Effect of the Anticancer Drug Tamoxifen on Catecholamine Transmitter Release and Storage from Single Cells

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ABSTRACT

Electrochemical measurements of exocytosis combined with intracellular vesicle impact electrochemical cytometry have been used to evaluate the effect of an anticancer drug, tamoxifen, on the nervous system at the single cell level. Tamoxifen has been used for over 40 years to treat estrogen receptor positive breast cancers during both early stages of the disease and in the adjuvant setting. Tamoxifen causes memory and cognitive dysfunction, but the reasons for the cognitive impairment and memory problems induced by this anti-cancer drug are not well known. We show that tamoxifen, through a nongenomic mechanism, can modulate both exocytosis and vesicle catecholamine storage in a model cell line. The results indicate that exocytosis is inhibited at high concentrations of tamoxifen and is stimulated at low levels. Tamoxifen also elicits a significant concentration dependent change in total catecholamine content of single vesicles, while sub-nanomolar concentrations of the drug have stimulatory activity on catecholamine content of vesicles. In addition, it has profound effects on storage at higher concentrations. Tamoxifen also reduces the intracellular free Ca^{2+}, but only at micromolar concentration, by acting on voltage–gated Ca^{2+} channels, which likely affects neurotransmitter secretion.

KEYWORDS: Tamoxifen, Single cell amperometry, Exocytosis, Vesicle content, Ca^{2+} channels

INTRODUCTION

Tamoxifen (TAM) is the most widely used anti-estrogen to treat advanced and early stage breast cancer and reduce the incidence of breast cancer in high-risk women.[1] The drug is classified as a selective estrogen receptor modulator (SERM), because it is a mixed estrogen agonist/antagonist that alters hormone action by competing with estrogen to bind to its receptor and partially blocking endogenous estrogen action.[2] Because of its anti-estrogenic effects, tamoxifen was used initially to treat estrogen receptor-positive breast cancers. Later, the use of the drug has been developed to include all sorts of breast cancer, and more recently has been used for the prevention of breast cancer for healthy women at high risk.[3]

Tamoxifen targets are any tissues that possess estrogen receptors including breasts, uterus, bone, liver, and brain. It has been reported that tamoxifen causes symptoms common to menopause and postpartum depression including sleep disturbance, anxiety, panic, memory and cognitive dysfunction.[4-6] Several observational studies and clinical trials on tamoxifen treated women have shown that the anti-estrogen effect of this drug causes specific impairments in cognition, processing speed, and verbal memory.[1,7] It has been also reported that women taking tamoxifen have more memory problems and poorer performance on narrative writing tasks compared to women who have never used the drug.[2]

Some regions of the brain, important in memory and cognition, are rich in estrogen receptors. Because estrogen is known to be a modulator of cognitive function, the anti-estrogenic effects of tamoxifen treatment appear to cause negative side effects for cognition and memory, especially when used in the long-term.[8] However, the investigation of side effects caused by tamoxifen on the central nervous system is very limited, and only a few studies have been carried out to study these effects.[9,10]

An adverse effect on cognition might be an acceptable risk in the context of cancer treatment, where positive impacts on survival have been shown. However, possible risks related to the side effects of the drug should be taken into account when considering its administration as a preventative agent to healthy individuals, especially for older women who begin to experience cognitive decline. Therefore, a better understanding of how tamoxifen modulates chemical signal transduction between cells is vital to reveal new possibilities or limitations for its use in clinical applications.

SERMs trigger multiple molecular/cellular signaling pathways through interaction with membrane receptors, ion channels and modulation of calcium and messenger systems through which they contribute to the regulation of transmitter secretion and storage in targeted cells.[11] Activation of each pathway has been shown to both stimulate and inhibit neuronal activity

depending on cell type and the drug concentration. It has been reported that SERMs allosterically interact with plasma membrane estrogen receptors and can either positively or negatively influence neurotransmitter secretion from rat and bovine adrenal medullary cells.[12]

Neurotransmitter secretion from cells occurs through exocytosis, a vital process in cellular communication that involves all cell types and all animal species from yeast to humans.[13] During exocytosis, the membranes of secretory vesicles containing neurotransmitters merge with the cell membrane to release chemical transmitters. Individual exocytotic events from single cells can be easily monitored by applying real time electrochemical methods e.g. single-cell amperometry (SCA). In this method, a carbon fiber microelectrode is placed on the cell membrane to oxidize and quantify released electroactive catecholamines during exocytotic events.[14,15] In order to examine how the vesicular neurotransmitter content changes upon drug treatment, intracellular vesicle impact electrochemical cytometry (IVIEC) can also be carried out by pushing a nanotip carbon-fiber microelectrode through the cell membrane. This allows the whole catecholamine content of individual vesicles in live cells to be measured.[16,17] Here, we have chosen pheochromocytoma (PC12) cells as a model to assess the effects of nanomolar and micromolar concentration of tamoxifen on exocytotic release and vesicle storage in cultured cells. PC12 cells have been used frequently to study presynaptic exocytosis machinery and are also an appropriate model system to study both intracellular calcium release mechanisms[18] and effects of estrogens on neuronal function.[19]

In this paper, PC12 cells were first treated with different concentrations of drug for specific periods of time. Single-cell amperometry was subsequently used to monitor individual exocytotic events and catecholamine release (mostly dopamine for PC12 cells). Tamoxifen elicited a significant concentration dependent change in both exocytotic release and vesicular contents of PC12 cells. We found that the amount of vesicular catecholamine released during exocytosis and the chemical content of vesicles are significantly decreased after treatment with high levels of tamoxifen. Low concentration of tamoxifen changes the number of molecules released during exocytosis oppositely to high concentrations, and vesicle catecholamine storage changes are modest. The drug influences not only the amount of released catecholamines, but also the dynamics of exocytosis during single exocytotic processes, thus appearing to regulate the size of the fusion pore and the duration of its opening and closure in completely different directions depending on its concentration. Finally, we examined the effect of tamoxifen on depolarization-evoked calcium entry into cells using fluorescence imaging measurements. Micromolar concentration of the drug inhibits Ca^{2+} influx, a prerequisite for

catecholamine release, via voltage dependent Ca^{2+} channels, and appears to have functional consequences on secretion.

RESULTS AND DISCUSSION

SCA and IVIEC to Study the Nongenomic Regulation of Chemical Transmitter Release and Storage by 4OH-TAM: Event Frequency

Tamoxifen is metabolized in peripheral tumor tissues and transformed into the activated antiestrogen such as 4-mono- hydroxytamoxifen (4OH-TAM), which has greater affinity for the estrogen receptor than its precursor.[20] Therefore, we have used 4OH-TAM to treat cells in all the experiments. We first carried out single-cell amperometry on PC12 cells to examine the effect of 4OH-TAM to modulate individual exocytotic events. Cells were treated with different amounts of the drug prior to the experiments, including 10 and 100 nM as low concentrations and 1,10 and 100 μM as high concentrations. In order to perform the SCA measurements, a nanotip conical carbon-fiber microelectrode was placed on top of a single PC12 cell and held at +700 mV versus Ag/AgCl reference electrode. We used a nanotip electrode for exocytosis measurements instead of a disk electrode to maintain the same signal to noise ratio when later comparing to the IVEIC measurements (vide infra). Stimulated release from the cell after application of high K+ solution was recorded as amperometric current transients (Figure 1, scheme A). The current spikes can be attributed to vesicular release of dopamine from cells pre-incubated with vehicle or different amounts of the drug. Figure 1A (a, b, c) shows representative amperometric traces from single-cell amperometry of exocytotic dopamine release obtained for control (cells incubated in HEPES physiological saline solution and vehicle), 100 nM, and 10 μM 4OH-TAM treated cells, respectively. The number of events generated per cell and the transient currents increased after treatment with low concentration of drug (Fig, 1A, b), whereas an inhibitory effect was observed at high concentration (10 μM) with less events and lower currents (Figure 1A, c). Data showing the differences between the number of events for control and 100 nM, and 10 μM 4OH-TAM treated cells are shown in Figure S1 in the Supporting Information.

To compare vesicle content to release, we then performed intracellular measurements. A nanotip electrode was pushed through the cell membrane exposing the active conical electrode surface to catecholamine-containing vesicles in the cell interior (Figure 1, scheme B). Without any chemical stimulation, amperometric spikes related to the content of individual vesicles were recorded continuously (Figure 1B (a, b, c)). The data in Figure 1B, (b, c) show that

exposure to a nanomolar level of OH-TAM leads to more vesicle events compared to the control, whereas exposure to higher level of 4OH-TAM in the micromolar range significantly attenuates the number of events (see Supporting Information, Figure S1, B). This difference in vesicles measured with IVIEC seems to indicate that the explanation for the different number of stimulated exocytosis events observed results from a different number of vesicles available after each treatment or at least a change in the process leading to the initiation of vesicle fusion that is similar for both exocytosis and vesicle opening on the electrode. The mechanism leading to a larger number of events after low 4OH-TAM treatment remains unclear, but it appears to involve vesicle structure in some way.

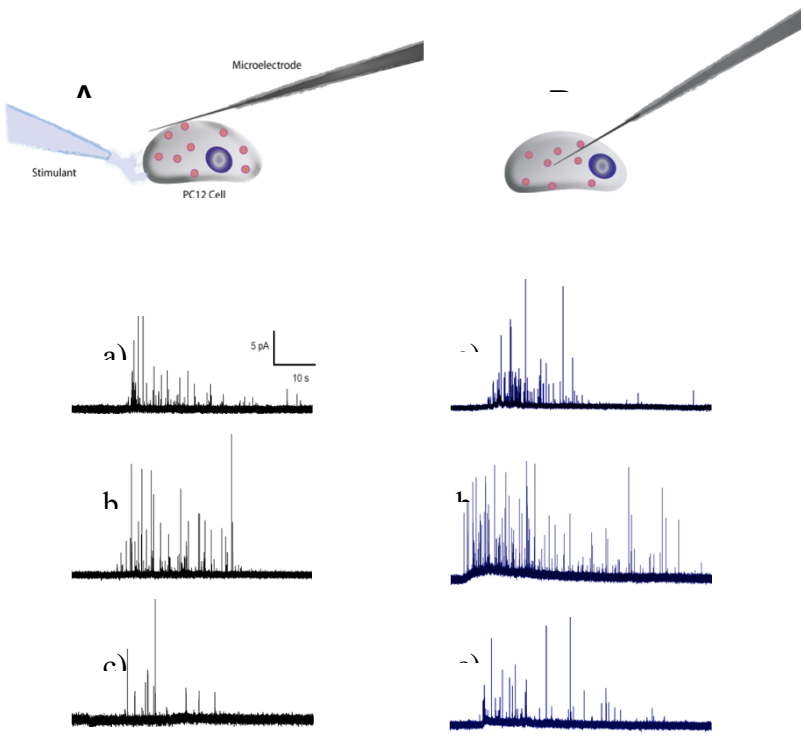

Figure 1. (A) Illustration of the use of a carbon fiber nanotip electrode for single cell amperometry. Representative amperometric traces of exocytotic release from cells a) without (control) and, with (b) 100 nM and (c) 10 μM 4OH-TAM. (B). Illustration of the use of a carbon fiber nanotip electrode for intracellular vesicle impact electrochemical cytometry. Representative amperometric traces of vesicle content in cells a) without (control) and, with (b) 100 nM and (c) 10 μM 4OH-TAM.

4OH-TAM Regulates the Amount of Release and Dynamics of Exocytosis in a Dose-Dependent Manner

For each technique, Faraday's law (N=Q/nF) was used to calculate the average number of released dopamine molecules per spike, N, from the charge, Q, passed at the electrode surface given the number of electrons, n, exchanged in the oxidation reaction and the Faraday constant (96 485 C mol^{-1}). The results for the amount released during exocytosis from all the transients for cells treated with different concentrations of 4OH-TAM are presented as normalized frequency histograms in Figure 2. The distribution of molecules provides a near Gaussian distribution for the molecules released from control cells with some difference for 4OH-TAM

treated cells. Concurrent with these histograms are plots of the number of catecholamine molecules released for control and 4OH-TAM treated cells. To reduce the inherent variation between different cells, the means of median values are used for both control and treated cells. Compared to control, there is a significant difference between the number of released molecules for cells treated with low and high concentrations of the drug. Due to the properties of tamoxifen as an anticancer drug, concerns regarding the viability of PC12 cells after 4OH-TAM treatment had to be addressed to exclude the possibility of lowered viability being the reason for the effects observed on exocytosis. A MTT cell viability assay was performed for all the 4OH-TAM concentrations used in this paper with the results shown in Figure S2 (see Supporting Information). The cell viability did not change upon 10 nM to 10 μM 4OH-TAM treatment, which is in agreement with previous studies.[21] Thus, the data suggest that tamoxifen differentially regulates exocytosis at low and high concentrations but does not influence the cell viability. However, it should be noted that at the highest concentration examined (100 μM) 4OH-TAM exerted a dramatic effect on cell viability decreasing it by approximately 50%. Data at this concentration were discarded.

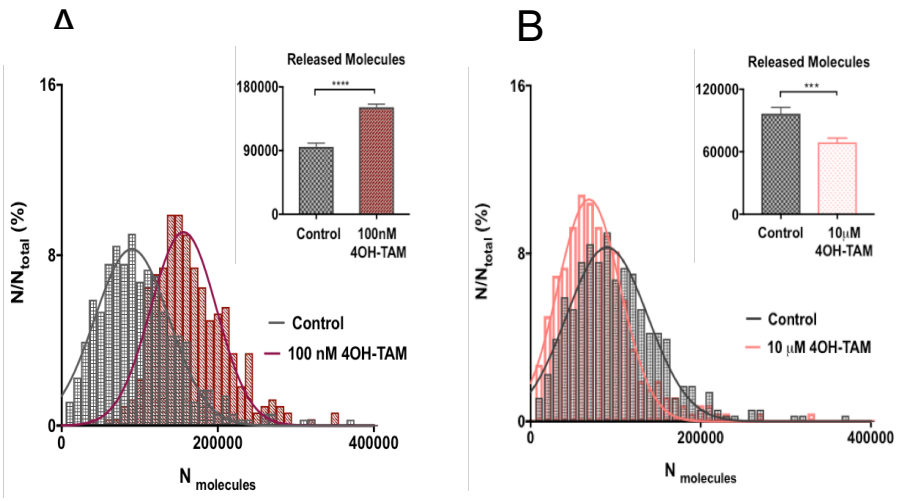

Figure 2. Distributions of catecholamines released shown in normalized frequency histograms. (A) Comparison of control cells (35 cells) and 100 nM 4OH-TAM. (B) Comparison of control and 10 μM 4OH-TAM treated cells (28 cells). Bin size: 1.0 $\times 10^4$ molecules. A Gaussian distribution of the data was employed to obtain fits. Insets show the exocytotic released molecule values from mean of median for control and 4OH-TAM treated PC12 cells. Data sets have been compared with a two-tailed Mann-Whitney rank-sum test; ****, $p < 0.0001$; ***, $p < 0.001$.

To further investigate the 4OH-TAM effect on fusion pore dynamics and exocytotic release, we analyzed the amperometric spikes from single cell amperometry. Several parameters related to the dynamics of the release event can be obtained from each exocytotic signal including the peak current, I_{max}, the 25% to 75% of the maximum on the ascending part of the transient, t_{rise}, the peak half width t_{half}, and the 75 % to 25 % of the of maximum on the descending part of the

transient, t_{fall} (see Supporting Information, Figure S3). The values of t_{rise} and t_{fall} are typically thought to be characteristics of the pore opening and closing, respectively.

All the pooled parameters obtained from exocytotic spikes are presented in Figure 3. The data reveal that low concentrations of 4OH-TAM slow down exocytotic release with significant increases in characteristic peak times, t_{rise} and t_{half}, compared with control cells which seems to indicate the formation of a much more stable fusion pore during vesicle opening. This changes the rate of neurotransmitter release to lengthen the events, which might regulate synaptic strength. However, t_{fall} did not change significantly, which suggests the closing of the fusion pore is not affected with low amounts of the drug. An increase in peak current, i_{max}, might indicate that the pore size is larger after 100 nM 4OH-TAM treatment. In contrast, the opposite effects were observed for t_{fall} and peak current, I_{max}, (with no significant change in t_{rise} and t_{half}) when the 4OH-TAM concentration was increased to 10 µM. Here, we conclude that the fusion pore closing process and pore size are now affected by the drug but not the pore opening. Similar effects were observed for cells treated with 1 µM 4OH-TAM (data not shown). This is very interesting as the lower values of released molecules at high drug concentrations appear to result from a shorter lifetime of the pore, causing smaller depletion of the vesicle content during exocytosis. Of course, tamoxifen might also have some effects on vesicle structure and content. Moreover, the lower I_{max} is an indication of a narrower fusion pore during release when the cells are treated with a high concentration of the drug. This leads to a decrease in the number of released molecules compared with control.

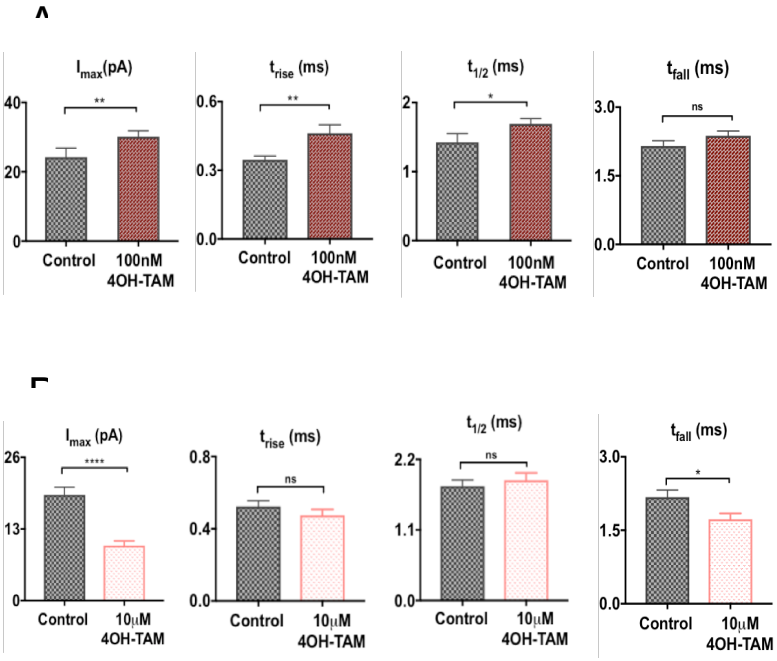

Figure 3. Average values for amperometric peak parameters including I_{max}, $t_{1/2}$, t_{rise} and t_{fall} obtained from single cell amperometry for PC12 cells treated without or with 100 nM (A), and 10 µM 4OH-TAM (B). Data sets have been compared with a two-tailed Mann-Whitney rank-sum test; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.1$.

We also investigated the amperometric feet in these recordings of control and 4OH-TAM treated cells. The pre-spike feet are seen as a small increase in oxidative current at the very beginning of an amperometric spike. These features are commonly thought to be an artifact of the first stage of fusion pore formation, when the pore has formed but not yet expanded. The results are summarized in Table 1. The probability to observe a peak with a pre-spike foot was higher for cells treated with 100 nM 4OH-TAM and lower for cells treated with 10 µM 4OH-TAM than for the control cells. For cells treated with 100 nM 4OH-TAM, i_{foot} and the duration were considerably increased, consistent with the data for exocytosis shown in Figure 3. The value of t_{foot} has been found to be related to the dynamics of the fusion pore, whereas i_{foot} has been found to be related to the geometry of the fusion pore, thus it appears that 100 nM 4OH-TAM treatment induces a more stable fusion pore that is prone to dilate and close slower than in the control.

Upon 10 µM 4OH-TAM treatment, both i_{foot} and Q_{foot} decreased substantially, oppositely to 100 nM concentration, which is also consistent with the overall trend for exocytosis events presented above. These results here further suggest that tamoxifen has different mechanisms in the regulation of creation of pore during exocytosis. Different amounts of the drug not only influence the number of released molecules during single exocytotic process, but also the dynamics of exocytosis.

Table 1. Values of foot parameters following K^+-stimulation of PC12 cells (i_{foot}> 2 pA), for control (30 cells, 18 peaks with foot) to 4OH-TAM treated cells (100 nM: 26 cells, 44 peaks with foot and 10 µM: 20 cells, 7 peaks with foot). [a]

Treatment	i_{foot} [pA]	t_{foot} [ms]	Q_{foot} [10^3 molecules]	Peaks with a foot
Control	3.1 (2.3-3.8)	2.3 (1.5-2.6)	26 (18-34)	9%
100 nM 4OH-TAM	3.8 (2.9-4.6)	2.9 (2.1-4.1)	31 (23-47)	12%
Variation	+22%**	+26%*	+19%	
10 µM 4OH-TAM	2.2 (2- 2.3)	2.2 (2-2.5)	18 (15-27)	2%
Variation	-29%****	-4%	-31%**	

[a] Data are listed as the median (1st quartile–3rd quartile). Data pairs have been compared using a two-tailed Mann-Whitney rank-sum test. ****, p< 0.0001; **: p<0.01; *: p<0.05.

IVIEC Shows that 4OH-TAM Alters Vesicle Content During Exocytosis.

To further investigate whether these effects are attributed to changes in the vesicular content of treated cells, IVIEC was carried out with the same conditions as those for exocytosis measurements. IVIEC traces for cells treated with low and high amounts of 4OH-TAM were analyzed and compared to control cells. The number of catecholamine molecules in single vesicles was calculated from all the transients, and these values are plotted in Figure 4. These results reveal that the vesicular content is moderately affected by 4OH-TAM exposure, depending on the amount of the drug. According to the data in Figure 4, 4OH-TAM at 100 nM (and 10 nM, data not shown) increases the total vesicular content, whereas at higher concentrations it suppresses dopamine loading and decreases vesicular content. The normalized frequency histograms in Figure 4A, with similar standard deviation of the Gaussian for control and 100 nM 4OH-TAM treated cells, implies that 4OH-TAM has equally increased the catecholamine content of all vesicles.

Other specific spike parameters of IVIEC measurements from control cells and cells treated with low and high concentrations of 4OH-TAM are shown in Figure 5, which reveal that 100 nM 4OH-TAM treatment significantly increases the t_{rise}, t_{half}, and t_{fall}. Interestingly, 10 μM 4OH-TAM also leads to a substantial decrease in t_{half} and t_{fall}, without changing the t_{rise} indicating that the drug affects the vesicle structure. Since electroporation is the main mechanism of vesicle rupture in the IVIEC technique, any change in the dynamic of the process, is due to the possible changes in the vesicle membrane (lipids) and not because of alteration in the typical fusion machinery.[22,23] One explanation for this could be the concentration dependent behavior of tamoxifen on membrane fluidity. Spectroscopic studies have shown that tamoxifen causes opposite effects on membrane fluidity at low and high concentrations (1 and 30 mol%). The fluidity increases at high concentrations and decreases at low amounts.[24]

Overall, the exocytotic catecholamine release and total vesicular catecholamine content increased after nanomolar tamoxifen treatment and decreased when the high concentrations, in micromolar range were used. Taken together these results suggest that 4OH-TAM has a stimulatory effect at sub-nanomolar but an inhibitory effect at higher concentrations for both exocytosis and IVIEC measurements, but with very different sensitivities.

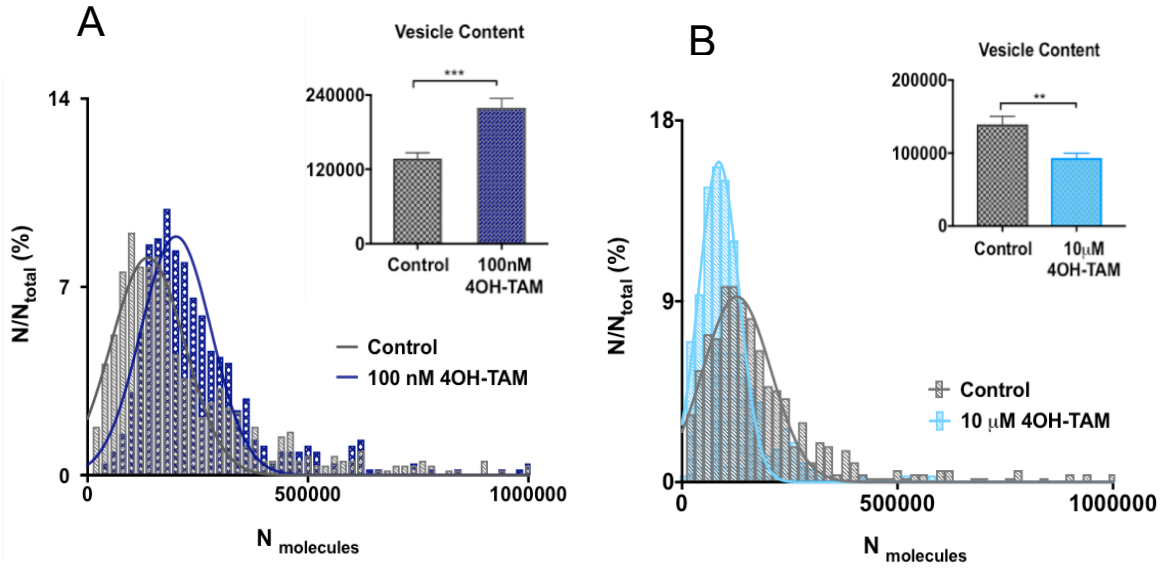

Figure 4. Normalized frequency histograms presenting the distributions of the molecules measured in vesicles of control cells and 100 nM (A) and 10 μM (B) 4OH-TAM treated cells (N= 20 cells). Bin size: 2.0 $\times 10^4$ molecules. A Gaussian distribution of the data was employed to obtain fits. Insets: Vesicle content from mean of median for control and 100 nM and 10 μM 4OH-TAM treated cells. Data sets have been compared with a two-tailed Mann-Whitney rank-sum test; ***, $p < 0.001$, **, $p < 0.01$.

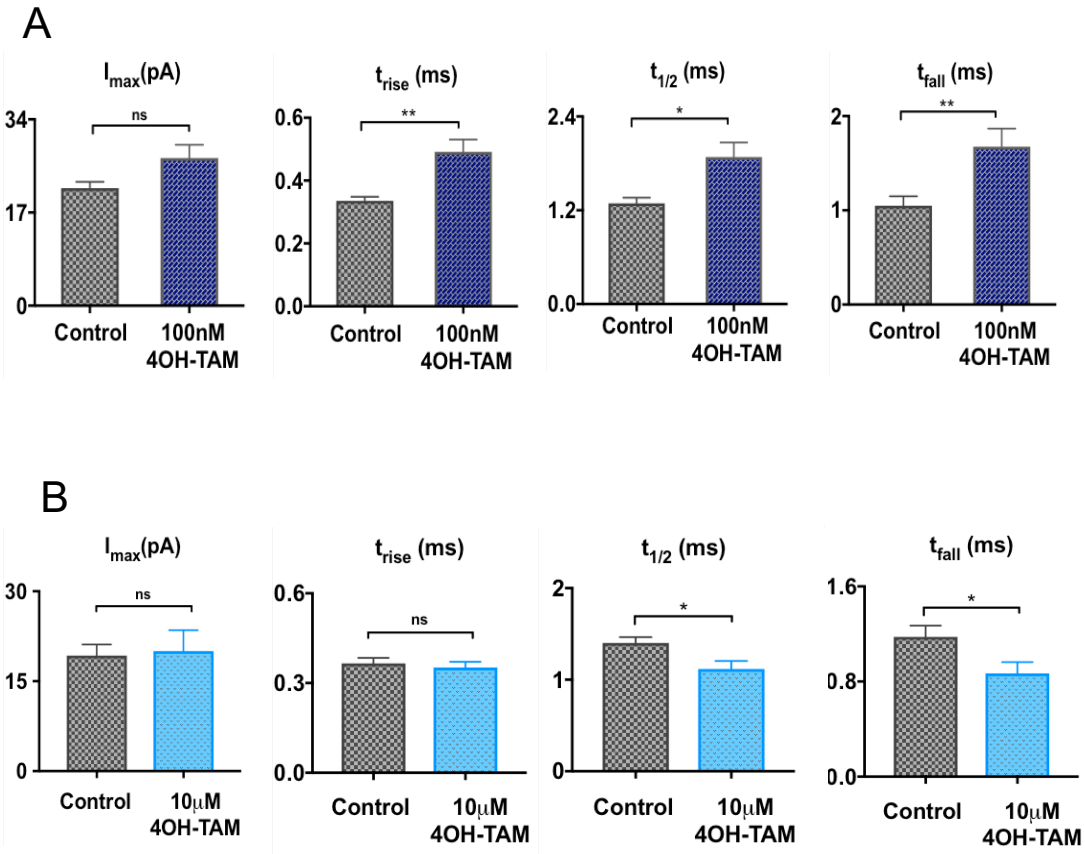

Figure 5. Comparisons of I_{max}, $t_{1/2}$, t_{rise}, t_{fall} and N obtained from intracellular vesicle electrochemical cytometry results for PC12 cells treated without or with 100 nM (A), and 10 μM 4OH-TAM (B). Data sets have been compared with a two-tailed Mann-Whitney rank-sum test; **, $p < 0.01$; *, $p < 0.1$.

Micromolar Concentration of 4OH-TAM Reduces Intracellular Free Calcium

Intracellular calcium is known to be involved in the regulation of exocytosis and is known to affect both the number of transmitters released as well as the frequency of events.[25] Thus, we investigated the possibility that the effects of tamoxifen are mediated through this secondary messenger. Tamoxifen might act on voltage-gated calcium channels and subsequently influence catecholamine exocytosis. We used the calcium chelator and fluorophore Fura-2 to measure intracellular calcium.[26,27] This dye is used for ratiometric imaging, thereby reducing issues with uneven dye distribution between cells and other factors.[28] Figure 6 shows the average calcium response after overnight treatment with 4OH-TAM for the different treatments. Treatment of tamoxifen appears to decrease the cellular calcium response to stimulation. This is particularly true for the higher concentration of tamoxifen, 10 μM. Even with 100 nM 4OH-TAM treatment; however, the level of the calcium response is reduced slightly. These data suggest that tamoxifen can act on voltage gated calcium channels and introduces fast changes in intracellular calcium signaling and probably stores, especially at higher drug concentrations.

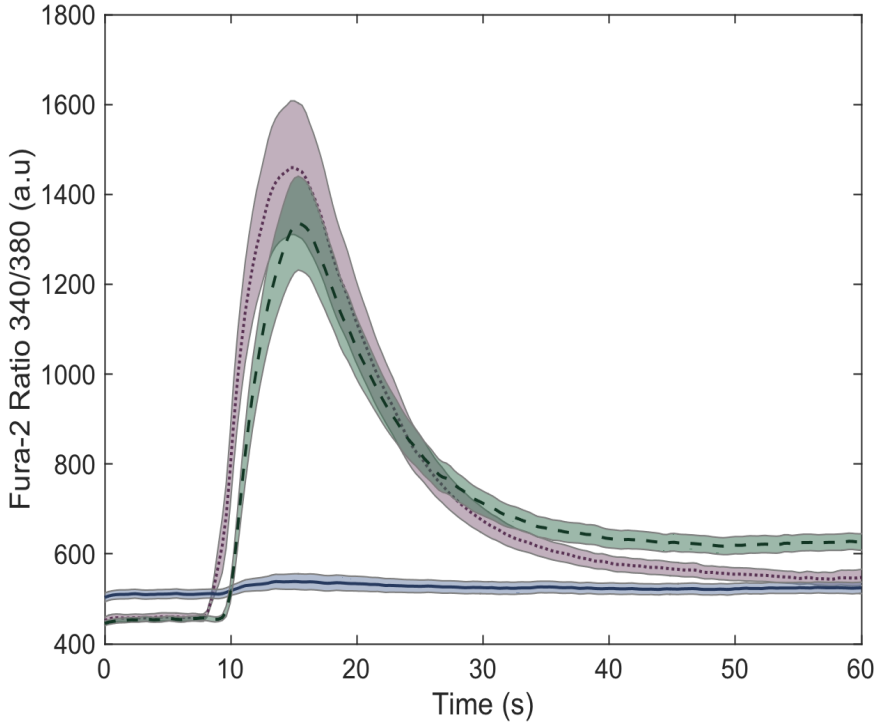

Figure 6. Intracellular calcium responses after 5-s potassium stimulations with 4OH-TAM incubation. The purple dotted line represents the average calcium response for control cells, the green dashed line represents the average response for cells treated overnight with 100 nM 4OH-TAM, and the blue solid line represents the average response for cells treated overnight with 10 μM 4OH-TAM. Shaded areas around the lines indicate SEM for the measurements (n= 17-19 cells).

Possible Mechanisms for the Action of TAM on Exocytosis and Vesicular Content

Tamoxifen acts via multiple cellular/molecular pathways in the target cells. Figure 7 summarizes the possible mechanisms of action of tamoxifen. The thick solid-line arrows in Figure 7 represent extracellular targets of the drug including plasma membrane estrogen receptors, the dopamine transporter (DAT), and ion channels. Thick dashed-line arrows are the intracellular targets of the drug such as nuclear estrogen receptors, protein kinase C (PKC) and calmodulin.

TAM interaction with the membrane and nuclear estrogen receptors is thought to mediate many of the biological and chemotherapeutic effects of this drug during long-term exposures,[29] which are out of the scope of this work. However, TAM has many alternative mechanisms of action, which occur independently of the compound actions at the estrogen receptor that might affect catecholamine storage and secretion. It has a direct effect on ion channels and subsequent cellular functions in a non-genomic manner. For instance, it has been shown that TAM induces fast changes in intracellular calcium movements and inhibits Ca^{2+} influx.[30] Our calcium imaging data are consistent with this and confirm reduction of Ca^{2+} currents at certain concentrations of the drug, which subsequently affect secretion of catecholamines. As shown in Figure 7, TAM also directly interacts with the dopamine transporter (DAT),[31] a key mediator of dopamine uptake, and calmodulin,[32] a calcium- dependent regulatory protein involved in intracellular membrane fusion.

Ample evidence exists supporting the therapeutic potential of tamoxifen as a protein kinase C (PKC) regulator in a time dependent manner. TAM can inhibit or activate PKC,[33] that is involved in regulating multiple stages in exocytosis such as the fusion pore opening and closing processes.[34] Several PKC substrates have been shown to be involved in exocytosis, including SNAP-25, synaptotagmin, calcium-dependent activator protein for secretion (CAPS), and nsec1 protein,[35] all of which are present on the vesicles and intracellular side of the cell membrane. Previous studies have shown that low concentrations of TAM enhance PKC activity in rat brain cells relative to control levels, and an inhibitory effect is observed at high concentrations.[33]

Activation of PKC in-vitro requires the presence of calcium ions,[36] that play a central role in the formation of the active complex; therefore, the ability of tamoxifen to activate PKC appears to depend strongly on the Ca^{2+} concentration. This property is likely to explain the increase we observe in release and vesicular dopamine content for cells treated with low concentration of 4OH-TAM, because generally the PKC activation facilitates neurotransmitter release by increasing the Ca^{2+}-sensitivity of vesicle fusion. It also might promote actin fragmentation and

causes a loose actin network at the fusion pore, which results in a stable fusion pore and longer exocytotic events.[37] Again, this is consistent with our observations here. However, by decreasing intracellular calcium concentration in cells treated with high concentration of the drug (μM), tamoxifen-mediated PKC activation is then decreased, becoming inhibitory.

At low concentrations, TAM appears to work by acting on other cellular targets than PKC. For example, an increase in cyclic AMP (cAMP) is involved in the presynaptic effects of low concentration of tamoxifen.[38,39] As shown in Figure 7, some of the membrane estrogen receptors are coupled to a G-protein-stimulating adenylate cyclase that catalyzes the conversion of ATP to cAMP. Any increase of intracellular cAMP caused by low amount of tamoxifen slows the dynamics of exocytosis and increases the amount of released catecholamines.[40]

Most SERMs, particularly tamoxifen, are highly lipophilic and protein-bound compounds that can accumulate in the lipid part of cell membranes and change the lipid bilayer organization and physical membrane properties.[41] During exocytosis, a dynamic high curvature shape transition occurs on the membrane of the lipidic fusion pore between the vesicle membrane and the plasma membrane. These changes in the local chemical environment and the physical forces acting upon the cell membrane can modify exocytotic release.[42] At high concentrations, tamoxifen might cause changes in lipidic membrane composition or structure, resulting in a narrower fusion pore with fewer molecules released and fewer events. There are conflicting results on the effect of tamoxifen on membrane dynamics. One of the factors in this discrepancy is the concentration of tamoxifen used in different studies, because the drug causes opposite effects on membrane fluidity at low and high concentrations.[41]

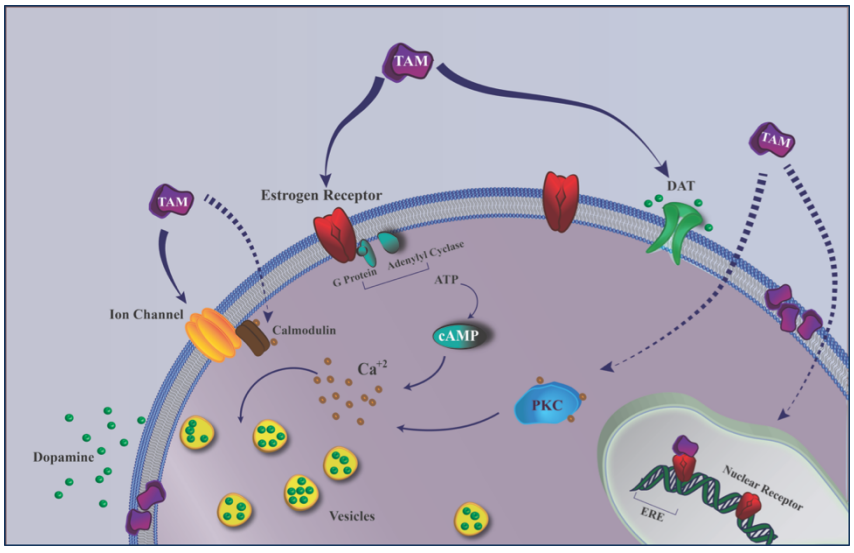

Figure 7. Proposed scheme for the possible mechanisms of the action of tamoxifen on target cells and its effect on exocytosis. Thick solid-line arrows represent extracellular targets of the drug including plasma membrane estrogen receptors, the dopamine transporter (DAT) and ion channels. Thick dashed-line arrows are the intracellular targets of the drug including nuclear estrogen receptors, protein kinase C (PKC) and calmodulin.

Conclusions

In this study, we evaluated the effects of the anticancer drug, tamoxifen, on exocytosis and the catecholamine content of vesicles in PC12 cells, using single cell amperometry and IVIEC techniques to measure both exocytotic release and vesicle content, respectively. At lower concentrations (nM), 4OH-TAM treatment increases both catecholamine release and its vesicular content. However, a clear inhibitory effect is observed in the presence of high tamoxifen concentrations (μM), as fewer events generated per cell and less catecholamine molecules released for both exocytosis and IVIEC measurements. Pre-spike foot analyses further suggest that tamoxifen treatment regulates exocytosis differentially at low and high concentrations. We suggest that 4OH-TAM has an inhibitory on intracellular Ca^{2+} levels and can also change membrane lipid properties, which can modify the dynamics of exocytosis. Testing the effect of anticancer drugs on neurotransmitter secretion and storage will help to understand the actions of these drugs in regulation of vesicles and exocytosis and the communication between nerve cells, and importantly, could have implications for the clinical care of the patients that use them long term.

METHODS

Chemical and Solutions. All chemicals were of analytical grade and obtained from Sigma-Aldrich (Sweden) unless otherwise specified. 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 aqueous solutions were prepared using 18 MΩ cm^{-1} water from a Purelab Classic purification system (ELGA, Sweden).

4-Hydroxytamoxifen (4OH-TAM), the active form of the drug, was used in all the experiments. The stock solution of 4OH-TAM was prepared at 1 mM in ethanol (96%) the day of the experiment. Diluted solutions were prepared from these stocks just before the incubation. For each concentration of 4OH-TAM tested during the experiment, the amount of ethanol in the final solution was set to less than 0.02%.

Nano-Tip Conical Carbon Fiber Microelectrode Fabrication. The fabrication of nano-tip conical carbon fiber microelectrodes was done as described previously.[16,43] A 5 μm carbon fiber was aspirated into a borosilicate glass capillary (1.2 m O.D., 0.69 mm I.D., no filament, Sutter Instrument Co., USA). The capillaries were subsequently pulled to a taper with a

micropipette puller (PE-21, Narishige, Japan). In the following step a scalpel was used to cut the fiber extending from the glass 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 fibers, with needle-sharp tips (about 100-200 nm in diameter, 50-100 μm in length, were sealed with epoxy (Epoxy Technology, Billerica, MA). Prior to recording, each electrode was tested by performing cyclic voltammetry at 200 mVs^{-1} in a solution of 100 μM dopamine in PBS (pH 7.4). Only electrodes with stable *I-E* curves were used for the experiments.

Cell Culture and Drug Treatment. Cultures of PC12 cells were maintained similarly to those in previous studies in our laboratory.[22,44] PC12 cells, a gift from LIoyd Greene (Columbia University), were maintained in RPMI-1640 media (Lonza™ BioWhittaker) supplemented with 10% donor equine serum and 5% fetal bovine serum 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 experiments, PC12 cells were sub-cultured sparsely on mouse collagen coated culture dishes (type IV, BD Biosciences, Bedford, MA) 3 days before the experiment and cell medium was replaced every day.

For tamoxifen treatment, the cells were treated with vehicle (as control) and different concentrations of 4OH-TAM by rinsing the dishes three times with RPMI-1640 media and then incubating the cells in low serum (1% horse serum and 0.5% FBS) medium in a humidified incubator overnight. After incubation, the cells were rinsed 3 times with warm HEPES physiological saline and then 4 mL of isotonic saline (pH 7.4) was added to the cells prior to experimentation.

Single Cell Amperometry and Intracellular Impact Cytometry. Electrochemical recordings from single PC12 cells were performed on an inverted microscope (IX81, Olympus), in a Faraday cage, as previously described.[16,43] A constant potential of +700 mV (vs. Ag|AgCl) was applied to the working electrode under the control of an Axopatch 200B potentiostat (Molecular Devices, Sunnyvale, CA, USA). The output was digitized at 5 kHz and filtered with an internal low pass Bessel filter at 2.1 kHz. The signal was displayed in real time (AxoScope 10.4, Axon Instruments, USA Inc., Sunnyvale, CA, USA) and stored digitally.

For exocytosis experiments, the nano-tip electrode was moved slowly by a PatchClamp Micromanipulator (PCS-5000, Burleigh Instruments, Inc., USA) to place it on the membrane of a PC12 cell without causing any damage to the surface. A high-K^+ stimulating solution in a glass micropipette was injected into the surrounding volume around the PC12 cell with a single 5-s injection pulse at 5 s after the start of recording. For IVIEC, the tip of the carbon fiber electrode was first placed on top of the PC12 cell membrane. The tip was slowly pressed through the membrane of the PC12 cell while the current was recorded. Stimulation is not needed in this method. All cell experiments were performed at 37 °C.

Data Acquisition and Statistical analysis. Data analysis was similar to that in previous studies in our laboratory.[43,44] The amperometric traces were processed using Igor Pro 6 (Version 6.3.7.2; WaveMetrics, Lake Oswego, OR) designed for analysis of quantal release by the group of David Sulzer at Columbia University. Peaks were detected if exceeding a threshold of 3 times the RMS noise and digitally smoothed using a binominal filter at 1 kHz. The traces were carefully inspected after peak detection and false positives were manually removed. 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 the median of molecule number was used. In this way, the impact of the cell-to-cell variations was minimized, as the value is less sensitive to outliers. Pairs of data sets were compared with a two-tailed Mann-Whitney rank-sum test; ****, $p < 0.0001$, ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.1$.

Calcium Imaging. PC12 cells were cultured and grown as previously described for electrochemical experiments. Cells used as control were first rinsed 3 times with warm isotonic solution followed by incubation (20 min, 37°C) with the calcium sensitive, fluorescent dye Fura-2 (0.4 μM, Invitrogen, Sweden). Treated cells were incubated with specified amounts of 4OH-TAM (100 nM or 10 μM) in addition to the dye. These particular concentrations of tamoxifen were chosen as high and low level of the drug. After loading of the dye, cells were again rinsed 3 times and experiments were subsequently performed in isotonic solution.

An inverted microscope with an MT-20 illumination system was used for the fluorescent imaging (Olympus, Japan). The light source was a 150 W Xenon arc lamp and images were obtained using an ORCA-ER camera (Hamamatsu, Japan). Fura-2 was imaged ratiometrically at 380 nm and 340 nm for a total of 60 s, with K^+-solution stimulation occurring after a 10-s baseline collection. The experiment setup, background subtraction and analysis of regions of

interest were all done in the Cell-R software (Olympus). The responses for control and different treatments were pooled and further analyzed and plotted in MATLAB (The MathWorks Inc.). Between 17-19 cells were analyzed for both control and treatments.

MTT assay. The MTT assay involves the conversion of the water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to an insoluble formazan which is impermeable to the cell membranes and therefore accumulates in viable cells. The formazan crystals are then solubilized, and the concentration determined by optical density.

After incubation with different concentration of 4OH-TAM, the cell medium was removed and replaced with 100 µL of fresh culture medium. A volume of 10 µL of 12 mM MTT stock solution was added to each well and incubated at 37°C for 3 h. The MTT media was removed and 50 µL of DMSO was added to each well to dissolve the formazan crystals. After mixing thoroughly with the pipette, it was incubated at 37°C for 10 min. The optical density (OD) value for each well was measured using a microplate absorbance reader (FLUOstar Omega plate reader, BMG LABTECH, Germany) at the wavelength of 540 nm and the cell survival rate was calculated.

ASSOCIATED CONTENT

* Supporting Information

Average number of events recorded per cell obtained from exocytosis (A) and intracellular vesicle electrochemical cytometry results (B) upon treatment with 100 nM and 10 µM of 4OH-TAM.
Cell viability as measured by MTT assay for PC12 cells treated with different concentrations of 4OH-TAM.
Scheme showing the different parameters used for the peak analysis in this work.

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Author Contributions

All authors have given approval to the final version of the manuscript. ZT conceived of the idea to examine Tamoxifen on exocytosis and performed most of the experiments, interpreted the data, and wrote the manuscript. AL carried out the calcium imaging experiments, interpreted the data, was involved in intellectual discussions, and editing the manuscript. AGE was involved in supervising and

determining the experimental plan, discussions of data interpretation, additional experiments, outlining the manuscript, revisions and editing.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

SCA single-cell amperometry; IVIEC intracellular vesicle impact electrochemical cytometry; PC12 pheochromocytoma; 4OH-TAM 4-mono- hydroxytamoxifen.

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