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The real catecholamine content of secretory vesicles in the CNS revealed by electrochemical cytometry

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Resolution of synaptic vesicle neurotransmitter content has mostly been limited to the study of stimulated release in cultured cell systems, and it has been controversial as to whether synaptic vesicle transmitter levels are saturated *in vivo*. We use electrochemical cytometry to count dopamine molecules in individual synaptic vesicles in populations directly sampled from brain tissue. Vesicles from the striatum yield an average of 33,000 dopamine molecules per vesicle, an amount considerably greater than typically measured during quantal release at cultured neurons. Vesicular content was markedly increased by L-DOPA or decreased by reserpine in a time-dependent manner in response to *in vivo* administration of drugs known to alter dopamine release. We investigated the effects of the psychostimulant amphetamine on vesicle content, finding that vesicular transmitter is rapidly depleted by 50% following *in vivo* administration, supporting the "weak base hypothesis" that amphetamine reduces synaptic vesicle transmitter and quantal size.

Synaptic vesicles are the primary intracellular organelles responsible for the storage and release of chemical messengers from neurons and other secretory cells. As such, they are key determinates in regulating synaptic signaling. Extrapolating data from a 2004 paper by Sulzer and coworkers, repeated complex flickering events measured at cultured midbrain neurons that mammalian vesicular content might be considerably higher than previously thought with approximately 30,000 molecules released[1]. We recently developed a novel method, electrochemical cytometry[2], whereby the total transmitter content of individual nanoscale vesicles can be quantified in a high throughput manner enabling the study of statistically relevant populations of vesicles. In cultured pheochromocytoma (PC12) cells, an immortalized adrenal cell line, we compared large dense core vesicle content with the amount of transmitter detected at individual release events and found that, at most, only 40% of vesicular transmitter is released during exocytosis[3], which would be consistent with transient large dense core vesicle fusion. Whether transient fusion with neurotransmission might occur with brain synaptic vesicles, however, has remained a contentious issue.

Here, we use electrochemical cytometry to count dopamine neurotransmitter molecules in individual mouse brain synaptic vesicles before and after pharmacological challenges. We find that the dopamine is present at average levels of 33,000 molecules per vesicle, an order of magnitude greater than often estimated by previous methods, which have relied primarily on measuring neurotransmitter release[4–7]. In vesicles isolated from mouse brain tissue, we show that the number of dopamine molecules per striatal vesicle are significantly reduced following *in vivo* administration of reserpine, a prodepressant and high blood pressure medication that inhibits vesicular monoamine neurotransmitter uptake. By contrast, dopamine vesicular content increases after peripheral administration of L-DOPA, the dopaminergic precursor widely used to treat Parkinson's disease. Effects of both drugs are time dependent, enabling *in vivo* pharmacokinetics with single vesicle resolution. The contrast between estimates of quantal size based on release and the present results, as well as the drug-induced variations in vesicle content, suggest that transmitter release during exocytosis might be regulated at the level of single release events. This has implications for understanding synaptic plasticity and highlights vesicular neurotransmitter levels as an important pharmaceutical target. In fact, the importance of this technique to study the effects of drugs and animal models should be immense. In Parkinson's disease for example, data are all based on release and there are not any direct data related to actual vesicle content.

Results

Counting transmitter molecules in individual vesicles. Electrochemical cytometry of neuronal vesicles was performed in a high-throughput manner quantifying transmitter molecules in thousands of individual vesicles per experiment. Although exocytosis is quantal such that a discrete amount of transmitter is associated with each vesicle, the number of molecules per vesicle is distributed across a broad range. Vesicles were isolated from mice striatal tissue and dopamine content was quantitatively investigated. Vesicle suspensions were separated with capillary electrophoresis[2,3,8,9], followed by lysing of individual vesicles to expel their contents at a carbon-fiber electrode enclosed in a microchannel (Figure 1A). We have previously shown that nearly all the electroactive transmitter in a vesicle is oxidized at the electrode surface[2]. Oxidation of dopamine results in an increase in current at the electrode (Figure 1B). The area under each current spike represents the total amount of transmitter per vesicle, which is quantified using Faraday's law ($N = Q/nF$). Here, N is the total number of moles of transmitter oxidized, Q is equal to the total charge during oxidation, n is number of electrons transferred during oxidation, and F is the Faraday constant. We integrated the current-time transients for individual vesicles obtained from mouse striatal tissue and plotted the number of vesicles versus the amount of transmitter contained in each vesicle (binned into groups) to produce histograms (Figure 1C). The amount of transmitter in each vesicle is proportional to the vesicle volume and here, vesicle radii are nearly normally distributed[10]. Therefore, data are plotted as transmitter amount in zeptomoles to the one-third power, providing nearly Gaussian plots for analysis.

Measurement of the content of mammalian synaptic vesicles requires sub-picoampere sensitivity and precision (Figure 1B). The species oxidized at the electrode is predominantly dopamine, since it is the prevalent electroactive neurotransmitter in striatum, as confirmed by voltammetric release experiments in rats[11,12] and HPLC analyses of striatal tissue[5,13]. Integration of the current and relation using Faraday's Law yields an average mole amount of 55 ± 0.4 zmol/vesicle ($N = 20{,}331$ vesicles from 3 isolation experiments). This corresponds to 33,000 ± 300 dopamine molecules per vesicle. The mean of the distribution in the frequency histogram in Figure 1C is 3.7 $zmol^{1/3}$, $r^2 = 0.99$ for a single Gaussian fit. Additionally, in the overall isolation experiments, the dopamine count per vesicle was reproducible with 33,000, 34,000 and 33,000 molecules for isolation 1, 2, and 3, respectively.

The supernatant from isolated vesicle suspensions was re-centrifuged for an additional 3 h and the remaining vesicles were analyzed to verify that the analysis included smaller vesicles that might take longer to capture by centrifugation. Vesicles from this second isolation contained an average of 30,000 dopamine molecules, not significantly different from those quantified in the first isolation. Electrochemical cytometry provides markedly low limits of transmitter detection. A current transient that is representative of the smaller subset of vesicles is shown on the right side of Figure 1B. Integration of this peak yields 0.58 fC, which is equivalent to 3 zmol or 1,800 dopamine molecules. This is comparable to the smallest amounts quantified for vesicular dopamine content at small synaptic vesicles (SSVs) using electroanalytical methods[1].

Manipulating dopamine content in neuronal vesicles. Electrochemical cytometry was used to quantify changes in the numbers of dopamine molecules in individual vesicles in response to drugs that modify dopamine neurotransmission at different times after parenteral administration. Figure 2A shows a representative current transient for dopaminergic striatal vesicles isolated from mice treated with 20 mg/kg reserpine 12 h prior to sacrifice. Reserpine inhibits the transport of dopamine into synaptic vesicles via vesicular monoamine transporters. A separate group of mice was administered 50 mg/kg L-DOPA and sacrificed 2 h later. L-DOPA is the endogenous dopamine precursor and is used as standard treatment in Parkinson's disease. Data are compared to a representative current spike from an untreated mouse.

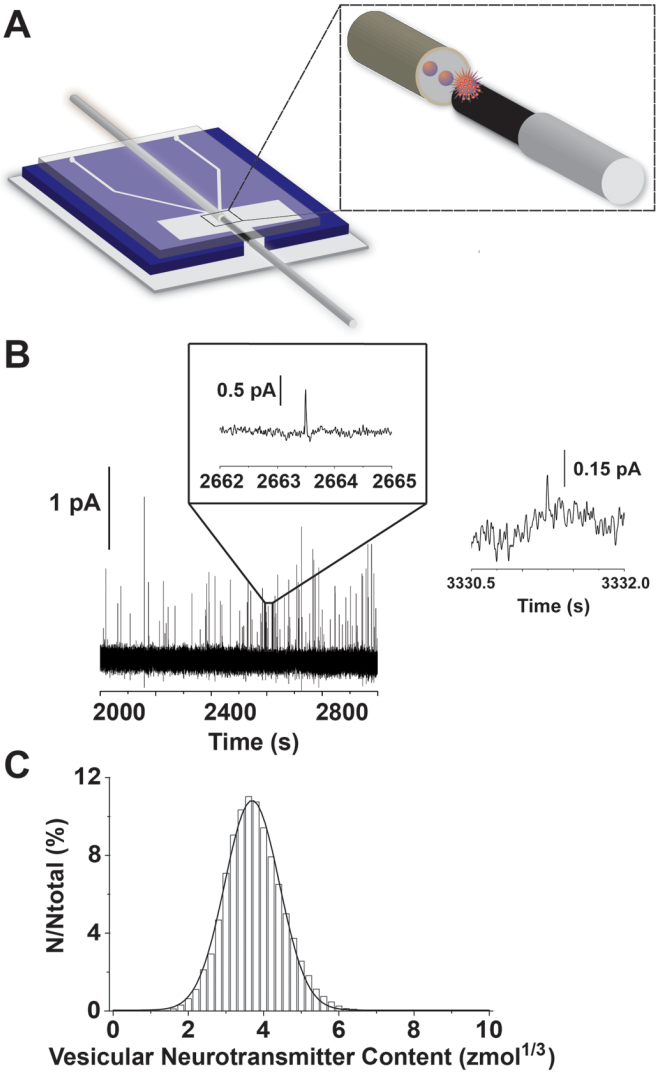

Figure 1 | Electrochemical cytometry of mouse striatal vesicles (A) Schematic of electrochemical cytometry. Electrochemical cytometry uses capillary electrophoresis to separate individual neurotransmitter vesicles. The electrophoresis capillary terminates into a PDMS-based microfluidic device where individual vesicles undergo chemical lysis as they exit and strike the carbon-fiber detection electrode. The vesicle contents are quantitatively detected using amperometry. (B) Representative data from electrochemical cytometry analysis of mouse synaptic vesicles isolated from striatum. Presented is a 1000-s portion of an electropherogram showing lysis and subsequent electrochemical detection of total neurotransmitter content in synaptic vesicles. The inset depicts an expanded axis to view the typical peak characteristics. To the right is shown an event representative of the smallest vesicles quantified on the electrochemical cytometry device. The peak integral is equal to 0.58 fC (1,841 molecules, 3 zmol), and falls in line as one of the smaller vesicular neurotransmitter amounts quantified by amperometry. (C) Normalized frequency histogram for vesicular neurotransmitter amounts quantified from mouse striatal vesicles by electrochemical cytometry. Data are plotted as the cube root transform. Bin size = 0.2 $zmol^{1/3}$. Fit was obtained from a Gaussian distribution of the data. Distribution mean is 3.7 $zmol^{1/3}$. The correlation coefficient for a single Gaussian fit of these data is 0.99.

The numbers of vesicular dopamine molecules following drug administration were calculated using Faraday's law, binned, and

plotted as normalized frequency histograms of cube root transforms. The distribution for vesicles from reserpine-treated mice, as seen in Figure 2B (red), is shifted to the left of control (black). The distribution for L-DOPA treatment (blue) is shifted to the right of control on the amount axis. The means of each distribution from single Gaussian fits of the data were 2.9 zmol$^{1/3}$, 3.7 zmol$^{1/3}$, and 4.7 zmol$^{1/3}$ for reserpine-treated, untreated, and L-DOPA-treated mice, respectively. Reserpine lowered the average number of dopamine molecules in striatal vesicles to 18,000, whereas L-DOPA resulted in an increase in average vesicular dopamine to 71,000 molecules ($p < 0.001$ versus control).

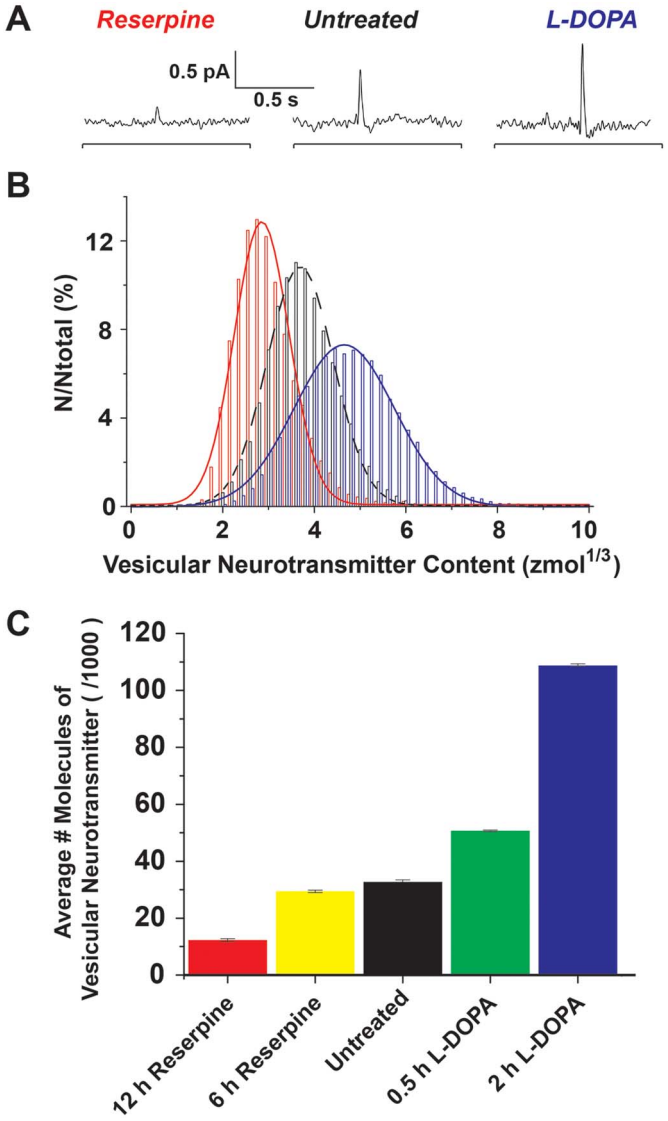

Figure 2 | Pharmacological manipulation of striatal vesicle neurotransmitter content (A) Representative current spikes for mice receiving reserpine treatment (left, 12 h, 20 mg/kg), untreated (center), or L-DOPA treatment (right, 2 h, 50 mg/kg). (B) Normalized frequency histograms for reserpine-treated (red, 12 h, 20 mg/kg, distribution mean = 2.9 zmol$^{1/3}$) and L-DOPA-treated (blue, 2 h, 50 mg/kg, distribution mean = 4.7 zmol$^{1/3}$) striatal vesicular dopamine content versus control (black, 3.7 zmol$^{1/3}$). Data are plotted as cube root transforms. Bin size = 0.2 zmol$^{1/3}$. Fits were obtained from a Gaussian distribution of the data. (C) Cumulative analysis of striatal vesicles from mice with respect to drug and time after treatment. The time after pharmacological treatment is associated with regulation of neurotransmitter content in mouse synaptic vesicles. Mean numbers of molecules of vesicular dopamine for mice dosed with 20 mg/kg reserpine for 12 h and 6 h treatments were 18,000 ± 100 (N = 7,602) and 29,000 ± 400 (N = 3,029) molecules, respectively. Mean amount of vesicular dopamine from untreated mice striatal tissue was 33,000 ± 100 (N = 20,331) molecules. Mean numbers of molecules of vesicular dopamine for mice dosed with 50 mg/kg reserpine for 0.5 h and 2 h were 51,000 ± 200 (N = 23,700) and 71,000 ± 400 (N = 22,047) molecules, respectively. Mean values are significantly different from each other (one-way ANOVA, $p < 0.001$).

The pharmacokinetics of treatments to alter vesicular dopamine can be resolved at the level of single striatal vesicles. After it was determined that changes in vesicular dopamine content could be measured, temporally related changes in vesicular content were investigated using electrochemical cytometry. These data are summarized in a cumulative statistical data analysis in Figure 2C. Mice were injected with reserpine or L-DOPA and sacrificed at shorter intervals following dosing. The mean number of dopamine molecules per vesicle 6 h after reserpine was 29,000 ± 400. This represents a 12% decrease in transmitter compared to a 45% decrease at 12 h post-treatment. An earlier time point after treatment was also investigated for L-DOPA. Here, 51,000 ± 200 dopamine molecules were present in vesicles isolated from mouse striata 0.5 h after treatment. This represents a 50% increase in transmitter compared to a 115% increase 2 h after L-DOPA administration.

Effects of amphetamine on vesicle content. Mice were administered 10 mg/kg (+)-amphetamine 1 h prior to isolating striatal vesicles for analysis of dopamine content (Figure 3). Amphetamine-treated mice showed lower average vesicular dopamine content of 28 ± 0.1 zmol or 17,000 ± 100 molecules per vesicle ($p < 0.001$ versus control).

Discussion

The advent of amperometric recordings of synaptic vesicular content has indicated that quantal size can be modulated in neuronal culture systems, for example by L-DOPA and amphetamine[14–17]. Nevertheless, it is possible that this type of modulation might not occur *in vivo*, where synaptic vesicle content might be saturated. By adapting cytometry to synaptic vesicles directly isolated from brain tissue following systemic drug treatment, we show that widely used medications modulate synaptic vesicle transmitter content. These findings increase understanding of the effects of these drugs in term of their subcellular effects. They further suggest that control of synaptic vesicle transmitter levels provides one avenue for synaptic plasticity.

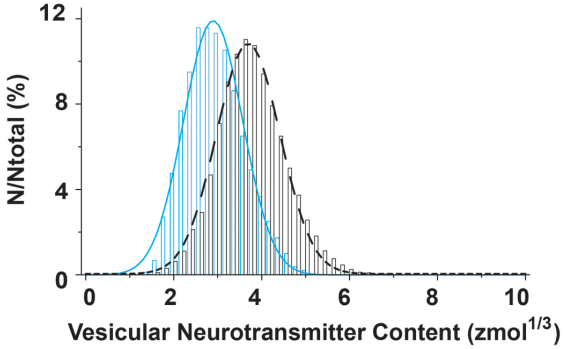

Figure 3 | **Effects of amphetamine on dopamine content of striatal vesicles Mice were treated with (+)-amphetamine (10 mg/kg i.p.), sacrificed after 1 h, and striata were dissected for vesicle isolation.** Plotted are normalized frequency histograms of vesicular data for amphetamine-treated mice (blue) versus untreated mice (gray). The distribution means were 2.9 zmol$^{1/3}$ and 3.7 zmol$^{1/3}$, for the amphetamine and untreated vesicles, respectively. Data plotted as the cube root transform. Bin size = 0.2 zmol$^{1/3}$. Fits were obtained from a Gaussian distribution of the data.

Dopamine transmission pathways in the striatum are involved in controlling a variety of behaviors including motor function, reward, addiction, cognition, and are compromised in neurodegenerative disorders, including Parkinson's and Huntington's disease[18]. However, investigating vesicular content from CNS synapses is challenging owing to the small volume and content of these subcellular organelles. Striatal synapses arising from ventral midbrain dopamine neurons are mostly populated with electron-lucent small synaptic vesicles (SSVs), which have been estimated by electron microscopy to be ~50 nm in diameter[19], although occasional dense-core vesicles have also been observed. Dopamine neurons have been reported to possess small amounts of vesicular neurotransmitter, yielding 1000–10,000 transmitter molecules per vesicle with an intravesicular concentration projected to be ~0.1 M[10,20]. These estimates have been based primarily on extrapolations from experiments measuring release from cells and tissues.

Here we use electrochemical cytometry to count molecules in synaptic vesicles isolated directly from striatal brain tissue providing content numbers that can be compared to *in vitro* electroanalytical studies of exocytotic release performed at CNS dopaminergic neurons[1,5,6,21,22]. Sulzer and co-workers investigated quantitative release at single primary mouse midbrain neurons using amperometry and recorded an average of 3,000 vesicular dopamine molecules released via chemical stimulation of cells in culture[5]. Interestingly, five larger release events with an average of 48,900 ± 12,000 molecules were quantified by amperometric measurements of neurons in culture but were excluded from the analysis data set. It was later demonstrated that vesicular release could be increased by overexpressing the vesicular monoamine transporter (VMAT2) on vesicle membranes[16]. Quantal size of released transmitter increased from an average of 7,800 molecules at control sites to 11,800 molecules at transfected sites, and the frequency of exocytosis increased 10-fold. Chow and co-workers performed amperometric measurements of dopaminergic secretory vesicle quantal size using rat substantia nigra slices[22]. In that study, exocytotic release was quantified in an environment that more closely represents an *in vivo* experimental setting when compared to primary cultures. However, in nigral slices, release was somatodendritic rather than synaptic. Released dopamine measured from the soma of nigral neurons yielded an average vesicle content of 14,000 molecules following chemical stimulation. Benzekhroufa et al., also reported amperometric detection of serotonin release from rat raphe tissue slices citing ~28,000 molecules from varicosities and ~34,000 molecules from cell bodies and in some cases as much as 800,000 molecules released[23]. These data further endorse the measurements observed in the current study at midbrain neurons. Interestingly, extrapolations from the amount of released dopamine molecules per vesicle at synapses range from 10 to 40% when compared to the electrochemical cytometry average (~33,000 molecules), thus supporting the notion of partial vesicular release during exocytosis[3]. This begs the question, is exocytotic release predominantly transient or "kiss and run" fusion?

Several experiments in the literature support the concept that in exocytosis, what most consider full release is really partial. Using quartz crystal microbalance gravimetry to examine exocytosis from cell populations, we found that the large majority of release events were immediately followed by endocytosis[24]. Looking at "complex" kiss and run events from mammalian cells, Sulzer and coworkers suggested that the complex events represented consecutive rapid transient fusion events from the same vesicle and each event was reduced by an amount for which extrapolation would suggest a total vesicular content of about 30,000 molecules[1]. Later, Borges and coworkers showed in adrenal cells that patch amperometry of large dense core vesicle exocytosis yielded approximately three times the release amount compared to non-patched amperometry of similar cells[25], suggesting that the suction from the patch pipette pulls out the remaining transmitter in an exocytosis event. Finally, Amatore and coworkers have recently found that the fusion pore does not need to open fully to accurately model the amperometric release transient observed during exocytosis[26]. We have shown that only 40% of the molecules in PC12 cell vesicles are released during amperometric detection of exocytosis[3] and evidence for vesicles closing again after exocytosis[27]. If exocytotic release is not all or none, even in the full exocytosis mode (in contrast to kiss and run release), then this presumes that release and transmission can be regulated at the level of a single event by altering the released amount.

To manipulate vesicle count *in vivo* mice were treated with pharmacological agents to alter vesicular neurotransmitter levels. The dose used in the L-DOPA experiments is within the therapeutic dose range and it causes a large increase in the number of dopamine transmitter molecules in the average vesicle. Thus, synaptic vesicles *in vivo* are not saturated with neurotransmitter, and considerable plasticity is possible. This response might partially explain the clinical action of L-DOPA, and is consistent with predictions made from quantal recordings in cultured neurons. This result is also interesting in light of the progression of Parkinson's disease with the possibility of oxidative stress increased by L-DOPA treatment versus its neuroprotective potential[28–31]. Here, reserpine reduces vesicular content by at least 45% (and perhaps more as the smallest content vesicles might not be observed) and both of these drugs can be monitored for how they affect vesicle content as a function of time. We note that a smaller number of vesicles were measured from the electrochemical cytometry of striatal tissue from reserpine-treated mice versus control and L-DOPA-treated groups (7,602 events versus ~20,000 events for control and L-DOPA groups). Perhaps some vesicles from reserpine-treated mice fall below the detection threshold (3× RMS noise). It is also possible that the smallest population of vesicles is lost during the isolation procedures, although the results of experiments involving additional centrifugation of supernatants do not support this explanation. Alternately, reserpine might cause decreased numbers of vesicles available for release.

Amphetamine is a psychostimulant that has been reported to act as a substrate for both VMAT2 and dopamine transporters[32,33]. An action at synaptic vesicles has been proposed to occur by displacement of dopamine from synaptic vesicles via a "weak base mechanism" that collapses the acidic vesicular pH gradient and/or interactions with the vesicular transporter[34]. However, the hypothesis that amphetamine affects synaptic vesicle transmitter stores under pharmacologically relevant conditions remains controversial and it has been reported that amphetamine does not, in fact, redistribute dopamine from vesicles[35]. The mechanism of amphetamine has also been investigated by performing measurements at PC12 cells and at the dopaminergic nerve of *Planorbis corneus*[32]. Interestingly, amphetamine was shown to cause an increase in extracellular dopamine release from *Planorbis*, but a decrease in vesicular quantal size (>50%) from PC12 cells. These data, in combination with whole cell capillary electrophoresis measurements of cytosolic dopamine levels, led to the hypothesis that amphetamine causes vesicles to redistribute dopamine to the cytosol and supported the weak-base hypothesis. Most experiments designed to investigate this mechanism have involved model cell lines and *in vitro* systems. Here, we adapted electrochemical cytometry to count dopamine molecules in mammalian vesicles directly after peripheral amphetamine treatment. The number of dopamine molecules decreased by half at 1 h following a moderate dose of amphetamine. Thus, we show evidence demonstrating that vesicular dopamine can be depleted by this pharmacologically relevant dose and short exposure of amphetamine. This could be due to redistribution of vesicular neurotransmitter to the cytosol in accordance with the weak base hypothesis observed in the *in vitro* analyses discussed *a priori*. Clearly this and other doses of this drug will be interesting to study in the future.

These findings indicate that the number of dopamine molecules per vesicle is considerably larger than anticipated based on prior

experiments using release measurements. This suggests that during typical exocytosis, the full vesicle content is not released for transmission and points to the possibility for regulation of the amount released at the single vesicle level. Electrochemical cytometry provides a means to quantify transmitter molecules as a function of individual vesicles in animal models of relevance for pharmacological studies and for studies of drugs of abuse. The concept of partial vesicular release raises the possibility for new pharmacological targets at the vesicular level, and defines both the transmitter count in a vesicle and the fraction released as having the potential to be plastic. If this is a general phenomenon, it has implications for short-term regulation where the number of transmitter molecules and the kinetics of release might be modified from one event to another.

Methods

Reagents. N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), sodium hydroxide (NaOH), sodium dodecyl sulfate (SDS), Trizma® hydrochloride (Tris-HCl), reserpine, and 3,4-dihydroxy-L-phenyl-alanine (L-DOPA), benserazide hydrochloride, (+)-amphetamine, glacial acetic acid, hydrofluoric acid (HF, aq. 48%), sodium chloride (NaCl), potassium chloride (KCl), glucose, calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), and Synaptic Vesicles Isolation Kits were obtained from Sigma Aldrich (St. Louis, MO). All chemicals were used as received. All buffer constituents for isolation procedures are listed as proprietary knowledge of Sigma Aldrich (*e.g.*, homogenization, lysis, and storage buffers). The electrophoretic separation buffer for electrochemical cytometry experiments consisted of 50 mM TES with 2% 1-propanol. The lysis buffer for electrochemical cytometry was 50 mM TES with 5% (w/v) SDS. All buffers were made in ultrapure water, adjusted to pH 7.4 using NaOH, and filtered through 0.2-μm pore size filters (Nalgene, Rochester, NY).

Animals and treatments. Male and female Crl:CD-1(ICR)BR mice were obtained from Charles River Laboratories (Wilmington, MA) and bred in-house for 2–4 generations. Mice were 2- to 5-months old (average age 3 months) when they were used for these experiments. Animals were group housed by sex (2–4 mice per cage) in a temperature and humidity controlled room on an automatic 12-h light/dark cycle. Food and water were available *ad libitum*. Experimental protocols adhered to National Institutes of Health Animal Care guidelines and were approved by the Pennsylvania State University Institutional Animal Care and Use Committee.

Benserazide hydrochloride was dissolved in distilled water and administered at a dose of 10 mg/kg 30 min before treatment with L-DOPA to inhibit peripheral decarboxylation of L-DOPA. L-DOPA was dissolved in 0.1 M HCl and administered at 50 mg/kg. Reserpine was dissolved in 20-μL glacial acetic acid and then diluted to volume with distilled water and administered at 20 mg/kg. (+)-Amphetamine was dissolved in distilled water and injected at as dose of 10 mg/kg. Drug doses were calculated as the free base and were administered intraperitoneally (i.p.) in a 0.2-mL volume. Time periods prior to sacrifice were either 0.5 h or 2 h for L-DOPA, 6 h or 12 h for reserpine, and 1 h for amphetamine. Mice were euthanized by cervical dislocation and brains were rapidly removed and dissected over ice to isolate the bilateral striatal samples. All animals were sacrificed during the light portion of the light/dark cycle.

Microfluidic device fabrication for the electrochemical cytometry of individual vesicles. A hybrid capillary-microfluidic device was developed as previously described to investigate transmitter amounts from individual isolated vesicles[2,3]. Briefly, microfluidic channels were fabricated using conventional photo- and soft-lithography methods. A master mold was developed by spin-coating 125 μm of SU-8 100 negative photoresist (MicroChem Corp., Newton, MA) on a 3-inch silicon wafer (Silicon Quest International, Inc., Santa Clara, CA). A photolithographic mask was placed over the wafer, exposed to ultraviolet light, and developed according to the resist manufacturer protocol. Soft lithography was carried out using a Sylgard® 184 silicone elastomer kit (Dow Corning Corp., Midland, MI). A 10 : 1 ratio of poly(dimethylsiloxane) (PDMS) prepolymer base to curing agent was cast onto the master mold and cured at 70°C for 2 h. The PDMS layer was then peeled from the master, revealing an impression of microfluidic channels. The center channel secured the separation capillary and the other two channels, each 200-μm wide, were set at a 30° angle to the center channel and used to direct lysis buffer to the capillary outlet in a sheath-flow format. The three channels converge into a 2-mm channel where the electrode was placed for detection of catecholamine quantified from individual lysed vesicles. A buffer reservoir for capillary electrophoresis was cut into a 2-mm layer of PDMS and plasma-bonded to a glass micro slide (Corning Inc., Corning, NY). The layer containing imprinted microfluidic channels was then plasma-bonded onto the reservoir layer to assemble the device (100 W, 1 min).

Separation and detection of isolated vesicles. Fused-silica capillaries (45 cm in length, 15-μm i.d./150-μm o.d., Polymicro Technologies, Phoenix, AZ) were prepared for electrochemical detection by removing 2 mm of the polyimide coating with a flame and subsequently etching the exposed fused silica by purging He (250 psi) for 15 min in an HF bath. This resulted in an etch with a frustum geometry measuring approximately 40-μm wide at the base, which served to both ease placement of the electrode at the capillary outlet, as well as to decouple the applied separation voltage from the electrochemical cell[36]. Electrokinetic injections of vesicles were performed for 5 s at 111 V/cm and separations were carried out at 333 V/cm using a high voltage supply (Spellman, Hauppauge, NY). Capillaries were conditioned before each separation to prevent nonspecific binding of the vesicle membrane to the fused-silica by rinsing at 333 V/cm with 1 M NaOH for 2 min, Ultratrol™ Dynamic Pre-Coat-HN (Target Discovery, Palo Alto, CA) for 5 min, and separation buffer for 10 min. A syringe pump (KD Scientific, Holliston, MA) was used to control volumetric flow of lysis buffer via 1-mL plastic syringes continuously through the microchannels at a rate of 2 μL/min (0.05 cm/s). Amperometric electrochemical detection was carried out in a two-electrode format. A 5-μm-diameter carbon fiber was sealed in a glass capillary and cut to a length of approximately 50 μm from the glass seal to fabricate a cylindrical microelectrode as previously described[37]. The electrode was held at 0.90 V versus a silver wire quasi reference electrode (Ag QRE, 0.25-mm-diameter, Alfa Aesar, Ward Hill, MA) and the carbon-fiber electrode was positioned at the outlet of the capillary using a micromanipulator (Newport, Irvine, CA). Previous measurements and modeling indicate that this system provides a coulometric efficiency >95%[2]. Current was measured using a Keithly model 427 (15 Hz bandpass) current amplifier (Cleveland, OH) and digitized at 5000 Hz with a National Instruments PCI-6221 DAQ card using LabView 8.0 software (National Instruments, Austin, TX) written in-house. OriginLab 8.0 (Northampton, MA) was used to generate electropherograms and Mini Analysis (Synaptosoft Inc., Fort Lee, NJ) was used for peak identification and subsequent integration. Events were quantified if the signal exceeded a threshold greater than three times the RMS baseline noise.

Western blot of isolated vesicles. Vesicles isolated from brain tissue were investigated for the presence of synaptophysin, a known integral membrane protein present in synaptic and neuroendocrine vesicles. A 50-μL suspension of vesicles was combined with 50 μL of lysis solution containing 5% (w/v) SDS in 50 mM TES buffer. The mixture was vortexed vigorously for 1 min. Aliquots of this solution containing ~20 μg of protein were loaded into four lanes of a 12% SDS-PAGE gel. Separations were carried out by applying a 200 V potential for 2 h. A SeeBlue® Plus2 pre-stained standard protein kit (Invitrogen, Carlsbad, CA) was used to visualize the separation. The gel was then transferred onto nitrocellulose paper, followed by blocking and incubation with the primary antibody monoclonal anti-synaptophysin (2-h incubation at room temperature; 1 : 1000 dilution of antibody. Monoclonal anti-synaptophysin antibody was provided in the Synaptic Vesicle Isolation Kit). The secondary antibody, HRP-conjugated anti-mouse IgG, was used for chemiluminescent detection of the targeted protein (ProteoQwest ChemiluminescentWestern Blotting Kit, Sigma Aldrich). Results revealed a prominent band at 38 kDa, confirming the presence of synaptophysin in the vesicle lysate.

Statistical analysis. Histograms of vesicular neurotransmitter amounts are plotted as a function of vesicle volume, as the cubed root transforms of these amounts approximate normal distributions of the data[10]. Fits of histograms were obtained from Gaussian distributions of the data. The number of events recorded (or vesicles measured) were counted as the *N* value for hypothesis testing. Data in Figure 2C were analyzed by ANOVA of the means. The means of the data in Figure 2B and Figure 3 possessed unequal variances and, therefore, were analyzed by a two-tailed unpaired Student's t-test.

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Author contributions

D.M.O. contributed to the experimental design, collected and analyzed data, interpreted the data and models and wrote the first drafts of the manuscript. A.J.B. worked with the mice and carried out in vivo experimental aspects, helped analyze data and edit the manuscript. A.S.C. interpreted experimental results and edited parts of the manuscript. A.M.A. provided expertise in pharmacology, mouse experimental protocols, edited the manuscript and interpreted data. M.L.H. aided in experimental design for cytometry, interpretation of data, and writing the manuscript. A.G.E. participated in design and interpretation of the experiments, writing of the manuscript, and acquired the resources and environment for the work to be done.

Additional information

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