Comparison of Disk and Nano-tip Electrodes for Measurement of Single-Cell Amperometry during Exocytotic Release

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ABSTRACT: We compare single-cell amperometric measurements of exocytosis from PC12 cells between two types of electrodes, carbon fiber disk microelectrodes and nano-tip conical-shape carbon fiber microelectrodes. During the exocytotic process, individual exocytotic release events, measured as current spikes at the electrode offer quantitative and dynamic information about chemical release from cells. Using two electrodes gives rise to unequal distance between the fusion pore and the electrode as well as fusion pore size, which leads to different average spike shapes. Nano-tip electrodes show a higher but narrower spike than disk electrodes when measuring exocytosis. The estimated pore-electrode distance and fusion pore size for disk electrodes are 239 nm and 11.5 nm, while for nano-tip electrodes these are 215 nm and 18.2 nm, respectively. The data show that nano-tip electrodes, despite showing different dynamics for release, are quantitative in measuring the number of molecules released and can be used for quantitative comparison between exocytosis and vesicular content in intracellular vesicle impact electrochemical cytometry.

Exocytosis, the cellular process that is conserved among multicellular organisms, occurs through merging of a pre-synaptic vesicle membrane with the cell membrane to release vesicular messengers to the synaptic cleft or extracellular environment. The messenger or transmitter content that is expelled diffuses and eventually binds to and thus activates certain receptors on post-synaptic cells. The pheochromocytoma (PC12) cell line was first established in 1976 and the relative ease of culture and use makes it an attractive model to study neurotransmitter secretion and neuronal differentiation.[1-2] PC12 cells contain large dense-core vesicles which are enriched by catecholamines with dopamine being the dominant species, followed by norepinephrine.

Owing to the fact that exocytosis occurs on the millisecond time scale, it is highly desirable that techniques applied for monitoring exocytotic release offer sufficient temporal resolution. Applying single-cell amperometry (SCA) with a carbon fiber disk electrode to measure exocytosis was first reported by the Wightman group in the 1990s.[3-4] SCA provides the capabilities to dynamically resolve individual vesicle release events and to quantify the amount being released to better understand the mechanism of exocytosis.[5]

Nano-tip electrodes were fabricated to perform intracellular vesicle impact electrochemical cytometry (IVIEC) to quantify transmitter storage in single vesicles as it preserves the natural cellular environment during the measurement.[6-7] By piercing the sharp tip carefully through the membrane of a cell, the intracellular vesicles can rupture on the electrode tip to release the transmitter stored, and the electroactive transmitters can be quantified. When the same nano-tip electrode is placed above a single cell, the amount of release can be quantified upon applying chemical stimulation. The combination of SCA and IVIEC makes it possible to compare exocytotic release and transmitter content, and to calculate the fraction of release, which is release divided by content, from a cell population upon pharmacological treatment or activity-induced plasticity and it shows that exocytosis does not occur through an all-or-none process, instead only a fraction of vesicular transmitter is released.[8-14] However, a careful quantitative evaluation of the use of nano-tip electrodes to measure SCA has not been presented yet.

In this paper, we compare the response of nano-tip electrodes with traditional disk electrodes to verify the suitability of using nano-tip electrodes for the study of exocytotic release. Our results quantitatively confirm that the amount of neurotransmitter released measured by nano-tip electrodes is the same as at disk electrodes. This is significant as when comparing the dynamics of release events, nano-tip electrodes offer a higher current and faster event than observed at disk electrodes. The distribution of exocytotic release events varies between the two types of electrodes due to the distinct noise levels. However, as the noise level of the electrode determines peak selection criteria, by setting criteria the same for both electrodes, the distributions show no significant difference.

EXPERIMENTAL SECTION

More details of chemicals and solutions, cell culture, data processing and statistics, finite element simulation, and estimation of pore-electrode distance and fusion pore size are included in the supporting information. The new experimental aspects needed for the novel work in this paper are presented here.

Microelectrode fabrication. The fabrication of carbon fiber disk and carbon fiber nano-tip microelectrodes was carried out as previously described.[6, 15] Both electrodes share several fabrication steps. In brief, a carbon fiber with a diameter of 5 μm was aspirated into a glass capillary (Sutter Instrument Co., Novato, CA, U.S.A.), which was then pulled into two electrodes with a commercial micropipette puller (model PE-21, Narishige Inc., Japan). For disk electrodes, the carbon fiber was cut with

a scalpel to the edge of the glass and the electrode was subsequently sealed with epoxy (G A Lindberg ChemTech AB, Sweden) and baked at 100 °C overnight. After that, the electrode was beveled at a 45 °C angle with a commercial beveller (EG-400, Narishige Inc., London, UK). For nano-tip electrodes, after pulling, a scalpel was used to cut the carbon fiber outside the glass to about 100-150 µm under a microscope. The carbon fiber was then flame etched with a butane gas burner (Clas Ohlson, Sweden) until it had a needle-sharp tip under a microscope (about 100-200 nm diameter and 30-100 µm length). The electrode was subsequently sealed with epoxy and cured at 100 °C overnight. Both types of electrodes were tested before the experiment with cyclic voltammetry (-0.2 to 0.8 V versus Ag/AgCl, 100 mV/s) in a solution of 100 µM dopamine in PBS (pH 7.4). Electrodes used for experiments were required to show good reaction kinetics and stable steady-state currents.

Single-cell amperometry experiments. Before performing single-cell experiments, the culture medium was removed from the culture dish and the cells were rinsed 3 times with isotonic solution. During the experiment, the cells were kept at 37 °C in isotonic solution. Single-cell amperometry was performed at single PC12 cells on an inverted microscope (IX71, Olympus). An Axopatch 200B potentiostat (Molecular Devices, Sunnyvale, CA, U.S.A.) was used to hold the working electrode at a constant potential of +700 mV versus an Ag/AgCl reference electrode. The output of the signal was filtered at 2 kHz and digitized at 5 kHz. To study single-cell exocytosis, a disk or nano-tip electrode was placed carefully over the PC12 cell membrane without causing visible damage to the cell surface. Then a glass micropipette filled with high K+ stimulation solution was placed next to the cell. To stimulate the cell for exocytosis, the micropipette was connected to a microinjection system (Picospritzer II, General Valve Corporation, Fairfield, NJ, U.S.A.) and each injection was triggered via a 5-s 20 psi injection pulse.

RESULTS AND DISCUSSION

Quantification of exocytotic release by disk and nano-tip electrodes is the same. Exocytosis is measured using SCA. By placing a carbon fiber microelectrode over a single cell, which is illustrated in Figure 1, upon stimulation with an increased K+ concentration solution, neurotransmitters are triggered to be released from vesicles and the electroactive transmitters are oxidized on the electrode surface. The oxidation of transmitters for each vesicle is detected as current transients as a function of time, in which each spike corresponds to a single exocytotic event. The typical current traces measured by disk and nano-tip electrodes are shown in Figure 1A and 1B, and the average spike shapes from both electrodes are depicted in Figure 1C and 1D. Useful information can be found by analyzing the amperometric traces. We observed that the traces obtained with a disk electrode are smoother with a lower background noise compared to those obtained using a nano-tip electrode, which has a higher noise level. This is not surprising as the noise level in amperometry is generally proportional to the electrode area and the nano-tip electrode has the larger overall area with its conical shape leading to the tip. The exocytotic peak measured by the disk electrode is also wider and has slightly lower current when compared to that measured by the nano-tip electrode.

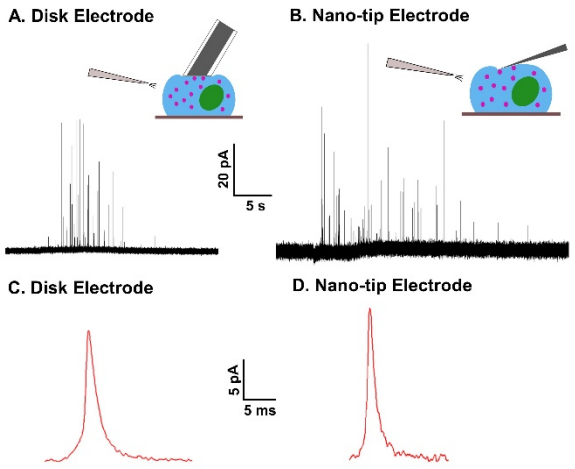

Figure 1. Representative amperometric traces of SCA with (A) a disk and (B) a nano-tip electrode and average spike shape obtained by (C) disk and (D) nano-tip electrodes at PC12 cells.

The amount of neurotransmitter release during exocytosis can be quantified using Faraday's equation, N = Q/nF, with Q being the charge of the spike obtained by integrating the area under the spike, n being the number of electrons transferred during the oxidation reaction which is 2 for dopamine oxidation, and F is Faraday's constant which is 96485 Cmol-1. The numbers of moles of released molecules during single exocytotic event measured using disk and nano-tip electrodes are nearly the same (Figure 2), with p value equal to 0.86, demonstrating no significant difference.

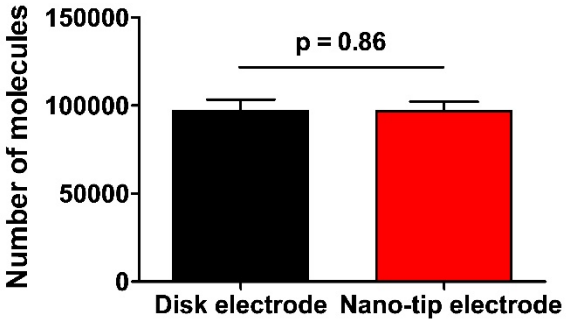

Figure 2. Average number of molecules quantified per exocytotic release event with disk and nano-tip electrodes at PC12 cells. Data are shown as mean of medians ± SEM. Numbers of cells measured with disk and nano-tip electrode were both 12. A nonparametric, two-tailed Mann-Whitney test was applied to compare the two data sets and the p value is included above the bar.

Dynamics of exocytotic spikes are influenced by pore-electrode distance and fusion pore size. We investigated the difference between exocytotic spike shapes measured by disk and nano-tip electrodes in order to gain useful information regarding release dynamics. Here, peak analysis was carried out for SCA at the two different electrodes. The parameters utilized for peak analysis are depicted in Figure 3A, including I_{max}, representing the amplitude of peak, $t_{1/2}$, the width of peak at its half amplitude, t_{rise}, the time from 25% to 75% of amplitude on the rising phase of peak, and t_{fall}, the time from 75% to

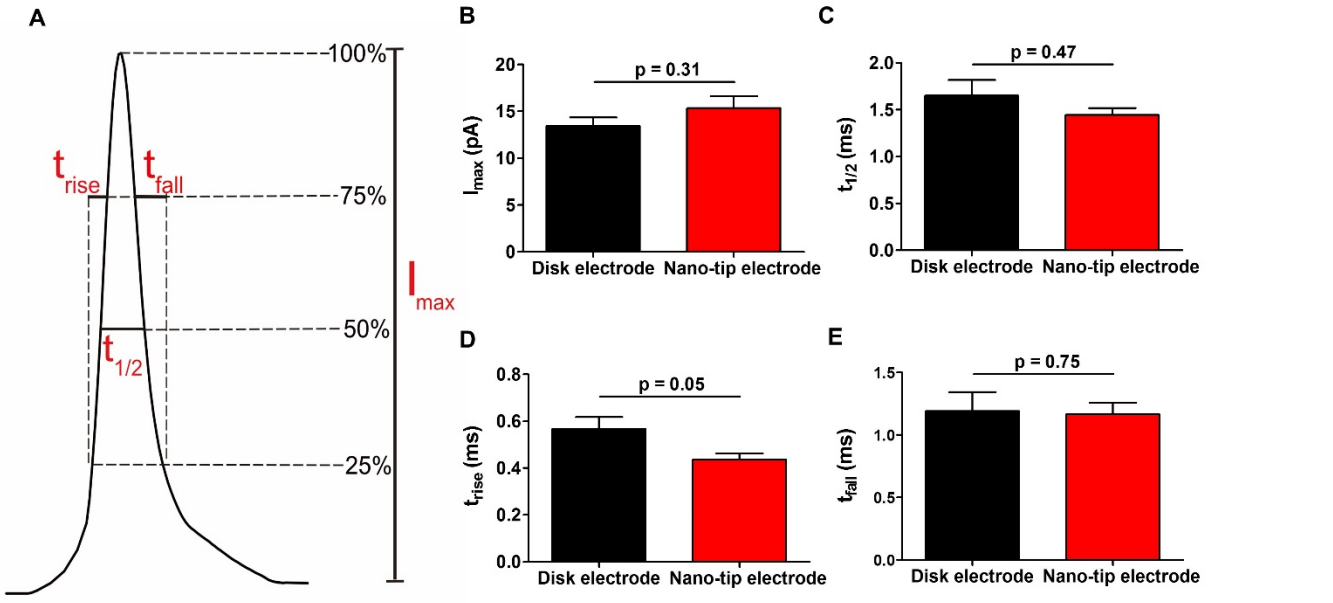

Figure 3. Different SCA peak parameters describing release dynamics to compare disk and nano-tip electrodes. (A) Scheme showing different parameters used for peak analysis. Parameters including (B) peak amplitude, (C) peak half width, (D) rise time, and (D) fall time from exocytotic events were analyzed to compare SCA measured by disk and nano-tip electrodes from PC12 cells. Data represent mean of medians ± SEM. 12 cells were used for both disk and nano-tip electrodes. Data sets were compared with a nonparametric, two-tailed Mann-Whitney test and the p values are shown above each bar.

25% of amplitude on the falling phase of peak. As shown in Figure 3B, the average peak current for exocytotic events measured by nano-tip electrodes appears higher than that measured with disk electrodes. However, the difference is not significant with a p value equal to 0.31. Nano-tip electrodes also appear to have a smaller $t_{1/2}$ for exocytotic measurements compared to disk electrodes (Figure 3C), but again the p value is only 0.47. As $t_{1/2}$ indicates the duration of an exocytotic event or the duration of the fusion pore,[16] which is formed during exocytotic process, it can be broken down into two other fusion pore related parameters, t_{rise} and t_{fall}, which are presented in Figure 3D and 3E. Here, t_{rise} is the time for the fusion pore opening or expansion and t_{fall} is related to closing of the fusion pore in combination with diffusion of neurotransmitter to the extracellular space. When measuring exocytosis with a nano-tip electrode compared to a disk electrode, the closing time, t_{fall}, does not differ between electrodes, but the fusion pore opens significantly faster.

It is quite interesting that release events at nano-tip electrodes are faster than at disk electrodes and the factors contributing to this difference can be speculated, including geometry of diffusional space induced by the shape of electrode, distance between exocytotic fusion pore and electrode (pore-electrode distance), and the size of fusion pore. To further study these factors, we used COMSOL 5.3 to apply finite element simulations to construct a corresponding model, and this is shown in Figure 4A and 4B (methods are included in supporting information), similar to our previous work.[17]

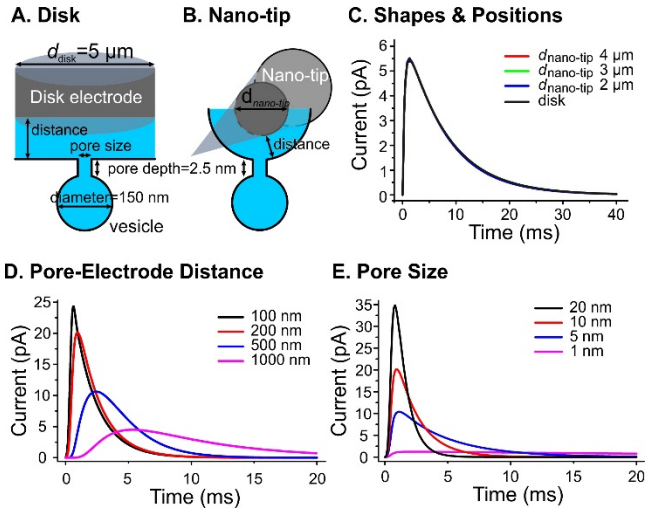

Figure 4. Schematic and parameters of diffusional space between fusion pore and (A) a disk or (B) a nano-tip electrode. d_{disk} is the diameter of the disk electrode and $d_{nano\text{-}tip}$ is the diameter of the nano-tip electrode cross-section where exocytosis occurs. Picture is not drawn according to scale. (C) Simulated spikes of exocytosis at a disk electrode and with different cross-section diameters, related to different positions, on a nano-tip electrode. (D) Simulated spikes of exocytosis with different distances between fusion pore and electrode surface, ranging from 100 to 1000 nm. (E) Simulated spikes of exocytosis with different fusion pore sizes (radius), including 1, 5, 10 and 20 nm. Details regarding simulation methods are included in the supporting information.

By setting the fusion pore size and pore-electrode distance the same for both electrodes, the simulation is only based on dif

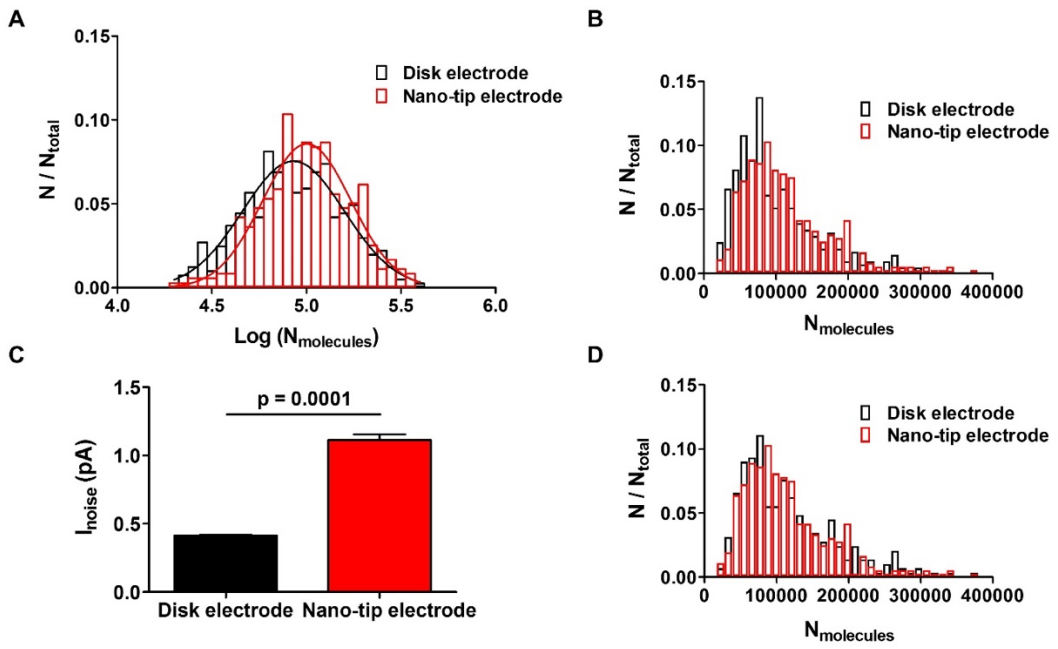

Figure 5. Distribution of exocytotic release from SCA and comparison of noise levels of electrodes. Normalized frequency histograms comparing (A) the distribution of number of molecules, (B) the distribution of log (number of molecules) and (C) the distribution of molecules with same peak selection criteria from exocytotic release measured by disk and nano-tip electrodes from PC12 cells. 12 cells were used for each electrode type. Numbers of exocytotic events analyzed for (A) and (B) were 406 and 358, and for (C) were 288 and 358 by disk and nano-tip electrodes, respectively. (D) Average standard deviation of noise from SCA amperometric traces measured by disk and nano-tip electrodes. Data are mean ± SEM. Number of cells used for both disk and nano-tip electrodes were 12. Data sets were compared with a nonparametric, two-tailed Mann-Whitney test and the p value is given above the bar.

ferent shapes of the electrode surface and the corresponding simulated spike shapes show no obvious changes, as can be seen in Figure 4C. Although the geometry of the diffusional space between exocytotic fusion pore and the electrode canvary due to the shape of electrode surface to be either a completely flat disk (disk electrode) or a somewhat arced cylindrical wall (nano-tip electrode), the fusion pore size (smaller than several tens of nanometers) is much smaller than the radius of electrode (2 to 5 μm). Hence, the diffusional space is still approximately a rectangular shape within a short period of time. When setting pore-electrode distance to be the only changing factor, it can be observed from Figure 4D that with increasing distance, the simulated spike tends to have a decreased I_{max} but increased $t_{1/2}$ and t_{fall}. Due to the smaller diameter of the tip of the nano-tip electrode, it may exert a larger physical force on the cell membrane in comparison to a disk electrode when placed on the cell. A hypothesis that fits the simulated data is that this external force compresses the protein matrix or glycocalyx outside the cell membrane holding the pore-electrode distance smaller and accelerating the vesicular neurotransmitter efflux.[18] The external force might also enlarge the size of the fusion pore by changing membrane tension, as shown in Figure 4E, which could subsequently increase the flux of transmitter, similar to the effects brought about by a smaller pore-electrode distance.[19]

To further evaluate the most likely factors causing the difference between disk and nano-tip electrodes, least-square regression analysis was applied to provide the pore-electrode distance, as well as the fusion pore size, that is best fitted to the experimental average amperometric peaks (shown in Figure 1C and 1D) obtained with these two types of electrodes. The results are shown in Figure S1A and S1B, and the approach is included in the supporting information. The estimated pore-electrode distance and fusion pore size for a disk electrode are 239 nm and 11.5 nm, while those for a nano-tip electrode are 215 nm and 18.2 nm, respectively. Therefore, we suggest that both pore-electrode distance and fusion pore size contribute to the difference between disk and nano-tip electrodes with pore size contributing relatively more (-58%) than distance (+10%). This is consistent with a previous study showing that with larger distance between the electrode and cell surface, the area of the spike remains constant due to the decreased spike height and increased spike width.[20]

Amperometric spike detection affected by the noise levels of different electrodes. The average release amounts per exocytotic event measured by both disk and nano-tip electrodes are basically the same, as mentioned above. However, by examining the distribution of the number of molecules from all exocytotic release events, it appears that the two distributions have similar range but do not completely overlap owing to the criteria for signal to noise and peak detection. The data in Figure 5A and 5B show that disk electrodes enable the detection of a higher fraction of small release events, especially for the events with less than 77000 molecules, when the criteria for peak selection are the same for both electrodes (five times the standard deviation of the noise of the electrode). We further removed all the small events with less than 77000 molecules and the distributions of these small events are shown in Figure S2, with a p value for the difference of 0.06 between disk and nano-tip electrodes. Since the criteria of peak selection is based on the background level of the amperometric trace, the different background levels observed in the amperometric traces in Figure 1A and 1B lead to the difference in the distribution of molecule

number observed. To examine this further, the background levels of each amperometric trace were averaged for all traces for both types of electrodes. The background current for the disk electrodes is significantly lower than that for nano-tip electrodes (Figure 5C), with a p value of 0.0001. Assuming unequal background level as the main reason leading to the different distributions of molecule number, by setting peak selection criteria for amperometric traces measured with disk electrodes higher than the criteria for nano-tip electrodes, the two distributions of molecule now overlap (Figure 5D). This confirms that the different distributions of the number of molecules arise from the unequal noise levels provided by the two electrodes and not a real difference in release. The lower-noise-level disk electrode favors small release events and the relatively higher-noise-level nano-tip electrode leads to rejection of these events in the selection criteria.

CONCLUSIONS

By comparing two different electrode types, conventional disk electrodes and conical shaped nano-tip electrodes, for measuring exocytotic release, we show that the amounts quantified with both electrodes are the same. However, simulation results show that the distance between the electrode surface and the release fusion pore can contribute to a faster rate of release measured with nano-tip electrodes, at least the initial stage of release. Additionally, the size of fusion pore, which might change with membrane tension when a nano-tip electrode is placed against a cell, might be important here. Taking the different noise levels of the disk and nano-tip electrodes into consideration it is clearly important to examine the peak selection criteria when comparing data between electrodes. We furthermore suggest that combining SCA with IVIEC using the same electrode to study partial exocytotic release should be done with the same electrode.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Additional experimental details; mean experimental spikes compared to respective best-fitting spikes for disk and nano-tip electrodes; normalized frequency histograms showing the molecule distribution from small exocytotic events measured with disk and nano-tip electrodes at PC12 cells (PDF)

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Author Contributions

All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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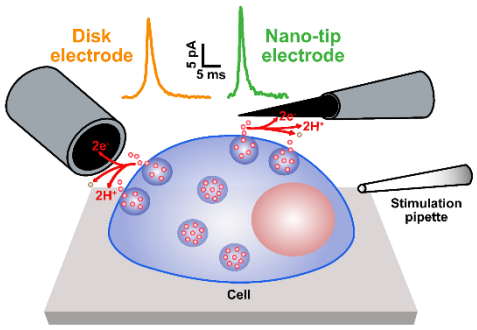

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Supplementary information:

Comparison of Disk and Nano-tip Electrodes for Measurement of Single-Cell Amperometry during Exocytotic Release

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ADDITIONAL EXPERIMENTAL DETAILS

Chemicals and solutions

Unless stated, all chemicals were of analytical grade and purchased from Sigma-Aldrich. The isotonic solution consisted of 150 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM glucose and 10 mM HEPES. The high K^+ stimulation solution was made of 55 mM NaCl, 100 mM KCl, 1.2 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM glucose and 10 mM HEPES. The pH of both solutions was adjusted to 7.4 with 3 M NaOH and the solutions were filtered before use.

Cell culture

PC12 cells were received as a gift from Lloyd Greene from Columbia University. To maintain them in culture, RPMI-1640 medium (Lonza, Fisher Scientific, Sweden) supplemented with 10 % donor equine serum and 5 % fetal bovine serum was used and the culture was kept in an incubator at 37 °C with 7 % CO_2 and 100 % humidity. For the optimal growth of the cells, collagen type IV coated cell culture flasks (Corning BioCoat, Fisher Scientific, Sweden) were used and the cells were sub-cultured every 7-9 days or when confluence was reached. Medium was replaced every 2 days for the whole culture time. For single-cell experiments, cells were sub-cultured on 60 mm collagen type IV coated cell culture dishes (Corning BioCoat, Fisher Scientific, Sweden) sparsely and experiments were performed 4-5 days afterwards.

Data processing and statistics

All the amperometric data were analyzed using an Igor Pro 6.37 routine originating from David Sulzer's group at Columbia University. A 1 kHz (binomial sm.) filter was applied to all amperometric traces. The threshold criteria for exocytotic peak selection was normally five times the standard deviation of the noise of the electrode, but was changed for quantitative comparison between electrodes. All traces were examined manually and carefully after peak selection to avoid false positives. For calculating numbers of molecules and different peak parameters, the mean of medians from single cells was used to reduce possible impact from extreme values. Datasets were compared with a nonparametric, two-tailed Mann-Whitney rank-sum test.

Finite element simulation

The models of vesicles releasing neurotransmitters to the electrode surface were built and calculated by finite element analysis software COMSOL Multiphysics® (version 5.3). The models were built in the 3D domain as shown in Figure 4.

Since convection was neglected, the concentration distribution of dopamine was only controlled by diffusion. Meanwhile, the electrode potential was kept at 700 mV which was higher than the oxidation peak potential of dopamine on carbon fiber, so the surface concentration of dopamine was nearly 0. The flux density of dopamine reaching the electrode area can be calculated by Fick's law which was built in

COMSOL. The total current was obtained by surface integrating the flux density of dopamine across electrode surface and solving Faraday's equation to get the following,

$$I(t) = \oiint_{0}^{S_{\text{electrode}}} z_{\text{e}} \text{F} J(t)\, \text{d}S$$

where $J(t)$ is the flux density of dopamine across the electrode surface, z_{e} is the charge transfer number of the electrochemical reaction which is 2, F is Faraday's constant.

Due to the highly similar simulated spikes for exocytosis that occur at disk and nano-tip electrodes, even in different positions of nano-tip electrodes (Figure 4C), the "disk" model could be used to represent two different electrodes. The input parameters are listed in Table S1.

Table S1. Model input parameters

Parameters	Description	Value	Unit
D	diffusion coefficient of dopamine	6×10^{-11}	$m^2 \cdot s^{-1}$
c_{dopamine}	initial concentration of dopamine inside a vesicle	150	mmol/L
z_{e}	number of electrons transferred in the electrochemical reaction	2	
d_{ves}	the diameter of vesicle	150	nm
fusion pore depth	depth of the fusion pore	2.5	nm
d_{disk}	diameter of disk electrode	5	μm
distance	distance between fusion pore and electrode surface (pore-electrode distance)	200; 100, 200, 500, 1000[a]	nm
R_{pore}	radius of fusion pore	3; 1, 5, 10, 20[b]	nm
$d_{\text{nano-tip}}$	diameter of cross-section of nano-tip electrode where exocytosis occurs	2, 3, 4[c]	μm
$h_{\text{nano-tip}}$	height of nano-tip electrode	20	μm
d_{base}	base diameter of nano-tip electrode	5	μm

a: the parameter set in the distance-dependent spike simulation.

b: the parameter set in the fusion pore size-dependent spike simulation.

c: the parameter set in spike simulations of exocytosis occurred on different positions on nano-tip electrodes.

Estimation of pore-electrode distance and fusion pore size

A parameter combination based on the least-squares method was utilized to inversely estimate the pore-electrode distance and fusion pore size related to respective average spike of disk and nano-tip electrodes (shown in Figure 1C and 1D). The simulation was based on the disk electrode model, where a combination of a set of pore-electrode distances ($10^{0 \text{ to } 2.72}$, step size is 0.04 ×5 nm) and fusion pore sizes ($10^{0 \text{ to} 1.8}$, step size is 0.02 ×0.5 nm) were used as the initial conditions. The other parameters were consistent with Table S1. After comparing each parameter combination to the average spike shape, the goodness of fitting (R^2,

higher means better fitting) was used to find the best-fitting parameter combination to each experimental average spike,

$$R^2 \stackrel{\text{def}}{=} 1 - \frac{\sum(\varphi_{\text{exp}} - \overline{\varphi_{\text{exp}}})^2}{\sum(\varphi_{\text{exp}} - \varphi_{\text{sim}})^2}$$

where φ_{exp} and φ_{sim} are normalized experimental spike and simulated spike without variance of vesicular content.

The parameter combination with highest R^2 is considered to give the most possible parameters reflecting the real conditions of the experimental data. To further improve the precision of the estimated parameter combination, the same program was applied again with more specific parameters (50 % to 200 % of best-fitting pore-electrode distance and fusion pore size, step size was 10 %) around the 1st order best-fitting parameter combination. The 2nd order best-fitting parameter combination was considered to be the final estimated result. The R^2 values of the best-fitting parameter combinations were 0.988 and 0.975 for disk and nano-tip electrodes, respectively. Considering background noise and the pre-spike foot on rising phase of the spike, the R^2 was quite high and shows the reliability of the estimation. The whole estimation was done with a home-built MATLAB program and COMSOL 5.3.

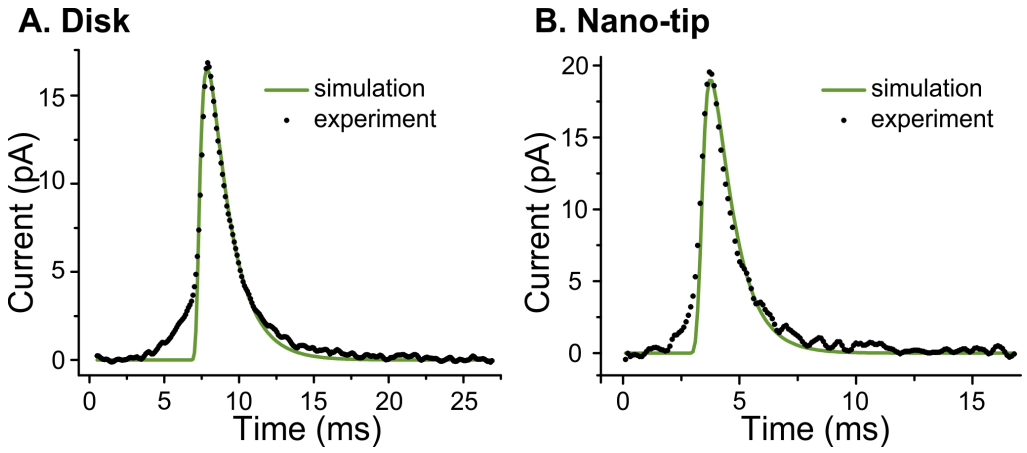

Figure S1. Mean experimental spikes compared to respective best-fitting simulated spikes for (A) disk and (B) nano-tip electrodes.

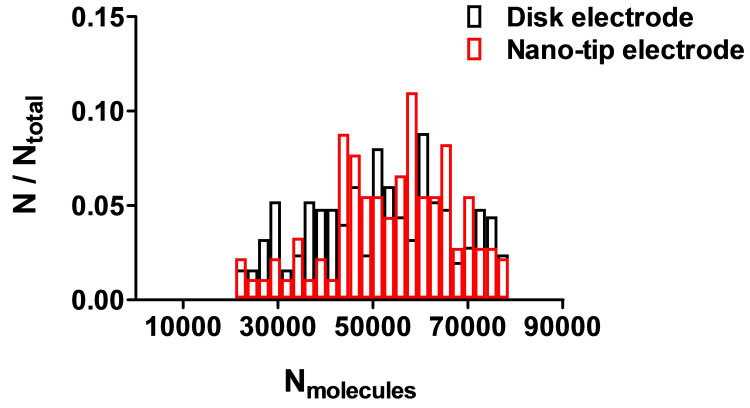

Figure S2. Normalized frequency histograms showing the molecule distribution from small exocytotic events measured with disk and nano-tip electrodes at PC12 cells. Small release events refer to the events with less than 77000 molecules being released. 12 cells were used for both electrode types and exocytotic events analyzed were 70 and 76, respectively, for disk and nano-tip electrodes.