On the Action of General Anesthetics on Cellular Function: Barbiturate Alters the Exocytosis of Catecholamines in a Model Cell System

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Abstract. General anesthetics are essential in many areas, however, the cellular mechanisms of anesthetic-induced amnesia and unconsciousness are incompletely understood. Exocytosis is the main mechanism of signal transduction and neuronal communication through the release of chemical transmitters from vesicles to the extracellular environment. Here, we use disk electrodes placed on top of PC12 cells to show that treatment with barbiturate induces fewer molecules released during exocytosis and changes the event dynamics perhaps by inducing a less stable fusion pore that is prone to close faster during partial exocytosis. Larger events are essentially abolished. However, use of intracellular vesicle impact electrochemical cytometry using a nano-tip electrode inserted into a cell shows that the distribution of vesicle transmitter content does not change after barbiturate treatment. This indicates that barbiturate selectively alters the pore size of larger events or perhaps differentially between types of vesicles. Alteration of exocytosis in this manner could be linked to the effects of general anesthetics on memory loss.

Keywords: barbiturate; exocytosis; catecholamine; vesicle, amperometry; IVIEC

1. Introduction

General anesthetics, which are essential to both medical practice and experimental neuroscience, have potent and selective effects on neurotransmission, including both presynaptic actions and postsynaptic actions.[1] The molecular and cellular mechanisms of

anesthetic-induced amnesia and unconsciousness are incompletely understood.[2] And for some anesthetics, they can cause loss of motivation to follow commands.[2a] Knowledge of the fundamental cellular-level synaptic effects of anesthetics is therefore essential to our molecular and physiological understanding of anesthetic mechanisms, and to the development of more selective and safer anesthetics. Barbiturates are among the most extensively studied central nervous system depressants and are commonly used as anesthetic.[3] It also can cause severe depression with suicidal indications after a period of treatment.[4] This it is important to study the role of barbiturate in altering exocytosis to help further understand the mechanism of this drug.

In order to have a better understanding the role of barbiturate on neurotransmitter release at the single cell level, we used a combination of amperometric detection of exocytotic release with intracellular vesicle impact electrochemical cytometry (IVIEC)[5] for vesicle content. These methods have the requisite speed, sensitivity, spatial resolution, and are reliable Up to now, several techniques, including optical spectroscopy, patch-clamp capacitance detection, as well as electrochemical methods have been developed. [6] Among these, electrochemical methods offer the unique advantages of providing quantitative information about the amount of released molecules and precise dynamic characteristics with high sensitivity and submillisecond time resolution. In a typical experiment, a carbon fiber microelectrode held at a constant potential (usually 700 mV vs Ag| AgCl) is placed on top of the cell surface, and exocytosis is triggered by stimulating the cell with highly concentrated K^+ solution. The released neurotransmitters are oxidized at the surface of a microelectrode in a diffusion-limited manner. Recently, several reports using amperometry have proposed various cellular or molecular mechanisms regarding the control of exocytosis pharmacological and physicochemical changes.[7]

Pheochromocytoma (PC12) cells provide an excellent model for the study of different aspects of neuronal physiology and biochemistry. So, in this study, PC12 cells have been used to investigate the mechanism of barbiturate action on exocytosis. We show that for this system, barbiturate influences not only the amount of the transmitter released but also the

dynamics of exocytosis during single exocytotic process. Moreover, this drug appears to turn off release from larger vesicles or to differentially change the larger release events. Hence, we used intracellular vesicle impact electrochemical cytometry with a nano-tip electrode placed in the cell to measure vesicle catecholamine storage content. Here we found that barbiturate does not change the content of vesicle. This suggests that there are different pools of vesicles[8], and that barbiturate affects the release events from these differentially. Interestingly, the IVIEC experiment is affected by barbiturate in that it changes the kinetics of the vesicle opening on the electrode surface. We can only speculate on the meaning of this phenomenon. Perhaps barbiturate stops release from one pool of vesicles by changing their structure in a way that also affects their interaction with a carbon electrode.

2. Materials and Methods

2.1. Chemicals and solutions.

Analytical grade chemicals were obtained from Sigma-Aldrich (unless stated otherwise) and used as received. Sodium barbiturate was used in this work. The HEPES physiological saline contains 150 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES, and 2 mM $CaCl_2$. The high K^+ stimulating solution consists of 55 mM NaCl, 100 mM KCl, 1.2 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES and 2 mM $CaCl_2$. All solutions were made with 18 MΩ·cm water from a Purelab Classic purification system (ELGA, Sweden), and the solution pH was adjusted to 7.4 with concentrated NaOH (3.0 M).

2.2. Fabrication of carbon fiber disk microelectrodes

Disk-shaped microelectrodes were made as described before[9]. Briefly, 5-μm diameter carbon fibers by aspiration into a borosilicate capillary (1.2 mm O.D., 0.69 mm I.D., Sutter Instrument Co., Novato, CA, U.S.A.). The capillaries were subsequently pulled with a micropipette puller (model PE-21, Narishige, Inc., Japan). After that the really long carbon fiber out of the glass was cut at the place 20-100 μm far from the glass junction. Electrodes were sealed by dipping the tip in a solution of epoxy (Epoxy Technology, Billerica, MA, U.S.A). The glued electrodes were cured at 100 °C overnight and subsequently cut at the glass junction and beveled at 45° angle (EG-400, Narishige Inc., London, UK). Prior to the

experiments, the electrode response was tested with cyclic voltammetry (- 0.2 to 0.8 V vs. Ag/AgCl, 100 mV/s) in 100 μM dopamine in PBS (pH 7.4). Only electrodes showing good reaction kinetics and stable steady-state currents, which were in agreement with theoretically calculated values for 5-μm disk electrodes, were used for experiments.

2.3. Chemicals fabrication of nano-tip conical carbon fiber microelectrodes.

Nano-tip conical carbon fiber microelectrodes were fabricated as described previously[5]. Brielfy, a 5-μm carbon fiber was first aspirated into a borosilicate glass capillary (1.2 mm o.d., 0.69 mm i.d., Sutter Instrument Co., Novato, CA). The glass capillary was then pulled into two separate electrodes with a commercial micropipette puller (model PE-21, Narishige, Inc., Japan). The fiber extending from the glass was cut to 100-150 μm with a scalpel under a microscope. To flame etch the carbon fiber, the electrodes were held on the edge of the blue part of a butane flame (Multiflame AB, Hässleholm, Sweden) for about 3 s. As soon as the end of the tip became red, the electrode was pulled out from the flame and checked under the microscope. The electrodes obtained with needle-sharp fiber tips (about 100-200 nm in diameter, 30-100 μm in length) were sealed with epoxy (Epoxy Technology, Billerica, MA). Each electrode was then tested by performing cyclic voltammetry at 100 mV/s in a solution of 100 μM dopamine in PBS (pH 7.4). Only electrodes showing good reaction kinetics and a steady-state diffusion limited current were used for the experiments.

2.4. Cell culture

PC12 cells, a gift from Lloyd Greene (Columbia University), were maintained in phenol red-free RPMI-1640 media (PAA Laboratories, Inc. Australia) supplemented with 10% donor equine serum (PAA Laboratories) and 5% fetal bovine serum Gold (PAA Laboratories) in a 7% CO_2, 100% humidity atmosphere at 37 °C. The cells were grown on mouse collagen coated cell culture flasks (collagen type IV, BD Biosciences, Bedford, MA) and were sub-cultured every 7-9 days. The media was replaced every 2 days throughout the lifetime of all cultures. For barbiturate treatment, the cells were incubated with 0.1 mM barbiturate HEPES physiological saline for 10 min before experiments.

2.5. Single cell amperometry and intracellular impact cytometry.

In preparation for either single cell amperometry or IVIEC, the medium solution was removed and the cells were rinsed three times with new HEPES physiological saline. The cells were then kept at 37°C in the solution of HEPES physiological saline during the whole experimental process. Electrochemical recordings from single PC12 cells were performed on an inverted microscope (IX81, Olympus), in a Faraday cage. The working potential was +700 mV versus Ag/AgCl reference electrode (Scanbur, Sweden) under the control of an Axopatch 200B potentiostat (Molecular Devices, Sunnyvale, CA). The output was filtered at 2.1 kHz and digitized at 5 kHz (Axoscope 10.4 software, Axon Instruments Inc., Sunnyvale, CA, USA). All the experiments were observed under an inverted microscope (IX81, Olympus) with 10x and 40x objectives. For single cell exocytosis, the disk carbon fiber microelectrode was moved slowly by a Patch-Clamp Micromanipulator (PCS-5000, Burleigh Instruments, Inc., USA) to place it on the membrane of a PC12 cell without causing any damage to the surface. Four seconds after the start of recording, high K^+ stimulating solution in a glass micropipette was injected into the surrounding of the PC12 cells with a single 5-s injection pulse. For IVIEC, the tip of the electrode was first placed on top of a PC12 cell membrane then it was slowly pressed through the membrane of a PC12 cell while the current was recorded. Stimulation is not needed in this method.

2.6. Data acquisition and analysis.

The amperometric traces were processed using an Igor Pro 6.22 routine originating from David Sulzer's group at Columbia University. The filter for the current was 1 kHz (binomial sm.). The threshold for peak detection was three times the standard deviation of the noise. The traces were carefully inspected after peak detection and false positives were manually rejected. The number of molecules released by single cells was pooled, and the median of the data was calculated for each experimental condition. To compare between different conditions, the mean of median of molecular number was used. The responding cells were also calculated from each experiment. Pairs of data sets were compared with a two-tailed Mann-Whitney rank-sum test; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

3. Results and Discussion

3.1. Barbiturate decreases the amount of release but increases dynamics in exocytosis.

We carried out single-cell amperometry on PC12 cells after incubation for 10 min in HEPES physiological saline solution containing 0.1 mM of barbiturate. A 5 μm electrode was placed on top of a selected PC12 cell and held at a +700 mV potential versus Ag/AgCl reference electrode and the system arrangement is shown in Figure 1A. Typical traces obtained from the control (10-min incubation in HEPES physiological saline solution) and barbiturate (10-min incubation in HEPES physiological saline solution with 0.1 mM barbiturate) treatments are shown in Figure 1B. In both cases, the 5-s stimulation is followed by a train of current spikes, where each spike corresponds to a single exocytotic release event. We analyzed the spikes, and determined the following parameters from the exocytotic events: the peak current, i_{max} ; the 25− 75% rise time, t_{rise} ; the half peak width, $t_{1/2}$; the 75− 25% fall time, t_{fall} ; and the number of molecules released, N , obtained by integrating the area under the peak, as shown in Figure 1C.

When the data for the amount released is plotted as a normalized frequency histogram during stimulated exocytosis (Figure 2), there is significant difference in the number of molecules released during exocytotic release for barbiturate-treated cells versus control; fewer molecules were released from cells after they were treated with barbiturate. Furthermore, in order to minimize the impact of cell-to-cell variation, the mean of median values (N) obtained from every current transient for both control and barbiturate-treated cells were compared. Using this alternative data reduction, we come to the same conclusion that fewer molecules were released from cells after barbiturate treatment.

The rate of release is also potentially important in determining the effect of presynaptic effects on synaptic strength. We examined the dynamics of amperometrically measured release with $t_{1/2}$, t_{fall}, t_{rise} and i_{max} of the current transients. As shown in Figure 3, a decrease in $t_{1/2}$ was observed after barbiturate incubation, which means that the vesicle opening and closing process becomes faster with perhaps a less stable fusion pore being formed after barbiturate treatment. The values of t_{rise} and t_{fall} are characteristic of pore

opening and closing, respectively. Following barbiturate, t_{rise} did not change significantly, indicating that the opening process is not affected by barbiturate. However, t_{fall} significantly decreased, which suggests that the closing of the fusion pore is significantly faster. As the pore size appears to remain the same (i_{max} does not change significantly), it is important to determine if the vesicle content is changed by the drug. This will allow us to determine if the reduced release observed is attributed to the dynamic changes of exocytosis after barbiturate treatment or vesicle content.

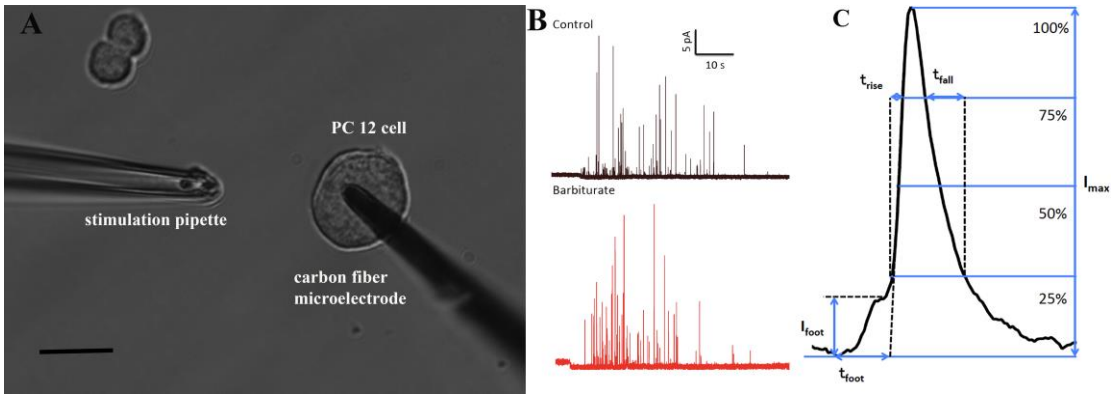

Figure 1. A) Optical micrograph showing the experimental setup. Scale bar: 20 µm. B) Typical amperometric traces obtained for a single K^+-stimulated PC12 cell with and without barbiturate treatment. C) Scheme showing the different parameters used for the peak analysis in this work. I_{max}=peak current, t_{rise}=rise time, $t_{1/2}$=half peak width, t_{fall}=fall time, i_{foot}=foot current, t_{foot}=foot duration.

3.2. IVIEC shows that barbiturate does not alter vesicle content.

We used IVIEC to measure the catecholamine storage of PC12 cell vesicles with and without barbiturate. Figures 4 A and B show the typical traces for control cell and barbiturate treated cell, respectively. From all the traces, the numbers of catecholamine molecules in single vesicles were calculated and the results are presented in a normalized frequency histogram. From Figure 4C and D, we can see that the number of catecholamine molecules in the vesicles did not change significantly compared with the control cells. However, barbiturate changes the values of i_{max}, $t_{1/2}$ and t_{rise} during the IVIEC experiment indicating that. the drug does have some effect on vesicle structure.

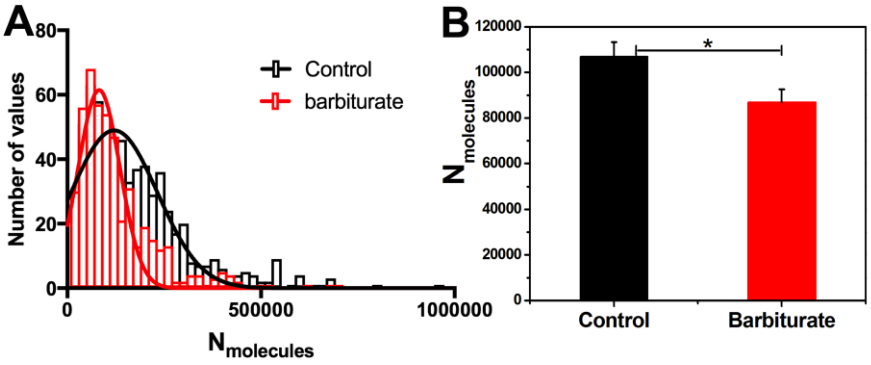

Figure 2. A) Normalized frequency histograms describing the distributions of the molecules released from control cells (1424 spikes from 52 cells) and barbiturate treatment cells (693 spikes from 32 cells), bin size: 2.0×10^4 molecules. Fits were obtained from a Gaussian distribution of the data. B) exocytotic release molecule values from mean of median for control and barbiturate treated cells.

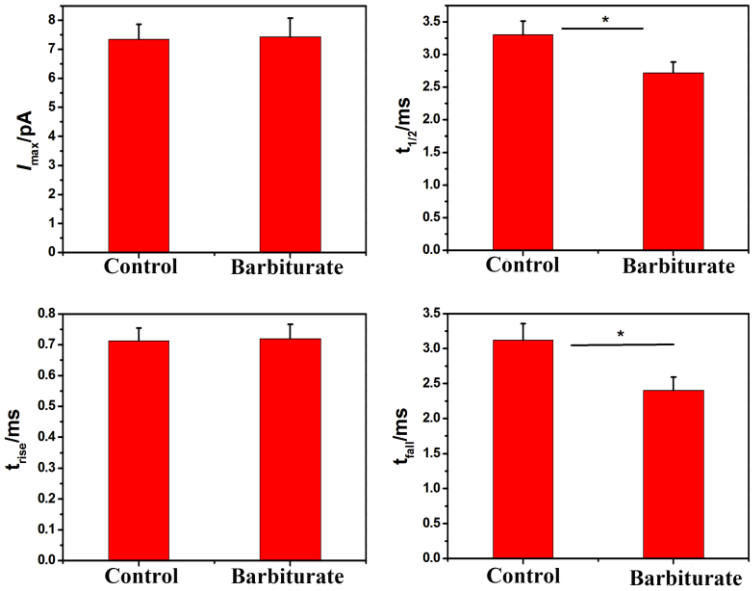

Figure 3. Experimental results for exocytosis events obtained from K^+ -stimulated PC12 Cells with (693 spikes from 32 cells) and without barbiturate (1424 spikes from 52 cells) treatment. *, $p < 0.05$.

The data in Figure 3 show that cells exposed to 0.1 mM barbiturate have a decrease in t_{fall}. The IVIEC data in Figure 4C and D show that the vesicular content is not affected by barbiturate pre-incubation. Thus, this decay arises from the closing of the pore, which is faster in presence of barbiturate. In general, synaptotagmin influences the stability of opening fusion pores and vesicles containing excess synaptotagmin IV formed less stable fusion pores.[10] Changes in fusion pore dynamics have recently been proposed to play a role in synaptic plasticity.[11]

3.3. Barbiturate selectively changes the larger release events.

Another possible mechanism for the effect of barbiturate on exocytosis is that it differentially affects multiple pools of vesicles. The data in Figure 2A indicate that an increased proportion of the large released events are eliminated after barbiturate treatment. When we could only measure release amounts, we might have guessed that barbiturate decreases vesicular levels. However, the distribution of vesicular content shown in Figure 4C, measured with IVIEC, is clearly unchanged. It appears that barbiturate affects the release events from different vesicles differentially without changing their content. To look if there are two pools of vesicles in control cells, we examined the time of release separating the small events and large events below and above the point in the histogram where the barbiturate-treated cells seemed to have a drop in number, respectively (indicated by the line in Figure 6A). We then compared the corresponding $t_{1/2}$ and t_{fall} for these different events (Figure 6B and C). Large events take a longer time to release their content, with the release pore closing more slowly compared to small events. This sheds light on the report by Westerink et al.[8] that the releasable pool of vesicles in PC12 cells is heterogeneous. It also suggests that these vesicle pools might have subtle differences in how they undergo exocytosis in addition to different vesicle content. Barbiturate appears to turn off release from larger vesicles and thereby differentially changes the larger release events, further suggesting that the different pools create different mechanisms for transmission from presynaptic release.

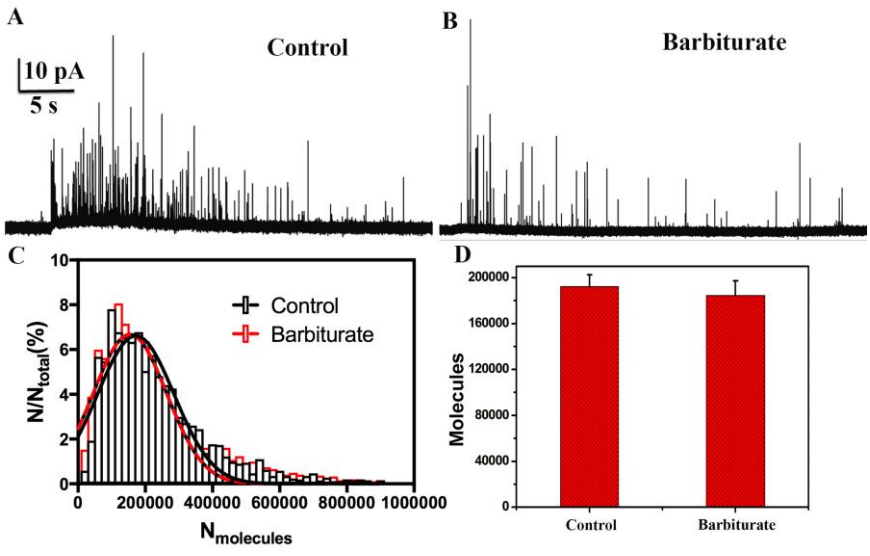

Figure 4. Representative amperometric traces of vesicle content in cells A) without and B) with 0.1 mM

barbiturate treatment for 10 min; C) Normalized frequency histograms describing the distribution of the molecules observed in vesicles of control cells (2511 spikes from 40 cells) versus barbiturate-treated cells (2416 spikes from 48 cells), bin size: 2×10^4 molecules. Fits were obtained from a Gaussian distribution of the data. D) Vesicle content from mean of median for control and barbiturate treated cells.

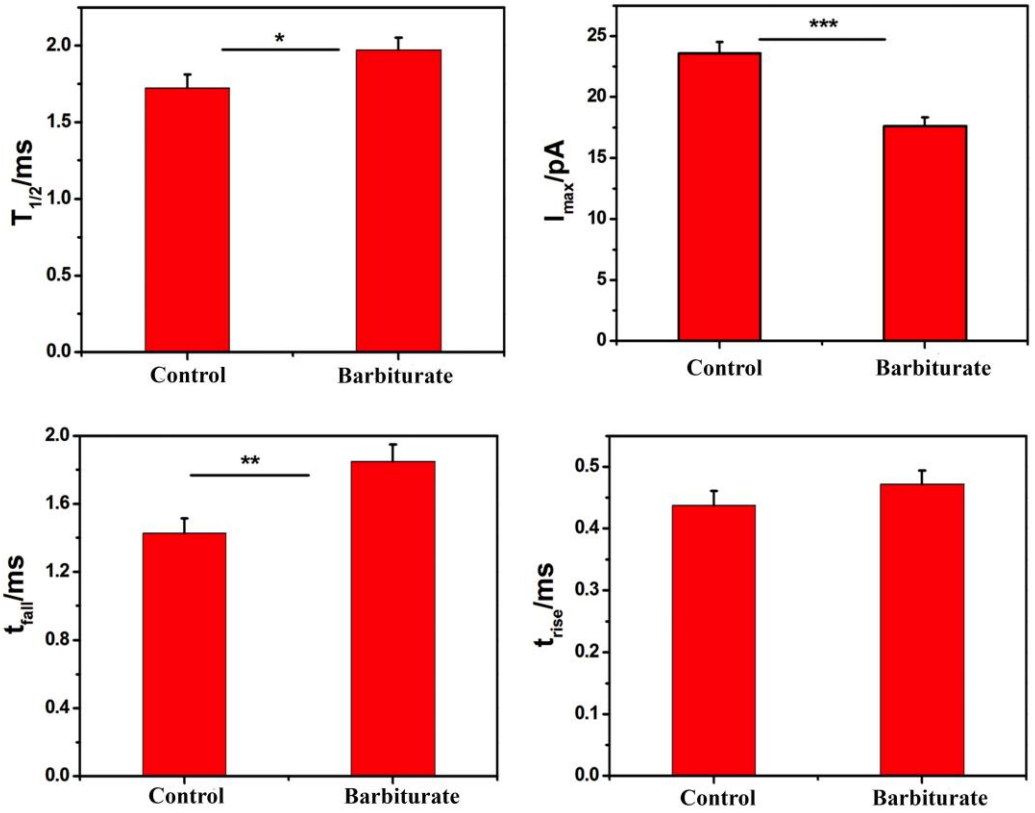

Figure 5. Experimental results for intracellular vesicle impact electrochemical cytometry events, control cells (2511 spikes from 40 cells), barbiturate-treated cells (2416 spikes from 48 cells). ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

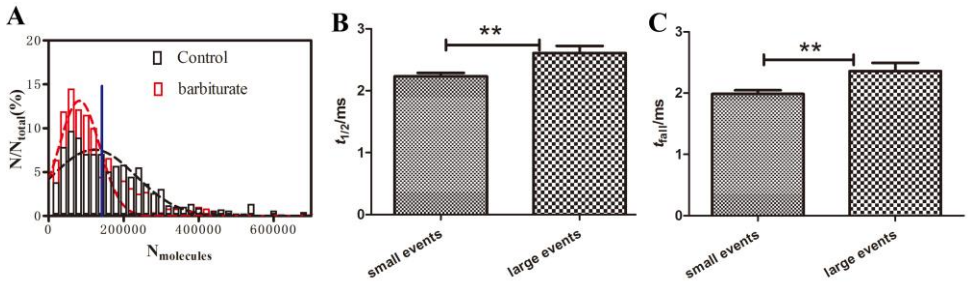

Figure 6. A) Normalized frequency histograms describing the distributions of the molecules released from control cells (1424 spikes from 52 cells) and barbiturate treatment cells (693 spikes from 32 cells), bin size: 2.0×10^4 molecules. Fits were obtained from a Gaussian distribution of the data. The blue line separates the small vesicles and large vesicles for control cells and was chosen as it is the point where the histograms for barbiturate-treated and control cells overlap. B) Average of $t_{1/2}$; C) t_{fall} from small and large events for control cells.

Traditionally, general anesthetics have been thought to act by perturbing the lipid bilayer.[12] However, accumulating evidence casts serious doubt on this idea. An alternative view is that general anaesthetics act directly on proteins.[13] Some general anesthetics appear to interact with multiple release machinery proteins. Zheng et al. [14] suggested that general anesthetics inhibit the neurotransmitter release machinery by interacting with multiple SNARE and SNARE-associated proteins. The SNARE (soluble NSF attachment protein receptor) proteins include synaptobrevin, synaptotagmin I, syntaxin, SNAP-23 and SNAP-25, which are present on the vesicles and the intracellular side of the cell membrane. The formation of a complex of these proteins is essential for triggering exocytosis. Knockdown of synaptotagmin I attenuated the inhibitory effects of isoflurane and propofol on neurotransmitter release.[14] Knockdown of SNAP-25 and SNAP-23 expression also changed the ability of these anesthetics to inhibit neurotransmitter release.[14] Here, we consider the effect of barbiturate on exocytosis in dynamics. Dynamin has a direct effect on the duration and kinetics of exocytotic release. The inhibition of this specific feature of dynamin with dynasore leads to shorter duration of exocytosis events, with a decrease in $t_{1/2}$ and t_{fall} and fewer molecules released. This means that if the pore is not framed by the dynamin coil, the pore tends to collapse more rapidly. [15] Interestingly, this result is quite similar to that with the effect of 0.1 mM barbiturate on exocytosis, suggesting that barbiturate might block the action of dynamin on the pore opening. There are also some results shown that anesthetics alter the function of many cytoplasmic signaling proteins, including protein kinase C.[3a] When protein kinase C was activated with phorbol ester, the amperometric spikes showed a significant decrease in their total charge due to a decrease in their mean half-width, [16] which is also consistent with our results. However, Mikawa et al [17] reported in 1990 that barbiturates have an inhibitory effect on protein kinase C activation, but only at very high concentration.

3.4. Barbiturate alters the dynamics of the exocytosis foot.

We also investigated the amperometric feet in these recordings without and with barbiturate. The value of t_{foot} is also related to the dynamics of the fusion pore. These feet represent the

early stage fusion pores formed during exocytosis and are recorded as a small current increase at the beginning of amperometric detection. The parameters i_{foot}, t_{foot}, and Q_{foot} for the pre-spike feet were analyzed according to Figure 1C, and the results are summarized in Table 1. After barbiturate treatment, i_{foot} remains the same, but the duration is considerably decreased which is consistent with the effect of dynamin inhibition by dynasore on the foot. Additionally, Q_{foot} decreased significantly, consistent with the data for exocytosis shown in Figure 3. This further indicates that barbiturate treatment induces a less stable fusion pore that is prone to close faster.

Table 1. Foot parameters obtained from K+-stimulated PC12 cells (i_{foot} >2 pA), comparing control (52 cells, 124 peaks with foot) to 0.1 mM barbiturate-treated cells (32 cells, 31 peaks with foot). [a]

	i_{foot} /pA	t_{foot} /ms	$Q_{foot}/10^3$ molecules	Peaks with a foot
Control	2.9 (2.7-3.1)	2.4 (1.3-4.8)	28 (13-57)	6.6%
Barbiturate	2.9 (2.3-3.8)	1.7 (1.2-2.8)	21 (12-32)	4.4%
Variation	0%	-29%*	-25%*	-2.2%

[a] The data are presented as the median (1st quartile–3rd quartile). The pairs of data sets were compared using a two-tailed Wilcoxon–Mann–Whitney rank-sum test, and the result is indicated next to the variation. *: $p<0.05$

4. Conclusions

Single cell amperometry has been used to investigate the effect of barbiturate on exocytosis in PC12 cells. The results show that barbiturate treatment results in fewer molecules released during exocytosis. A significant decrease in t_{fall} for exocytosis spikes suggests that the closing of the fusion pore is significantly faster making the release time shorter and resulting in fewer molecules released. Intracellular vesicle impact electrochemical cytometry shows that the total catecholamine content of vesicles in PC12 cells after barbiturate does not change the vesicle content. Thus, the fraction of content released during exocytotic release is decreased after barbiturate treatment. This is in consistent with the release dynamics. Pre-spike feet analyses further suggest again that barbiturate treatment induces a less stable fusion pore that

is prone to close faster. We suggest that, at least in part, barbiturate might interact with dynamin and protein kinase C as part of its action. The data indicating that barbiturate differentially affects the larger release events is fascinating as it implies that the changes in the pore closing time are vesicle pool dependent. Future studies will follow up this line of enquiry.

Accumulating evidence now indicates that there is no universal target that explains all the actions of every general anesthetic, or even of a single anesthetic agent. Of course, other proteins might be involved in this process. These fundamental data might be helpful for understanding anesthetic-induced amnesia and unconsciousness at the single-cell level.

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Conflict of interest

The authors declare no conflict of interest.

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