Behavioral/Systems/Cognitive

Synaptic Overflow of Dopamine in the Nucleus Accumbens Arises from Neuronal Activity in the Ventral Tegmental Area

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Dopamine concentrations fluctuate on a subsecond time scale in the nucleus accumbens (NAc) of awake rats. These transients occur in resting animals, are more frequent following administration of drugs of abuse, and become time–locked to cues predicting reward. Despite their importance in various behaviors, the origin of these signals has not been demonstrated. Here we show that dopamine transients are evoked by neural activity in the ventral tegmental area (VTA), a brain region containing dopaminergic cell bodies. The frequency of naturally occurring dopamine transients in a resting, awake animal was reduced by a local VTA microinfusion of either lidocaine or (±)2-amino,5-phosphopentanoic acid (AP-5), an NMDA receptor antagonist that attenuates phasic firing. When dopamine increases were pharmacologically evoked by noncontingent administration of cocaine, intra-VTA infusion of lidocaine or AP-5 significantly diminished this effect. Dopamine transients acquired in response to a cue during intracranial self-stimulation were also attenuated by intra-VTA microinfusion of AP-5, and this was accompanied by an increase in latency to lever press. The results from these three distinct experiments directly demonstrate, for the first time, how neuronal firing of dopamine neurons originating in the VTA translates into synaptic overflow in a key terminal region, the NAc shell.

Key words: in vivo **voltammetry; neurotransmission; carbon-fiber microelectrode; cocaine; intracranial self-stimulation; burst firing**

Introduction

Dopaminergic neurons provide a critical modulatory influence in reward seeking (Everitt and Robbins, 2000; Phillips et al., 2003a), prediction error (Schultz et al., 1997) and reinforcement (Wise, 2004). Real-time dopamine neurotransmission in awake animals, monitored with fast-scan cyclic voltammetry at carbon-fiber microelectrodes, has revealed naturally occurring, subsecond dopamine concentration fluctuations (transients) in the nucleus accumbens (NAc) (Robinson et al., 2002; Wightman et al., 2007). Under basal conditions these transients occur at highly variable frequencies with amplitudes of ~50 nM and durations of ~1 s (Wightman et al., 2007). They are enhanced upon administration of drugs of abuse (Stuber et al., 2005), and become time-locked to cues that predict reward availability (Phillips et al., 2003a; Roitman et al., 2004; Day et al., 2007; Owesson-White et al., 2008). Despite their importance, the origin of dopamine transients in the NAc is unclear.

The most likely cause of dopamine transients is phasic firing of dopaminergic neurons in the ventral tegmental area (VTA). These neurons normally fire in a tonic pattern (~5 Hz) and periodically discharge in short bursts (~20 Hz). Bursts are particularly apparent at presentation of primary rewards or their associated cues (Schultz et al., 1997; Hyland et al., 2002). The activity of dopaminergic neurons is regulated by multiple inputs (Floresco et al., 2003; Lodge and Grace, 2006), and in brain slices that lack these inputs, phasic activity is not observed (Overton and Clark, 1997). In the intact brain the transition from tonic to phasic firing is caused by excitatory amino acids in the VTA (Overton and Clark, 1992; Chergui et al., 1993). Consistent with this, microdialysis studies revealed that activation of NMDA receptors in the VTA causes an increase in NAc extracellular dopamine (Karreman et al., 1996; Kretschmer, 1999). However, a direct assessment of the release consequence of phasic firing in the VTA requires rapid dopamine measurements.

Although VTA cell firing is a likely origin of dopamine transients, other factors may contribute. First, dopamine release is not always directly proportional to the degree of VTA activation, but can exhibit facilitation or depression (Montague et al., 2004; Kita et al., 2007). Moreover, terminal mechanisms may alter release. For example, glutamatergic inputs from the basolateral amygdala to the NAc modulate dopamine efflux (Howland et al., 2002), and nicotinic and opiate receptors on dopamine terminals can locally influence dopamine release probability (Zhou et al., 2001; Rice and Cragg, 2004; Britt and McGehee, 2008). Reverse transport via the dopamine transporter could also generate extracellular dopamine (Falkenburger et al., 2001). Here, we investigate the origin of dopamine transients in the NAc shell, a region that exhibits dopamine transients in animals at rest (Wightman et al., 2007), following pharmacological manipulation (Stuber et

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al., 2005; Cheer et al., 2007b), and in response to cues that predict reward (Phillips et al., 2003a; Roitman et al., 2004; Stuber et al., 2005; Cheer et al., 2007a; Owesson-White et al., 2008). Intra-VTA microinfusion of neuronal firing inhibitors establishes that dopamine transients in the NAc shell require ongoing phasic activity in the VTA.

Materials and Methods

Electrodes

Glass-encased, carbon-fiber microelectrodes were constructed as previously described with T-650 carbon fiber (Phillips et al., 2003b). The reference electrodes were chloridized silver wires (0.5 mm diameter, Sigma-Aldrich) in 0.1 N HCl. All potentials reported are versus Ag/AgCl.

Animals and surgery

Male Sprague Dawley rats ($n = 22$; Charles River Laboratories; 250–350 g) some of which were implanted with a jugular vein catheter ($n = 11$) were individually housed on a 12:12 h light cycle with *ad libitum* access to food and water. Rats were anesthetized with ketamine hydrochloride (100 mg/kg, i.p.) and xylazine hydrochloride (20 mg/kg, i.p.) and stereotaxic surgeries were performed as described previously (Phillips et al., 2003b). The Ag/AgCl reference electrode was placed in the forebrain, and a guide cannula (Bioanalytical Systems) was positioned above the contralateral NAc (1.7 mm anterior, 0.8 mm lateral, 2.5 mm ventral relative to bregma). A combination bipolar stimulating electrode/steel guide cannula (26 gauge; Plastics One) was implanted unilaterally into the VTA at a 6° angle toward the midline to avoid the midline sinus (5.4 mm posterior, 1.2 mm lateral, 7.8 mm ventral relative to bregma). The components were permanently affixed with dental cement. The animals recovered for 3 d. All procedures were performed in accordance with the University of North Carolina Animal Care and Use Committee.

Data acquisition

The cyclic voltammetric waveform was generated and the resulting signal was collected using LabVIEW (National Instruments) and a multifunction data acquisition board (PCI-6052E, National Instruments). PCI-6711E and PCI-6601 (National Instruments) boards were used to synchronize waveform acquisition, data collection, and stimulation delivery. Waveform processing and current transduction used custom-built instrumentation (University of North Carolina, Department of Chemistry Electronics Facility).

Recording sessions

A fresh carbon-fiber microelectrode was lowered into the NAc. The electrodes were connected to a head-mounted amplifier attached to a commutator (Crist Instrument Company). Electrodes were conditioned at 60 Hz for 15 min with a triangular waveform (−0.4 V to 1.3 V vs Ag/AgCl, 400 V/s), followed by 15 min of cycling at 10 Hz. The microelectrode position was optimized by monitoring naturally occurring and electrically evoked (biphasic, 2 ms/phase, 24 pulses, 60 Hz, 125 μA) dopamine release. All data included in this work were from subjects whose electrically evoked dopamine release exhibited a signal-to-noise ratio of at least 30. Stimulated dopamine release was evoked at the end of each session to ensure neuronal viability, and the electrode response was calibrated *in vitro*. A separate set of animals exhibited naturally occurring dopamine transients but minimal or no stimulated release, suggesting a surgical misplacement of the combination bipolar stimulating electrode/steel guide microinjection cannula in the VTA. These were used as misplacement controls. Drugs (Sigma Aldrich) were administered with a syringe pump (Kent Scientific Corporation, 0.5 μl for 60 s) unilaterally via an infusion cannulae (33 gauge) inserted into the implanted guide.

Experiment 1. Experiments consisted of 2 min of baseline collection, 4 min of recording during and after microinfusion, and an electrical stimulation that evoked dopamine release ($n = 6$). The first microinfusion into the VTA consisted of saline (0.9%), and the process was repeated 1 hour later with microinfusion of lidocaine (350 nmol/0.5 μl, dissolved in sterile saline; pH 6). On the next day, a similar experiment was done with these animals to evaluate NMDA receptor effects. Microinfusions of saline into the VTA were followed 1 hour later by microinfusion of NMDA (0.2 nmol) or (±)2-amino,5-phosphopentanoic acid (AP-5; 5 nmol) (dissolved in 0.5 μl of sterile saline), randomly selected. Two hours later the other NMDA active compound was microinfused in the same way. Values were expressed as a ratio of postinfusion to preinfusion measurements. The onset of behavioral activation served as the initial time for the frequency measurement following NMDA or AP-5 microinfusion.

Experiment 2. After 2 min of baseline collection saline was microinfused into the VTA ($n = 11$). Thirty seconds into the microinfusion the animal received a computer-controlled, 3.0 mg/kg (i.v.) cocaine administration and recording continued for 90 s. The microinfusion needle was then removed. Following a 2 h rest period, the experiment was repeated with a second 0.5 μl of microinfusion of saline ($n = 5$) or lidocaine ($n = 6$, 350 nmol/0.5 μl, dissolved in saline; pH 6) and an identical systemic cocaine administration. On the next day, a similar experiment was done with the saline control animals to evaluate the effects of NMDA receptors on the cocaine-elicited dopamine release. The first part of the experiment was identical to that done on the previous day. The second part of the experiment (following the 2 h rest period) consisted of an intra-VTA microinfusion of AP-5 (5 nmol, dissolved in 0.5 μl of sterile saline), paired with the systemic cocaine administration

Experiment 3. Rats ($n = 5$) were trained to perform intracranial self-stimulation (ICSS) on an FR1 reinforcement schedule as described previously (Owesson-White et al., 2008). The stimulation current was selected to optimize operant responding (100–150 μA, 60 Hz, 24 biphasic pulses, 2 ms/phase). Initially, the lever was continuously extended and the rats pressed freely. Once criterion responding was achieved (30 consecutive presses), the lever was retracted. Rats were then trained to perform ICSS on an FR1, variable time-out reinforcement schedule. Lever extension was accompanied by simultaneous presentation of an audiovisual cue (67 dB, 1 kHz) tone coupled with a change in the lighting of the experimental chamber) for the first 50 trials. The audiovisual cue was then set to precede lever extension by 2 s for the next 150 trials (Owesson-White et al., 2008) with a random time-out between trials (5–25 s). This paradigm was used for the recording session.

Following training, a carbon-fiber microelectrode was lowered into the NAc and an optimal recording site was found as described above. Animals were allowed to resume operant responding for ICSS. Once a reproducible, cue-evoked increase in dopamine was detected the behavioral session was briefly stopped and saline (0.9%, 0.5 μl) was microinfused into the VTA over 60 s. The needle was removed 60 s after the end of the infusion, and the behavioral session resumed for 50 trials. Next, AP-5 (5 nmol, 0.5 μl) was microinfused in an identical manner.

Data analysis

Background subtraction and digital filtering were done with locally written programs. A nonlinear color scale was used to represent the current (Wightman et al., 2007). Substances were resolved with principal component regression using MATLAB (The MathWorks) (Heien et al., 2005). Dopamine concentration transients were events with a signal-to-noise ratio greater than five, and were characterized with Mini Analysis Software (Synaptosoft).

Statistical analysis

Two-tailed paired Student's *t* tests were used to determine statistical differences in all experiments except in experiment 3, where dopamine fluctuations were analyzed using a one-way ANOVA with Tukey's *post hoc* test for repeated measures. Statistical significance was designated at $p < 0.05$. Statistical analyses were performed using GraphPad Prism 4 Software Version 4.03 for Windows (Graphpad Software).

Histology

Animals were anesthetized with sodium urethane (2 g kg^{-1}, i.p.). NAc recording locations were marked via an electrical lesion. 0.5 μl of a 2% Chicago Sky Blue solution was microinfused into the VTA. Animals were transcardially perfused with saline followed by 10% formalin. Brains were removed, frozen, coronally sectioned at 40 μm, stained with thionin and visualized under a microscope.

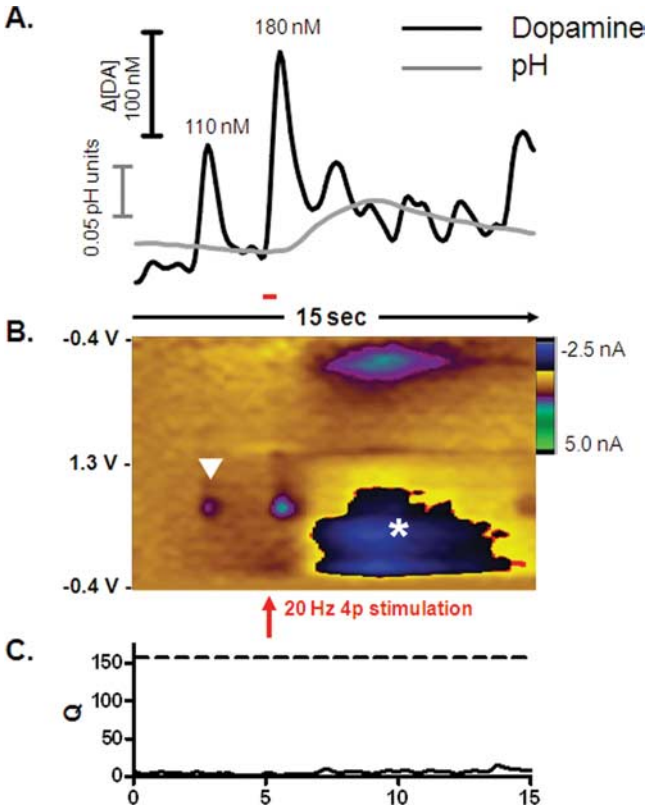

Figure 1. Voltammetric recording from an awake rat showing both naturally occurring (indicated with an inverted white triangle) and electrically evoked (arrow) dopamine release, and a pH shift (white asterisk). ***A***, Dopamine concentration changes and pH shift extracted from the voltammetric data using principal component analysis. ***B***, The color plot contains 150 background-subtracted cyclic voltammograms recorded over 15 s. The ordinate is the potential applied to the carbon fiber electrode, the abscissa is time, and the current is depicted in false color. 4 p, Four pulse. ***C***, The residual shows the principal components describe the data well. The dashed line is the threshold for noise predicted by the principal components.

Results

Dopamine signals in the NAc shell

Transient changes in dopamine concentration that occur following electrical stimulation of the VTA, and in absence of such a stimulation, can be measured using fast-scan cyclic voltammetry (Wightman et al., 2007). Figure 1 shows an example of voltammetric recordings obtained in the NAc shell of a resting, awake rat. At this recording site, frequent dopamine transients were observed that resemble dopamine release evoked by a four pulse, 20 Hz stimulation of the VTA (Fig. 1*A*,*B*). Principal component analysis with a training set made up of representative, background-subtracted cyclic voltammograms for dopamine and pH was used to extract the data from the color plot shown. The low residual (Fig. 1*C*) shows that the retained principal components describe the data well. The current fluctuations due to dopamine release are evident on the positive portion of the voltage scan at the potential where dopamine is oxidized (~0.6 V vs Ag/AgCl). Here, both naturally occurring (indicated by the inverted white triangle) and electrically evoked (red arrow) dopamine fluctuations are evident in the area surrounding the sensor, as well as a delayed and longer-lasting basic pH shift (white asterisk). The current fluctuation due to the pH shift is evident as a blue patch spanning ~ −0.3 V to +0.3 V. pH shifts are typically seen after electrical stimulation of dopaminergic neurons (Cheer et al., 2006).

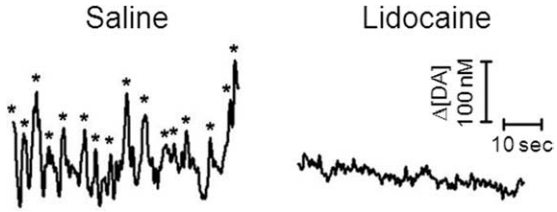

Figure 2. Dopamine transients in the NAc shell of an awake rat are suppressed or enhanced by select agents microinfused into the VTA (experiment 1). Representative dopamine signals after microinfusion of saline (left) and after microinfusion of lidocaine (350 nmol, right). Dopamine transients (with a signal-to-noise ratio >5, asterisks) are eliminated after microinfusion of lidocaine. Dopamine concentration changes were extracted from background-subtracted voltammograms.

Experiment 1: dopamine signaling in the NAc shell is dependent on neuronal activity in the VTA

After the electrode was positioned at a site where transients occurred and dopamine was electrically elicited, we assessed the effects of inactivation of the VTA on these signals. When the sodium channel blocker lidocaine was microinfused into the VTA (350 nmol), both electrically evoked release ($p < 0.05$) and naturally occurring dopamine transients ($p < 0.001$) were significantly attenuated relative to their values following saline infusions. Lidocaine eliminates dopamine fluctuations as shown in a representative location in Figure 2, and the averaged effects are quantified and reported as ratios in Table 1. Intra-VTA lidocaine also reduced the electrically evoked, basic pH shifts in all animals tested (Table 1, $p < 0.05$). In the absence of electrical stimulation, pH shifts are less obvious and were not quantitated further. Dopamine and pH signals recovered in 1–2 h (data not shown). In a separate set of animals ($n = 3$) that did not exhibit electrically stimulated release and were thus used as misplacement controls, the effects of lidocaine microinfusion on naturally occurring dopamine transients were not significantly different from saline control (data not shown).

To investigate whether NMDA receptors in the VTA modulate the occurrence of dopamine transients in the NAc, we applied NMDA receptor-specific agents to the VTA. Local administration of NMDA into the VTA induces locomotor activity; thus we used doses shown to be most effective in generating locomotion to ensure an effective dose (Cornish et al., 2001; Ikemoto, 2004). In Figure 3*A*, four transients (indicated by asterisk) occurred during the 60 s interval shown before intra-VTA microinfusion. Following treatment with the selective NMDA receptor antagonist AP-5 (5 nmol, right panel) dopamine transients were abolished. The recording shown in Figure 3*B* shows data collected from the same animal 2 h later. Three dopamine transients are evident before intra-VTA microinfusion (left), and 9 dopamine transients are evident immediately following the microinfusion of NMDA (right, 0.2 nmol). Figure 3*C* quantifies these effects for all animals following microinfusion of saline ($n = 6$), AP-5 ($n = 6$), or NMDA ($n = 5$). The frequency of transients was unchanged by saline infusions. In contrast, AP-5 significantly decreased the frequency of dopamine transients ($p < 0.05$), whereas the trend following NMDA was an increase in transients that did not achieve statistical significance. For both drugs, the neurochemical and behavioral effects were temporally coincident, with the peak effects lasting for 2 min before neurochemical rebound (data not shown). In the misplacement control subjects ($n = 3$), the effects of AP-5 or NMDA microinfusion on naturally occurring dopamine transients or behavior were not significantly different from saline control (data not shown).

Experiment 2: NMDA receptors in the VTA regulate tonic and phasic dopamine fluctuations elicited by cocaine

A carbon fiber electrode was positioned in the NAc shell at a site that exhibited both naturally occurring dopamine transients occurring at >1 per min and electrically evoked dopamine release. A 1 min microinfusion of saline vehicle was administered, and thirty seconds later cocaine was administered intravenously at a dose (3 mg/kg) demonstrated previously to have robust effects both on dopamine extracellular concentrations and behavior (Di Chiara and Imperato, 1988; Heien et al., 2005).

Administration of cocaine caused a gradual increase in extracellular dopamine concentration in the NAc that appeared to plateau during the 90 s observation time with superimposed phasic dopamine transients. An example is shown in Figure 4*A* (gray); it resembles that reported for an intravenous cocaine injection without an intra-VTA saline microinfusion (Heien et al., 2005). The increase in frequency and amplitude of dopamine transients is termed the phasic response while the more gradual increase in dopamine concentration is termed the tonic response. One hour later, a second intra-VTA saline microinfusion was administered followed 30 s later by a second cocaine administration ($n = 5$). Dopamine concentration changes due to this second cocaine challenge (Fig. 4*A*, black) are the same as those following the initial intravenous cocaine injection. Figure 4, *B* and *C*, shows representative examples using the same protocol but the second VTA microinfusion was of either lidocaine ($n = 6$, 350 nmol) (Fig. 4*B*) or AP-5 ($n = 5$, 5 nmol) (Fig. 4*C*). In both cases the dopamine responses were attenuated. In a separate set of misplacement control subjects ($n = 2$), the effects of AP-5 or lidocaine microinfusion on the cocaine-evoked dopamine response were not significantly different from saline control (data not shown).

Table 1. Effects of VTA inactivation on spontaneous and stimulated responses in the NAc shell (experiment 1)

Treatment	Stimulated DA	Stimulated pH	Relative frequency of spontaneous DA
Saline	1.12 ± 0.28	0.92 ± 0.09	0.94 ± 0.07
Lidocaine	0.01 ± 0.01*	0.15 ± 0.07*	0.08 ± 0.04***

Average effects of intra-VTA saline or lidocaine ($n = 5$ for each microinfusion) on electrically stimulated dopamine release, electrically stimulated basic pH shifts, and relative frequency of naturally occurring dopamine transients (right, averaged over 2 min epochs). Values are expressed as a ratio of postinfusion to preinfusion measurements (*$p < 0.05$, ***$p < 0.001$, Student's *t* test).

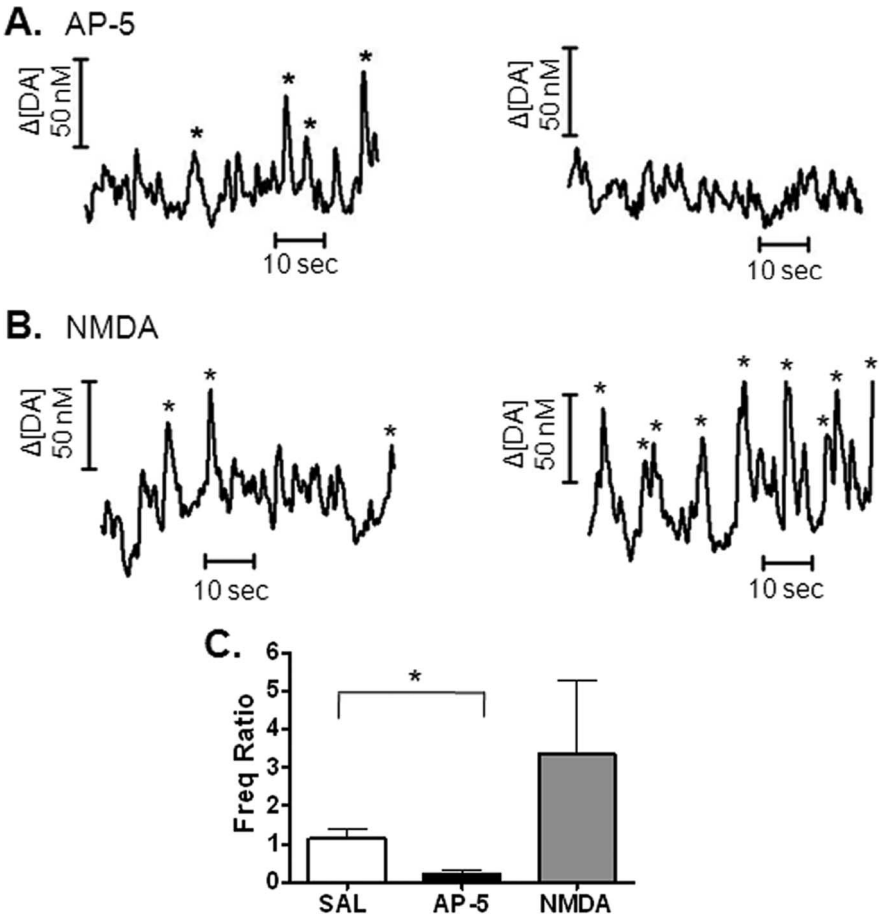

Figure 3. Phasic dopamine signaling is modulated by NMDA receptor-dependent agents in the VTA (experiment 1). ***A***, ***B***, Representative voltammetric data collected in the NAc shell of an awake animal at rest before (left) and immediately after (right) an intra-VTA microinfusion. Dopamine fluctuations are indicated by the asterisks, and are attenuated by microinfusion of AP-5 (***A***) and enhanced by microinfusion of NMDA (***B***). Dopamine concentration changes were extracted from the voltammetric data. ***C***, Average dopamine transient frequency ratio (measured over 2 min) before and after microinfusions of saline ($n = 6$), AP-5 (5 nmol, $n = 6$, *$p < 0.05$), and NMDA (0.2 nmol, $n = 5$).

The tonic increase in dopamine concentration ($[DA]_T$) was quantified as the average cocaine-induced increase in concentration during a 10 s epoch at the end of the sampling period (50–60 s after cocaine administration) relative to that measured in the 10 s epoch immediately before cocaine (Fig. 4, epochs boxed in gray, left). Phasic fluctuations in dopamine concentration were quantitated as the number of dopamine transients measured during the second epoch. The averaged tonic and phasic dopamine responses for all animals ($n = 5$ saline, $n = 5$ AP-5, $n = 6$ lidocaine) are shown in Table 2. Both types of cocaine-induced dopamine responses were significantly diminished by intra-VTA microinfusion of lidocaine or AP-5, but not saline.

Experiment 3: NMDA receptors in the VTA regulate cue-induced phasic dopamine fluctuations

We previously reported enhanced rapid dopamine signaling relative to cues during ICSS (Owesson-White et al., 2008). Rats were trained to depress a lever to deliver an electrical stimulation (60 Hz, 24 biphasic pulses, 125 μA each phase) to their VTA. Lever extension was preceded by 2 s with an audio-visual cue. Consistent with our prior reports, presentation of the cue predicting ICSS availability elicits a time-locked, transient increase in extracellular dopamine concentration in the NAc shell that precedes the electrically stimulated dopamine response (Fig. 5*A*). Intra-VTA microinfusion of saline did not significantly alter the cue-evoked dopamine release (Fig. 5*A*, left) while intra-VTA microinfusion of AP-5 dramatically decreased it (Fig. 5*A*, right). Interestingly, intra-VTA application of AP-5 also decreased electrically stimulated dopamine release in the NAc (Fig. 5*A*, right). Across all animals ($n = 5$), intra-VTA microinfusion of AP-5 produced a significant attenuation ($p < 0.001$) (Fig. 5*B*) in the cue-evoked response that exhibited postinfusion recovery and a significant attenuation in stimulated release ($p < 0.001$) (data

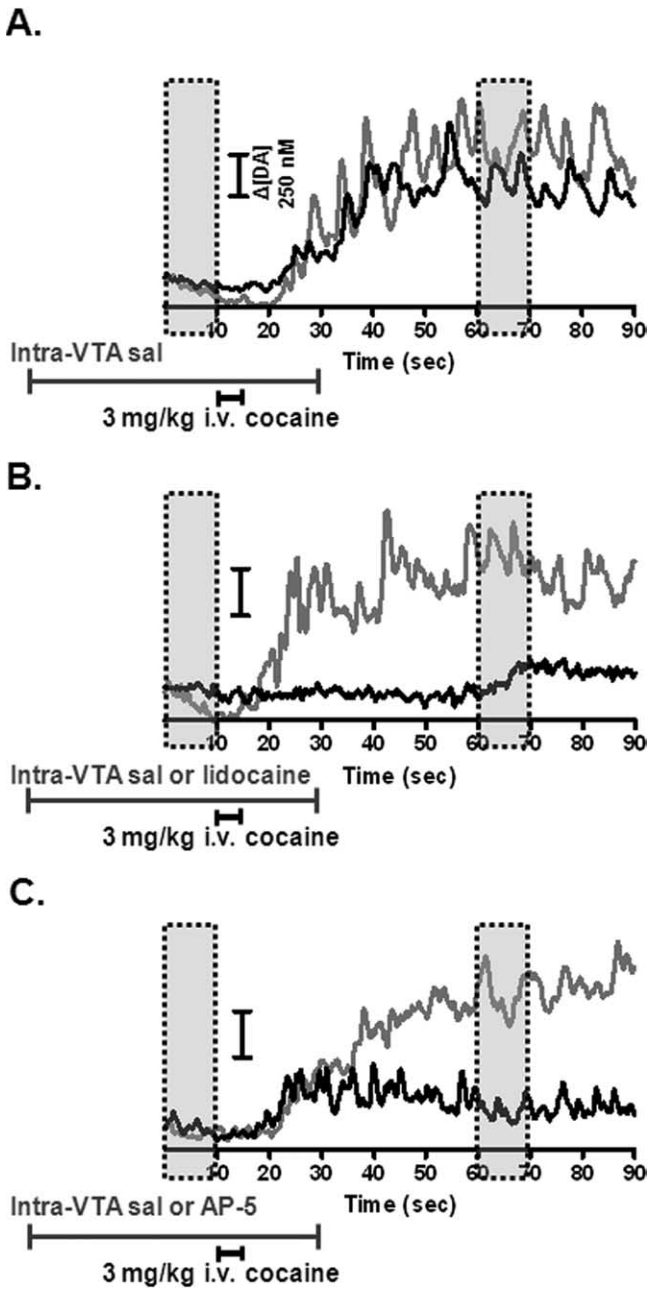

Figure 4. NMDA receptors in the VTA regulate phasic and tonic dopamine in the NAc shell elicited by intravenous cocaine administration (experiment 2). ***A–C***, Each panel contains two superimposed concentration traces collected in a single animal. The animals first received an intra-VTA saline microinfusion during which cocaine was administered intravenously (gray). A second systemic cocaine administration was given 2 h later while saline ($n = 5$) (***A***), lidocaine (350 nmol, $n = 5$) (***B***), or AP-5 (5 nmol, $n = 6$) (***C***) was infused into the VTA (black). Scale bar is the same in all traces.

Table 2. NMDA receptors in the VTA regulate phasic and tonic dopamine in the NAc shell elicited by intravenous cocaine administration (experiment 2)

Treatment	$[DA]_T$ (nM)	Transient count/10 s
Saline 1	396 ± 140	2.5 ± 0.6
Saline 2	262 ± 61	2.3 ± 0.9
Saline	266 ± 73	3.6 ± 0.6
Lidocaine	36 ± 27*	0.6 ± 0.4**
Saline	371 ± 58	2.6 ± 0.5
AP-5	74 ± 59*	1.0 ± 0.4*

Intra-VTA microinfusion of AP-5 or lidocaine significantly decreased both the average dopamine concentration increase ($[DA]_T$) and the number of phasic events elicited by cocaine compared with microinfusion of saline (*$p < 0.05$, **$p < 0.01$, Student's t test).

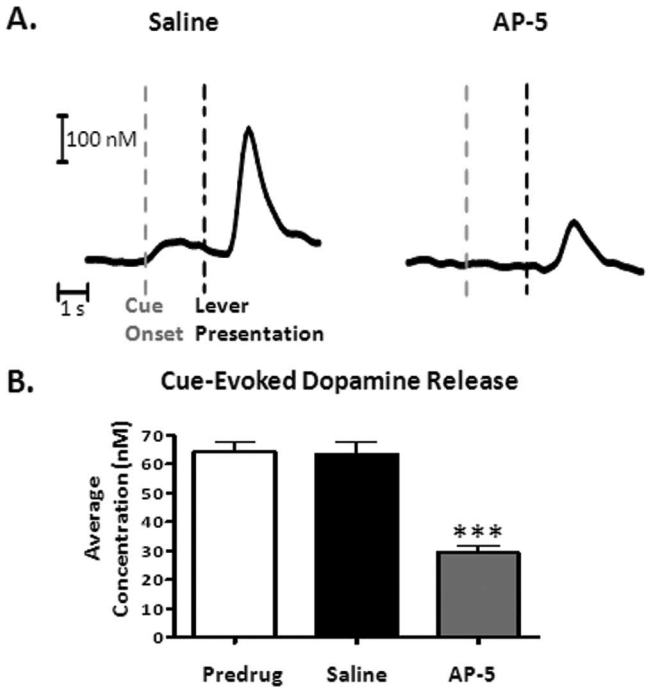

Figure 5. NMDA receptors in the VTA regulate phasic dopamine elicited by reward-predictive cues (experiment 3). ***A***, Representative dopamine concentration trace after microinfusion of saline (left) is attenuated after AP-5 microinfusion (right). ***B***, Average cue-evoked dopamine concentration changes for 50 trials, $n = 5$ rats. Intra-VTA microinfusion of AP-5, but not saline, significantly decreased the dopamine concentration change elicited by the cue (***$p < 0.001$).

Table 3. Average latency to press for ICS (experiment 3)

Treatment	Latency
Saline	0.89 ± 0.18
AP-5	1.89 ± 0.38**

The average latency to lever press was significantly increased by intra-VTA AP-5 (**$p < 0.01$, Student's t test) but not saline. Values are expressed as a ratio of postinfusion to preinfusion measurements.

not shown). The average latency to lever press following lever extension was significantly increased after microinfusion of AP-5 relative to saline values ($p < 0.01$) (Table 3).

Histology

Histological examination of electrode placements in select animals revealed that recordings were made in the NAc shell; see Figure 6*A* for details. For visualization of the region of the VTA affected by microinfusion, 0.5 μl of a 2% Chicago Sky Blue solution was microinfused immediately before fixation. The location of each cannula tip is shown in Figure 6*B*. Subjects included in this work ($n = 14$, black) showed dye covering ~70% of the VTA, defined as the paranigral, the parainterfascicular, the parabrachial pigmented nuclei and the rostral VTA (Paxinos and Watson, 2005). In misplacement control subjects that did not exhibit stimulated dopamine release ($n = 5$, gray), dye covered less than ~10% of the VTA.

Discussion

Using fast-scan cyclic voltammetry at carbon fiber microelectrodes in freely moving rats, subsecond, dopamine-

concentration fluctuations (transients) can be detected in select locations of the NAc (Wightman et al., 2007). Dopamine transients become more pronounced following administration of drugs of abuse (Cheer et al., 2007b), and they become time locked to cues that predict reward availability (Phillips et al., 2003a; Roitman et al., 2004; Stuber et al., 2005; Day et al., 2007; Owesson-White et al., 2008). Here, we establish that dopamine transients observed during these three distinct conditions depend on neuronal activity in the VTA. Although factors such as neuronal firing history and presynaptic mechanisms may modulate synaptic dopamine overflow, this work confirms that NMDA receptor-mediated firing of dopamine neurons in the VTA is a major factor underlying rapid dopamine neurotransmission in the NAc shell.

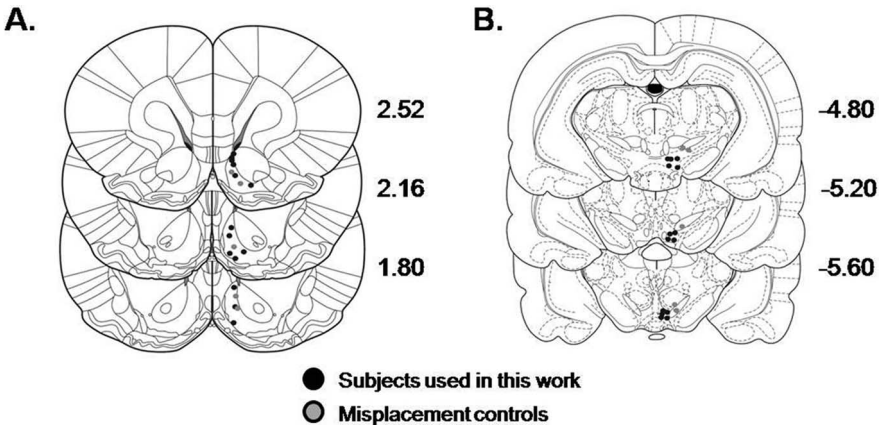

Figure 6. ***A***, Distribution of carbon fiber microelectrode placements in the NAc shell. Coronal diagrams show electrode tip locations for 14 subjects used in this study (black) and for 5 VTA misplacement control subjects (gray). ***B***, Distribution of combination bipolar stimulating electrode/steel guide cannulae placements in the VTA for the same animals. Numbers to the right indicate the anteroposterior coordinates (±0.2 mm) relative to bregma. Coordinates and drawings were taken from a stereotaxic atlas (Paxinos and Watson, 1997).

We previously hypothesized that dopamine transients arise from phasic firing of dopaminergic neurons (Wightman et al., 2007). Modeling of dopamine terminal activity during phasic firing predicts increased extracellular dopamine (Arbuthnott and Wickens, 2007) due to a decreased time between action pulses that allows less time for uptake (Venton et al., 2003), consistent with the results of Figure 1. Such an accumulation of dopamine enables activation of both low affinity as well as high affinity dopamine receptors (Richfield et al., 1989), and suggests functionally distinct roles arising from tonic and phasic firing modes. Further support that dopamine transients arise from phasic firing comes from the similarity between the rate of transients observed and the rate of action potential bursts exhibited by the majority of dopaminergic neurons in the VTA of ambulant rats (Hyland et al., 2002). Consistent with this evidence, naturally occurring dopamine transients, as well as electrically stimulated release, were abolished by nonspecific inactivation of the VTA via lidocaine microinfusion in experiment 1 (Fig. 2, Table 1). Furthermore, the pH changes that are evoked by electrical stimulation, and are a consequence of changes in local blood flow and metabolism (Cheer et al., 2006), were also abolished by microinfusions of lidocaine (Fig. 2, Table 1). pH changes occur independently of dopamine release (Cheer et al., 2006); however, the present data suggests that they also originate from presynaptic activity of VTA neurons that project to the NAc.

As reviewed by Fields et al. (2007), excitatory projections to the VTA include glutamatergic inputs from the lateral hypothalamus (LH) (Rosin et al., 2003), bed nucleus of the stria terminalis (Georges and Aston-Jones, 2002), the superior colliculus (Geisler and Zahm, 2005) and a large excitatory input from the prefrontal cortex (PFC) (Sesack and Pickel, 1992). Additionally, two groups of mesopontine tegmental area neurons provide a large excitatory input to the VTA: the pedunculopontine tegmental nucleus (PPTg) and the laterodorsal tegmental nucleus (LDT) (Semba and Fibiger, 1992). The NMDA receptors located on dopaminergic neurons are major targets of these inputs, and electrophysiological studies have shown that application of NMDA into the VTA produces phasic firing in putative dopamine neurons (Johnson et al., 1992; Chergui et al., 1993). *In vitro* studies in VTA slices have shown that the non-NMDA agonists kainate or quisqualate do not induce bursts (Johnson et al., 1992), and have also shown that application of NMDA antagonists block glutamate-induced firing rate increases (Wang and French, 1993). Iontophoretic ejections of NMDA receptor antagonists into the VTA potently regularized the discharge pattern of phasic firing cells (Overton and Clark, 1992; Chergui et al., 1993). However similar ejections of CNQX, a competitive AMPA/kainate glutamate receptor antagonist, were unable to affect the firing pattern (Chergui et al., 1993). The effects of microinfusion of NMDA were compared with those induced by non-NMDA excitatory agonists. While all agonists increased the firing rate of putative dopaminergic neurons; only NMDA evoked a phasic firing pattern (Suaud-Chagny et al., 1992; Chergui et al., 1993). Using differential pulse amperometry in anesthetized rats treated with pargyline, NMDA was shown to be twice as potent as quisqualate at evoking NAc dopamine release (Suaud-Chagny et al., 1992).

Since the control of bursting activity by NMDA receptors is well documented, we microinfused the NMDA antagonist, AP-5 into the VTA. Like lidocaine, AP-5 inhibited the occurrence of dopamine transients (Fig. 3). This result is consistent with prior microdialysis studies in freely moving animals that showed a decrease in extracellular dopamine levels following intra-VTA administration of AP-5 (Karreman et al., 1996; Kretschmer, 1999). The diminished transient frequency after AP-5 microinfusions demonstrates that NMDA receptors are occupied with endogenous ligand in freely moving animals, and the lack of significance in our NMDA microinfusion results may indicate that further activity is difficult to promote with NMDA microinfusion alone. Indeed, only small dopamine increases were found with microdialysis using similar doses of NMDA (Karreman et al., 1996; Kretschmer, 1999).

Dopamine transients are significantly enhanced when the dopamine transporter is inhibited (Stuber et al., 2005; Cheer et al., 2007b; Aragona et al., 2008). Specifically, intravenous injection of cocaine increases the transient frequency in the NAc and also causes a gradual increase in extracellular dopamine (Heien et al., 2005). The gradual increase is consistent with uptake inhibition accompanying continued tonic firing (Venton et al., 2003). While it has long been known that dopamine release by cocaine requires neuronal action potentials (Nomikos et al., 1990), the cocaine-induced increase in rapid dopamine transients was not originally anticipated because cocaine tends to decrease the firing rate of VTA neurons (Einhorn et al., 1988), presumably due to D_2

autoreceptor-mediated inhibition of firing (Bunney et al., 2001; Shi et al., 2004). The results of experiment 2 show that both tonic ($[DA]_T$) and phasic (transients) cocaine-induced increases in dopamine concentrations in the NAc shell are virtually eliminated with VTA inactivation by lidocaine (Fig. 4*B*, Table 2). Thus, ongoing firing of VTA neurons is necessary for cocaine to elevate dopamine concentrations in the NAc. Similarly, NMDA-receptor blockade attenuated the effects of cocaine on dopamine concentrations, demonstrating that glutamatergic activation of the VTA is a necessary component of cocaine-induced dopamine changes in the NAc (Fig. 4*C*, Table 2). Indeed, it has been shown by microdialysis that acute cocaine administration elevates extracellular VTA glutamate (Kalivas and Duffy, 1995). Consistent with this, intra-VTA application of ionotropic glutamate receptor antagonists reduces the rewarding effects of cocaine (Harris and Aston-Jones, 2003; Sun et al., 2005; You et al., 2007). Even a single exposure to cocaine potentiates NMDA receptor function in the VTA (Schilström et al., 2006), and with repeated cocaine treatments or cocaine self-administration this effect may become even more prominent.

Like the burst firing of dopaminergic neurons (Schultz, 1998), dopamine transients in the NAc become time-locked to cues that predict reward availability in well trained animals (Phillips et al., 2003a; Roitman et al., 2004; Stuber et al., 2005; Cheer et al., 2007a; Day et al., 2007; Owesson-White et al., 2008). Furthermore, NMDA receptors in the VTA play a key role in the acquisition of reward-related learning (Zellner et al., 2008). Thus, experiment 3 examined whether cue-evoked dopamine signals and behavior during ICSS were altered by pharmacological inhibition of VTA phasic firing. Intra-VTA microinfusion of AP-5 produced a significant decrease in cue-evoked dopamine release in the NAc (Fig. 5*A*,*B*). At the same time the latency to lever press for ICSS was increased (Table 3). The NAc is thought to act as a limbic-motor interface, integrating information from limbic and cortical afferents and influencing goal-directed behavior via its efferent projections (Goto and Grace, 2005; Nicola et al., 2005). Thus, fluctuations in NAc dopamine are anticipated to influence behavioral output. The simultaneous attenuation of cue-induced dopamine transients and delayed lever pressing behavior caused by AP-5 microinfusion provide direct support for this hypothesis. The application of AP-5 to the VTA also significantly attenuated electrically stimulated dopamine release in the NAc, suggesting that electrically stimulated dopamine release is an indirect response resulting from glutamatergic innervation of the VTA. Indeed, direct activation of dopamine neurons is unlikely to occur with electrical stimulation to the cell bodies because they have thin, unmyelinated axons and exhibit high thresholds of activation (Ranck, 1975; Yeomans et al., 1988; Yeomans, 1989; Anderson et al., 1996; Nowak and Bullier, 1998).

Much of our knowledge of the role of dopamine has come from single-unit electrophysiological recordings. However, identification of VTA dopamine neurons by electrophysiological characteristics can be ambiguous (Margolis et al., 2006). Here we provide unequivocal confirmation that dopamine release in terminal fields follows the expectations of the classic electrophysiological studies. In the NAc shell, extracellular dopamine levels fall when phasic activity in the VTA is disrupted or when the VTA is inactivated. Even more intriguing, this relationship is maintained whether dopamine fluctuations in the NAc are spontaneous in animals at rest, pharmacologically induced by cocaine administration, or evoked by ICSS reward-predictive cues. This suggests that NMDA-dependent phasic firing of dopamine cells is a mechanism that is broadly operant and not solely associated with reward-related stimuli. Dopamine neurons are conditional output neurons capable of switching between tonic and phasic firing patterns (Floresco et al., 2003), and this work shows that activation of NMDA receptors is necessary to enable the switch. Further research into other neuronal mechanisms underlying phasic firing will increase our understanding of reward-related behaviors and disease states, including addiction.

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