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Short communication

Dopamine levels of two classes of vesicles are differentially depleted by amphetamine

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Abstract

Differential depletion of neurotransmitter by amphetamine in two classes of vesicles, termed large vesicles and small vesicles, has been studied with amperometry. Carbon fiber microelectrodes have been used to monitor and quantify exocytotic events. Current transients, corresponding to individual exocytotic events, have been obtained from the cell body of the dopamine-containing neuron of *Planorbis corneus*. The dopamine released from individual vesicles of these cells has been compared for cells treated with D-amphetamine vs. control cells. Our results show that amphetamine has differential effects on the release of dopamine from the two classes of vesicles. Thus, it is concluded that at low concentrations, amphetamine preferentially depletes the large vesicles with a minimal effect on the small vesicles. At high concentrations, amphetamine depletes small vesicles more strongly than large vesicles although amphetamine continues to deplete the large vesicles in a dose-dependent manner. Our data appear to indicate that the two classes of vesicles observed in the *Planorbis* dopamine neuron might have different mechanisms associated with transmitter depletion. © 1998 Elsevier Science B.V.

Keywords: Dopamine; Exocytosis; Amperometry; Vesicle type; Amphetamine; *Planorbis corneus*

Recent advances in electron microscopy and ultrastructural analysis have shown that different classes of vesicles are localized in neurons [30,43]. Small synaptic vesicles (SSV) are usually densely packed near the active zones of presynaptic terminals. Large, dense-cored vesicles (LDCV) are often distributed throughout the cell bodies, axons, dendrites, as well as varicosities. However, both LDCVs and SSVs are observed in some of the same localized areas, such as axonal terminals and cell bodies [15,19,29,30,43–45,51]. These different classes of vesicles have been shown to contain different neuropeptides [2,3,29,42] and/or membrane binding proteins [2,7,16,52] even though they contain the same classical neurotransmitter. The occurrence of different neuropeptides has been reported in certain terminals of the rat dorsal horn, glutamate and calcitonin-gene related peptide (CGRP) have been co-localized in SSVs, whereas glutamate and substance P have been found in LDCVs [30]. The variance in neuropeptide and/or protein composition in multiple classes of vesicles may be an indication of specialized vesicular functions. For instance, in *Torpedo* electromotor nerve terminals, three classes of vesicles have been shown to possess selective uptake of acetylcholine, ATP and Ca^{2+} [19,45].

Evidence has been accumulated recently that the transmitter concentration and/or size of vesicles can be altered by administration of different stimulants [3,8,14,20–22,48–50]. Increase in both vasopressin concentration and vesicle size has been observed from neurosecretory vesicles of paraventricular corticotropin releasing factor (CRF) neurons after adrenalectomy [20]. Larger quantal size of catecholamine vesicles after application of the catecholamine precursor L-dihydroxyphenylananine (L-dopa) has also been seen by measuring individual exocytotic events [47]. In contrast, decrease in transmitter content and depletion of the number of vesicles has been shown from a number of systems by applying lanthanum, [14] reserpine, [21] and amphetamine [8,48–50]. In particular, amphetamine, a lipophilic molecule, has been shown to redistribute dopamine from vesicles to the cytosol and promote reverse transport [49]. Although the above evidence indicates that some stimulants can alter the function of vesicular proteins that control the accumulation of transmitters

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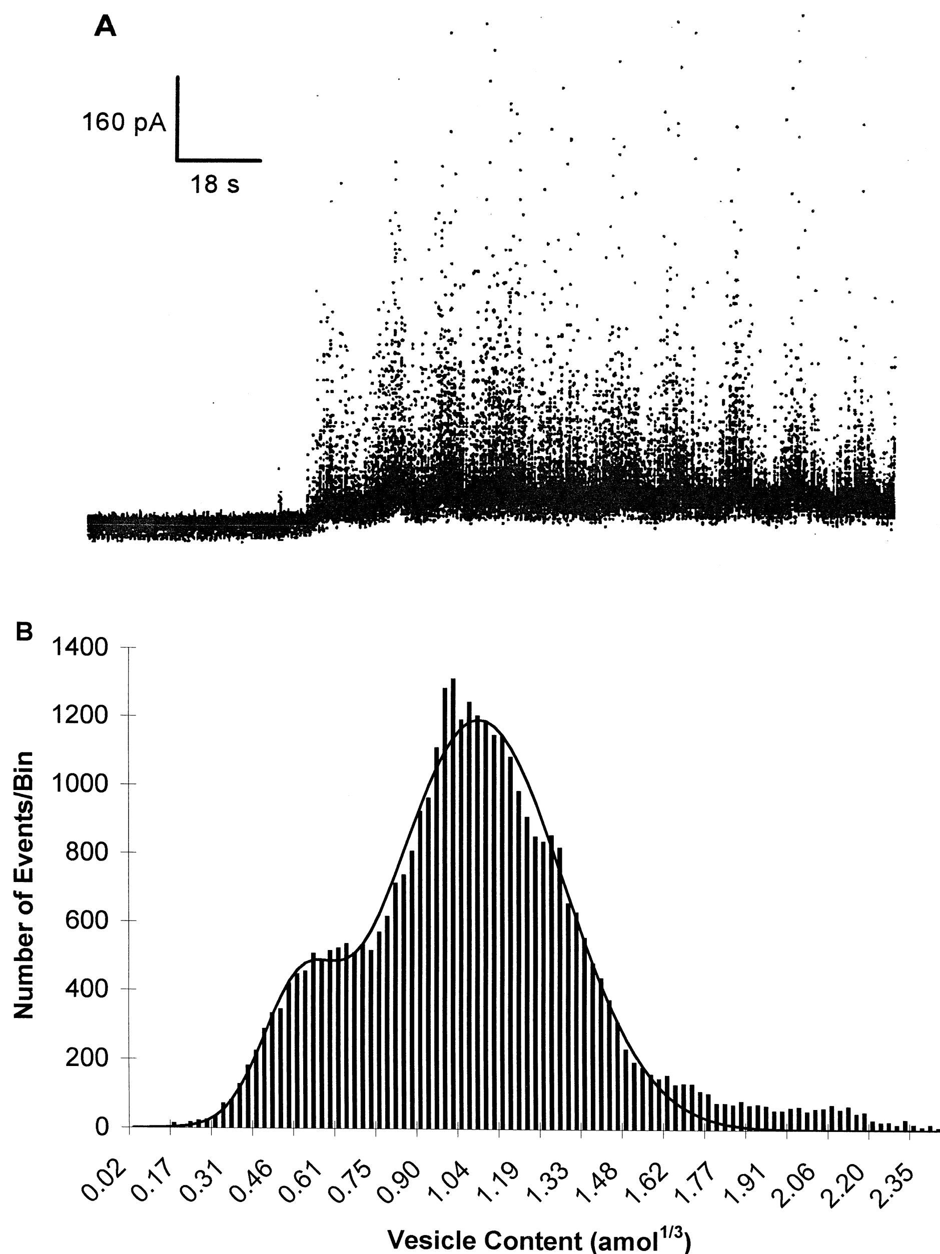

Fig. 1. An example current–time trace (A) detected by a carbon fiber electrode (10 μm) from a 140-μm diameter cell following a 6-s stimulation with 1 M KCl (500 nl). A glass pipette (10 μm) placed about 15 μm away from the cell body was used to deliver the KCl solution to depolarize the cell membrane. (B) The dopamine content for the data from 11 cells was calculated on a per vesicle basis and plotted as a cube root of vesicle amount histogram (see below). The bimodal distribution was fit with a double Gaussian (Eq. (1)). The fit is shown as the solid line. *Procedures and data analysis*: Carbon fiber working microelectrodes [8,9,17] were used on dissected *P. corneus* to reveal the identified dopamine neuron [4,5,8,9,24,28,32–35,46]. D-Amphetamine solutions (Sigma, St. Louis, MO) prepared in snail Ringer solution were used to incubate the whole dissected snail for 20 min, washed away and replaced by the snail Ringer solution with a perfusion system built-in-house. A 5-min waiting time was used after changing the solutions and before the next stimulation with 1 M KCl. Experiments were carried out at room temperature. Data were filtered with a 500-Hz two-pole low pass filter and digitized at 2 kHz. Transient current responses obtained in the amperometric mode were evaluated by locally developed peak-detection–integration software [9]. Only transients with peak widths between 10 ms and 2 ms, and peak heights larger than twice the peak-to-peak noise were considered. The vesicle content was calculated by $N = Q/nF$ [9]. Given a constant vesicular neurotransmitter concentration, the number of moles of dopamine in each vesicle should depend on the volume of each vesicle. Since concentration is moles/volume (or moles/radius^3), the cube root of the number of moles detected will be proportional to the vesicle radius or size [13]. Thus, histograms are plotted as frequency vs. the cube root of moles detected per event. All histograms have been fitted with a double Gaussian function (Eq. (1)) using Sigma Plot for Windows (Jandel Scientific, San Rafael, CA).

$$y = \frac{k}{s} e^{-0.5\left(\frac{x-m}{s}\right)^2} + \frac{P}{t} e^{-0.5\left(\frac{x-q}{t}\right)^2} \quad (1)$$

Here k, s, m and p, t, q are parameters defining two Gaussians, respectively, whereas x and y are two axes specified by the cube root histograms as described above.

into vesicles, little is known as to whether these stimulants have differential action in depleting different classes of vesicles.

Preliminary data [8] suggest that amphetamine (10 μM) preferentially depletes one of the two classes of vesicles defined by two distributions of the amount of dopamine released from the giant dopamine cell (GDC) of the pond snail *Planorbis corneus* when it is stimulated. In this paper, a more detailed investigation of the dose-dependent action of amphetamine on exocytosis events from two

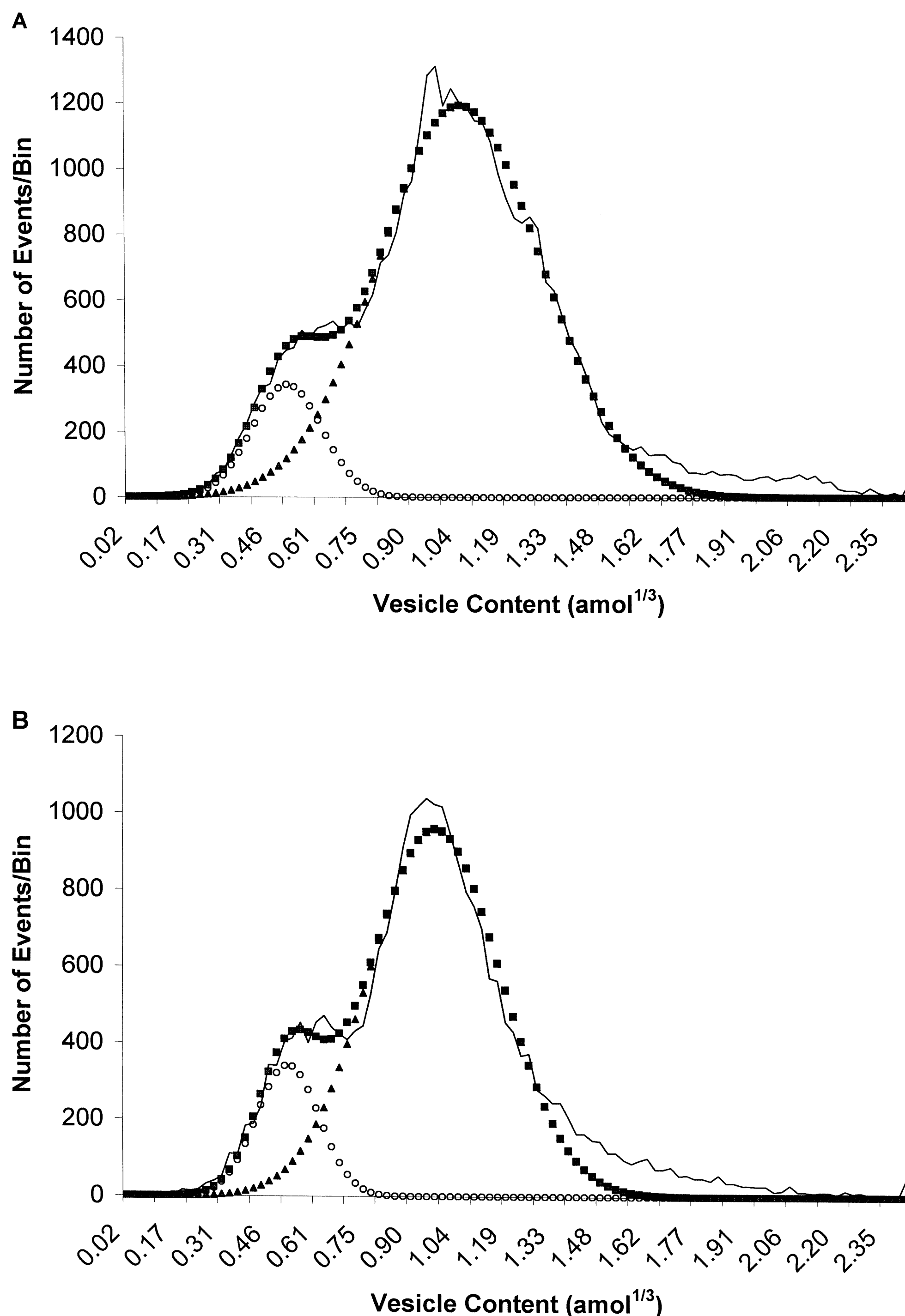

Fig. 2. Histogram (100 bins 0.024 amol/bin) of the frequency of release events vs. the cube root of attomoles of dopamine released per vesicle. The original data is represented by the solid line. The small Gaussian (open circles), the large Gaussian (closed diamonds) and the sum of the large and small Gaussian (closed boxes), as calculated by Eq. (1), are also presented in each histogram. (A) Control data (no amphetamine incubation) showing a histogram representing data from 36,304 detected transients from 11 cells. (B) Histogram representing data obtained from seven cells (25,438 transients) after treatment with 10 μM amphetamine. (C) Histogram representing data obtained from four cells (2878 transients) after treatment with 50 μM amphetamine. (D) Histogram representing data obtained from five cells (8875 transients) after treatment with 100 μM amphetamine.

classes of vesicles in the GDC is studied with amperometry using carbon fiber microelectrodes [1,6,8–11,16,18,23,49,53,54]. The results reveal two conclusions. First, there is a differential effect of amphetamine on the two types of vesicles. Second, there is an apparent amphetamine concentration-dependent depletion of both types of vesicles. Our results thus appear to indicate that these two classes of vesicles have substantially different tendencies to resist stimulants and perhaps different mechanisms of transmitter depletion are involved.

It has been shown [8,9] that current transients corresponding to individual dopamine exocytotic events can be detected with a carbon fiber microelectrode placed at the cell body of the GDC. Calcium-dependent dopamine exocytosis is evoked by stimulating the cell with elevated extracellular potassium to depolarize the cell membrane.

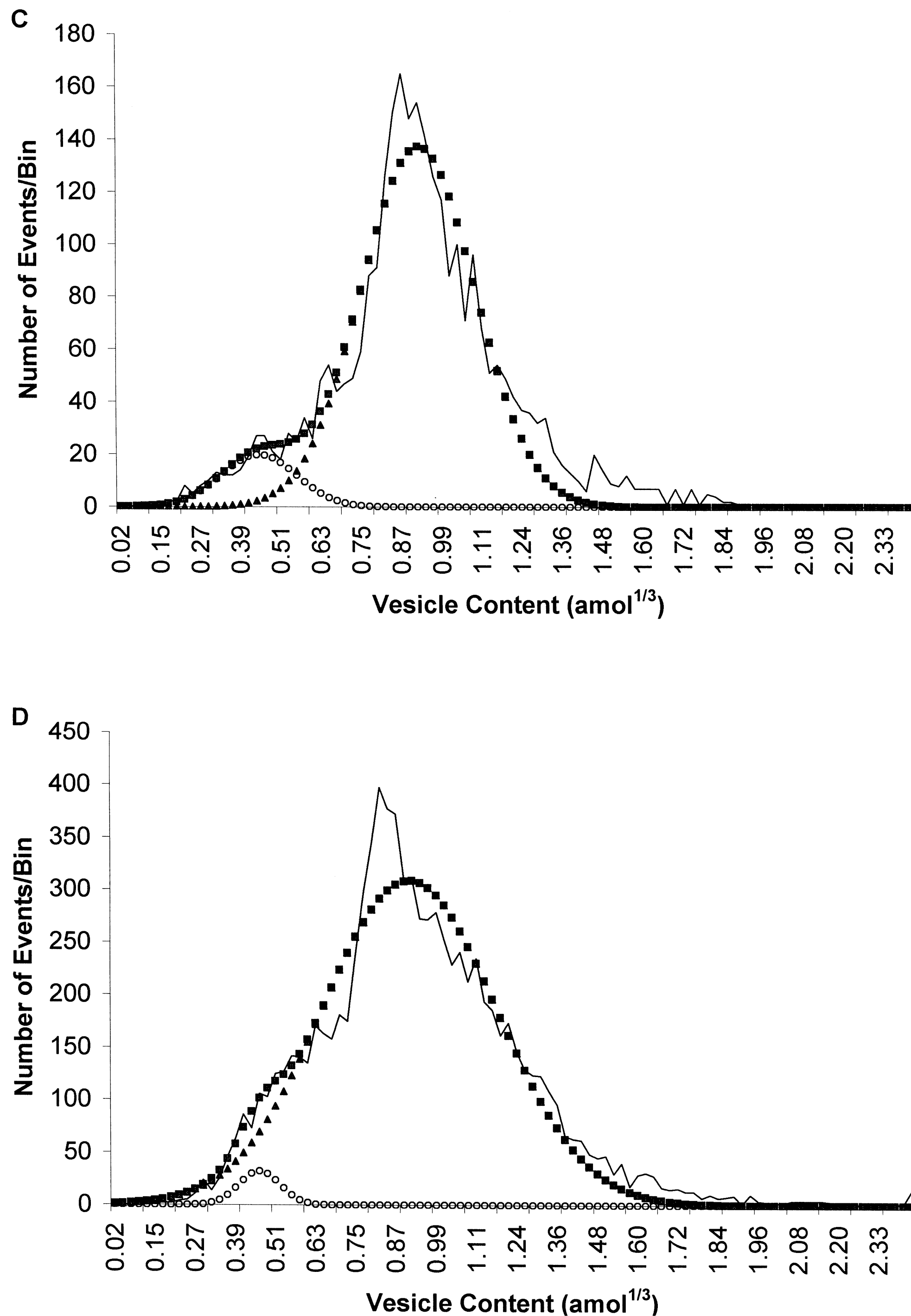

Fig. 2 (continued).

Fig. 1A shows a representative response obtained from a 140-μm diameter neuron after a 6-s stimulation with elevated potassium. The amount of transmitter released per vesicle has been quantified for 11 cells under the same conditions. Fig. 1B shows a histogram of the frequency of the detected transients vs. the cube root of the amount of dopamine released from individual vesicles (see Fig. 1 legend for a description of the cube root histogram). A bimodal distribution is obtained. A double Gaussian (Eq. (1)) can be fitted to the bimodal distribution in Fig. 1B (solid line) suggesting that the two distributions are a result of different size vesicles [13]. Although it is still not clear that these two distributions represent different vesicle diameters, we refer to these vesicle classes throughout this manuscript as large vs. small vesicles. This most likely represents vesicle size but might also represent differences in packaging of neurotransmitter in the vesicles. A bimodal distribution of vesicle amount is also observed in data obtained from the leech serotonin-containing neuron [6]. The presence of the bimodal distribution of vesicle amount in the GDC makes it an ideal model for investigating different effects of chemical stimulants.

The psychostimulant amphetamine has been shown to deplete vesicular dopamine stores by affecting the pH gradient across the vesicular membrane [48–50]. To examine if D-amphetamine has a differential effect on the two classes of vesicles, we treated *Planorbis* dopamine neurons with different concentrations of amphetamine before amperometric measurements were performed. Fig. 2A–D shows four histograms of the cube root of the amount of dopamine released from individual vesicles obtained from cells after 0 μM (control, 11 cells), 10 μM (seven cells), 50 μM (four cells) and 100 μM (five cells) amphetamine treatment, respectively. The deconvolution of these histograms into two Gaussians is also shown.

The midpoints of the small and large Gaussians are defined as the mean dopamine content of small and large vesicles. For the non-treated, control cells (Fig. 2A), the mean dopamine content is 0.51 and 1.04 amol$^{1/3}$ for the small and large vesicles, respectively. The number of small vesicles represents 11% of the total number of events detected which agrees with the data reported earlier [8]. In the case of cells treated with 10 μM amphetamine (Fig. 2B), the small Gaussian represents about 14% of the total transients which is slightly greater than that of control and the mean dopamine content released from the small vesicles shows no difference from that of control. However, the mean dopamine content of the large vesicles is about 20% smaller than that of control. For cells treated with 50 μM and 100 μM amphetamine (Fig. 2C and D), the small Gaussian corresponds to 8.8 and 2.4% of the total transients, respectively.

Although it has been previously shown that stimulants such as lanthanum [14] and β-bungarotoxin [26] have preferential effects on two classes of miniature end plate potentials (MEPP), bell-MEPPs and small skew-MEPPs, little is known as to whether stimulants have differential effects on different classes of vesicles. Therefore, the data presented here are the first demonstration of differential depletion of transmitter content from two classes of vesicles by a psychostimulant. Since the percentage of small exocytosis events is increased after exposure to a low concentration of amphetamine (10 μM) (14%, Fig. 2B) compared to control (11%, Fig. 2A), it is reasonable to suggest that some large vesicles (defined by the dopamine content released) are partially depleted by amphetamine. Thus, these partially depleted large vesicles are included in the group of small vesicles, resulting in an increase in the percentage of small exocytosis events. At high amphetamine concentration, small vesicles appear to be preferentially depleted as evidenced from the data in Fig. 2C and D.

To evaluate the dose-dependent effect that amphetamine has on the two classes of vesicles, the amount of dopamine released per exocytosis event at the midpoint of the small and large distributions following incubation with amphetamine is compared in Table 1. Although the peak dopamine content of each distribution decreases following incubation with amphetamine, there is no clear dose dependence. As amphetamine appears to decrease the amount of dopamine in vesicles, [21,37,41,48–50] the change in the small distribution is difficult to assess since release events in the large distribution of control cells might well fall into the small distribution following incubation with amphetamine. In addition, the slight variation in the peak of the large distribution might result from amphetamine only affecting a subpopulation of these vesicles. This is supported by previous data suggesting the possibility of three classes of vesicles in this cell [8]. An effect of amphetamine incubation on the peak of the small distribution is not observed at the low (10 μM) concentration. However, there is an apparent difference between control values and 50 and 100 μM amphetamine treatment. Thus, it appears the vesicles represented by the small distribution

Table 1
Comparison of the dopamine content of vesicles at the peak of each distribution following incubation with amphetamine

Amphetamine concentration (μM)	Dopamine content in the large distribution (amol)	Dopamine content in the small distribution (amol)
Control	1.13	0.13
10	0.91	0.13
50	0.78	0.083
100	0.78	0.097

are resistant to depletion by lower concentrations of amphetamine.

The average amount of dopamine from all exocytosis events recorded from all cells has been quantified to be 1.49 ± 0.27 amol for control, 1.15 ± 0.40, 0.97 ± 0.23 and 0.95 ± 0.30 amol for 10, 50 and 100 μM amphetamine-treated cells, respectively. The control value is statistically different from those for amphetamine-treated cells ($p =$ 0.04, 0.005 and 0.003 for 10, 50 and 100 μM amphetamine-treated cells compared to control, one-sided Student's t-test).

Differential depletion of dopamine from vesicles could be a result of the action of amphetamine on the vesicular monoamine transporter (VMAT). VMAT is responsible for accumulation of amines into the secretory vesicles [40]. There are two isoforms of VMAT (VMAT1 and VMAT2) with VMAT2 being the primary form for the CNS [12,25,31,36]. VMAT2 is likely to be present in *P. corneus* CNS since dopamine synthesis and metabolism is similar to that in vertebrate neurons [32]. Amphetamine could therefore act on VMAT2 as a dose-dependent antagonist to produce differential depletion.

Data presented by Pifl et al. [37] suggest that amphetamine at least in part, acts at the binding site of the vesicular transporter. It has also been shown that amphetamine derivatives in the range of about 1–3 μM can block vesicular transport of 5-HT in chromaffin granules [41]. If VMAT binding for amphetamine is similar to its derivatives, the amphetamine concentrations used in the present study could inhibit vesicular dopamine transport by a transporter antagonist mechanism. In fact, Scherman and Henry [39] showed an active and inactive form of VMAT present in chromaffin granules. Thus, one could speculate that the VMAT on the smaller vesicles might be inactive or otherwise unaffected by amphetamine. In contrast, the VMAT on the large class of vesicles is likely affected over the range of amphetamine employed here leading to the dose-dependent depletion observed.

There is also significant data showing that amphetamine and its derivatives dissipate the pH gradient that drives vesicular monoamine uptake and, therefore, results in efflux of transmitter from vesicles and cells [27,37,38,41,48–50]. Sulzer and Rayport [48] and Schuldiner et al. [41] have both shown that amphetamine alkalinizes isolated and reconstituted chromaffin granules. Interestingly, amphetamine alkalinizes the chromaffin granules in a dose-dependent manner. Based on these previous findings, the higher amphetamine concentrations used in the present study (50 and 100 μM) probably deplete vesicular dopamine in the GDC by the weak base mechanism.

The present data suggest that two classes of dopamine-containing vesicles from the cell body of the *P. corneus* dopamine neuron are differentially depleted by amphetamine. At a low dose, amphetamine preferentially depletes large vesicles without significant effect on small vesicles. At high doses, amphetamine almost completely depletes small vesicles while it continues to deplete large vesicles in a apparent dose-dependent manner. It is likely the depletion of vesicles observed here is a combination of amphetamine binding to VMAT and alkalinization of the vesicle interiors by the weak base action of amphetamine. Since the transporter antagonist action occurs at a lower concentration of amphetamine, incubation of the GDC with 10 μM amphetamine could be primarily affecting the transporter binding properties and, at higher amphetamine concentration incubations, the weak base effect is taking over to deplete dopamine in a dose-dependent manner. This is an exciting result as it could provide a mechanism to explain the differential effects of different doses of psychostimulants on behavior.

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