Counting the Number of Glutamate Molecules in Single Synaptic Vesicles

Yuanmo Wang,[†] Hoda Fathali,[†] Devesh Mishra,[‡,⊥] Thomas Olsson,[‖] Jacqueline D. Keighron[#], Karolina P. Skibicka,[⊥,‡] and Ann-Sofie Cans*[,†]

[†]Department of Chemistry and Chemical Engineering, Chalmers University of Technology, Kemigården 4, SE-412 96 Gothenburg, Sweden;

[‡]Department of Physiology/Metabolic Physiology, Institute of Neuroscience and Physiology, The Sahlgrenska Academy at the University of Gothenburg, Medicinaregatan 11, SE-413 90 Gothenburg, Sweden;

[#]Department of Chemical and Biological Sciences, New York Institute of Technology, Old Westbury, NY 11568, USA

[‖]Department of Physics, Chalmers University of Technology, Kemigården 4, SE-412 96 Gothenburg, Sweden;

[⊥] Wallenberg Centre for Molecular and Translational Medicine, University of Gothenburg, SE-405 30 Gothenburg, Sweden

Supporting Information Placeholder

ABSTRACT: Analytical tools for direct quantitative measurements of glutamate, the principal excitatory neurotransmitter in brain, are lacking. Here, we introduce a new enzyme-based amperometric sensor technique for direct counting of the number of glutamate molecules stored inside single synaptic vesicles. An ultra-fast enzyme-based glutamate sensor is placed into a solution of isolated synaptic vesicles, which stochastically rupture at the sensor surface in a potential dependent manner by applying a constant negative potential. High-speed (10 kHz) amperometry is used to record sub-millisecond current spikes, which represent glutamate release from single vesicles that burst open. Glutamate quantification is achieved by a calibration curve that is based on measurements of glutamate release from vesicles pre-filled with various concentrations of glutamate. Our measurements show that a single synaptic vesicle encapsulates about 8000 glutamate molecules, which is comparable to the measured exocytotic quantal glutamate release in the nucleus accumbens of mouse brain tissue. Hence, this new methodology introduces the means to quantify ultra-small amounts of glutamate and to study synaptic vesicle physiology, pathogenesis and drug treatments for neuronal disorders where glutamate is involved.

Synaptic vesicles are organelles used by brain cells to tightly pack and store neurotransmitters in the cell cytoplasm.[1] Neurotransmitter signals are exchanged between neurons through electrical depolarization which trigger these vesicles to fuse with the cell plasma membrane and release the signaling molecules into the synaptic cleft in a process called exocytosis. From here the neurotransmitters diffuse and bind to receptors at the cell surface of neighboring neurons to relay the chemical message. The amount of neurotransmitter released and the frequency of exocytosis activity are how synapses are thought to control communication strength through plasticity. This process underlies the basics of brain functions such as memory and learning, and also relates to brain disorders including drug abuse and addiction, diseases still not fully understood.[2] Therefore, many drugs are designed to affect the quantity of neurotransmitters released and residing in the extracellular space to enhance the chemical signaling process.

Glutamate is the major excitatory neurotransmitter involved in major brain functions such as cognition, memory and learning as well as many neurological disorders.[1] As such, glutamate regulation through pharmacological agents is of specific interest in managing disease states. Specifically, the amount of neurotransmitters stored in a synaptic vesicle, often referred to as the vesicle "quantal size", is an important quantitative measure for neuropharmacology and drug development research.[3-7] However, there is still an analytical challenge for direct, reliable quantitative measurements of glutamate stored in the ultra-small volume of a synaptic vesicle with an average diameter of 40 nm.[8,9] Methods such as radioreceptor assay (3640 glutamate molecules/vesicle)[10] and immunoisolation of synaptic vesicles from synaptosomes (60 mM)[11] have been used to try to solve the debate about how many glutamate molecules are stored inside synaptic vesicles. While methods such as fluorescence imaging techniques are powerful tools for studying the mechanisms for vesicular glutamate loading and storage.[9,12,13] To gain a better understanding of neurophysiology, neurological diseases and possible treatments, methods for direct quantification of synaptic vesicle glutamate storage and its release is needed. Recently, new methodologies have been developed for the quantitative analysis of catecholamine containing vesicles using amperometry.[14,15] These techniques place a carbon fiber microelectrode into a solution of isolated secretory vesicles and apply a positive oxidation potential that triggers vesicles in contact with the electrode surface to stochastically rupture. Catecholamines that leak out from vesicles that bursts open and onto the electrode surface are electrochemically oxidized and these events are recorded as isolated current spikes by the amperometric electrode. The integrated total charge (Q) for each current spike is used to calculate the vesicle neurotransmitter concentration, through Faraday´s law.[14,16] The mechanism for this method is influenced by a combination of vesicle adsorption and a positive applied potential, which initiates vesicle membrane pore formation that finally shatters the organelle.[17] Granting amperometry is a quantitative method that displays excellent temporal resolution, the limitation is that it only applies for neurotransmitters that are electroactive.

Glutamate is a non-electroactive neurotransmitter that cannot directly be detected amperometrically, but this becomes feasible by functionalizing the electrode surface with an enzyme, glutamate oxidase (GluOx), creating a glutamate biosensor.[18] Here, the enzyme works to catalyzes glutamate into forming hydrogen peroxide, H_2O_2, which serves as an electroactive redox reporter molecule for glutamate detection. Glutamate sensing has long suffered from low temporal resolution and this prevented applications such as monitoring neuronal single exocytosis activity in real time. To solve this, an ultrafast glutamate sensor, made of a gold nanoparticle (AuNP) modified carbon fiber microelectrode with an ultrathin coating of GluOx, was recently developed in our lab.[19] This invention helped to push the temporal limits for a chemically selective glutamate sensor from previous sub-second to current sub-millisecond time scale.[19] The sensor detection scheme (Figure 1A) is based on the electrochemical reduction reaction for H_2O_2 detection by applying an amperometric reduction potential at -0.5 V (vs a Ag/AgCl reference electrode) at the sensor surface. By utilizing this technical achievement, the dynamics of individual sub-millisecond exocytosis events during spontaneous glutamate activity at the core region of the nucleus accumbens in rodent brain tissue was temporally resolved.[19] Although relative quantitative measures were feasible, the sensor was not validated for absolute quantification. In order to address the lack of methods for quantifying ultra-small amounts of glutamate, we first developed a new method for quantification of glutamate in small synaptic vesicles. This was achieved by implementing our ultrafast enzyme-based glutamate sensor and adopting to previous protocols for quantitative analysis of vesicles containing easily oxidized neurotransmitters.[14,15,17] Briefly, we placed our sensor into a solution of either glutamate-filled large unilamellar vesicles (LUVs) or isolated synaptic vesicles from mouse brain. In contrast to the catecholamine analysis protocols using positive oxidation potentials at a bare carbon fiber microelectrode, we applied a constant

negative reduction potential of -0.5 V (vs a Ag/AgCl reference electrode) at the sensor surface to avoid oxidative interfering analytes and produce chemically selective measurements. This strategy resulted in amperometric time traces that demonstrated stochastic bursts of individual sub-millisecond reduction current spikes corresponding to detection of single LUVs (Figure 1B) and synaptic vesicles (Figure 1C) randomly bursting and releasing their internal glutamate content onto the sensor surface. In both of these amperometric time traces, the current spikes displayed a rapid current rise and a slower decay versus time (Table S1 and S2), which are typical shape characteristics for detection of vesicle content release (Figure S1A) and indicates that a similar mechanism is acting to rupture these different vesicle compartments.[20]

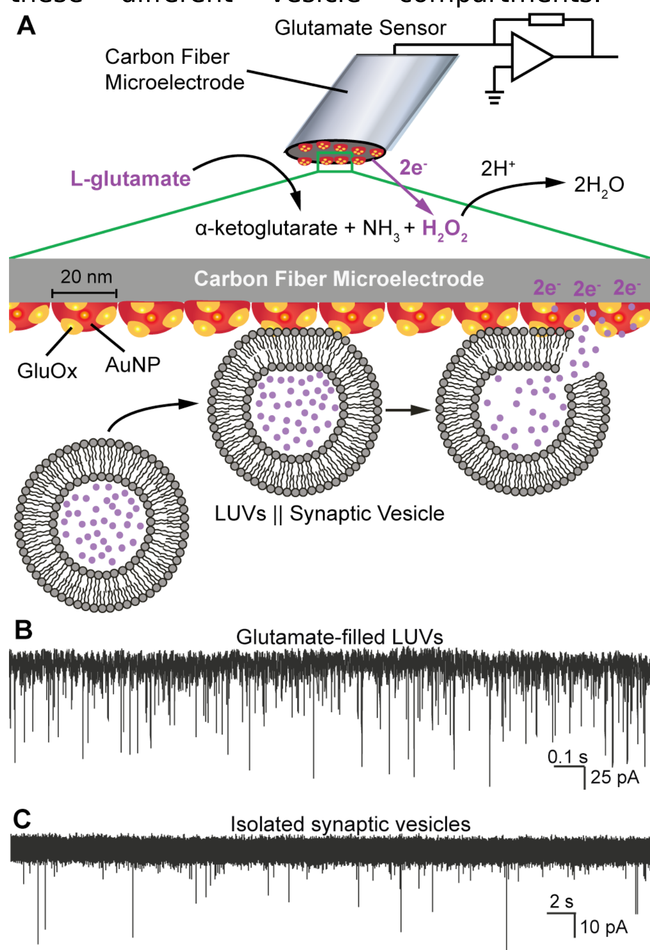

Figure 1. (A) Schematic of the ultrafast glutamate sensor detection scheme used for counting the number of glutamate molecules in single synaptic vesicles as vesicles rupture and release their glutamate content at the sensor surface, modified with AuNPs (red hemispheres) and an ultra-thin coating of GluOx (yellow hemispheres). Schematic is not drawn to scale. (B) Amperometric reduction current recording of glutamate release from LUVs encapsulating 100 mM glutamate, and (C) isolated synaptic vesicles from mouse brain.

Empirical titration of the LUVs and synaptic vesicle samples was performed to find the threshold of vesicular concentration needed to initiate vesicle rupture at the electrode surface. A significantly higher frequency was observed for LUVs compared to synaptic vesicles, which is in agreement with the idea that the protein-rich synaptic vesicle membrane is substantially more rigid and more difficult to rupture than protein-free lipid-based LUVs.[17]

To further characterize and validate this method, the glutamate sensor was exposed to LUVs encapsulating 5 different concentrations of glutamate (100, 150, 200, 250 and 300 mM). The LUVs were prepared by a gentle swelling method, using five rounds of freeze-thaw cycles to encapsulate glutamate concentration and multiple extrusion passages to ensure a homogenous LUV size (Figure 2C).[20,21] Through altering the applied potential from -0.5 V to -0.7 V and -0.9 V, we observed vesicles rupturing at the sensor surface in a potential dependent manner. The average frequency of vesicles bursting was significantly increasing with potential amplitude (Figure 2B), whereas for each potential applied, the average frequency remained constant over the time course for measurements (Figure 2A). It was also noted that the concentration of the encapsulating glutamate solution, regardless of the potential applied, could enhance likelihood of vesicle shattering, possibly as a result of glutamate interactions with the vesicle membrane, which may alter the vesicle membrane elastic properties and favor membrane rupture (Table S1).[22–28] These observations suggest that the electric field near the sensor surface induces membrane pore formation at vesicles in close contact with the sensor surface, which is in agreement with previous observations for analysis of catecholamine vesicles when applying potentials of opposite polarity.[14,15]

A common issue with amperometric enzyme-based sensors, is that the detection efficiency is often less then 100%, and therefore appropriate calibration methods are needed for quantitative analysis. Previous characterization of our ultrafast glutamate sensor demonstrated a linear concentration range between 10-100 μM, as determined by steady state current amplitudes in bulk solution system.[19] However, the experimental conditions for this new method analyzing glutamate content in synaptic vesicle differ significantly from saturated sensor surface measurements. In these measurements we only detect sub-millisecond events of nanosized vesicles emptying onto the surface of a 33-μm (in diameter) sensor their 20 zL internal solution containing on the order of only a few thousands of neurotransmitter molecules.[8,29] We therefore designed a calibration system that mimics the experimental conditions used for synaptic vesicle analysis. This was realized by preparation of LUVs pre-filled with various physiologically relevant concentrations of glutamate solution and performing analysis of them using our ultrafast glutamate sensor. By plotting the charge density for each current spike detected (Q/LUV volume) versus the glutamate concentration used to load the LUVs, we found a linear relationship that was valid for quantitative glutamate analysis of single synaptic vesicle (Figure 2D). Although neither the applied potential nor the encapsulated glutamate concentration affected the rupture kinetics (Figure S1, Table S2), we noted that Q and the current spike maximal amplitude (I_{max}) were amplified with the increasing applied potential, which is in agreement with sensor voltammograms for H_2O_2.[19,30] However, for each constant potential applied, linear calibration graphs were achieved (Figure 2D and S2), validating the LUV calibration method for quantitative glutamate analysis of single synaptic vesicles.

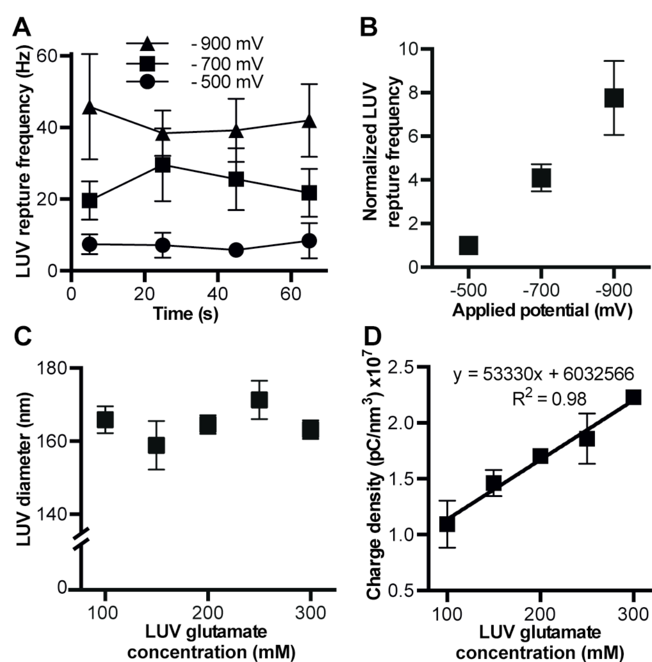

Figure 2. Characterization of synaptic vesicle glutamate analysis method by amperometrically detecting rupture of LUVs loaded with 5 different glutamate concentrations. (A) The average frequency of LUV rupture versus time when applying a -0.5, -0.7 or -0.9 V potential. Error bars are standard deviation of the mean (SEM). (B) The average frequency of LUV rupture versus the applied sensor potential. Each data point was averaged over the initial 65 s of recording and normalized to the frequency recorded at -0.5 V. Error bars are SEM. (C) The average LUV size versus concentration of the encapsulating glutamate solution (n=3), measured by nanosized tracking analysis system. Error bars are standard deviation. (D) Calibration curve constructed from the average charge detected from vesicle glutamate release (n=4-5) versus the LUV glutamate concentration. Error bars are SEM.

To determine the average number of glutamate molecules stored inside of single synaptic vesicles, quantitative measurements using the ultrafast glutamate sensor was performed immediately after isolation of synaptic vesicles that was performed in the presence of ATP to minimize glutamate leakage before analysis. Assuming a vesicle internal volume of 20 zL, these measurements showed that an average of 8300 ± 600 glutamate molecules are stored in each synaptic vesicle and corresponds to a concentration of ~ 0.7 ±

0.05 M glutamate. We note that this is a magnitude of approximately 3-10 times larger concentration than previously stated for this neurotransmitter that has been so difficult to quantify.[10,11] This is in line with recent estimations using postsynaptic receptor saturation measurement and computer modeling of quantal release.[33] This result could be related to that our newly developed ultrafast glutamate sensor allows direct measurement of glutamate at single synaptic vesicle level and perhaps also that we might have used a synaptic vesicle protocol that better retain the vesicle glutamate content before analysis. Interestingly, this neurotransmitter concentration then matches the concentrations previously measured for catecholamine containing vesicles.[31]

These experimental conditions also resemble those for amperometric recording of exocytosis at secretory cells, where typically a microelectrode is placed in close proximity to a cell surface to capture isolated current spikes from single vesicle neurotransmitter release events. Therefore, we also applied the LUV-based calibration to our recordings of spontaneous glutamate in the core region of the nucleus accumbens of mouse (Figure 3A) and rat (Figure S3B) brain slices. Analysis of the sub-millisecond kinetics (Table S3 and S4) and the charge, Q, for each category of spike detected in brain tissue, and the average number of glutamate molecules released from exocytotic events was determined. Except for "funky spikes" that we believe may represent multiple vesicle detection and "overlap" which might represent co-detection, all other categories of spikes were analyzed according to their shape and assumed to represent single exocytosis events.[19] This analysis revealed that about 5200 ± 850 (average of means ± SEM) glutamate molecules were released when sharp spikes, which were the most frequently found spikes (50%), were detected. [19] While an average of 8500 ± 1100 glutamate molecules were measured at events that can be characterized as more complex plateau shaped spikes (Figure 3A). Assuming that a full exocytosis model dominates these recordings, , this predicts an original vesicular glutamate concentration of 0.7 ± 0.05M. By comparing the measured exocytotic glutamate release with the number of glutamate molecules stored in the isolated synaptic vesicles, we note that only part of the glutamate content is detected when monitoring sharp current spikes, whereas the more complex current spikes seem to represent that the entire vesicle glutamate content is being emptied (Figure 3B). Similar variability in current spike dynamics were previously described by amperometric dopamine recording from neurons of the rat ventral tegmental area and suggested to represent different modes of exocytosis as well as multi-vesicle release events. Sharp spikes were denoted by less dopamine detection and more complex shaped spikes represented a larger number of dopamine molecules released per vesicle.[32] However, more work is needed to characterize the mechanisms that are responsible for the different current spike dynamics observed in brain tissue, as variations in quantal release might be a result of glutamate release from different cell types or different modes of exocytosis.

The experiments shown here present a methodology to count the number of glutamate molecules loaded inside single isolated synaptic vesicles and provide new means to quantify the amount glutamate released by single exocytosis events in brain tissue during neuronal communication. We found that by counting the number of glutamate molecules stored inside isolated synaptic vesicles and determining the number of glutamate molecules released at exocytosis, the glutamate concentrations are similar to concentrations of catecholamines previously found in catecholamine-containing vesicles. Collectively, this novel method offers new means to study the dynamics of glutamate vesicle loading, storage and regulatory aspects of synaptic glutamate transmission that can help to gain a clearer view of mechanisms of the glutamate system involved in neurotransmission of both healthy and disease states.

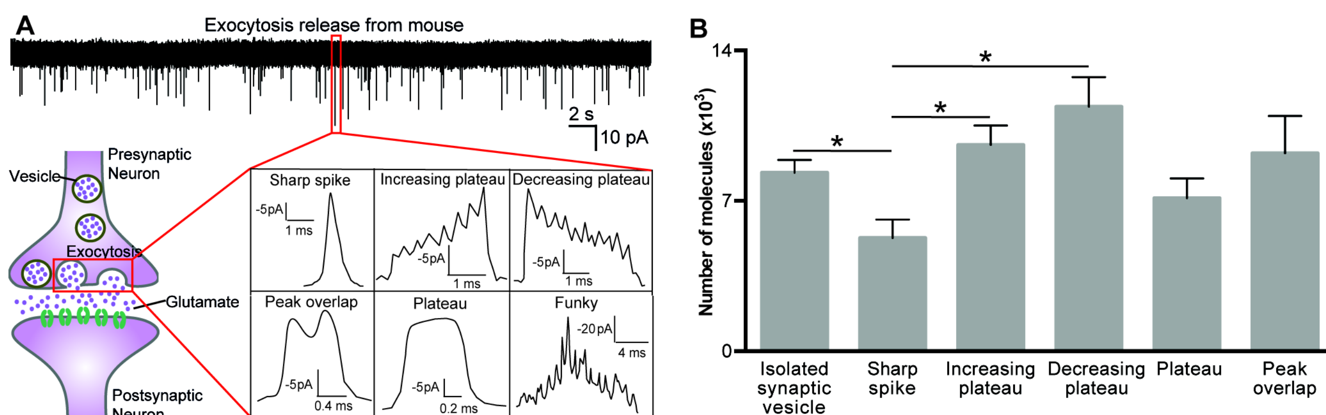

Figure 3. (A) A representative amperometric recordings (10 kHz) of exocytotic glutamate release events in the core region of the nucleus accumbens of mouse brain slice (n=4) with an illustration of the 6 different current spike types associated with glutamate release in rodent brain tissue. Schematic is not drawn to scale. (B) Comparison of the average number of glutamate molecules stored in synaptic vesicles isolated from mouse brain (n=4) and the average number of glutamate molecules released by exocytosis in the mouse brain slice (n=4) and as by the 5 different categories of current spike shapes detected. All amperometric data was collected at a -0.5 V potential vs Ag/AgCl reference electrode. Data is presented as average of the means ± SEM. Two-tailed paired student's t-test was performed to compare the glutamate number, *$p < 0.05$.

ASSOCIATED CONTENT

Supporting Information. Additional data including lipid vesicle current spike kinetics, frequency of vesicle rupture at different applied potential with time, kinetics of amperometric spike in rat brain, and methods in experimental section.

AUTHOR INFORMATION

Corresponding Author

* cans@chalmers.se

Funding Sources

We appreciate financial support from The Swedish Research Council, The Swedish Brain Foundation, Ragnar Söderberg Foundation, The Novo Nordisk Foundation, The Wallenberg Center for Molecular and Translational Medicine at the University of Gothenburg, Ernst and Fru Rådman Colliander Stiftelse, Wilhelm and Martina Lundgren Stiftelse and Magnus Bergvall Stiftelse.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

We thank Dr. Eric Hanse for the use of microscope and low-current potentiostat.

REFERENCES

(1) Zhou, Y.; Danbolt, N. C. Glutamate as a Neurotransmitter in the Healthy Brain. *J. Neural Transm.* **2014**, *121*, 799–817.
(2) Hyman, S. E.; Malenka, R. C.; Nestler, E. J. NEURAL MECHANISMS OF ADDICTION: The Role of Reward-Related Learning and Memory. *Annu. Rev. Neurosci.* **2006**, *29*, 565–598.
(3) Takamori, S. Presynaptic Molecular Determinants of Quantal Size. *Front. Synaptic Neurosci.* **2016**, *8*.
(4) Sulzer, D.; Cragg, S. J.; Rice, M. E. Striatal Dopamine Neurotransmission: Regulation of Release and Uptake. *Basal Ganglia* **2016**, *6*, 123–148.
(5) McEntee, W. J.; Crook, T. H. Glutamate: Its Role in Learning, Memory, and the Aging Brain. *Psychopharmacology (Berl).* **1993**, *111*, 391–401.
(6) Meldrum, B. S. Glutamate as a Neurotransmitter in the Brain: Review of Physiology and Pathology. *J. Nutr.* **2000**, *130*, 1007S-1015S.
(7) Djuricic, B. Glutamate in brain: transmitter and poison. *Glas. Srp. Akad. Nauka Med.* **2002**, No. 47, 55–76.
(8) Takamori, S.; Holt, M.; Stenius, K.; Lemke, E. A.; Grønborg, M.; Riedel, D.; Urlaub, H.; Schenck, S.; Brügger, B.; Ringler, P.; et al. Molecular Anatomy of a Trafficking Organelle. *Cell* **2006**, *127*, 831–846.
(9) Budzinski, K. L.; Allen, R. W.; Fujimoto, B. S.; Kensel-Hammes, P.; Belnap, D. M.; Bajjalieh, S. M.; Chiu, D. T. Large Structural Change in Isolated Synaptic Vesicles upon Loading with Neurotransmitter. *Biophys. J.* **2009**, *97*, 2577–2584.
(10) Riveros, N.; Fiedler, J.; Lagos, N.; Mun˜oz, C.; Orrego, F. Glutamate in Rat Brain Cortex Synaptic

Vesicles: Influence of the Vesicle Isolation Procedure. *Brain Res.* **1986**, *386*, 405–408.

(11) Burger, P. M.; Mehl, E.; Cameron, P. L.; Maycox, P. R.; Baumert, M.; Lottspeich, F.; De Camilli, P.; Jahn, R. Synaptic Vesicles Immunoisolated from Rat Cerebral Cortex Contain High Levels of Glutamate. *Neuron* **1989**, *3*, 715–720.

(12) Budzinski, K. L.; Zeigler, M.; Fujimoto, B. S.; Bajjalieh, S. M.; Chiu, D. T. Measurements of the Acidification Kinetics of Single SynaptopHluorin Vesicles. *Biophys. J.* **2011**, *101*, 1580–1589.

(13) Farsi, Z.; Preobraschenski, J.; van den Bogaart, G.; Riedel, D.; Jahn, R.; Woehler, A. Single-Vesicle Imaging Reveals Different Transport Mechanisms between Glutamatergic and GABAergic Vesicles. *Science (80-.).* **2016**, *351*, 981–984.

(14) Omiatek, D. M.; Bressler, A. J.; Cans, A. S.; Andrews, A. M.; Heien, M. L.; Ewing, A. G. The Real Catecholamine Content of Secretory Vesicles in the CNS Revealed by Electrochemical Cytometry. *Sci. Rep.* **2013**, *3*, 1–6.

(15) Dunevall, J.; Fathali, H.; Najafinobar, N.; Lovric, J.; Wigström, J.; Cans, A.-S.; Ewing, A. G. Characterizing the Catecholamine Content of Single Mammalian Vesicles by Collision–Adsorption Events at an Electrode. *J. Am. Chem. Soc.* **2015**, *137*, 4344–4346.

(16) Hochstetler, S. E.; Puopolo, M.; Gustincich, S.; Raviola, E.; Wightman, R. M. Real-Time Amperometric Measurements of Zeptomole Quantities of Dopamine Released from Neurons. *Anal. Chem.* **2000**, *72*, 489–496.

(17) Lovrić, J.; Najafinobar, N.; Dunevall, J.; Majdi, S.; Svir, I.; Oleinick, A.; Amatore, C.; Ewing, A. G. On the Mechanism of Electrochemical Vesicle Cytometry: Chromaffin Cell Vesicles and Liposomes. *Faraday Discuss.* **2016**, *193*, 65–79.

(18) Day, B. K.; Pomerleau, F.; Burmeister, J. J.; Huettl, P.; Gerhardt, G. A. Microelectrode Array Studies of Basal and Potassium-Evoked Release of l-Glutamate in the Anesthetized Rat Brain. *J. Neurochem.* **2006**, *96*, 1626–1635.

(19) Wang, Y.; Mishra, D.; Bergman, J.; Keighron, J. D.; Skibicka, K. P.; Cans, A.-S. Ultrafast Glutamate Biosensor Recordings in Brain Slices Reveal Complex Single Exocytosis Transients. *ACS Chem. Neurosci.* **2019**, *10*, 1744–1752.

(20) Simonsson, L.; Kurczy, M. E.; Trouillon, R.; Hook, F.; Cans, A.-S. A Functioning Artificial Secretory Cell. *Sci. Rep.* **2012**, *2*, 824.

(21) Mayer, L. D.; Hope, M. J.; Cullis, P. R.; Janoff, A. S. Solute Distributions and Trapping Efficiencies Observed in Freeze-Thawed Multilamellar Vesicles. *Biochim. Biophys. Acta* **-** *Biomembr.* **1985**, *817*, 193–196.

(22) Wang, C.; Ye, F.; Velardez, G. F.; Peters, G. H.; Westh, P. Affinity of Four Polar Neurotransmitters for Lipid Bilayer Membranes. *J. Phys. Chem. B* **2011**, *115*, 196–203.

(23) Tsong, T. Y. Electroporation of Cell Membranes. *Biophys. J.* **1991**, *60*, 297–306.

(24) Lipowsky, R. Bending of Membranes by Anchored Polymers. *Europhys. Lett.* **1995**, *30*, 197–202.

(25) Simon, J.; Kühner, M.; Ringsdorf, H.; Sackmanna, E. Polymer-Induced Shape Changes and Capping in Giant Liposomes. *Chem. Phys. Lipids* **1995**, *76*, 241–258.

(26) Döbereiner, H.-G.; Selchow, O.; Lipowsky, R. Spontaneous Curvature of Fluid Vesicles Induced by Trans-Bilayer Sugar Asymmetry. *Eur. Biophys. J.* **1999**, *28*, 174–178.

(27) Kakorin, S.; Neumann, E. Kinetics of the Electroporative Deformation of Lipid Vesicles and Biological Cells in an Electric Field. *Berichte der Bunsengesellschaft für Phys. Chemie* **1998**, *102*, 670–675.

(28) Reimhult, E.; Höök, F.; Kasemo, B. Intact Vesicle Adsorption and Supported Biomembrane Formation from Vesicles in Solution: Influence of Surface Chemistry, Vesicle Size, Temperature, and Osmotic Pressure. *Langmuir* **2003**, *19*, 1681–1691.

(29) Newton, A. J. H.; Wall, M. J.; Richardson, M. J. E. Modeling Microelectrode Biosensors: Free-Flow Calibration Can Substantially Underestimate Tissue Concentrations. *J. Neurophysiol.* **2017**, *117*, 937–949.

(30) Keighron, J. D.; Wigström, J.; Kurczy, M. E.; Bergman, J.; Wang, Y.; Cans, A.-S. Amperometric Detection of Single Vesicle Acetylcholine Release Events from an Artificial Cell. *ACS Chem. Neurosci.* **2015**, *6*, 181–188.

(31) Bruns, D.; Jahn, R. Real-Time Measurement of Transmitter Release from Single Synaptic Vesicles. *Nature* **1995**, *377*, 62–65.

(32) Staal, R. G. W.; Mosharov, E. V.; Sulzer, D. Dopamine Neurons Release Transmitter via a Flickering Fusion Pore. *Nat. Neurosci.* **2004**, *7*, 341–346.

(33) Budisantoso, T.; Harada, H.; Kamasawa, N.; Fukazawa, Y.; Shigemoto, R.; Matsui, K. Evaluation of Glutamate Concentration Transient in the Synaptic Cleft of the Rat Calyx of Held. *J. Physiol.* **2013**, *591*, 219–239.

Table of Contents