Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2012 | 121 | 252–262

doi: 10.1111/j.1471-4159.2012.07677.x

ORIGINAL ARTICLE

Sources contributing to the average extracellular concentration of dopamine in the nucleus accumbens

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Abstract

Mesolimbic dopamine neurons fire in both tonic and phasic modes resulting in detectable extracellular levels of dopamine in the nucleus accumbens (NAc). In the past, different techniques have targeted dopamine levels in the NAc to establish a basal concentration. In this study, we used *in vivo* fast scan cyclic voltammetry (FSCV) in the NAc of awake, freely moving rats. The experiments were primarily designed to capture changes in dopamine caused by phasic firing – that is, the measurement of dopamine 'transients'. These FSCV measurements revealed for the first time that spontaneous dopamine transients constitute a major component of extracellular dopamine levels in the NAc. A series of experiments were designed to probe regulation of extracellular dopamine. Lidocaine was infused into the ventral tegmental area, the site of dopamine cell bodies, to arrest neuronal firing. While there was virtually no instantaneous change in dopamine concentration, longer sampling revealed a decrease in dopamine transients and a time-averaged decrease in the extracellular level. Dopamine transporter inhibition using intravenous GBR12909 injections increased extracellular dopamine levels changing both frequency and size of dopamine transients in the NAc. To further unmask the mechanics governing extracellular dopamine levels we used intravenous injection of the vesicular monoamine transporter (VMAT2) inhibitor, tetrabenazine, to deplete dopamine storage and increase cytoplasmic dopamine in the nerve terminals. Tetrabenazine almost abolished phasic dopamine release but increased extracellular dopamine to ~500 nM, presumably by inducing reverse transport by dopamine transporter (DAT). Taken together, data presented here show that average extracellular dopamine in the NAc is low (20–30 nM) and largely arises from phasic dopamine transients.

Keywords: basal level, carbon-fiber, Dopamine, FSCV, transients.

J. Neurochem. (2012) **121**, 252–262.

Dopaminergic neurons normally fire in a slow (3–7 Hz), tonic fashion, but periodically they fire more rapidly (≥ 20 Hz) in a phasic manner for a brief interval before resuming tonic firing (Grace and Bunney 1984a,b; Schultz *et al.* 1993; Hyland *et al.* 2002; Floresco *et al.* 2003). Similar terms (e.g. tonic/phasic) have been used to describe extracellular concentrations of dopamine. However, it is unclear how different firing modes relate to extracellular dopamine levels. Phasic firing of dopaminergic neurons is

Received September 9, 2011; revised manuscript received January 25, 2012; accepted January 26, 2012.

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Abbreviations used: DAT, dopamine transporter; FSCV, fast scan cyclic voltammetry; NAc, nucleus accumbens; VMAT2, vesicular monoamine transporter; VTA, ventral tegmental area.

triggered in several ways, including activation of NMDA receptors in the cell body regions (Chergui *et al.* 1993), and often occurs in response to salient stimuli that are associated with reward (Schultz *et al.* 1997; Pan *et al.* 2005). Similar stimuli result in dopamine concentration transients in regions with dopamine terminals (Robinson *et al.* 2002; Phillips *et al.* 2003; Stuber *et al.* 2005; Day *et al.* 2007; Roitman *et al.* 2008), and these transients are both necessary (Zweifel *et al.* 2009) and sufficient (Tsai *et al.* 2009) for reward related behaviors. Like phasic firing, dopamine transients also depend on functional NMDA receptors in the ventral tegmental area (VTA) and they can be evoked by electrical stimuli that mimic phasic bursts (Sombers *et al.* 2009). Thus, phasic firing and dopamine concentration transients are tightly coupled.

The overall contribution of phasic and tonic firing establishes a basal level of extracellular dopamine. Complete removal of extracellular dopamine leads to Parkinson-like conditions (Berke and Hyman 2000) supporting a functional role for basal dopamine. However, establishing the magnitude of extracellular dopamine has proved technically difficult (reviewed in Watson *et al.* 2006). Early estimates with slow electrochemical techniques for the extracellular striatal dopamine concentration were ~25 nM (Gonon and Buda 1985). More modern techniques such as FSCV, as well as other rapid electrochemical methods, have a large background that must be removed before the current due to dopamine is resolved. Thus, these methods only yield differential concentrations. Estimates of the striatal extracellular dopamine concentration from these types of experiments range from 6 nM (Kawagoe *et al.* 1992) to 2.5 μM (Kulagina *et al.* 2001; Borland and Michael 2004), with intermediate values also proposed (Chen and Budygin 2007). Basal concentrations of striatal dopamine evaluated by no-net flux microdialysis sampling have been reported to be in the low nanomolar range (Justice 1993; Shou *et al.* 2006). However, these values may be an underestimate due to tissue damage caused by probe insertion (Yang *et al.* 1998; Bungay *et al.* 2003). Thus, while there is considerable variability in reports of the average extracellular level, data seem to have coalesced around the low nanomolar range (Dreyer *et al.* 2010). It remains unclear, though, whether this level is established largely by tonic or phasic dopamine neuronal firing.

Here, we address the contribution of dopamine transients to the average extracellular concentration of dopamine in the NAc with FSCV. To accomplish this, we recorded dopamine transients in the brain of awake rats at rest. When signal averaged over multiple minutes, we obtained an extracellular level similar to those previously reported using microdialysis. To suppress both tonic and phasic firing, lidocaine was microinfused into the ventral tegmental area. This microinfusion significantly lowered the time-averaged extracellular dopamine level without affecting the instantaneous level. We then characterized how inhibition of dopamine transporter (DAT) and increased cytoplasmic dopamine altered extracellular dopamine levels and dopamine transients. Together, these experiments indicate that under normal circumstances, extracellular dopamine is quite low (20–30 nM) and a significant fraction of this amount is due to time-averaged dopamine transients evoked by phasic firing.

Methods

Animals

Male Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN, USA) weighing 275–350 g were used as subjects and individually housed with a 12 : 12 light : dark cycle (lights on at 6 AM) with access to food and water *ad libitum*. All experiments were conducted between 9:00 AM and 5:00 PM. For some experiments, rats (n = 10) purchased with intravenous catheters were used (Charles River, Wilmington, MA, USA). All procedures were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

Surgical procedures

Rats were surgically prepared for voltammetric recordings as described previously (Phillips *et al.* 2003; Roitman *et al.* 2004). Rats were anesthetized with isoflurane; rats were induced at 4% and maintained at 1.5–2.0% during surgery. Rats were secured in a stereotaxic frame for implantation of a guide cannula (Bioanalytical Systems, West Lafayette, IL, USA) that was positioned 2.5 mm deep into the brain over the core subregion of the NAc (+1.3 AP, 1.3 ML from bregma). A chlorinated silver electrode (Ag/AgCl) was placed contralateral to the guide cannula in the forebrain to serve as a reference. A bipolar stimulating electrode (Plastics One, West Lafayette, IN, USA) was positioned just dorsal to the ventral tegmental area (−5.2 AP, 1.0 ML from bregma and 7.8 mm ventral from the dural surface of the brain). For lidocaine infusion experiments, the microinfusion guide cannula (C315G-MS303/2/SPC; Plastics One) was located between the bipolar tips to ensure introduction of the drug into the appropriate region. Stainless steel skull screws and dental cement were used to secure all items. Postoperatively, rats were closely monitored and received ibuprofen (15 mg/kg).

Fast-scan cyclic voltammetry

Following surgery, animals were allowed 2 days to recover to pre-surgery body weight. A detachable micromanipulator containing a glass-sealed carbon-fiber electrode (75–100 μm exposed tip length, 7 μm diameter, T-650; Amoco, Greenville, SC, USA) was inserted into the guide cannula and the electrode was lowered into the NAc core. Electrical stimulations (24 biphasic pulses, 60 Hz, 125 μA, 2 ms per phase) to the VTA were applied as the carbon-fiber microelectrode was lowered to ensure it was in a location that supported robust dopamine release (Wightman *et al.* 2007).

The carbon-fiber and Ag/AgCl electrodes were connected to a head-mounted voltammetric amplifier attached to a commutator (Crist Instrument Co., Hagerstown, MD, USA) at the top of the experimental chamber. Electrochemical data were digitized and

stored using computer software written in LabVIEW (National Instruments, Austin, TX, USA). The potential of the carbon-fiber electrode was held at −0.4 V versus the Ag/AgCl reference electrode. Voltammetric recordings were made by applying a triangular waveform that drove the potential to +1.3 V and back at a rate of 400 V/s. To minimize current drift and to enhance sensitivity, the carbon-fiber electrode was allowed to equilibrate for 30–45 min with voltammetric scanning at 60 Hz prior to the start of the experiment (Takmakov *et al.* 2010). During data acquisition, the repetition rate was 10 Hz. Dopamine release was electrically evoked by stimulating the VTA (24 biphasic pulses, 60 Hz, 125 μA, 2 ms per phase).

Drugs and local microperfusion

Local infusion of drugs (Sigma Aldrich, St Louis, MO, USA) employed a syringe pump (Kent Scientific Corp., Torrington, CT, USA; 0.5 μL injected for 60 s) with an infusion cannulae (33 gauge) inserted into the unilaterally implanted guide. Microinfusions were of saline (0.9%) or lidocaine (350 nmol/0.5 μL, dissolved in saline with the pH adjusted to 6.0 with NaOH). The microinfusion needle was inserted before the experiment started to reduce handling or other sources of noise. After 2 min of baseline lidocaine was infused for 1 min. Following infusion the needle was removed.

GBR12909-HCl (Tocris Bioscience, Ellisville, MO, USA) was dissolved in 0.1 mL of distilled water and then diluted to 1 mL with saline. The vehicle was prepared similarly. The dose of GBR12909 selected (1.5 mg/kg i.v.) caused a moderate increase in locomotor activity. Tetrabenazine was dissolved in 50% ethanol and 50% distilled water. Vehicle was the 50 : 50 ethanol : distilled water that was used to dissolve tetrabenazine. The dose of tetrabenazine used (1 mg/kg) was selected because it caused immobility of the animal without inducing catalepsy. All injection volumes were 0.3 mL.

Signal analysis

Background currents were removed by subtraction of cyclic voltammograms recorded over 1 s. For the evaluation of electrically stimulated release, the background was taken in the interval before the stimulation. To determine the time averaged concentration arising from dopamine transients, cyclic voltammograms were continuously collected for 15 min. These data were broken into 30 s epochs and for each epoch the set of cyclic voltammograms during 1 s around the local minimum served as the background. For each rat, all 30 s epochs (30 epochs) were averaged together to obtain an average 30 s concentration trace. Each rat's average trace was then averaged together to obtain means and standard errors.

To understand how time-averaging alters the variability of dopamine concentration, we calculated the coefficient of variation of dopamine concentration for each rat when data were averaged across different bin sizes. For the 0.1s bin, all 9000 CVs were averaged for each rat and a mean and standard deviation was obtained. For the 1-s bin, first every 10 cyclic voltammograms were averaged to obtain a single value for each 1-s bin and then data were averaged across 900 bins to obtain a mean and standard deviation for each rat. For the 5-s bin, the procedure was similar except 50 cyclic voltammograms were averaged to obtain a single value for each 5-s bin. Likewise, every 100 cyclic voltammograms were averaged for the 10 s bin followed by averaging over 90 bins to obtain a mean and standard deviation for each rat. Identical procedures were followed to compute the 15 and 30 s bins. The coefficient of variation (standard deviation/mean) was determined for each bin size for each rat.

Before and after *in vivo* recordings, dopamine release was evoked by VTA stimulation to provide a training set for principal component regression (Heien *et al.* 2004; Keithley *et al.* 2009, 2010a,b). Stimulation of the VTA leads to two well established electrochemical events: an immediate but transient increase in dopamine and a delayed but longer-lasting basic pH shift. The training set was constructed from representative, background-subtracted cyclic voltammograms for dopamine and pH. This training set was used to perform principal component regression on data collected during the recording session. The calibration factors used to convert cyclic voltammetry currents into concentration were obtained after in vivo use in physiological buffer (Heien *et al.* 2003). Because the electrodes were used to make electrical lesions at the end of the experiment, the calibration factor (12 nA/μM for dopamine, −40 nA/pH unit) used were the average obtained from more than 25 electrodes used in prior work. A residual analysis procedure was used to verify that the cyclic voltammograms of the trials being predicted were consistent with the analyte cyclic voltammograms used for calibration. Any trials containing uncharacteristic variance larger than 95% of the noise of the training set were discarded (Keithley *et al.* 2009, 2010b). Dopamine events were termed transients if their concentration was 5 times the standard deviation of the noise. The noise was calculated as the root mean square amplitude in the first 1 s (10 scans) obtained in the beginning of the 30 s epoch. If this interval contained a dopamine transient, the time for the noise calculation was moved to a later time. One-way repeated measures ANOVAS were used to test for significant changes in dopamine transient frequencies and for changes in stimulated dopamine release in different treatment groups compared with naïve (pre-drug) values.

Histological verification of electrode placement

Upon completion of each experiment, rats were deeply anesthetized with urethane. To mark the placement of electrode tips, the carbon-fiber microelectrode was removed and replaced with a tungsten electrode (Owesson-White *et al.* 2009). A 500-μA current was passed through the electrode for 5 s to make a visible lesion. Transcardial perfusions were then performed using physiological saline and 10% formalin, and the brains were removed. The lesion site was assessed by visual examination of successive coronal sections. Placement of an electrode tip within the NAc core was determined by examining the relative position of the lesion to visual landmarks using the anatomical organization of the NAc represented in a stereotaxic atlas (Paxinos and Watson 2007).

Results

Time-averaged phasic dopamine signaling

Naïve rats (n = 9) were surgically prepared for voltammetric recordings. After recovery (at least 2 days), rats were placed in a standard experimental chamber (Med Associates, St Albans, VT, USA) and cyclic voltammograms were recorded in the NAc for 15 min. Dopamine concentration changes during each 30 s interval were determined from the back-

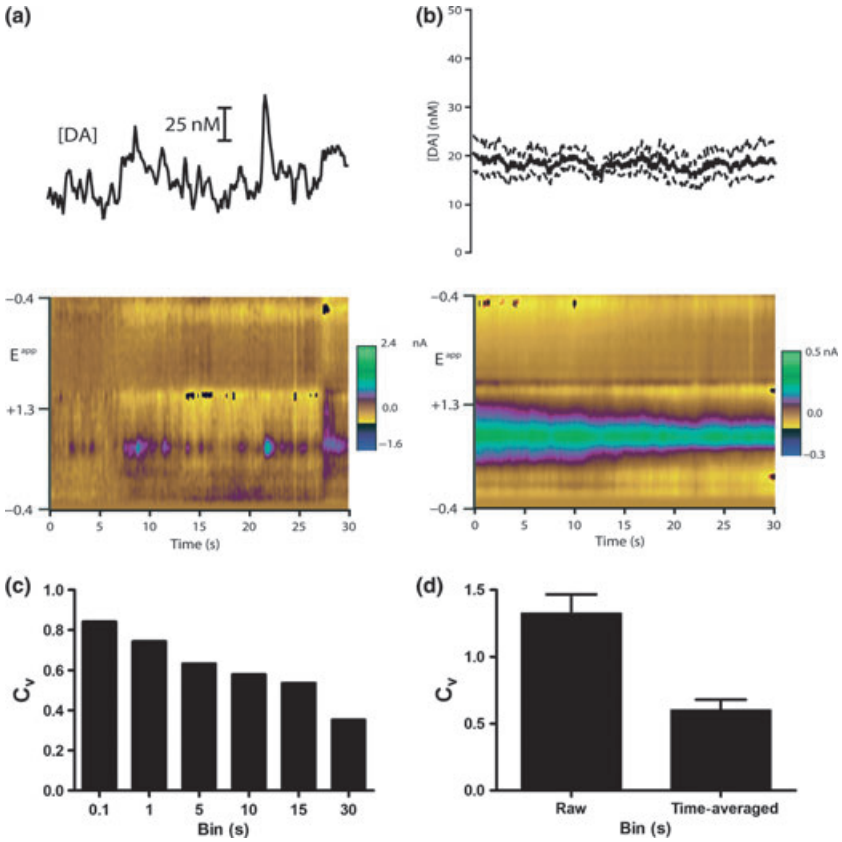

Fig. 1 Time-averaged phasic dopamine signaling. (a) Top, dopamine concentration fluctuations over a single 30 s epoch extracted from raw data using principal component regression. Bottom, 30 s of background-subtracted cyclic voltammograms showing dopamine transients at various time points. (b) Top, the mean ± SEM dopamine concentration over the 30 s epoch averaged first across 15 min for each rat and then across all nine rats. Bottom, color representation of the averaged, background-subtracted cyclic voltammograms. (c) The coefficient of variation (C_V) for different time bins (0.1–30 s) for one rat. (d) the average C_V across rats (n = 9) when bins are either 0.1 (raw) or 30 (time averaged) s.

ground-subtracted cyclic voltammograms using principal component regression (Fig. 1). Figure 1a shows the dopamine concentration fluctuations (top) extracted from these data (bottom) by principal component regression. Occasional dopamine transients can be seen. These spontaneous dopamine transients have been reported previously (Wightman *et al.* 2007), and arise from brief phasic firing of dopamine neurons (Sombers *et al.* 2009).

The microdialysis technique, the approach that has been most used for measuring basal dopamine concentrations, samples the extracellular fluid on a minutes time scale whereas the background-subtracted cyclic voltammograms in Fig. 1 were sampled at 0.1-s intervals. To allow comparison of these data with microdialysis, we averaged together 30 s epochs of cyclic voltammograms over 15 min. Figure 1b shows the average dopamine concentration fluctuation (top) as well as a color representation of this average (bottom). The averaging procedure smoothed out the transients and yielded an average concentration of approximately 20 nM (18 ± 2, mean ± SEM). This is the average concentration over 15 min that is contributed by dopamine transients.

To illustrate how signal averaging results in the loss of variability in dopamine fluctuations we examined the coefficient of variation for dopamine transients averaged over different time epochs. For each rat, we calculated the concentration of dopamine over 15 min (30 consecutive 30 s samples) in different intervals (1, 5, 10, 15, and 30 s). For the individual example shown in Fig. 1c, the coefficient of variation decreased as the duration used to time-average the data increased ($\beta = -0.96$; CI = −1.36 to −0.56, $p < 0.01$), although there was no effect on the average concentration of dopamine (not shown, 18.7 ± 2.0 nM; $\beta = -0.38$; CI = −1.67 to 0.89, $p = 0.44$). For each rat, we calculated the coefficient of variation for 15 min of data, averaged in 0.1, 1, 5, 10, 15 and 30 s epochs. Figure 1d shows the average coefficient of variation of nine rats tested, with the unaveraged data yielding a higher coefficient of variation than data time-averaged in 30 s bins. Across the population of rats tested, average concentration of dopamine did not depend on the time interval used to sample the data ($\beta = 0.00$; CI = −0.23 to 0.23, $p = 0.99$), but the coefficient of variation decreased with increasing duration of interval ($\beta = -0.53$; CI = −0.74 to −0.32, $p < 0.0001$). These data support the idea that sampling over time, as in microdialysis, reduces the native variability of dopamine concentration fluctuations.

Intra-VTA injection of lidocaine

Previously we have shown that inactivation of the VTA with microinfusions of lidocaine significantly diminishes electrically stimulated release of dopamine and lowers the

frequency of spontaneous dopamine transients (Sombers *et al.* 2009). We repeated these experiments here to examine more closely any concentration changes that occurred at the time of the local microinfusions (instantaneous) as well as to examine the effects of lidocaine on the average concentration of dopamine. A separate group of rats (n = 7) was surgically prepared as described above with the addition of an injection cannula between the poles of the bipolar stimulating electrode. This modification ensures that the microinjection site is at a location that supports electrically evoked release. After recovery from surgery, rats were placed in the experimental chamber and a fresh carbon-fiber microelectrode was inserted into the NAc at a location that supported stimulated release.

First, we recorded for 10 min to gather pre-drug voltammograms and these were grouped into 30 s epochs. Immediately before microinfusion of saline or lidocaine, electrically evoked dopamine release was measured. Then, 2 min later, a 1-min microinfusion of either saline or lidocaine was made while recording voltammograms. The voltammograms collected during the microinfusion and for 9 min thereafter were also grouped into 30 s epochs. Then, electrically evoked dopamine was measured again. Figure 2a–c shows a representative example of the events measured around a single lidocaine microinfusion. Electrically evoked dopamine release before lidocaine is accompanied by a small pH change (black line for dopamine, blue trace for pH change, Figure 2a).

Figure 2b shows the events that occurred in this representative animal during the 1-min lidocaine microinfusion and the subsequent minute. Neither dopamine nor pH changed appreciably during the infusion or in the following minute. When evaluated in all seven animals, the average dopamine concentration change in the 2-min interval during and after lidocaine infusion was 0.5 ± 6.4 nM. As no significant change was determined immediately after the lidocaine microinfusion, the tonic level must be below the limits of detection of fast-scan cyclic voltammetry. Ten minutes after lidocaine microinfusion, both electrically evoked dopamine and pH were significantly attenuated (Fig. 2c).

To evaluate the contribution of dopamine transients to the average concentration during the 10 min before microinfusions, the 30 s epochs were background sub-

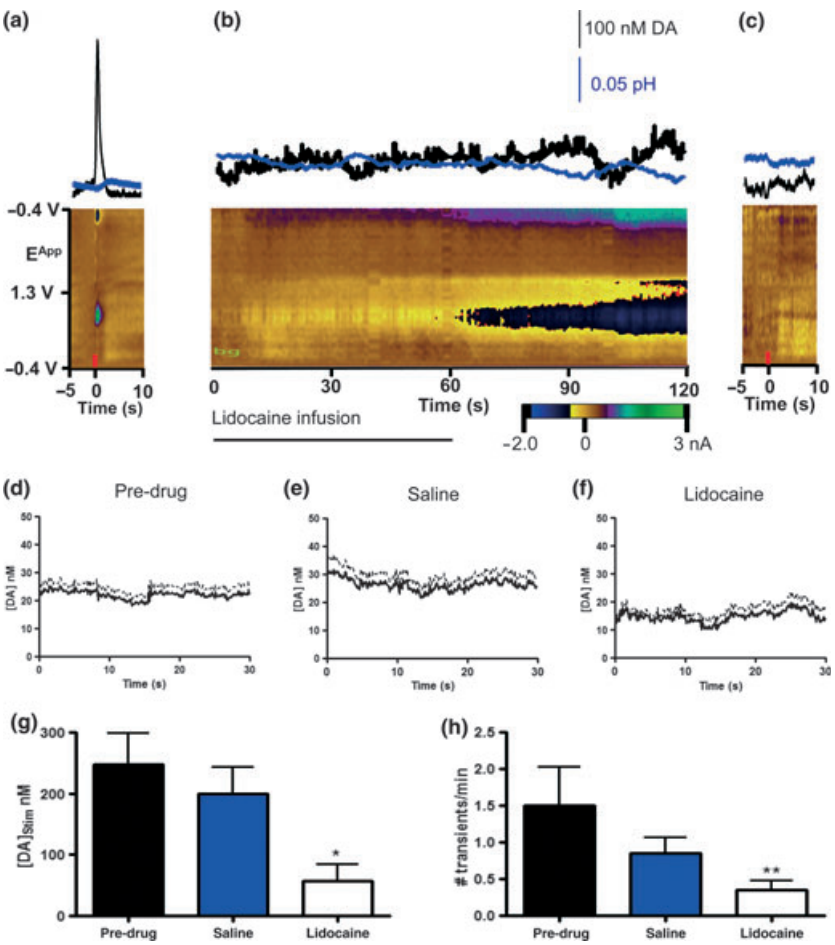

Fig. 2 Intra-VTA injection of lidocaine. (a) Stimulated dopamine release and pH - changes are shown with the color plot from which they were extracted by principal component analysis. (b) Dopamine and pH with corresponding colorplot, during and after a 1-min lidocaine microinfusion. (c) Dopamine and pH response to electrical stimulation post-lidocaine infusion. (d) The average dopamine baseline pre saline and lidocaine ~22 nM. (e) Average dopamine baseline after saline microinfusion into the VTA. (f) Average dopamine baseline after lidocaine infusion. (g) $[DA]_{stim}$ measured pre-drug, post-saline and post-lidocaine. Lidocaine significantly reduced dopamine release, one-way ANOVA, $^{*}p < 0.05$, $F_{5.54} = 2$. (h) Frequency of spontaneous transient dopamine release transients measured pre-drug, post-saline and post-lidocaine. Lidocaine significantly reduced the frequency of transients, one-way ANOVA, $^{**}p < 0.01$, $F_{4.61} = 2$ ($n = 7$).

tracted and the dopamine concentration was extracted as described above. These dopamine concentrations were then averaged together from all animals. Similar processing was done for the data collected in the 10 min during and after saline microinfusion and during and after lidocaine infusion. In this group of animals, the average dopamine concentration arising from transients was 23 ± 2 nM (Fig. 2d), a value in good agreement with the previous experiment (Fig. 1). Following saline the average dopamine concentration was unchanged (26 ± 2 nM, Fig. 2e). However, following lidocaine infusion, the average dopamine concentration due to transients was decreased (15 ± 2 nM, Fig. 2f). The frequency of dopamine transients was also compared across the three conditions. The number of transients before saline was significantly higher than after lidocaine, and was reduced to 23% of the pre-drug value. There was a small reduction in transient frequency following saline but it was not significantly different from the pre-drug value (Fig. 2h). The maximal dopamine concentration evoked by electrical stimulation, $[DA]_{stim}$, was averaged and compared across the three conditions. Saline caused an insignificant attenuation of release relative to pre-infusion levels, but, following lidocaine microinfusions into the VTA, electrically evoked dopamine release was significantly diminished, 23% of pre-drug (Fig. 2g).

Dopamine extracellular levels after DAT inhibition

To examine the role of DAT in maintaining extracellular levels, we inhibited its function with GBR12909. The intravenous route was used to allow rapid access of drug to the brain. Results from a single animal during GBR12909 are shown in Fig. 3a–c. DAT inhibition increased stimulated dopamine release measured 10 min after drug administration (compare Fig. 3a and c), a result that was seen in all animals (Fig. 3d) and that is consistent with measurement in anesthetized animals (Wightman *et al.* 1988). GBR12909 caused a rise in extracellular dopamine that was characterized by multiple, overlapping transients that summed to give a substantial dopamine change that approached 400 nM (Fig. 3b). This response, increased transients and a large increase in extracellular dopamine, is also seen following cocaine (Wightman *et al.* 2007). Figure 3d and e summarize the effect on electrically evoked dopamine release and frequency of transients, $n = 5$. GBR12909 significantly elevated both stimulated dopamine release and number of transients observed. Administration of vehicle did not affect stimulated release or transient frequency (Fig. 3d and e).

Dopamine extracellular levels following blockade of the vesicular transporter

Rats with intravenous catheters were also used to evaluate inhibition of vesicular dopamine storage by tetrabenazine, a

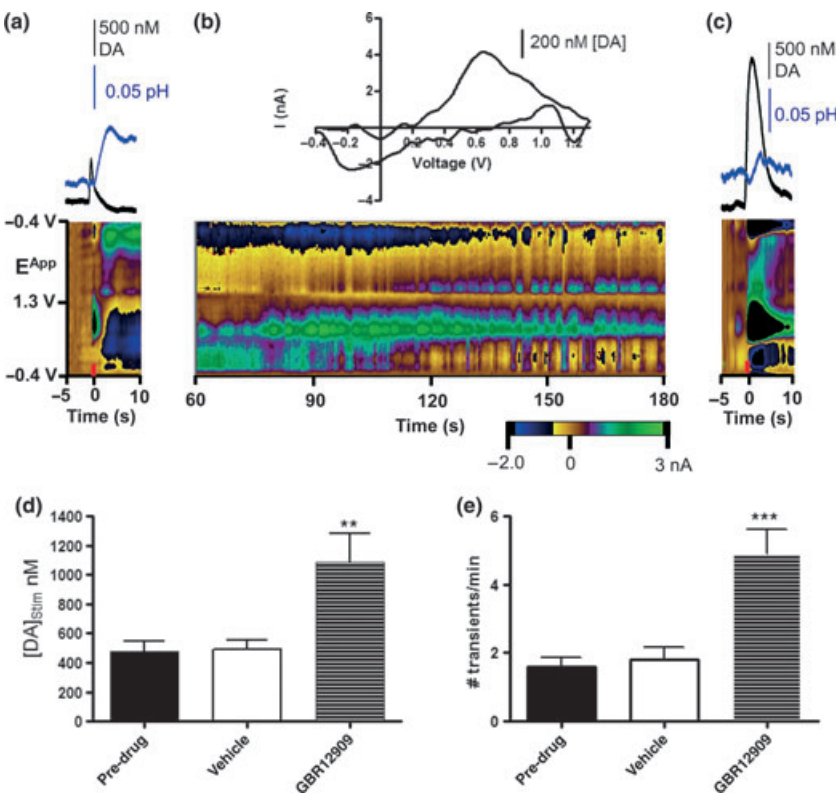

Fig. 3 Intravenous GBR12909. (a) Electrically evoked dopamine and pH response pre GBR12909 injection with corresponding colorplot. (b) Colorplot representation of extracellular dopamine. The cyclic voltammogram was recorded 5 min after GBR12909 administration. (c) Dopamine and pH response to electrical stimulation 30 min after GBR12909 injection. (d) Electrically evoked dopamine release pre-drug, after vehicle and after GBR12909. GBR12909 significantly increases dopamine release, one-way ANOVA, $^{**}p < 0.01$, $F_{6.25} = 2$, compared with pre-drug. (e) Frequency of spontaneous transient dopamine release events, pre-drug, post-vehicle and post-GBR12909. GBR12909 significantly increase the frequency of transients, one-way ANOVA, $^{***}p < 0.001$, $F_{12.78} = 2$ ($n = 5$).

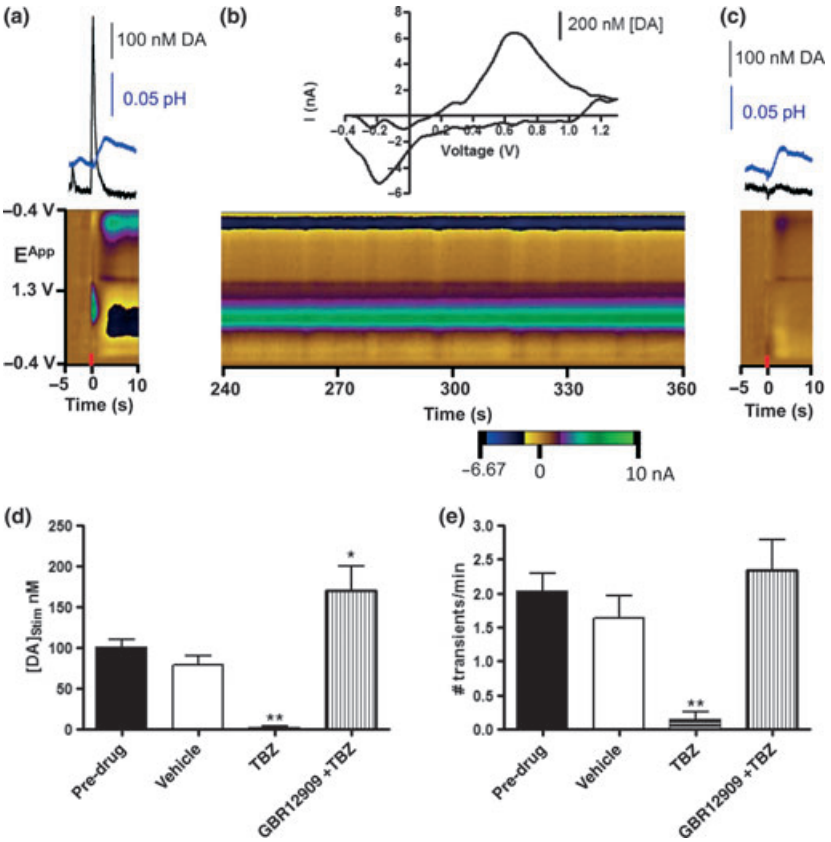

Fig. 4 Intravenous tetrabenazine. (a) Stimulated dopamine release and pH - changes are shown with the color plot from which they were extracted by principal component analysis. (b) Color plot of voltammograms recorded 4- to 6-min after an i.v. injection of tetrabenazine. (c) Dopamine and pH response to electrical stimulation 30 min after tetrabenazine. (d) $[DA]_{stim}$ measured pre-drug, after vehicle, after tetrabenazine, and after GBR12909 followed by tetrabenazine. Tetrabenazine significantly reduced stimulated dopamine release, one-way ANOVA, $^{**}p < 0.01$, $F_{17.75} = 2$, whereas GBR12909 with tetrabenazine significantly increased stimulated dopamine release, $^{*}p < 0.05$. (e) Frequency of spontaneous transient dopamine release transients measured pre-drug, post-vehicle, after tetrabenazine and after GBR12909 followed by tetrabenazine. Tetrabenazine significantly reduced the frequency of transients, one-way ANOVA, $^{**}p < 0.01$, $F_{20.24} = 2$ ($n = 5$).

vesicular monoamine transporter (VMAT2) blocker. By increasing cytoplasmic dopamine, VMAT2 blockade increases the likelihood of reverse transport of dopamine by DAT (Jones *et al.* 1998, 1999; Leviel 2001). Rats received an intravenous injection of vehicle and voltammograms were recorded for 10 min.

Events around the tetrabenazine injection from a single animal are illustrated in Fig. 4a–c. Thirty minutes after tetrabenazine administration, electrically evoked dopamine release was attenuated without affecting the simultaneously measured pH change (compare Fig. 2a and c). The decrease in stimulated dopamine release was statistically significant when evaluated in all animals (Fig. 4d). Following tetrabenazine injection, dopamine changes can be seen both in the color plot recorded 4–6 min after tetrabenazine and in the cyclic voltammogram constructed by subtracting those recorded 5 min after tetrabenazine from those recorded before its administration (Fig. 4b). Data were continuously collected for an additional 30 min following injection and analyzed as described above. In this interval, spontaneous dopamine transients disappeared (Fig. 4e). Vehicle injection did not alter stimulated release or the number of spontaneous dopamine transients observed (Fig. 4d and e).

In a separate group of animals, GBR12909 was injected i.v. followed 10 min later by tetrabenazine. Our intention was to use a DAT inhibitor to reduce the possibility of reverse transport. The subsequent injection of tetrabenazine restored both the electrically evoked stimulated dopamine release and frequency of transients to pre-GBR 12909 levels, Fig. 4d and e. However, in the first 5 min after tetrabenazine, dopamine continued to increase (data not shown).

Discussion

Dopamine neurons exhibit both phasic and tonic cell firing patterns (Grace and Bunney 1984a,b; Schultz *et al.* 1993; Floresco *et al.* 2003). The extracellular level of dopamine concentration could reflect tonic or phasic firing of dopamine neurons or both. As transients reflect phasic firing (Sombers *et al.* 2009), we assayed their contribution to the extracellular level of dopamine. We determined the mean dopamine concentration contributed by phasic firing by signal averaging spontaneous dopamine transients over multiple minutes, a time scale compatible with microdialysis sampling. In two separate groups of rats, we found that the average dopamine concentration contributed by phasic firing is ~20 nM for an awake rat at rest. The contribution of dopamine cell firing in general was probed on a shorter time scale by inactivation of dopamine cell bodies in the VTA with lidocaine microinfusion. The absence of an appreciable decrement in dopamine

concentration during the infusion and immediately thereafter indicates that the contribution of tonic firing to the extracellular level was below our detection limit. The lidocaine microinfusions were clearly effective because electrically evoked dopamine release was blocked and the frequency of transients was diminished. Indeed, when signal averaged over many minutes, the dopamine concentration contributed from phasic firing was significantly lower after lidocaine treatment. Inhibition of either DAT or VMAT2 caused increases in extracellular dopamine in the 500 nM range, indicating the important role that these two transporters play in maintaining low extracellular dopamine concentrations.

Attempts to measure the basal concentration of dopamine have led to a variety of estimates. The value of the extracellular dopamine concentration depends upon the time and space scale of the measurement. For example, if the level was taken as that at the exocytotic fusion pore, a value of greater than 100 mM would be obtained (Kawagoe *et al.* 1992). However, this concentration rapidly decreases with distance from the release site due to dilution in the extracellular fluid. Here, we are interested in the extracellular concentration found on a length scale of 100′ s of micrometers and a time scale of minutes. This value is fundamentally important to understand normal receptor occupancy and the changes that can occur during dopamine transients (Arbuthnott and Wickens 2007; Dreyer *et al.* 2010). One approach has been to evaluate the competition between endogenous dopamine and radio-labeled agonists (Ross 1991; Delforge *et al.* 2001). This procedure, which depends upon known values for agonist binding, has led to estimates of basal dopamine concentrations in the 50–100 nM range. A more direct approach is the use of *in vivo* microdialysis in which the extracellular fluid is sampled with an implanted dialysis membrane (Watson *et al.* 2006). To minimize the effects of concentration gradients, two variants of this technique have been employed. The no-net flux method, which employs a known amount of the analyte of interest in the perfusion buffer, has yielded extracellular dopamine concentration of 5 nM (Parsons and Justice 1992). The extrapolation to zero flow method employs multiple flow rates to allow extrapolation to the value in the absence of flow. The Kennedy group reported a striatal extracellular dopamine concentration of 18 nM using a low flow rate procedure (Shou *et al.* 2006). However, when the damage caused by probe insertion is considered, theoretical models of microdialysis have suggested that these values are an underestimate of the true extracellular dopamine concentration (Peters and Michael 1998; Bungay *et al.* 2003).

Carbon-fiber microelectrodes are much smaller than microdialysis probes so they cause less tissue damage (Peters *et al.* 2004). Unlike classic, slow-scan techniques, fast-scan cyclic voltammetry provides a distinct signal for dopamine that differs from that for dihydroxyphenylacetic acid, the major dopamine metabolite (Heien *et al.* 2004). Furthermore, the method provides subsecond time resolution for catecholamine concentration changes. However, measurements with rapid electrochemical methods have a large background that requires background subtraction to reveal dopamine changes (Millar *et al.* 1985; Keithley *et al.* 2011). This prevents direct measurement of the extracellular concentration. For example, the amplitude of the current for 100 nM dopamine is only ~0.1% of the background current. In addition to the large background, drift in the background signal prevents accurate background subtraction unless drift is accounted for in the principal component regression (Hermans *et al.* 2008).

It has previously been suggested that a portion of the dopamine collected with microdialysis arises from dopamine released by phasic firing (Floresco *et al.* 2003; Robinson *et al.* 2009). To estimate this fraction on the time scale of microdialysis, we averaged multiple recordings over 15 min. Before averaging, each 30 s epoch of data was background subtracted using the cyclic voltammograms that had the lowest current at 0.65 V, the peak potential for dopamine oxidation. This averaging procedure accounts for all of the rapidly fluctuating dopamine extracted by principal component regression. The result shown in Fig. 1 (and replicated in Fig. 2 with a completely different group of subjects) strongly suggests that microdialysis estimates of the basal dopamine concentration include a significant contribution from dopamine released by phasic firing. Indeed, our averaged dopamine concentration, ~20 nM, is well within the range of multiple estimates made by microdialysis. Note, however, this concentration does not include the contribution from tonic firing because of the background subtraction procedure.

The concentration measured by the averaging procedure is the average working range for normal dopamine signaling contributed by phasic firing. We used lidocaine microinfusions into the VTA to distinguish whether tonic or phasic firing further contributes to the average extracellular dopamine concentration. Inactivation of dopamine neurons with lidocaine resulted in a significant decrease of extracellular dopamine in the NAc when measured on a minutes time scale with microdialysis (Howland *et al.* 2002). Indeed, lidocaine acts rapidly as shown following injection into the primate cerebral cortex where a 60% decrease in neuronal firing rates occurred within 60 s (Tehovnik and Sommer 1997). As we have previously shown, lidocaine microinfusions into the VTA rapidly eliminates spontaneous transients and stimulated release (Sombers *et al.* 2009). We anticipated that the cessation of release arising from inhibition of firing of dopaminergic neurons would lead to an immediate disappearance of extracellular dopamine contributed by tonic firing, leading to a rapid decrease in dopamine concentration that would not be distorted by the drift of the electrode response. Without ongoing tonic release activity, the DAT would remove all extracellular dopamine in less than a second. However, the voltammetric recordings showed no

detectable change in the dopamine signal in the 60-s interval following the microinfusion. Thus, the contribution from tonic firing must be in the low nanomolar range, below our previous theoretical estimate of 30 nM (Venton *et al.* 2003). Our estimate was based on a release amplitude that was obtained during electrically stimulated release of dopamine. However, the measurements following lidocaine microinfusion in the VTA, with a method that has a detection limit of ~10 nM dopamine for single voltammetric scans (Heien *et al.* 2003), clearly indicate that extracellular dopamine concentration is very low. When the time scale is increased to 10 min by averaging dopamine responses following microinfusion, a reduction in the dopamine concentration was found relative to pre-microinfusion levels and the frequency of transients diminished. As the frequency of transients was not totally suppressed following lidocaine, the time-averaged concentration after lidocaine likely reflects a phasic contribution.

We used intravenous pharmacological treatments to block two of the central transporters involved in the regulation of extracellular dopamine. Global inhibition of both DAT and VMAT2 led to a substantial increase in extracellular dopamine that approached 500 nM. Following DAT inhibition with GBR12909 the increased extracellular level was accompanied by robust dopamine transients. This behavior is also seen for NAc dopamine following nomifensine (Robinson and Wightman 2004) or cocaine (Heien *et al.* 2005). For cocaine, the transients have been shown to depend on continued phasic firing of dopaminergic neurons in the VTA (Sombers *et al.* 2009). Following VMAT2 inhibition with tetrabenazine, the substantial dopamine increase is not accompanied by dopamine transients. The absence of transients is due to the different mechanism of release evoked by tetrabenazine: the continuous outpouring of dopamine arises from its reversed transport from the cytoplasm by the DAT (Jones *et al.* 1999), a process that is independent of neuronal firing. Consistent with this, when tetrabenazine was given after inhibition of the DAT, transients were still apparent and stimulated dopamine release was not suppressed. Reverse transport has been suggested to maintain high extracellular levels under normal conditions (Borland and Michael 2004). Our results demonstrate that reverse transport can lead to high extracellular dopamine levels but only when there are abnormally high cytoplasmic levels of dopamine.

Taken together, our data suggest that the extracellular level of dopamine – observed as a function of averaging over time and space – is largely established by phasic dopamine release. The data indicate that the sum of phasic and tonic dopamine in the NAc of awake freely moving rats is in the range of 20–30 nM when measured on a time scale of multiple minutes which is in accordance with the range reported by many quantitative microdialysis studies (Justice 1993; Shou *et al.* 2006). Thus, these results reconcile the results from microdialysis with those measured by *in vivo* voltammetry. The contribution from tonic firing was difficult to quantify with the methods used here. A basal dopamine level certainly exists because the delivery of some aversive stimuli, known to cause an inhibition of firing of dopamine neurons (Mirenowicz and Schultz 1996), cause a decrease in dopamine levels of approximately 10 nM (Roitman *et al.* 2008; Wheeler *et al.* 2011). The high affinity forms of dopamine receptors are activated by relatively low concentrations (approximately 10 nM), but activation of the low affinity forms requires a much higher (on the order of 1 μM) dopamine concentration (Richfield *et al.* 1989; Neve and Neve 1997). Thus, for phasic transient events, which are in the 40–100 nM range (Wightman *et al.* 2007), to have physiological importance, the extracellular, steady state concentration of dopamine needs to be fairly low. Our data are consistent with several theoretical models that predict a normally low extracellular dopamine concentration that is controlled by the balance between release and uptake (Arbuthnott and Wickens 2007; Dreyer *et al.* 2010).

Acknowledgements

This research was supported by the National Institutes of Health (DA 10900 to RMW and DA025634 to MFR). The authors declare no conflicts of interest.

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