Analysis of Exocytotic Events Recorded by Amperometry

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Supplementary Methods

Digital filters

Gaussian filters are similar to Bessel filters (albeit much easier to program) in that they both have constant group delay and do not produce ripples in the time domain[1,2]. We employ two types of digital low-pass Gaussian filters, a genuine infinite impulse response (IIR) Gaussian filter and a finite impulse response (FIR) binomial smoothing. For Gaussian IIR filter, the fast Fourier transform of an amperometric trace is multiplied by a Gaussian of selected width to achieve -3dB attenuation of frequency at a desired cutoff (F_c, Hz). The advantage of this filter is that its corner frequency can be adjusted precisely in a wide range of values. The Gaussian is computed as:

$$G(x) = \exp\left(-\frac{x^2}{\sigma^2}\right); \text{ where } \sigma = \frac{Ampl_{cutoff}}{\sqrt{-\ln(F_c)}} \text{ and } Ampl_{cutoff} = \frac{1}{\sqrt{2}}$$

Binomial FIR filter convolves the data with normalized coefficients derived from Pascal´s triangle at a level equal to the smoothing parameter[3]. Since this filter does not employ a Fourier transforms of the data, it requires much less computation time and is easier to use with large data files, such as neuronal amperometric traces that are recorded at a high sampling rate and may consist of millions of data-points. As a FIR filter, binomial smoothing has a more stable frequency response that an IIR Binomial filter. The following empirical formula can be used to express the level of binomial smoothing from Igor Pro (*Coeff*, the half-width of the base of Pascal's triangle) in the units of cutoff frequency:

$F_c[Hz] = A \times SR \times Coeff^B$, where A = 0.1809, B = -0.4815 and SR is sampling rate in Hz.

As seen from the formula, the dependence between the binomial coefficients and desired F_c is a power function. This and the fact that *Coeff* is an integer create the major drawbacks of this filter: it has an F_c limit (Binomial 1 smoothing), and its steps can become too wide at higher frequencies making it difficult to accurately adjust the cutoff.

The choice of an 'optimal' filter for a particular type of recording is a complicated issue[2]. The two-step filtering protocol shown in **Box 1** of the manuscript, however, simplifies the task by allowing to choose one filter (F_{c1} -3dB cutoff frequency higher than the signal frequency) that does not distort spike shapes and another one (lower F_{c2}) that increases signal-to-noise ratio sufficiently for events detection. The initial estimate for an appropriate filter cutoffs can be made from the maximum of the first derivative of amperometric current[4], which corresponds to a spike with the fastest rising slope. The ratio of dI/dt amplitude to the amplitude of the corresponding spike on the non-differentiated trace (the latter is roughly estimated as delta current between two time-points at the intercepts of dI/dt with zero to the left and to the right of dI/dt maximum) represents twice the frequency of this 'fastest' spike (F_{sp}(max)). Next, 2*F_{sp}(max) and 0.5*F_{sp}(max) can be used as the initial F_{c1} and F_{c2}, correspondingly. In practice, because of the presence of very high frequency noise, F_{sp}(max) values found on an unfiltered amperometric trace are often overestimated for relatively slow spikes found on LDCV recordings. To counter this, the routine incorporated in our program filters the recording with 3*F_{sp}(max) Gaussian filter, which approximately equals the cutoff of a filter that will not affect the amplitude of the spike with a width =1/F_{sp}(max) [2], and then repeats the procedure for finding the F_{sp}(max) described above. Finally, if the differentiated trace is used for spike detection, the differential is filtered at F_{c3} = F_{sp}(max), as dI/dt frequency is approximately two fold higher than that of the non-differentiated trace.

Although this procedure helps to choose initial values for the filters cutoffs, further adjustments are usually required, including visual examination of a trace generated by subtracting the recording before and after the filtering - appearance of profound maxima on this trace indicates reduction of signal amplitude. It should be emphasized that all amperometric traces in an experimental series have to be filtered using the same set of filters. Therefore, the choice of the filters should be made prior to analysis using representative recordings from all experimental groups and the filter with the highest cutoff frequency should be used for all traces. Empirical values for LDCV and SSV recordings are as follows. For amperometric recordings from rat chromaffin cell (sampling frequency 10-25 kHz): F_{sp}(max) = 200-1000 Hz, F_{c1} = 500-1000 Hz, F_{c2} = 100-400 Hz, F_{c3} = 200-1000 Hz. For recordings from dopamine midbrain neurons: (sampling frequency 100-200 kHz): F_{sp}(max) = 5-10 kHz, F_{c1} = 10-20 kHz, F_{c2} = not used, F_{c3} = 5-10 kHz.

Algorithms for finding spike baseline

The first parameter determined for a spike, the location of its maximum T_{max}, can be reliably evaluated by a protocol described by Borges's laboratory where it is found between $T(dI/dt)_{max}$ and the time point having the same current value on the descending segment of the spike[5] (**Supplementary Fig. 1a**, points *1* and *1'*). To improve the accuracy of T_{max} approximation on spikes with low signal-to-noise ratio, later in the analysis the whole spike or its topmost segment can be fit with exponentially modified Gaussian curve[6] (**Supplementary Fig. 1b**).

If spikes are detected using SD_I and the background current is stable with an average value I_{bkg}, the algorithms for finding spike beginning (T_{bkg1}), and end (T_{bkg2}) are straightforward - these are the first time-points to the left and to the right of T_{max} at the I_{bkg} current level[7]. Similarly, if dI/dt is used for spike detection, in the simplest scenario, T_{bkg1} is

at the interception of dI/dt with zero to the left of $T(dI/dt)_{max}$ (**Supplementary Fig. 1a**, point *2*) and T_{bkg2} is the time-point at the same as T_{bkg1} current level to the right of T_{max}. The variability of possible spikes shapes and arrangements and the presence of noise however make it rather difficult to define spike baseline in all cases in a completely user-independent fashion. While additional algorithms presented below help to account for some possible complications during analysis, it is always advisable to visually examine the spikes and readjust their baselines if necessary.

The first algorithm improves baseline positioning on spikes with low signal/noise or those having slow-rising PSF, when dI/dt=0 fails to represent the true T_{bkg1} (**Supplementary Fig. 1c**). The program searches for a steady state that persists for certain duration ΔT_{min}, by first dividing the trace into ΔT_{min} segments and then comparing the average current values between the adjacent segments. Searching from T_{max} or $T(dI/dt)_{max}$ and reiteratively moving to the left, the steady state is found if two segments have the currents within one SD_I of each other. Next, T_{bkg1} is set to the first time-point at the steady state current. Spike width at $T(dI/dt)_{max}$ can be used as the initial ΔT_{min} value and it is essential to repeat the procedure with $2*\Delta T_{min}$ or longer increments to account for the presence of PSF with a steady state. If longer increments result in lower steady state values, the latter should be used for T_{bkg1} calculation (**Supplementary Fig. 1d**). Obviously, some PSF with extremely long steady states may still be missed by this routine and their baseline should be readjusted manually. Additionally, it should be noted that this method employs SD_I to find the steady state and therefore is dependent on the degree of filtering applied to the trace.

When determining T_{bkg2}, an increasing contribution of high-frequency noise complicates the assessment of the time-point when the transmitter concentration reaches background

levels (**Supplementary Fig. 1e**). For the smallest spikes, this may underestimate the amperometric charge by 10-20% and introduce as much as a two-fold error in the exponential decay constants of the falling phase fits. To counter this, the search for T_{bkg2} can be performed on the trace additionally filtered with F_{c2} binomial filter (see above), which does not affect low frequencies present in the decaying phase of the spike but substantially decreases the artifacts associated with the presence of white noise.

Another difficulty that may interfere with proper baseline positioning is the presence of background drifts, such as in overlapping spikes; even if the overlaps will be discarded during the analysis, first they need to be recognized as such (see *Analysis of overlapping spikes* in the main text). While spikes with uneven baseline may not be preceded by steady states and the current at their baseline may not be at the I_{bkg} level, the beginning of these events can be found at dI/dt=0 (**Supplementary Fig. 1f**, spike 2). Similarly, the presence of background drifts may complicate the search for T_{bkg2} since it might not be at the same current level as T_{bkg1} or within one SD_I of this value (**Supplementary Fig. 1f**, spike 1). A simple solution that works in this case is to set T_{bkg2} to the minimum of current that follows the spike. This minimum is found between the spike's T_{max} and the next spike maximum (if found) or the end of the trace. Although the above protocols for finding the baselines of the overlapping spikes are not very accurate on traces with high noise levels, their purpose is only to locate such events. Subsequently, the overlaps are either discarded or analyzed by separating them (**Supplementary Fig. 3** online). In the latter case, the beginning and the end of the overlap-containing event are refined using the same algorithms as for a singlet spike: T_{bkg1} is at the steady state current to the left of the first spike, and T_{bkg2} is at the same as T_{bkg1} current to the right of the last spike.

Primary cell cultures

Chemicals were purchased from Sigma (Milwaukee, WI). Rat chromaffin cells were prepared and cultured as previously described[8]. Cells were maintained in a 5% CO_2 incubator at 37°C. All measurements were conducted between days 3-4 post-plating. Animal protocols were approved by the Columbia University Institutional Animal Care and Use Committees.

Amperometric recordings

Solutions used for amperometric recordings were as follows. The bath saline contained (in mM): 128 NaCl, 2 KCl, 1 NaH_2PO_4, 2 $MgCl_2$, 1.2 $CaCl_2$, 10 glucose, 10 HEPES (pH 7.4). Stimulation solution was the same except for 90 NaCl and 40 KCl. A 5 μm diameter carbon fiber electrode held at +700 mV was positioned over a cell (Newport micromanipulator MX300R, Irvine, CA) and lowered until the latter was slightly depressed. No events were recorded when the applied voltage was adjusted to 0 mV or when the electrode was transiently lifted from the cell. The current was filtered using a 4-pole 5 kHz Bessel filter built into an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) and sampled at 25 kHz (ITC-18, Instrutech, Great Neck, NY). Secretagogue was applied by local perfusion through a pressurized glass micropipette (Picospritzer, General Valve Corp., Fairfield, NJ) for 5 sec at ~10 μm from the cell. After secretagogues application, the amperometric current was recorded for 60 sec using a locally written routine in Igor Pro (Wave Metrics, Lake Oswego, OR). Data files were saved in Igor binary format for further analysis.

For catecholamine release, it is convenient to present the quantal size as the number of released molecules, N, which is related to electric charge, Q, as $N = Q / (n \times F)$, where F is Faraday's constant and n is the number of electrons donated by each catechol moiety[9,10]:

$$N = Q\left[pC\right] \times 10^{-12}\left[\frac{C}{pC}\right] \times \frac{6.023 \times 10^{23}\left[\frac{electrons}{mole}\right]}{2\left[\frac{electrons}{molecule}\right] \times 96{,}485\left[\frac{C}{mole}\right]} = Q\left[pC\right] \times 3.121 \times 10^{6}\left[\frac{molecule}{pC}\right]$$

Statistical analysis

Datasets in the tables were compared by Mann-Whitney rank sum test. The goodness of Gaussian fits was analysed by Wald-Wolfowitz (runs) test in Prism 4 (GraphPad Software Inc.).

1. Heinemann, S.H. in *Single-Channel Recording* (eds. Sakmann, B. & Neher, E.) 53-91 (Plenum Press, New York, 1995).
2. Colquhoun, D. & Sigworth, F.J. in *Single-Channel Recording* (eds. Sakmann, B. & Neher, E.) 483-587 (Plenum Press, New York, 1995).
3. Marchand, P. & Marmet, L. Binomial smoothing filter: A way to avoid some pitfalls of least-squares polynomial smoothing. *Rev. Sci. Instrum.* **54**, 1034-1041 (1983).
4. Gomez, J.F. *et al.* New approaches for analysis of amperometrical recordings. *Ann. NY Acad. Sci.* **971**, 647-654 (2002).
5. Segura, F. *et al.* Automatic analysis for amperometrical recordings of exocytosis. *J. Neurosci. Methods* **103**, 151-156 (2000).
6. Schroeder, T.J. *et al.* Temporally resolved, independent stages of individual exocytotic secretion events. *Biophys. J.* **70**, 1061-1068 (1996).
7. Chow, R.H. & von Ruden, L. in *Single-channel recording* (eds. Sakmann, B. & Neher, E.) 245-276 (Plenum Press, New York, 1995).
8. Mosharov, E. *et al.* Intracellular Patch Electrochemistry: Regulation of Cytosolic Catecholamines in Chromaffin Cells. *J. Neurosci.* **23**, 5835–5845 (2003).
9. Kissinger, P.T., Hart, J.B. & Adams, R.N. Voltammetry in brain tissue-a new neurophysiological measurement. *Brain Res.* **55**, 209-213 (1973).

10. Baur, J.E. *et al.* Fast-scan voltammetry of biogenic amines. *Anal. Chem.* **60**, 1268-1272 (1988).