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Dopamine release is heterogeneous within microenvironments of the rat nucleus accumbens

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Abstract

Many individual neurons within the intact brain fire in stochastic patterns that arise from interactions with the neuronal circuits that they comprise. However, the chemical communication that is evoked by these firing patterns has not been characterized because sensors suitable to monitor subsecond chemical events in micron dimensions have only recently become available. Here we employ a voltammetric sensor technology coupled with principal component regression to examine the dynamics of dopamine concentrations in the nucleus accumbens (NAc) of awake and unrestrained rats. The sensor has submillimeter dimensions and provides high temporal (0.1 s) resolution. At select locations spontaneous dopamine transient concentration changes were detected, achieving instantaneous concentrations of ~50 nM. At other locations, transients were absent even though dopamine was available for release as shown by extracellular dopamine increases following electrical activation of dopaminergic neurons. At sites where dopamine concentration transients occur, uptake inhibition by cocaine enhances the frequency and magnitude of the rapid transients while also causing a more gradual increase in extracellular dopamine. These effects were largely absent from sites that did not support ongoing transient activity. These findings reveal an unanticipated spatial and temporal heterogeneity of dopamine transmission within the NAc that may depend upon the firing of specific subpopulations of dopamine neurons.

Introduction

The brain is comprised of billions of neurons that are in constant communication with one another by the secretion of neurotransmitters. These neurons are organized into specific networks that regulate the behavioral state of the animal. The electrical firing of individual neurons can be monitored with electrophysiological microelectrodes, and such recordings have shown that many neurons fire randomly in animals at rest, while the firing pattern of neurons within circuits becomes synchronized during certain behaviors (Nicholls *et al*., 1992). Until recently the chemical communication that is an anticipated consequence of increased neuronal firing was only inferred. However, voltammetric microelectrodes provide an approach to monitor neurotransmitter concentrations in the extracellular fluid of the brain in real time (Adams, 1990). We have used carbon-fiber microelectrodes with fast-scan cyclic voltammetry for the detection of dopamine, a neurotransmitter with a large number of terminals in the nucleus accumbens (NAc) whose cell bodies originate in the ventral tegmental area (VTA). These neurons have been extensively studied because they play a central role in the mediation of reward-related behaviors (Schultz, 1998; Ikemoto & Panksepp, 1999; Wise, 2004; Dommett *et al*., 2005).

When used in behaving rats at rest, spontaneous periodic increases in dopamine concentration occur, termed dopamine transients (duration of 1–2 s), that increase in frequency in the presence of another rat (Robinson *et al*., 2002). Recent technological advances have improved sensitivity to the nanomolar range (Venton *et al*., 2002; Heien *et al*., 2003), and the resolving power has been improved by the use of principal component regression (Heien *et al*., 2004, 2005). These improvements have allowed the measurement of dopamine transients synchronized to specific cues during drug seeking (Phillips *et al*., 2003b) and the acquisition of natural rewards (Roitman *et al*., 2004). We have also shown that dopamine transients increase in amplitude following administration of drugs that inhibit uptake by the dopamine transporter (DAT; Robinson & Wightman, 2004; Stuber *et al*., 2005a).

In this work we further characterize the temporal and spatial dynamics of extracellular dopamine in the brain of freely moving animals measured with this technology. Although the identification of naturally occurring dopamine transients associated with reward seeking has provided important insight into the role of rapid dopamine release in reward-related processing (Carelli & Wightman, 2004), a number of critical questions remain unanswered regarding the fundamental chemical dynamics of rapid dopamine transients in awake animals at rest. For example, are spontaneous dopamine release events ubiquitous or are they spatially localized within discrete microenvironments in the NAc? During behavioral tasks dopaminergic

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neurons are thought to fire synchronously, causing increased dopamine release throughout the striatum (Schultz, 1998); does this occur in animals at rest? What are the characteristics of these transients in terms of concentration and frequency? How do transients change following uptake inhibition with a pharmacological agent such as cocaine? Here, we address these questions and provide critical insight into the fundamental properties and organization of rapid dopamine release events in awake rodents.

Materials and methods

Animals and surgery

Protocols were in accordance with NIH guidelines (publication 86–23) and approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill. Male Sprague–Dawley rats ($n = 23$, 250–350 g, Charles River Laboratories, Wilmington, MA, USA), implanted with a jugular vein catheter (Stuber *et al*., 2005a), were housed individually on a 12 : 12 h light cycle (lights on at 07.00 h), with *ad libitum* access to food and water. Three days before experiments, rats were anesthetized with ketamine hydrochloride (100 mg/kg i.p.) and xylazine hydrochloride (20 mg/kg i.p.), and placed in a stereotaxic frame for implantation of electrodes and cannula (Phillips *et al*., 2003a). A guide cannula (Bioanalytical Systems, West Lafayette, IN, USA) was positioned above the NAc shell (1.7 mm anterior, 0.8 mm lateral, −2.5 mm ventral relative to bregma). The Ag/AgCl reference electrode was placed contralateral to the guide cannula beneath the meningeal membrane, and both it and the guide cannula were permanently attached with cranioplastic cement. A locally constructed microdrive (University of North Carolina at Chapel Hill, Department of Chemistry Instrument Shop) containing a carbon-fiber electrode was inserted into the guide cannula and the electrode was lowered into the NAc. In all but four animals, a bipolar stimulating electrode (Plastics One, Roanoke, VA, USA) was lowered into the VTA (5.2 mm posterior, 1.0 mm lateral, 7.5 mm ventral relative to bregma) until electrically evoked dopamine release was observed at the carbon-fiber electrode. The electrical stimulation was a biphasic (2 ms/phase), 60 pulse, 60 Hz, 125 μA train delivered with a stimulus isolator (A-M Systems, Carlsborg, WA, USA). Once dopamine release was observed, the stimulating electrode was permanently secured in place.

Fast-scan cyclic voltammetry

Glass-encased carbon-fiber (5 μm diameter, 50–100 μm length) electrodes were constructed as previously described (Phillips *et al*., 2003a). The Ag/AgCl reference electrodes were prepared from silver wires (0.5 mm diameter, Sigma-Aldrich, St Louis, MO, USA) chloridized in 0.1 N HCl.

Cyclic voltammograms were obtained with a triangular waveform (−0.4–1.3 V vs Ag/AgCl, 400 V/s) repeated every 100 ms. The carbon-fiber electrode was held at −0.4 V between scans. Waveform generation and data collection employed a multifunction data acquisition board (PCI-6052E, National Instruments, Austin, TX, USA) controlled with programs written in LabVIEW (National Instruments). A PCI-6711E board (National Instruments) was used to synchronize waveform acquisition, data collection and stimulation delivery (Heien *et al*., 2003). Waveform processing and current transduction employed locally built instrumentation (University of North Carolina Department of Chemistry Electronics Facility). Data were collected at 107 kHz, and the output signal was low pass filtered at 30 kHz before being digitized.

Positioning of the carbon-fiber electrode

Following recovery from surgery, a fresh carbon-fiber electrode was lowered into the NAc with the microdrive. Typically the electrode was lowered to the upper extent of the NAc at a rate of 1 mm/min. One revolution of the wheel on the microdrive lowers the electrode by 300 μm. In some experiments, the electrode was lowered in 80-μm increments through the NAc. At each location the frequency of spontaneous transients was determined as well as the maximal dopamine concentration electrically evoked (biphasic pulses, 2 ms/phase, 24 pulses, 60 Hz, 125 μA). On average, the electrode was equilibrated at each site for at least 3 min. In other experiments, the electrode was lowered until a site was found that supported robust electrically stimulated release. The frequency of spontaneous transients was recorded, and then saline and cocaine were administered and the frequency of transients was re-examined.

Cocaine infusion experiments

After 15 min of baseline collection, saline was infused (i.v., 20 s/infusion), followed by 20-s infusions of 0.3 mg/kg, 1.0 mg/kg and 3.0 mg/kg cocaine at 15-min intervals. Cocaine evoked significant changes in dopamine concentration at all doses ($P < 0.05$, Wilcoxon signed rank test); in this work only the results from the higher two doses are presented. During drug administration, data files (90 s duration) were collected continuously. After the experimental session, the carbon-fiber electrode was removed and calibrated with dopamine and ±0.1 pH unit changes in a flow-injection analysis system (Phillips *et al*., 2003a).

Data analysis

Cyclic voltammograms were digitally filtered (4-pole Bessel filter, 2 kHz) and background subtracted using 10 voltammograms collected at the beginning of a file (Phillips *et al*., 2003a) with locally written LabVIEW programs. To initially evaluate results, data sets were viewed with the current in false color, the abscissa as time and the applied potential as the ordinate (Michael *et al*., 1998). Changes in dopamine concentration were quantified with principal component regression to remove contributions from pH changes (Heien *et al*., 2005). Principal component regression employed MATLAB (The MathWorks, Natick, MA, USA; Heien *et al*., 2004) using training data for dopamine and pH changes measured *in vivo* and generated by electrical stimulation (Heien *et al*., 2005). Prior work has shown that these two components are the primary contributors to changes in current following cocaine. The number of factors retained, typically five, was selected so that >99.5% of the variance could be captured. The quality of the fit was evaluated by reproducing the data set with the regression values and evaluating the residual (Heien *et al*., 2005). The residuals for all reported results were at a level beneath the 95% confidence level. pH changes resolved by this procedure have been reported elsewhere (Heien *et al*., 2005). While changes in dopamine concentration can be measured with this technique, basal levels cannot be determined because the use of background subtraction makes the method a differential one (Michael *et al*., 1998).

Fluctuations in the dopamine signal obtained from principal component regression were considered concentration transients if they had a signal-to-noise ratio greater than five. The transient characteristics [amplitude ([DA]), half-width ($t_{1/2}$), intertransient interval (ITI)] were determined with Mini Analysis (Synaptosoft, Decatur, GA, USA), a program written to characterize transient events. In the animals without stimulating electrodes, dopamine transients

were evaluated by manual comparison of voltammograms using a differential method described previously (Heien *et al.*, 2005). The cyclic voltammograms were from *in vitro* calibrations and responses following cocaine. This procedure is less sensitive than the principal component regression method.

Immunocytochemistry

Rats of similar age to those used with cyclic voltammetry recordings were anesthetized with urethane (1.5 g/kg) and perfused transcardially with saline followed by 4% paraformaldehyde. Their brains were removed and postfixed for 1 h in the same fixative and then stored at 4 °C in phosphate-buffered solution (PBS, pH 7.4) for 24 h. Coronal brain slices (150 μm thick) containing the NAc shell were prepared using a vibratome. Slices were preblocked for 2 h with agitation at room temperature in PBS with 1% bovine serum albumin, 10% normal goat serum and 0.3% Triton X-100. Slices were then agitated at 4 °C for 20 h in the block solution containing a rabbit anti-DAT polyclonal antibody (1 : 100) and an anti-glutamic acid decarboxylase 67-kDa isoform monoclonal antibody (1 : 1000). Slices were rinsed with PBS and then incubated overnight at 4 °C with Cy5 or fluorescein isothiocyanate goat anti-rabbit/mouse secondary antibody (1 : 50). The slices were then washed thoroughly with PBS and mounted on slides using Bio-Rad Fluoroguard Antifade Reagent mounting media. Slices were visualized using a Leica SP2 AOBS laser-scanning confocal microscope. Primary antibodies were obtained from Chemicon International (Temecula, CA, USA), and secondary antibodies from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Statistical evaluation

Data were analysed in GraphPad Prism. Correlations were obtained by linear regression analysis. Tukey's multiple comparison *post hoc* tests were used to elucidate significant differences across treatments ($P < 0.05$).

Chemicals

Unless stated, all chemicals were purchased from Sigma-Aldrich, and were used as received. Solutions were prepared with doubly distilled deionized water (Megapure system, Corning, NY, USA). Stock solutions of dopamine were prepared in 0.1 N $HClO_4$, and 1 μM solutions were prepared in TRIS buffer for calibration on the day of use. The TRIS buffer contained (in mM): TRIS, 15; NaCl, 126; KCl, 2.5; $NaHCO_3$, 25; $CaCl_2$, 2.4; NaH_2PO_4, 1.2; $MgCl_2$, 1.2; Na_2SO_4, 2.0; and was adjusted to pH 7.4.

Histology

Rats were anesthetized with 1.5 g/kg urethane. Rat brains were prepared for verification of electrode placements as described previously (Robinson & Wightman, 2004). In some cases, an electrolytic lesion was made at the electrode tip to ease visualization. In all cases the carbon-fiber was in the NAc.

Results

Signals and noise during fast-scan cyclic voltammetry

Figure 1 shows an example of voltammetric recordings obtained at two locations in an awake unrestrained rat at rest, and these are compared with recordings collected in physiological buffer using the same electrode and recording apparatus after the *in vivo* experiment. In Fig. 1B, the color plot contains 600 background-subtracted cyclic voltammograms recorded for 60 s following saline infusion. Occasional small fluctuations (dark vertical streaks) spontaneously occur on the positive portion of the voltage scan at the potential where dopamine is oxidized (~0.6 V vs Ag/AgCl). Principal component regression is used to extract the dopamine concentration (Fig. 1A), providing a residual (Fig. 1C) that indicates the confidence level of the fit exceeds 95%. The recordings in Fig. 1D–F show more pronounced dopamine transients. These data were collected in the same brain region but at a location 300 μm below the recording site in Fig. 1A–C. At both sites, dopamine release could be electrically evoked,

FIG. 1. Voltammetric recordings from a carbon-fiber electrode implanted in the NAc of an awake animal at rest compared with data collected in physiological buffer. Left panels: recording in an area of the NAc where there is little dopamine activity; middle panels: recording at a site 300 μm ventral to the recording site shown in the left panels where naturally occurring dopamine fluctuations in the NAc are clearly evident; right panels: *in vitro* data collected with the same electrode and recording apparatus after completion of the *in vivo* experiment. (A, D, G) Dopamine concentration extracted from the voltammetric recordings with principal component analysis. (B, E, H) The color plots (Michael *et al.*, 1998) each contain 600 background-subtracted cyclic voltammograms recorded for 60 s. The current is in false color, the abscissa is time, and the ordinate is the applied potential. (E) Occasional fluctuations (dark vertical streaks) occur on the positive portion of the voltage scan at the potential where dopamine is oxidized (~0.6 V vs Ag/AgCl). (C, F, I) The residual provides an index of the confidence level of the fit, which exceeds 95%.

FIG. 2. Spatial heterogeneity of dopamine release. (A) The f_{DA} in a single animal monitored as the carbon-fiber electrode was lowered in 80-μm increments. (B) The maximal dopamine release ($[DA]_{max}$) caused by an electrical stimulation of the VTA was monitored at the same positions represented in (A). (C) The track of the recording sites for (A and B), obtained from histological examination of the tissue, is shown by the dots. (D) Confocal fluorescence micrograph of a coronal slice from the NAc that is cytochemically labeled for both the DAT (green) and the 67-kDa form of GAD67 (purple). We have drawn to scale a 100-μm cylindrical carbon-fiber microelectrode on this micrograph. (E) An expanded dopamine transient. Its amplitude, [DA], was measured as the difference between the maximum (indicated by the red x) and its baseline measured for 1.5 s before the transient (green bar). The red dot marks the beginning of the transient. The width at half-amplitude ($t_{1/2}$) was also measured. (F) Summed green fluorescence intensity (corresponding to DAT labeling) for two rectangles (5 × 100 μm) measured at two locations in the micrograph shown in (D), one 80 μm below the first.

FIG. 3. Increase in dopamine following cocaine administration. Left panels: response after a 20-s i.v. infusion of saline (solid bar). (A) The solid line shows the dopamine concentration changes extracted by principal component regression from the data in the color plot. (B) The color plot contains 900 cyclic voltammograms recorded over the 90-s interval. (C) The residual from the principal component regression remains below the 95% confidence limit (dashed line). Right panels: responses after a 20-s i.v. infusion of 1 mg/kg cocaine (solid bar). (D) The dopamine concentration changes obtained by principal component regression of the data in the color plot are shown as a solid line. (E) Color representation of the voltammograms in the 90-s measurement interval. Note that changes occur at the peak oxidation potential for dopamine (~0.6 V) as well as at other potentials. (F) The residual for the data following cocaine that remains below the 95% confidence limit. In panels (A) and (D) the scale bars are 500 nM.

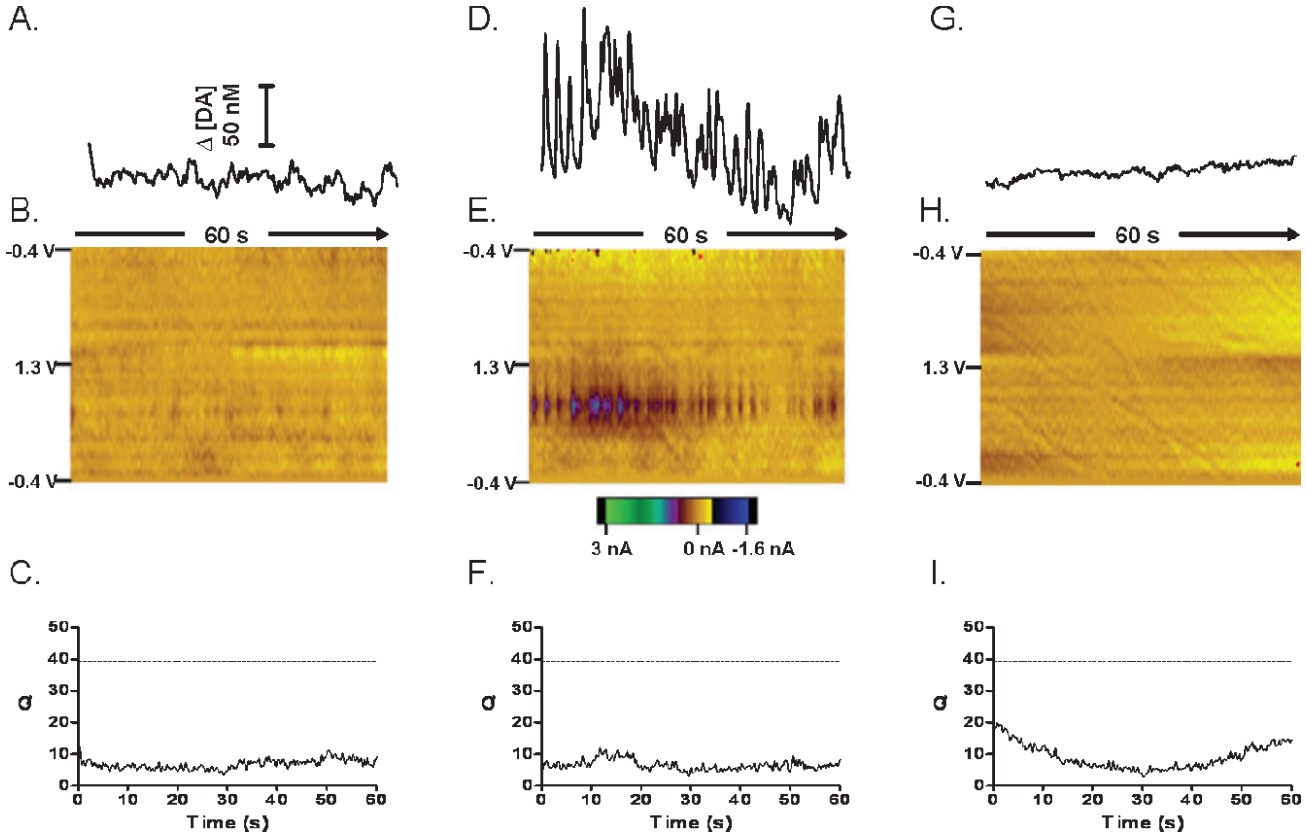

FIG. 1.

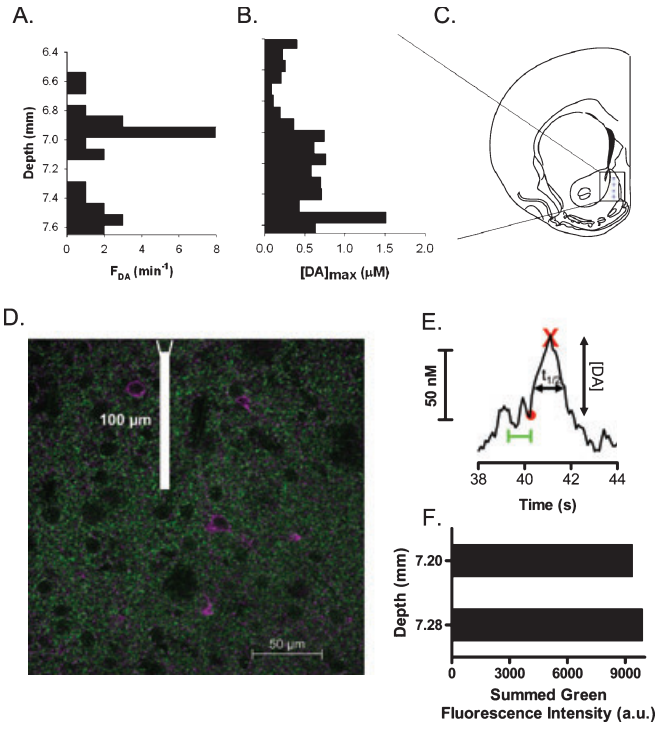

FIG. 2.

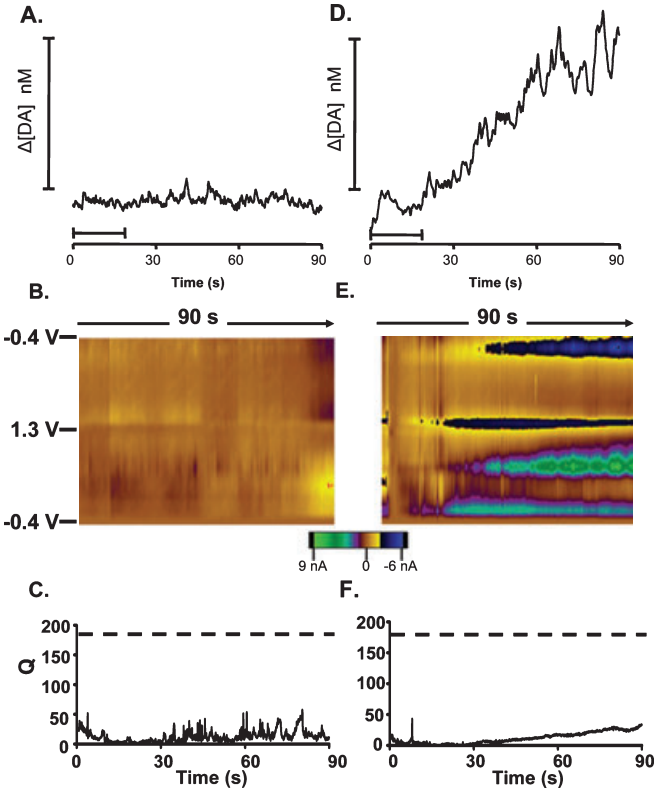

FIG. 3.

demonstrating that releasable neurotransmitter was available. Figure 1G–I was made with the same electrode in a flow injection analysis system with the electrode in physiological buffer. The root-mean-square (rms) value of the trace in Fig. 1A is not statistically different from that measured in the flow cell (Fig. 1G), but the rms value in Fig. 1D is statistically greater ($P < 0.01$, Student's paired *t*-test). These data reveal that the noise inherent to the system is far below the dynamic concentration fluctuations (chemical variability) measured in select locations within the NAc of a rat at rest.

Spatial heterogeneity of dopamine transients

At each site evaluated, dopamine release was evoked by VTA electrical stimulation that was intended to cause synchronous firing of most dopaminergic neurons, a condition that occurs upon presentation of a cue that predicts reward presentation (Schultz, 1998). The electrical stimulation causes a high-frequency burst of firing of dopamine neurons, and the measurement of release provides an indication that the electrode tip is positioned near a site that can support dopamine release. However, when the naturally occurring and spontaneous dopamine transients were recorded at these sites, they showed extraordinary variability, ranging from a robust nature to their complete absence. Figure 2E shows an expanded view of a single dopamine transient and indicates the way in which it can be quantified. Its onset is marked with a red dot. The amplitude of this transient, the maximal dopamine concentration that reaches the voltammetric sensor, is marked with an X. The time before the transient where the baseline was taken is indicated with a green bar, and the width at half-height ($t_{1/2}$) is indicated with a double arrow.

The degree of variability in site-specific transient frequency is demonstrated in Fig. 2A. In this example, when the carbon-fiber electrode was lowered in small (80-μm) increments, transient frequency measured for 60 s at each location varied dramatically (between 0 and 8 transients/min). This was not a function of altered electrode sensitivity, because locations that supported robust stimulated release did not necessarily exhibit a high frequency of dopamine transients, and vice versa (taken from the maximal evoked release, $[DA]_{max}$, example series of measurements in Fig. 2B). Note that at 6.9 mm and 7.2 mm, where the frequency of transients was at a maximum and minimum, respectively, stimulated release is approximately the same. Similar variability was found in the NAc of four other animals examined in this way (data not shown). Furthermore, the spatial variability was not a result of damage to the cell body region caused by implantation of the stimulating electrode, because it was also encountered in another four animals without these electrodes (data not shown).

The high variability of dopamine transient frequency is in contradiction to the apparent homogenous distribution of dopamine terminals when viewed at the 100 μm size of the electrode. Dopamine terminals have dimensions in the micrometer range with a density of $\sim 1 \times 10^8/mm^3$ in this brain region (Arbuthnott & Wickens, 2007). These features can be qualitatively viewed in the confocal fluorescence micrograph in Fig. 2D that shows a coronal slice from the NAc that was cytochemically labeled for both the DAT protein (green) and the 67 kDa form of glutamate decarboxylase (GAD67, purple). DAT is located ubiquitously on dopaminergic terminals (Nirenberg *et al.*, 1997), and thus it provides a measure of the density and distribution of dopamine terminals in this brain region. The label for GAD67 is not co-localized, but rather binds to a different population of nerve processes including cell bodies of medium spiny neurons onto which dopaminergic neurons synapse in the NAc. Drawn to scale on this micrograph is a representation of a 100-μm cylindrical carbon-fiber microelectrode. The length of the microelectrode is much greater than the size scale of dopaminergic terminals that exist in this region. Indeed, when the DAT-labeled green fluorescence intensity was summed over consecutive rectangles (80 μm apart) with similar dimensions to the sensing area of the microelectrode, adjacent regions showed no heterogeneity (Fig. 2F). Thus, we conclude that, in animals at rest, some dopaminergic terminals are actively releasing dopamine while others are releasing nothing or are releasing below the detection limit.

Alteration of dopamine transients by cocaine

Cocaine exerts its behavioral effects, in part, by inhibiting the DAT, the protein that removes dopamine from the extracellular space. This prolongs the lifetime of extracellular dopamine and thus facilitates transient detection (Stuber *et al.*, 2005b). We examined the effects of cocaine on transients at sites that supported electrically stimulated dopamine release but otherwise were randomly selected. At some electrode locations, as in the example in Fig. 3, cocaine caused a slow increase in the baseline concentration of dopamine that reaches an enhanced level. At such sites enhanced dopamine transients are superimposed on the gradually increasing baseline (Fig. 3D; Heien *et al.*, 2005). However, at other locations, cocaine caused little change in dopamine levels when compared with that measured before the injection (not shown). To probe these differences, data gathered from sites that were initially poor in naturally occurring spontaneous dopamine transients were evaluated and compared with sites that exhibited robust naturally occurring transient dopamine release (Fig. 4A and B; data from single sites in 23 animals). We correlated the mean frequency of phasic dopamine transients (f_{DA}) evident immediately following an i.v. cocaine injection (1.0 mg/kg) at each location with f_{DA} after a comparable saline injection. A high correlation was obtained ($r = 0.76$, $P < 0.0001$; Fig. 4A), confirming that locations with frequent dopamine transients after saline are more likely to show an enhanced frequency of transients following cocaine. However, the frequency of transients was uncorrelated with the amplitude of stimulated release ($[DA]_{max}$) at that location both before ($r = 0.10$ following saline, $P > 0.05$) and after cocaine infusion (1.0 mg/kg, $r = 0.08$, $P > 0.05$; Fig. 4B). Collectively, the results reveal that even though sites are capable of release (as shown by the electrical stimulation), extracellular dopamine concentrations are dynamic and heterogeneous throughout the NAc even in an animal at rest.

Characteristics of dopamine transients

Next, we examined the transient characteristics in a subset of the animals used in the cocaine experiment. Of the 23 animals examined before and after cocaine injection, recordings in seven animals were at sites where transient frequencies following saline were greater than 1/min (mean value 1.6 ± 0.2/min). These animals were given a subsequent dose of cocaine (3 mg/kg), and the properties of the transients following this dose were compared with those following saline injection while voltammetrically recording at the same sites. The ITI following saline infusion had a broad distribution (between 1 and >60 s) with a mean of 11 s (Fig. 5A). This distribution became more compact following cocaine infusion, and the average ITI decreased significantly ($P < 0.0001$) to a mean of 4 s. Consistent with this, the observed mean transient frequency following cocaine increased to 10.4 ± 1.4 transients/min. The amplitude distribution

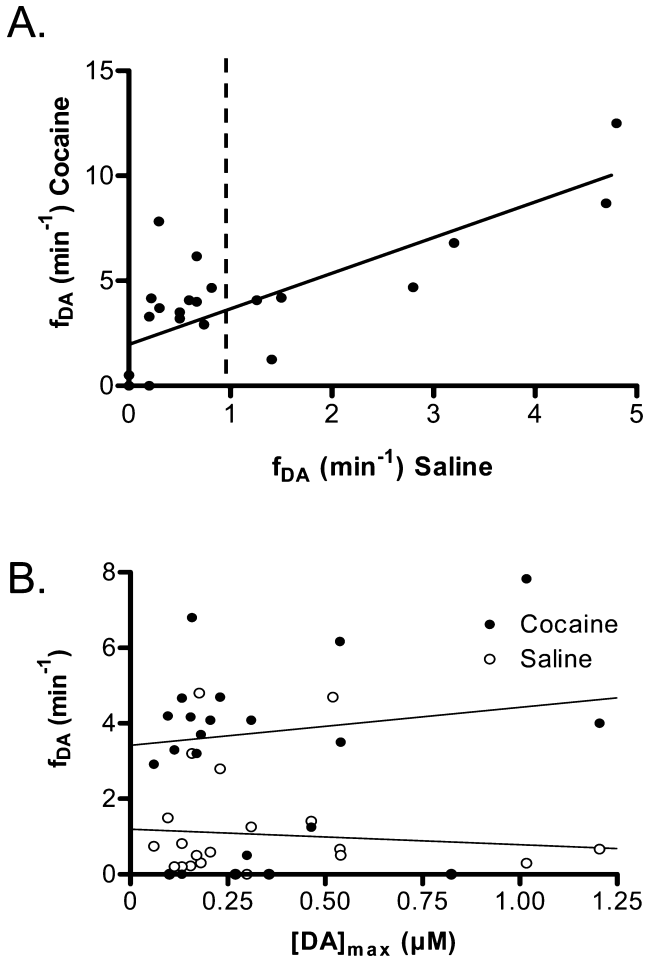

FIG. 4. Characterization of dopamine transient frequency. (A) The f_{DA} evoked by cocaine is correlated with f_{DA} during saline. (B) f_{DA} following saline and following cocaine as a function of the maximal amplitude of electrically stimulated release ($[DA]_{max}$). Measurements from 23 different animals.

of the dopamine transients following saline was skewed, suggesting events occur with amplitudes that are less than our detection limit (Fig. 5B). After cocaine, the distribution was shifted to the right with mean amplitudes increasing from 48 to 72 nM ($P < 0.0001$). The variances before and after cocaine also significantly differ ($P < 0.001$), with $F_{167,1086} = 0.23$. The half-width ($t_{1/2}$) of the transients varied over a broad range (0.4–2.6 s) following saline and significantly increased after cocaine, with mean values increasing from 1.03 s to 1.39 s ($P < 0.0001$, Fig. 5C). The increases in both amplitude and $t_{1/2}$ of the dopamine transients following cocaine resemble those seen for stimulated release of dopamine, indicating they are due to dopamine uptake inhibition by cocaine (Samaha *et al.*, 2004).

Discussion

The results of this study provide a direct view of fundamental aspects of the neurochemical dynamics of the concentration of dopamine in the extracellular fluid of awake rats. Specifically, the results reveal that dopamine concentrations within the NAc are

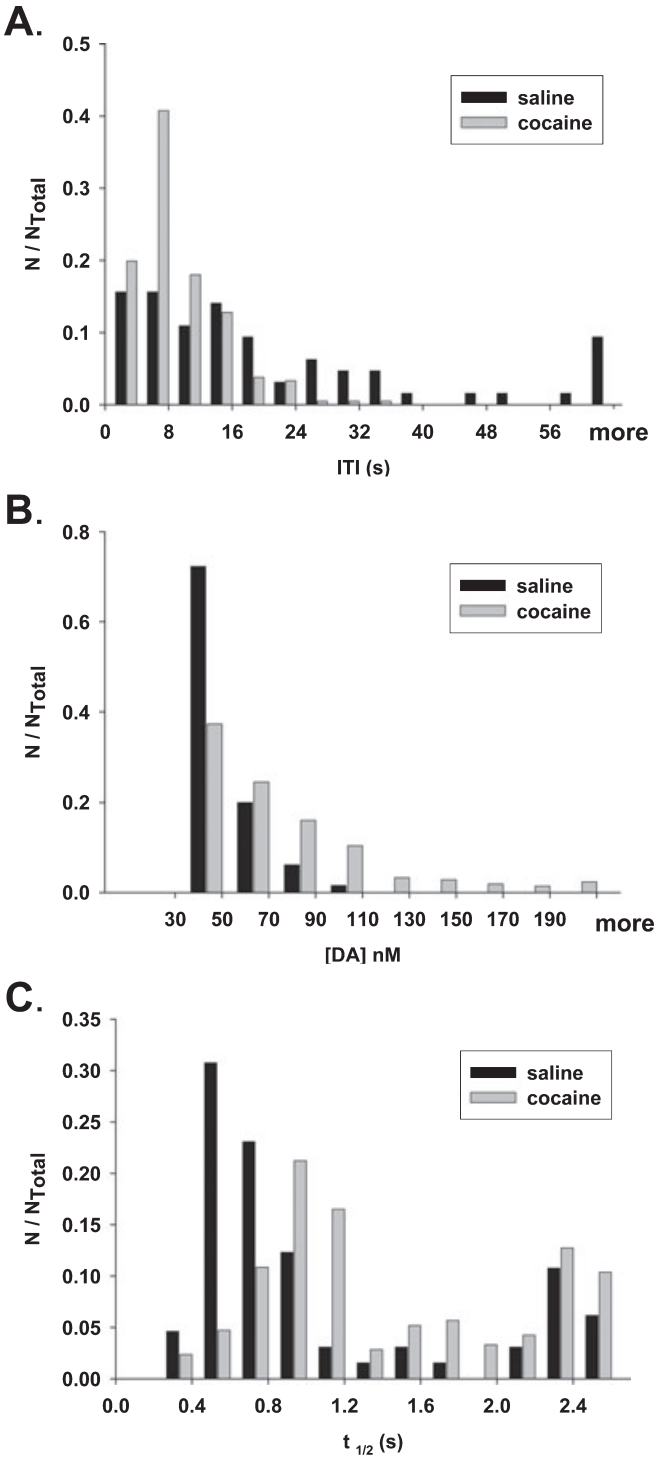

FIG. 5. Properties of dopamine transients in the NAc. (A) The intertransient interval (ITI) for dopamine transients in response to 20-s infusion of saline and 3 mg/kg cocaine. (B) Amplitude histogram of dopamine transients [DA] following both saline and cocaine. (C) Histogram of half-width ($t_{1/2}$) of dopamine transients following both saline and cocaine. Transients are from $n = 7$ rats and were measured at sites that exhibited >1 transient/min following saline. There were 168 transients during the saline epoch and 1087 during the cocaine (3 mg/kg) epoch. Dopamine transients were determined using principal component regression from three consecutive blocks of data, each 90 s long.

spatially heterogeneous and, in some sites, exhibit high-frequency fluctuations, and in others exhibit no activity even though dopamine release can be evoked in these locations. These unforeseen results provide the first direct evidence that dopamine transmission within the NAc depends upon the activation of discrete subpopulations of dopamine terminals in this brain region. Until recently, chemical sensors for all neurotransmitters were either too slow, too insensitive, too large or insufficiently selective to record the naturally occurring fluctuations that occur on the time scale of neuronal firing (Wightman & Robinson, 2002) that are revealed in this work. The spatial heterogeneity is shown by the dramatic variance in frequency of dopamine transients according to the position of the 100-μm-long electrode within the NAc. The temporal heterogeneity is illustrated by the dynamic changes in dopamine over a short time interval as shown in the representative examples of Figs 1 and 3. Collectively, the data reveal both a dynamic signaling profile and a spatial heterogeneity of dopaminergic neurotransmission that have been unrecognized until now.

Since the initial development of *in vivo* voltammetry (Conti *et al*., 1978; Gonon *et al*., 1980), selectivity has been a concern due to the presence of multiple easily oxidized substances. Fast-scan cyclic voltammetry used with principal component regression provides greatly improved selectivity (Heien *et al*., 2004) and can be used in freely moving animals (Garris *et al*., 1997; Rebec *et al*., 1997). Simultaneous improvements in the detection limit for dopamine (Heien *et al*., 2003) now allow detection of naturally occurring dopamine concentration changes in the NAc. However, because the method used requires background subtraction, the measurements are necessarily differential and only reveal the concentration fluctuations around the mean extracellular dopamine value. While this value has been the subject of some controversy (Watson *et al*., 2006), recent microdialysis measurements indicate that the basal level of dopamine is of the order of 20 nM (Shou *et al*., 2006). Because the amplitudes of the transients reported here are sufficiently higher than this reported basal concentration, reaching a concentration sufficient to activate D1 receptors (Richfield *et al*., 1989), naturally occurring transient dopamine concentration fluctuations likely play an important role in normal dopamine communication.

Voltammetric measurements in freely moving animals avoid anesthesia confounds and permit rapid chemical measurements in the brain during behavior. Indeed, dopamine transients are not seen in anesthetized animals, and in that preparation can only be induced by robust pharmacological stimulation (Venton & Wightman, 2007). This indicates that their occurrence requires intact brain circuitry with ongoing neuronal activity. In an awake animal at rest or given saline, they are only observed at select locations, even though sites that lack dopamine transients can support release as shown by their response to electrical stimulation. In locations where dopamine transients are detected, dopamine concentrations fluctuate continually with transients that have a mean amplitude of ~50 nM. This amplitude is about 1/10th of that evoked by 24 pulse, 60 Hz electrical stimulations at sites that support robust release. However, the amplitude difference should not effect the detectability of such events because the evoked concentration is insufficient to fully saturate the DAT (K_m = 0.2 μM), and the time course for diffusion between sites is independent of concentration (Bard & Faulkner, 2001).

The amplitude and half-width of transients exhibit considerable variance, and they occur at irregular intervals. In these locations cocaine not only induces an increase in the tonic extracellular concentration of dopamine in the NAc, but also increases the frequency (Fig. 4), amplitude and half-width of the phasic dopamine concentration transients (Fig. 5). Although the amplitude distribution for data collected after cocaine injection has a greater variance, the two distributions are similarly shaped, suggesting that the frequency of transients before drug may be underestimated. The increased half-width of the transients following cocaine is consistent with its actions on stimulated release because of inhibition of dopamine uptake (Samaha *et al*., 2004). These data directly demonstrate that dopamine transmission can operate in multiple temporal modalities.

The results show that sites that lack dopamine concentration transients are least likely to show them after cocaine (Fig. 4A). Although uptake blockade allows released dopamine to diffuse further and interact with a greater population of neurons (Venton *et al*., 2003; Cragg & Rice, 2004), the prolonged diffusion length is insufficient to eradicate the spatial heterogeneity of dopamine signaling even with the highest dose of cocaine used. Consistent with this observation, in prior work we estimated the diffusion distance to increase from 5 μm to 8 μm following a similar degree of uptake inhibition (Venton *et al*., 2003). Thus, dopamine heterogeneity is maintained even when uptake is inhibited by cocaine. However, to further understand the actions of cocaine, additional experiments are required because the effects of cocaine on dopamine neurotransmission are complex (Lupica *et al*., 2004), affecting both uptake of dopamine and release mechanisms (Venton *et al*., 2006).

Because dopamine communicates extrasynaptically (Cragg & Rice, 2004), the region sampled by the carbon-fiber electrode, these dopamine fluctuations are a normal component of its neurotransmission. The similarity of the characteristics of naturally occurring dopamine transients to dopamine concentration profiles evoked by short, high-frequency stimulations (Venton *et al*., 2003) suggest that dopamine transients originate from phasic firing of dopamine neurons. Indeed, the two–five transients/min observed at some locations is quite similar to the reported frequency of bursts of action potentials exhibited by the majority of dopaminergic neurons that have been termed 'low-bursting' cells (Hyland *et al*., 2002). Bursting of dopamine neurons can originate from inputs such as endocannabinoid retrograde transmission (Cheer *et al*., 2007b) at the cell body level. However, other origins for dopamine transients cannot yet be eliminated. For example, excitatory inputs onto dopaminergic terminals could evoke tonic or phasic dopamine directly within the NAc (Howland *et al*., 2002).

We find it surprising that sites within the NAc that support stimulated release do not always support dopamine transients. In well-trained non-human primates, dopaminergic neurons fire in high synchrony during reward-related tasks (Schultz, 1998). Based on the concept of synchronous firing, the anatomical distribution of dopamine terminals and the kinetics of dopamine uptake with its coupled diffusion, we previously developed a dynamic model of the expected heterogeneity of extracellular dopamine based on findings from electrically stimulated release (Venton *et al*., 2004). This model captures the temporal heterogeneity we observe in naturally occurring spontaneous dopamine release but dramatically underestimates the spatial heterogeneity. Here we find that naturally occurring spontaneous dopamine concentration transients in animals at rest are highly heterogeneous in their spatial occurrence, even when there is little change in the amplitude of stimulated release. Because not all VTA dopaminergic neurons burst fire (Hyland *et al*., 2002), and some are suspected to be silent (Harden & Grace, 1995), the lack of dopamine transients in sites that support stimulated release could indicate that the carbon-fiber electrode is situated near terminals of electrically silent neurons or the terminals of neurons that are not synchronously firing. Alternatively, these findings could reflect the high level of microanatomical heterogeneity of the NAc. For example, striosome compartments exist over spatial dimensions similar to the largest dimension of our electrode (Voorn *et al*., 1988), and these regions have been shown

to differ in their sensitivity to the release of dopamine evoked by *N*-methyl-D-aspartate (NMDA; Krebs *et al*., 1991). In either case, the temporal and spatial heterogeneity supports the concept that there are distinct microcircuits within the NAc that play quite different roles depending upon the behavioral state of the animal (Carelli & Wightman, 2004). Most importantly, this chemical functional heterogeneity could only be revealed using the carbon-fiber-based microsensor. Future investigations into the role of dopamine in the regulation of specific behaviors will need to account for these spatial and temporal fluctuations that lead to inhomogenous dopamine concentrations.

The results of this investigation reveal unanticipated heterogeneities in the spatial and temporal aspects of dopamine neurotransmission. Thus, in animals that are awake but unchallenged by behavioral tasks, extracellular dopamine concentrations are dynamic and fluctuating and are regulated in heterogeneous locations, consistent with the site-specific patterns of neuronal firing seen in behaving animals (Carelli & Wightman, 2004). Furthermore, the heterogeneity, both tonic and phasic, persists following cocaine. This spatial and temporal heterogeneity may become more uniform during goal-directed behaviors because, in response to reward-based stimuli, dopaminergic neurons have been shown to fire synchronously (Schultz, 1998). However, in a recent study in the NAc shell of dopamine during intracranial self-stimulation, we found some sites exhibited cue-evoked dopamine transients while others did not, even at sites where dopamine release was supported, indicating that the release heterogeneity is maintained even during goal-directed behaviors (Cheer *et al*., 2007a). Because the same carbon-fiber microelectrode can be used to measure neural activity simultaneously with chemical fluctuations (Cheer *et al*., 2005), these new tools will enable these components to be unravelled.

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Abbreviations

DAT, dopamine transporter; GAD, glutamate decarboxylase; ITI, intertransient interval; NAc, nucleus accumbens; PBS, phosphate-buffered solution; rms, root-mean-square; VTA, ventral tegmental area.

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