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An inverse correlation between apparent rate of dopamine clearance and tonic autoinhibition in subdomains of the rat striatum: a possible role of transporter-mediated dopamine efflux

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Abstract

The dopaminergic terminal field in the rat striatum is compartmentalized into subdomains that exhibit distinct dynamics of electrically evoked dopamine release. The fast striatal domains, where dopamine release is predominantly vesicular, exhibit conventional dopaminergic activity. However, vesicular dopamine release is tonically autoinhibited in the slow domains, which suggests that dopamine reaches the autoreceptors via a non-vesicular route. Hence, it appears that the domains use distinct mechanisms to regulate the basal dopamine concentration available to activate, or not, presynaptic autoinhibitory receptors. However, direct detection of local variations in tonic extracellular dopamine concentrations is not yet possible. So, the present study employed voltammetry to test the hypothesis that the apparent rate of dopamine clearance from the extracellular space should be domain-dependent. The apparent rate of dopamine clearance is equal to the difference in the rates of dopamine release and uptake that determine extracellular dopamine concentrations. This study confirms that the apparent rate of dopamine clearance is slower in the slow striatal domains where vesicular dopamine release is tonically autoinhibited. These findings support the view that the basal concentration in slow domains is maintained by a non-vesicular release process, possibly transporter-mediated efflux.

Keywords

dopamine transporter; dopamine efflux; basal dopamine; D2 receptor; autoinhibition; in vivo voltammetry

The dopamine (DA) transporter (DAT), a chloride-dependent member of the SLC6 family of neurotransmitter:sodium symporters, intimately contributes to the regulation of extracellular DA concentrations in the brain (Iversen 1971; Amara and Kuhar 1993). Understanding DAT function is significant because it is targeted by a variety of antidepressant and psychoactive drugs (Torres *et al.* 2003; Mandt and Zahniser 2010). Prior studies have established that DAT function is dynamically regulated by protein trafficking, interactions with the DA D2 receptor (D2R), protein-lipid interactions, membrane voltage, and ligand and substrate binding (Ingram *et al.* 2002). DAT's primary function appears to be reuptake of DA from the extracellular space, but it also releases DA in the presence of exogenous DA-releasers, such as amphetamine (Sulzer *et al.* 1993). The present study, however, contributes to an

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emerging body of evidence suggesting that the DAT may also release DA in the absence of amphetamine-like drugs.

In vitro studies have demonstrated that striatal terminals release DA via DAT-mediated DA efflux (DDE) (Cheramy *et al.* 1986; Lonart and Zigmond 1991; Leviel 2001). In vivo studies likewise suggest that DDE is functionally relevant within certain specific subdomains of the rat striatum (Moquin and Michael 2009; Wang *et al.* 2010). Voltammetric recordings show that the striatum is compartmentalized into spatial domains that yield distinct fast-type and slow-type responses during electrical stimulation of midbrain DA axons. An autoinhibitory tone on DA terminals contributes to determining whether a given recording site corresponds to a fast or slow domain. The autoinhibitory tone is minimal in fast domains (Garris *et al.* 1993; Benoit-Marand *et al.* 2001) but intense in slow domains (Moquin and Michael 2009; Wang *et al.* 2010), which suggests that the domains operate under different basal DA concentrations. Since electrically evoked DA release, a vesicular event, is autoinhibited in the slow domains, it appears that the DA responsible for the autoinhibition reaches the extracellular space via a non-vesicular route. This concept is supported by the finding that the striatum contains a tonic pool of extracellular DA that is both tetrodotoxin-insensitive and nomifensine-sensitive (Borland and Michael 2004), which are classic hall-marks of DDE.

This emerging evidence that DDE has a functional role in the striatum is at odds with a considerable body of literature. According to microdialysis, for example, DA release is exclusively vesicular (Westerink *et al.* 1987; Santiago and Westerink 1990) except in the presence of DA releasers (Carboni *et al.* 1989; Schmitz *et al.* 2001). However, microdialysis is an averaging technique (Bungay *et al.* 2007) that does not detect spatially localized concentration differences. At present, no technology has the demonstrated ability to directly detect localized variations of basal extracellular DA concentrations. For this reason, the present study was designed to examine this issue via an alternate route.

We tested the hypothesis that the fast and slow striatal domains should exhibit distinct values of the apparent rate of DA clearance, V_{app} , a quantity that depends on the rates of DA uptake and release that determine the extracellular DA concentration (Chen 2005; Michael *et al.* 2005; Dreyer *et al.* 2010). We used voltammetry to quantify V_{app} in vivo after electrically evoking the release of endogenous DA (Wu *et al.* 2001) and after ejecting exogenous DA from a pipet (Cass *et al.* 1993; Kiyatkin *et al.* 2000; Sabeti *et al.* 2002). Our findings confirm that the rat striatum exhibits the hypothesized regional variation in V_{app} and, as expected, that V_{app} is significantly slower in those striatal domains where extracellular dopamine concentrations appear to be sufficiently high to tonically autoinhibit vesicular DA release.

Materials and Methods

Carbon fiber microelectrodes were constructed by inserting 5- μ m diameter carbon fibers (T650, Cytac Carbon Fibers LLC., Piedmont, SC) into single barrel borosilicate glass capillaries (0.4 mm ID, 0.6 mm OD, A-M systems Inc., Sequim, WA), pulling the capillaries to a fine tip with a vertical micropipette puller (Narishige, Los Angeles, CA), backfilling the tubes with a low-viscosity epoxy (Spurr Epoxy, Polysciences Inc., Warrington, PA), and connecting the fiber to a hook-up wire with a droplet of mercury. The exposed fibers were cut to 400 μ m and sonicated in reagent-grade isopropyl alcohol (Sigma, St. Louis, MO) containing activated carbon (Fisher Scientific, Fair Lawn, NJ) for 30 minutes (Bath *et al.* 2000).

Voltammetric recording was performed by fast scan cyclic voltammetry using an EI 400 high-speed potentiostat (originally constructed by Ensman Instruments but presently available from ESA Inc., Chelmsford, MA) and the “CV Tar Heels v4.3” software package (courtesy of Dr. Michael Heien, present address Department of Chemistry, University of Arizona, Tucson AZ, USA). The rest potential was 0 V vs Ag/AgCl and the voltammetric waveform consisted of three linear potential sweeps to +1 V, -0.5 V, and back to 0 V at a sweep rate of 400 V/s. The scans were repeated at 10 Hz. The DA oxidation current was recorded between 0.5 and 0.7 V during the first sweep. DA voltammograms were obtained by background subtraction.

Male Sprague-Dawley rats (250–350g) (Hilltop, Scottsdale, PA) anesthetized with isoflurane (2% by vol.) and placed in a stereotax (David Kopf Instruments, Tujunga, CA) with the incisor bar raised 5 mm above the interaural line (Pellegrino *et al.* 1979). A heating blanket (Harvard Apparatus, Holliston, MA) maintained body temperature at 37°C. A twisted, bipolar, stainless steel stimulating electrode was placed over the medial forebrain bundle (from bregma: 1.6 mm lateral, 2.2 mm posterior, 8.0 mm below dura) (Pellegrino *et al.* 1979). A carbon fiber microelectrode was placed in the ipsilateral striatum (from bregma: 2.5 mm lateral, 2.5 mm anterior, initially 4.5 mm below dura) (Pellegrino *et al.* 1979). The final position of the stimulating electrode was determined by lowering it until evoked DA release was observed in the striatum: this is a well-established protocol for identifying effective midbrain stimulus sites (Ewing *et al.* 1983; Kuhr *et al.* 1984; Heien *et al.* 2005). As appropriate, the striatal recording location was optimized to permit detection of hybrid evoked responses (explained further in the Results section): the optimization procedure involved lowering the carbon fiber electrode deeper into the striatum until a hybrid stimulus response was observed.

Electrical stimulation was delivered as an optically isolated, constant-current, biphasic waveform (frequency 60 Hz, pulse height 270 μ A, pulse width 2 ms). The stimulus duration was 200 ms (12 pulses) during experiments involving fast-type responses, 3 s during experiments involving slow-type responses, and 3–5 s during experiments involving hybrid responses.

Pressure-ejection of exogenous substances (raclopride and dopamine) into the extracellular space of the striatum was by means of a double-barreled micropipet: one barrel contained a carbon fiber microelectrode and the other was used for pressure-ejection (Fig 1). The open barrel was pre-loaded with either 25 mM DA or 2 mM raclopride dissolved in artificial cerebral spinal fluid (1.2mM Ca^{2+} , 152mM Cl^- , 2.7mM K^+ , 1.0mM Mg^{2+} , 145mM Na^+ , pH 7.4). The solution was pressure ejected (~20 psi) with a Picospritzer III (Parker Hannifin, Fairfield, NY). In the case of experiments involving exogenous DA, the ejection time was adjusted between 0.1 and 0.5 s so that the amplitude of the voltammetric response to exogenous DA matched the amplitude of the electrically evoked response at the same recording location (see Results section for further details). In the case of experiments involving the pressure-ejection of raclopride, the ejection lasted for 2 s and occurred 5 min prior to the next electrical stimulus.

Microelectrode DA calibration was performed after the microelectrodes were removed from brain tissues and mounted into a gravity-fed flow-through cell. Conversion of in vivo oxidation currents to DA concentrations was based on post-calibration results. Standard solutions were prepared by dissolving DA in artificial cerebrospinal fluid.

Drugs were dissolved in phosphate buffered saline (155mM Na^+ , 155mM Cl^- , 100mM phosphate, pH 7.4). Nomifensine maleate (Sigma Aldrich, St. Louis, MO) was administered

at a dose of 20 mg/kg i.p. Raclopride tartrate (Sigma Aldrich, St. Louis, MO) was administered at a dose of 2 mg/kg i.p.

DA clearance profiles were constructed as follows: The time of the first voltammetric measurement after the termination of an electrical stimulus or pressure ejection was defined as $t = 0$ (x-axis). The time required for the completion of DA clearance after electrical stimulation or pressure ejection was identified by using an Excel spreadsheet to determine the linear regression slope of sequential 5-point line segments on the clearance profile. The midpoint of the first line segment with a slope below $-0.1 \mu\text{M/s}$ was taken as a measure of a) the extracellular lifetime of DA and b) the zero-point of the change in DA concentration (y-axis): this procedure eliminated contributions from occasional small baseline shifts that occurred between the onset of the stimulus and the completion of DA clearance.

The DA clearance profiles for each group were averaged together ($n=4$) and compared using two-way ANOVA (1st factor – group; 2nd factor – time point) and a Tukey post hoc test. Extracellular DA lifetimes were averaged together ($n=4$ per group) and compared using one-way ANOVA and a Tukey post hoc test. Throughout this study, the intensity of the stimulus was sufficient to cause the initial segment of the clearance profile to be linear ($r^2 > 0.96$): the initial linear clearance rates were averaged ($n=4$ per group) and compared using one-way ANOVA and a Tukey post hoc test. In all figures, the error bars indicate s.e.m.

Results

Voltammetric identification of fast and slow DA domains

Voltammetric recordings of electrically evoked DA release in the rat striatum yield distinct responses, classified as fast-type and slow-type according to previously explained criteria (Moquin and Michael 2009). The slow-type responses are characterized by a delay in the onset of evoked release when the stimulus begins and short-term facilitation of release with continued stimulation (Fig 2a). Systemic administration of raclopride, a selective D2-antagonist, abolishes the delay in the onset of evoked release (Fig 2a), confirming that the slow-type response is a consequence of an autoinhibitory tone on DA terminals. This tone is established prior to the onset of the stimulus, indicating that it derives from non-evoked DA release.

The position of the voltammetric electrode can be optimized to locate recording sites that yield hybrid responses (Fig 2b) with a fast-type initial component and a subsequent slow-type component. The fast-type responses are characterized by evoked release that starts without delay and then exhibits short-term depression, which sometimes produces a ‘dip’ between the fast and slow components of the hybrid response (Fig 2b). The short-term depression is attributable to the rapid onset of autoinhibition caused by the evoked DA release (Dugast *et al.* 1997; Benoit-Marand *et al.* 2001; Phillips *et al.* 2002). The initial component sometimes appears as a leading shoulder on the response (Fig 2c), rather than a stand-alone peak (Fig 2b), because different hybrid sites have different fast and slow contributions. Pressure-ejection of raclopride from a pipet adjacent to the microelectrode (Fig 1) had no effect on the amplitude of the initial fast component but significantly increased the amplitude of the subsequent slow component (Fig 2c and 2d: see figure legend for ANOVA results). Because the fast component was refractory to raclopride, it appears to derive from DA terminals that are not autoinhibited prior to the stimulus. This finding is consistent with previous reports of fast evoked signals refractory to D2R antagonists (Garris *et al.* 1994). These results, which show that autoinhibitory D2Rs are present and functional in both fast and slow domains, point to the tonic DA concentration present in the extracellular space prior to the stimulus as a principle determinant of the autoinhibitory tone.

Apparent DA clearance rates in fast and slow striatal domains

During the remainder of this study, fast-type responses were obtained by optimizing the electrode position to find hybrid sites and then shortening the stimulus to 200 ms (12 pulses). The difference between V_{app} within fast and slow domains of the striatum of an individual rat is readily apparent even from the illustrative responses in Fig 3a. DA was cleared from the fast domain within 0.5 s, whereas clearance from the slow domain required more than 2 s. To obtain Fig 3a, the duration of the stimuli was adjusted to obtain similar response amplitudes at the two recording sites: the time needed to clear the same DA concentration from each site is obviously different. The remainder of this study is devoted to a quantitative evaluation of the DA clearance profiles in fast and slow striatal domains.

The clearance profiles obtained in fast and slow domains are significantly different (Fig 3b, see figure legends for ANOVA results). The clearance profiles in slow domains persist for a longer duration and exhibit a slower initial slope. Fig 3c reproduces the data of Fig 3b but, for clarity, omits the error bars and includes the linear regression lines that best fit the initial linear segment of each clearance profile. In generating Fig 3c, responses were only included in the data set if at least three data points on the descending phase of the response produced a straight line with $r^2 > 0.96$. All slow-type clearance profiles met this criterion, whereas only 4 of the 7 fast-type profiles did so. At the other 3 fast sites, DA returned to baseline within 200 ms and was thus too fast to quantify by our method. It is likely that omitting these 'very fast' profiles leads to an underestimate of the apparent clearance rate in fast domains. Nevertheless, the initial clearance slope (Fig 3d) in fast and slow domains, $4.8 \pm 1.2 \mu\text{M/s}$ and $1.4 \pm 0.46 \mu\text{M/s}$, respectively, are significantly different (see the figure legend for ANOVA details).

We compared DA clearance profiles obtained from evoked responses in slow domains with those obtained after pressure ejection in the same recording location. We adjusted the duration of the pressure ejection so that the initial amplitude of the profiles obtained after evoked release and pressure ejection were similar. The clearance profiles after evoked release and pressure ejection are essentially identical (Fig 3e). The evoked responses in slow domains are sensitive to the DAT-inhibitor, nomifensine, which confirms the DAT is active within slow domains (Fig 3e). The extracellular DA lifetime (Fig 3f) is shortest after fast-type evoked release (0.8 s). The lifetime is longer after slow-type evoked release (2.1 s), similar to that observed after pressure-ejection (2.3 s), and further extended after nomifensine administration (3.7 s).

Effect of evoked DA concentration on clearance

The clearance profiles of Fig 3c are re-plotted in Fig 4a normalized with respect to the initial DA amplitude. The normalized initial clearance slopes in the two domains are significantly different (Fig 4b), which further confirms that the difference in slope is not dependent on the initial DA amplitude. The regression lines that give the best fit to the initial linear segments of the clearance profiles from 8 individual experiments, 4 from fast domains and 4 from slow domains (Fig 4c), allow examination of the time at which the regression lines intersect the x-axis (i.e. the time at which they reach $\Delta[\text{DA}] = 0 \mu\text{M}$): in fast domains the x-intercepts are all less than 0.5 s, while in slow domains they are all more than 1.2 s. The x-intercept times are significantly shorter in the fast domains compared to slow domains (Fig 4d: see the figure legend for ANOVA details). Fig 4 confirms that apparent DA clearance is domain-dependent.

Discussion

The present study extends our on-going efforts to characterize the fast and slow domains of the striatal DA terminal field. It is well-known that the striatum is heterogeneous with respect to DA (May and Wightman 1989; Kawagoe *et al.* 1992; Garriss and Wightman 1995; Wightman *et al.* 2007). However, the compartmentalization of DA terminals into distinctive domains is a relatively recent concept (Moquin and Michael 2009; Wang *et al.* 2010). Our prior studies demonstrated that the domains are distinguished by the autoinhibition-dependent dynamics of evoked DA release. The autoinhibition of evoked DA release (Fig 2), an accepted index of vesicular release, suggests that DA reaches the extracellular space of slow domains via a non-vesicular mechanism. So, the domains apparently exhibit different basal DA concentrations as a consequence of different rates and mechanisms of DA release. Much as we would like to, thus far we have not been able to examine this implication by directly measuring basal DA concentrations in the two domains. So, we have examined instead the related prediction that the domains should exhibit different apparent rates of DA clearance.

Different basal DA concentrations in the two domains

The raclopride-sensitivity of the initial delay in the onset of evoked DA release (Fig 2a) indicates that DA terminals within slow domains are tonically autoinhibited, while the raclopride-insensitivity of the fast-type evoked release (Fig 2c and 2d) indicates that the terminals are not tonically autoinhibited. However, the fast domains exhibit autoreceptor function in the form of short-term depression of evoked release (Fig 2b) and sensitivity to quinpirole, a D2R agonist (Wang *et al.* 2010). Since autoreceptors are functional in both domains, it appears that the domain difference in autoinhibitory tone derives from a difference in the basal DA concentration available to activate, or not, the D2Rs.

Prospects for direct detection of basal DA in striatal domains

Voltammetry measures extracellular DA with sufficient spatial and temporal resolution (May and Wightman 1989; Kawagoe *et al.* 1992; Garriss *et al.* 1994; Peters and Michael 2000; Greco and Garriss 2003) to, for instance, expose the fast and slow striatal domains. But, it is poorly suited to determining steady-state DA concentrations (e.g. Heien *et al.* 2005). Although this limitation might eventually be resolved, voltammetry in its present form does not have the demonstrated ability to measure basal DA concentrations. Microdialysis is widely adopted for basal measurements but does not have the necessary spatial resolution to selectively sample DA from one domain or the other. So, the present study takes advantage of voltammetry's ability to quantify DA clearance, which reflects the balance in the rates of DA uptake and release that is ultimately responsible for the extracellular DA concentration.

The two striatal domains exhibit significant differences in both apparent clearance rate, deduced from the slope of the initial linear segments of the clearance profiles, and the extracellular DA lifetime (Fig 3 and 4). The DAT is saturable, so a difference in the rate of clearance could arise if the DAT were saturated during some measurements but not others. All the clearance profiles, however, exhibited an initial linear segment, which shows that clearance was zero-order in all cases, so the DAT was saturated in all cases. Differences in clearance time could be due to differences in the initial amplitude, i.e. it might take longer to clear DA after large-amplitude evoked responses simply because there is more of it to clear. This does not seem to be the main source of differences in lifetime: the lifetimes were clearly distinct in the two domains (Fig 3a), even though there was considerable overlap in the initial amplitude (Fig 4c and 4d). Differences in the apparent rate of DA clearance could also arise from D2R activation (Cass and Gerhardt 1994; Dickinson *et al.* 1999; Wu *et al.*

2002). However, these studies demonstrate that D2R activation accelerates the apparent rate of DA clearance, which is not what we observed: we found slower clearance in the presence of tonic autoinhibition. Although DA clearance is known to be heterogeneous in the striatum (Garris *et al.* 2003), this is the first study to show significant differences in DA clearance between sites associated with fast-type and slow-type evoked responses.

Defining the apparent rate of DA clearance

The apparent rate of DA clearance is measured with voltammetry as the extracellular DA concentration returns to its basal level following a transient increase induced either by electrically evoking the release of endogenous DA (Wu *et al.* 2001) or by ejecting exogenous DA into the extracellular space via a pipet (Cass *et al.* 1993; Sabeti *et al.* 2002). The apparent rate of clearance is the net rate at which DA is delivered to, and removed from, the recording electrode (Chen 2005; Michael *et al.* 2005). Since clearance occurs after the electrical stimulus or pressure-ejection, the delivery process of interest is the spontaneous (i.e. non-evoked) release of endogenous DA. DA removal occurs by uptake and diffusion (Wightman *et al.* 1988; Venton *et al.* 2002; John and Jones 2007). However, the linearity of the initial segment of the clearance profiles (Fig 3c and 4a) indicates zero-order rate processes. So we can conclude that diffusion, which is not a zero-order rate process (Hrabetova and Nicholson 2007), does not contribute substantially to the initial linear stage of clearance.

Thus, during the initial linear stage of clearance, the apparent rate of DA clearance, V_{app}^0 , is the difference between the rate of DA uptake, V_u^0 , and the rate of DA release, V_r^0 (Michael *et al.* 2005):

$$V_{app}^0 = V_u^0 - V_r^0 \quad (\text{Equation 1})$$

where the superscripts “0” indicate zero-order rates. In some cases, V_u^0 might correspond to the V_{max} of uptake from the Michaelis-Menton model (Wu *et al.* 2001; Montague *et al.* 2004; Kita *et al.* 2007), i.e. the maximal possible rate of uptake when the extracellular concentration of DA is sufficient to saturate the DAT and all of the DAT protein is available to participate in uptake. But, if DDE occurs, then some of the DAT protein would not be available for uptake and the zero-order uptake rate would be less than V_{max} . To leave open this possibility, we have used V_u^0 rather than V_{max} to denote the intrinsic zero-order uptake rate in Equation 1.

Equation 2 explains how basal DA concentrations depend upon the rates of release and uptake (Chen 2005; Michael *et al.* 2005):

$$C^* = \frac{V_r^0 K_m}{V_u^0 - V_r^0} \quad (\text{Equation 2})$$

where C^* represents the basal DA concentration, K_m is the DAT's Michaelis constant, and the other terms were defined earlier. As explained above, we have replaced V_{max} with V_u^0 to leave open the possibility that some DAT proteins are not available for uptake. Since the denominator of Equation 2 is $V_u^0 - V_r^0$, it predicts an inverse relationship between V_{app}^0 and C^* .

The inverse relationship between C^* and V_{app}^0

The absence of tonic autoinhibition in the fast domains might be due to a low basal DA concentration. According to Equation 2, a low C^* would derive from slow release (the numerator) and/or fast apparent clearance (the denominator). When release is slow, the apparent clearance rate should approach the maximal intrinsic rate, V_{max} , i.e. $V_{app}^0 \approx V_u^0$ and $V_u^0 \approx V_{max}$. In a less-likely scenario, a low C^* could derive from fast release if V_{app}^0 were correspondingly even faster (this scenario is unlikely because it implies a high expenditure of energy for no apparent reason). In either case, rapid clearance implies a low concentration. Therefore, fast DA clearance in fast domains supports the hypothesis that tonic autoinhibition is absent because the basal DA concentration is low, presumably too low to activate D2Rs.

Tonic autoinhibition in slow domains might be due to a high basal DA concentration. According to Equation 2, a high C^* would derive from fast release and/or slow apparent clearance. When release is fast, apparent clearance should be slow, because $V_{app}^0 = V_u^0 - V_r^0$ (Equation 1). As explained above, basal release in slow domains is likely non-vesicular. If this were to involve DDE, then $V_u^0 \ll V_{max}$ and apparent clearance would be slowed further. So, slow apparent clearance implies a high concentration, supporting the hypothesis that the autoinhibitory tone on DA terminals in slow domains is a consequence of a high basal DA concentration, presumably high enough to tonically activate D2Rs.

Does DDE contribute to release in slow domains?

Equation 2 allows the alternate scenario that a high C^* could derive from slow release if clearance were correspondingly even slower (note, however, there must be some release or C^* goes to zero). Slow release and slow clearance might derive from a striatal location with a low density of DA terminals. In this scenario, again, the apparent clearance rate would approach the maximal intrinsic uptake rate, V_{max} , i.e. $V_{app}^0 \approx V_u^0$ and $V_u^0 \approx V_{max}$, but V would be small because there are few DATs. This scenario, in which slow clearance still implies a high C^* , upholds our hypothesis that the autoinhibitory tone is due to a high basal DA concentration. However, if there are so few DATs then DDE might not be possible and vesicular release, even though it is autoinhibited, would have to be sufficient to tonically activate D2Rs. As discussed next, there are several reasons to think this scenario is unlikely.

A low density of DA terminals is inconsistent with the large amplitude of evoked release once autoinhibition is alleviated with raclopride (Fig 2c and 2d). The nomifensine-sensitivity of the slow-type evoked responses (Fig 3e and 3f) shows that the slow domains contain DAT, which is expressed by DA terminals (Ciliax *et al.* 1995; Nirenberg *et al.* 1996). Our finding that diffusion is not a major contributor to the initial segment of clearance (Fig 3c and 3d) implies the close proximity of DATs to the recording electrode. The rapid onset of DA clearance when the stimulus ends also indicates the presence of functional DATs near the electrode (Moquin and Michael 2009). Moquin and Michael (2009) thoroughly established that the slow-type responses exhibit multiple features that cannot be explained by diffusion.

Equation 2 shows that a domain difference in basal DA concentration requires a difference in the relative rates of release and uptake. Apparent clearance is approximately 5-fold slower in slow domains compared to fast (Fig 3c). Meanwhile, Moquin and Michael (2009: Figure 5c) found that raclopride increases the rate of slow-type evoked release approximately 5-fold, suggesting that autoinhibition slows vesicular release approximately 5-fold. Thus, the relative rates of clearance and vesicular release are not sufficiently different to produce

different basal concentrations. So, we conclude that autoinhibited vesicular release is insufficient to drive an increase in basal DA concentrations, which supports the hypothesis that a non-vesicular process is responsible for the autoinhibitory tone.

Ancillary observations further support a role for DDE. For example, raclopride promoted slow-domain evoked release when administered directly to the striatum (Fig 2c and 2d), which confirms that the low amplitude of slow-type evoked release stems from presynaptic events rather than a low-density of terminals. Furthermore, Borland and Michael (2004) reported a tetrodotoxin-insensitive, nomifensine-sensitive pool of extracellular DA in the striatum, while Wang et al (2010) found that this pool correlates with the domain type. Collectively, these various findings confirm that the type of domain is determined by the rate and mechanism of the processes that control the basal DA concentration, rather than the local density of DA terminals.

Clearance after evoked release and pressure-ejection

Several studies have discussed why DA clearance after pressure-ejection appears to be slower than after evoked release (Cass *et al.* 1993; Cass and Gerhardt 1994; Kiyatkin *et al.* 2000; Sabeti *et al.* 2002). For example, since the DAT is electrogenic, there is a concern that the membrane depolarization associated with the use of electrical stimulation might bias the DAT kinetics. However, we obtained close agreement between clearance after evoked release and pressure-ejection (Fig 3e) by establishing several experimental conditions. First, clearance after evoked release and ejection were measured at the same recording site. Second, the initial amplitude of the clearance profiles after ejection and evoked release were made similar by placing the ejection pipet immediately adjacent to the recording microelectrode (Fig 1) and by adjusting the ejection time. Third, by placing the ejection pipet adjacent to the microelectrode, we kept the ejection time brief so that the ejection was finished prior to the clearance measurement. Fourth, we performed the measurements in slow domains. Under these conditions, clearance after evoked release and pressure-ejection was indistinguishable (Fig 3e): this corroborates the slow apparent clearance rate and confirms that no bias was introduced by the use of a longer stimulus to overcome the autoinhibition of evoked release in the slow domains.

Thus, it appears that the domains may have contributed to the discrepancy between DA clearance rates after evoked release and pressure-ejection noted in prior studies. Voltammetry protocols usually involve a procedure to optimize the placement of the recording electrode. As we have shown, optimization seeks out those domains where DA clearance is inherently fast. The pressure-ejection technique does not seek out the fast domains and so likely reflect slow-domain clearance.

We have not, however, attempted to evaluate DA clearance after pressure-ejection into fast domains. Thus far, we have not been able to identify purely fast recording sites: rather the electrodes straddle fast and slow domains, giving rise to hybrid responses. We assume that pressure ejection would not permit the delivery of exogenous DA only into the fast subdomain of a hybrid site. However, we do not expect the electrogenic properties of the DAT to be a key factor in the faster DA clearance because we only stimulated the fast sites for 200 ms: thus, we evoked far less depolarization in the fast sites and, presumably, far less disruption of ion activities.

Conclusion

Figure 5 schematically summarizes the DAergic function of the fast and slow striatal domains that emerges from these studies. The fast domains operate under the control of an avid uptake process that rapidly clears DA from the extracellular space and maintains a low

basal DA concentration. The DAT primarily engages in uptake, so DDE is negligible and DA release is dominated by the vesicular mechanism. This is the conventional view of DAergic function deduced from voltammetric studies wherein the placement of the recording electrode was optimized to find fast-type DA. The fast-type responses reported here are similar to the optimized responses reported by other investigators. For example, the DA clearance rate we report in fast domains is similar to conventional values (Wu *et al.* 2001; Venton *et al.* 2003) and prior studies have demonstrated that short-stimulus responses at optimized recording locations are refractory to D2R antagonists (Garris *et al.* 1994). On the other hand, DA clearance is 5-fold less-avid in the slow domains and this coincides with a higher basal DA concentration to tonically autoinhibit vesicular release. Autoinhibited vesicular release is insufficient to increase the extracellular DA concentration in the slow domains (Equation 2), so it appears that non-vesicular release contributes to the basal DA tone. This supports the conclusion that the DAT, even though it is conventionally viewed as a DA reuptake site, contributes to functionally relevant DA release.

The existence of distinct fast and slow DA domains accords well with the concept that DA functions on multiple time courses (Schultz 2007). Recent studies have highlighted high-speed DA transients (Robinson *et al.* 2001; Phillips *et al.* 2003; Robinson *et al.* 2003; Cheer *et al.* 2004; Wightman *et al.* 2007; Sombers *et al.* 2009; Addy *et al.* 2010; Wanat *et al.* 2010). While fascinating, it is unlikely that transients account for DA's slower signaling as exemplified, for example, by the efficacy of L-DOPA as a therapy for Parkinson's disease. The non-DAergic elements of the striatum that convert L-DOPA to DA are incapable of phasic DA release yet manage deliver a DA tone that reinstates motor function in parkinsonian individuals (Zigmond *et al.* 1992). There are indications that tonic firing of midbrain DA neurons drives tonal vesicular DA release (Floresco *et al.* 2003; Venton *et al.* 2003), but presumably this is relevant in the fast domains where vesicular release is not autoinhibited. The slow domains, wherein DA release is dominated by non-vesicular processes, appear to operate along-side the fast domains. The physiological implications of the domain architecture remain to be fully elucidated but are a matter of on-going interest.

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Abbreviations

DA	dopamine
D2R	dopamine D2 receptor
DAT	dopamine transporter
DDE	DAT-mediated DA efflux
V_{max}	maximal rate of DA uptake
K_m	the Michaelis constant
V_{app}	apparent rate of DA clearance
V_u^0	steady state rate of DA uptake
V_r^0	steady state rate of DA release

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Figure 1. SEM image of a double barrel carbon fiber electrode tip used for local administration of raclopride or exogenous DA. One barrel contains the carbon fiber microelectrode, while the open barrel is used to deliver drugs directly at the recording site by means of the Picospritzer III pressure-ejection system.

Figure 2.

Voltammetric recordings of evoked DA release demonstrate the presence of slow and fast domains in the rat striatum. (a) In slow domains, evoked DA release is delayed and exhibits facilitation as the stimulus proceeds. Raclopride (2 mg/kg i.p.) abolishes the delay, confirming the role of autoinhibition. (b) When the recording electrode straddles both fast and slow domains, hybrid responses are obtained. The initial component exhibits no delay in evoked release: a robust DA signal is detected on the first voltammetric measurement, which occurs 100 ms after the onset of the stimulus. The initial component exhibits depression as the stimulus continues, which is attributable to the onset of autoinhibition in the fast domain caused by the evoked rise in DA concentration. The fast component is isolated by limiting the stimulus duration to 200 ms (gray line). (c) Local picospritzer ejections of raclopride confirm the role of autoinhibition on the hybrid-type responses curve profile. (d) In hybrid-type responses, raclopride did not alter the initial amplitude of evoked DA following the first 0.2s of stimulation ($n=4$, One-way ANOVA, $F_{(1,6)}=0.202$, $p=0.67$), but significantly increased the amplitude following 3s of stimulation ($n=4$, One-way ANOVA, $F_{(1,6)}=353.1$, $\dagger$ $p<0.0001$).

Figure 3.

Voltammetric recordings of evoked DA release in the rat striatum yield fast-type and slow-type responses. (a) Evoked responses (stimulus start - circle, stimulus end - triangles) from two recording sites in one rat. Fast-type responses exhibit immediate DA release when the stimulus begins. Due to autoinhibition, slow-type responses exhibit a delayed onset of evoked release and short-term facilitation. (b) Fast- and slow-type responses exhibit differential apparent DA clearance rates ($n=4$, Two-way ANOVA, $F_{(1,180)}=118.9$, $\dagger$ $p<0.00001$). (c) Linear regression lines fit to the averaged clearance curves in panel (b) exhibit distinct slopes. (d) The clearance slopes observed in fast and slow domains are significantly different ($n=4$, One-way ANOVA, $F_{(1,6)}=6.93$, $\dagger$ $p=0.039$), with slow domains exhibiting the slower apparent rate of DA clearance. (e) Slow-type apparent DA clearance is identical to the apparent clearance of exogenous DA and is DAT-dependent (nomifensine-sensitive) ($n=4$, Two-way ANOVA – Tukey post-hoc, $F_{(2,270)}=56.18$, $\dagger$ $p<0.00001$). (f) The extracellular lifetime of DA in fast and slow domains is significantly different; in slow domains, the lifetime of DA is identical to the lifetime of exogenous DA and is DAT-dependent ($n=4$, One-way ANOVA – Tukey post-hoc, $F_{(3,12)}=20.70$, $\dagger\dagger$ $p=0.00005$).

Figure 4.

Fast and slow striatal domains exhibit significantly different apparent rates of DA clearance and significantly different extracellular DA lifetimes. (a and b) Normalized clearance profiles exhibit significantly different apparent DA clearance rates ($n=4$, One-way ANOVA, $F_{(1,6)}=28.38$, $\dagger p=0.0018$). (c) Individual regression lines for the data set used to construct figure (3c) demonstrate that the distinction in apparent DA clearance rates is independent of the initial amplitude. The categorization of a response as fast-type or slow-type is not based on the amplitude of the evoked response but rather its temporal profile, as explained by Fig 2. Thus, fast-type and slow-type evoked responses often have similar amplitudes (Fig 2b, 3a). Even when the amplitudes are similar, the distinction in apparent clearance rate is maintained. (d) The fast-type regression lines intersect the x-axis significantly faster than the slow-type regression lines independent of initial evoked amplitude ($n=4$, One-way ANOVA – Tukey post-hoc, $F_{(1,6)}=64.17$, $\dagger p=0.0002$).

Figure 5.

The interaction of DAT, basal DA, D2R mediated autoinhibition, and evoked DA release in fast and slow domains. (a) In fast domains, the DAT favors DA uptake, leading to rapid evoked DA clearance. This rapid DA clearance maintains a basal DA concentration below D2R activation. Thus, tonic autoinhibition is off, enabling terminals to release DA immediately upon stimulation. (b) In slow domains the DAT favors DA efflux, leading to slowed evoked DA clearance. Constant DDE maintains a basal DA concentration large enough to activate D2Rs. Tonic D2R activation creates a constant autoinhibitory tone on these terminals, preventing evoked DA release at the onset of stimulation.