is known to have greater electrically evoked dopamine release and uptake than the shell (*12*). *In vivo* microdialysis data also confirm that extracellular dopamine levels are greater in

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the core than the shell (*13*, *14*). However, it is not clear whether these observations are solely due to the reduced expression of the dopamine transporter from the dorsal to ventral striatum or if they are impacted by varied expression levels of dopamine autoreceptors.

Dopamine binds to at least five known receptor subtypes, which are divided into two distinct classes: D1-like (includes D1 and D5), which stimulate adenylate cylase and neuronal activity, or D2-like (includes D2, D3, and D4), which inhibit adenylate cyclase and neuronal activity (*2*, *15*, *16*). Stimulation of D2 receptors on presynaptic terminals results in feedback inhibition, reducing extracellular levels of dopamine via the regulation of dopamine synthesis and release (*17*, *18*). Additionally, there is evidence, which implies that the dopamine D3 receptor also regulates dopamine release in terminal regions such as the NAc (*19*−*21*). Importantly, the anatomical distribution of the D2 and D3 receptors is very distinct. Specifically, the D3 receptor shows localization in the mesolimbic pathway, including the NAc, Islands of Calleja, and olfactory tubercles (*18*, *22*, *23*). The highest levels of this receptor in the striatum appear to be in the NAc shell instead of the core (*24*, *25*). In contrast, evidence suggests that the dopamine D2 receptors are more homogeneously distributed throughout the striatum (*26*, *27*).

There is intense interest in characterizing the functional effects of dopamine D2 and D3 agonists because of their potential therapeutic involvement in schizophrenia and Parkinson's disease. However, a major problem with characterizing these agonists is their lack of selectivity for a given receptor. The development of D2 and D3 receptor knockout animals has facilitated the characterization and classification of D2 and D3 receptor agonists. Both microdialysis and voltammetric techniques have been used to examine D2-like agonists in D2 and D3-receptor knockout mice (*28*−*32*). However, conflicting neurochemical results obtained in these experiments, which used D2 or D3 knockout mice highlights a concern with respect to using knockout mice: Life long constitutive reduction could easily alter other facets of the dopamine system, which may make it more difficult to characterize selective D2-like agonists as either D2 or D3 specific (*28*, *29*). Therefore, it is critical to develop new strategies to better identify the functional properties of potential D2 and D3 agonists.

Current tools to evaluate dopamine D2 and D3 receptor function include microdialysis, voltammetry, and magnetic resonance imaging (MRI) (*33*). Typically, microdialysis is used in neurochemical studies because of its ability to sample numerous neurotransmitters and provide greater sensitivity of these baseline levels, but the limitation with using microdialysis to characterize dopamine autoreceptor function is its poor temporal resolution and inability to discriminate subanatomical brain regions such as the NAc core from shell, especially in mice. Recently, Chen et al. demonstrated that pharmacological MRI is a valuable tool for characterizing dopamine receptor function since it is a noninvasive technique that allows for multiple simultaneous measurements in a variety of brain regions and provides the ability to perform longitudinal studies (*33*). However, few laboratories have the expertise or MRI equipment to perform these studies. However, voltammetry has been used routinely *in vitro* and *in vivo* to characterize the functionality of dopamine receptors (*31*, *32*, *34*−*39*). The advantages to using voltammetry are its fast temporal resolution (100 ms), which allows for the measurements of both release and uptake in the presences of D2-like agonists, and the small size of the carbon fiber microelectrodes, which allows for the ability to sample from discrete subanatomical regions such as the NAc core versus shell. Additionally, it is well established that D2-like agonists mediate dopamine release, and there is increasing evidence that D2-like autoreceptors may mediate dopamine uptake (*20*, *40*−*45*). Thus, voltammetry is particularly useful to characterize both of these parameters that are influenced by dopamine agonists.

The present study characterizes the functional effects of dopamine D2 and D3 receptor agonists in the CPu, NAc core, and shell. Fast scan cyclic voltammetry (FSCV) with a carbon fiber microelectrode (~7 $\mu$m in diameter) was used, allowing for discrete anatomical detection of electrically stimulated dopamine. We have chosen to use *in vitro* (slice) experiments to eliminate contributions from the dopamine cell bodies. Commercially available D2, D3, or mixed D2/D3 agonists (e.g., quinpirole, 7-OH-DPAT, and (+)-PD 128907) have been used to evaluate autoreceptor function (*28*, *31*, *34*, *36*, *38*, *46*). Additionally, the effect of B-HT 920 was also examined since it is a reported dopamine D2 agonist, but with very limited use in voltammetry and microdialysis studies (*47*). Although, there are many voltammetric studies that have examined autoreceptor functionality, to our knowledge this is the first reported study that examines anatomically distinct brain regions to characterize D2-like agonists as either selective D2 or D3 agonists, or mixed D2/D3 agonists (*31*, *32*, *34*−*38*). The results here suggest that the mode of action for dopamine agonists' functionality can be specifically assigned upon the basis of their potency and efficacy within the discrete anatomical subregions. The utility of this functional voltammetric assay will assist future characterization of selective agonists that could be used as potential therapeutic agents.

## Results and Discussion

### Effect of Dopamine D2 Agonists on Electrically Stimulated Dopamine in the Striatum

Dopamine autoreceptors regulate the extracellular levels of dopamine through a negative feedback mechanism where increasing agonist concentration results in a



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reduction in extracellular dopamine. The most common method for evaluation of dopamine receptor density is autoradiography, which employs the use of radioactive ligands to quantify receptor levels (*27*, *48*−*51*). Within the striatum (including the CPu and NAc), it is well known that the D2 density is fairly homogeneous, while the D3 receptor density is greatest in the NAc shell region (*16*, *23*, *27*, *52*, *53*). The striatum was chosen as the region of interest to take advantage of this divergent D2 and D3 receptor distribution in order to better differentiate D2 and D3 receptor agonists and antagonists. Using FSCV, the activity of dopamine release-regulating autoreceptors was evaluated in the dorsal CPu and separately in the core and the shell of the NAc. In all brain regions evaluated, increasing concentrations of the D2 or D3 receptor agonists (0.001−10 $\mu$M) were added to slices at 30-min intervals. Upon addition of each agonist, a plateau in dopamine release was reached within 15−25 min. The peak dopamine release was determined during this plateau and expressed as a percent of the predrug (control) concentration.

The two D2 agonists, quinpirole and B-HT 920, were evaluated by first examining their effects on dopamine release stimulated by a single electrical pulse in the CPu, NAc core, and NAc shell. The observed responses for these two agonists were nearly indistinguishable (Figures 1 and 2). The amount of dopamine evoked before drug application was approximately 2 $\mu$M ($n$ = 10), 1 $\mu$M ($n$ = 10), and 0.6 $\mu$M ($n$ = 10) for CPu, NAc core, and NAc shell, respectively. Agonist concentrations greater than 10 nM significantly inhibited electrically stimulated dopamine release throughout the striatal brain regions examined ($P$ < 0.0001). Representative voltammetric traces of electrically evoked dopamine in the CPu in the absence or presence of quinpirole (0.03, 0.1, and 1 $\mu$M) are shown in Figure 1A. The quinpirole dose response and B-HT 920 showed a decrease in stimulated dopamine release and could be fitted to a monophasic curves (Figures 1B and 2, respectively). The half maximal response effective concentration ($EC_{50}$) values for quinpirole and B-HT 920 are summarized in Table 1. No difference was observed for quinpirole (one-way ANOVA; $F_{2,19}$ = 0.60; $P$ = 0.56) or B-HT 920 ($F_{2,14}$ = 0.52; $P$ = 0.61) between their $EC_{50}$ values in the CPu, NAc core, or NAc shell. The results with both D2 agonists show that dopamine D2 receptors have a fairly homogeneous expression throughout the striatum (from the CPu to the NAc) as evidenced by similar functional effects of D2 agonist on dopamine release across the striatum. Our results correlate with autoradiography studies that have shown that dopamine D2 receptor density is fairly homogeneous throughout the striatum (*16*, *23*, *27*, *52*, *53*).

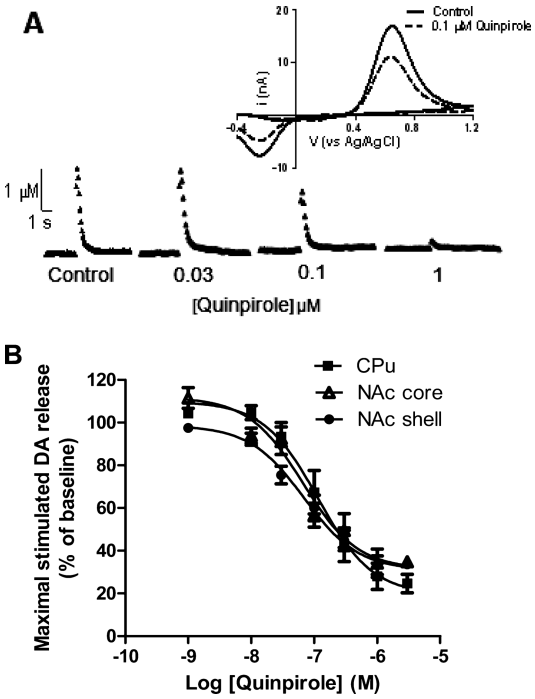


**Figure 1.** Effect of increasing concentrations of quinpirole on maximal evoked dopamine release in the striatum. (A) Representative dopamine concentration versus time plots showing the concentration dependent effect of the D2 agonist quinpirole on stimulated dopamine release in the CPu. The inset is two representative background subtracted cyclic voltammograms of control (solid line) and 0.1 $\mu$M quinpirole (dashed line) taken at the peak response. (B) The log concentration of quinpirole (M) versus the amount of dopamine released expressed as a percentage of the baseline (prior to agonist exposure), which is defined as 100%. As increasing concentrations of quinpirole are applied to the slice, a decrease in the electrically evoked dopamine is observed. Concentration−response relationship of quinpirole on inhibiting electrically stimulated dopamine release in the CPu (■), NAc core (Δ), and shell (●) is shown.

## Effect of Dopamine D3 Agonists on Electrically Stimulated Dopamine

The effect of the D3 agonists 7-OH-DPAT and (+)-PD 128907 on a single pulse of electrically stimulated dopamine release was evaluated as described above for D2 agonists. Concentrations of 7-OH-DPAT greater than 30 nM significantly reduced electrically stimulated dopamine release in all striatal regions ($P$ < 0.0001). Representative voltammetric plots of dopamine concentration versus time in the NAc shell in the absence and presence of 7-OH-DPAT (0.03, 0.1, and 1 $\mu$M) are shown in Figure 3A. Similar to the dopamine D2 agonists, dose−response curves were analyzed by curve fitting analysis, which revealed the potency ($EC_{50}$) and efficacy of the dopamine D3 agonist to decrease electrically stimulated dopamine release in the striatum. The $EC_{50}$ values for 7-OH-DPAT are presented in Table 1. Unlike the dopamine D2 agonists, the D3 agonist 7-OH-DPAT $EC_{50}$ values were significantly different across the brain regions in the striatum as analyzed with one-way ANOVA



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($F_{2,14}$ = 7.0; $P$ < 0.01) (Figure 3B). A Tukey posthoc test revealed a significant leftward shift in the $EC_{50}$ values observed between the dorsal CPu and the NAc core ($P$ < 0.05) and the dorsal CPu to the NAc shell ($P$ < 0.05). However, the Tukey posthoc test revealed no difference between the 7-OH-DPAT $EC_{50}$ values in the core versus the shell. This shift in $EC_{50}$ values indicates that the dopamine D3 receptors function at a higher level in the NAc (includes both the core and shell), suggesting higher dopamine D3 receptor density in the NAc versus the CPu. These results are also consistent with autoradiography experiments, which showed that the dopamine D3 density is greater in the NAc compared to the that in CPu (*18*).

The effect of the dopamine D3-preferring agonist (+)-PD 128907 on electrically stimulated dopamine release was similar to that of 7-OH-DPAT. Increasing concentrations of (+)-PD 128907 decreased electrically stimulated dopamine in a dose responsive manner (Figure 4). The $EC_{50}$ values for (+)-PD 128907 are summarized in Table 1. The (+)-PD 128907 $EC_{50}$ values across these striatal brain regions were significantly different as analyzed by a one-way ANOVA ($F_{2,14}$ = 11.24; $P$ < 0.01). A Tukey posthoc test revealed that the $EC_{50}$ values in the NAc shell exhibited the greatest shift to the left compared to that of the CPu ($P$ < 0.01) and NAc core ($P$ < 0.05). However, the Tukey posthoc test showed no difference between the $EC_{50}$ values for (+)-PD 128907 in the CPu and NAc core. On the basis of the $EC_{50}$ values, 7-OH-DPAT and (+)-PD 128907 had significant but different effects on the brain regions studied. The ability of these dopamine D3 agonists to lower the concentration of stimulated dopamine was greatest in the NAc shell and least effective in the CPu. This suggests regional difference in potency of these agonists to inhibit electrically stimulated dopamine release, which may reflect D2/D3 receptor selectivity.

This is the first report that we are aware of that has compared the response of dopamine agonist in

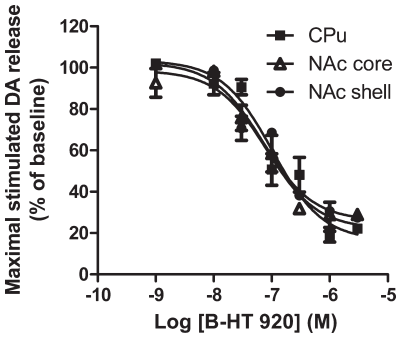


**Figure 2.** Effect of increasing concentrations of the D2 agonist B-HT 920 on maximal evoked dopamine release in the striatum. Concentration−response relationship of the dopamine D2 agonist B-HT 920 on inhibiting electrically stimulated dopamine release in the CPu (■), NAc core (Δ), and shell (●).

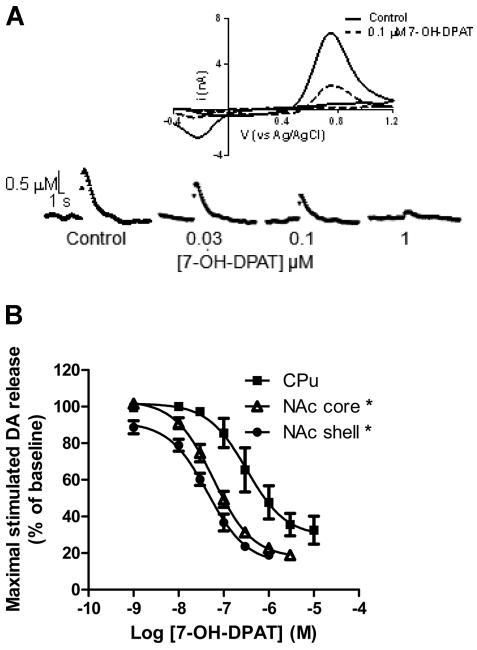


**Figure 3.** Effect of increasing concentrations of 7-OH-DPAT on maximal evoked dopamine release in the striatum. (A) Representative dopamine concentration versus time plots showing the concentration dependent effect of the D3 agonist 7-OH-DPAT on stimulated dopamine release in the NAc shell. The inset represents two background subtracted cyclic voltammograms of the control (solid line) and 0.1 μM 7-OH-DPAT (dashed line) taken at the peak response. (B) Concentration−dopamine response relationship of increasing concentrations of 7-OH-DPAT on inhibiting electrically stimulated dopamine release in the CPu (■), NAc core (Δ), and shell (●). Each log $EC_{50}$ value from the dose−response curves were analyzed with one-way ANOVA (* $P$ < 0.05 vs CPu).

**Table 1.** Voltammetrically Determined Potency ($EC_{50}$) and Efficacy Values for Dopamine D2 and D3 Agonists in the Striatum

| | caudate putamen | | nucleus accumbens core | | nucleus accumbens shell | |
|---|---|---|---|---|---|---|
| drug | $EC_{50}$ ± SEM (nM) | mean ± SEM efficacy (%) | $EC_{50}$ ± SEM (nM) | mean ± SEM efficacy (%) | $EC_{50}$ ± SEM (nM) | mean ± SEM efficacy (%) |
| 7-OH-DPAT | 325 ± 119 | 48 ± 9 | 59 ± 9 | 23 ± 1 | 44 ± 8 | 19 ± 2 |
| (+)-PD 128907 | 250 ± 77 | 43 ± 5 | 163 ± 47 | 36 ± 6 | 65 ± 12 | 29 ± 3 |
| quinpirole | 114 ± 35 | 28 ± 6 | 66 ± 33 | 34 ± 6 | 69 ± 16 | 33 ± 4 |
| B-HT 920 | 102 ± 32 | 18 ± 3 | 82 ± 29 | 28 ± 6 | 70 ± 18 | 30 ± 2 |



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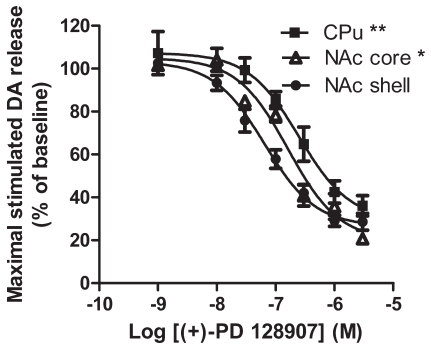


**Figure 4.** Effect of increasing concentrations of the D3 agonist (+)-PD 128907 on maximal evoked dopamine release in the striatum. Concentration−response relationship of the D3 agonist (+)-PD 128907 on inhibiting electrically stimulated dopamine release in the CPu (■), NAc core (Δ), and shell (●). The log $EC_{50}$ values from the dose−response curves were analyzed with one-way ANOVA (* $P < 0.05$; ** $P < 0.01$ vs shell).

subanatomical striatal brain regions to distinguish their selectivity for D2- or D3-autoreceptors. Agonists for the dopamine D2 and D3 receptors, when perfused across a slice, can bind and activate their respective receptors located on both pre- and postsynaptic surfaces. In this study, voltammetry was used to characterize dopamine release during agonist perfusion; however, only presynaptic autoreceptors which regulate dopamine release were evaluated. Shifts in voltammetric dose−response curves are most often associated with receptor functionality, but changes in receptor sensitivity and density cannot be ruled out (*34*). Using *in vitro* FSCV, dopamine D2 and D3 agonists give distinct dose−response curves and $EC_{50}$ values, which are dependent on the brain region examined. The dose−response curves and $EC_{50}$ of dopamine D2 agonists are more similar across the CPu, NAc core, and shell, compared to the more D3 selective agonists, which demonstrated a significant leftward shift in their dose−response curves and a reduction in $EC_{50}$ values from the CPu to the NAc shell. Additionally, the $EC_{50}$ values obtained from these voltammetry studies (for D2 and D3 agonists) directly correlate with D2 and D3 receptor density, as measured by autoradiography (*18*, *22*, *23*, *27*, *48*−*51*). This correlation suggests that voltammetry can be used to determine receptor density in different regions of the brain. Taken together, these results indicate that dopamine D2 agonists are relatively more potent in the CPu than in dopamine D3 agonists. These results suggest that combining *in vitro* voltammetry and receptor localization maybe a novel method to characterize agonists as more D2- or D3-preferring.

## Efficacy of Dopamine D2 and D3 Agonists

The relative maximum response of dopamine D2 and D3 agonists in the dorsal and ventral striatum were to determine if there was a difference in efficacy between these agonists across these regions. In order to directly compare the maximum inhibition of each of the agonists for decreasing dopamine release, the efficacy of each agonist at a concentration of 1 $\mu$M was compared. This concentration of 1 $\mu$M was chosen to evaluate drug efficacy because all drugs responded to this agonist concentration. This comparison was used to determine the relative activity of each agonist to decrease dopamine release in each of these brain regions, which we believe reflects the preference of these drugs to activate D2 or D3 receptors, which predominate in the CPu or NAc, respectively. This comparison was conducted for each brain region, and the efficacies are expressed as percent of the drug effect relative to the predrug value (Table 1). Thus, a low percentage reflects high efficacy for the given agonist. The dopamine D3 agonists exhibited the highest efficacy at 1 $\mu$M (greatest inhibition of electrically stimulated dopamine release) in the NAc shell, with 19% and 29% maximal stimulated dopamine release as a percent of predrug values (defined as a 100%) for 7-OH-DPAT and (+)-PD 128907, respectively. The efficacy of the dopamine D3 agonists in the CPu showed an efficacy of 48% for 7-OH-DPAT and 43% for (+)-PD 128907, which suggests that these D3 agonists have the ability to decrease dopamine release, but when compared to the ability, D2 agonists do not produce a maximum effect at this concentration. In contrast but consistent with the homogeneous distribution of the D2 receptor, the efficacy of D2 agonist quinpirole was approximately the same across the different brain regions, 28% for CPu, 34% for NAc core, and 33% NAc shell. Just like quinpirole, B-HT 920 exhibited a similar effect with values of 18%, 28%, and 30% for the CPu, NAc core, and shell, respectively.

## Effect of Dopamine D2 and D3 Agonists on Dopamine Uptake in the Striatum

The main mechanism by which D2 and D3 agonists regulate extracellular dopamine levels is by inhibiting dopamine release, although D2 receptors are also known to influence dopamine synthesis as well. However, there is considerable evidence indicating that both dopamine D2 and D3 receptors regulate dopamine transporter function (*19*, *20*, *40*−*45*). If dopamine D2 or D3 agonists modulate the activity of the dopamine transporter, then this would suggest another mechanism for these agonists to regulate extracellular dopamine levels. Many of the initial findings that linked the ability of dopamine D2/D3 agonists to modulate $V_{max}$ of the dopamine transporter used rotating disk voltammetry or chronoamperometry (*19*, *42*, *43*). An advantage of using electrochemical techniques is their rapid data collection rate, which is on the order of seconds, and provides the temporal resolution to discriminate differences



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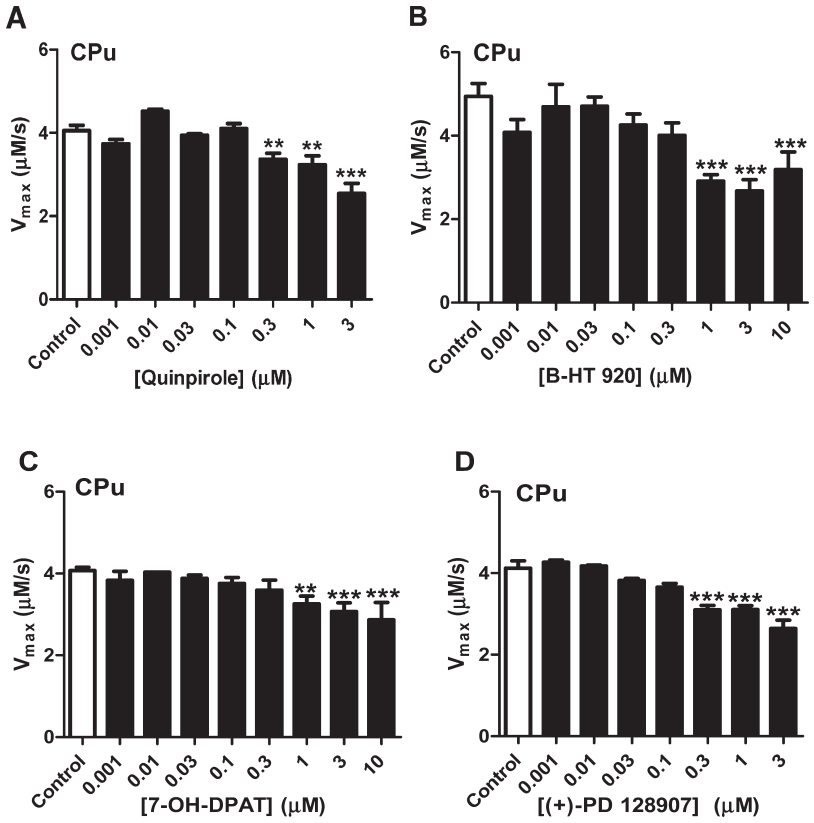


**Figure 5.** Effect of dopamine D2 and D3 agonist concentration on electrically evoked dopamine uptake rates in the CPu. (A) Quinpirole, (B) B-HT 920, (C) 7-OH-DPAT, (D) (+)-PD 128907. Dopamine uptake rates were analyzed using a one-way ANOVA by comparing control $V_{max}$ values to dopamine agonist treatment (** $P < 0.01$; *** $P < 0.0001$ compared to control).

in uptake rates. The objective of the following experiments was to evaluate dopamine uptake rates in the presence of increasing concentrations of dopamine D2 and D3 agonists.

As described in the Methods (see data section), the Michaelis−Menten based kinetic model was used to evaluate release ([DA]$p$) and uptake kinetics ($V_{max}$ and $K_m$). When analyzing dopamine current versus time plots, $K_m$ values were fixed to 0.16 $\mu$M allowing for manipulation of dopamine peak amplitude (release) and dopamine uptake ($V_{max}$) in the presence or absence of a dopamine D2 or D3 agonist in striatal regions (*54*, *55*). The effect of 0.001, 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 $\mu$M quinpirole, B-HT 920, 7-OH-DPAT, or (+)-PD 128907 on dopamine uptake was evaluated. The results show a significant decrease in $V_{max}$ in the presence of the D2 or D3 receptor agonist only in the CPu and at very high concentrations of the agonist (quinpirole, $F_{7,90} = 13$; $P < 0.0001$; B-HT 920, $F_{8,71} = 8.9$; $P < 0.0001$; 7-OH DPAT, $F_{8,117} = 4.8$; $P < 0.0001$; (+)-PD 128907, $F_{7,66} = 16$; $P < 0.0001$) (Figures 5 and 6). The clearance of dopamine by the dopamine transporter in NAc (core and shell) was not affected by the presence of the dopamine agonists quinpirole, B-HT 920, 7- OH-DPAT, or (+) PD-128907 (see Supporting Information, Figure S1).

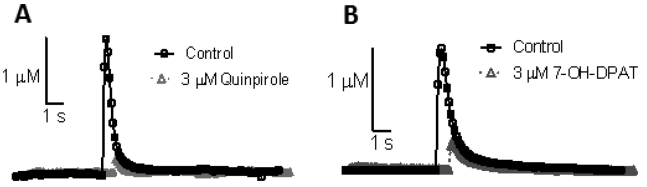


**Figure 6.** Representative concentration versus time plot showing the electrically evoked dopamine release and uptake profiles before (□) and after 3 $\mu$M perfusion (Δ) of quinpirole (A) and 7-OH-DPAT (B). In both cases, the agonist curve is shifted to the right to illustrate dopamine uptake between the control (predrug) and agonist.

Our results from mouse brain slices show only a decrease in $V_{max}$ at the highest concentrations of agonists applied in the CPu. However, previous electrochemical studies reported an increase in dopamine clearance in the presence of a D2 agonist and a decrease in dopamine clearance by a D2-like receptor antagonist (*42*, *43*, *56*). However, these data are not conclusive because Dickinson et al. showed no difference in dopamine clearance in the presence of raclopride, a known D2 antagonist (*30*). These previous studies, which evaluated the effect of dopamine agonists or antagonists on dopamine clearance, did not use FSCV but used other electrochemical methods. The discrepancy in uptake



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rates between our results and these previous studies could be a result of different experimental parameters used in FSCV compared to those in chronoamperometry or rotating disk voltammetry such as brain slices versus an intact system (*in vivo*) or inducing dopamine depolarization by employing one-pulse stimulation (endogenous dopamine release) versus exogenously applying dopamine. However, most FSCV experiments that measure the effect of D2-agonists on dopamine peak amplitude do not report uptake rates (*31*, *36*, *37*, *39*, *57*−*60*). This approach is most likely due to an a priori assumption that only electrically stimulated dopamine release (or peak height or dopamine amplitude) has been altered in the presence of agonists (*35*). A study by Joseph et al. measured dopamine uptake using FSCV and in the presence of quinpirole noted that uptake in CPu was not different (*31*). Joseph et al. suggested that alterations in dopamine uptake kinetics are not observed because (1) dopamine uptake rates are maximally accelerated or (2) temporal resolution of FSCV is not adequate to resolve these elevated dopamine uptake rates (*31*).

Dopamine transporter activity is regulated by either receptors or second-messenger linked signal transduction pathways. Briefly, activation of protein kinase C (PKC), extracellular signal-regulated kinases 1 and 2 (ERK1/2), and phosphoinositide 3 kinase (PI3K) have all been shown to influence dopamine transporter activity (*61*−*63*). Although voltammetric studies suggest modulation of the dopamine transport via dopamine receptors, only recently have the second-messenger pathways between dopamine receptors and transporters have been examined (*20*, *41*, *44*). Specifically, Bolan et al. demonstrated that D2 receptor activation enhanced cell surface expression of the dopamine transporter by ERK1/2 (*41*). Additionally, Lee et al. demonstrated a direct protein−protein interaction between the D2 receptor and the dopamine transporter, and this direct physical coupling promoted dopamine transporter expression to the cell surface (*44*). Specifically, an increase in dopamine transporter velocity ($V_{max}$) was observed with no difference in $K_m$ (*44*). In a subsequent study, acute D3 receptor activation modulates dopamine transporter activity by both ERK1/2 and PI3K, but prolonged D3 receptor activation induced a reduction in the cell surface dopamine transporter expression (*20*). In our study, cumulative dose−response curves are used to evaluate dopamine uptake, and as a result, the slice is bathed with an agonist for at least 2 h before concentrations greater than 0.1 $\mu$M are applied. Our agonist results suggest low concentrations do not influence dopamine uptake, which may represent acute activation. However, a combination of prolonged exposure and agonist concentrations greater than 0.1 $\mu$M do demonstrate a significant decrease in dopamine uptake in the CPu, which agrees with previous findings (*20*). Since this decrease in dopamine uptake was observed with both D2 and D3 agonists, we would speculate that a possible mechanism for receptors regulating transporter expression and/or function may be through the ERK1/2 pathway. However, future studies would have to asses this proposed mechanism.

The fact that dopamine uptake is influenced only by high concentrations of agonist in the CPu, while no difference in uptake is observed in the NAc core and shell (see Supporting Information, Figure S-1), suggests this may be a brain region specific phenomena. The dopamine transporter density within the striatum is known to vary depending on the subanatomical location, with the CPu having the greatest density of dopamine transporters, while the NAc core and shell have considerably less (*8*−*11*). We hypothesize that this lack of agonist effect on uptake in the accumbens may be a result of fewer dopamine transporters compared to that in the CPu. The density of dopamine transporters is reduced in the NAc compared to that in the CPu, while D2-like receptor density remains the same or is increased in the NAc. Taken together, these data suggest that fewer dopamine transporters are coupled and/or not responsive to D2-like receptor agonists in the accumbens. Hence, no effect of these agonists in the NAc core or shell is observed versus the CPu.

## Effect of Dopamine Antagonists in the CPu

Dopamine D2 and D3 receptor antagonists block their respective receptors and activate dopamine synthesis and release in presynaptic terminals (*64*−*66*). To demonstrate the reversibility of the electrically evoked dopamine signal, an antagonist was applied to brain slices immediately after agonist application. The objective was to determine if dopamine D2-like and D3 antagonists can selectively reverse their respective agonist response. The CPu was chosen as the brain region to characterize these antagonist effects because it is known to have the greatest discrepancy between dopamine D2 and D3 receptor levels. In these studies, only one concentration of the dopamine D2 or D3 receptor agonist (300 nM) was applied to the slice. This agonist concentration was chosen on the basis of the dose−response curves that we generated demonstrating approximately 40−60% decrease in the dopamine release. Immediately after agonist application, a nonselective dopamine D2 or selective D3 antagonist (10 $\mu$M) was applied to the slice. As shown by Schmitz et al., even after a 10 min perfusion with 500 nM quinpirole, the dopamine peak amplitude as recorded by FSCV was attenuated for at least an additional 22 min after the removal of quinpirole demonstrating that the response of the agonist was not washed out when the buffer was changed to artificial cerebral spinal fluid (aCSF) (*32*).



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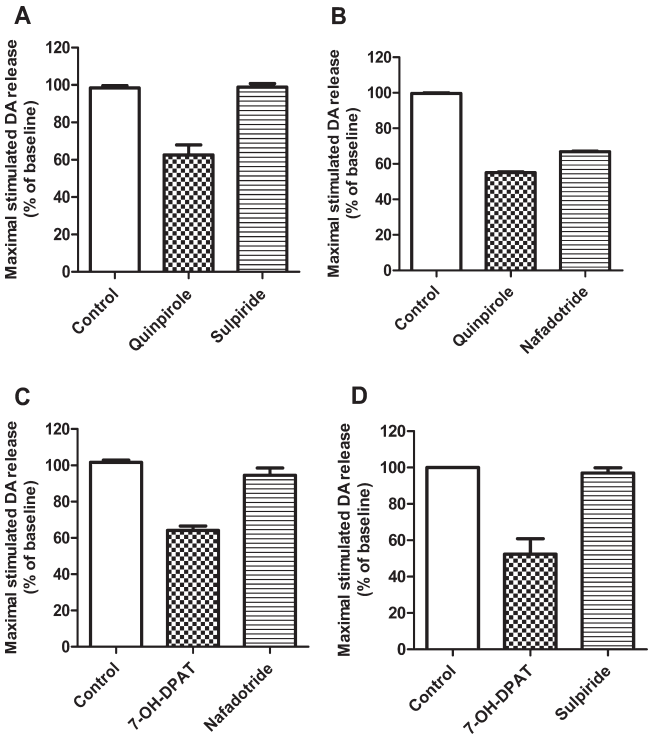


**Figure 7.** Effect of 300 nM quinpirole (A,B) or 7-OH-DPAT (C,D) on electrically evoked dopamine in the dorsal CPu. In both cases, quinpirole or 7-OH-DPAT decreased electrically evoked dopamine. When the nonselective D2 antagonist sulpiride was applied after quinpirole, it has the ability to fully reverse the dopamine response (A), but when nafadotride, a selective D3 antagonist was added, it was unable to reverse the electrically evoked dopamine response. However, when both the nonselective D2 antagonist sulpiride and nafadotride were applied after 7-OH-DPAT, they were both able to fully reverse the response (C,D).

Immediately after agonist application, either sulpiride, a nonselective dopamine antagonist, or nafadotride, a selective dopamine D3 antagonist, was perfused over the slice. The D2 agonist effect in the CPu was reversed fully only by the sulpiride (Figure 7A), while nafadotride increased electrically stimulated dopamine levels to approximately 70% of the predrug value (Figure 7B). Hence, the D3 antagonist only had the ability to increase maximal stimulated dopamine release by 10% in the CPu. Similarly, after 7-OH-DPAT perfusion in the CPu an approximately 40% decrease in the electrically stimulated dopamine response was observed. However, upon perfusion of sulpiride or nafadotride the electrically stimulated dopamine response returned to predrug levels (100%) (Figure 7C and D).

Thus, it appears that the effect of a dopamine D2 agonist is only reversible after infusion of a nonselective D2 antagonist suggesting that nafadotride is acting primarily at either available dopamine D3 receptors within the CPu, where there is low density of these receptors present, or alternatively, nafadotride is elevating dopamine levels by interacting at available dopamine D2 receptors (*18*, *22*, *23*). However, we believe that nafadotride is not acting at the D2 receptors because with a high concentration (10 $\mu$M) being applied, we would expect to observe a greater response with more D2 receptor functionality and/or density present within the CPu. Nafadotride is unable to fully reverse the effect of quinpirole because of the lower abundance of D3 receptors available within this brain region, and the levoisomer of nafadotride is known to have a greater affinity to the dopamine D3 receptors than to the D2 receptors (*67*). Within the CPu, we have demonstrated that sulpiride can fully reverse the effects of quinpirole, while nafadotride is unable to reverse these effects. Since only the nonselective dopamine antagonist reversed the agonist response, this further supports the hypothesis that the dopamine D2 receptors are more functional in the CPu than the D3 receptors.

However, when the dopamine D3 agonist, 7-OH-DPAT, is applied to the CPu both antagonists, sulpiride and nafadotride, reverse the dopamine response. The ability of both antagonists to reverse the dopamine D3 agonist is a result of sulpiride being a nonselective dopamine antagonist, with a high affinity for D2 and D3 receptors. As a result of sulpiride's promiscuity, it is able to reverse the effect of the D3 agonist in the CPu. Previous work using *in vitro* FSCV showed that higher concentrations of sulpiride and similar nonselective dopamine antagonists such as clozapine and haloperidol had the ability to attenuate the 7-OH-DPAT-induced inhibition of electrically stimulated dopamine release in the NAc core (*36*). Our results with quinpirole−sulpiride and 7-OH-DPAT−sulpiride demonstrate that sulpiride is indeed a nonselective dopamine antagonist with high affinity for both the D2 and D3 receptors. In order to demonstrate exclusive receptor reversibility of the D3 receptor in the CPu, a very selective D2 antagonist would have to be applied; however, many of the classic antipsychotic dopamine antagonist are not selective enough for the D2 receptor.

The D3 antagonist nafadotride is described as a highly potent, preferential D3 antagonist. When an excess of nafadotride is applied to the CPu, it easily reverses the agonist effects, and this reversal is most likely a result of its ability to compete with 7-OH-DPAT for available dopamine D3 receptors. Additionally, on the basis of the results with quinpirole−nafadotride in the CPu (Figure 7B), it appears that nafadotride is not very effective at activating the dopamine D2 receptor suggesting that nafadotride is a more selective D3 receptor antagonist. Taken together, these agonist−antagonist treatments suggest that within the CPu a nonselective dopamine antagonist in excess concentration can easily reverse the inhibition of D2- and D3-receptor agonists, but a selective D3 antagonist can reverse only the effects of a D3 agonist.



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## Conclusions

The results presented here demonstrate that the striatal region of the brain can be used as a tool to determine whether agonists are selective for D2- or D3-autoreceptors. The advantage of studying these effects in the striatum is the distinct localization of D2 and D3 receptors. Using *in vitro* FSCV, we demonstrated that the D2 receptor functionality is uniform in the striatum. Specifically, commercially available D2 agonists (quinpirole and B-HT 920) showed similar $EC_{50}$ values throughout the striatum. However, the D3 receptor functionality is localized in the NAc shell. More specifically, dopamine agonists with more D3-like properties (7-OH-DPAT and (+)-PD 128907) demonstrated a significant leftward shift in their dose−response curves and $EC_{50}$'s from the dorsal CPu to the NAc shell. Our results, which examine autoreceptor function, complement the autoradiography work that has mapped the distribution of dopamine D2 and D3 receptors. Although FSCV cannot distinguish receptor density from sensitivity, these results demonstrate a simple and fast method for determining dopamine functionality with D2 and D3 receptors. We believe that by exploiting the unique receptor density within the striatum voltammetry may be used as a tool to characterize D2-like agonists as either D2- or D3-preferring. Mapping these receptors can offer powerful insight into the neuropathology of disorders involving these receptors as well as the drugs' mode of action.

## Methods

### Animals

Male C57Bl/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME) at 3−5 weeks old and housed in the animal care facilities at Wayne State University. All animals were allowed to acclimate to the animal care facilities for at least one week before they were used in any experiment. The mice were kept in groups of either 5 or 10 animals per cage with food and water ad libtum on a 12 h light−dark cycle. Experimental protocols adhered to the National Institues of Health Animal Care Guidelines and were approved by the Wayne State University Institutional Animal Care and Use Committee.

### Brain Slices

Mice 8−12 weeks old were anesthetized with $CO_2$, and the brains were rapidly removed and cooled in preoxygenated (95% $O_2$/5% $CO_2$) high sucrose-aCSF buffer for 10 min. The sucrose-aCSF buffer consisted of 180 mM sucrose, 30 mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.2 mM $NaH_2PO_4$, and 10 mM D-glucose (*68*). The brain was sectioned with a vibrating tissue slicer (Vibratome, St. Louis, MO) into 400-$\mu$m-thick coronal slices. Brain slices containing dopamine rich regions of interest such as CPu and NAc were obtained. Slices were maintained in oxygenated aCSF at room temperature for 1 h. A slice was transferred to a custom-made submersion recording chamber (Custom Scientific, Denver, CO) and allowed to equilibrate in oxygenated aCSF at 32 °C for 30 min before dopamine measurements were made.

### Fast Scan Cyclic Voltammetry

Carbon fiber microelectrodes were fabricated in house using a previously described method with minor modifications (*69*). First, a 7 $\mu$m diameter carbon fiber (Goodfellow, Oakdale, PA) was aspirated through a glass capillary (A-M Systems, Carlsborg, WA) using vacuum suction. The capillary was heated and pulled using a micropipet puller (Narishige, Tokyo, Japan) to form two microelectrodes with a tight glass seal around the carbon fiber. The exposed carbon fiber was trimmed to a length of 50−200 $\mu$m beyond the glass carbon fiber seal. The electrode was backfilled with 150 mM KCl, and a lead wire (Squires Electronics, Cornelius, OR) was inserted into the microelectrode to make an electrical connection with the carbon fiber. The silver/silver chloride (Ag/AgCl) reference electrode was made from a 250 $\mu$m silver wire (A-M Systems, Carlsborg, WA). The silver wire was coated with a thin layer of silver chloride by anodizing (+1 V) in a solution of 1 M hydrochloric acid for 5−10 min. The potential at a carbon fiber microelectrode was held at −0.4 V versus the reference electrode, then ramped to +1.2 V, and back to −0.4 V (400 V/s) every 100 ms (10 Hz) (*37*, *60*, *70*−*73*). All electrode and stimulation parameters were controlled by TH software (ESA Inc., Chelmsford, MA). When the triangle waveform is applied, a stable background current is produced before dopamine is released, and this background is digitally subtracted from the voltammograms following dopamine stimulation (*74*). Characteristic background subtracted voltammograms for dopamine demonstrate peak oxidation currents for dopamine at approximately +600 mV and the peak reduction currents for dopamine-ortho-quinone at approximately −200 mV. A low-noise ChemClamp potentiostat (Dagan Corporation, Minneapolis, MN) was used for FSCV measurements. The slice chamber was perfused at 1 mL/min with 32 °C oxygenated aCSF. Dopamine was evoked every 5 min by one pulse stimulation (monophasic, 350 $\mu$A, 60 Hz, and 4 ms pulse width) from the adjacent stimulating tungsten electrode (Plastics one, Roande, VA) and generated by a Neurolog stimulus isolator (Digitmeter, Hertfordshire, England). The stimulating electrode was placed directly on the slice, approximately 100−200 $\mu$m away from the carbon fiber electrode, which was placed ~75 $\mu$m below the surface of the slice (*70*, *72*). After a stable baseline recording (≥30 min), 0.001−10 $\mu$M dopamine agonist solutions of (−)-quinpirole hydrochloride, (4*aR*,10*bR*)-3,4*a*,4,10*b*-tetrahydro-4-propyl-2*H*,5*H*-[1]benzopyrano-[4,3-*b*]-1,4-oxazin-9-ol hydrochloride ((+)-PD 128907), 5,6,7,8-tetrahydro-6-(2-propen-1-yl)-4*H*-thiazolo[4,5-*d*]azepin-2-amine dihydrochloride (B-HT 920), or (±)-7-hydroxy-2-dipropylaminotetralin hydrobromide (7-OH-DPAT) were perfused over the slice for 30 min. A cumulative dose−response curve was chosen because previously John and Jones demonstrated that cumulative concentrations of drugs do not alter release or uptake compared to that when applying only a single concentration of the drug (*72*). The effect of each drug concentration was recorded for 30 min. In the reversing experiments, agonists and antagonist were used. A single dose of the D2 or D3 agonist (300 nM) was perfused over the slice for 30 min, then to determine if the



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antagonist could reverse the effects of the agonist, 10 $\mu$M (*S*)-(−)-sulpiride or nafadotride was perfused over the slice immediately after the agonist for 30 min. The peak oxidation current for dopamine was converted into concentration from a postelectrode calibration with 3 $\mu$M dopamine. The electrode calibration buffer used was similar to the aCSF buffer with the absence of D-glucose and ascorbic acid.

### Data Analysis

The resulting current versus time plot obtained from the slice was fit by nonlinear regression as described by Jones et al. in software written in LabVIEW (National Instruments, Austin, TX) (*11*, *75*, *76*). The electrically stimulated dopamine (DA) release and uptake rates were determined using a set of Michaelis−Menten based equations (*11*, *72*, *77*, *78*).

$$\frac{d[DA]}{dt} = f[DA]_p - \frac{V_{max}}{(K_m/[DA]+1)}$$

The apparent $K_m$ is a constant, which correlates the affinity of dopamine to the dopamine transporter, and was set during the analysis of stimulated [DA] and maximal uptake rates as maximum velocity ($V_{max}$). The $K_m$ value used in all data analyses was obtained from a representative average of $K_m$ values from the literature, which was determined to be 0.16 $\mu$M (*54*). Data analysis of stimulated dopamine release per pulse ([DA]*p*) in a given stimulation frequency *f* and maximal uptake rates were then evaluated as maximal velocity ($V_{max}$). It has been well characterized that the rise in electrically stimulated dopamine signal during FSCV is a competition between release and uptake, where release dominates, while the decay phase of the electrical stimulated dopamine is mainly due to uptake (*75*, *76*).

### Chemicals

All chemicals were used as received and purchased from Sigma (St. Louis, MO) unless otherwise stated. ACSF consisting of 108 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 8.2 mM $MgCl_2$, 4 mM $NaHCO_3$, 1 mM $NaH_2PO_4$, 11 mM D-glucose, and 0.4 mM ascorbic acid (pH 7.4) was made using ultrapure (18 MΩ cm) water. Nafadotride, sulpiride, (+)-PD 128907, B-HT 920, and 7-OH-DPAT were purchased from Tocris Bioscience (Ellisville, MO). All solutions of the drugs and dopamine were diluted in the aCSF from the stock solutions unless otherwise stated.

### Statistics

All statistical analyses were carried out using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). Data are shown as the mean ± standard error of the mean (SEM) of at least five brain slices, which were from different animals. When dopamine agonists were used, the change in the current versus time profile was evaluated as a change in [DA]*p*, which is the inhibition of dopamine release via the D2-like autoreceptors. This change in electrically stimulated dopamine release was compared to predrug values (each animal served as their own control) leading to a percent change in stimulated dopamine release. Using GraphPad Prism, we plotted the dose−response curve as a log concentration (M) of quinpirole versus percent of baseline (maximal stimulated dopamine release), and the data was fitted using a nonlinear regression curve fit to determine $EC_{50}$ concentrations. The log $EC_{50}$ obtained after the administration of dopamine D2 or D3 agonists were subjected to a one-way ANOVA, Tukey post hoc test by comparing the CPu, NAc core, and shell. Effect of dopamine D2 or D3-like agonists on dopamine uptake was analyzed using a one-way ANOVA, Dunnett's post hoc test by comparing predrug $V_{max}$ values to dopamine agonist treatment. In all cases, statistical significance was set at $P < 0.05$.

## Supporting Information Available

Dopamine uptake in the NAc in the presence of D2 or D3 agonists. This information is available free of charge via the Internet at http://pubs.acs.org/

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### Notes

The content is solely the responsibility of the authors and does not represent the official views of the NIAAA or the National Institutes of Health.

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## Abbreviations

CPu, caudate putamen; NAc, nucleus accumbens; aCSF, artificial cerebral spinal fluid; FSCV, fast scan cyclic voltammetry; B-HT 920, 5,6,7,8-tetrahydro-6-(2-propen-1-yl)-4*H*-thiazolo[4,5-*d*]azepin-2-amine dihydrochloride; (+)-PD 128907, (4*aR*,10*bR*)-3,4*a*,4,10*b*-tetrahydro-4-propyl-2*H*,5*H*-[1]benzopyrano-[4,3-*b*]-1,4-oxazin-9-ol hydrochloride; 7-OH-DPAT, (±)-7-hydroxy-2-dipropylaminotetralin hydrobromide; MRI, magnetic resonance imaging; $EC_{50}$, half maximal effective concentration.

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