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Electrochemical Quantification of Transmitter Concentration in Single Nanoscale Vesicles Isolated from PC12 Cells

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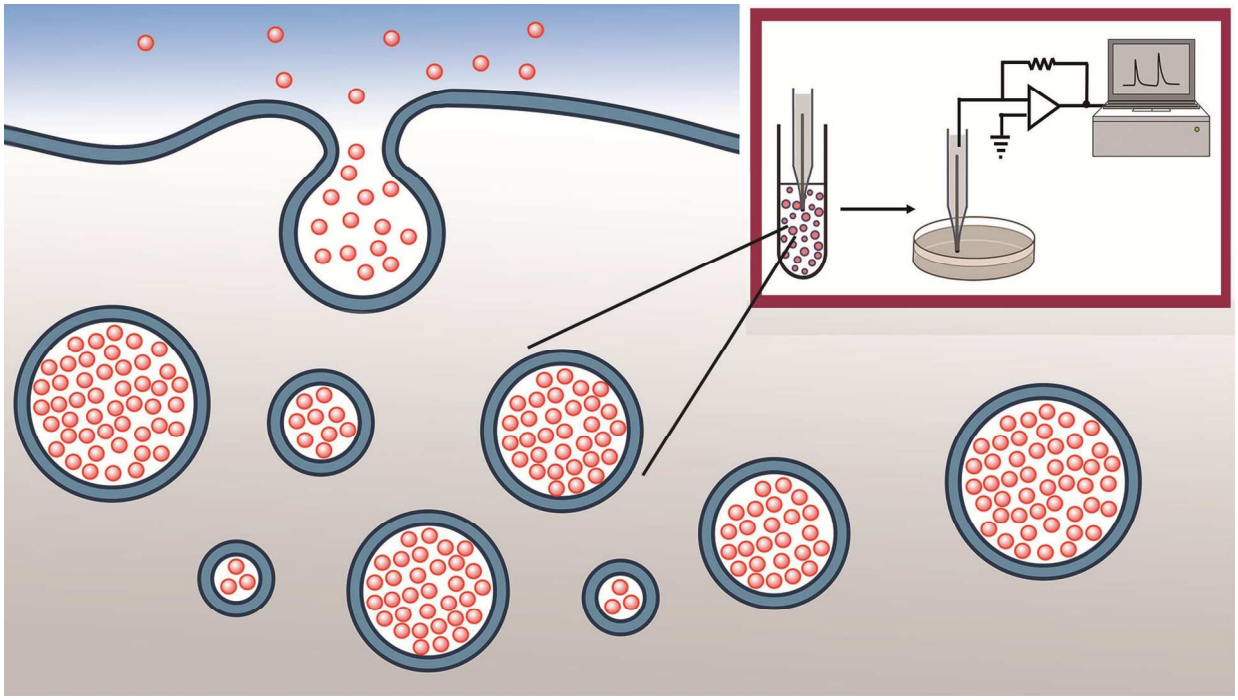

ABSTRACT

We use an electrochemical platform, nanoparticle tracking analysis, and differential centrifugation of single catecholamine vesicles to study the properties of nanometer transmitter vesicles, including the number of molecules, size, and catecholamine concentration inside. Vesicle impact electrochemical cytometry (VIEC) was used to quantify the catecholamine content of single vesicles in different batches isolated from pheochromocytoma (PC12) cells with different ultracentrifugation speeds. We show that, vesicles containing less catecholamine are obtained at subsequent centrifugation steps with higher speed (force). Important to quantification, the cumulative content after subsequent centrifugation steps is equivalent to that of one-step centrifugation at the highest speed, 70 000 g. Moreover, as we count molecules in the vesicles, we compared molecular numbers from VIEC, flow VIEC, and intracellular VIEC to corresponding vesicle size measured by nanoparticle tracking analysis to evaluate catecholamine concentration in vesicles. The data suggest that vesicular catecholamine concentration is relatively constant and independent of the vesicular size, indicating vesicular transmitter content as a main factor regulating the vesicle size.

Introduction

Higher mental brain functions, including perception, motivation, emotion, learning, and memory, are operated by neuronal connections in the brain. Neurons communicate with chemical signals through the fundamental process called exocytosis in the synapse. During exocytosis, nanometer vesicles filled with neurotransmitter molecules fuse with the presynaptic membrane with the assistance of SNARE proteins and release transmitters into the extracellular space through a fusion pore.[1, 2] These transmitters diffuse across a nanometer synaptic gap and bind to specific receptors on the postsynaptic membrane where depolarization or hyperpolarization occurs and an electrical signal that has triggered exocytosis can be transmitted further or inhibited, respectively. Hence, the synaptic vesicle is the essential cell organelle that stores and releases neurotransmitter molecules in the brain. Owing to its critical involvement in neuron communication, the dynamic process of the synaptic vesicle during exocytosis and its properties have drawn a lot of attention to the understanding of the molecular mechanisms that control the process of chemical communication between neurons.

Single cell amperometry and fast-scan cyclic voltammetry (FSCV) have proven to be powerful techniques to study the release of catecholamine from single vesicles in neurons and endocrine cells.[3, 4] Amperometric results at neuronal cell models and midbrain neurons have consistently shown that L-3,4-dihydroxyphenylalanine (L-DOPA) increases the quantal size and consequently the amount of catecholamine released[5-8], whereas use of the FSCV method revealed that the relative concentration of catecholamine released from single PC12 cell vesicles is unchanged after exposure to L-DOPA.[9] In the later reports, comparison between the number of catecholamine released from single vesicle in L-DOPA and reserpine treated cells quantified with single cell amperometry and corresponding vesicle volume quantified with transmission electron

microscopy suggested that vesicles regulate a constant concentration of neurotransmitter internally.[10, 11] This was concluded by assuming the amount released during exocytosis is the full content stored in vesicles initially based upon the "all-or-none" exocytosis that was widely accepted at that date.[2, 12] However, recent studies have shown that the process of exocytosis most likely includes three or even more potential modes of release, namely the "kiss-and-run", "open and closed" or "partial", and "full release".[13-18] The most significant and general release process, being open and closed exocytosis with up to 65% of the vesicular transmitter contents released, partial exocytosis, makes the closing of the vesicle an important part for regulating individual events and synaptic strength.[19] Moreover, different physiological conditions and chemical treatments can regulate the fraction of vesicular catecholamine released.[6, 20-26] As a consequence, the concentration of transmitter in single vesicles and its related questions need to be re-considered.

Owing to the nanometer-sized structure of vesicles, direct measurement of the transmitter content in single vesicles has been a challenge requiring development of new analytical techniques. Recently, vesicle impact electrochemical cytometry (VIEC) was developed for quantification of the catecholamine content in single mammalian vesicles.[27-30] In this process, isolated single vesicles are adsorbed at the electrode surface and, subsequently, the vesicles rupture stochastically in a potential dependent manner, suggesting electroporation as a major mechanism of rupturing.[30, 31] The electric field across the vesicle membrane induces the formation of a pore in the vesicular membrane causing the expulsion of the vesicular content towards the electrode surface. The electroactive content of the vesicles is quantified by electrochemical oxidation at the electrode surface.[31, 32] By placing a nanotip electrode into

cytoplasm, we created a new method, intracellular VIEC or IVIEC, to measure vesicular catecholamine content inside single living cells.[19, 33-36]

In this paper, we used VIEC to quantify the catecholamine content in single vesicles isolated from PC12 cells, a widely used cell model for neuronal communication. Differential centrifugation was used to collect different batches of vesicles with different sizes that were measured with nanoparticle tracking analysis (NTA) in solution without any labeling. The comparison of the vesicular content and the size of the vesicles reveals that vesicles containing more catecholamine represents the larger size and the concentration of catecholamine is kept relatively constant for all vesicle fractions collected by differential centrifugation.

Experimental section

Ultramicroelectrode (UME) Fabrication

UMEs were fabricated as previously described.[33, 37] Briefly, 33-μm diameter carbon fibers were aspirated into borosilicate capillaries (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 P-1000, Sutter Instruments Co., Novato, CA, U.S.A.). The electrode was sealed by dipping the pulled tip in a solution of epoxy (Epoxy Technology, Billerica, MA, U.S.A). The glued electrodes were cured in an oven at 100°C overnight and subsequently cut at the glass junction and beveled at 45° angle (EG-400, Narishige Inc., London, U.K.). Electrode responses were tested by performing cyclic voltammetry (-0.2 to 0.8 V vs. Ag/AgCl, 100 mV/s) in solution of 100 μM dopamine in PBS (pH 7.4). Only electrodes showing good reaction kinetics and stable steady-state currents in agreement with theoretically calculated values were used for experiments.

Cell Culture and Vesicle Isolation

PC12 cells were a generous donation by Lloyd Greene (Columbia University). The cells were grown on mouse collagen coated cell culture flasks (collagen type IV, Corning) , and maintained in phenol red-free RPMI-1640 media (Lonza) supplemented with 10% donor equine serum (Sigma) and 5% fetal bovine serum Gold (Sigma) in a 7% CO_2, 100% humidity atmosphere at 37 °C. The medium was replaced every 2-3 days throughout the lifetime of all cultures. The cells were sub-cultured every 7−9 days.

Confluent PC12 cells from five 75 cm^2 flasks (collagen type IV, Corning) were treated with TrypLE^{TM} Express (Gibco) and subsequently detached from surface by tapping on surface gently. The cell suspensions were combined into one fraction and then centrifuged at 300 g for 5 min to pellet the cells. The supernatant containing growth media was then removed and discarded. The cells were suspended with homogenization buffer (381 mOsm/kg, contains 0.3 M sucrose, 1 mM EDTA, 1 mM $MgSO_4$, 10 mM HEPES, 10 mM KCl and cOmplete Protease Inhibitor) and homogenized in a glass homogenizer (WHEATON) at 4 °C until the cell membrane was broken as observed under a microscope. The procedure for isolation of vesicles with subsequent centrifugations is shown in Fig. 1a, unless stated otherwise. First, the homogenate was centrifuged at 1000 g for 10 min to remove cell debris. The supernatant was subsequently centrifuged at 3000g for 30 min. The pellet was kept for measurement and the supernatant was used for the next centrifugation at 6000 g for 30 min. Similarly, the pellet was kept for measurement and the supernatant was used for next trial run at 10000 g for 30 min. Two more runs of centrifugation were operated at 70000 g and 100000 g for 45 min each. All the pellets were re-suspended separately in homogenizing buffer and used as vesicle stock solution. The centrifugation was run at 4°C to minimize the degradation of vesicles by protease.

Nanoparticle Tracking Analysis

Vesicle diameter and concentration were measured at 37°C with a NanoSight LM10 (Malvern Instruments Ltd, UK) equipped with a 488 nm laser and Hamamatsu C11440-50B/A11893-02 camera. The software used for capturing and analyzing the data was NTA 3.1. Samples were diluted in homogenizing buffer with NaCl (315 mOsm/kg) to obtain 30–60 particles per frame. For each sample, 5 runs for 90 s were performed.

Vesicle Impact Electrochemical Cytometry (VIEC)

Disk UMEs were placed in a concentrated PC12 vesicle stock solution for 10 min at 4°C to allow adsorption of vesicles. This was followed by recording signal in homogenizing buffer for 10 min at 37°C. The electrodes were re-beveled and re-loaded with vesicles for each experimental run. The electrochemical recording of catecholamine content in individual vesicles was performed by applying a constant potential of +700 mV (*vs.* Ag/AgCl) to the working electrode using a potentiostat (Axopatch 200B, Molecular Devices, Sunnyvale, CA, U.S.A.). The recorded signal was filtered at 2 kHz using an analog 4-pole Bessel filter and digitized at 10 kHz using a Digidata model 1440A interfaced by the Axoscope 10.3 software (Axon Instruments Inc., Sunnyvale, CA, U.S.A.).

Data Processing

The amperometric traces were processed using an IgorPro 6.22 routine originating from the Sulzer group.[38] The traces obtained were digitally filtered at 1 kHz using binomial smoothing. The threshold for peak detection was five times the standard deviation of the noise. The traces were carefully inspected after peak detection and false positives were manually rejected. Only traces with more than 20 peaks were used in the analysis in order to minimize the variance of the

means.

Scanning Electron Microscopy

Characterization of the vesicles on disk UMEs was performed on a scanning electron microscope (Leo Ultra 55). Electrodes were loaded with PC12 vesicles as described above, quickly rinsed twice with PBS, subsequently dipped in 4 % formaldehyde for 15 min to fix the vesicles and dried at room temperature. The electrodes were mounted onto standard aluminum specimen stages with the help of conductive carbon adhesive tabs, and then a copper wire was threaded into the glass capillaries for electrical contact and was grounded to the scanning electron microscopy stub using conductive carbon adhesive tabs.

Results and discussion

1. Vesicular Catecholamine Content *versus* Centrifugation Force.

VIEC was first introduced to characterize the catecholamine content in single vesicles isolated from adrenal glands.[27] During this process, single vesicles adsorb on the electrode surface, and rupture stochastically on the electrode with a pore formed by electroporation. Through this pore, vesicular catecholamines are expelled to the electrode and detected electrochemically.[19, 32] The existence of proteins on the vesicle membrane acts as a barrier for vesicle opening in VIEC through decreasing the probability of electroporation.[31]

In this study, VIEC has been applied to quantify the vesicular catecholamine content in single vesicles isolated from PC12 cells. Samples obtained by differential centrifugation were carefully analyzed by VIEC to check the existence of vesicles. Fig. 1a illustrates the procedure for obtaining different batches of vesicle samples and the schematic VIEC experimental setup used

in this study. Different batches of vesicles were first collected from homogenized PC12 cells by continuous differential centrifugation, and subsequently adsorbed on an UME by dipping the UME into a concentrated vesicle suspension for 10 min. Finally, the UME was moved to a bulk buffer without vesicles and the current was measured continuously for at least 10 min at + 0.7 V (*vs.* Ag/AgCl).

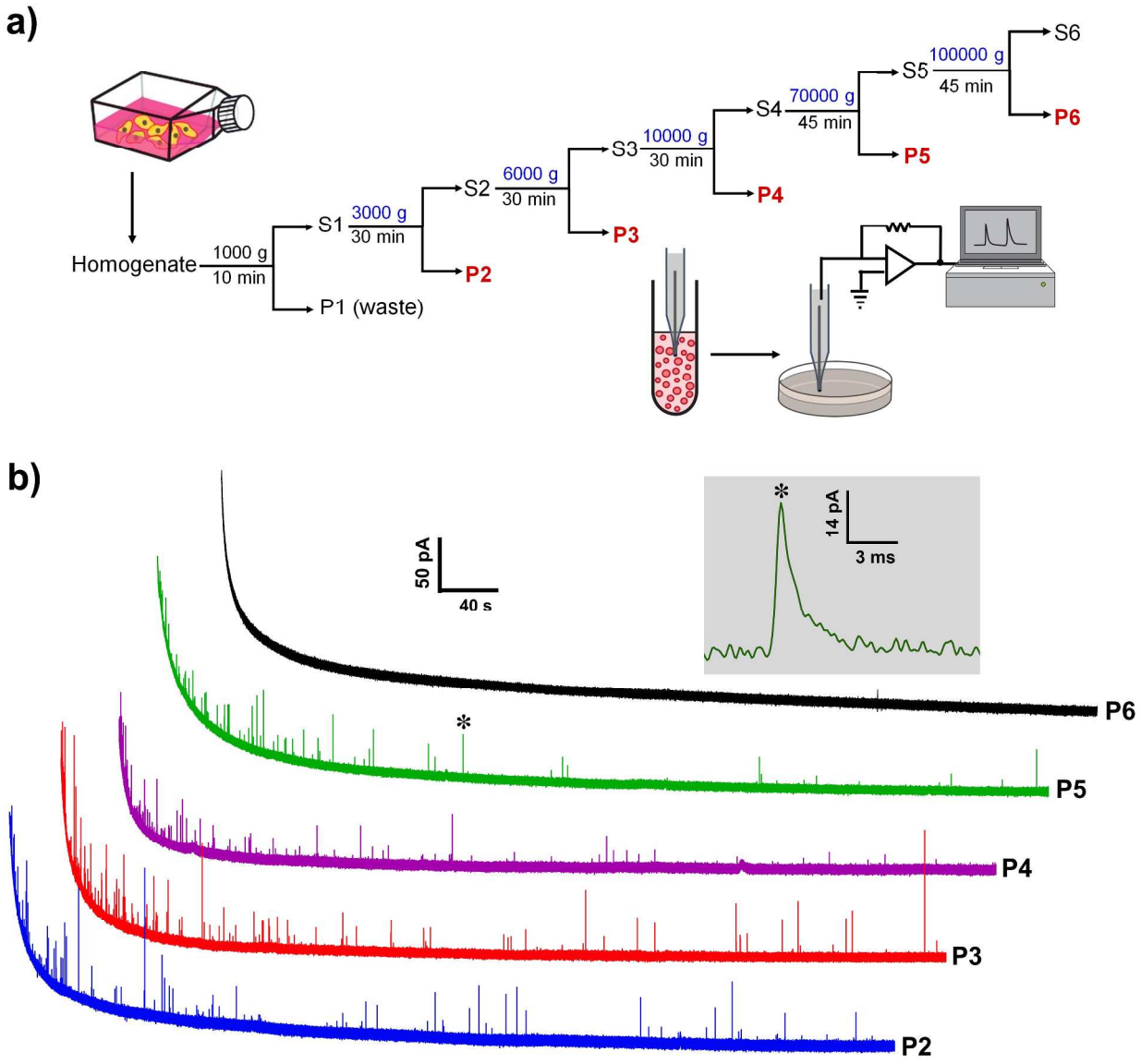

Fig. 1. Schematic illustrating the procedure for isolating fractional vesicles from PC12 cells with differential centrifugation and the experimental process of vesicle impact electrochemical cytometry (VIEC) **(a)**, and corresponding typical amperometric traces of fractional PC12 vesicles obtained in VIEC

(b). Inset (grey), an enlarged view of a typical amperometric spike marked with an asterisk in the amperometric trace for P5.

To exclude most of the nuclei, mitochondria, and other larger organelles existing in the cell homogenization suspension, centrifugation was carried out at 1000g for 10 min and the pellet was discarded. Then the supernatant was used for subsequent centrifugation runs (3000g, 6000g, 10000g, 70000g, and 100000g sequentially on the same sample), as shown in Fig. 1a. Pellets obtained in each centrifugation were re-suspended with homogenization buffer and run for VIEC. Typical amperometric traces for each vesicle fraction are displayed in Fig. 1b. For samples collected with centrifugation at 3000g, 6000g, 10000g and 70000g, significant numbers of amperometric transients (105±9, 145±19, 125±18, and 82±13, correspondingly, mean±SEM) were recorded in VIEC (Fig. 3a). However, amperometric transients were rarely seen during recordings for the sample obtained at 100000g, which suggests that very few vesicles are present in this fraction and that the majority of all vesicles have been collected in the previous steps of centrifugation.

To confirm that PC12 vesicles were adsorbed on the carbon disk UME, scanning electron microscopy (SEM) was used to image the vesicles on the electrodes. At UMEs exposed to homogenizing buffer, only a few bright dots with diameter less than 100 nm appeared. These most likely were residual salt crystals formed in chemical fixation process (Fig. 2a). However, when UMEs were placed in a concentrated PC12 vesicle suspension for 10 min, large numbers of vesicles with diameter 100-300 nm were adsorbed on the UME even after rinsing two times with PBS buffer to exclude the weak interaction (Fig. 2b). Moreover, as shown in SEM, vesicles adsorbed on the disk carbon UME spread individually, i.e. without aggregation. This is important for vesicle opening one at a time and quantification of content in single vesicles with VIEC. It is

worth noting that the shapes of vesicles sitting on the UME are irregular, which may be due to the rough surface of the UME. This clearly shows that the vesicles interacts and adsorbs to the carbon electrode surface individually leading to the high probability of rupture one at a time in amperometric recording.

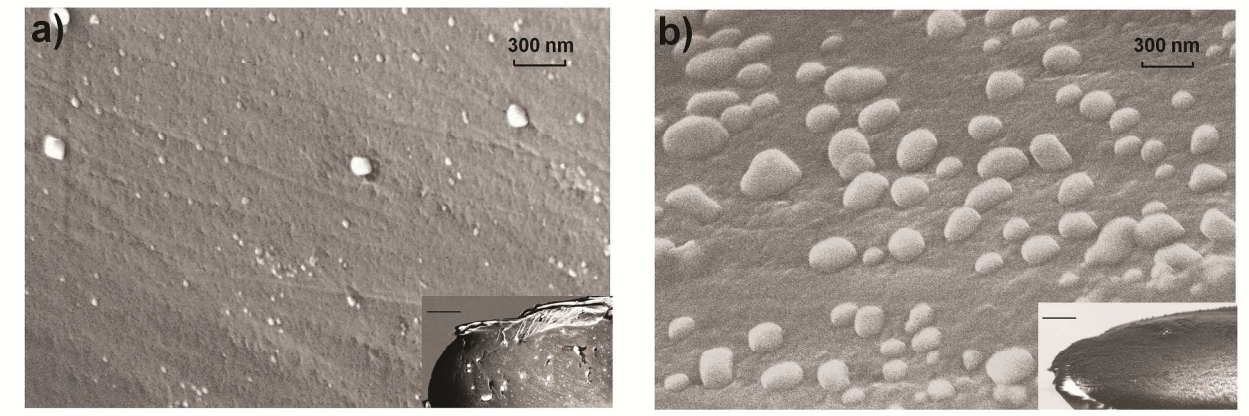

Fig. 2. SEM images of a carbon UME without (a) or with (b) isolated PC12 vesicles after fixation with 4% formaldehyde for 15 min. Both electrodes went through the chemical fixation process. Insets: corresponding images in a big view, scale bar, 5 μm. The bright dots in (a) are probably residual salt crystals formed during chemical fixation process.

We compared the sum of the four-step sequential centrifugation (Fig. 1a) to a one-step centrifugation (70000g for 45 min directly after 1000g for 10min) to evaluate if leakage occurs in relatively long-term subsequent differential centrifugation steps. As shown in Fig. 3b, the distributions of the number of vesicular catecholamine content ($N_{molecules}$) match quite well between the two different schemes of centrifugation. This indicates that no significant leakage occurs over the 2.3 hours of the four-step sequential centrifugation and this scheme captures all the vesicles in the PC12 cell homogenate.

The distribution of $N_{molecules}$ strongly deviates from normality but can be fitted well with a lognormal distribution. Fig. 3c represents the distributions of the base 10 logarithm of vesicular $N_{molecules}$ for different batches of vesicles isolated with differential centrifugation. As the

centrifugation force is increased, the distribution moves to smaller values of $N_{molecules}$. The average catecholamine content in vesicles is compared in Fig. 3d and Table 1. More apparently, the average $N_{molecules}$ decreased gradually and significantly from low to high centrifugation force, indicating vesicles containing more catecholamine are spun down much easier compared to those containing less catecholamine.

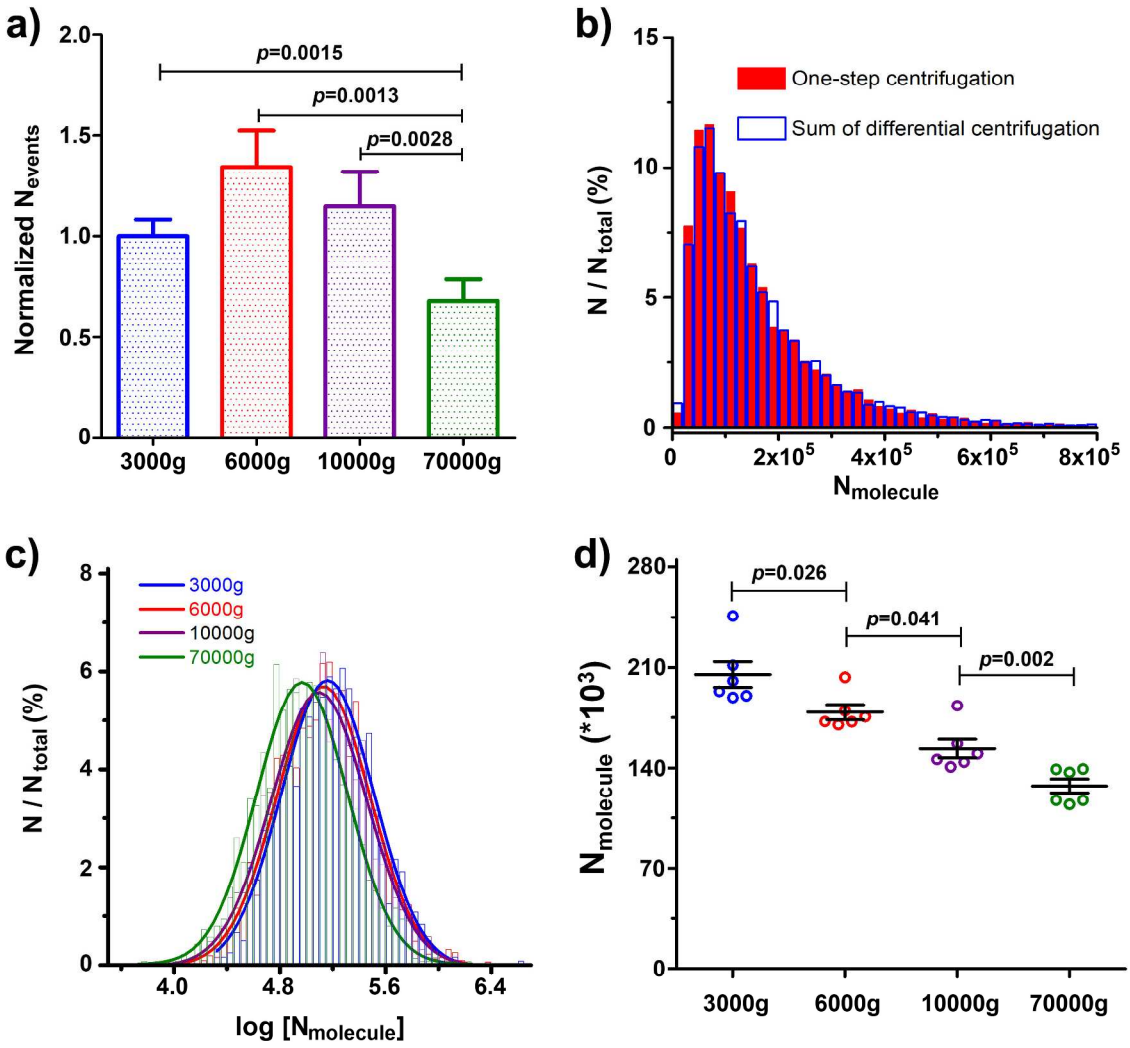

Fig. 3. (**a**) The average number of the amperometric events recorded in 10 min with VIEC for isolated fractional vesicles obtained with differential centrifugation. (**b**) Comparison of normalized frequency histograms describing of $N_{molecules}$ quantified for PC12 vesicles isolated with continuous differential centrifugation (summarizing VIEC events of vesicle samples obtained at 3000g, 6000g, 10000g and 700000g, blue columns) and one-step centrifugation (70000g for 45min directly after 1000g for 10 min,

red columns). (**c)** Normalized frequency histograms describing of log [$N_{molecules}$] quantified for PC12 vesicles isolated with differential centrifugation. Fits were obtained from Gaussian distributions of the data. (**d)** Average number of catecholamine molecule quantified in individual isolated vesicles obtained with differential centrifugation. N = 6 isolations, 4 amperometric traces for each isolation. The pairs of data sets were compared using a two-tailed Wilcoxon–Mann–Whitney rank-sum test, and *p*-values are indicated in the Fig. 3a and 3d.

Table 1. Vesicular catecholamine content ($N_{molecules}$) quantified with VIEC (N = 6), vesicle diameter (D_{ves}) measured with NTA independently (N = 5) and corresponding vesicular catecholamine concentration ($C_{molecules}$) calculated with equation, $C_{molecules} = \frac{N_{molecules}}{N_A \cdot \frac{4}{3}\pi(\frac{D_{ves}}{2})^3}$, where $N_A = 6.02\times10^{23}$ mol^{-1}).

	3000g	6000g	10000g	70000g	Full fraction
$N_{molecules}$	205100 ± 8800[a]	178700 ± 4100[a]	153400 ± 4400[a]	127400 ± 4100[a]	159000±3800[a, b]
D_{ves}/ nm	215 ± 3.2[a]	211 ± 2.7[a]	193 ± 0.5[a]	156 ± 2.5[a]	197 ± 1.3[a, b]
$C_{molecules}$ /mM	62	61	68	106	66

[a] The data are presented as mean ± SEM.

[b] Vesicles obtained with one-step centrifugation at 70000g for 45 min directly after 1000g for 10 min.

2. Characterization of the Size of Isolated Vesicles with Nanoparticle Tracking Analysis.

To measure the size of individual secretory vesicles obtained in differential centrifugation, independent measurements of vesicle size were carried out by NTA. NTA is used to visualize and track the Brownian motion of nanoparticles in solution, which is inversely proportional to the hydrodynamic radius of the particles, as described by the Stokes Einstein equation. Compared with TEM, which necessitates fixing cells and staining the vesicles chemically, NTA analysis allows the measurement of vesicle size in buffer at physiological temperature without any chemical modification. This helps to keep their initial size at physiological conditions. Based upon NTA measurements, vesicle size is roughly a Gaussian distribution indicating that the vesicles obtained with the procedures here exhibit a relatively high purity. The average diameter of isolated vesicles is in the range of 150-220 nm, and this is consistent with results reported

before,[15, 39] suggesting that individual secretory vesicles are obtained from the differential centrifugation process. As shown in Table 1 and Fig. S1, the average diameter of the vesicles is essentially identical when the centrifugation force is increased from 3000g to 6000g, whereas this value decreases gradually with statistical significance when the centrifugation force increases to 10000g and 70000g.

3. Analysis of Vesicular Transmitter Concentration

By measuring the number of catecholamine molecules in each vesicle with VIEC and the vesicle size with NTA, the vesicular catecholamine concentration can be calculated. As shown in Table 1, for larger vesicles obtained at 3000g, 6000g and 10000g centrifugation speeds, the catecholamine concentration in the vesicles is constant, in the range of 60-70 mM. However, the catecholamine concentration in vesicles is 106 mM for the smaller vesicles obtained at 70000g centrifugation speed. NTA measurements show that ~ 3 times more vesicles exist in the 70000g fraction compared to the 10000g fraction (Fig. S2). However, the ratio of the number of amperometric spikes obtained by VIEC between 10000g and 70000g is 7:10 (Fig. 3a). Assuming vesicles in different fractions have roughly the same probability to rupture at the electrode surface during VIEC recordings, this result suggests 77% of the vesicles existing in the fraction of 70000g are not detectable by VIEC. This might result from non-catecholamine vesicles or other small objects in the cell that do not contain catecholamine and that are separated at this high centrifugation speed. This could bias the vesicular catecholamine concentration calculated with molecule number detected with VIEC and vesicle size with NTA. We obtained a whole fraction of vesicles from PC12 cells by running the cell homogenate only at 70000g for 45 min directly after discarding the cell debris pelleted at 1000g for 10min, and the value of D_{ves} was 197 nm for this sample. With the vesicular content measured in VIEC, the vesicular catecholamine

concentration was calculated as 66.0 mM, which is close to that obtained at 3000g, 6000g and 10000g (Table 1). This suggests that the vesicles maintain their catecholamine concentration inside regardless of size. This is consistent with previous reports comparing the exocytotic release amount from control cells and drug-treated cells, and comparing vesicular catecholamine content of control cells and hypertonic stressed cells.[10, 11, 35] Here, with direct measurement of vesicular transmitter content and comparison of vesicular catecholamine concentration for vesicles isolated from untreated cells, the results can reflect the native properties of vesicles more effectively.

Combining our data here with that in the literature where both vesicle content and size were measured, it is possible to compare the vesicular catecholamine content in PC12 cells quantified with electrochemical cytometry, including flow electrochemical cytometry,[15] and intracellular VIEC[19, 34] reported before with the content reported by VIEC in this study (Table 2). Using the vesicle diameter measured in this study, D_{ves} = 197nm, we calculate the vesicular catecholamine concentration for each approach. Although the number of vesicular catecholamine molecule varies in these reports, the concentration of catecholamine in vesicles is in the range from 60 to 100 mM. It is worth noting that this concentration is slightly lower than that reported previously, and appears to result from the larger vesicle size measured by NTA in isotonic solution in this study compared with TEM values reported before.[15]

Table 2. Vesicular catecholamine content quantified with electrochemical cytometry and vesicular catecholamine concentration calculated.[a]

	Flow cytometry[15]	**IVIEC[19]**	**IVIEC[34]**	**VIEC**
$\mathbf{N_{molecules}}$	220000± 1100	188000± 28100	201000±20400	159000±3800

$c_{molecules}$ **(mM)**	91.3	78.0	83.4	66.0

[a] To compare fairly, the mean value of $N_{molecules}$ from all reports was used. D_{ves} was set as 197 nm for calculation of $c_{molecules}$.

Summary and conclusions

We describe the application of vesicle impact electrochemical cytometry for quantification of transmitter content in single nanoscale vesicles isolated from PC12 cells. We have used differential centrifugation to collect vesicles into batches for which the distribution of the vesicle size is slightly changed. By comparing the vesicle size and vesicular catecholamine content in each batch, we conclude that catecholamine concentration in vesicles with different sizes is relatively constant. These results suggest that transmitter content is a major factor in regulating vesicle size and maintaining vesicle homeostasis. This study builds upon an electrochemical platform capable of determining the properties of single electroactive neurotransmitter vesicles including storage, leakage, growth, etc.

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Notes

The authors declare no competing financial interest.

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References

1. J. R. Monck and J. M. Fernandez, *J. Cell Biol.*, 1992, **119**, 1395-1404.
2. A. Albillos, G. Dernick, H. Horstmann, W. Almers, G. A. de Toledo and M. Lindau, *Nature*, 1997, **389**, 509-512.
3. K. T. Kawagoe, J. A. Jankowski and R. M. Wightman, *Anal. Chem.*, 1991, **63**, 1589-1594.
4. R. M. Wightman, J. A. Jankowski, R. T. Kennedy, K. T. Kawagoe, T. J. Schroeder, D. J. Leszczyszyn, J. A. Near, E. J. Diliberto and O. H. Viveros, *Proc. Natl. Acad. Sci. USA*, 1991, **88**, 10754-10758.
5. J. Wang, R. Trouillon, Y. Lin, M. I. Svensson and A. G. Ewing, *Anal. Chem.*, 2013, **85**, 5600-5608.
6. L. A. Sombers, M. M. Maxson and A. G. Ewing, *J. Neurochem.*, 2005, **93**, 1122-1131.
7. E. Pothos, M. Desmond and D. Sulzer, *J. Neurochem.*, 1996, **66**, 629-636.
8. E. N. Pothos, V. Davila and D. Sulzer, *J. Neurosci.*, 1998, **18**, 4106-4118.
9. K. D. Kozminski, D. A. Gutman, V. Davila, D. Sulzer and A. G. Ewing, *Anal. Chem.*, 1998, **70**, 3123-3130.
10. T. L. Colliver, S. J. Pyott, M. Achalabun and A. G. Ewing, *J. Neurosci.*, 2000, **20**, 5276-5282.
11. L.-W. Gong, I. Hafez, G. Alvarez de Toledo and M. Lindau, *J. Neurosci.*, 2003, **23**, 7917-7921.
12. E. V. Mosharov, L. W. Gong, B. Khanna, D. Sulzer and M. Lindau, *J. Neurosci.*, 2003, **23**, 5835-5845.
13. L. Ren, Mellander, L.J., Keighron, J., Cans, A-S., Kurczy M. E., Svir, I., Oleinick, A., Amatore, C., Ewing, A. G., *Q. Rev. Biophys.*, 2016, **49**, e12.
14. X. Li, J. Dunevall and A. G. Ewing, *Acc. Chem. Res.*, 2016, **49**, 2347-2354.
15. D. M. Omiatek, Y. Dong, M. L. Heien and A. G. Ewing, *ACS. Chem. Neurosci.*, 2010, **1**, 234-245.
16. C. Amatore, A. I. Oleinick and I. Svir, *Chemphyschem*, 2010, **11**, 159-174.
17. R. G. W. Staal, E. V. Mosharov and D. Sulzer, *Nat. Neurosci.*, 2004, **7**, 341-346.
18. E. Ales, L. Tabares, J. M. Poyato, V. Valero, M. Lindau and T. Alvarez de, *Nat. cell Biol.*, 1999, **1**, 40-44.
19. X. Li, S. Majdi, J. Dunevall, H. Fathali and A. G. Ewing, *Angew. Chem. Int. Ed.*, 2015, **54**, 11978-11982.
20. R. Borges, E. R. Travis, S. E. Hochstetler and R. M. Wightman, *J. Biol. Chem.*, 1997, **272**, 8325-8331.
21. M. Camacho, J. D. Machado, M. S. Montesinos, M. Criado and R. Borges, *J. Neurochem.*, 2006, **96**, 324-334.
22. C. L. Haynes, L. N. Siff and R. M. Wightman, *BBA-Mol. Cell Res.*, 2007, **1773**, 728-735.
23. J. D. Machado, A. Morales, J. F. Gomez and R. Borges, *Mol. Pharmacol.*, 2001, **60**, 514-520.
24. E. Calvo-Gallardo, Á. López-Gil, I. Méndez-López, C. Martínez-Ramírez, J. F. Padín and A. G. García, *J. Neurochem.*, 2016, **139**, 722-736.
25. E. N. Pothos, E. Mosharov, K.-P. Liu, W. Setlik, M. Haburcak, G. Baldini, M. D. Gershon, H. Tamir and D. Sulzer, *J. Physiol.*, 2002, **542**, 453-476.
26. A. Elhamdani, H. C. Palfrey and C. R. Artalejo, *Neuron*, **31**, 819-830.

27. J. Dunevall, H. Fathali, N. Najafinobar, J. Lovric, J. Wigström, A.-S. Cans and A. G. Ewing, *J. Am. Chem. Soc.*, 2015, **137**, 4344-4346.
28. W. Cheng and R. G. Compton, *Angew. Chem. Int. Ed.*, 2014, **53**, 13928-13930.
29. E. Lebègue, C. M. Anderson, J. E. Dick, L. J. Webb and A. J. Bard, *Langmuir*, 2015, **31**, 11734-11739.
30. X. Li, J. Dunevall, L. Ren and A. G. Ewing, *Anal. Chem.*, 2017, **89**, 9416-9423.
31. J. Lovric, N. Najafinobar, J. Dunevall, S. Majdi, I. Svir, A. Oleinick, C. Amatore and A. Ewing, *Faraday Discuss.*, 2016, **193**, 65-79.
32. N. Najafinobar, J. Lovrić, S. Majdi, J. Dunevall, A.-S. Cans and A. Ewing, *Angew. Chem. Int. Ed.*, 2016, **55**, 15081-15085.
33. X. Li, J. Dunevall and A. G. Ewing, *Angew. Chem. Int. Ed.*, 2016, **55**, 9041-9044.
34. L. Ren, M. D. Pour, S. Majdi, X. Li, P. Malmberg and A. G. Ewing, *Angew. Chem. Int. Ed.*, 2017, **56**, 4970-4975.
35. H. Fathali, J. Dunevall, S. Majdi and A.-S. Cans, *ACS. Chem. Neurosci.*, 2017, **8**, 368-375.
36. N. T. N. Phan, X. Li and A. G. Ewing, *Nature Review Chemistry*, 2017, **1**, 0048.
37. X. Li, A. S. Mohammadi and A. G. Ewing, *J. Electroanal. Chem.*, 2016, **781**, 30-35.
38. E. V. Mosharov and D. Sulzer, *Nat. Methods*, 2005, **2**, 651-658.
39. L. A. Sombers, H. J. Hanchar, T. L. Colliver, N. Wittenberg, A. Cans, S. Arbault, C. Amatore and A. G. Ewing, *J. Neurosci.*, 2004, **24**, 303-309.

Supporting Information

Electrochemical Quantification of Transmitter Concentration in Single Nanoscale Vesicles Isolated from PC12 Cells

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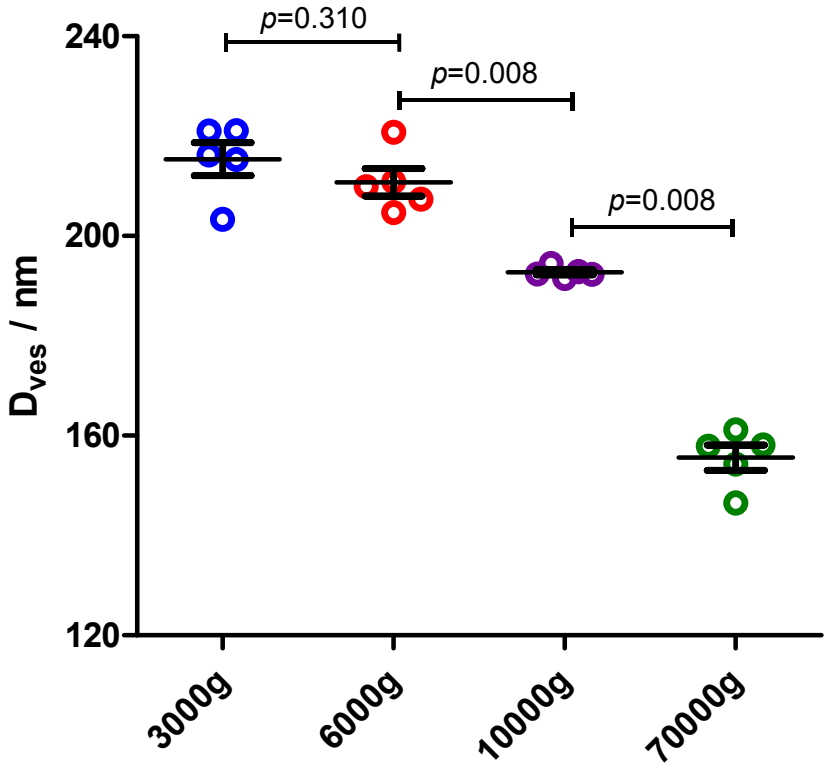

Fig. S1. Average diameters of individual vesicles obtained with differential centrifugation quantified with nanoparticle tracking analysis (NTA). The data are presented as mean ± standard error of the mean (SEM). Blue, 3000 g; red, 6000 g; purple, 10000 g; green, 70000 g. N = 6 isolations. The pairs of data sets were compared using a two-tailed Wilcoxon–Mann–Whitney rank-sum test, and *p*-values are indicated in the figure.

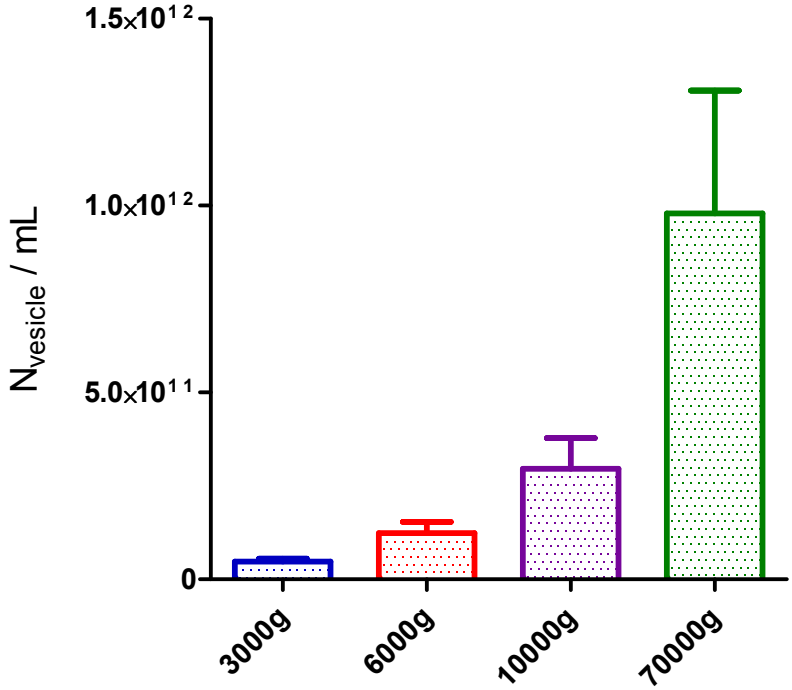

Fig. S2. Average number of vesicles per milliliter detected in NTA for vesicle samples obtained with differential centrifugation. Error bar, SEM. N = 6 isolations. Blue, 3000 g; red, 6000 g; purple, 10000 g; green, 70000 g.