Presynaptic Recording of Quanta from Midbrain Dopamine Neurons and Modulation of the Quantal Size

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The observation of quantal release from central catecholamine neurons has proven elusive because of the absence of evoked rapid postsynaptic currents. We adapted amperometric methods to observe quantal release directly from axonal varicosities of midbrain dopamine neurons that predominantly contain small synaptic vesicles. Quantal events were elicited by high K+ or α-latrotoxin, required extracellular Ca2+, and were abolished by reserpine. The events indicated the release of 3000 molecules over 200 μsec, much smaller and faster events than quanta associated with large dense-core vesicles previously recorded in vertebrate preparations. The number of dopamine molecules per quantum increased as a population to 380% of controls after glial-derived neurotrophic factor (GDNF) exposure and to 350% of controls after exposure to the dopamine precursor L-dihydroxyphenylalanine (L-DOPA). These results introduce a means to measure directly the number of transmitter molecules released from small synaptic vesicles of CNS neurons. Moreover, quantal size was not an invariant parameter in CNS neurons but could be modulated by neurotrophic factors and altered neurotransmitter synthesis.

Key words: α-latrotoxin; amperometry; dopamine; electrophysiology; evocability; GDNF; L-DOPA; midbrain; quantal analysis; quantal release; recycling; synaptic vesicles; VMAT

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paper thus provides a new means for analysis of the fundamental units of CNS transmission.

MATERIALS AND METHODS

Primary cultures. Postnatally derived cultures of midbrain neurons were prepared as described earlier (Rayport et al., 1992) except that serum-free medium was used (Mena et al., 1997). Recordings were performed 3–6 weeks postplating. We did not observe amperometric spikes in younger cultures, perhaps because of delayed maturation of the synaptic machinery after the dissociation procedure and continued neurite outgrowth during this period (Burke et al., 1998); such delays are not unusual in studies of synaptic function in neuronal cultures. The medium contained the antioxidants selenium and catalase and did not contain ascorbic acid. The major released oxidizable species detected by HPLC with electrochemical detection (HPLC-EC) were identified as DA and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid. The cultures did not contain other monoaminergic cell types (Rayport et al., 1992). For glial-derived neurotrophic factor (GDNF)-treated cultures, GDNF (10 ng/ml; Intergen, Purchase, NY) was added to the normal growth medium on the day of plating.

Electrochemical recording. Electrochemical recordings (HPLC-EC) were performed using carbon fiber electrodes manufactured at Instrutech (Great Neck, NY). The resulting current showed exponential decay on time constants of 4–5 sec duration in excess of 20 μsec, depending on the filter. However, a 100 kHz filter was typically sufficient to resolve points spaced at 0.9 μsec apart, at least two orders of magnitude less than the typical rms noise of the system is below resolution; the 16 bit digital-to-analog converter across the range we recorded (± 250 pA) resolves points ± 7.5 fA apart, at least two orders of magnitude less than the typical rms electrode noise.

Elevated temperatures. Normal recording medium contained (in mM) 150 NaCl, 2 KCl, 10 HEPES, 1 MgCl₂, pH 7.4. Ca²⁺-free medium eliminated CaCl₂, and included 1.2 mM EGTA. Stimulation media were used for 20–80 mM K⁺ iso-osmotically substituted for NaCl. A background medium or α-latroxin (a-LTX; 20 μM) was provided good membrane preservation (Sulzer and Rayport, 1990; Rayport et al., 1992). Secretagogues were applied from a distance of 20–40 μm to the recording electrode by using gentle (3–6 psi) air pressure (Picospritzer, General Valve, Fairfield, NJ) for 2–6 sec for a total application of ~7–20 μl. Therefore, final secretagogue levels at the neuron were more dilute. Because quantal size was not different for the different secretagogues (see Fig. 2B), α-LTX sometimes was prepared in high K⁺ medium. Recordings took place at room temperature (25°C), and experiments were completed within 90 min of removal from the incubator. For the data reported in Table 2 and Figure 6, the dihydroxyphenylalanylamine (1-DOPA) exposures were for 30 min at 36.7°C, and the 1-DOPA-containing medium was replaced with fresh physiological saline before recording.

Electrochemical recording. Carbon fiber electrodes were manufactured as described, placed in a Leica 13.1 numerical aperture oil immersion lens and standard fluorescein filter sets (Zeiss, Oberkochen, Germany) were used. Digital images were obtained with a Photometrics SenSys PVCAM 12-bit camera (Tucson, AZ) using IPLab Spectrum software (Signal Analytics, Vienna, VA), with additional software for quantification. Two images were acquired in Ca²⁺-free medium (with 1.2 mM EGTA) 5 min apart. Subsequent images were taken, the first after a single extracellular exposure to 40 mM K⁺ for 10 sec (local perfusion as above) and the other after three additional 10 sec exposures to 40 mM K⁺. The fluorescence arbitrary units are the mean emission intensity of pixels of ROIs as indicated by the digital camera, with a maximum emission of 4096 units.

Electrophysiological recording. Carbon fiber electrodes were manufactured as described (Potheos et al., 1996), and potential was applied via an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Electrodes with background >0.9 pA root mean square (rms; four-pole 5 kHz Butterworth filter) or unstable cyclic voltammograms when tested in a Butterworth filter) or unstable cyclic voltammograms when tested in a 1 kHz four-pole Bessel filter were initially filtered by a four-pole Bessel filter to improve signal/background at low temporal resolution. The bottom traces of Figure 2B were filtered digitally at 10 kHz to provide a sharp cutoff at that frequency and to eliminate a few extremely rapid (1 data point) current transients in intervals between events.

Amperometric spikes were identified as events with ±5 data points (i.e., a 40 μsec interval) >4.5 times the rms noise of the baseline. This cutoof excluded transients observed during background recordings in the bath. The cutoff amplitude was typically ~7 pA above baseline (rms noise with a four-pole 10 kHz Bessel filter was typically ~1.4 pA). The event width was the duration between (1) the baseline intercept of the maximal baseline to the baseline from first point that exceeded the cutoff and (2) the first data point after the maximal amplitude that registered a value less than or equal to baseline. The maximum amplitude (I_peak) of the event was the highest value within the event. The width at half height (t½) was determined by the duration of the spike trace at one-half of the maximal amplitude. To resolve quantal size, we determined the total charge of the event between the baseline intercepts and the number of molecules estimated by the relation n = Q/F, where Q is the number of electrons donated per molecule, and F is Faraday’s constant (96,485 coulombs per equivalent). Quantal sizes reported in this study were based on an assumption of two electrons donated per oxidized molecule of DA (Ciolkowski et al., 1994), although monoamines donate four electrons under conditions of restricted diffusion (Ciolkowski et al., 1994; Bruns and Jahn, 1995); the lower estimate was derived in a study of DA oxidation in an analogous system (Ciolkowski et al., 1994). HPLC with electrochemical detection. Monoamines and metabolites were measured by HPLC-EC. For measurement of DA and metabolites in whole cultures, sister cultures (n = 4–5 per condition) were exposed first to high K⁺ (40 mM, 2 min), and the extracellular medium was stored at −80°C for injection. Then the cells were scraped and extracted with 0.1N perchloric acid for the measurement of the remaining intracellular levels. Separation was obtained by a Velosel 10 cm RP-18 3 μm column (Applied Biosystems, Foster City, CA). Detection was performed by a coulometric detector (Coulonich II, ESA, Bedford, MA) with a guard cell set at −40 mV and an analytical cell at 400 mV. The mobile phase contained 6.9 gm/l sodium phosphate, 80 mg/l EDTA, 750 mg/l heptane sulfonic acid, and 4.5% methanol, pH 3.6 (phosphoric acid).

Immunostaining. Tyrosine hydroxylase (TH) immunostaining was performed as described (Przedborski et al., 1996). Rabbit anti-vesicular monoamine transporter (VMAT2) primary polyclonal antibody was obtained from Dr. Robert Edwards (University of California, San Francisco). After fixation (4% paraformaldehyde for 30 min), cultures were incubated for 48 hr with an anti-VMAT2 antibody diluted 1:100 and visualized with biotinylated goat anti-rabbit antibody and diaminobenzidine/horseradish peroxidase reaction (Vectorstain Elite kit, Vector Laboratories, Burlingame, CA). Digital images were acquired with a CCD camera (Photometrics). The measurement of VMAT-labeled varicosity dimensions and distributions was performed with National Institutes of Health Image software.

Electron microscopy. For electron microscopic observations, the preparation was as reported (Sulzer and Rayport, 1990) except that the ice-cold fixative (1.5% glutaraldehyde in 0.1 m phosphate buffer) was exchanged rapidly with the culture medium over 5 sec. This provided for better preservation of 5-hydroxydopamine (5-OHDA) label than slower room temperature fixation in the presence of 400 μM CaCl₂, which provided good membrane preservation (Sulzer and Rayport, 1990; Rayport et al., 1992).

Statistics. Comparison of populations used the Kolmogorov–Smirnov statistic (GBStat, Dynamic Microsystems, Silver Spring, MD), a standard nonparametric procedure routinely used for the analysis of quantal populations detected by postsynaptic recording (Van der Kloot, 1991). ANOVA with repeated measures was used to compare changes in fluorescence emission.
RESULTS

Amperometric recording from axonal varicosities

To observe directly the quantal release from a presynaptic site of a central neuron, we examined axonal varicosities of midbrain DA neurons derived from rat neonates and cultured for 3–6 weeks. DA neurons identified by immunoreactivity to TH antibodies (Fig. 1A1) exhibited neurites that commonly were observed to extend over 1 mm in length. The axons displayed presynaptic varicosities that were 0.5–4 μm in length (mean = 1.7 μm; Table 1), as indicated by immunostaining for VMAT2, and often were in apparent contact with cell bodies (Fig. 1A2; Table 1).

Such sites showed exocytosis of recycling structures, as observed with the stimulation-dependent destaining of the fluorescent endocytic tracer FM1-43 (Fig. 1B). The mean fluorescence was reduced (Fig. 1C) from 1004 ± 129 [mean ± SEM, mean fluorescence arbitrary units per region of interest (ROI)] in the first baseline image (cultures maintained in Ca2+-free medium) and 967 ± 100 units in the second baseline image (Ca2+-free) to 743 ± 88 units after one exposure to 40 mM K+ (10 sec) and 562 ± 105 units after three additional exposures to 40 mM K+ (10 sec each). The observed reduction was significant, as shown by one-way ANOVA with repeated measures (n = 23 ROI; F1,21.915 = 12.9868; p < 0.0001). Post hoc Student’s t test pairwise comparisons showed that the fluorescence reduction was significant only in the images after 40 mM K+ exposure (t = 3.53 and 4.82 with the first image after 40 mM K+ paired to each of the two baselines, respectively, p < 0.01; t = 5.30 and 4.87 for the subsequent post-40 mM K+ image, p < 0.01). Furthermore, there was a significant reduction in fluorescence when the first post-40 mM K+ images were compared with the subsequent images (t = 3.77; p < 0.01). No significant difference was found between the two baseline images per site, which suggests that the observed destaining after stimulation is attributable to exocytosis and not to bleaching of the fluorescent signal.

We used two techniques that assisted in the identification of sites for recording: (1) intracellular labeling to illuminate candidate axonal varicosities and (2) the selection of areas where multiple processes clearly overlap, because these showed a higher incidence of VMAT2 stain (see below). The detection of exocytic release of DA was performed by applying +700 mV to a 5-μm-diameter carbon fiber electrode placed directly on the exposed upper surface (~20% of surface area) of such varicosities. When we used these techniques, ~5–10% of the sites that were examined showed amperometric events. The low fraction of responding areas was not attributable to a low representation of DA neurons, which range from ~20–40% of the neuronal population and display extensive axonal branching. We suspect that many DAergic axonal varicosities were not electrochemically active because the releasing site was not oriented directly to the electrode surface or was occluded by other neurites or glial sheaths.

All of the sites that showed amperometric events and subsequently were fixed and processed for immunocytochemistry were found to be TH-immunolabeled (n = 12). In instances of apparent release at cell bodies, subsequent TH or VMAT2 immunostaining indicated that DA varicosities were under the electrode in close contact with the cell bodies.

In some cases, neurites were labeled with Lucifer yellow (0.3%) that was injected during whole-cell patch clamp to visualize varicosities (Fig. 2A). Depolarization by high K+ (20–80 mM) evoked generally low numbers of amperometric events (2.6 ± 1.2 events, mean ± SEM; n = 13 sites where events were observed) (Fig. 2A3, top trace). Because DA diffuses rapidly and the events were mostly <500 μsec in duration, only events directly under the electrode face were likely to have produced distinct amperometric spikes (Schröder et al., 1992). An elevated baseline was observed sometimes, presumably because of diffusional broadening of DA release from nearby fusion events not directly under the electrode. Amperometric events were dependent on extracellular Ca2+ (Fig. 2A4, middle and bottom traces). No events were elicited by the application of physiological saline, and event detection was abolished reversibly when the voltage applied to the electrode was decreased to 0 mV. Although we found Lucifer yellow label to be useful for identifying varicosities associated with given cells, the protocol was inefficient, and we generally selected sites that displayed several overlapping neurites.

Amperometric events also were elicited by application of α-LTX (Fig. 2B), a secretagogue that induces massive synaptic vesicle exocytosis and sustained transmitter release (Petrenko, 1993). High K+ or α-LTX elicited amperometric events of identical size (Fig. 2B) although apparent overlapping events were observed with α-LTX (see below), whereas such events were rare (<1% of events) with high K+ stimulation. The average number of release events per stimulation was 4 ± 2 (n = 19 sites). However, in subsequent observations there were rare instances of massive release with >200 quanta after a single α-LTX application. After subsequent immunostaining for VMAT2 or TH, we could discern no morphological differences between sites that released many events and those that released few.

Amperometric spikes were almost never observed after the application of high K+ in Ca2+-free conditions (one event from 50 sites, 4400 molecules) or after exposure to the VMAT inhibitor reserpine (no events from 23 sites after 1 μM reserpine for 90 min), a treatment that depletes intracellular and releasable DA stores by 97% in this system (Sulzer et al., 1996). However, in the case of α-LTX, release events could be observed in the absence of extracellular Ca2+ (n = 2 sites in 16 trials), as has been reported for other neuronal preparations after exposure to this secretagogue (Zhou and Misler, 1995).

In 80 sites where amperometric spikes were observed, only three spontaneous events in control cultures (3700 ± 700 molecules, mean ± SEM) were recorded during a ≥6 sec period before secretagogue application, consistent with the tendency of midbrain DA neurons to not produce spontaneous action potentials in vitro (Rayport et al., 1992). Hippocampal neurons were used as a control preparation because they do not release detectable monoamines and are not TH-immunopositive (TH⁺; data not shown); no current spikes were detected from hippocampal processes after high K+ or α-LTX stimulation (n = 35 sites).

There were a few very large events (48,900 ± 12,000 molecules; n = 5) in the control group that were ≥10-fold greater than the SD of the rest of the population distribution. These large events were evoked by α-LTX (n = 4) and high K+ (n = 1). Analogous infrequent large postsynaptic potentials, termed “giants” miniature end plate potentials, have long been noted at the neuromuscular junction (Van der Kloot, 1991).

To examine the vesicle population capable of accumulating DA in our system, we identified DA synaptic vesicles under electron microscopy by uptake of the DA analog 5-OHDA, using a rapid glutaraldehyde fixation technique that we found to be very effective at fixing vesicular 5-OHDA. This analog produces an electron-dense reaction product after exposure to osmium tetroxide. We found that a majority (>99%) of vesicles in the DA varicosities in this system are small synaptic vesicles with diamete-
Figure 1. A1, Ventral midbrain dopamine (DA) neurons in culture were immunostained for tyrosine hydroxylase (TH), using a fluorescein-conjugated secondary antibody. A2, The same cell pair immunostained for the brain vesicular monoamine transporter (VMAT2) and visualized by a horseradish peroxidase/diaminobenzidine reaction product. The VMAT2-labeled varicosities (examples indicated by arrows) measured 0.5–4 μm in diameter at their longest axis and occurred both with and without obvious contact to other neurons. Scale bar, 5 μm. B1, B2, Presumed presynaptic compartments (examples indicated at arrows) were labeled by the endocytic tracer FM1-43 (4 μM in the presence of 6 μM nicotine for 1 min; the nicotinic acetylcholine receptor is presynaptic at midbrain DA neurons) (Marshall et al., 1997). The neurons were maintained in Ca²⁺-free medium for 10 min with little decrement in label. The figures display the label at 5 and 10 min. The perfusion pipette (P) extends from the base and appears green in the pseudocolored images because of the autofluorescence. B3, B4, Then these sites were stimulated with 40 mM K⁺ medium (10 sec), decreasing the label at several sites in the path of the perfusion. Three further applications of 40 mM K⁺ medium (10 sec) induced further decrement of FM1-43 label, demonstrating that the sites studied contain organelles that recycle on stimulation. The site indicated by the top left arrow, which is farther from the perfusion pipette, shows less decrement than sites closer to the pipette. Scale bar, 5 μm. C, Quantitation of decrease in mean fluorescence per region of interest (ROI) after stimulation (n = 23 sites). Each box and whiskers symbol corresponds to the exposures as indicated in the figure above. The mean is indicated by the black horizontal cross on the vertical bars, the limits (whiskers) at the ends of the bars indicate the maximum and minimum values for the data set, and the box limits indicate the 5th and 95th percentiles of the data set.
of TH expression and the potentiation of stimulation-dependent (Hebert et al., 1996) and results in a delayed (1–3 week) elevation 

activates the receptor tyrosine kinase Ret (Durbec et al., 1996; 

release is significantly higher in GDNF-treated cultures, there are no changes in VMAT 

m 1 at normal Ca 2 

probability of simultaneous exocytosis, the quantal sizes were 

events that were recorded were monoquantal. 

postsynaptic response to the secretion of neuro-

aptic quantal size may be modulated is to examine neurotrophic 

factors, which are known to facilitate transmitter release as well as 

to promote neuronal survival (for review, see Kang and Schuman, 1995; Thoenen, 1995; Stoop and Poo, 1996). 

After the initial characterization of quantal release, we examined this issue under conditions designed to mimic effects reported in vivo. In the rodent a single injection of GDNF, which activates the receptor tyrosine kinase Ret (Durbec et al., 1996; Jing et al., 1996), is neurotrophic for midbrain DA neurons (Hebert et al., 1996) and results in a delayed (1–3 week) elevation of TH expression and the potentiation of stimulation-dependent DA release (Beck et al., 1996). Therefore, to examine the effects of GDNF on presynaptic modulation, we cultured ventral midbrain neurons for 3–6 weeks with or without 10 ng/ml GDNF in the extracellular medium at the time of plating; the factor was not replaced. 

GDNF-exposed cultures displayed a twofold increase in the presence of TH -labeled cell bodies, similar to studies conducted with embryonically derived cultures (Lin et al., 1993) and postnatally derived cultures (Burke et al., 1998) (Fig. 4.A,B, Table 1). To determine the effect of GDNF exposure on overall depolarization-evoked DA release, we used HPLC-EC. GDNF increased the DA release evoked by high K+ to 280% of control levels (Table 1) and the corresponding intracellular DA levels to 400% (from 6.10 ± 0.79 to 24.54 ± 3.43 pmol per culture), as measured by HPLC-EC. 

To determine whether GDNF increased the density of DA synaptic varicosities, we immunostained for VMAT2 (Fig. 4C,D, Table 1). The number of varicosities that might contribute to amperometric detection was estimated by two methods. First, the number of varicosities per axonal length and the intervaricosity distance along the axon were measured and were found not to change with GDNF exposure (Table 1). However, if more axonal branching occurred, more varicosities might be found under the electrode. Therefore, we counted the number of VMAT2-immunostained varicosities present within 5-μm-diameter circles at sites with the morphology from which we typically record, i.e., where neurites cross; this was found to be identical under both conditions (Table 1). Finally, the two-dimensional morphology of the varicosities (maximum length along the process axis, maximum width tangential to the process, and area) was not changed by GDNF exposure (Table 1). Therefore, whereas GDNF increased the total number of surviving DAergic neurons and overall axonal outgrowth, it did not alter significantly the morphology or distribution of VMAT2-expressing varicosities along the axons. 

We used amperometric recordings from varicosities of GDNF-exposed neurons and control cultures to determine the basis of increased DA release. GDNF-treated neurons had a mean quantal size 380% greater than controls (Table 2; p < 0.0001). The combination of a higher presence of DA neurons and greater quantal size would suggest that GDNF exposure elevates total DA release per culture to even greater levels than that measured by HPLC. The relatively lower levels of potentiation measured when extracellular levels are examined by HPLC-EC are likely attributable to differences in experimental conditions. In particular, total extracellular DA would underestimate overall quantal 

Table 1. Effects of one-time GDNF exposure on DA release, distribution of VMAT2-stained varicosities, and varicosity morphology

<table>
<thead>
<tr>
<th>GDNF</th>
<th>DA release</th>
<th>Percentage of TH+ cell bodies</th>
<th>VMAT+ intersite distance</th>
<th>VMAT+ /area</th>
<th>VMAT+ surface area</th>
<th>VMAT+ length</th>
<th>VMAT+ width</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.54 ± 0.14</td>
<td>18.11 ± 0.0</td>
<td>24.7 ± 0.15</td>
<td>1.69 ± 0.13</td>
<td>0.66 ± 0.04</td>
<td>1.70 ± 0.04</td>
<td>0.37 ± 0.01</td>
</tr>
<tr>
<td>GDNF</td>
<td>1.52 ± 0.19</td>
<td>37.90 ± 1.4</td>
<td>22.9 ± 0.11</td>
<td>1.69 ± 0.13</td>
<td>0.46 ± 0.03</td>
<td>1.42 ± 0.04</td>
<td>0.31 ± 0.01</td>
</tr>
</tbody>
</table>

DA release reports extracellular levels of DA (pmol/culture) after exposure to high K+ medium (40 mM, 2 min; n = 4 and 5 cultures, respectively; p < 0.003 by one-way ANOVA; F(1,5) = 19.2199). Percentage of TH+ cell bodies reports the percentage of neurons in the cultures that were TH-immunostained (n = 18,745 total neurons in three cultures and 24,368 total neurons in four cultures, respectively; pc < 0.0001 by one-way ANOVA; F(1,5) = 441.1639). VMAT+/intersite distance reports the axonal distances (μm) between neighboring VMAT-labeled varicosities (n = 105 intersite measurements for both groups). To determine VMAT+/area, we placed 5 μm diameter circles, the approximate surface area of the electrodes used in this study, over the locations of the type that we typically recorded from, i.e., sites where multiple processes clearly overlap, and the number of VMAT+ sites underneath was counted; the units are the number of varicosities per 5-μm-diameter circle, thereby estimating the number of varicosities under the electrode (n = 29 circles for each condition). VMAT+ surface area represents the two-dimensional area (μm2) of the labeled varicosities as determined by the limits of the VMAT label; the length (μm) and width (μm) of these also are reported (n = 63 varicosities for each condition). Although the proportion of TH+ neurons and DA release is significantly higher in GDNF-treated cultures, there are no changes in VMAT+ sites under the electrode, distribution along the axon, surface area, or varicosity length or width.
release, because stimulation of the culture is measured after 2 min of depolarization so that autoreceptors likely have been activated, and there has been significant DA reuptake by the plasma membrane uptake transporter.

**Effects of L-DOPA**

TH catalyzes the rate-limiting step for catecholamine synthesis in neurons. If cytosolic levels of DA are rate-limiting for synaptic vesicle uptake, quantal size should be increased by the TH product L-DOPA, which is converted to DA by the cytosolic enzyme aromatic acid decarboxylase. We exposed ventral midbrain DA neurons to 100 μM L-DOPA (30 min at 25°C; Fig. 5) or 20 μM L-DOPA (30 min at 36.7°C; Fig. 6, Table 2), which rapidly increases intracellular DA levels in this system as measured by HPLC-EC (HPLC-EC identifies the principal compound released by high K + after L-DOPA as DA; Pothos et al., 1996). In the present study we found that preincubation with L-DOPA elevated quantal size (Table 2), demonstrating that vesicular accumulation is controlled in part by the pool of cytosolic transmitter.

**Population distribution of the quantal size**

The protocol used to analyze presynaptic modulation of quantal size could compare either single sites observed before and after a given treatment (e.g., Figs. 2B, 5) or multiple sites exposed to a single stimulation (e.g., analysis in Table 2 and Fig. 6). Although the examination of single sites would eliminate synapse-to-synapse variation, the low number of events typically induced by high K + makes statistical analysis difficult. Although α-LTX induced greater release, and occasionally elicited large numbers of events, it also produces massive Ca 2+ currents that may strongly influence subsequent responses, including excitotoxicity. Moreover, catecholamine release is very susceptible to influence from previous stimulations, and chromaffin cells show potentiated quantal release after single high K + exposures (Finnegan et al., 1996). Therefore, although observations at single sites indicated that quantal size was modulated, we suggest that the more stringent analysis in this case was to examine quantal size in groups of sites exposed to the secretagogue once only and to bin the events. Because the effects on quantal size were large, this approach demonstrated highly significant differences between groups.

Individual examples of typical monoquantal events recorded after the first stimulation at neurite varicosities are displayed (Fig. 6A–C). Occasional spike features that may be analogous to the “foot signals” reported for large dense-core vesicle exocytosis may be present. The total population distributions of the quantal events detected in control, GDNF, and L-DOPA conditions are indicated (Fig. 6D). The quantal size, amplitude, and width are each highly significantly increased in the GDNF and L-DOPA groups in comparison to controls (p < 0.0001 in each case; see Table 2).

The distribution of the GDNF and L-DOPA quantal sizes was shifted to the right, and the data indicate a wider variance of values. The untransformed quantal size distribution of the populations displayed a prominent right-hand skew (Fig. 6D) so that the population modes were smaller than the population means (Table 2).

**DISCUSSION**

In this study, quanta from midbrain DA neurons exhibited a unimodal size population that was modulated by exposure to neurotrophic factors and altered transmitter synthesis. The quanta were of far more rapid duration and represented fewer molecules than those from neuroendocrine cells and peripheral neurons, which release 3 × 10 4–10 6 molecules over a period of several milliseconds. The events we recorded were attributable to exocytosis from small synaptic vesicles because (1) small synaptic vesicles are abundant in this preparation, whereas large dense-core vesicles are rare, (2) the small synaptic vesicles accumulated a DA analog and so would be competent to provide exocytic release, and (3) in the striatum in vivo, DA axonal varicosities nearly exclusively display small synaptic vesicles, and VMAT2 expression is limited to small synaptic vesicles (Nirenberg et al., 1997); therefore, small synaptic vesicles are the likely sites for the storage and release of DA. Significantly, the leech Retzius cell contains high numbers of both small synaptic vesicles and large dense-core vesicles and exhibits two subpopulations of amperometric events (Bruns and Jahn, 1995). One set exhibits the millisecond-long durations seen with large dense-core vesicle preparations, whereas a group of rapid events exhibits durations similar to those we report in midbrain DA neurons. Regulation of the quantal size and the duration of the events were not examined in the leech preparation.

We conclude that amperometric events in midbrain DA varicosities were attributable to monoquantal release because (1) randomly coupled events would be expected to be very rare, because only one to three varicosities were in contact with the electrode and the number of release events recorded was low; (2) the patterns of amperometric release events elicited by α-LTX or high K + were different, but the events were not of different size; (3) the few spontaneous events recorded were the same size as events elicited with secretagogues; (4) quantal size was not different at 0, 1.2, and 6 mM Ca 2+; and (5) the quantal size 1/3 of the GDNF and L-DOPA groups approached a unimodal normally distributed population. Nonetheless, apparent overlapping quanta occasionally were recorded at some sites with α-LTX, and a small number of more closely overlapping nonrandom events seem likely to have occurred. To appear as single events, they would have to be highly temporally coordinated, i.e., within 100
Figure 2. A1, A combined fluorescent/incandescent image of a neuron labeled with Lucifer yellow by whole-cell patch clamp. Magnification bar, 10 μm. A2, Fluorescent image of a detail corresponding to the area indicated by the broken box in A1. The placement of the 5 μm (vertical axis) electrode is indicated by the oval. Two labeled varicosities are apparent under the electrode surface. A3, The top trace shows amperometric events at the site in A2 after 3 sec of high K⁺ in the presence of 1.2 mM Ca²⁺ (the arrow indicates the application of high K⁺ for each trace). The events were not evoked by high K⁺ in Ca²⁺-free medium (middle trace). Recovery occurred when high K⁺ in the presence of 1.2 mM Ca²⁺ was reapplied (bottom trace). Of 14 neurons labeled by Lucifer yellow injection, five showed amperometric spikes from apparent varicosities. For each of three sites so examined, the removal of Ca²⁺ resulted in the complete cessation of spikes, and spikes resumed after the readministration of high K⁺ with Ca²⁺. All sites where quanta were observed had TH⁺ processes underneath the electrode, although only three of the five dye-filled neurons in which amperometric events were evoked were themselves TH⁺; therefore, nondye-filled dopaminergic processes were sometimes under the electrode. B, Exemplar amperometric recording contrasting two secretagogues. This culture was exposed to both GDNF and L-DOPA (50 μM for 30 min) to promote elevated levels (Figure legend continues).
Therefore, the ability to distinguish between “simultaneous” events is at least an order of magnitude better than with previous quantal recording techniques that measure postsynaptic responses of millisecond durations.

An advantage of this approach is that it is adapted for the study of catecholamine release, which is not measured by postsynaptic recording. Quantal release from DA presynaptic varicosities was observed with the electrode placed at the upper surface, clearly indicating that the varicosities can provide extrasynaptic overflow. The amperometric events we report are attributable to the release of DA because (1) other catecholamines were not released in this system as shown by HPLC-EC, (2) the events were abolished rapidly by reserpine, a VMAT inhibitor that depletes vesicular DA but not ascorbic acid (Rebec and Pierce, 1994), (3) events were not observed unless sufficient oxidation potential was applied to the electrode, (4) all sites that provided amperometric events and were processed for immunocytochemistry were TH+, (5) quantal size was increased by exposure to L-DOPA, which promotes synthesis of cytosolic DA, and (6) hippocampal neurons did not produce such events. DA metabolites such as dihydroxyphenylacetic acid (DOPAC) were unlikely to contribute to the signal because there is no evidence that they are sequestered in synaptic vesicles, they are not released in a Ca2+-dependent manner, and they are not substrates for VMAT2 and hence would not be reserpine-sensitive.

**Alteration of quantal size**

A basic presynaptic property by which quantal size could be elevated is by the release of more neurotransmitter molecules. We recently have observed this with large dense-core vesicle exocytosis of DA release. Medium containing 40 mM K+ was applied for 2 sec (first arrow). Nineteen spikes (amplitudes >4.5 × rms background) were elicited, of which 13 occurred during the perfusion plus 1 sec. α-LTX (20 nm) in normal recording medium was applied for 2 sec (second arrow). Then 51 spikes followed, beginning 6.9 sec from the start of the perfusion. A portion of the trace marked by the dashed lines is shown with increased time resolution in the two lower contiguous traces. The dashed lines extending from the lowest contiguous trace indicate those spikes with amplitudes greater than 4.5 × rms background noise. The lowest trace shows these spikes at further increased time resolution. The rightmost pair of events that are displayed appear to overlap slightly at the base. Interevent intervals were 1000 ± 492 msec (mean ± SEM) for high K+ and 1935 ± 556 msec for α-LTX. The quantal sizes elicited by the two secretagogues were not significantly different (p = 0.24; KS-Z = 1.0302, Kolmogorov–Smirnov test). The top trace in B was digitally filtered at 1 kHz to provide better signal-to-noise resolution at low temporal resolution; the lower traces were digitally filtered at 10 kHz. Note that the same spikes are visible with both filters.
tosis in peripheral secretory cell lines by using amperometric recordings of PC12 cells (Sulzer et al., 1995; Pothos et al., 1996), and analogous effects have been reported at the neuromuscular junction (Van der Kloot and Molgo, 1995; Song et al., 1997). In the case of quantal catecholamine release, it is possible that transmitter release from large (150–1000 nm in diameter) dense-core vesicles in PC12 and other neurosecretory cells that contain prominent electron-dense precipitates of transmitter and associated anions differs in fundamental ways from the release caused by exocytosis of small (50 nm in diameter) “clear” synaptic vesicles.

An alternate explanation for elevated quantal size could be that treatments changed the distance of the recording electrode from the release site. This possibility is inconsistent with the following observations. (1) The charge measured with a ~5-μm-diameter electrode surface remains the same for distances of up to ~1 μm (Schroeder et al., 1992). The present experiments used an electrode in physical contact with the plasma membrane so that the

Table 2. Comparison of amperometric events from control neurons and neurons exposed to GDNF or 20 μM l-DOPA for 30 min (mean ± SEM)

<table>
<thead>
<tr>
<th></th>
<th>Number of molecules (mean)</th>
<th>Number of molecules (mode)</th>
<th>$i_{\text{max}}$ (pA)</th>
<th>$t_{1/2}$ (μsec)</th>
<th>Width (μsec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3000 ± 300</td>
<td>2300</td>
<td>14.6 ± 1.4</td>
<td>120 ± 40</td>
<td>180 ± 40</td>
</tr>
<tr>
<td>GDNF</td>
<td>11,400 ± 700</td>
<td>4300</td>
<td>26.8 ± 1.6</td>
<td>130 ± 80</td>
<td>430 ± 50</td>
</tr>
<tr>
<td>20 μM l-DOPA</td>
<td>10,600 ± 400</td>
<td>5400</td>
<td>23.7 ± 1.0</td>
<td>130 ± 10</td>
<td>370 ± 20</td>
</tr>
</tbody>
</table>

$i_{\text{max}}$ represents maximum amplitude, $t_{1/2}$ the width at half height, and the width the duration of the event. Controls represent $n = 69$ events (20 sites); the 20 μM l-DOPA-treated group represents $n = 295$ events (9 sites); the GDNF-treated group represents $n = 109$ events (14 sites). Five large outlying events (see Results) and apparent overlapping events (e.g., rightmost pair of Figure 2B, bottom expansion) are not included. GDNF and l-DOPA increased the mean quantal size to 380 and 350% of control levels, and both were significantly different from controls ($p < 0.0001$ in both cases; KS-Z = 3.027, KS-Z = 5.0931, respectively). GDNF and l-DOPA increased the maximum amplitude ($p < 0.0001$; KS-Z = 4.0202 and KS-Z = 4.6284, respectively) and width ($p < 0.0001$; KS-Z = 2.9962, KS-Z = 4.8912, respectively). The modal values of the quantal size are smaller than the means because of the right-hand skew of untransformed quantal size populations. The population distribution of the same events is displayed in Figure 6D.

Figure 4. Morphological effect of GDNF on midbrain DA neurons in culture. A, Three weeks after plating, TH immunostaining indicates a dense plexus of neurite outgrowth. The arrows indicate TH-unlabeled cell bodies. B, Cultures exposed to GDNF display twofold more stained cell bodies (see Table 1) and also maintain a dense plexus of neurites. C, VMAT2 immunolabel in a control culture indicates sites containing dopaminergic synaptic vesicles. Arrowheads indicate examples of VMAT-labeled varicosities. D, Shown is a VMAT2 stain of a culture exposed to GDNF. The distribution of VMAT2-labeled varicosities along the axis of the axon and the two-dimensional structure of the varicosities were not altered by GDNF exposure (see Table 1). Scale bar, 50 μm.
electrode is much closer than 1 μm to release sites. (2) As the distance between the electrode face and release site increases, the event amplitude decreases, whereas width increases. In contrast, larger quantal sizes result in both an increased amplitude and broader duration (Schroeder et al., 1992; Chow and von Ruden, 1995). Therefore, the results with GDNF and L-DOPA are consistent only with increased quantal size. (3) Control and GDNF-treated DA neurons display VMAT2-labeled axonal varicosities with identical length, width, total area, and intervaricosity distances. Therefore, the surface in contact with the electrode is unchanged. (4) GDNF- and L-DOPA-mediated increases in quantal size were consistent with potentiated total release that was measured independently by HPLC-EC. This technique measures all DA in the culture, distinguishing stimulation-dependent release and intracellular levels. In summary, we suggest that the evidence weighs heavily in support of an increase in quantal size caused by the elevated release of transmitter.

Figure 5. Amperometric recording from a presumed axonal varicosity of a GDNF-exposed neuron before and after exposure to L-DOPA. α-LTX (20 nM) was perfused for 3 sec (first dotted line); the trace displays the period of 45–210 sec that follows (events at this site were not observed until 45 sec after α-LTX stimulation). The mean quantal size was 10,300 ± 1000 molecules (n = 49). Then the culture was exposed to 100 μM L-DOPA for 30 min, and α-LTX was reapplied (second dotted line); the trace displays the period of 45–210 sec that follows. Of the total events elicited (n = 317), the mean size was 39,700 ± 3700 molecules; of these, n = 90 appear to be overlapping events. If apparent overlapping events (e.g., rightmost pair of Fig. 2B, bottom expansion) are removed from consideration, the mean quantal size was increased to 18,300 ± 1600 molecules (p < 0.0001 different from control; KS-Z = 2.3389).

The previous difficulty in demonstrating presynaptic modulation of quantal size in the CNS is attributable in part to the saturation of postsynaptic sites from the contents of a single vesicle (Tang et al., 1994; Edwards, 1995). Nevertheless, regulation of quantal size could play a role in synaptic plasticity for synapses that are either not saturated by a quantum (Bekkers et al., 1990; Tong and Jahr, 1994; Frerking et al., 1995; Liu and Tsien, 1995; Forti et al., 1997) or from which extrasynaptic overflow occurs (Garris et al., 1994; Kullmann et al., 1996). The present results with L-DOPA suggest that the well known control of TH activity in vivo by second messenger-mediated phosphorylation, neuronal activity, and antipsychotic drugs (Kumer and Vrana, 1996), as well as GDNF (Beck et al., 1996), modulates quantal size. These findings thus indicate a possible physiological role for the regulation of transmitter synthesis.

The results indicate that GDNF can modulate synaptic function even in relatively mature neurons, suggesting a basis for the potential therapeutic use of this factor for Parkinson’s disease and other neurodegenerative disorders. In a culture model of synapse formation at the neuromuscular junction, neurotrophins elicit an elevated amplitude of postsynaptic currents (Wang et al., 1995) along with an increased probability of quantal release (Stoop and Poo, 1996). Neurotrophic factors can increase transmitter release via a variety of mechanisms, including potentiation of the number of release events, promotion of additional synapse formation, and elevated quantal size. The present results indicate that elevated quantal size plays a prominent, if not major, role in GDNF-
Figure 6. A, Sample amperometric events evoked from control neurons. B, Examples of events from GDNF-treated preparations. C, Examples of events after exposure to 20 μM L-DOPA (30 min). D, Distributions of the untransformed (molecules/1000) and cubed root transformations (molecules^{1/3}) of quantal sizes, maximum amplitude (pA), and width (μsec) of the events reported in Table 2. In each case, exposure to L-DOPA or GDNF shifted the population parameters to higher values. Note that the control untransformed quantal sizes distribution uses a different y-axis scale than the GDNF and L-DOPA groups.
evoked increased release in this preparation. A variety of mech-

anisms may underlie this effect by GDNF, such as elevated

VMAT per vesicle, potentiation of the vesicle electrochemical

gradient, or increased TH activity. It is consistent with results in brain slice preparations (Garris et al., 1994).

In summary, amperometric recordings from presynaptic sites demonstrate that quantal size in a central neuron can be modu-

lated presynaptically by altering the number of molecules re-

leased per synaptic vesicle exocytic event. This would affect

synapses adapted for transmitter overflow by increasing the spa-

tial and temporal exposure of extrasynaptic sites to a given

quantum. Both the advantages (high temporal resolution, useful-

ness for catecholamine detection, and direct measurement of

quantal size) and drawbacks (low number of events recorded) of

this technique follow from the detection of electroactive events only immediately under the electrode surface, because diffusional broadening would effectively filter the distal events. If the entire contents of a 50-nm-diameter synaptic vesicle were released during a single exocytic event, the intravesicular concentration in DA neurons exposed to GDNF or 20 μM l-DOPA would be ~300 mM; although this level is high, supraosmotic levels within vesicles may be achieved by precipitation with intravesicular anions (Rahamimoff and Fernandez, 1997).

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