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In vivo measurement of somatodendritic release of dopamine in the ventral tegmental area

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Abstract

The ventral tegmental area (VTA), the locus of mesolimbic dopamine cell bodies, contains dopamine. Experiments in brain slices have demonstrated that VTA dopamine can be released by local electrical stimulation. Measurements with both push-pull cannula and microdialysis in intact animals have also obtained evidence for releasable dopamine. Here we demonstrate that dopamine release in the VTA can be evoked by remote stimulations of the medial forebrain bundle (MFB) in the anesthetized rat. In initial experiments, the MFB was electrically stimulated while a carbon-fiber electrode was lowered to the VTA, with recording by fast-scan cyclic voltammetry. While release was not observed with the carbon fiber 4 to 6 mm below dura, a voltammetric response was observed a t 6-8 mm below dura, but the voltammogram was poorly defined. At lower depths, in the VTA, dopamine release was evoked. Immunohistochemistry experiments with antibodies for tyrosine hydroxylase (TH) confirmed that dopamine processes were primarily found below 8 mm. Similarly, tissue content determined by liquid chromatography revealed serotonin but not dopamine dorsal to 8 mm with both dopamine and serotonin at lower depths. Evaluation of the VTA signal by pharmacological means showed that it increased with inhibitors of dopamine uptake, but release was not altered by D2 agents. Dopamine release in the VTA was frequency dependent and could be exhausted by stimulations longer than 5 s. Thus, VTA dopamine release can be evoked *in vivo* by remote stimulations and it resembles release in terminal regions, possessing a similar uptake mechanism and a finite releasable storage pool.

Keywords

carbon-fiber electrode; stimulated dopamine release; dopamine uptake; dopamine release; anesthetized rat

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The mesolimbic dopamine pathway projects from the ventral tegmental area (VTA) to the nucleus accumbens and plays an important role in reward processing (Pan et al., 2005). It runs parallel to another dopaminergic pathway, the nigrostriatal pathway, which regulates locomotion and originates in the substantia nigra (SN). Dopamine release at the terminals of both of these pathways has been extensively characterized. Terminal release is evoked by action potentials, is highly regulated, and has a release capacity that is regulated by dopamine stores in the active pool (Garris et al., 1993; Montague et al., 2004; Nicolaysen et al., 1988). Following release, dopamine is cleared from the extracellular space by the dopamine transporter (DAT), which transports it back into the neuron (Gainetdinov and Caron, 2003). Because uptake is time dependent, whereas release is dependent on the frequency of action potentials, dopamine overflow into the extracellular space is highly

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dependent on the frequency of action potentials. At high frequencies, uptake has little time between pulses to sequester dopamine leading to greater overflow than at low frequencies (Heien and Wightman, 2006; Wightman et al., 1988). As well as interacting with postsynaptic receptors, dopamine binds to presynaptic autoreceptors that serve to inhibit subsequent dopamine release (Garris et al., 2003; Phillips et al., 2002).

Dopamine is also stored in the dendrites of the SN and VTA (Bjorklund and Lindvall, 1975; Cuello and Kelly, 1977). In the SN, Ca^{2+} dependent somatodendritic dopamine release was first reported in brain slices (Geffen et al., 1976). This finding was confirmed by *in vivo* measurement with a push-pull canula (Cheramy et al., 1981). Subsequent microdialysis studies also demonstrated dopamine release in the VTA (Kalivas and Duffy, 1988). Voltammetric measurements in VTA containing slices from guinea pig brain (Rice et al., 1994), from rat (Iravani et al., 1996), and from mouse (John et al., 2006) also confirm somatodendritic dopamine release evoked by local electrical stimulation. While dopamine release was suggested to occur via reverse transport via the DAT (Falkenburger et al., 2001), further research has shown that dendritic DA release is exocytotic in both the SN and VTA (Chen and Rice, 2001; John and Jones, 2006). Somatodendritic dopamine release has a presynaptic regulatory role as well as a postsynaptic role (Beckstead et al., 2007; Beckstead et al., 2004), although it does not appear to play a role in regulating release in the VTA (Cragg and Greenfield, 1997).

While neurons normally transmit action potentials from the cell bodies to the terminals, it has been demonstrated that midbrain dopaminergic neurons can also backpropagate action potentials (Gentet and Williams, 2007; Hausser et al., 1995) to the dendritic field. In this work we have tested the hypothesis that back-propagation of action potentials in dopaminergic neurons can evoke dopamine release. To achieve this goal, a stimulating electrode was lowered in an anesthetized rat to the level of the MFB, and a carbon-fiber microelectrode was placed in the VTA. Dopamine release in the VTA was measured during stimulations with fast-scan cyclic voltammetry (Robinson et al., 2008). The stimulated release of dopamine resembled its evoked release in terminal regions except it was lower, uptake was slower, and it was insensitive to drugs that inhibit dopamine autoreceptor functioning.

MATERIALS AND METHODS

Animals and surgery

Male Sprague-Dawley rats (225-350g; Charles River, Wilmington, MA) were anesthetized with urethane (1.5 g/kg, i.p.) and placed in a stereotaxic frame (Kopf, Tujunga, CA). A heating pad (Harvard Apparatus, Holliston, MA) maintained a constant body temperature of 37°C. Holes were drilled in the skull for the working, reference, and stimulation electrodes at coordinates selected from a rat brain atlas (Paxinos and Watson, 1986). A Ag/AgCl reference was inserted in the contralateral region of the brain.

Electrochemistry

Cylindrical carbon fiber microelectrodes were prepared using T650 carbon fibers (3 μm radius, Amoco) and encased in glass capillaries (A-M Systems, Sequim, WA) and pulled with a micropipette puller (Narashige, East Meadow, NY). The protruding fiber was then cut to a length of 50-100 μm (Cahill et al., 1996). On the day of use, the electrode was soaked for 10 minutes in isopropanol purified with activated carbon (Bath et al., 2000). To make contact with the carbon fiber, a potassium acetate solution was injected into the glass capillary and a silver wire was inserted into the open end of the capillary and twisted to

ensure solid contact with the carbon fiber. The reference electrode was chloridized by placing a silver wire in a HCl solution and applying +5 V.

Fast-scan cyclic voltammetry was used in all experiments. The potential of the working electrode was held at -0.4 V vs Ag/AgCl between scans and was ramped to +1.3 V at 400 V/s and repeated at a frequency of 10 Hz. All cyclic voltammograms were background subtracted using a voltammogram obtained immediately before the concentration change evoked by the stimulation. Voltammograms were correlated with authentic dopamine signals as described previously (Troyer et al., 2002). After the experiment the working electrode was calibrated *in vitro* using dopamine solutions of known concentration (Cahill et al., 1996).

Electrical stimulation

An untwisted bipolar stimulating electrode (Plastics One, Roanoke, VA) was used to stimulate dopaminergic neurons. The stimulus was delivered via a stimulus isolator (A-M Systems, Sequim, WA). The stimulation train consisted of biphasic pulses (± 300 μA, 2 ms/phase). The frequency and number of pulses per train were varied as noted in the text. The pulses were generated by a computer and applied between the cyclic voltammograms to avoid electrical interference.

The position of the stimulating electrode in the MFB was optimized in the following way. A carbon-fiber microelectrode was placed in the caudate-putamen (AP +1.2, ML +2.0, and DV -4.5, all stereotaxic coordinates in mm according to a stereotaxic atlas (Paxinos and Watson, 1986)). The stimulating electrode was lowered in a series of 100 μm increments from a hole in the skull (AP -1.8, ML +2.0) toward the MFB. An electrical stimulation was delivered at each location until dopamine release was measured in the caudate-putamen, typically at a depth of 8 mm. Once a robust dopamine signal was obtained with cyclic voltammetry, the stimulating electrode remained in place. The carbon-fiber microelectrode was further lowered to the nucleus accumbens core to ensure that dopamine release was evoked in that region. Next, the carbon-fiber microelectrode was removed and reinserted into a hole drilled over the VTA (AP -5.2, ML +1.0). For the mapping studies, the electrode was lowered to the VTA while stimulations were made in the MFB to map out the events as a function of depth. For experiments involving the pharmacology and physiology of VTA release, the electrode was lowered to 8 mm below the surface of the skull, the depth of the VTA.

Immunohistochemistry

Six rats were anesthesized with urethane (1.5 g/kg, i.p.) and perfused transcardially with 80 ml of 0.9% saline, followed by 80 ml of 4% paraformaldehyde. The brains were removed immediately after fixation and post-fixed for one hour in 4% paraformaldehyde. After one hour, the brains were transferred to phosphate buffered saline (pH 7.4) and kept in the refrigerator overnight. The brains were then cut on a microtome into 200 μm slices and collected in artificial cerebral spinal fluid. The slices were transferred to a pre-blocking solution containing 10 mL of PBS at pH 7.4, 0.1 g of bovine serum albumin (BSA), 1.0 mL of normal goat serum, and 30 μL of Triton-X 100 and allowed to incubate for 2 hours. The slices were then incubated in a solution of primary tyrosine hydroxylase (TH) antibody (rabbit anti-TH polyclonal, Chemicon cat# AB 152) or (rabbit anti-serotonin polyclonal, Invitrogen, Carlsbad, CA) at a concentration of 1:200. The slices were then kept on a shaker plate overnight at 4°C. The primary antibody was rinsed off with six aliquots of PBS at pH 7.4 for one hour. The slices were then incubated in a solution of secondary antibody (Invitrogen Alexa Fluor(R) 555 goat/anti-rabbit) at a concentration of 1:200 for 24 hours on a shaker plate at 4°C. The secondary antibody was rinsed off with six aliquots of PBS at pH 7.4 for one hour. The slides were mounted on glass slides using BioRad Fluoroguard

Antifade Reagent mounting media and analyzed on a Leica SP2 Laser Scanning Confocal Microscope (Leica Microsystems Inc., Bannockburn, IL).

The specificity of the serotonin antibody was evaluated in the dorsal raphe nucleus that has a high concentration of serotonin (Michelsen et al., 2007). Fluorescent labeling, demonstrated regionally specific labeling of the serotonin cell bodies in the dorsal raphe nucleus (data not shown).

High performance liquid chromatography (HPLC)

Tissue samples were dissected from brain slices containing the area of interest (500 μm thickness). The tissue was wet weighted in a pre-tared volume of extraction solution of 200 μL 0.1 N $HClO_4$ spiked with 1 μM hydroquinone (HQ). The tissue was homogenized with a sonic dismembrator (Fisher Sci., Model 60, Pittsburgh, PA, USA). The homogenized tissue was centrifuged at 6000 rpm for 10 min, and the supernatant was removed and filtered using a 0.2-μm syringe microfilter (Millex-LG). Injections (10 μL) were made onto a reverse phase column (C-18, 5 μm, 4.8 × 250 mm, Waters symmetry 300). The mobile phase (prepared in HPLC grade water) contained 0.1 M citric acid, 1 mM hexyl sodium sulfate, 0.1 mm EDTA, and 10% methanol (pH 3.5) at a flow rate of 1 mL/min. Catecholamines were detected with a thin-layer radial electrochemical flowcell (BASi, West Lafayette, IN, USA) at a potential of 700 mV versus a Ag/AgCl reference electrode. Catecholamine standards were prepared from 10 mM stock solutions in 0.1 N perchloric acid. A labview stripchart recorder program (Jorgenson Lab, UNCCH) was used for data collection through home-built electronic equipment.

Data analysis

The shape of the stimulated release and uptake curve was evaluated with a simple kinetic model (Wightman et al., 1988) using nonlinear regression (Wu et al., 2001). Each stimulus pulse was considered to release a fixed amount of dopamine, and in the time between pulses and after the stimulus train uptake was assumed to follow Michaelis-Menten kinetics characterized by a V_{max} and a K_m. Since all of the uptake inhibitors that were used are competitive, changes in the K_m value following their administration were determined. Data were evaluated in Graph Pad Prism (Graph Pad Software, San Diego, CA) and are expressed as mean ± SEM. Statistical significance was determined using a two-way ANOVA, and posthoc comparisons were performed using the method of least squares with a Bonferroni correction.

HPLC data analysis was performed using custom Igor programs for baseline correction and peak area analysis (Jorgenson Lab, UNC-CH). The peak area of the analyte was divided by the peak area of the internal standard HQ which is present in the extraction buffer at a known concentration. After correction for differential detector response, the amount of analyte in the brain tissue could be calculated.

Chemicals and solutions

All chemicals and drugs were purchased from Sigma/Aldrich (St. Louis, MO) and used as received. Solutions were prepared using doubly distilled deionized water (Megapure system, Corning, NY). The TRIS buffer solution for flow cell analysis was prepared using 12 mM TRIS, 140 mM NaCl, 3.2 mM KCl, 1.2 mM $CaCl_2$, 1.25mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.0 mM Na_2SO_4 at pH 7.4. All drugs were dissolved in physiological saline and injected intraperitoneally. To aid in dissolution, nomifensine was prepared by adding small amounts of tartaric acid to the saline solution as described previously (Garris et al., 2003).

RESULTS

Detection of dopamine release in the VTA

The stimulating electrode was positioned in the MFB at a site where it could elicit robust dopamine release in the nucleus accumbens core as measured with a carbon-fiber microelectrode (Garris and Wightman, 1994; Kuhr et al., 1986). Then, the carbon-fiber microelectrode was extracted from the brain and placed above the VTA. It was lowered towards the VTA in 200 μm increments (Fig. 1a, dashed line). At each location, the MFB was stimulated with a pulse train of 60 biphasic, 300 μA electrical pulses delivered at 60 Hz. The maximal response in the background-subtracted voltammogram at 0.6 V (the potential where dopamine is oxidized) observed during stimulation was normalized to the maximal response observed on the whole track, and plotted as a function of electrode depth (Fig. 1b).

Between 4 and 6 mm along the track, the stimulation evoked little release. An electroactive substance was evoked at depths of 6.0 - 8.0 mm, regions that correlate approximately to the location of the interstitial medial longitudinal fasciculus (iMLF), a subregion of the thalamus, and the red nucleus that lies below it. However, the cyclic voltammograms in these regions showed a poor match for dopamine (example in Fig. 1c, middle panel, r^2 = 0.541 for comparison of a cyclic voltammogram measured in this region to a cyclic voltammogram obtained during stimulated release in the nucleus accumbens core). At depths of between 8 and 9 mm, the coordinates of the VTA, release of an electrochemically active substance was also elicited. At these depths, the background-subtracted cyclic voltammograms had an oxidation peak at +0.6 V, and a reduction peak at -0.2 V (example in Fig. 1c, right panel), identical to that expected for a catecholamine (Heien et al., 2003). These cyclic voltammograms showed a high correlation with the cyclic voltammograms for dopamine recorded in the nucleus accumbens core (r^2 = 0.916 for the cyclic voltammogram recorded in the VTA compared to one obtained in the nucleus accumbens core).

Dopamine distribution in the midbrain

To evaluate whether the voltammetric signals obtained at various depths were coincident with dopaminergic neurons, TH localization was examined with immunohistochemistry. A secondary antibody conjugated to a fluorescent marker, Alexa-555/anti-rabbit, was used to visualize TH localization. The region indicated by the white box in Figure 2a shows the area examined by the confocal image in Figure 2b. The image shows TH antibody binding only in the region ventral to 8 mm, the VTA. In this region, the fluorescence TH antibody binding can be seen in cell bodies surrounded by wispy projections, likely corresponding to dendrites (Fig. 2b). When the confocal image (Fig. 2b) was correlated with the electrochemical depth profile (Fig. 1b), it is clear that release with a signature dopamine cyclic voltammogram occurs where TH antibody binding is localized, below 8 mm, but not in the more dorsal regions where another electroactive species is observed.

To examine whether serotonin could contribute to the measured signal, additional immunohistochemical experiments were done with a rabbit/anti serotonin primary antibody. Again, the Alexa-555 conjugated goat/anti-rabbit secondary antibody was used to visualize the serotonin antibody binding with fluorescence. The region that contains the red nucleus is shown by the dashed black square in Fig. 2a, and it displayed strong fluorescence due to the antibody labeling with a roughly circular shape corresponding to the red nucleus (Fig. 2c). In this image, the midline is located at the left edge of the image, and the VTA and SN are located along the lower edge of the image. Both dopaminergic cell body regions showed weaker, but measurable, serotonin reactivity. No fluorescent labeling was apparent in the iMLF (Fig. 2d, black square from Fig. 2a). The midline and ventricle are located on the left

edge of this image, and possess only faint labeling; however, the region containing the iMLF is almost black.

To determine the amount of electroactive neurotransmitters in the VTA and the more dorsal regions, we dissected these regions and analyzed their content by liquid chromatography with electrochemical detection. The dissected tissue was sonicated in perchloric acid to liberate small molecule neurotransmitters from the tissue. Neurotransmitter amounts were obtained by ratioing the peak areas with hydroquinone that was used as an internal standard. In the VTA, dopamine and serotonin were detected (Table I) while epinephrine and norepinephrine levels were below detection limits. However, in the more dorsal region, which includes the red nucleus, serotonin was observed but dopamine was not.

Pharmacological characterization in the VTA

The remaining experiments were conducted in the VTA (histological marking of the carbon-fiber sites is given by the black dots in Figure 1a) where all of the evidence indicates dopamine is the species released. Dopamine release was evoked by electrical stimulation (60 pulse, 60 Hz, 300 μA) delivered at three-minute intervals, and, once stable release was obtained, the effects of various pharmacological agents on this signal were examined. As seen in Figure 3b, the maximal amplitude of stimulated release is very reproducible both before and after saline. The maximal release of dopamine in the VTA with this stimulation corresponded to ~150 nM, which is significantly less than the 1 μM commonly seen in the nucleus accumbens core with less intense stimulation parameters (Heien et al., 2005). The V_{max} (0.13 ± 0.03 μM/s) was in the range reported previously in brain slices containing the VTA, 0.15 μM/s (John et al., 2006).

Cocaine can inhibit uptake of serotonin, norepinephrine, and dopamine (Iversen, 2006). At a dose of 20 mg/kg, cocaine dramatically inhibits dopamine uptake in the nucleus accumbens (Suaud-Chagny et al., 1995). Using a slightly higher dose (30 mg/kg i.p.), cocaine significantly increased the amplitude of neurotransmitter released, as well as inhibited the clearance. The apparent value of K_m increased 820% ± 110% ($n = 7$, $p < 0.01$) (representative trace in Fig. 3a). Twenty minutes after administration of cocaine, maximal release was increased approximately three-fold ($n = 7$, $p < 0.007$) (Fig. 3b).

Nomifensine selectively inhibits uptake by catecholamine transporters (Lengyel et al., 2008). At a dose of 20 mg/kg, nomifensine inhibits dopamine uptake in the NAc (Suaud-Chagny et al., 1995). Using this dose, stimulated release in the VTA was increased by 250% ($n = 5$, $p < 0.004$) and the apparent value of K_m was increased 610% ± 140 ($n = 5$, $p < 0.05$) (Fig. 3b). The increase in release following nomifensine persisted for approximately 40 minutes; and the effects of the drug wore off approximately 2 hours after administration (data not shown).

The effect of a selective uptake blocker of the serotonin transporter, citalopram, was also examined. At a dose of 5 mg/kg i.p., citalopram has been shown to increase extracellular serotonin concentrations in the NAc (Weikop et al., 2007). Increasing the dose four-fold to 20 mg/kg was insufficient to affect the concentration of evoked neurotransmitter in the VTA (Fig. 3b), even though a 20 mg/kg injection of nomifensine 30 minutes later in the same animal was able to increase release (Fig. 3c, $p < 0.009$). Desipramine, a selective norepinephrine transport inhibitor, has been shown to increase stimulated norepinephrine release by as much as 200% in the NAc (Brun et al., 1993) at a dose of 10 mg/kg i.p. Using a higher dose of 20 mg/kg produced no significant change in the VTA (Fig. 3b). Application of a 20 mg/kg injection of nomifensine 30 minutes after administration of desipramine at the same location increased the maximal release by approximately 225% (Fig. 3c, $p < 0.01$).

The subsequent application of nomifensine verifies that uptake control was present at the site being recorded.

In the NAc, dopamine release can be modulated by drugs that interact with autoreceptors. Specifically, the D2 autoreceptor agonist, quinpirole, and the D2 autoreceptor antagonist, raclopride, decrease and increase, respectively, the maximum evoked dopamine release in the NAc (Cragg and Greenfield, 1997). Intraperitoneal injections of either the agonist, quinpirole or the antagonist, raclopride (both at 1 mg/kg) were given, and the amplitude of release was then measured in the VTA 20 minutes after administration of the drug. There was no significant change in the signal in the VTA upon administration of either drug (Fig. 3b). These data suggest that dopamine release was not under autoregulation by the dopamine D2 autoreceptor, consistent with prior reports (Cragg and Greenfield, 1997).

Physiological characterization

To probe the characteristics of release in the VTA, the frequency of electrical stimulation was varied (Fig. 4). During stimulation, the overflow of dopamine in terminal regions shows a frequency dependent behavior (Wightman et al., 1988;Wu et al., 2001), where the temporal changes in extracellular dopamine concentration can be described by the release of dopamine minus the amount cleared via uptake that follows Michaelis-Menten kinetics. Thus, high stimulation frequencies should lead to greater amounts of release than lower frequencies. A plot of dopamine concentration versus stimulation frequency (Fig. 4d) revealed such a relationship. The approximately linear relationship between release amplitude and stimulation frequency are consistent with dopamine release seen at the terminals of dopaminergic neurons (Garris and Wightman, 1994). The shape of release at 10 Hz (Fig. 4a) approaches a steady state indicating that the release and uptake components are balanced at this frequency. The release profile at 50 Hz increases during the stimulation, demonstrating that release overwhelms uptake at this frequency (Fig. 4c). The slower dopamine uptake is consistent with measurements performed in VTA brain slices (John et al., 2006).

To probe the effect of extended stimulation, dopamine release was measured during a 50 s electrical stimulation (60 Hz). The amplitude of release rose until approximately 5 seconds after the initiation of stimulation. After this, overflow actually diminished, even though the stimulation was maintained, demonstrating depletion of the neurotransmitter available for release (Fig. 5a). A second identical stimulation train immediately following the completion of the depletion stimulation led to a marked decrease in amplitude when compared to the first stimulation (Fig. 5b). These depletion results are consistent with the long-lasting depression observed previously in the caudate-putamen (Montague et al., 2004;Nicolaysen et al., 1988).

DISCUSSION

The release of dopamine in the VTA has been well documented to occur with local electrical stimulation in brain slices (Chen and Rice, 2002; Cragg and Greenfield, 1997; John et al., 2006; Rice et al., 1997). The goal of this work was to evaluate the effect of backward-propagating action potentials on the somatodendritic release of dopamine in the VTA of an intact, anesthetized animal. Electrophysiological studies have provided evidence that stimulations can propagate through the axon and should evoke dendritic release (Gentet and Williams, 2007; Hausser et al., 1995). To this end, the stimulating electrode was placed in the MFB more than 3 mm away from the VTA and at a site that elicited release in terminal regions. Robust catecholamine release was observed in the VTA, and chromatographic analysis of the tissue established that dopamine was the only catecholamine present in this region that could contribute to the voltammetric signal. Subsequent pharmacological studies

established that dopamine release in this region is counterbalanced by uptake, and it was unaffected by agents that lacked pharmacological activity at the dopamine transporter. Release was found to be similar to that in terminal regions in most respects. Overflow of dopamine increased with increasing frequency. Furthermore, the amount of dopamine available for release was limited (Figure 5), a behavior also seen in striatal terminal regions (Garris et al., 1993), because only a portion of dopamine stores reside in releasable pools. Release differed from that in striatal regions in that it was insensitive to autoreceptor regulation, a feature also observed in slices of the VTA (Cragg and Greenfield, 1997). Otherwise, the data show that dopamine released from somatodendritic sources in the VTA undergoes similar regulation as found in terminal striatal regions.

The strategy used in this work to evoke release of dopamine in the VTA has been used to map release in terminal regions previously (Garris et al., 1993; Stamford et al., 1988). Electrical stimulation of the MFB of an anesthetized animal elicits release in a wide range of dopamine terminal regions. We anticipated that electrical stimulations of the MFB would also elicit release from dopamine cell bodies because stimulation pulses have been shown to back propagate to dopamine cell bodies (Kuhr et al., 1987). Furthermore, at dopamine neurons in the SN, the axon emerges from a dendrite (Hausser et al., 1995). Because of this arrangement, action potentials generated in the axons show little attenuation in the dendritic tree (Gentet and Williams, 2007), and should cause exocytotic release. Consistent with these expectations, MFB stimulations were sufficient to evoke dopamine release in the VTA, suggesting that these neurons have similar architecture. However, we did not anticipate the evoked electrochemical responses at locations dorsal to the VTA. Was this dopamine released in the VTA that diffused to more dorsal locations? Alternatively, was it release of other substances from local terminals that were activated by the MFB stimulation? The voltammetric signals in the regions dorsal to 8 mm were insufficient to identify the measured substance(s) because of poor signal to noise. All catecholamines have a similar signature with fast-scan cyclic voltammetry, and the cyclic voltammogram for serotonin only differs from catecholamines in that its reverse wave is at more positive potentials (Heien et al., 2003; John et al., 2006).

To provide further evidence to support the voltammetric identity of dopamine release in the VTA, we used antibody binding for TH and chromatographic analysis of tissue content of the mesencephalic subregions probed during the descent of the electrode to the VTA. As expected from prior histochemistry for dopamine (Bjorklund and Lindvall, 1975), the VTA was clearly stained with antibodies to TH, however, TH was not seen in the region immediately dorsal to the VTA, the red nucleus In contrast, an antibody to serotonin showed considerable binding in the red nucleus with weaker, but observable, binding in the VTA. Consistent with our chromatographic findings, the red nucleus has been shown to contain significant amounts of serotonin (Palkovits et al., 1974) and it receives serotonergic innervation through the MFB (Pierce et al., 1976), suggesting a possible mechanism for its release with our protocol. Although dopamine was the predominant electroactive species found in the VTA, serotonin was also present, consistent with the documented serotonin receptors (Liu et al., 2006) and releasable serotonin (Yan et al., 2005) found there. The voltammetric recordings, however, showed no evidence for concurrent serotonin release. Thus, if the stimulation evoked serotonin releases, its concentration was much less than dopamine, and its presence was masked. No binding of the serotonin antibody was evident in the iMLF although there was weak labeling for TH (data not shown). Thus, a catecholamine may have contributed to the voltammetric signal evoked by stimulation in that region.

In brain slices, dopamine uptake in the VTA has been reported to follow Michaelis-Menten kinetics with a similar K_m as found in terminal regions. However, V_{max} values in striatal

areas are ~30 times greater than those in the VTA (John et al., 2006). Our results show that the uptake of dopamine in the VTA is by the dopamine transporter because it was inhibited with agents that inhibit the dopamine transporter (cocaine, nomifensine), but it was unaffected by agents specific for inhibition of the serotonin (citalopram) or norepinephrine (desipramine) transporters. These results are in agreement with prior measurements in slices (John and Jones, 2007). Diffusion of dopamine from the site of release to other sites can occur with slow uptake as in the VTA. This so-called volume transmission (Garris and Rebec, 2002) is proportional to the square root of the residence time of dopamine in the extracellular space. Even though this time may be 30 times longer than in striatal regions, we would anticipate diffusion distances of less than 30 μm based on these rates. Thus, diffusion distances are much less than the 100's of microns needed for VTA dopamine to diffuse to the red nucleus and IMLF and contribute to the signals measured in those regions. However, despite the fact that dopamine can diffuse in the VTA, its actions on cells are short-lived indicating that proximal interactions are important as well (Beckstead et al., 2004).

In terminal regions, electrically evoked dopamine release is under control of autoreceptors that can be blocked by raclopride (Cragg and Greenfield, 1997; Kita et al., 2007). Such regulation was not found for dopamine VTA release in the intact animal, consistent with findings in brain slices (Beckstead et al., 2007; Cragg and Greenfield, 1997). This is a rather surprising result since D2 receptors on the dendrites of dopaminergic neurons are present and regulate cell firing (Lacey et al., 1987). It may be that the hyperpolarization accompanying D2 receptor activation (Lacey et al., 1987) is simply overcome by the electrical stimulation. However, regulation of cell firing would also regulate dopamine release under physiological conditions without directly inhibiting it.

When examined in brain slices, electrically evoked somatodendritic release of dopamine shows a maximum at 10 Hz (Rice et al., 1997). A quite different response is obtained *in vivo* (Figure 4) with evoked release continuing to increase up to 60 Hz, a frequency dependence that is similar to that found in terminal regions (Garris et al., 1993). Indeed, the short term plasticity characterized in brain slices (Beckstead et al., 2007) simply is not present in the intact brain. Similar differences have been reported in terminal regions when release *in vivo* and in brain slices are compared (Kennedy et al., 1992), although the origins are not understood. One suggestion for the differences found between brain slices and *in vivo* may be due to a lack of endogenous tone in the former preparation where inputs have been severed. The results of this study clearly show that, when *in vivo* results are compared, somatodendritic and terminal release of dopamine are quite similar, including the restricted amount of dopamine available for stimulated release.

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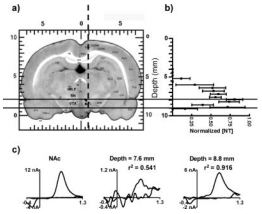

Figure 1.

Location of carbon-fiber electrode in the midbrain. The position of the carbon-fiber electrode was determined by lowering it through the midbrain with a stereotaxic micromanipulator, while the stimulating electrode was in the MFB. Signals were recorded in the midbrain during a 60 Hz, 60 p, 300 μA electrical stimulation of the MFB. **(a)** Brain slice obtained -5.20 mm posterior from bregma. Black squares indicate final position of four of the electrodes determined histologically. The areas under investigation include the iMLF, RN and the VTA. Regions labeled in the left hemisphere are structurally identical to the regions in the right hemisphere. The dashed line represents the track of the working electrode, and the solid lines represent the depth of the VTA. **(b)** The normalized concentration of evoked neurotransmitter (NT) was measured in 200 μm increments as the working electrode was lowered. In each animal, the signal at each data point was normalized to the maximum amplitude of release obtained from the animal. (n = 6) **(c)** Representative cyclic voltammograms obtained from the NAc (left panel) and depths of 7.6 mm (middle panel) and 8.8 mm (right panel). R^2 values correspond to the correlation between CV's from midbrain and the dopamine CV obtained from the NAc.

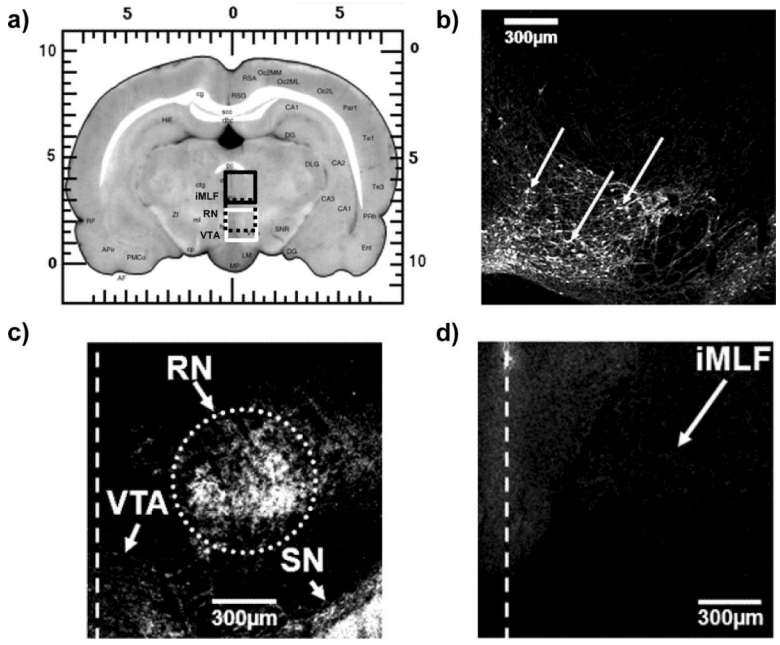

Figure 2.

Dopaminergic structures in the ventral tegmental area (VTA). To identify dopamine innervation in the midbrain, TH was labeled with a primary antibody (rabbit/anti-TH). A fluorescent secondary antibody, Alexa-555/anti-rabbit, was used to provide a marker for TH. **(a)** Brain slice from the midbrain used to orient regions displayed in immunohistochemical images. White square corresponds to panel (b), the dashed square to panel (c), and the dark bordered square to panel (d). **(b)** A 10X fluorescent image showing the white fluorescent labeling of TH in the right hemisphere of the VTA. The bright dots indicate cell bodies (white arrows). **(c)** A 10X fluorescent image of the RN following labeling with an antibody to serotonin. Serotonin labeling is shown in white. **(d)** A 10X fluorescent image of the iMLF following labeling with an antibody to serotonin.

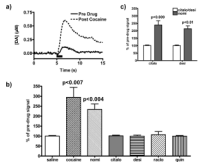

Figure 3.

Effect of uptake inhibitors and D2 agents on evoked release in the VTA. Release was evoked via a 60 Hz, 60 p, 300 μA electrical stimulation of the MFB. Signals were recorded in the VTA before and after administration of saline, cocaine (n = 7, 30 mg/kg i.p.), nomifensine (nomi, n = 5, 20 mg/kg i.p.), citalopram (citalo, n = 5, 20 mg/kg i.p.), desipramine (desi, n = 5, 20 mg/kg i.p.), raclopride (raclo, n = 5, 1 mg/kg i.p.), and quinpirole (quin, n = 5, 1 mg/kg i.p.). **(a)** Representative trace of dopamine release before and after administration of cocaine. **(b)** Saline injection had no significant effect. Cocaine administration significantly increased the signal by 300% ($p < 0.007$). Administration of nomifensine significantly increased the signal by 250% ($p < 0.004$). The selective serotonin reuptake inhibitor, citalopram, had no significant effect. The selective norepinephrine reuptake inhibitor, desipramine, had no effect. **(c)** Nomifensine administered 30 minutes after either citalopram (n = 5, $p < 0.009$) or desipramine (n = 5, $p < 0.01$) in the same rat significantly increased the signal.

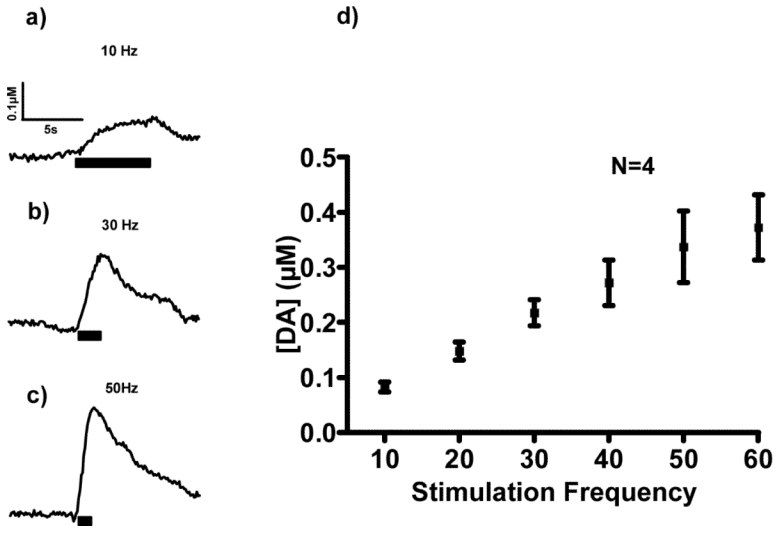

Figure 4.

Effect of stimulation frequency on signal amplitude in the VTA. Representative traces obtained for a 60 p, 300 μA stimulation at **(a)** 10 Hz, **(b)** 30 Hz, **(c)** 50 Hz. **(d)** The effect of stimulation frequency can be seen with [DA] (μM) plotted as a function of the stimulation frequency (Hz).

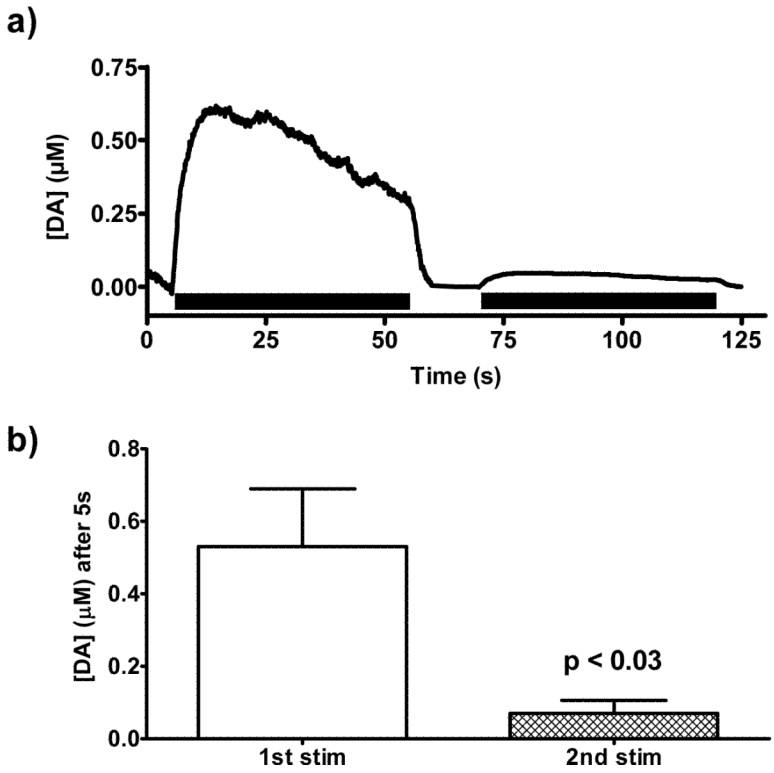

Figure 5.

Effect of prolonged stimulation on electrically evoked dopamine release. Dopamine release was evoked by a 60 Hz, 3000 p, 300 μA electrical stimulation. **(a)** Representative trace of two identical stimulations. During the first stimulation dopamine release reached a steady state within 5 seconds and slowly returned to baseline over the course of 50 seconds. The second identical stimulation event occurred 10 seconds after the completion of the first stimulation. **(c)** The amplitude of dopamine release after 5 s of stimulation is significantly less during the second stimulation ($n = 5$, $p < 0.03$)

Table I

Neurotransmitter content obtained from the VTA and RN by HPLC. The content of both dopamine and serotonin was obtained by dissecting brain tissue from the RN and VTA and analyzing the supernatant from the homogenized tissue using HPLC. Norepinephrine and epinephrine were not detectable in either region.

Brain Region	Dopamine (μg/g)	Serotonin (μg/g)
RN	ND	0.69 ± 0.01
VTA	1.26 ± 0.08	0.86 ± 0.08