Published in final edited form as:

Anal Chem. 2013 May 7; 85(9): 4822–4828. doi:10.1021/ac4008682.

Single Cell Amperometry Reveals that the Glycocalyx Hinders the Release of Neurotransmitters During Exocytosis

Raphaël Trouillon^a and Andrew G. Ewing^{a,b,*}

^aDepartment of Chemistry and Molecular Biology, University of Gothenburg, S-41296, Gothenburg, Sweden

^bDepartment of Chemical and Biological Engineering, Chalmers University of Technology, S-41296 Gothenburg, Sweden

Abstract

The diffusional hindrance of the glycocalyx along the cell surface on exocytotic peaks, observed with single cell amperometry, was investigated. Partial digestion of the glycocalyx with neuraminidase led to the observation of faster peaks, as shown by varied peak parameters. This result indicates that diffusion of small molecules in the partially digested glycocalyx is 2.2 faster than in the intact glycocalyx. Similarly, neutralization of the negative charges present in the cell microenvironment led to faster peak kinetics. The analysis of the vesicular efflux indicates that the diffusion coefficient of dopamine at the cell surface is at most 45 % of the diffusion coefficient in free solution. This study shows that the glycocalyx plays an important role in the diffusion kinetics of processes along the cell surface, including exocytotic events.

Introduction

Glycocalyx, a complex and densely packed mixture of glycoproteins and glycolipids coating the cell surface,¹ is crucial for mediating cellular interactions. It has been reported to be involved in immune response² and mechanical transduction.^{3,4} This chemical environment is also a main structural component of the body, responsible for cell adhesion and therefore the formation of tissues and organs.⁵ The density of macro-molecules is above 30,000 molecules per μm^2 .^{6,7} Unfortunately, this very thin layer of biopolymer is experimentally hard to characterize. For a variety of cells, the distance separating the membrane of cells from their substrate, which takes into account the geometry of the cell membrane as well as the thickness of the glycocalyx, was found to be in the 10 - 160 nm range.^{7–13} In the case of endothelial cells, this layer is almost an order of magnitude thicker.^{7,14}

Components of the glycocalyx can be digested with specific enzymes. For instance, neuraminidase, a glycoside hydrolase primarily produced by the influenza virus, catalyzes the cleavage of a sugar residue from a terminal sialic acid.^{15,16} This enzyme has been shown to reduce the gap separating adherent macrophages and red blood cells by 21 %.¹⁷ In endothelial cells, where the glycocalyx is much thicker than in other cell types and can be observed with confocal microscopy, neuraminidase was used to decrease by 60-70 % the fluorescence signal from labeled glycocalyx in vascular and glomerular endothelial cells without impairing cell viability.^{14,18}

*Corresponding author andrew.ewing@chem.gu.se, tel: +46(0)31-7869113.

Notes The authors declare no competing financial interest.

Exocytosis is the basic phenomenon underlying synaptic communication. In this process, vesicles filled with neurotransmitters fuse with the cell membrane, release their content in the synaptic cleft through a fusion pore,^{19–21} eventually stimulating the post synaptic cell and transmitting the signal.²² This phenomenon involves analyte diffusion in the vicinity of the cell membrane and is therefore prone to hindrances from the glycocalyx.

Single cell amperometry is a real time, quantitative chemical method that can be used to analyze exocytosis.^{23–25} In this approach, an artificial synapse is formed between a disk microelectrode, typically made from a 5 μm carbon fiber, and the surface of a cell. Individual exocytotic events can be observed by holding the electrode at a potential sufficient to oxidize neurotransmitters (typically 700 mV). The analytes are released through a fusion pore, diffuse away from the cell, and are amperometrically detected at the electrode. The effects of pharmacology^{26–28} and changes in physico-chemical conditions^{29–31} on the release of neurotransmitters can be investigated by analyzing the traces.³² This method has contributed to the elucidation of the fundamental mechanisms of exocytosis and underlined, in particular, the role of the fusion pore formed during vesicle fusion in neurotransmitter release.^{33,34}

In this article, the diffusional hindrance of the glycocalyx at the cell surface on the exocytotic release of dopamine from PC12 cells is examined. The cells were pre-incubated with neuraminidase to partially digest the glycocalyx. In a second set of experiments, the negative charges of the macro-molecules composing the glycocalyx were neutralized with the polycation protamine sulfate.^{35–37} In both cases, these treatments led to the recording of faster exocytotic peaks during single cell amperometry. This observation indicates that the cell microenvironment decreases the apparent diffusion coefficient of dopamine. The analysis of the vesicular efflux for the treated cells *vs.* control indicates that the diffusion of dopamine at the vicinity of the cell surface is at most 45 % of the diffusion coefficient in free solution.

Experimental Section

Chemicals and solutions

The chemicals, of analytical grade, were obtained from Sigma-Aldrich (unless stated otherwise) and used as received.

The HEPES physiological saline contains 150 mM NaCl, 5 mM KCl, 1.2 mM MgCl_2 , 5 mM glucose, 10 mM HEPES, and 2 mM CaCl_2 . The K^+ stimulating solution consists of 55 mM NaCl, 100 mM KCl, 1.2 mM MgCl_2 , 5 mM glucose, 10 mM HEPES, and 2 mM CaCl_2 .

All solutions were made using 18 M Ω .cm water from a Millipore purification system and the solutions pH was adjusted to 7.4 with concentrated (3 M) NaOH.

Fabrication of the disk microelectrodes

The fabrication of these electrodes was previously described.³⁸

The carbon fiber working electrodes were fabricated by aspirating 5 μm diameter carbon fibers into borosilicate glass capillaries (1.2 mm O.D., 0.69 mm I.D., Sutter Instrument Co., Novato, CA). The capillaries were subsequently pulled with a commercial micropipette puller (Model PE-21, Narishige, Inc., London, UK) and sealed with epoxy (Epoxy Technology, Billerica, MA). After beveling (Model BV-10, Sutter Instrument Co., Novato, CA) at 45 degrees, each electrode was then tested by performing cyclic voltammograms in a solution of 0.1 mM dopamine in PBS (pH 7.4). Only the electrodes showing good reaction

kinetics and a diffusion limited current in agreement with the theoretical value calculated for a 5 μm diameter disk were used for the experiments.

Cell culture

PC12 cells were purchased from the American Type Culture Collection (Manassas, VA). The cells were maintained in phenol red-free RPMI-1640 media (PAA Laboratories, Inc. Australia) supplemented with 10 % donor equine serum (PAA Laboratories), 5 % fetal bovine serum Gold (PAA Laboratories), 2 mM L-glutamine and 0.4 % penicillin streptomycin solution (PAA Laboratories) in a 7 % CO_2 , 100 % humidity atmosphere at 37 °C. The cells were grown on mouse collagen coated cell culture flasks (collagen type IV, BD Biosciences, Bedford, MA) and were sub-cultured every 7-9 days. The medium was replaced every 2 days throughout the lifetime of all cultures. For stimulated exocytosis experiments at single cells, PC12 cells were grown on mouse collagen coated culture dishes (type IV, BD Biosciences, Bedford, MA) 4-5 days before experiment and cell media was replaced every day.

Single cell experiments

Electrochemical recordings of exocytotic events from single PC12 cells were performed as previously described³⁴ on an inverted microscope (IX71, Olympus), in a Faraday cage. The working electrode was held at + 700 mV versus a Ag|AgCl reference electrode (Scanbur, Sweden) using an Axon 200B potentiostat (Molecular Devices, Sunnyvale, CA). The output was filtered at 2.1 kHz using a Bessel filter and digitized at 5 kHz. Before experiments, the cells were rinsed three times with HEPES buffer at 37 °C and were maintained under these conditions throughout the experiment. A glass micropipette containing K^+ stimulating solution was positioned 60 μm from the cell. Each cell was then stimulated once with a single 5 s K^+ injection (20 psi) through the micropipette coupled to a microinjection system (Picospritzer II, General Valve Corporation, Fairfield, NJ). A constant potential (700 mV) was applied to the microelectrode with respect to the Ag|AgCl reference electrode placed in the cell bathing solution throughout the experiment. All cell experiments were performed at 37 °C.

The cells were exposed to neuraminidase (type V from *Clostridium perfringens*) by rinsing the dishes 3 times with HEPES saline and incubating the cells for one hour, in the humid incubator, with a 1 U mL^{-1} solution of neuraminidase in HEPES buffer.¹⁴ In a set of experiments, the effect of a lower concentration (0.1 U mL^{-1}) was investigated. The cells were exposed to protamine sulfate (grade X from salmon) by rinsing the dishes 3 times with HEPES buffer and incubating the cells for 30 minutes, in the humid incubator, with a 1 $\mu\text{g mL}^{-1}$ solution of protamine sulfate in HEPES buffer.³⁷ The cells were then rinsed 3 times with HEPES buffer and tested immediately with amperometry. In the case of the protamine sulfate treatment, it was found that the effect of the drug was reversed after few minutes, and only the traces obtained during the first 10 minutes of the experiments were used for the analysis.³⁹

Data processing and statistics

All the data processing routines were performed with IgorPro 6.21. The amperometric traces were processed using an IgorPro 6.21 routine originating from David Sulzer's group.³² The filters for the current and differentiated current traces were 2 and 0.8 kHz, respectively. The threshold for peak detection on the differentiated trace was three times the standard deviation of the noise. The traces were carefully inspected after peak detection and false positive were manually rejected. The fitting of the peak parameters was adjusted. All the peaks larger than 2 pA (about four times the noise of the smoothed signal, between 0.5 and 0.7 pA in our experiments, based on 2- to 4-s baseline acquisitions at the beginning of the

trace) were collected. Feet showing a foot current higher than 2 pA were selected for analysis.

The parameters obtained from the peaks are shown on Figure 1A, B. These parameters, for the body of the peak, are the peak current i_p , the rise time t_{rise} , defined as the time separating 25 % of the maximum from 75 % of the maximum on the ascending part of the spike, the half peak width $t_{1/2}$, defined as the width of the exocytotic at half of its magnitude, the fall time t_{fall} , defined as the time separating 75 % of the maximum from 25 % of the maximum on the descending part of the spike, and the charge n , *i.e.* the area under the curve, expressed as a number of molecules. For the feet, the parameters are the foot current i_{foot} , defined as the average of the measured current over the duration of the foot or as the plateau current when a steady-state is reached, the foot duration t_{foot} the foot charge n_{foot} , *i.e.* the area under the curve defining the foot, expressed as a number of molecules.

These parameters were pooled, and the median of the data was calculated for each experimental condition. The distribution of the sample was evaluated using the 1st quartile-3rd quartile (or 25 %-75 %) interval. The distribution of the exocytotic parameters is asymmetric and strongly deviates from normality, hence motivating the use of the median in place of the mean.^{32,40–42} Additionally, the use of the median was found to minimize the impact of the cell-to-cell variations, as the value is less sensitive to outliers. Pairs of datasets were compared with the two-tailed Wilcoxon-Mann-Whitney rank-sum test, ***: $p < 0.001$.⁴³

Results and discussion

The partial digestion of the glycocalyx increases the release kinetics

If the glycocalyx hinders the diffusion of dopamine at the cell surface, the digestion or denaturation of this polymer is expected to increase the mass transport of the neurotransmitter in the artificial synapse. By considering Fick's first law, where $\vec{J}$ is the flux of analyte, D is the diffusion coefficient and c is the concentration of the species of interest⁴⁴

$$\vec{J} = -D \overrightarrow{\text{grad}}(c) \quad (1)$$

and the relationship between the distance Δx over which a bolus of analyte has diffused, in N dimensions, over the time t ⁴⁵

$$\Delta x = \sqrt{2NDt} \quad (2)$$

an apparent increase in the diffusion coefficient of dopamine in this system will result in higher (from Equation 1) and sharper (from Equation 2) peaks, for the same amount of neurotransmitter released (*i.e.* the area under the curve, see Figure 2).

In this report, PC12 cells were incubated for 1 hour with neuraminidase (0.1 or 1 U mL⁻¹ in HEPES buffer, pH 7.4). The traces obtained were compared to control cells incubated for 1 hour in HEPES buffer before the experiment, in the absence of neuraminidase (the duration of the HEPES incubation was not found to significantly alter the results, 30 minutes *vs.* 1 hour, $p > 0.12$, *vide infra*). These two experimental conditions were then compared (control: 12 cells, 345 peaks; 1 U mL⁻¹ neuraminidase: 15 cells, 646 peaks). Figures 3A and B show two typical traces obtained from control and neuraminidase (1 U mL⁻¹) treated PC12 cells. In both cases, clear, sharp exocytotic peaks were obtained upon K⁺ stimulation. Neuraminidase has been shown to have a limited impact on cell viability.¹⁴ This enzyme cleaves glycoside moieties, and should therefore have a limited impact on the protein machinery controlling exocytosis. Indeed, neuraminidase did not impair the exocytotic

ability of the PC12 cells, as the average number of peaks recorded per cell is higher than the one obtained from the control cells (control: 29 peaks per cell, neuraminidase: 43 peaks per cell).

Figures 3D and E show averaged peaks obtained from the traces shown in Figures 3A and B. The averaged peak obtained from the neuraminidase treated cells shows a higher peak current, and is sharper compared to control. The rate of dopamine release associated with these peaks appears to be much faster. Since the mechanisms controlling the formation of the fusion pore and the vesicular efflux should not be impaired by neuraminidase, faster diffusion in the digested glycocalyx appears to account for the observed changes in release kinetics.

Interestingly, as shown on Figures 4A, B and D, peaks were usually only observed during the 10 s following stimulation for the control treatment, and up to 25 s after the exposure to K^+ for the neuraminidase treated cells. In particular, from the end of the main sequence of peaks ($t = 15$ s) to 25 s, on average, 0.02 ± 0.05 (mean $\pm$ SD) events were recorded per cell per 0.2 ms time bin for the control treatment. The average number of peaks observed over this time range for the neuraminidase treated cells was significantly higher (0.1 ± 0.09 events are recorded per cell per 0.2 ms time bin, mean $\pm$ SD, $p < 0.001$, double-tailed Wilcoxon-Mann-Whitney rank-sum test). It would appear that this observation is a consequence of the facilitated K^+ diffusion through the partially digested glycocalyx. The presence of a higher concentration of K^+ in this region extends the duration of the cell membrane depolarization, thus triggering the fusion of vesicles for a longer period.

The peaks were analyzed as shown in Figure 1A. The corresponding exocytotic parameters are summarized in Table 1. No significant changes were observed when a low (0.1 U mL^{-1}) concentration of neuraminidase was used. However, all the parameters were significantly altered by the 1 U mL^{-1} neuraminidase treatment. Overall, the characteristic times (t_{rise} , $t_{1/2}$, t_{fall}) decreased, and i_p and n increased. The 28 % increase in n could not account for the larger (+124 %) increase in i_p , thus indicating that these changes mostly arise from faster diffusion kinetics in the partially digested glycocalyx. This is in good agreement with the fact that neuraminidase digestion of the glycocalyx increases by 207 % the albumin flux across glomerular endothelial cell monolayers.¹⁸

Furthermore, using an electrochemical cytometric setup⁴⁶ and post spike feet analysis,⁴⁷ it has been found that PC12 cells release 40 % of their vesicular content during exocytosis. In this case, the higher n measured at the electrode could be a consequence of the faster diffusion, facilitating the depletion of the vesicle during partial exocytotic release.

Foot currents, *i.e.* leakage currents recorded during the first milliseconds of the formation of the fusion pore,⁴⁷ have been observed in the data here. Only peaks with a foot current higher than 2 pA are discussed in this report. The feet have been analyzed according to the procedure presented on Figure 1B, and the results are summarized on Table 2. The probability to observe a foot is higher for the neuraminidase treated cells, as expected owing to the better signal to noise ratio induced by the faster diffusion, giving rise to higher amperometric currents. However, this increase is limited compared to the one observed for i_p . This can be explained by the fact that i_{foot} actually arises from a quasi-steady state flux established by a very narrow fusion pore, thus limiting the impact of the diffusion in the glycocalyx on the measured exocytotic peak. In comparison to the main part of the peak, the pore has a more restrictive influence on the concentration gradient, and controls mostly the exocytotic release. Hence, the role of the glycocalyx is limited here, and the digestion treatment has less impact. The duration of the foot, t_{foot} is not changed by the neuraminidase treatment. As this value is an indicator of the stability of the fusion pore

before it expands, thus giving rise to the body of the peak, this important result shows that the neuraminidase treatment does not alter the fusion dynamics of the pore. A characteristic of the pore, n_{foot} was not significantly altered, thus again indicating that neuraminidase does not interfere with the fusion pore.

The charge of the microenvironment hinders the kinetics of the exocytotic events

The effect of the electrostatic charges in the glycocalyx over the kinetics of exocytotic release was investigated by treating the cells with the positively charged protamine sulfate. The cells were exposed to $1 \mu\text{g mL}^{-1}$ protamine sulfate for 30 minutes, washed with HEPES buffer and exocytosis was triggered and detected as previously described. These results were compared to the ones obtained for cells incubated in HEPES buffer for one hour. Indeed, the duration of the incubation (30 minutes vs. 1 hour) was not found to produce any significant difference on the exocytotic data (data not shown, $p > 0.12$).

Despite the low ($1 \mu\text{g mL}^{-1}$) concentration of protamine sulfate used, the frequency of exocytosis was found to be decreased, and on average only 9 events were recorded per cells (12 cells, 108 peaks, see Figure 3C). Exocytotic events were observed only during the seconds following the onset of the stimulation (Figures 4C and D). At high concentrations ($< 40 \mu\text{g mL}^{-1}$), protamine sulfate is known to inhibit the activity of the ryanodine calcium receptors.³⁹ Such an effect, even in a limited capability, is expected to reduce the frequency of exocytosis. This observation could also indicate that the removal of the negative charges in the cell microenvironment decreases the effect of the K^+ stimulation, as this is thought to reduce the affinity of the positively charged K^+ for this matrix. Therefore, the treatment should decrease the local K^+ concentration at the vicinity of the cell membrane and hinder its ability to trigger exocytosis. An alternative explanation could be that the neutralization of the negative surface charges makes the membrane potential more negative, hence increasing the magnitude of the stimulation threshold required to elicit exocytosis.

The peak parameters obtained for the protamine sulfate treated cells are reported on Table 1. As with neuraminidase, protamine sulfate was found to increase the release kinetics of dopamine during exocytosis (Figure 3F), as indicated by the significant increase in i_p and the significant decreases in the characteristic times. Furthermore, some of these results are significantly different from the ones obtained after partial digestion of the glycocalyx with neuraminidase. In particular, the increase in i_p is more important in the case of the neuraminidase treatment. This fact indicates that the effect of neuraminidase is more potent in increasing the apparent diffusion coefficient of dopamine in the cell microenvironment than protamine sulfate. In good agreement with this finding, no significant change in n is observed after treatment with protamine sulfate, thus supporting the idea that only a large increase in diffusion coefficient can induce a higher depletion of the vesicle during exocytosis.

Again, no significant variation were observed in the foot parameters, indicating the dynamics of the geometry of the fusion pore are unaffected by this treatment (Table 2).

Diffusion dynamics

The measured current is a direct observation of the flux of analytes at the surface of the electrode. Fick's first law (Equation 1) establishes that this diffusive flux is proportional to the gradient of the concentration and to the diffusion coefficient D . The pore dynamics, and therefore the amount released, are not expected to be altered by neuraminidase or protamine sulfate, as shown by the limited increase in the number of molecules released and the constant foot values. Hence, comparing the control case to the neuraminidase or protamine sulfate treated case is similar to comparing the diffusion of a bolus of analytes released from

the pore in solutions of varying apparent diffusion coefficients, even though this apparent change in D arises from different causes (tortuosity of the matrix or electrostatic interactions).

Fick's first law indicates that the ratio of the measured current (*i.e.* the fluxes reaching the electrode) for the control and treated cells is equal to the ratio of the apparent diffusion coefficients in the intact or treated glycocalyx. The experimental data here indicates that the apparent diffusion coefficient is 2.2 times larger after partial digestion of the glycocalyx with neuraminidase (1 U mL⁻¹), and 1.4 times larger after removal of the static charges with protamine sulfate.

Even though the removal of the negative charges carried by the sialic acids may contribute to the apparent increase in diffusion coefficient observed after the neuraminidase treatment, this enzymatic digestion is more efficient than the exposure to protamine sulfate. This observation suggests that the activity of neuraminidase is not limited to reducing the electrostatic interactions, but that its main effect is to decrease the density and the tortuosity of the matrix.⁴⁸ This is in good agreement with the reported decrease in glycocalyx thickness after exposure to neuraminidase.¹⁷ Similarly, sialic acid moieties do not account for all the charges present in the glycocalyx. Incubation with neuraminidase is indeed known to decrease the electrophoretic mobility of cells,⁴⁹ but this effect could not be only attributed to the removal of the sialic acid moieties.⁵⁰ Hence, it can be assumed that these two treatments are mostly independent, and affect two different physico-chemical parameters (*i.e.* tortuosity of the matrix *vs.* electrostatic interactions). As neuraminidase and protamine sulfate, respectively, induce 2.2 and 1.4 increases in the apparent diffusion coefficient, probably mostly *via* independent phenomena, this indicates that the diffusion coefficient of dopamine at the vicinity of the cell surface is at least $2.2 \times 1.4 = 3.1$ times lower than in free buffer. This value is an under-estimate as the neuraminidase treatment does not completely remove the glycocalyx, but is still in good agreement with the previous finding that the diffusion coefficient of epinephrine is 8 times lower than in free solution at the vicinity of chromaffin cells.⁵¹ This result also fits well with the observation that exocytosis is slower than expected, when the diffusion coefficient in free buffer is used to analyze the data.^{40,52,53} This delay is usually attributed to the affinity of the neurotransmitters for the intravesicular dense core.^{54,55} The treatments used here are not expected to modify the intravesicular machinery. So, the intravesicular processes are not different from control, and their role cannot be clearly elucidated. As the treatments used in this report alter the dynamics of the exocytotic bolus once it has left the fused vesicle, it is hard to conclude anything about the contribution of the intravesicular components to the dynamics of exocytosis.

Biological significance

The composition and variation of the glycocalyx may be an important contributor to the maturity of the nervous system and to neurological pathologies.

Sialic acids are important components of the brain, involved in cognition⁵⁶ and aging.^{56–59} The total of concentration of sialic acid moieties is found to increase strongly in the early stages of the neonatal development, and to slightly decrease in the older individuals.⁶⁰ Electrochemical investigations of neuronal aging, comparing exocytosis at young and adult cells,^{61–63} have revealed variations in the kinetics of the release of neurotransmitters. In particular, the cells from young individuals, where the concentration of sialic acids is expected to be lower, have shown faster exocytotic kinetics. This is in very good agreement with the finding that exposure to neuraminidase, which is expected to cleave these sialic acids, increases the apparent diffusion coefficient of dopamine.

Additionally, the level of glycosylation⁶⁴ and the biochemistry of glycosaminoglycans⁶⁵ have been found to be involved in neurodegenerative diseases. Adhesion molecules, present in the synapse,^{66,67} may therefore play an important role in the mechanisms of these pathologies by altering the transport efficiency of the exocytotic bolus.

Conclusion

This report underlines the role of the glycocalyx in slowing down and hindering the bolus of analyte released during exocytosis. This fact has to be considered when analyzing the rate of exocytosis with single cell amperometry and should be important in models of neurotransmitter diffusion along the outer membrane in restricted environments like the synapse. Thus, the presence of the glycocalyx in the synapse is likely to be an important chemical factor controlling or hindering neuronal mediation at cell-to-cell interfaces.

Acknowledgments

We acknowledge the European Research Council (Advanced Grant), Knut and Alice Wallenberg Foundation, the Swedish Research Council (VR) and the National Institutes of Health (USA) for financial support.

References

1. Hay, ED. Collagen and other matrix glycoproteins in embryogenesis. In: Hay, ED., editor. Cell biology of extracellular matrix. Plenum Press; New York: 1991. p. 419-462.
2. Springer TA. Nature. 1990; 346:425-434. [PubMed: 1974032]
3. Kjær M. Physiol. Rev. 2004; 84:649-698. [PubMed: 15044685]
4. Ingber DE. Circ. Res. 2002; 91:877-887. [PubMed: 12433832]
5. Berrier AL, Yamada KM. J. Cell. Physiol. 2007; 213:565-573. [PubMed: 17680633]
6. Anstee DJ. J. Immunogenet. 1990; 17:219-225. [PubMed: 2093725]
7. Mager MD, LaPointe V, Stevens MM. Nat. Chemistry. 2011; 3:582-589.
8. Giebel KF, Bechinger C, Herminghaus S, Riedel M, Leiderer P, Weiland U, Bastmeyer M. Biophys. J. 1999; 76:509-516. [PubMed: 9876164]
9. Izzard CS, Lochner LR. J. Cell Sci. 1976; 21:129-159. [PubMed: 932106]
10. Izzard CS, Lochner LR. J. Cell Sci. 1980; 42:81-116. [PubMed: 7400245]
11. Lo CM, Glogauer M, Rossi M, Ferrier J. Eur. Biophys. J. 1998; 27:9-17. [PubMed: 9463886]
12. Pierres A, Benoliel AM, Touchard D, Bongrand P. Biophys. J. 2008; 94:4114-4122. [PubMed: 18234815]
13. Zeck G, Fromherz P. Langmuir. 2003; 19:1580-1585.
14. Barker AL, Konopatskaya O, Neal CR, Macpherson JV, Whatmore JL, Winlove CP, Unwin PR, Shore AC. Phys. Chem. Chem. Phys. 2004; 6:1006-1011.
15. Varghese JN, Laver WG, Colman PM. Nature. 1983; 303:35-40. [PubMed: 6843658]
16. Kim CU, Lew W, Williams MA, Liu H, Zhang L, Swaminathan S, Bischofberger N, Chen MS, Mendel DB, Tai CY, Graeme Laver W, Stevens RC. J. Am. Chem. Soc. 1997; 119:681-690. [PubMed: 16526129]
17. Foa C, Soler M, Benoliel AM, Bongrand PJ. Mater. Sci.- Mater. 1996; 7:141-148.
18. Singh A, Satchell S, Neal C, McKenzie E, Tooke J, Mathieson P. Journal of the American Society of Nephrology. 2007; 18:2885-2893. [PubMed: 17942961]
19. Nanavati C, Markin V, Oberhauser A, Fernandez J. Biophys. J. 1992; 63:1118-1132. [PubMed: 1420930]
20. De Toledo GA, Fernandez-Chacon R, Fernandez J. Nature. 1993; 363:554-558. [PubMed: 8505984]
21. Oberhauser AF, Robinson IM, Fernandez JM. Biophys. J. 1996; 71:1131. [PubMed: 8842250]
22. Heuser JE, Reese TS, Dennis MJ, Jan Y, Jan L, Evans LJ. Cell Biol. 1979; 81:275-300.

23. Lin Y, Trouillon R, Safina G, Ewing AG. *Anal. Chem.* 2011; 83:4369–4392. [PubMed: 21500835]
24. Wightman RM, Jankowski JA, Kennedy RT, Kawagoe KT, Schroeder TJ, Leszczyszyn DJ, Near JA, Diliberto EJ, Viveros OH. *Proc. Natl. Acad. Sci. U.S.A.* 1991; 88:10754–10758. [PubMed: 1961743]
25. Trouillon R, Passarelli MK, Wang J, Kurczy ME, Ewing AG. *Anal. Chem.* 2013; 85:522–542. [PubMed: 23151043]
26. Colliver TL, Pyott SJ, Achalabun M, Ewing AG. *J. Neurosci.* 2000; 20:5276–5282. [PubMed: 10884311]
27. Amatore C, Arbault S, Bouret Y, Guille M, Lemaître F, Verchier Y. *ChemBioChem.* 2006; 7:1998–2003. [PubMed: 17086558]
28. Uchiyama Y, Maxson MM, Sawada T, Nakano A, Ewing AG. *Brain Res.* 2007; 1151:46–54. [PubMed: 17408597]
29. Amatore C, Arbault S, Bonifas I, Lemaître F, Verchier Y. *ChemPhysChem.* 2007; 8:578–585. [PubMed: 17243189]
30. Sombers LA, Wittenberg NJ, Maxson MM, Adams KL, Ewing AG. *ChemPhysChem.* 2007; 8:2471–2477. [PubMed: 17966970]
31. Haynes CL, Siff LN, Wightman RM. *BBA- Mol. Cell Res.* 2007; 1773:728–735.
32. Mosharov EV, Sulzer D. *Nat. Methods.* 2005; 2:651–658. [PubMed: 16118635]
33. Amatore C, Oleinick AI, Svir I. *ChemPhysChem.* 2009; 11:159–174. [PubMed: 19937905]
34. Sombers LA, Hanchar HJ, Colliver TL, Wittenberg N, Cans AS, Arbault S, Amatore C, Ewing AG. *J. Neurosci.* 2004; 24:303–309. [PubMed: 14724228]
35. Sunnergren KP, Fairman RP, Glauser FL. *J. Appl. Physiol.* 1987; 63:1987–1992. [PubMed: 3693232]
36. Swanson JA, Kern DF. *Am. J. Physiol.- Heart Circ. Physiol.* 1994; 266:H1300–H1303.
37. Ueda A, Shimomura M, Ikeda M, Yamaguchi R, Tanishita K. *Am. J. Physiol.- Heart Circ. Physiol.* 2004; 287:H2287–H2294. [PubMed: 15256377]
38. Adams KL, Engelbrektsson J, Voinova M, Zhang B, Eves DJ, Karlsson R, Heien ML, Cans AS, Ewing AG. *Anal. Chem.* 2009; 82:1020–1026. [PubMed: 20039639]
39. Koulen P, Ehrlich BE. *Mol. Biol. cell.* 2000; 11:2213–2219. [PubMed: 10888663]
40. Wightman RM, Schroeder TJ, Finnegan JM, Ciolkowski EL, Pihel K. *Biophys. J.* 1995; 68:383–390. [PubMed: 7711264]
41. van Kempen GTH, Vanderleest HT, Van Den Berg RJ, Eilers P, Westerink RHS. *Biophys. J.* 2011; 100:968–977. [PubMed: 21320441]
42. Mosharov, EV. Exocytosis and Endocytosis. In: Ivanov, A., editor. *Methods in Molecular Biology.* Vol. Vol. 440. Humana Press; 2008. p. 315–327.
43. Sulzer D, Pothos EN. *Rev. Neurosci.* 2000; 11:159–212. [PubMed: 10718152]
44. Crank, J. *The mathematics of diffusion.* 2nd Ed. Clarendon Press; Oxford, UK: 1979.
45. Schroeder TJ, Jankowski JA, Senyshyn J, Holz RW, Wightman RMJ. *Biol. Chem.* 1994; 269:17215–17220.
46. Omiatek DM, Dong Y, Heien ML, Ewing AG. *ACS Chem. Neurosci.* 2010; 1:234–245. [PubMed: 20368748]
47. Mellander LJ, Trouillon R, Svensson MI, Ewing AG. *Sci. Rep.* 2012; 2 DOI: 10.1038/srep00907.
48. Mackie JS, Meares PP. *Roy. Soc. Lond. A Mat.* 1955; 232:498–509.
49. Mayhew E. *J. Gen. Physiol.* 1966; 49:717–725. [PubMed: 5221375]
50. Wallach DF, De Perez Esandi MV. *Biochim. Biophys. Acta.* 1964; 83:363–366. [PubMed: 14236715]
51. Hafez I, Kisler K, Berberian K, Dernick G, Valero V, Yong MG, Craighead HG, Lindau M. *Proc. Natl. Acad. Sci. U.S.A.* 2005; 102:13879–13884. [PubMed: 16172395]
52. Chow RH, von Rüden L, Neher E. *Nature.* 1992; 356:60–63. [PubMed: 1538782]
53. Schroeder TJ, Borges R, Finnegan JM, Pihel K, Amatore C, Wightman RM. *Biophys. J.* 1996; 70:1061–1068. [PubMed: 8789125]
54. Nanavati C, Fernandez JM. *Science.* 1993; 259:963–965. [PubMed: 8438154]

55. Montesinos MS, Machado JD, Camacho M, Diaz J, Morales YG, de la Rosa DA, Carmona E, Castañeyra A, Viveros OH, O'Connor DT, Mahata SK, Borges RJ. *Neurosci.* 2008; 28:3350–3358.
56. Wang B. *Ann. Rev. Nutr.* 2009; 29:177–222. [PubMed: 19575597]
57. Sato Y, Endo T. *Geriat. Geront. Int.* 2010; 10:S32–S40.
58. Finne J. J. *Biol. Chem.* 1982; 257:11966–11970. [PubMed: 7118922]
59. Uslu E, Karagöz Güzey F, Oguz E, Güzey D. *Act. Neurochir.* 2004; 146:1337–1340.
60. Svennerholm L, Boström K, Fredman P, Månsson JE, Rosengren B, Rynmark BM. *BBA-Lipid Lipid Met.* 1989; 1005:109–117.
61. Zanin MP, Phillips L, Mackenzie KD, Keating DJ. *PloS one.* 2011; 6:e27820. [PubMed: 22125627]
62. Elhamdani A, Palfrey CH, Artalejo CR. *Neurobiol. Aging.* 2002; 23:287–293. [PubMed: 11804714]
63. Patel BA, Arundell M, Parker KH, Yeoman MS, O'Hare D. *Phys. Chem. Chem. Phys.* 2010; 12:10065–10072. [PubMed: 20625576]
64. Ohtsubo K, Marth JD. *Cell.* 2006; 126:855–867. [PubMed: 16959566]
65. Parnetti, L.; Cornelli, U. Glycosaminoglycans and Analogs in Neurodegenerative Disorders. In: Memo, M.; Stocchi, F.; Hanin, I., editors. *Advances in Alzheimers and Parkinsons Disease.* Springer; New York: 2008. p. 231-245.
66. Yamagata M, Sanes JR, Weiner JA. *Curr. Opin. Cell Biol.* 2003; 15:621–632. [PubMed: 14519398]
67. Dityatev A, Schachner M. *Nat. Rev. Neurosci.* 2003; 4:456–468. [PubMed: 12778118]

Figure 1. Peak analysis. A) Scheme showing the different peak parameters: i_p , t_{rise} , $t_{1/2}$, t_{fall} and the number of molecules released n (here shown as the gray area); B) parameters of the foot: t_{foot} , i_{foot} , and the number of molecules released during the foot n_{foot} (here shown as the gray area).

Figure 2. Peak dynamics in partially denaturated glycocalyx. In comparison to the intact glycocalyx (dark blue), the apparent diffusion coefficient and the flux of neurotransmitters are increased in the case of the looser glycocalyx (light blue). This results in the sharpening of the peak (see Equations 1 and 2). The schematic is not drawn to scale.

Figure 3.

Typical amperometric traces and corresponding averaged peaks obtained for a single K⁺ stimulated PC12 cell (A, D: control; B, E: after a 1 hour pre-incubation with 1 U mL⁻¹ neuraminidase; C, F: after a 30 min incubation with 1 μg mL⁻¹ protamine sulfate). The black bars indicate the 5 s stimulation.

Figure 4.

Average number of peaks N recorded per cell per 0.2 s bin at A) control cells (12 cells, 395 peaks), B) neuraminidase-treated cells (1 U mL^{-1} , 15 cells, 646 peaks), and C) protamine sulfate-treated cells (12 cells, 108 peaks). The black bars indicate the 5 s stimulation. The average number of peaks released per 0.2 s bin obtained for the control and neuraminidase treatments were averaged for $t = 15 \text{ s}$ to $t = 25 \text{ s}$. These results were tested for significant difference (double-tailed Wilcoxon-Mann-Whitney rank-sum test, ***: $p < 0.001$). D) Cumulated number of events obtained per cell as a function of time obtained for the three treatments.

Table 1

Peak parameters obtained from K⁺ stimulated PC12 cells. The data is presented as median (1st quartile-3rd quartile). The pairs of datasets were compared using a two-tailed Wilcoxon-Mann-Whitney rank-sum test, ***: $p < 0.001$. The variations of the median in comparison to the control (or to the 1 U mL⁻¹ neuraminidase treatment in the case of the exposure to protamine sulfate) are also reported

	i_p / pA	t_{rise} / ms	$t_{1/2}$ / ms	t_{fall} / ms	$n/10^3$ molecules
Control (1 hour, 12 cells, 345 peaks)	5.9 (3.7-10.9)	0.7 (0.5-1.8)	3.2 (2.3-4.5)	2.7 (1.9-4.2)	99 (65-148)
Neuraminidase, 0.1 U mL⁻¹ (12 cells, 419 peaks)	5.4 (3.8-8.7)	0.7 (0.5-1.4)	3.0(2.1-4.2)	2.6(1.7-3.9)	92 (61-131)
vs Control	-8 %	-2%	-6%	-4 %	-7 %
Neuraminidase, 1 U mL⁻¹ (15 cells, 646 peaks)	13.2 (7.8-28.9)	0.4 (0.3-0.8)	1.7(1.1-2.6)	1.2 (0.7-2.3)	127 (83-200)
vs Control	+ 124 %***	-39 %***	-46 %***	-55 %***	+28 %***
Protamine sulfate, 1 μg mL⁻¹ (12 cells, 108 peaks)	8.2(4.5-18.3)	0.4 (0.3-0.8)	1.7 (1.0-2.9)	1.4 (0.7-2.3)	85 (43-144)
vs Control	+39 %***	-38 %***	-45 %***	-48 %***	-14%
vs Neuraminidase, 1 U mL ⁻¹	-38 %***	-18 %***	+ 19 %	+15 %	-33 %***

Table 2

Foot parameters obtained from K⁺ stimulated PC12 cells. The data is presented as median (1st quartile-3rd quartile). The pairs of datasets were compared using a two-tailed Wilcoxon-Mann-Whitney rank-sum test, ***: $p < 0.001$. The variations of the median in comparison to the control are also reported

	i_{foot} / pA	t_{foot} / ms	$n_{foot} / 10^3 \text{ molecules}$	Peaks with a foot
Control (1 hour, 12 cells, 345 peaks)	2.5 (2.1-2.8)	2.9 (1.5-4.2)	26 (13-35)	6%
Neuraminidase, 0.1 U mL⁻¹ (12 cells, 419 peaks)	2.9 (2.3-3.4)	3.3 (2.5-5.0)	38 (29-50)	5%
vs Control	+ 15 %	+13 %	+43 %	
Neuraminidase, 1 U mL⁻¹ (15 cells, 646 peaks)	2.8 (2.4-3.7)	1.7 (1.0-3.0)	20(11-35)	30 %
vs Control	+ 13 %***	-41 %	-22 %	
Protamine sulfate, 1 $\mu\text{g mL}^{-1}$ (12 cells, 108 peaks)	2.5 (2.3-3.7)	2.1 (1.5-3.2)	24 (15-40)	14%
vs Control	-2 %	-27 %	-9 %	