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Functional Reorganization of the Presynaptic Dopaminergic Terminal in Parkinsonism

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Abstract

Whether dopamine release is compensated during the presymptomatic phase of Parkinson's disease is controversial. Here we use *in vivo* voltammetry in the parkinsonian rat and an electrical stimulation protocol established to fatigue nigrostriatal dopaminergic neurons to investigate the plasticity of dopamine release mechanisms. Amplitudes of evoked voltammetric signals recorded in intact rat striata decreased with repetitive, high-frequency stimulation (60 Hz, every 5 min. / 60 min.). Strikingly, dopamine levels were maintained during an identical "fatiguing" protocol in 6-hydroxydopamine-lesioned (<40% denervation) striata in the absence of enhanced dopamine synthesis. In contrast, more severely lesioned striata (>55% denervation) also appeared to sustain DA release, however, this was demonstrated in the presence of enhanced synthesis. Sustained release was replicated in intact animals after irreversible blockade of the dopamine transporter via RTI-76, implicating neuronal uptake as a trigger. We further demonstrate through kinetic analysis that lesions and compromised uptake target a "long-term" (time constant of minutes) presynaptic depression, which underlies the maintenance of release. Taken together, our findings identify a denervation-induced maintenance of dopamine release that was independent of activated synthesis and driven by altered uptake. This novel neuroadaptation may contribute to early preclinical normalization of function and help resolve discrepant findings regarding compensatory changes in dopamine release during progression of the parkinsonian state.

Keywords

Parkinson's disease; dopamine; compensation; release; synthesis; uptake

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Progressive loss of nigrostriatal dopaminergic (DAergic) neurons of the basal ganglia results in the clinical condition known as Parkinson's disease (PD). The extended preclinical phase of PD, during which cardinal symptoms of rigidity, resting tremor and akinesia do not present until neurodegeneration exceeds ~80%, suggests that potent compensatory responses actively stabilize motor function (Hornykiewicz and Kish, 1987; Galvan and Wichmann, 2008). Animal studies indicate that striatal levels of extracellular dopamine (DA) are successfully maintained across the same presymptomatic denervation range (Abercrombie et al., 1990; Robinson et al., 1994). To achieve this normalization of functional DA input to target cells, residual DAergic terminals are thought to undergo multi-level neurochemical adaptations. One prevailing hypothesis identifies up-regulated DA release as the central neuroadaptation (Zigmond et al., 1990; Zigmond, 1997). Through a concerted action, enhanced release, sustained by activated DA synthesis, and diminished uptake drive DA diffusion from innervated to denervated regions, thereby preserving DAergic tone throughout the striatum.

Despite the attractiveness of this model, definitive characterization of potential alterations in DA release during the preclinical phase of PD has proven to be particularly difficult. Indeed, two diametrically opposed findings have emerged, with some studies suggesting up-regulated DA release by surviving DAergic terminals in the striatum (Stachowiak et al., 1987; Snyder et al., 1990; McCallum et al., 2006; Perez et al., 2008), while others showing no compensation (Garris et al., 1997; Bezard et al., 2000; Bergstrom and Garris, 2003). Technical issues are a possible explanation for these discrepant results, because different means to elicit release (electrical, chemical and pharmacological) and animal models (rat, mouse and monkey) have been employed. It is thus tempting to speculate that different neurotransmitter storage pools (Richards et al., 2003; Rizzoli and Betz, 2005) are interrogated under different conditions and that these components of DA release are differentially sensitive to lesions.

Previous *in vivo* studies failing to demonstrate a denervation-induced compensatory up-regulation of DA release employed stimulus protocols during which instantaneous release was constant over time. In contrast to these “time-independent” release conditions, the pattern of applied stimulus trains can be altered to elicit both increases and decreases in DA release over time (Michael et al., 1987; Yavich, 1996; Montague et al., 2004). These “time-dependent” release conditions have previously been used to investigate key components of the DA release mechanism in intact animals (Yavich and MacDonald, 2000; Phillips et al., 2002; Cragg, 2003; Kita et al., 2007). We therefore examined the effects of DA depletions on time-dependent release to determine whether this mode exhibited plasticity *in vivo*. Although some *in vivo* microdialysis reports (Abercrombie et al., 1990; Zigmond et al., 1990) have offered indirect evidence suggestive of an increase in DA release; the dialysate DA levels obtained provide valuable information concerning changes in basal DA levels but do not provide a direct measurement of DA release as we do in the present study. Using a “fatiguing” protocol consisting of repetitive, high-frequency stimulation and voltammetry in the parkinsonian rat, we demonstrate, for the first time *in vivo*, a denervation-induced compensatory adaptation of DA release as reflected in reduced fatigue of DA release. Moreover, sustained release during early preclinical states appeared to be independent of activated DA synthesis and triggered by reduced recycling of DA through diminished presynaptic uptake. Furthermore, we confirm *in vitro* reports indicating sustained DA release is supported by enhanced synthesis in more advanced preclinical states.

1. Experimental Procedures

1.1 Experimental Design

Three experimental designs were employed in the present study. The 6-OHDA unilateral partial lesion model was utilized to generate all parkinsonian rats for two of the designs. The first design consisted of two groups and examined synthesis rates in lesioned and intact rats by administering the dopa-decarboxylase inhibitor NSD-1015. The second design examined time-dependent DA release utilizing the fatigue stimulation protocol both before and after depletion of the releasable pool of DA via the tyrosine hydroxylase inhibitor α MpT in both intact and lesioned animals. The third and final design examined time-dependent DA release using RTI-76 to produce a pharmacological “knock-out” of the DAT. This protocol repeated the fatigue stimulation protocol in two separate groups of animals; one group was pre-treated with RTI-76 alone and the other group was pre-treated with RTI-76 and administered α MpT.

1.2 Animals

Adult male rats (250-400g) were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and maintained in facilities approved by the Institutional Animal Care and Use Committee at Illinois State University. Rat chow and water were provided *ad libitum*. Animal care was in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication 86-23).

1.3 DA Synthesis

Lesioned and intact animals received the DOPA-decarboxylase inhibitor NSD-1015 (100 mg/kg, i.p.) and alterations in DA synthesis were determined as a function of DOPA accumulations. Animals were sacrificed 30 minutes after injection, and tissue DA and DOPA content was determined via HPLC-EC as described below.

1.4 RTI-76 Microinjections

RTI-76 was microinjected 1-3 d before voltammetric surgeries using a previously described method (Wu et al., 2001a). The rats received Equithesin (3 ml/kg, i.p.) and were placed into a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) after the anesthesia had taken effect. Stereotaxic coordinates were based on a flat skull and given in mm (Paxinos and Watson, 1986) for all surgeries performed. Bregma served as a reference point for anteroposterior (AP) and mediolateral (ML) stereotaxic coordinates. Dura mater served as the reference for dorsoventral (DV) stereotaxic coordinates. Internal body temperature was maintained at 37°C by Deltaphase Isothermal Pads (Braintree Scientific, Braintree, MA). A hole was drilled ipsilateral to the side voltammetric recordings would take place and a 30 gauge needle lowered to -0.25 AP, 1.4 ML, and -4.0 to 5.0 DV for intracerebroventricular injection. 100 nmol of RTI-76 was dissolved in 10 μ l of sterile saline and infused at a rate of 0.5 μ l/min with a microsyringe pump (KD Scientific model 100, Fisher Scientific, Fair Lawn, NJ). Once injection was completed, the needle remained in place for 5 min. After the needle was removed, bone wax was used to seal the hole and the scalp was sutured.

1.5 6-OHDA Unilateral Partial Lesion Surgery

Injections of 6-OHDA into the lateral substantia nigra were performed according to Bergstrom and co-workers (Bergstrom et al., 2001). This model provided a graded loss of DA neurons with the lateral regions being denervated to a greater extent than the medial regions of the CP. Equithesin (6 ml/kg i.p.) was used as anesthesia followed by injections of desipramine (25mg/kg i.p.) and pargyline (40 mg/kg i.p.). Stereotaxic coordinates were determined as described above. Internal body temperature was maintained at 37°C by

Deltaphase Isothermal Pads (Braintree Scientific, Braintree, MA). A single 7 μ g dose of the neurotoxin, 6-OHDA, was injected into the lateral substantia nigra (-5.4 AP, +3.0 ML, -8.2 DV) using a 30 gauge needle. After the needle was removed, bone wax was used to seal the hole and the scalp was sutured.

1.6 Voltammetry Surgery

Fast-scan cyclic voltammetry (FSCV) at carbon-fiber microelectrodes was performed as previously described (Bergstrom and Garriss, 2003). Urethane anesthesia (1.5 mg/kg i.p.) was administered before voltammetric surgery. After the animal was deeply anaesthetized, a rotary tool was used to drill three holes through the skull, each of which corresponded to placement of the reference, stimulating and recording electrodes. Reference electrodes were positioned contralateral to the lesion in superficial cortex, whereas recording and stimulating electrodes were positioned ipsilateral. The stimulating electrode was lowered over the medial forebrain bundle (-4.6 AP, 1.4 ML, -7.5 to -8.5 DV) to evoke DA release. Two carbon-fiber recording electrodes were positioned in the CP at 6° angles using two separate mediolateral positions (1.2 AP, 1.0, 4.0 ML). The dorsoventral regions of the CP sampled varied slightly depending upon signal quality (-4.3 to -4.9).

1.7 Electrochemistry

Carbon-fiber recording electrodes were locally constructed according to a previously described protocol (Cahill et al., 1996). Fibers ($r = 2.5 \mu\text{m}$, Thornel T-650 Amoco, Greenville, SC) extended approximately 75-150 μm past the pulled borosilicate capillary tube. All electrochemistry was performed by an EI 400 potentiostat (Ensmann Instruments, Bloomington, IN) and was under computer control (Wiedemann et al., 1991). Triangle waves were applied at 10 Hz. Voltage was ramped from -400 mV to 1000 mV back to -400 mV at a scan rate of 300 V/s. All signals collected in lesioned, intact, and RTI-76 pre-treated animals were identified as DA based on background subtracted cyclic voltammograms (data not shown) (Baur et al., 1988). Post-calibration enabled the conversion of measured current to DA concentration.

1.8 Fatigue Stimulation Protocol

Bipolar stimulating electrodes were used for all electrical stimulation with tips separated by 1 mm (Plastics One, Roanoke, VA). Biphasic, constant current square wave pulses were used during electrical stimulation. Phase amplitude and duration were $\pm 300 \mu\text{A}$ and 2 ms, respectively. Each stimulus pulse was under computer control, optically isolated (NL 800 Neurolog, Medical Systems, Great Neck, NY) and synchronized with each recording electrode. Stimulus pulse trains (60 Hz, 2 s) were delivered every 5 min for 60 min (see Figure 1). Sufficient time was allowed for signals to return to the pre-fatigue baseline (approximately 20 min) before αMPT (200 mg/kg i.p.) was administered and the stimulations were repeated (see Figure 3). Animals pre-treated with RTI-76 underwent one round of fatigue stimulations (see Figure 5).

1.9 Tissue DA and DOPA Content

HPLC-EC was used to quantify tissue DA and DOPA content. Animals were decapitated 2-4 weeks post-surgery and whole brain was extracted from each skull. After the brain was firm in ice-cold saline (150 mM NaCl) for approximately 10 minutes, tissue was sliced using a chilled aluminum brain block. Razor blades were used for slicing and yielded slices approximately 1 mm thick. The CP was isolated and further dissected to obtain the dorsal medial, mediolateral, and lateral regions of the CP. This slice corresponded to voltammetric recording sites and was evident due to the minor tissue damage resulting from implantation

of electrodes. All samples were weighed wet and frozen on dry ice. Storage at -20 °C ensured stability until the day of HPLC-EC.

Tissue samples were homogenized (Ultrasonic Processor, Sigma-Aldrich Techware, St. Louis, MO) in 0.1 N perchloric acid and frozen on dry ice in order to precipitate proteins. Samples were then centrifuged (Marathon micro H, Fisher Scientific) and supernatant was removed and run on HPLC-EC (BAS 200B with a Unijet detector and injector, Bioanalytical Systems, W. Lafayette, IN). HPLC-EC performed trace analysis of DA and DOPA using a microbore, reverse phase column (Phase -II ODS 3µm 100× 3.2mm, Bioanalytical Systems). The electrochemical detector was set at an oxidizing potential (+550 mV). HPLC mobile phase was made as previously described (Bergstrom et al., 2001)

1.10 Kinetic Analysis

Rate constants describing uptake were determined from the evoked signals measured by voltammetry. These signals are mathematically represented by a set of equations generally given by (Wightman et al., 1988):

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

where t is time, f is the frequency of stimulation, $[DA]_p$ is the concentration of DA elicited per stimulus pulse (i.e., fixed $[DA]_p$), and $V_{\max}$ and K_m are Michaelis-Menten rate constants for DA uptake, related to transporter affinity and number of transporter molecules, respectively. Measured parameters describe average DA activity in the region sampled by the voltammetric probe. Curve fitting used non-linear regression based on a simplex minimization algorithm (Wu et al., 2001b). When responses evoked by 60-Hz pulse trains were analyzed, K_m was fixed at 0.2 µM, the value previously determined in the striatum by *in vivo* voltammetry (Jones et al., 1995; Garriss et al., 1997) and by synaptosomal uptake of 3H -dopamine (Near et al., 1988; Horn, 1990).

Further kinetic analysis analyzed the assumption that $[DA]_p$ may vary with time (Montague et al., 2004):

$$[DA]_p = A_0 * f * d1 * d2 \quad (2)$$

where A_0 represents the initial value of DA release (original, fixed $[DA]_p$), f is short-term facilitation, $d1$ is short-term depression and $d2$ is long-term depression. The factors f , $d1$ and $d2$ are initially 1, but increase (f) or decrease ($d1, d2$) multiplicably by a kick factor with each stimulus pulse (1.01, 0.99, and 0.999, respectively). The factors f , $d1$ and $d2$ also decay back to 1 in the time between stimulus pulse as governed by a time constant (4.41, 3.23 and 840 s, respectively).

1.11 Drugs and Reagents

NSD-1015, 6-OHDA hydrochloride, and α MpT were used as received from Sigma (St. Louis, MO). RTI-76 was synthesized at Research Triangle Institute (Research Triangle Park, NC). All solutions were prepared using nanopure water (Barnstead/Thermolyne Corp., Dubuque IA).

1.12 Statistical Analysis

Where applicable data are expressed as the mean $\pm$ SEM where n is the number of animals. DA innervation level was determined from tissue DA content by taking the percent right (lesion or intact) over left (unmanipulated or control) side. Absolute values for tissue DA content are found in Table 1 for the voltammetry experiments and Table 2 for the synthesis experiments. DA synthesis was determined from tissue DOPA content and normalized to tissue DA content. Absolute values for tissue DOPA content are found in Table 2. Decay rates were determined through first-order rate kinetics as determined through Sigmaplot®. Statistical analysis included: *t*-test, one-way ANOVA, two-way ANOVA, and ANOVA with repeated measures and was performed using Microsoft Excel and SPSS. Bonferroni corrections were used where appropriate. The significance level for all comparisons was set at $p < 0.05$.

2. Results

2.1 Time-Dependent DA Release Dynamics

The present study employed a fatiguing protocol, in which pulse trains were applied more frequently in order to elicit a time-dependent DA release that decreased over time (Michael et al., 1987; Yavich, 1996; Montague et al., 2004). Voltammetric traces collected in an intact rat exemplify this time-dependent decrease in release (Fig. 1A, top). Evoked signals consist of an upward slope, which coincides with stimulus application and whose amplitude reflects DA release, and a downward slope after stimulation ceases governed by DA uptake. In sharp contrast, evoked signals collected in a unilateral partial 6-OHDA-denervated animal were sustained over the entire time course of the fatiguing protocol (Fig. 1A, bottom). The presence of a lesion, in this case a 46% innervation level determined by tissue DA content, is indicated in the pre-fatigue voltammetric trace collected at time 0 min by slower uptake and lower evoked amplitude compared to the intact signal.

Sustained DA release is confirmed by the average normalized responses shown in Figure 1B and reflected as reduced fatigue. In both the medial and lateral CP, evoked signals declined in intact animals to about 60% of control at 60 min (solid and open circles, respectively), but were sustained in lesioned animals over this same measurement period (solid and open triangles, respectively). Evoked signals were simultaneously recorded in the medial and lateral CP of each animal. Because there were no significant differences between the medial and lateral CP in either lesion or intact animals, these respective groups were pooled to increase power of the statistical analysis. A significant effect of both lesion ($F_{(12,288)}=5.4$; $p<0.001$) and time ($F_{(12,288)}=11.6$; $p<0.001$) was then demonstrated on evoked signals. As determined by tissue DA content, the average % innervation of the medial and lateral CP was 64.4 ± 15.3 and 45.2 ± 13.2 , respectively. The greater denervation in the lateral CP, the expected outcome of the unilateral partial 6-OHDA-lesion model employed (Bergstrom et al., 1999; 2003), suggests that this region encompasses the transition between the late preclinical and early symptomatic phases of PD, whereas the lesser denervation in the medial CP reflects the early preclinical phase (see Fig. 4A for individual lesion values). Although not found to be significantly different, the lateral CP did exhibit the highest maintenance of DA release. Since the average % innervation level coincided within the point of synthesis activation, sustained release is likely being supported by enhanced synthesis in these more severely denervated regions.

2.2 Synthesis Activation following Unilateral Partial 6-OHDA-Lesions

We further investigated whether the maintenance of evoked signals during the fatiguing protocol (Fig. 1) was similarly associated with enhanced synthesis in the unilateral partial lesion model employed in the present study. DA synthesis, estimated by the DOPA-

accumulation method, exponentially increases with decreased tissue DA content (Fig. 2A). Because this increase appeared to emerge at ~40% innervation, lesioned animals were subdivided into <40% and >40% groups and compared to intact animals. As shown in Figure 2B, there was a significant effect of innervation group on DA synthesis ($F_{(2,41)}=19.3$; $p<0.001$), with <40% innervation significantly elevated above both >40% innervation and intact ($p<0.001$). The latter two groups were not significantly different. For comparison, corresponding tissue DA content is shown in Figure 2C for each group. Taken together, these data indicate that DA synthesis is normal following mild lesions (~60% innervation on average), but becomes up-regulated when denervation becomes more severe (~20% innervation on average). Because data were pooled across the medial, mediolateral and lateral CP to construct Figure 2A, it was necessary to establish that DA synthesis and tissue content did not vary by striatal region in intact animals (Figs. 2D and E, respectively).

Most importantly, these results suggest that the denervation-induced maintenance of DA release in the medial CP (Fig. 1) was most likely not due to activated DA synthesis, because the 64% innervation degree is above the 40% threshold for synthesis compensation. With a lower 45% innervation level, maintenance of evoked signals in the lateral CP could be related to activated synthesis for some recordings. To test these hypotheses further, the DA synthesis inhibitor α MpT (200 mg/kg i.p.) was administered to determine the reliance of DA release on synthesis. As shown in Figures 3A and B, α MpT decreased the evoked amplitude of signals recorded in the medial and lateral CP of both intact and lesioned animals. However, consistent with a similar decline of release and hence a similar reliance on synthesis, statistical analysis found a significant effect of time ($F_{(12,264)}=72.5$; $p<0.001$) but not lesion ($F_{(12,264)}=0.90$; $p>0.54$). Although we were unable to identify a significant effect of lesion, it does appear that the lateral CP in lesioned animals declined more rapidly in the final portion of the protocol. This finding likely implicates increased dependence on synthesis in regions where lesions are more severe. Taken together, these results suggest that the observed maintenance of DA release in early preclinical states was autonomous of activated DA synthesis, whereas more severe lesions began to rely on synthesis.

2.3 Assessment of DAergic Innervation

Since releasable stores were altered in the fatiguing protocol, we examined the relationship between tissue DA content and another well established index of innervation degree, DA uptake. Baseline voltammetric responses collected before the fatiguing protocol and synthesis inhibition underwent kinetic analysis to determine $V_{\max}$ for DA uptake. $V_{\max}$ and tissue DA content exhibited a significant ($p<0.05$) positive correlation with a y-intercept near zero (Fig. 4A). The best-fit regression line also intersected the value for $V_{\max}$ determined in intact animals, further establishing a close relationship between these two indices across experimental groups. To additionally assess this relationship, relative decreases in $V_{\max}$ and tissue DA content in 6-OHDA-lesioned animals were compared. In excellent agreement with the positive correlation, the “lesion-to-intact” ratio for the two indices was consistent, whether data were subdivided into medial and lateral CP (Fig. 4B and C, respectively) or also pooled across the CP (Fig. 4D). Combined, these results suggest that $V_{\max}$ for DA uptake and tissue DA content provide a similar assessment of DAergic innervation in the present study.

2.4 Effects of RTI-76 on Time-Dependent DA Release

In considering possible origins for sustaining the time-dependent DA release observed in 6-OHDA-lesioned rats, we reasoned that as DA diffuses into denervated striatal regions, it may be more likely to be taken up by glial cells and metabolized as opposed to recycled in DAergic terminals via DAT and repackaged for re-release. In this scenario, the net loss of DA to glia drives the compensatory response. To test this hypothesis, we examined the

fatiguing stimulation protocol in intact rats treated with RTI-76, an irreversible DAT inhibitor (Fleckenstein et al., 1996). RTI-76 (100 nmol i.c.v.) caused a pronounced broadening of the evoked voltammetric signal due to the robust pharmacological blockade of DA uptake (Fig. 5A, top; compare with traces collected in intact rats and shown in Fig. 1A, top). More importantly and consistent with the denervated striatum, time-dependent DA release was sustained during the fatiguing protocol following DAT inhibition (Fig. 5A, top and 5B, closed circles).

Similar to drug-naïve intact and lesioned animals (Fig. 3), administration of α MpT in another set of RTI-76-treated animals decreased evoked signals (Fig 5A, bottom and 5B, open circles), also suggesting that DA synthesis contributes to time-dependent DA release after uptake inhibition. Statistical analysis showed a significant effect of time ($F_{(12,108)}=50.6$; $p<0.001$) and treatment ($F_{(12,108)}=34.8$; $p<0.001$). The average $V_{\max}$ determined for the recording at time 0 min in animals that received RTI-76 or RTI-76 and α MpT was 0.12 ± 0.085 and 0.08 ± 0.019 $\mu\text{M/s}$, respectively. These values, which were not significantly different ($p>0.25$), are considerably lower than the $V_{\max}$ reported in drug-naïve intact animals here (3.38 ± 0.46 $\mu\text{M/s}$) and previously (~ 3.5 $\mu\text{M/s}$; Bergstrom et al., 2003), indicative of potent uptake inhibition *in vivo* by RTI-76.

2.5 Decay Constants for Time-Dependent DA Release

Fitting to first-order kinetics reduced time courses to a single parameter (k , s^{-1}) and allowed for statistical comparison of time-dependent DA release across all treatments. This decay rate constant was significantly ($F_{(2,28)}=15.5$; $p<0.001$) greater in intact compared to lesioned and RTI-76-treated animals (Fig. 6A), confirming sustained DA release in the latter two groups. Moreover, there was no significant difference between lesioned and RTI-76-treated groups ($p>0.15$), which likewise suggests a similar level of adaptation. By comparison, all rate constants measured after α MpT administration were elevated by at least three fold, confirming a close relationship between DA release and synthesis, but no significant differences ($F_{(2,28)}=0.23$; $p>0.79$) in decay rates were found between groups (Fig. 6B). Taken together, these results suggest that time-dependent DA release has the same reliance on DA synthesis in intact, lesioned and RTI-76-treated animals and further indicate that the compensatory maintenance of DA release is autonomous of synthesis activation in the early preclinical state.

2.6 Modeling altered DA Release

The compensatory maintenance of DA release was further dissected using two models of evoked DA dynamics. Figure 7 (circles) replicates the pre-fatigue recordings in intact, lesioned and RTI-76-treated animals. Overlaid on each are two simulated curves, calculated either by the “fixed-release” model of Wightman et al. (1988; black line) or the “variable-release” model of Montague et al. (2004; red line). Both models described measured responses equally well. Each model used an identical K_m for DA uptake, an identical, but a group-specific $V_{\max}$ for DA uptake, and an identical, but group-specific initial DA release term, called the concentration of DA released per stimulus pulse or $[\text{DA}]_p$ (see figure legend for details). Observed differences in evoked DA dynamics are due to these group-specific parameters. The fixed $[\text{DA}]_p$ obtained from baseline recordings (time-independent) prior to the fatigue stimulation protocol is decreased in lesioned compared to intact recordings (24 ± 3 and 41 ± 3 nM, respectively or 60%) to a similar extent as tissue DA content (57 ± 10 and 106 ± 5 %, respectively or 54%) and $V_{\max}$ (1.78 ± 0.41 and 3.38 ± 0.46 $\mu\text{M/s}$, respectively or 53%), and is thus not compensated.

The difference between models is that $[\text{DA}]_p$ is fixed for each stimulus pulse in Wightman et al. (1988), but variable in Montague et al. (2004) according to three terms: short-term (time

constant of seconds) facilitation (f) and depression (d1), and long-term (time constant of minutes) depression (d2) (see Methods for details). Figure 8A explains why the two models so closely agree. Similar to the Wightman model, $[DA]_p$ is effectively constant for the duration of the 2-s stimulus train in the Montague model, with short-term facilitation offsetting short-term and long-term depression. Measured responses in Figure 7 can thus be considered time-independent release, because of the short train duration and because sufficient time was allowed before their collection for the Montague terms to be reset to 1 (no effect).

Such would not be the case if the train duration was increased beyond 2 s or with repetitive trains spaced more closely in time (Montague et al., 2004). As shown in Figure 8B, for example, while short-term facilitation (f) and depression (d1) quickly return to baseline (no effect), long-term depression (d2) lasts for several minutes. Hence, trains delivered at 5 min, as we do here, would elicit lower DA release that compounds over time. Indeed, Figure 9A simulates the profound effect long-term depression has on DA signals evoked in intact animals by the fatiguing protocol. Notice how well calculated time-dependent release agrees with experimental measurements (Fig. 1). Most importantly, when long-term depression is disengaged, calculated DA release is sustained across the fatiguing protocol, identifying this term as a potential target for compensation after denervation and uptake blockade. Indeed, as shown in Figure 9B, solely disengaging long-term depression captures the maintenance of DA release in these two experimental groups while preserving the distinct DA dynamics produced by the lesion (Fig. 9C) and RTI-76 (Fig. 9D) treatment (see Figs. 1 and 5, respectively).

3. Discussion

The central neuroadaptation proposed for the presymptomatic phase of PD, a compensatory maintenance of DA release, has been demonstrated in the following study by using a direct measure of DA release as indicated by decreased fatigue rates of DA release and through kinetic analysis. It thus appears that components of striatal DA release differentially respond to partial DA depletion, with time-dependent, but not time-independent, mechanisms adapting. Our data also suggest that maintaining release in the early preclinical state is autonomous of activated synthesis. This response is a consequence of denervation and is triggered by reduced recycling of DA through diminished presynaptic uptake. This novel neuroadaptation may contribute to preclinical normalization of function and may help resolve discrepant findings regarding compensatory changes in DA release during progression of the parkinsonian state.

3.1 Enhanced DA synthesis

Activation of DA synthesis in the DA-depleted striatum is unequivocal. This compensatory up-regulation has been demonstrated by tyrosine hydroxylase activity (Zigmond et al., 1984) and message (Wolf et al., 1989). Using DOPA accumulation, the present study confirms this enhancement and further establishes its denervation profile in the unilateral partial lesion. Identifying the threshold for up-regulation (<40% innervation) and assessing the effects of α MpT on DA release were critical to eliminating synthesis as a contributing factor in sustaining time-dependent DA release in mildly lesioned striata. These data therefore question, in part, the prevailing hypothesis that activated synthesis supports up-regulated release during the entire preclinical phase of PD (Zigmond et al., 1990; Zigmond, 1997), leaving it without a prescribed role only in early preclinical stages (<40% denervation). However, as seen in responses in the lateral CP where DA depletions are more severe and there is a subsequent greater activation of synthesis (Zigmond et al., 1984) the hypothesis is in fact supported and demonstrates the enhanced reliance of DA release on synthesis that has also been observed *in vitro* (Snyder et al., 1990). Had the present study focused more

specifically on severe lesions as opposed to milder lesions as we do here, we would have likely demonstrated stronger evidence for this adaptation. Up-regulated synthesis may thus play a compensatory role in the late preclinical and symptomatic stages of PD.

3.2 Adaptation in Time-Dependent DA Release

Substantive evidence supports the notion that instantaneous DA release depends upon release history. For example, both increases and decreases in DA release can be elicited by manipulating the pattern of applied stimulus trains (Michael et al., 1987; Yavich, 1996). Montague et al. (2004) characterize this “dynamic gain control” of release in terms of short-term facilitation and depression with a time constant of seconds and long-term depression with a time constant of minutes (f, d1 and d2, respectively, Fig. 8). Autoreceptors underlie short-term depression, as well as facilitation via rapid desensitization (Kita et al., 2007). While not established, long-term depression most likely involves replenishing readily releasable neurotransmitter pools from recycling and reserve pools (Richards et al., 2003; Rizzoli and Betz, 2005). In sharp contrast to these time-dependent changes in DA release, a very different result emerges when the interval between stimulus trains is sufficient to permit a return of the factors controlling release to a stable baseline. In this case, release is constant over time or time-independent (Montague et al., 2004; Kita et al., 2007).

We used repetitive, high-frequency stimulation to investigate the effects of DA depletion on time-dependent DA release. Notably, amplitudes of voltammetric signals were maintained in unilateral partially 6-OHDA-lesioned striata in response to a fatiguing protocol that significantly decreased evoked levels in intact striata. Moreover, the transition from diminished to sustained release was fully captured by the Montague et al. (2004) model by solely eliminating long-term depression. While our results confirm the tight association between synthesis and release (Yavich and MacDonald, 2000), perhaps because newly synthesized DA is preferentially released (Herdon et al., 1985), activated synthesis is not required for release to adapt in this condition. Reduced autoreceptor control is also proposed to support up-regulated release (Snyder et al., 1990; Ekesbo et al., 1999). Although short-term facilitation and depression were unaltered to simulate responses collected in lesioned striata, which agrees with studies challenging down-regulated autoreceptors in PD (Richard and Bennet, 1995; Schneider, 1997), this hypothesis was not directly tested. To do so requires manipulating stimulus trains on the order of seconds, the time constant for these effects (Kita et al., 2007), not of minutes as we do here.

While this study thus describes the first direct demonstration of a denervation-induced maintenance of DA release *in vivo*, it remains to be determined what exactly underlies this central neuroadaptation in preclinical PD. Clearly, the effective compensatory response is an increase in the effective size of the releasable DA pool. It has recently been shown that DAergic neurons do not always release neurotransmitter with each action potential and that the probability of DA release depends on the recycling pool (Daniel et al., 2009). The psychostimulant, cocaine, has also been shown to mobilize a synapsin-dependent storage pool to maintain DA release during long-duration stimulation (Venton et al., 2006). Consequently, rapidly replenishing releasable stores via more efficient recycling and mobilization of long-term storage pools are attractive hypotheses.

3.3 Controversy of Compensated DA Release

Ostensibly, the present study establishing sustained DA release in denervated striata *in vivo* contradicts previous work failing to demonstrate such an adaptation in this preparation (Garris et al., 1997; Bezard et al., 2000; Bergstrom and Garris, 2003). We argue instead that findings are consistent, but that different components of the DA release mechanism were interrogated and that these components are differentially sensitive to lesions. Our previous

investigations measured DA release in 6-OHDA-lesioned rats under time-independent conditions and found that $[DA]_p$ decreased in proportion to denervation degree (Garris et al., 1997; Bergstrom and Garris, 2003). A similar result was obtained in the present study when similar recordings were analyzed identically by the fixed-release model of Wightman et al. (1988). In these same animals, however, voltammetric traces collected under time-dependent conditions revealed sustained DA release. Moreover, the variable-release model of Montague et al. (2004) predicts both adapted and non-adapted DA release.

The present study may also shed light on another discrepancy. Although time-independent release conditions have been utilized both *in vivo* and *in vitro*, compensated DA release has only been demonstrated in the later preparation (Stachowiak et al., 1987; Snyder et al., 1990; McCallum et al., 2006; Perez et al., 2008). One possible explanation is that different components of the DA release mechanism were interrogated by the different stimulation procedures employed. For example, a novel autoreceptor-independent and short-term depression of DA release has been reported *in vitro* utilizing paired-pulse designs that rely on intense local stimulation (Phillips et al., 2002; Cragg, 2003). It thus could be that direct stimulation of the dopaminergic terminal triggers a more fast-acting component *in vitro* but otherwise similar to the long-term depression governing time-dependent DA release *in vivo*. This notion may also serve to explain why up-regulated DA release has been observed *in vitro* when stimulation was alternatively provided by depolarizing K^+ or nicotine (McCallum et al., 2006; Perez et al., 2008).

3.4 Trigger for Adaptation of Time-Dependent DA Release

Pharmacological blockade of DAT by RTI-76 converted intact striata from presenting non-compensated to compensated time-dependent DA release. We thus propose reduced recycling of DA through diminished presynaptic uptake as the trigger for the denervation-induced maintenance of release. Indeed, it is well established that the loss of DAergic neurons in parkinsonian models leads to a concomitant reduction in DA uptake sites (Bezard et al., 2000) and rates measured either by 3H -DA *in vitro* (Zigmond et al., 1984; Altar et al., 1987) or voltammetry *in vivo* (Garris et al., 1997; Rothblat and Schneider, 1999; Bezard et al., 2000; Bergstrom and Garris, 2003). Moreover, both pharmacologically blocking DAT (Doucet et al., 1986; van Horne et al., 1992) and DAergic lesions (van Horne et al., 1992; Schneider et al., 1994) increase DA diffusion distances in the striatum. By diffusing farther and into more denervated regions, we submit that DA is less likely to be recovered and recycled for re-release by a DAergic terminal and more likely taken-up by glial cells, perhaps via the extraneuronal monoamine transporter (Russ et al., 1996; Grundemann et al., 1999), and metabolized. To compensate for reduced DA recycling, surviving DAergic terminals could increase the effective size of the readily releasable pool.

We have previously proposed that this same reduction of uptake and failure to recover released DA in the denervated striatum leads to activated DA synthesis (Bergstrom and Garris, 2003). Interestingly, DAT knockout mice display profound changes in presynaptic DA function, including increased DA synthesis, and decreased time-independent DA release and tissue DA content (Jones et al., 1998). We did not assess these indices in the RTI-76 experiment, but a future study could determine whether dose-dependent inhibition of DAT by RTI-76 elicits corresponding changes in DA synthesis and release that we observe here in 6-OHDA-lesioned rats. Because our previous work showed no significant reductions in time-independent DA release after pretreatment with RTI-76 (Wu et al., 2002a), it may also be difficult to directly compare between the relatively acute effects of pharmacological DAT inhibition (1-2 days) versus potential development effects in DAT knockout animals. Thus, investigating the relationships between DA release and synthesis in an inducible transgenic model may prove insightful as well. However, synthesis is clearly less sensitive to decreased uptake than release. Indeed, we observed fully compensated release with an average

innervation level of 65%, but compensatory synthesis only emerged at 40% innervation and exponentially increased with lower levels. Moreover, the threshold of synthesis activation that contributes to up-regulated DA release appears to require even greater levels of denervation (Snyder et al., 1990). Extraneuronal DA uptake may dominate under these conditions, and the combination of these severe DA depletions with compensated release may further activate synthesis to replenish the releasable DA pool.

3.5 Functional Significance of altered Time-Dependent DA Release

Extracellular DAergic signaling exists along a temporal spectrum, with steady-state tonic levels at one end and transient phasic events at the other (Schultz, 2007). Basal DA is maintained by presynaptic glutamate drive and low-frequency firing of DAergic neurons, whereas phasic release is elicited by high-frequency bursts in response to rewards and cues predicting these rewards (Grace, 2000; Carelli and Wightman, 2004). The present findings may have considerable implications for both signaling modes. It is well established that DAergic tone is normalized across the denervation range encompassing the preclinical phase of PD (Abercrombie et al., 1990; Zigmond et al., 1990; Robinson et al., 1994; Zigmond, 1997). Evoked tonic-like responses measured voltammetrically are similarly maintained without up-regulated DA release (Garris et al., 1997; Bergstrom and Garris, 2003). In contrast, DA responses exhibiting phasic-like dynamics decrease in proportion to denervation degree (Garris et al., 1997; Bezard et al., 2000; Bergstrom and Garris, 2003). An intriguing possibility is that the time-independent component of DA release is sufficient for normalizing DAergic tone without compensation (i.e., no change in the fixed $[DA]_p$ or long-term depression of the model), but that maintenance of time-dependent DA release is required to offset denervation-diminished phasic events (i.e., no change in the fixed $[DA]_p$ but a reduction in the long-term depression of the model). Sustaining this high-frequency, burst-evoked signaling mode by increasing the effective size of the readily releasable pool may be especially important to navigate complex environments that elicit multiple and repeated phasic patterns.

4. Conclusion

We establish a novel compensatory maintenance of DA release suggestive of a functional reorganization of the presynaptic DAergic terminal in the early parkinsonian state. Taken together, our results have broad implications for the neuroadaptation, progression, and treatment of PD.

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Highlights

1. First in vivo report of central compensatory mechanism in presymptomatic parkinsonism
2. Sustained dopamine release in early preclinical stage is independent of synthesis
3. Sustained dopamine release is mimicked by irreversible uptake inhibition
4. Sustained release is modeled by eliminating long-term presynaptic depression
5. First comparison of major kinetic models for dopamine release in parkinsonism

Figure 1. Representative and normalized responses of time-dependent DA release in lesioned and intact animals

The concentration of DA is measured every 100 ms and corresponds to each circle. Stimulus trains were applied every 5 min. **Panel A:** Time-dependent responses in intact (Top) and lesioned (Bottom) animals. Only recordings taken at baseline (time 0) 20-min intervals are shown. The lesioned animal possessed a 46 % innervation level. **Panel B:** Averaged normalized responses in the intact (n=6) and lesioned (n=7) animals. Control levels were averaged across stable baseline recordings prior to repetitive stimulations. The closed circle ● represents the intact medial CP. The open circle ○ represents intact lateral CP. The closed triangle ▲ represents lesioned medial CP, whereas open triangles represent lesioned lateral CP. Average innervation levels for lesioned animals in the medial CP was 64.4 ± 15.3 % and 45.2 ± 13.2 % in the lateral CP.

Figure 2. Synthesis activation after 6-OHDA-lesions

Panel A: DA synthesis via DOPA-accumulation method. L-DOPA/DA ratio was determined in the CP in intact (n=9) and lesioned (n=11) animals. The 33 determinations expressed in the figure for the lesioned animals utilized 3 striatal regions dissected. The open circle $\circ$ represents the average ratio observed in intact animals. The closed circles $\bullet$ represents individual ratios from lesions. Exponential fit utilized the following equation: $f = a \cdot \exp(-b \cdot x) + 0.16$, where 0.16 represents the average ratio in the > 40% group and the intact group. This analysis indicated significance ($r = 0.8$; $p < 0.01$). **Panel B:** Ratios were also subdivided into intact, < 40% innervation, and > 40% DAergic innervation. ANOVA analysis determined significant differences between < 40% innervation group and intact, > 40% innervation ($p < 0.01$). **Panel C:** Tissue DA content for each respective group as % control. **Panel D:** DA synthesis for medial (M), mediolateral (ML), and lateral (L) striata. **Panel E:** Tissue DA content as % control for the same regions listed in panel D.

Figure 3. Representative and normalized responses of time-dependent DA release in lesioned and intact animals after synthesis inhibition via α MPT

The concentration of DA is measured every 100 ms and corresponds to each circle. Stimulus trains were applied every 5 min. **Panel A:** Time-dependent responses in intact (Top) and lesioned (Bottom) animals. Only recordings taken at baseline (time 0) 20-min intervals are shown. The lesioned animal possessed a 46 % innervation level. **Panel B:** Averaged normalized responses in the intact (n=6) and lesioned (n=7) animals after α MPT. Control levels were averaged across stable baseline recordings prior to repetitive stimulations. The closed circle ● represents the intact medial CP. The open circle ○ represents intact lateral CP. The closed triangle ▲ represents lesioned medial CP whereas open triangles represent lesioned lateral CP. Average innervation levels for lesioned animals in the medial CP was 64.4 ± 15.3 % and 45.2 ± 13.2 % in the lateral CP.

Figure 4. Relationship between DA uptake and tissue DA content

Panel A: The open circle $\circ$ represents the average $V_{\max}$ and innervation level observed in intact animals ($n=6$). The closed circles $\bullet$ represents individual $V_{\max}$ and innervation values in lesioned animals ($n=7$). The two regions sampled in the CP account for the 14 determinations in lesions. Regression analysis showed a significant correlation ($p < 0.05$).

Panel B: Tissue content and $V_{\max}$ are plotted for the medial CP. **Panel C:** Tissue content and $V_{\max}$ are plotted for the lateral CP. **Panel D:** Tissue content and $V_{\max}$ are plotted for both medial and lateral CP. The numbers listed above each box represent the ratio of lesion to intact average value. The open squares $\square$ denote lesioned animals and the closed squares $\blacksquare$ represent intact animals.

Figure 5. Effects of pre-treatment with the non-competitive irreversible inhibitor RTI-76 on time-dependent DA release

Panel A: Representative responses in an animal pre-treated with RTI-76 alone ($-\alpha$ MPT; Top) and RTI-76 + α MPT (Bottom). The concentration of DA is measured every 100 ms and corresponds to each circle. **Panel B:** Averaged evoked DA signals plotted as percent control in RTI-76 animals (n=5) and RTI-76 + α MPT (n=6) animals. Control levels were averaged across as stable baseline recordings prior to repetitive stimulations.

Figure 6. First-order rate constant describing time-dependent DA release

Panel A: Decay rates observed prior to α MPT administration ($-\alpha$ MPT) in intact (n=6), lesioned (n=7), and RTI-76 (n=6) animals. Lesion and RTI-76 treated animals were significantly lower than intact animals ($p < 0.001$; *) yet lesion and RTI-76 were not significantly different from each other ($p > 0.15$). **Panel B:** Decay rates after α MPT administration ($+\alpha$ MPT) showing no significant differences between groups ($p > 0.79$).

Figure 7. Comparing fixed and variable release models

Voltammetric traces were taken from the time 0 min condition in Figure 1 for the intact group (Top left), Figure 4 for the lesioned animal (Top right; innervation level was 46%), and Figure 5 for RTI-76-treated animal (bottom). K_m was set at 0.2 μM for all simulations. $[\text{DA}]_p$ and V_{max} varied with group, but were treated identically in each model: intact - $[\text{DA}]_p = 0.054 \mu\text{M}$ and $V_{\text{max}} = 2.84 \mu\text{M/s}$; lesion - $[\text{DA}]_p = 0.025 \mu\text{M}$ and $V_{\text{max}} = 0.7 \mu\text{M/s}$; RTI-76 - $[\text{DA}]_p = 0.014 \mu\text{M}$ and $V_{\text{max}} = 0.065 \mu\text{M/s}$. The closed circles $\bullet$ represent pre-fatigue recordings in intact, lesioned, and RTI-76 animals. The black line represents the simulations calculated by the “fixed release” model whereas the red line indicates the calculation determined by the “variable release” model.

Figure 8. Variable release model simulation

Panel A: Simulation of variable release model with terms changing over time (60 Hz, 2 s, not repeated). **Panel B:** Same as panel A with expanded time scale. Variable release terms: f = short-term facilitation; d1 = short-term depression; d2 = long-term depression.

Figure 9. Effects of repetitive stimulation in the intact, lesioned, and RTI-76 treated striatum
Panel A: Simulation of repetitive stimulation in the intact striatum fit with long-term depression (d2). **Panel B:** Simulation of repetitive stimulation in the intact striatum fit without long-term depression. **Panel C:** Simulation of repetitive stimulation in the lesioned striatum without long-term depression. **Panel D:** Simulation of repetitive stimulation in RTI-76 treated animals without long-term depression.

Table 1
Tissue Dopamine Contents for Voltammetry Experiments

Dopamine (ng/mg protein)				
Animal	Striatal Region			
	Left Side		Right Side	
	M	L	M	L
Intact	182.6±41.1	166.9±43.1	192.9±43.1	162.8±29.2
Lesion	116.9±8.5	112.3±11.6	75.4±20.9	50.8±17.0

Where applicable, 6-OHDA was injected in the right side of the brain. All data are mean±SEM. Abbreviations: M, medial; L, lateral. Two-way ANOVA results for dopamine content: significant effect of treatment ($F_{(1,36)} = 19.62$, $P < 0.001$) but no significant effects of region ($F_{(3,36)} = 0.868$, $P = 0.466$) or interaction ($F_{(3,36)} = 0.652$, $P = 0.586$).

Table 2
Tissue Dopamine and DOPA Contents for Synthesis Experiments

Dopamine (ng/mg protein)						
Animal	Striatal Region					
	Left Side			Right Side		
	M	ML	L	M	ML	L
Intact	122.7±13.1	126.0±8.5	124.1±10.9	130.1±12.5	121.6±9.7	120.3±14.0
Lesion	135.8±17.4	163.3±21.3	134.2±17.0	103.0±15.2	84.6±16.6	36.3±8.5
DOPA (ng/mg protein)						
Animal	Striatal Region					
	Left Side			Right Side		
	M	ML	L	M	ML	L
Intact	20.0±3.2	21.9±1.1	19.4±1.2	21.3±1.8	21.9±1.6	19.8±2.4
Lesion	16.8±2.3	19.6±2.4	16.2±2.1	15.0±1.7	13.1±1.8	7.1±1.3

Where applicable, 6-OHDA was injected in the right side of the brain. All data are mean±SEM. Abbreviations: M, medial; ML, mediolateral; L, lateral. Two-way ANOVA results for dopamine content: no significant effect of treatment ($F(1,78) = 1.555, P = 0.216$) or interaction ($F(5,78) = 2.325, P = 0.051$) but a significant effect of region ($F(5,78) = 2.677, P = 0.028$). Two-way ANOVA results for DOPA content: significant effect of treatment ($F(1,78) = 17.595, P = 0.000$) but no significant effects of region ($F(5,78) = 1.783, P = 0.126$) or interaction ($F(5,78) = 1.308, P = 0.269$).