Correlating molecule count and release kinetics with vesicular size using open carbon nanopipettes

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1. Chemicals and solutions
All reagents were purchased from Sigma-Aldrich unless otherwise specified. Locke’s buffer (mM): 1540 NaCl, 56 KCl, 36 NaHCO₃, 56 glucose, 50 HEPES, 1% (v/v) penicillin, pH 7.4. This stock solution was diluted 10× with distilled water the day before the experiment.
CaCl₂ free isotonic solution (mM): 154 NaCl, 4.2 KCl, 0.7 MgCl₂·6H₂O, 11.2 glucose, 10 HEPES, pH 7.4, ~310 mOsm.
Cell culture medium: DMEM/Ham’s F12 1:1 supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin, 1% (v/v) cytosine β-Darabinofuranoside, 0.1% (v/v) 5-fuoro-2′-deoxyuridine. Homogenizing buffer (mM): 230 sucrose, 1 EDTA, 1 MgSO₄, 10 HEPES, 10 KCl, cOmplete enzyme inhibitor (Roche, Sweden), DNase I (10 μg/mL) (Roche), 0.001 oligomycin, pH 7.4, ~310 mOsm.

2. Chromaffin cell and vesicle isolation
Bovine adrenal glands were obtained from a local slaughterhouse, and the chromaffin cells were isolated as previously described. Briefly, the vein was perfused with Locke’s buffer to clear away blood cells. The medulla was isolated after collagenase (0.2%, Roche, Sweden) treatment, and cells were isolated using a series of homogenization and centrifugation steps. For single cell experiments, ~500000 cells were seeded on collagen (IV) coated plastic dishes (Corning Biocoat, VWR, Sweden) and maintained in a humidified incubator at 37 °C, 5% CO₂ for a maximum of 3 days prior to experiments.

For vesicle isolation, a protocol developed by the Borges group was used. Briefly, the medulla was mechanically homogenized in homogenizing buffer, and the vesicles were purified using a series of centrifugation steps: 1000g for 10 min to remove whole cells followed by 10000g to pellet vesicles. All centrifugation was performed at 4 °C. The final pellet of vesicles was resuspended and diluted in homogenizing buffer and subsequently used for electrochemical measurements the same day.

3. Fabrication of open carbon nanopipettes (CNPs) with chemical vapor deposition (CVD)
The nanopipettes with the tip radii from 50 to 600 nm were pulled from quartz capillaries (1.0 mm o.d., 0.5/0.7 mm i.d., Sutter Instrument Co.) by the laser pipette puller (P-2000, Sutter Instruments). Carbon was deposited on the inner wall of glass nanopipettes by chemical vapor deposition (CVD) at 900°C for 30 minutes, using methane as carbon source and argon as the protector, as described previously. Representative TEM images of open CNPs are shown in Figure S1.

4. Sample preparation of chromaffin cells for TEM imaging
The chromaffin cells cultured on collagen (IV) coated plastic dishes were treated with trypsin and then transferred to a centrifuge tube. After centrifugation, the upper solution was discarded. In the next step, the pellet was re-suspended in 1 mL of the fixative containing glutaraldehyde (2.5%) and paraformaldehyde (1%). Later, the cells were washed in phosphate buffer (0.1M, pH 7.4) and centrifuged. The pellets were then fixed in 2% osmium tetroxide (TAAB, Berks, England) in phosphate buffer (0.1M, pH 7.4) at 4°C for 2 h, dehydrated in ethanol followed by acetone and embedded in LX-112 (Ladd, Burlington, Vermont, USA). All sections were cut (around 50-60 nm) with a Leica Ultracut UCT/ Leica EM UC 6 (Leica, Wien, Austria), then sections were contrasted with uranyl acetate followed by lead citrate and examined in a Tecnai 12 Spirit Bio TWIN TEM (FEI Company, Eindhoven, The Netherlands) at 100 kV/Hitachi HT 7700 (Tokyo, Japan) at 80 kV. Representative TEM images of chromaffin cells are shown in Figure S4. Vesicle size and dense core size were measured with ImageJ.

5. Electrochemistry
In all amperometric experiments, a potential of +700 mV vs Ag/AgCl was applied using an Axon 200B potentiostat (Molecular Devices, San Jose, CA). For IVIEC measurement, CNP was carefully placed inside the cell by piercing the plasma membrane. VIEC measurement of vesicular content in samples of isolated vesicles was done as previously described. The CNP was placed in a vesicle suspension solution and current transients were recorded.

6. Data acquisition and analysis.

Supporting information
Current transients were recorded and digitized using a Digidata1440A (Molecular Devices) and digitized at 10 kHz or 100 kHz and filtered at 2 kHz using a 4-pole Bessel filter. The data were converted in Matlab (The MathWorks, Inc.) and processed in IgorPro (Wavemetrics, Lake Oswego, OR). Traces were manually checked for potential false detections done by the software. Spike characteristics were determined as number of molecules based on the charge measured in each spike, $t_{rise} =$ time from 25 to 75% of maximum during the increase of the spike, $t_{fall} =$ time from 75 to 25% of maximum during the decrease of the spike, $t_{1/2} =$ full spike width at half-maximum. The medians were calculated from all vesicles at the same size of CNPs.

**Figure S1.** Representative TEM images of open CNPs with 100, 200, 300 and 400 nm radius (from left to right)

**Figure S2.** Representative amperometric traces of VIEC test with 250, 300, 500 and 600 nm radius of open CNPs (from top to bottom)
Figure S3. Histograms of $t_{\text{rise}}$ and $t_{\text{fall}}$ derived from different size open CNPs

Figure S4. Representative TEM images of chromaffin cells

Figure S5. Image of an 18 µm diameter chromaffin cell after IVIEC measurement with a 400 nm open CNP
Figure S6. Amperometric trace of IVIEC measurement into a 13 µm diameter chromaffin cell with a 400 nm open CNP and cell images during and after IVIEC measurement (The red arrow indicated the moment the CNP was pushed through the cell membrane).

| CNP Diameter (nm) | N_molecules | t_{1/2} (ms) |
|-------------------|-------------|-------------|
| 200               | 1.07×10^6   | 12.33       |
| 250               | 3.64×10^6   | 18.37       |
| 300               | 3.21×10^8   | 22.66       |
| 400               | 4.19×10^8   | 33.79       |
| 500               | 5.91×10^8   | 18.15       |
| 600               | 4.83×10^9   | 18.68       |

Table S1. Medians of vesicular transmitters amount (N_molecules) and duration of transmitters release (t_{1/2}) derived from different size open CNPs.

References
1. O’Connor, D. T., Mahata, S. K., Mahata, M., Jiang, Q., Hook, V. Y., and Taupenot, L. Nat. Protoc. 2007, 5, 1248–1253.
2. Pardo, M. R., Estévez-Herrera, J., Castañeyra, L., Borges, R., and Machado, J. D. Anal. Biochem. 2017, 536, 1–7.
3. Hu, K. K.; Wang, Y. X.; Cai, H. J.; Mirkin, M. V.; Gao, Y.; Friedman, G.; Gogotsi, Y. Anal. Chem. 2014, 86, 8897-8901.
4. Dunevall, J.; Fathali, H.; Najafinobar, N.; Lovric, J.; Wigström, J.; Cans, A.-S.; Ewing, A. G. J. Am. Chem. Soc. 2015, 13, 4344-4346.
5. Mosharov, E., and Sulzer, D. Nat. Methods 2005, 9, 651–658.