Synaptic activity slows vesicular replenishment at excitatory synapses of rat hippocampus

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Abstract Short-term synaptic depression mainly reflects the depletion of the readily releasable pool (RRP) of quanta. Its dynamics, and especially the replenishment rate of the RRP, are still not well characterized in spite of decades of investigation. Main reason is that the vesicular storage and release system is treated as time-independent. If it is time-dependent all parameters thus estimated become problematic. Indeed the reports about how prolonged stimulation affects the dynamics are contradictory. To study this, we used patterned stimulation on the Schaffer collateral fiber pathway and model-fitting of the excitatory post-synaptic currents (EPSC) recorded from CA1 neurons in rat hippocampal slices. The parameters of a vesicular storage and release model with two pools were estimated by minimizing the squared difference between the EPSC amplitudes and simulated model output. This yields the ‘basic’ parameters (release coupling, replenishment coupling and RRP size) that underlie the ‘derived’ and commonly used parameters (fractional release and replenishment rate). The fractional release increases when $[Ca^{++}]_o$ is raised, whereas the replenishment rate is $[Ca^{++}]_o$ independent. Fractional release rises because release coupling increases, and the RRP becomes less able to contain quanta. During prolonged stimulation, the fractional release remains generally unaltered, whereas the replenishment rate decreases down to $\sim 10\%$ of its initial value with a decay time of ~ 15 s, and this decrease in the replenishment rate significantly contributes to synaptic depression. In conclusion, the fractional release is $[Ca^{++}]_o$ -dependent and stimulation-independent, whereas the

replenishment rate is $[Ca^{++}]_o$ -independent and stimulation-dependent.

Keywords Synaptic depression · Time-dependent system · Model fitting · Replenishment rate · Vesicular mobility

Introduction

Synapses can be plastic, their efficacy can change, and such plasticity is believed to be the basis of learning and memory. One of the most important brain areas related to learning and memory—hippocampus (Martin et al. 2000; Kaneki et al. 2009) has been the main model system for studying the synaptic plasticity. Two well-known phenomena of synaptic plasticity, long-term potentiation and long-term depression, which occur in response to tetanic stimuli of high and low frequencies have been first reported in hippocampus (Bliss and Lomo 1973; Lynch et al. 1977). Many other aspects of synaptic plasticity—the relation between spike-timing dependent plasticity and Ca^{2+} dynamics (Aihara et al. 2007), the spatiotemporal characteristics of synaptic EPSP summation (Yoneyama et al. 2011) and the interaction between the spatio-temporal learning rule and Hebb type (Tsukada et al. 2007) have been studied in hippocampus. A change in synaptic efficacy may occur over very different time scales, ranging from milliseconds to hours or days.

In this study we focus on short-time changes of synaptic efficacy. It has been shown long ago in neuro-muscular junction that the synaptic efficacy decreases during high frequency stimulation (Del Castillo and Katz 1954), and in many secretory cells the depletion model can be used as the basis for synaptic depression analysis (Kavalali 2007; Schneggenburger et al. 2002; Zucker and Regehr 2002).

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As the readily releasable pool (RRP) becomes depleted, its replenishment becomes a more important component of synaptic transmission (Birks and MacIntosh 1961; Larkman et al. 1991). The replenishment rate, which influences the fast component of synaptic depression and determines the steady state level, is typically unknown. Estimating the replenishment rate is difficult. In previous studies the assumption was made that the pool sizes are constant (Wu and Betz 1998) and that the replenishment rate remains constant with stimulation (Dittman and Regehr 1998; Sen et al. 1996), but the validity of these assumptions is problematic.

The ‘mobilization by liberation’ model holds that neuropeptide vesicles move slowly at rest, but become highly mobile following stimulation and Ca^{++} entry (Shakiryanova et al. 2007). It is unclear if the ‘mobilization by liberation’ model holds true at other types of synapses. However, some kinetic studies suggest that vesicular replenishment in fast central synapses may indeed occur via this model. Following high frequency stimulation, the recovery of excitatory post-synaptic current (EPSC) amplitudes is enhanced (Stevens and Wesseling 1998; Wang and Kaczmarek 1998). This suggests that the recovery is faster due to the accumulation of $[\text{Ca}^{++}]_i$, but it is unclear which parameters of the vesicular storage and release system are affected. For example, is enhanced recovery due to an increase in fractional release, an increase in replenishment rate, or some combination of both? Evaluating how $[\text{Ca}^{++}]_o$ and stimulation modulate the parameters of the system would clearly advance our understanding of vesicular replenishment.

In this study we use model-fitting to estimate what the parameters of the vesicular storage and release system are and how they change with stimulation or at elevated $[\text{Ca}^{++}]_o$. We fit an electric circuit model of vesicular dynamics to the EPSC amplitudes recorded in rat hippocampal pyramidal neurons. Such electric circuit models have been used for simulation in physics, chemistry, and biology (Kron 1948). They encapsulate the dynamics within a compact description, which is an important advantage when comparing storage and release properties across different synaptic types and from different species. This model assumes that the fluxes are driven by the difference in pool densities (i.e. vesicles move down their concentration gradient; Bielecki et al. 2008; Kruckenberg and Sandweg 1968; Li and Schwarz 1999). If the replenishment of the RRP is associated with vesicular movement, the flux described by this model will represent the random movement of vesicles. The vesicular fluxes that move against their concentration gradient (such as directed movement) were however not neglected, but captured as the ‘open system’ contribution (see “Methods”).

A preliminary account has appeared as an abstract (Krnjevic et al 2007).

Methods

Solutions and recordings

All procedures were in accordance with the regulations of McGill Animal Care. Post-natal (100–125 g) male Sprague–Dawley rats were decapitated under isoflurane anaesthesia. The brain was rapidly removed and immersed in oxygenated (96 % O_2 , 5 % CO_2) ice-cold artificial cerebrospinal fluid (ACSF) of the following composition (mM): NaCl 125, KCl 3, CaCl_2 2, MgCl_2 1.3, NaHCO_3 26, NaH_2PO_4 1.25 and glucose 10, and gassed with 95 % O_2 and 5 % CO_2 (pH 7.2–7.4). Hippocampal slices (400–450 μm thick) were cut using a vibrating microtome (Campden, United Kingdom) and incubated at room temperature in ACSF for at least 1 h before use. The slices were subsequently transferred to a recording chamber in which they were kept fully submerged in ACSF at 20–22 °C, flowing at 3–4 ml/min. Patch pipettes were filled with (mM): CsCl 130, NaCl 10, ATP-Mg 3, GTP 0.3, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid HEPES 10, ethyl-enebis(okonitrib) tetracetate 10; pH was adjusted to 7.2–7.3 with NaOH. Cesium was chosen as the main cation in order to block potassium conductances. DL-2-amino-5-phosphonovaleric acid (10 μM) was used to block NMDA-activated channels, and to suppress inhibitory (g-aminobutyric acid type A) synaptic currents bicuculline methiodide (10 μM) was added at least 10 min before recordings started.

EPSCs were recorded “blindly” at 20–22 °C (Blanton et al. 1989), in the deeper areas of the pyramidal cell layer of the CA1 region with whole-cell patch-clamp electrodes and an Axopatch-1D amplifier (Axon Instruments, USA). The holding potentials ranged from –110 to –70 mV. The recordings were anti-alias filtered with a Bessel filter whose corner frequency was set at 5 kHz. The series resistance ranged from 3 to 10 M Ω . EPSCs were digitized and recorded in real time on a PC, using a BNC 2120 interface card (National Instruments, USA). The sampling rate was 10 kHz. The interface card was also used to provide the timing pulses for the stimulus isolation unit and subsequent activation of the Schaffer collateral/commisural pathway. Various stimulation inputs were used—continuous stimulation of chosen frequency (1 or 10 Hz), a pair of high-low frequency trains (5–10 and 2.5–5 Hz trains, each lasting 5 s, or as specified), patterned stimulation (an alternating sequence of 10 and 5 Hz trains, each lasting 5 s and repeated 5–10 times).

Electrical circuit model of release

We simulate the dynamics of vesicular storage using an electrical circuit model (Kruckenberg and Sandweg 1968) with two vesicular pools—the RRP (C_1) and resting pool (RP; C_2). The differential equations which describe the system dynamics during release (i.e. when the switch is closed) are:

$$\frac{dU_1}{dt} = -\left(\frac{1}{R_1 \cdot C_1} + \frac{1}{R_0 \cdot C_1}\right) \cdot U_1 + \frac{1}{R_1 \cdot C_1} \cdot U_2 \quad (1)$$

and

$$\frac{dU_2}{dt} = \frac{1}{R_1 \cdot C_2} \cdot U_1 - \left(\frac{1}{R_2 \cdot C_2} + \frac{1}{R_1 \cdot C_2}\right) \cdot U_2 + \frac{1}{R_2 \cdot C_2} \cdot E. \quad (2)$$

The variables and parameters of the electrical model and those of the vesicular storage are related as follows. The capacitor size C indicates the ability of the pool to store vesicles (it gives the pool size), the charge Q gives the content (the number of vesicles in the pool), and the voltage U across the capacitor the vesicular density in that pool. If one vesicle corresponds to a charge q , there are n ($n = (C \cdot U)/q$) vesicles in the pool. The vesicular concentration difference between two pools (RP and RRP) drives the replenishment of the RRP, which is controlled by the replenishment rate of RRP. Likewise, the vesicular synthesis (shown in Fig. 1a as the battery E) drives the replenishment of the RP, which is controlled by the replenishment rate of RP. The release from the RRP (assuming no replenishment of the pool) at any time I_t is governed by the equation $I_0 \cdot \exp -t/(R_0 \cdot C_1)$, where I_0 is the release at time zero.

All parameters used to characterize vesicular storage and release by the rate-based models are also defined in the electrical model of vesicular storage and release. The fractional release is equal to $dt/(R_0 \cdot C_1)$, the replenishment rate of the readily releasable pool is $1/(R_1 \cdot C_1)$ and the replenishment rate of the resting pool is $1/(R_2 \cdot C_2)$. dt is the time interval during which an individual stimulus (i.e. an action potential) causes the vesicles to release their content and produce a post-synaptic response (1 ms). We used Simulink to simulate the circuit model (Aristizabal and Glavinovic 2004). An important advantage of this method is that its modular design provides information about the system's topology that is not easily accessible from differential equations.

In this model of vesicular storage and release, the vesicular fluxes are driven by the difference in pool densities. However, fluxes that move against their concentration gradient or are independent of it were not neglected,

but were captured as the 'open system' contribution. During prolonged stimulation, the vesicular parameters were estimated in 10 s windows. In a 'closed' type model, the estimate of the number of vesicles in each pool is taken as an initial condition for the next window until the end of stimulation, and synthesis is the only source of new quanta. In our model, however, the initial number of vesicles in each pool was estimated for every window (i.e. the initial voltages U_1 and U_2 across the capacitors are estimated), and the 'open system' contribution is assessed by comparing its release to a simulated 'closed' system. Finally, note that over 10 s windows used for the estimation of parameters of vesicular dynamics the 'open system' contribution is assumed to be constant, but this contribution may change from one window to the next. This 'open' vesicular model accounts also for the possibility that vesicle pools may not be in equilibrium at $t = 0$. If it is assumed that the vesicular pools are initially at equilibrium, the parameter estimates of the first 1–3 windows are sometimes quite inaccurate (data not shown).

Parameter estimation using optimization

The parameters of the vesicular storage and release model (R_0 , R_1 , R_2 , C_1 , C_2 , E , U_1 and U_2) were estimated by minimizing the distance, in a least squares sense, between the experimental data points (EPSC amplitudes) and the data points predicted by the Simulink model (the i_0 currents, when the switch is closed). Note that the least squares method, which minimizes the sum of the squared residuals, may either be linear or non-linear, depending on whether the residuals are linear. Whereas linear least-squares problems have closed-form solutions (i.e. there are formulas to evaluate them by a finite number of standard operations), non-linear problems have no closed-form solutions. They are solved by iterative refinement, where at each iteration the system is approximated by a linear one.

The MATLAB Optimization Toolbox function `lsqnonlin` was used to estimate the parameters in this study. `lsqnonlin`, a non-linear operator, which minimizes the sum of the squared residuals in an iterative manner, uses a subspace trust region method and is based on the interior-reflective Newton method (Coleman and Li 1996a, b). Briefly, the trust region method approximates the function to be minimized by a quadratic model within a region around the current search point. Within this region, the quadratic model is "trusted" to be correct (i.e. it well approximates the function for minimization). During the search, the size of the region is modified, based on how well the model agrees with the evaluations of the function. The steps are altered to remain within this region of trust. If the quadratic model approximates the function for minimization well, the trust region is expanded; if the approximation is poor

Fig. 1 Modeling a vesicular storage and release system.

a Diagram and equivalent electrical circuit of a secretory cell with two vesicular pools,—readily releasable and resting pool. **b** An example of the parameter space, with an optimization path that leads to the parameters that minimize the error. All parameters except two were fixed, since it is impossible to visualize a surface with more than three dimensions. **c** Understanding how the parameters affect synaptic depression. Higher release coupling (i.e. lower R_0) increases initial release of synaptic output, tetanic rundown and steady-state output. **d** As the ability of the readily releasable pool to contain transmitter decreases (i.e. lower C_1), the tetanic rundown becomes faster. **e** Higher replenishment coupling (i.e. lower R_1) results in higher steady-state output. **f** Changing the resting pool size (i.e. changing C_2) does not affect the above indexes. The initial parameters ($1/R_0$, $1/R_1$, C_1 and C_2) were chosen to be close to those that were estimated experimentally. Input (10 Hz) was constant during simulations

then the region is contracted. Trust region methods are in some sense opposite of line search methods. Line search methods first choose a step direction and then a step size, whereas trust region methods first choose a step size (the size of the trust region) and then a step direction. The algorithm is said to converge when one of the following criteria is met: (a) the cost function changes less than a tolerance value between iterations, or (b) a direction of negative curvature cannot be found. An example of the error surface, but with only two parameters changing, is shown in Fig. 1b. After each iteration, the error diminishes until the minimal error is achieved. The parameters of the vesicular dynamics that produce minimal error—the ‘best’ parameters—are taken to be ‘true’ parameters.

Results

How the parameters of a two pool vesicular system affect synaptic depression

The vesicular storage and release model used in this study consists of two pools, where C_1 and C_2 depict the RRP and RP, respectively (Fig. 1a). Note that the C_s represent the ability of various pools to contain quanta, whereas the actual number of quanta is given by charge q . $1/R_0$ gives the release coupling, $1/R_1$ the replenishment coupling between the resting and readily releasable pools, and $1/R_2$ the replenishment coupling between the synthesis and the resting pool. Once these parameters are known, all of the

variables (release, replenishment fluxes, number of quanta in each pool) can be calculated. To better understand how these parameters affect synaptic depression, we first simulated a time-independent (i.e. with constant parameters) system. A single parameter was then arbitrarily varied and the simulated output is shown. If release coupling ($1/R_0$) increases four times, the initial response also increases fourfold (Fig. 1c). Tetanic rundown (estimated from the synaptic output slope) and the steady-state output also all rise. If the RRP (C_1) changes in size, only the tetanic rundown is affected (Fig. 1d). Greater replenishment coupling ($1/R_1$) results in a higher steady-state output (Fig. 1e). Finally, a fourfold change of the RP (C_2) does not produce any visible changes (Fig. 1f), due to the fact that the RP is much greater than the RPP. Overall, the parameters that directly affect short-term depression are release coupling, replenishment coupling and RRP size.

The effect of $[Ca^{++}]_o$ on relative depression and relative recovery

To illustrate the limitations of previously used kinetic methods, we calculate the relative depression and recovery (Glavinovic and Narahashi 1988). Given a brief high (10 Hz, 5 s)—low (5 Hz, 5 s) frequency train, relative depression is defined as $(A_{t=0} - A_{t=5})/A_{t=0}$, and relative recovery is $(A_{t=5} - A_{t=10})/A_{t=0}$, where $A_{t=x}$ is the EPSC amplitude at x sec. Figure 2a, b show the release during five high-low frequency trains at physiological (2 mM), and at elevated (10 mM) $[Ca^{++}]_o$. $[Ca^{++}]_o$ clearly affects synaptic output, and we quantify the change of vesicular dynamics by evaluating the relative depression and recovery. Relative depression clearly rises with $[Ca^{++}]_o$ (Fig. 2c), suggesting that either the release coupling increases, or replenishment coupling decreases, or some combination of both occurs (see Fig. 1c, e). Relative recovery diminishes with $[Ca^{++}]_o$ (Fig. 2d) suggesting greater release coupling or lower replenishment coupling, or both. Thus, evaluating changes of relative depression and recovery, though useful, does not provide a clear answer on how the vesicular storage and release parameters change.

Using brief trains of stimulation to estimate the fractional release and replenishment rate

Brief high–low frequency stimulation trains were used to estimate the fractional release and replenishment rate. Fractional release ($dt/(R_0 * C_1)$) represents the fraction of the RRP that is released per impulse, and the replenishment rate ($1/(R_1 * C_1)$) represents the rate at which the RRP refills. Figure 3a shows the EPSC amplitudes and the model fits from four different trains (with stimulation frequencies as

indicated). Figure 3b, c give the parameter estimates if both high-low frequency trains were part of an estimation window, and Fig. 3d, e if just high frequency train was used. Although some variability is of biological origin, fractional release estimates are less accurate (they have greater standard deviations) when the mean stimulation frequency is low, and if brief low frequency trains are omitted (i.e. if just the brief high frequency trains are used). The replenishment rates are less sensitive to the choice of the trains used, if both high-low frequency trains are used, but become significantly less accurate if low frequency trains are omitted. Overall, pairs of high-low frequency trains 2, 3 and 4 in Fig. 3a provide reasonable parameter estimates, and in our study we thus used train pairs 2 (10 Hz for 5 s and 5 Hz for 5 s).

The number of activated boutons during stimulation of the Schaeffer collaterals is usually not known. We compared how the parameter estimates depend on stimulus voltage to ascertain that the vesicular dynamics are not affected by the interactions between collaterals or boutons. This is relevant because at guinea pig CA3-CA1 synapses, the induction of LTP depends on how many synapses are stimulated (Friedlander et al. 1990). The initial EPSC amplitudes expectedly increase with greater stimulus voltage (Fig. 3f), but the fractional release and replenishment rate estimates do not significantly change (Fig. 3g, h). Vesicular dynamics are thus independent of the stimulus voltage amplitude.

$[Ca^{++}]_o$ affects the fractional release, but not the replenishment rate

How does $[Ca^{++}]_o$ affect the parameters of a two pool vesicular storage and release system? Parameters were estimated from several trials in multiple cells at varying $[Ca^{++}]_o$. As $[Ca^{++}]_o$ rises, fractional release increases sharply (Fig. 4a), but there is no significant change of the replenishment rate (Fig. 4b). Note however the significant cell heterogeneity of both parameter estimates. Finally, the number of vesicles in the RRP at the beginning of stimulation is not dependent on $[Ca^{++}]_o$ (Fig. 4c). The numbers are high, since multiple active zones were likely involved during release. We also calculated the probability of release using quantal analysis, which is based on the binomial theorem (Chuhma and Ohmori 1998; Martin 1955). At physiological $[Ca^{++}]_o$ (2 mM), the probability of release values are much higher (0.92 ± 0.13 ; $n = 3$) than those of the fractional release. It may appear surprising that these values approach the theoretically maximal value of 1.0, but they are in good agreement with earlier studies at the neuromuscular junction (Glavinovic 1979). Note that estimates of the overall probability of release can be strongly biased by individual vesicles that have a high release probability

Fig. 2 Evaluating vesicular dynamics by calculating relative depression and recovery. **a–b** EPSC amplitudes at 2 and 10 mM $[Ca^{++}]_o$, during 5 brief high (10 Hz, 5 s)—low (5 Hz, 5 s) frequency trains with 1 min interval for recovery. **c** Relative depression increases as $[Ca^{++}]_o$ rises, with best-fitted line ($RD = 5.6 \cdot [Ca^{++}]_o + 26.8$; $r = 0.79$). **d** Relative recovery decreases as $[Ca^{++}]_o$ increases ($RR = -1.5 \cdot [Ca^{++}]_o + 20.4$; $r = 0.68$). Different symbols represent mean values (≥ 7 trials, with 1 min interval for recovery) from different cells. Vertical bars are standard errors. Stimulation consisted of pairs of brief high (10 Hz, 5 s) and low (5 Hz, 5 s) frequency trains

(Brown et al. 1976). This large discrepancy between the fractional release and probability of release thus suggests that the release probability of individual vesicles is fairly heterogeneous.

The model used here enables one to also determine the more ‘basic’ parameters (release coupling, replenishment coupling and RRP size) that underlie the ‘derived’ parameters (fractional release and replenishment rate). This is advantageous because these ‘basic’ parameters provide better insight into vesicular dynamics. As $[Ca^{++}]_o$ rises, release coupling increases (Fig. 4d), replenishment coupling remains unaltered (Fig. 4e), but the size of the RRP decreases (Fig. 4f). Note also that as $[Ca^{++}]_o$ increases, the number of vesicles in the pool does not significantly change, but the ability of the pool to contain vesicles decreases.

The replenishment rate decreases during long, high frequency stimulation

In addition to short trains of stimulation, we investigated how the parameters of a vesicular system behave during long, high frequency stimulation. Stimulation of long duration should lead to greater $[Ca^{++}]_i$ accumulation and this may affect the parameters of the system. The number of quanta released (EPSC amplitude/mean (mEPSC amplitudes)) during long patterned stimulation (repeating sequence of high (10 Hz, 5 s) and low (5 Hz, 5 s) frequency trains) and best model fit are shown in Fig. 5a. The corresponding Fit versus EPSC amplitudes scatterogram demonstrates that the model fit is good throughout the stimulation and for EPSC amplitudes of different sizes

Fig. 3 Estimating the fractional release and replenishment, using different patterned stimulation frequencies and stimulation voltages. **a** EPSC amplitudes with best model fits for different stimulation frequency combinations as indicated. **b–c** Fractional release and replenishment rate estimates using the stimulation trains from (**a**). **d–e** Same recordings, but with just high frequency trains being used. **f** Increasing stimulus voltage causes the initial EPSC amplitude to rise. **g–h** Neither fractional release nor replenishment rate estimates are significantly affected by changes to stimulus voltage. Different symbols represent mean values (≥ 7 trials, with 1 min interval for recovery) from different cells. Vertical bars are standard errors

(Fig. 5a₁). Figure 5a₂ shows the frequency histogram of mEPSC amplitudes. Parameters were estimated in 10 s intervals. To characterize how they change with stimulation, fractional release and replenishment rate estimates were fit with a line and exponential curve, respectively. Fractional release slightly increases with stimulation in this cell (Fig. 5b), but does not overall change, as the slopes of the best-fitted lines from pooled data are close to 0 (mean

and SD: -0.03 ± 0.12 %/s, $n = 13$). Unexpectedly, the replenishment rate decreases during stimulation (Fig. 5c). The exponential fits decay with a time constant of 14.65 ± 10.22 s, to a steady-state level of 0.07 ± 0.07 1/s ($n = 13$). Although our model may appear different from ‘traditional’ rate based models (Heinemann et al. 1993), they are kinetically the same. Given high frequency stimulation, the release in both cases displays a multi-

Fig. 4 Calcium affects the fractional release, but not the replenishment rate. **a** As $[Ca^{++}]_o$ increases, fractional release rises. **b** Replenishment rate is not significantly affected by $[Ca^{++}]_o$. **c** The number of quanta in the readily releasable pool does not change with $[Ca^{++}]_o$. **d** As $[Ca^{++}]_o$ increases, release coupling rises. **e** Replenishment coupling does not change with $[Ca^{++}]_o$. **f** The readily releasable pool size decreases with $[Ca^{++}]_o$. Stimulation consisted of brief high (10 Hz, 5 s) and low (5 Hz, 5 s) frequency trains. Each bar represents the mean of means (≥ 7 trials, with 1 min interval for recovery) of ≥ 4 cells, and the vertical bars are standard errors. $*p < 0.05$, significant compared with the parameter estimate at 1 mM $[Ca^{++}]_o$ (Student's *t* test)

exponential decay (if two pools are considered, this becomes bi-exponential decay). The 'baseline' release level is determined by the quantal synthesis and, if present, the 'open system' contribution. The parameters estimated by both methods should thus be the same. To reassure the reader that this is indeed so, we estimated the replenishment rates with both models, using the same biological data. As Fig. 5c demonstrates, the replenishment rate estimates obtained by both methods are essentially the same (subject to the variation due to the probabilistic nature of the estimation procedure), and moreover change similarly during stimulation.

In addition to providing the parameters of the vesicular system, the model used here also yields the variables (such as the release and replenishment fluxes), and these show how the different pools are emptied and re-filled during stimulation (Fig. 5d). The net vesicular flux from the RRP (dash dotted line), which dominates the release initially, decreases to a very low level during brief high frequency train (from 0 to 5 s). The replenishment flux from the RP (dotted line) rises from zero level and becomes the main contributor to release. During a subsequent brief low frequency train (from 5 to 10 s), the net vesicular flux of the RRP reverses, indicating that more vesicles are entering the

Fig. 5 The replenishment rate decreases during long, high frequency stimulation. **a** The number of quanta during long patterned stimulation with model fit. **a₁** Corresponding Fit versus EPSC amplitudes scatterogram, with best-fitted line (Fit = $0.88 \cdot A_{EPSC} + 29.30$; $r = 0.94$). **a₂** Frequency histogram of mEPSC amplitudes. **b** Fractional release increases marginally with stimulation in this cell, but does not overall change (FR = $0.01 \cdot t + 2.97$; $r = 0.38$). **c** The replenishment rate clearly decreases during stimulation, with best-fitted exponential curve (circles; RR = $0.10 + 0.82 \cdot \exp(-t/13.37)$; $r = 0.99$). The replenishment rate shows similar decrease if traditional rate model is used for fitting (crosses; RR = $0.19 + 0.59 \cdot \exp(-t/10.9)$; $r = 0.95$). The arrows depict the decay times, and the horizontal lines represent the steady-states. **d** Vesicular release (solid line), with the replenishment flux (dotted line), and the flux from the readily releasable pool (dashed line). Patterned stimulation consisted of 5 repeating trains of brief high (10 Hz, 5 s)—low (5 Hz, 5 s) frequencies

RRP than leaving. Note that the replenishment flux from the RP doubles at 5 s (these are per pulse estimates and reducing the stimulation frequency by half leads to a twice as long replenishment period), before decreasing to the lower steady-state level. Finally, both the net vesicular flux from the RRP and RP diminish and become slower with stimulation. Although the advantage of patterned stimulation is that the changes of vesicular dynamics are more easily assessed by visual inspection, the use of random stimulation should provide more accurate parameter estimates. However, given that both types of stimulation

provided sufficiently similar parameter estimates (data not shown), we used patterned stimulation in this study.

The depletion model is the basis for analysis of short-term synaptic depression, but other factors can also contribute to depression, and examples include lower quantal size or receptor desensitization. To investigate this, at least 50 miniature EPSC amplitudes were measured before and after long, high frequency stimulation (100 s) in three cells. Quantal size did not change, as the amplitudes before and after stimulation were not significantly different (Student's *t* test). Note however that changes in quantal size during

stimulation may escape detection, because the miniature EPSCs are drawn from a much larger population of afferent synapses.

Comparison of the replenishment rate decrease during random and patterned stimulation: effect of window length and initial parameter guess

As Fig. 6a, b show the synaptic output decreases similarly during random and patterned stimulation of the same mean frequency (7.5 Hz). The parameters that determine the change of synaptic output are also similar in both cases, and change similarly during stimulation (Fig. 6c, d). Note that in this cell the fractional release does change, but only modestly, whereas the replenishment rate diminishes markedly and quite rapidly. We have already shown that both the fractional release and the replenishment rate can be evaluated albeit less accurately, if the window is reduced by half (i.e. if the estimation window includes only a high frequency brief train; Fig. 3). We now compare the estimates and their change during long, but random stimulation with several estimation windows (10, 5 and 2.5 s). The values of the replenishment rate differ depending on the window length (Fig. 6e). Nevertheless, in all three cases the replenishment rate clearly diminishes with stimulation reaching very low values. Finally, we tested how the choices of the initial guesses of various parameters affect the estimates of the replenishment rate, and their change during stimulation. The initial guesses of individual parameters in two trials differed from each other greatly (by 10–100 times). Given that these choices influence the replenishment rate and their change only modestly, the open system contribution was also found to be similarly largely insensitive to the window length or initial guess (not shown).

Characterizing the underlying ‘basic’ parameters during long, high frequency stimulation

Since the replenishment rate changes during stimulation, the ‘basic’ parameters should also change and this is indeed the case. In individual recordings, the release coupling may decrease with stimulation (Fig. 7a), but overall, the slopes of the best-fitted lines are close to zero (Fig. 7a₁). The replenishment coupling diminishes mono-exponentially (Fig. 7b) and its time course is quite variable (Fig. 7b₁–b₂). Finally, the size of the RRP also decreases mono-exponentially (Fig. 7c), though its decrease is relatively small and variable (Fig. 7c₁–c₂). Thus, during long, high frequency stimulation, the decline in the replenishment rate is mainly due to the decrease in replenishment coupling.

The decrease in the replenishment rate during long, high frequency stimulation contributes significantly to synaptic depression

The model used here allows one to investigate how the decrease of replenishment during stimulation (see Fig. 5c) contributes to synaptic depression. Figure 8a depicts the EPSC amplitudes with the best model fit. Figure 8b shows the same model fit (solid line), together with the simulation of a time-independent system (dashed line). In this system, the parameters (as estimated from the first train in Fig. 8a) were held constant during stimulation. If the parameters do not change, synaptic depression is clearly less pronounced. To quantify this, the relative depression was calculated at 70 s ($(A_{t=0} - A_{t=70})/A_{t=0}$), for both cases. The difference between these values yields the contribution to synaptic depression by the variable parameters (mean and SD: $32.1 \pm 24.5\%$, $n = 15$). We also ‘dissected’ how changes in the fractional release and replenishment rate contribute to the depression (Fig. 8c). If the fractional release is held constant during stimulation (dashed line), release closely resembles synaptic depression as seen experimentally. Conversely, holding the replenishment rate constant attenuates the depression (dotted line). Overall, this demonstrates that synaptic depression during stimulation is partly due to the decrease of the replenishment rate. Finally, we estimated the replenishment flux that does not follow the vesicular concentration gradient (see “Methods”). As Fig. 8d shows this replenishment flux is significant. Without the ‘open system’ contribution (i.e. a ‘closed system’), the synaptic depression is more severe.

Discussion

The parameters that characterize the quantal release can be estimated by two methods—quantal analysis (Martin 1955) or kinetic analysis (as in this study). To avoid confusion, we use the term ‘probability of release’ for the quantal analysis estimate, and ‘fractional release’ for the kinetic estimate. At both the rat neuromuscular synapse (Glavinovic 1979), and at the excitatory synapse of rat hippocampus (this study), these two methods yield very different values. At physiological $[Ca^{++}]_o$ (2 mM), the probability of release estimates are very high (~ 80 – 90% at both rat neuromuscular synapse and at the excitatory synapse of hippocampus; see “Results”), whereas the fractional release estimates are much lower (2 – 5% at the neuromuscular synapse and $\sim 10\%$ at the excitatory synapse). This discrepancy is likely due to quantal analysis being strongly biased by vesicles with a high probability of release (Brown et al. 1976). Vesicles with a high probability of release may be those that are primed, or more

Fig. 6 Progressive decrease of the replenishment rate during long, high frequency stimulation is similar regardless of whether patterned or random stimulation is used, and is not very sensitive to the length of the estimation window, or the initial guess for parameters. **a–b** The amplitude of the excitatory post-synaptic currents during long random (**a**) and patterned stimulation (**b**) with model fit. **c** Fractional release changes, but only modestly irrespective of whether the patterned or random stimulation was used ($FR = 0.03 \cdot t + 1.83$; $r = 0.43$; patterned stimulation, and $FR = 0.04 \cdot t + 1.22$; $r = 0.26$; random stimulation). **d** The replenishment rate decreases markedly in both cases, with best-fitted exponential curves (patterned stimulation (*filled circles*); $RR = 0.03 + 1.35 \cdot \exp(-t/11.28)$; $r = 0.98$, and random stimulation (*empty circles*); $RR = 0.04 + 2.73 \cdot \exp(-t/5.68)$; $r = 0.99$). **e** Progressive decrease of the replenishment rate is clearly observed when the

estimation window is 10 s, but also when it is reduced to 5 or 2.5 s (random stimulation), with best-fitted exponential curves (10 s window, *filled circles*); $RR = 0.04 + 2.73 \cdot \exp(-t/5.68)$; $r = 0.99$; 5 s window, *empty circles*); $RR = 0.13 + 1.33 \cdot \exp(-t/17.05)$; $r = 0.88$, and 2.5 s window, *crosses*); $RR = 0.02 + 0.80 \cdot \exp(-t/7.66)$; $r = 0.92$). **f** Progressive decrease of the replenishment rate is also clearly present even when the initial guesses of the parameters to be estimated differ in two fitting trials by 10–100 times. The replenishment rates, their change during stimulation and the fits differ in two cases, but the decrease of the replenishment rate during stimulation is clearly observed in both cases. The fits are: $RR = 0.10 + 1.11 \cdot \exp(-t/25.61)$; $r = 0.92$, and $RR = 0.13 + 1.33 \cdot \exp(-t/17.05)$; $r = 0.88$

Fig. 7 The change of the underlying ‘basic’ parameters during long, high frequency stimulation. **a** Release coupling does not significantly change during stimulation, with best-fitted line ($1/R_0 = 0.0004 \cdot t + 0.2389$; $r = 0.29$). **a₁** Frequency histogram of slopes from pooled data (mean and SD: -0.0007 ± 0.0010). **b** Replenishment coupling decreases during stimulation, with best-fitted exponential curve ($1/R_1 = 0.001 + 0.005 \cdot \exp(-t/12.743)$; $r = 0.95$). **b₁–b₂** Frequency histograms of pooled decay times (mean and SD: 51.50 ± 72.08 s) and steady states (mean and SD: 19.75 ± 14.68 %). **c** The ability of

the readily releasable pool to contain quanta slightly decreases during stimulation, with best-fitted exponential curve ($C_1 = 0.00 + 0.01 \cdot \exp(-t/165.93)$; $r = 0.89$). **c₁–c₂** Frequency histograms of pooled decay times (mean and SD: 36.51 ± 59.71 s) and steady states (mean and SD: 88.32 ± 51.13 %). Steady-state values are represented as a percentage of the parameter estimate at $t = 0$ s. The arrows depict mean values. Patterned stimulation consisted of 5–10 repeating trains of brief high (10 Hz, 5 s)—low (5 Hz, 5 s) frequencies ($n = 18$)

closely positioned to the plasma membrane and/or of larger size that renders them more efficient barriers to calcium diffusion (Glavinovic and Rabie 2001). If such estimates are taken, the replenishment estimates will also be biased, but this study did not focus on such processes. Thus, we exclusively used kinetic analysis, which provides averaged parameter estimates. However, this method conceals the variability of synaptic release mechanisms. The variability

of synaptic mechanisms, which is unknown, may be high given the high frequency stimulation used in this study and the structure of the CA3-CA1 pathway. The recorded EPSC is not produced by a single vesicle (or quantum), but reflects the release of several vesicles, that probably originate from different synapses formed on the recorded neuron, and from multiple release sites at each synapse. Thus, the relationship between the EPSC and the vesicle

Fig. 8 The decrease of the replenishment rate during long, high frequency stimulation contributes significantly to synaptic depression, whereas the open-system contribution renders it less severe. **a** EPSC amplitudes with best model fit. **b** Release from a time-independent secretory system (i.e. with parameters that are constant and as estimated from the first pair of high-low frequency trains in **a**; dashed line), and time-dependent system (i.e. with parameters changing as observed experimentally in **a**; solid line). **c** Dissecting the contribution from each parameter change. Synaptic depression is rendered more severe because the replenishment rate decreases during stimulation. The fractional release changes marginally and contributes little to synaptic depression. **d** Dissecting the ‘open system’ contribution. Synaptic output including the ‘open system’ contribution (*Open system*), excluding such a contribution (*Closed system*) and their difference (*Open system contribution*). The open-system contribution renders synaptic depression less severe (see text). Patterned stimulation consisted of 9 repeating trains of brief high (10 Hz, 5 s)—low (5 Hz, 5 s)

pools is not confined to a single synapse, but to a multitude of synapses. Given the large difference between the probability of release and fractional release estimates, the variability of synaptic release mechanisms is probably quite pronounced. Nevertheless, note that the parameter estimates at the same recording site do not significantly change as the stimulation amplitude increases (Fig. 3g, h). Although it is not clear if the vesicular dynamics are the

same at all synapses, our results show that the recruitment of more synapses does not lead to different dynamics.

Early kinetic studies suggested the existence of multiple vesicular pools, since depression has 2–3 kinetic phases (Birks and MacIntosh 1961; Dittman and Regehr 1998; Kasai 1999), each taken to indicate the depletion of vesicles from the corresponding pool (Heinemann et al. 1993; Horrigan and Bookman 1994; Oheim et al. 1999; Parsons et al. 1995). The fractional release can be estimated using ‘tetanic rundown’ (Christensen and Martin 1970), but this method is accurate only if the replenishment of the RRP during the rundown plays just a minimal role. The replenishment rate can be estimated from the time constant of recovery (Stevens and Wesseling 1998; Wang and Kaczmarek 1998). The RRP is first quickly depleted (by a high frequency stimulation or hypertonic solution) and the response to a second stimulus given at a variable time thereafter reveals how much the pool has refilled. From a series of such measurements, the time course of recovery can be determined. However, if the replenishment rate changes during long, high frequency stimulation, it is also likely to change during recovery, and this will affect the recovery time estimates. As demonstrated in this study, at rat hippocampal excitatory synapses the replenishment rate decreases markedly during stimulation.

To avoid the limitations of the above methods, we used model fitting to estimate the parameters of the vesicular storage and release system. In our circuit model, the vesicular flux is driven by the difference in pool densities. The fluxes are density driven because there are, on average, more vesicles moving toward areas with lower density. Note that these models are general in nature, and can also describe secretory systems where replenishment does not occur by vesicular movement. This is important to stress since it remains unclear whether the vesicular pools, which are kinetically distinct, are also morphologically separate (Rizzoli and Betz 2005). If a large population of vesicles is already close to the membrane, the replenishment rate may simply represent the rate at which vesicle priming machinery operates, or be a function of the accumulation of free $[Ca^{++}]_i$ underneath the membrane (Glavinovic and Rabie 2001).

The model used here not only provides the ‘derived’ parameters (fractional release and replenishment), but also the underlying ‘basic’ parameters (release coupling, replenishment coupling and RRP size) that are more directly related to the vesicular storage and release processes and thus can provide additional insight (Goda and Stevens 1998; Aristizabal and Glavinovic 2004; Kruckenberg and Sandweg 1968). To illustrate the point, an increase in the replenishment rate may be due to an increase in replenishment coupling ($1/R_1$), decrease in RRP size (C_1), or both. Changes in the ‘basic’ parameters,

though rarely considered, have been reported. Modulation of the RRP size by serotonin was observed at the crayfish neuromuscular junction (Wang and Zucker 1998). Other processes may contribute to lower efficacy during high frequency stimulation, such as changes in quantal size (Highstein and Bennett 1975). However, the quantal size before and after long high frequency stimulation was not significantly different, arguing that post-synaptic receptor desensitization (Trussell et al. 1988) did not significantly contribute to synaptic depression. Additionally, the fractional release (and probability of release; data not shown) did not significantly change with stimulation, indicating that Ca^{++} entry per stimulus remains relatively constant. Therefore, factors that can affect Ca^{++} entry, such as Ca^{++} channel inactivation (Brehm and Eckert 1978), autoreceptor activation (Nistri and Cherubini 1991; Wu and Saggau 1994) or action potential conduction failures (Krnjevic and Miledi 1958), are unlikely to significantly contribute to the depression in this study.

Although different stimulation patterns can be used to evaluate the parameters of vesicular dynamics, we use only two extensively—a pair of brief high-low frequency trains and patterned stimulation (rapid sequence of such brief high-low frequency trains). Random stimulation was used for testing the accuracy of the estimated parameters. It provides more accurate parameter values, as it covers all, and not just two, stimulation frequencies (Aristizabal and Glavinovic 2004). Nonetheless, using either type of stimulation yielded similar parameters, and random stimulation does not allow for direct visual assessment of the changes to the vesicular dynamics from the EPSC amplitudes.

Several studies have shown that synaptic vesicles are associated with a variety of cytoskeletal and motor proteins (Dresbach et al. 2001; Evans et al. 1998; Miyamoto 1995; Morales et al. 2000; Prekeris and Terrian 1997; Ryan 1999). The synapsin family of phosphoproteins, which tethers vesicles to actin filaments, has been studied the most (Benfenati et al. 1989; Greengard et al. 1993; Pieribone et al. 1995). It is generally thought that in response to Ca^{++} entry, these proteins become phosphorylated, freeing vesicles from their cytoskeletal constraints and allowing them to replenish the RRP (Chi et al. 2001, 2003). However, there have been reports that challenge synapsin's role in vesicular mobility. Disruption of actin in frog motor nerve terminals showed no significant changes in vesicular mobility (Gaffield et al. 2006). In knockout mice lacking synapsins, the neurons display no gross anatomical abnormalities (Rosahl et al. 1995) or changes in synaptic vesicle trafficking (Gaffield and Betz 2007). In rat ribbon synapses (unique neurons that have a high rate of neurotransmitter release), there is an absence of synapsin, suggesting that vesicle clustering and mobilization involves a different process (Mandell et al. 1990). Thus, the role of

synapsin or the cytoskeleton in vesicular mobility needs to be reexamined, and the mechanisms that control vesicular replenishment are still not clear.

This study shows that as $[\text{Ca}^{++}]_o$ rises, fractional release rises steeply, while the replenishment rate is not significantly affected. However, the $[\text{Ca}^{++}]_o$ dependence of the basic parameters reveals a complex picture. The fractional release increases, not only due to increased release coupling, but also because the size of the RRP diminishes. The insensitivity of the replenishment rate to $[\text{Ca}^{++}]_o$ is puzzling, since recovery (following depression) increases with $[\text{Ca}^{++}]_o$ (Dittman and Regehr 1998; Wang and Manis 2008). This discrepancy may be due to the fact that the $[\text{Ca}^{++}]_o$ dependence of the replenishment rate may be different during depression and during recovery, and this warrants further investigation.

Figure 5c shows that at zero time (i.e. prior to long, high frequency stimulation) the replenishment rate is relatively high ($\sim 1 \text{ s}^{-1}$, which is equivalent to the replenishment time constant of 1 s), which is almost an order of magnitude faster than reported previously for excitatory synapses of rat hippocampus in culture (Stevens and Wesseling 1998). This may be due to the fact that the replenishment slows rapidly with stimulation (decay time of $\sim 15 \text{ s}$) and markedly (down to $\sim 10 \%$ of its initial value), to a steady state of $\sim 0.1 \text{ s}^{-1}$ (time constant of $\sim 10 \text{ s}$). Lower replenishment rate is largely due to the lower replenishment coupling (Fig. 6b), which decreases significantly (by $\sim 80 \%$) and rapidly (time constant of $\sim 10 \text{ s}$). Although synaptic depression is also a property of a time independent vesicular storage and release system, lower replenishment after long, high frequency stimulation significantly contributes ($\sim 30 \%$) to the total synaptic depression.

Because the slower replenishment we observe is difficult to reconcile with the 'mobilization by liberation' model, other explanations have to be considered. It may involve vesicles suitably positioned, but initially inapt for release, owing to their smaller size or less favorable situation in relation to calcium channels (Glavinovic and Rabie 2001). A combination of residual Ca^{++} from previous stimuli and Ca^{++} from a new stimulus may make them releasable. Alternatively, the replenishment of the RRP may be due to the re-deployment of the same vesicles that have recently undergone fusion (Aravanis et al. 2003; Ceccarelli et al. 1973; Pyle et al. 2000). This can be represented either as the replenishment of the RRP from the RP, or as part of the 'open system' contribution, or both. The 'open system contribution' indeed increases during long high frequency stimulation, as one would expect if it is (largely or entirely) due to endocytosis.

However, the endocytic vesicles may require spatial re-deployment before being subsequently released, and the re-deployment is likely to be influenced by the membrane tension. The membrane tension, which decreases during

stimulation owing to the incorporation of vesicular membrane into the plasma membrane of the secretory cell, will thus lead to the lower replenishment rate of the RRP. Note that the membrane tension also directly modulates the secretory system, stimulating the exocytosis and suppressing the endocytosis (Apodaca 2002).

The insensitivity of the fractional release (overall) to stimulation can be explained, if we consider that an increase of the fractional release caused by the $[Ca^{++}]_i$ accumulation may be counterbalanced by a comparable decrease of exocytosis due to lower membrane tension, but other factors unknown hitherto may also contribute. Further analysis of this problem requires additional pharmacological tests and this constitutes a separate study. Finally, our results suggest that the vesicular replenishment of RRP largely reflects changes near the plasma membrane. Interestingly, it has been recently reported that the activity-dependent regulation of presynaptic CaV2.1 channels by Ca^{++} sensor proteins (CaS proteins) may be a primary determinant of short-term synaptic plasticity and information-processing in the nervous system (Mochida et al. 2008). Such processes would also occur near plasma membrane. However, we find that it is the replenishment of the RRP, rather than fractional release, which changes during stimulation.

In conclusion this study demonstrates that the vesicular storage and release system is dynamically complex, with parameters that are both $[Ca^{++}]_o$ and time dependent. Interestingly, the fractional release is, but the replenishment rate is not strongly $[Ca^{++}]_o$ dependent. In contrast, during long, high frequency stimulation, the fractional release remains unaltered, but the replenishment rate decreases markedly and rapidly.

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