Recovery of vesicular storage and release parameters after high frequency stimulation in rat hippocampus

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Abstract The replenishment rates estimated from the recovery of synaptic efficacy following synaptic depression are known to be widely scattered. Given the importance of the replenishment during stimulation, especially if it is prolonged, it is important to better understand what influences the recovery of the synaptic efficacy following stimulation. We fit a two-pool model of vesicular secretion to the changes of the excitatory post-synaptic currents recorded in CA1 neurons of rat hippocampal slices to determine how the model parameters change during, and following, long stimulation. The replenishment rate at the end of stimulation inducing synaptic depression differs greatly from that at the beginning of stimulation. It decreases progressively and rapidly (by $\sim 75\%$ and with a time constant of <10 s) during stimulation, and this is followed by a similarly fast recovery (time constant of ~ 10 s), but to a steady-state that is approximately twice as large as its pre-stimulation value. Both $[Ca^{++}]_o$ and the duration of long stimulation influence the recovery of the replenishment rate. Its new steady-state is significantly higher, if either $[Ca^{++}]_o$ is higher or stimulation longer, but the recovery of the replenishment rate becomes clearly slower if $[Ca^{++}]_o$ is higher, and faster if stimulation is longer. Many factors thus influence the recovery of the replenishment rate and of the synaptic efficacy, but the stimulation induced $[Ca^{++}]_i$ accumulation cannot explain the change of the replenishment rate during recovery. Finally, okadaic acid, which speeds up vesicular trafficking, does not alter the recovery of the replenishment rate.

The vesicular replenishment of the RRP following stimulation is thus not likely to be associated with significant vesicular movement.

Keywords Synaptic depression · Replenishment rate · Model fitting · Recovery · Hippocampus

Introduction

Clinical studies have demonstrated long ago that the hippocampus, which receives many different types of sensory information via the entorhinal cortex, is involved in the formation of episodic and semantic memories (Scoville and Milner 1957; Zola-Morgan et al. 1986), but much remains to be done to fully understand the nature of the processes determining how the information of spatiotemporal sequence coming from the hippocampal CA3 area alters the synaptic responses of the hippocampal CA1 neurons (Kuroda et al. 2009). It is widely believed that the basis of learning and memory in neural systems occurs through the change of the synaptic strength, which is an index of their plasticity (Martin et al. 2000; Satel et al. 2009). The neurodynamics, which is the scientific branch that studies the dynamics of the states and properties of neural systems, has become an important sub-discipline within neuroscience (Atmanspacher and Rotter 2008). Synaptic plasticity can last for different lengths of time, and if the change lasts up to a few minutes, the plasticity is considered short-term. Long-term plasticity lasts up to a few hours or days, and some are thought to last much longer even, possibly a life-time (Clopath 2012). In this study we focus on the short-time changes of synaptic efficacy.

At high levels of release the synaptic efficacy decreases progressively during long, high frequency stimulation (Del Castillo and Katz 1954), and its kinetics during

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constant long high frequency stimulation can be well described by a depletion model consisting of two or several pools (Birks and MacIntosh 1961; Kavalali 2007; Zucker and Regehr 2002). It is generally accepted that as stimulation progresses the synaptic depression becomes more and more influenced by the replenishment of the readily releasable pool (RRP). The parameters determining the vesicular dynamics need to be evaluated from the changes of the synaptic output induced by the stimulation. The fractional release can be evaluated from the tetanic run-down at the beginning of stimulation, and the replenishment rate from the recovery of the total number of vesicles in the RRP following the stimulation (Christensen and Martin 1970; Stevens and Tsujimoto 1995; Wang and Kaczmarek 1998). If the RRP is first depleted (by high frequency stimulation or depolarizing solution) the response to a second stimulus given at a variable time thereafter reveals how much the RRP has refilled. From a series of such measurements, the time course of recovery and the replenishment rate are determined.

The estimation becomes more complex if the system is time dependent, i.e. if the rates of the secretory system—the replenishment rate and fractional release—change during stimulation. In such a case the replenishment rate estimated from the recovery of the synaptic output will not correspond to the value at the beginning, but at the end of stimulation. The parameters of the secretory system do not remain constant, but recover following the stimulation and this will compromise the replenishment rate estimates, especially if the recovery is fast relative to the time course of the replenishment rate at the end of stimulation. Finally, the stimulation is not only needed to induce the synaptic depression, but also to test the subsequent recovery of the synaptic output, and this further complicates the estimation of the replenishment rate. Any stimulation changes the parameters of the secretory system, and the stimulation during recovery, though reduced is not zero. It is thus of some interest to assess how much such stimulation alters the recovery of the synaptic output and of the replenishment rate.

The factors controlling the replenishment rate are unknown, but it has been speculated that the accumulation of $[Ca^{2+}]_i$, which renders vesicles free from their cytoskeletal constraints (Stevens and Wesseling 1998; Wang and Kaczmarek 1998) plays an important role. It is thus important to test how $[Ca^{2+}]_o$ affects the recovery of the replenishment rate when $[Ca^{2+}]_i$ accumulation is very pronounced. Finally, it is unclear whether the recovery of the replenishment rate following stimulation is associated with vesicular movement. If it is the phosphatase inhibitor okadaic acid, a compound which increases vesicular mobility (Betz and Henkel 1994; Gaffield et al. 2006) should affect the recovery of the replenishment rate

following stimulation. In frog motor nerve terminals okadaic acid disrupted vesicle clusters by mobilizing synaptic vesicles and unmasking a vesicle translocation mechanism (Betz and Henkel 1994). Vesicles in the reserve pool (RP), which were immobile, and vesicles in the recycling pool (RRP), which were mobile, both increased their mobility to the same level following application of okadaic acid (Gaffield et al. 2006).

A preliminary account has appeared as an abstract (Krnjevic et al. 2008).

Methods

Solution and recordings

All experiments were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee of McGill University. Post-natal (100–125 g) male Sprague–Dawley rats (Charles River Ltd., Canada) were decapitated under isoflurane anaesthesia (Baxter Corporation, Canada). The brain was rapidly (~ 1 min) removed from the cranial vault 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). The brain was then glued onto a mounting block in a cutting chamber, filled with ice-cold oxygenated ACSF. Hippocampal slices (400–450 μm thick) were cut using a vibrating microtome (Campden, United Kingdom) and subsequently transferred to a container with oxygenated ACSF and incubated at room temperature (20–22 $^{\circ}C$) for at least 1 h before the recordings. In the recording chamber they were kept fully submerged in ACSF, flowing at 3–4 ml/min. Patch pipettes (0.8 – 1.1 $\times$ 100 mm; Kimble Chase, United States) were filled with (mM): CsCl 130, NaCl 10, ATP-Mg 3, guanosine-5'-triphosphate (GTP) 0.3, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid HEPES 10, ethyl-ene-bis(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. Bicuculline methiodide (10 μM) was added at least 10 min before the recordings, to suppress inhibitory (*g*-aminobutyric acid type A) synaptic currents, whereas DL-2-amino-5-phosphonovaleric acid (10 μM) was added to reduce NMDA-activated channels (Taniike et al. 2008), to ensure no induction of the long-term potentiation, although such an induction was unlikely to occur given comparatively low frequency of stimulation (5–10 Hz). Indeed the difference in quantal size of miniature excitatory post-synaptic currents prior to

and after long patterned stimulation was not statistically significant (Bui and Glavinovic 2013).

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) and displayed on an oscilloscope (BK Precision, US). The holding potentials ranged from –110 to –70 mV. The series resistance ranged from 3 to 10 MΩ and the cell was discarded if the resistance changed by more than 15 %. EPSCs were anti-alias filtered with a Bessel filter whose corner frequency was set at 5 kHz, and were digitized and recorded in real time on a computer, using a BNC-2120 interface card (National Instruments, United States) with a sampling rate of 10 kHz. The interface card was also used to provide the timing pulses for the stimulus isolation unit (Digitimer Ltd., UK), and subsequent activation of the Schaffer collateral/commissural pathway. Stimulation pulse duration was 0.2 ms, whereas the voltage was chosen such that the initial EPSC amplitude was approximately half of the maximum response. 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 with two vesicular pools—the readily releasable pool (C_1) and resting pool (C_2) (Fig. 1a) (Aristizabal and Glavinovic 2004; Kruckenberg and Sandweg 1968). The resistor R controls the vesicular flux, the capacitance C indicates the ability of the pool to store vesicles, and the voltage U across the capacitor gives the vesicular density in the pool. Synthesis of new vesicles is represented by the battery E . If one vesicle corresponds to a charge q , then $(C * U)/q$ represents the number of vesicles in that pool. The parameters of the processes close to the release locus (R_0 , R_1 and C_1) can be reliably estimated using the model-fitting (see below), but the estimates of the processes that are further away (R_2 , C_2 and E) are less reliable (data not shown). An important advantage of this modeling is that it encapsulates the dynamics within a compact description, which is an important advantage when comparing synapse dynamics across different synaptic pathways and/or species.

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 vesicular concentration difference between two pools (RP and RRP) drives the replenishment of the RRP and is controlled by the replenishment rate of RRP. Likewise, the vesicular synthesis (shown in Fig. 1a as E) drives the replenishment of the RP and is controlled by the replenishment rate of RP. Note that 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 release from the RRP (assuming no replenishment of the pool) at any time t is governed by the equation $I_0 * \exp -t/(R_0 * C_1)$, where I_0 is the release at time zero. The fractional release is equal to $dt/(R_0 * C_1)$, the replenishment rate of the readily releasable pool is $1/(R_1 * C_1)$ and the replenishment rate of the resting pool is $1/(R_2 * C_2)$ 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). In all model-fitting of our experimental data we used Simulink for simulation of the vesicular storage and release system (Aristizabal and Glavinovic 2004). This type of modeling is based on two-port linear network theory (Chirilian 1969), and has an important advantage of being modular and thus providing visual information about the system’s topology that is not easily accessible from differential equations.

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 sum of squared errors (residuals) 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). The least squares methods, which minimize the sum of squared residuals, may be either linear or non-linear, depending on whether or not the residuals are linear in all unknowns. If they are linear least-squares problems have closed-form solutions (i.e. there are formulas to evaluate them by a finite number of standard operations). However, if they are non-linear problems they have no closed-form solutions, and have to be solved by iterative refinement, where at each iteration the system is approximated by a linear one.

Fig. 1 The replenishment rate decreases with long, high frequency stimulation and recovers subsequently.

a Diagram and equivalent electrical circuit of a secretory cell with two vesicular pools,—readily releasable and resting pool. **b** EPSC amplitudes with best model fits. Synaptic depression was induced with 100 s of stimulation (10 repeating trains of 10 Hz for 5 s and 5 Hz for 5 s). To study the recovery of the system, the synapse was stimulated with single trains at specific times afterwards. For comparison, dashed lines represent the model fit during the first 10 s of stimulation. **c** Fractional release estimates are similar during both long stimulation and subsequent recovery. **d** The replenishment rate decreases markedly and subsequently recovers

Given that the MATLAB Optimization Toolbox function `lsqnonlin` used for optimization is non-linear operator, the optimization problem is solved by iteration. `lsqnonlin` uses a subspace trust region method and is based on the interior-reflective Newton method (Coleman and Li 1996a, b). Briefly, a 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 for minimization is “trusted” to be valid (i.e. it approximates the function for minimization adequately well). During the search the size of the region is modified, based on how well the model agrees with the evaluations of the function, but choosing the steps to remain within this region of trust. If quadratic model approximates well the function for minimization within the trust region then the region is expanded. However, if the approximation is poor, the region is contracted. Trust region methods and line search methods may thus be considered as dual. Whereas trust region methods first choose a step size (the size of the trust region) and then its direction, line search methods first choose a step direction and then its size. The procedure is

repeated until convergence. The algorithm is taken to converge when one of the following criteria is met: a) the cost function changes less between iterations than a tolerance value, 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, illustrating how the error diminishes progressively during search 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.

The question may be raised why use model-fitting instead of traditional curve-fitting to determine the parameters of the vesicular storage and release, both derived (fractional release and replenishment rate of the RRP, and other replenishment rates) and basic (R 's and C 's). Curve fitting is an awkward approach lacking flexibility. It does not yield directly either derived or basic parameters. Consequently these parameters have to be calculated following curve-fitting by solving a system of algebraic equations, a task that becomes more difficult if patterned stimulation is used.

‘Open system’ contribution

The model of vesicular storage and release we used rests on the assumption that the vesicular fluxes are driven by the differences in vesicular pool densities (Kruckenberg and Sandweg 1968). However, if there are fluxes that move against their concentration gradient (such as directed movement), they were not neglected, but were captured as the ‘open system’ contribution. 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 vesicular synthesis is the only source of new quanta. In our model, however, the initial number of vesicles in each pool (i.e. the charges Q_1 and Q_2) was estimated for every window, but also the capacitors C_1 and C_2 and the initial voltages U_1 and U_2 across these capacitors. The ‘open system’ contribution is then assessed by subtracting the release of a simulated ‘closed’ system from the release of an ‘open system’.

Note that over 10 s windows used for the estimation of parameters of vesicular dynamics the ‘open system’ contribution is considered constant, but may change from one window to another. Finally, note that choosing the model of the vesicular storage and release system to be ‘open’ takes also into account the possibility that vesicle pools may not initially be in equilibrium. The parameter estimates of the first 1–3 windows are sometimes quite inaccurate (data not shown) if an equilibrium assumption is made, not surprisingly since vesicular pools do not have to be necessarily in equilibrium initially. Finally, note that regardless of whether the typically used rate-based models or our model are used, the estimated rate values are the same (not shown).

All graphs and all regression fits through the data were performed using Origin 8.0 software (Origin Lab, USA). Comparisons between two means were performed using Student’s paired or unpaired t test, as appropriate.

Results

The replenishment rate recovers rapidly after long, high frequency stimulation

The vesicular storage and release model used in this study consists of two pools, where C_1 and C_2 depict the readily releasable pool (RRP) and resting pool (RP), respectively (Fig. 1a). The parameters of the system are however not constant during prolonged stimulation (Bui and Glavinovic 2013). The fraction of the RRP that is released per impulse [fractional release; calculated as $1/(R_0 * C_1)$] remains unaltered, whereas the rate at which the RRP is refilled [replenishment rate; calculated as $1/(R_1 * C_1)$] decreases

markedly and rapidly. It is unclear, however, how the replenishment rate recovers after such stimulation. Figure 1b depicts the stimulation paradigm used in this study. The stimulation was divided into two parts: ‘Onset’—ten repeating high-low frequency trains that induces depression; ‘Recovery’—single trains at specific times afterwards, which probe the recovery of the system. Figure 1c, d give the fractional release and replenishment rate estimates (taken every 10 s) of the above data. The fractional release does not change during stimulation, whereas the replenishment rate decreases and subsequently recovers.

Figure 2 shows the pooled results of the replenishment rate estimates. During the initial depressing stimulation, the replenishment rate is 0.72 s^{-1} and decreases to 0.18 s^{-1} by the tenth train (Fig. 2a_{1–2}). To quantify the recovery of the replenishment rate, the estimates were fit with an

Fig. 2 Characterizing the decline and recovery of the replenishment rate. **a**_{1–2} Frequency histograms of the replenishment rate during the 1st (mean and SD: 0.72 ± 0.29) and 10th trains (mean and SD: 0.18 ± 0.22), showing its decrease during long, high frequency stimulation. **b** The replenishment rate recovers mono-exponentially, but to a steady-state that is greater than the 1st train estimate (data from Fig. 1d), with best-fitted curve ($\text{Fit}_{\text{recov}} = 1.11 - 0.94 \times \exp(-t/16.21)$; $r = 0.91$) **c**_{1–2} Frequency histogram of the steady-states (mean and SD: 1.58 ± 0.91) and time constants (mean and SD: 10.56 ± 15.07) of the replenishment rate recovery fits. Pooled data ($n \geq 12$). Triangles depict mean values

exponential curve (Fig. 2b), and histograms of the steady-state values and time constants are shown (Fig. 2c_{1–2}). Overall, the replenishment rate recovers to a steady state of 1.58 s^{-1} , with a rise time of 15.07 s. Note that the replenishment rate recovers rapidly to a steady state that is ~ 2 times higher than its initial value (during the first train of the ‘onset’ stimulation),

Estimating the replenishment from the time course of recovery of synaptic output

Traditional way of estimating the replenishment rate at the end of long high-frequency stimulation is to estimate the time of recovery of synaptic output. To compare our results to previous methods, we used this method. Synaptic depression was induced as before (ten repeating high-low frequency trains), but instead of brief trains, recovery was probed with 1 Hz stimulation (Fig. 3a₁). The recovery of EPSC amplitudes was then fit with an exponential curve, and the frequency histograms depict the steady-states and time constants of the fits (Fig. 3a_{2–3}). Using the same data

from Fig. 2, recovery was also estimated by fitting exponential curves to the first EPSC amplitude of the single trains (Fig. 3b_{1–3}). Both methods yield similar time constants ($\sim 3\text{--}4 \text{ s}$), that are clearly shorter than the replenishment time constant at the end of long stimulation, which is 5.6 s, but the difference is not surprising. As shown above (Fig. 1) the replenishment rate rapidly increases during recovery. Estimating the replenishment rate from the recovery of the synaptic efficacy, though very widely used inaccurately estimates the replenishment rate. The replenishment rate is not stationary during recovery of synaptic output and this alters the time course of recovery of EPSC amplitudes.

The stimulation used to evaluate the recovery of the vesicular system itself influences the recovery measurements. To better understand how important this effect might be, a secretory system was simulated with the parameters as determined in the experiment shown in Fig. 3b₁. The stimulation used to evaluate recovery was either with various constant frequencies (Fig. 4a) or brief high-low frequency trains separated by various times

Fig. 3 Estimating replenishment using the recovery time course. **a**_{1–3} Synaptic depression was induced as in Fig. 1b, but instead of single trains, low frequency stimulation (1 Hz) was used to measure the recovery time course. The EPSC amplitudes were fit with an exponential curve ($\text{Fit}_{\text{Recov}} = 209.23 - 117.11 \times \exp(-t/1.84)$; $r = 0.78$), and the frequency histograms depict the steady-states (mean and SD: 106.5 ± 53.5) and time constants (mean and SD: 3.01 ± 3.42) of the fits ($n = 8$). **b**_{1–3} Similar to **a**, but the recovery time course was calculated using the first EPSC amplitude from the single trains. The EPSC amplitudes were fit with an exponential curve ($\text{Fit}_{\text{Recov}} = 135.31 - 81.14 \times \exp(-t/3.41)$; $r = 0.73$), and the frequency histograms depict the steady-states (mean and SD: 220.11 ± 108.42) and time constants (mean and SD: 3.62 ± 3.40) of the fits ($n = 16$). Triangles depict mean values

Fig. 4 The stimulation used to evaluate the recovery of the vesicular dynamics itself influences the recovery parameters. **a_{1–2}** The recovery was simulated with the parameters as determined in Fig. 3B₁, using constant stimulation frequencies ranging from 0.1 to 3 Hz. The EPSC amplitudes were fit with an exponential curve and the steady-state and rise times of the fits are shown ($n = 8$). **b_{1–2}** Same as above, but using the first EPSC amplitude of brief high-low frequency trains separated by various times, ranging from 5 to 40 s ($n = 16$). Arrow represents the experimental stimulation. The dashed horizontal line represents the true steady-state and rise time value. Bars represent SE

(Fig. 4b). The recovery EPSC amplitudes were fit with an exponential curve, and the steady-state and rise time values were determined. As the stimulation frequency during recovery increases, the EPSC amplitudes recover to a lower steady state, but with a faster time course. The effect is significant at the frequency range of stimulations needed to estimate the recovery time with change of the time constant being especially pronounced.

The effect of calcium on the recovery of parameters

The fractional release is higher at high $[Ca^{++}]_o$, but is stimulation-independent, whereas the replenishment rate is $[Ca^{++}]_o$ -independent, but diminishes during stimulation (Bui and Glavinovic 2013). However, it is unclear if the recovery of the replenishment rate depends on $[Ca^{++}]_o$. To

clarify this we used the same stimulation paradigm as before, but at 2 and 10 mM $[Ca^{++}]_o$ (Fig. 5a). Parameters were estimated every 10 s, and the change of the fractional release and replenishment rate characterized by fitting the lines and exponential curves respectively. The means of these fits are displayed in Fig. 5b, c. The fractional release is as expected higher at high $[Ca^{++}]_o$ (higher intercept), and remains unchanged during both ‘onset’ and ‘recovery’ stimulation. The replenishment rate, which decreases with the ‘onset’ stimulation, is not significantly altered by changes of $[Ca^{++}]_o$. However, higher $[Ca^{++}]_o$ (10 mM) causes the replenishment rate to recover more slowly (time constant becomes ~ 40 s), but to a higher steady-state (~ 2 times greater), demonstrating that the replenishment eventually becomes faster after long, high frequency stimulation if $[Ca^{++}]_o$ is higher.

To better understand the effects of $[Ca^{++}]_o$ on recovery, we also examined how ‘basic’ parameters of the system recover following stimulation. Release coupling is stimulation-independent. It remains so following stimulation and $[Ca^{++}]_o$ does not alter that (Fig. 6a_{1–2}). Replenishment coupling, which decreases during the ‘onset’ stimulation in a $[Ca^{++}]_o$ -independent manner, however recovers more slowly, but to a higher steady-state at high $[Ca^{++}]_o$ (Fig. 6b_{1–2}). Finally, the RRP size, which is lower at higher $[Ca^{++}]_o$ (lower intercept), changes little with stimulation (Fig. 6c_{1–2}).

The recovery of parameters depends on stimulation duration

To induce synaptic depression, we have been using an ‘onset’ stimulation of 100 s. We now evaluate how stimulation duration affects the recovery of the parameters. We shortened the ‘onset’ stimulation duration, but kept the ‘recovery’ protocol as before. Figure 7a shows the EPSC amplitudes with best model fits, consisting of either 5 or 10 trains during the ‘onset’ stimulation. As expected, the fractional release estimates remain relatively constant and are unaffected by the number of initial trains (Fig. 7b_{1–2}). The replenishment rate declined to a similar extent with 5 or 10 initial trains (Fig. 7b₁). However, with shorter ‘onset’ stimulation, the replenishment rate recovers more slowly (time constant becomes ~ 85 s), and to a lower steady-state ($\sim 50\%$ less) (Fig. 7c₂). Thus, longer stimulation causes the replenishment rate of RRP to recover faster and to a higher steady-state.

Figure 8 demonstrates how the duration of ‘onset’ stimulation affects the change of ‘basic’ parameters of the vesicular system. Release coupling does not change during stimulation or afterwards (Fig. 8a_{1–2}). The replenishment coupling decreases to a similar level with 5 or 10 trains of stimulation, but it recovers more slowly (time constant is

Fig. 5 Higher calcium causes the replenishment rate to recover slower, to a higher steady-state. **a** EPSC amplitudes with best model fits, at 2 and 10 mM calcium. **b**_{1–2} Fractional release increases with calcium, but does not change during onset ($Fit_{2Ca} = 0.02 \times t + 5.56$ and $Fit_{10Ca} = 0.002 \times t + 13.63$) or recovery ($Fit_{2Ca} = 0.01 \times t + 5.87$ and $Fit_{10Ca} = 0.01 \times t + 12.58$) stimulation. **c**_{1–2} The replenishment rate decreases with long, high frequency stimulation,

and its time course is independent of calcium concentration [$Fit_{2Ca} = 0.34 + 0.000004 \times \exp(-t/8.34)$ and $Fit_{10Ca} = 0.22 + 0.000004 \times \exp(-t/8.13)$]. However, greater calcium causes the replenishment rate to recover to a higher steady-state [$Fit_{2Ca} = 1.56 - 0.91 \times \exp(-t/11.00)$ and $Fit_{10Ca} = 4.03 - 3.34 \times \exp(-t/49.16)$]. Each circle represents the mean of parameter fits, and bars represent SE ($n \geq 9$)

~25 s longer) and to a significantly lower steady-state level (~2/3 less) with shorter stimulation (Fig. 8b_{1–2}). Finally, the RRP size (the ability to contain quanta) changes little during stimulation or afterwards regardless of the stimulation duration (Fig. 8c_{1–2}). Overall, the change of the replenishment coupling largely determines change of the replenishment rate during or following stimulation.

Okadaic acid does not affect the fractional release or replenishment rate during recovery

Okadaic acid, an inhibitor of phosphatases 1 and 2A, has been shown to increase vesicular trafficking (Betz and Henkel 1994; Gaffield et al. 2006). We tested whether the recovery of the parameters is dependent on this drug, and used the same stimulation paradigm as before, both in

control and with 1 μ M okadaic acid in the bath. The recovery of either the fractional release (Fig. 9a) or the replenishment rate of RRP (Fig. 9b) is not affected by okadaic acid.

Discussion

We evaluated the vesicular fluxes between pools of the secretory cell, and the replenishment and release rates from the dynamics of the vesicular release by fitting an electric circuit model of vesicular dynamics to the EPSC amplitudes recorded in rat hippocampal pyramidal neurons (Aristizabal and Glavinovic 2004; Bui and Glavinovic 2013; Kruckenberg and Sandweg 1968). The dynamics of vesicular storage and release has been in the past analyzed

Fig. 6 The effects of calcium on the recovery of basic parameters. **a₁₋₂** Release coupling does not change during or following stimulation and $[Ca^{++}]_o$ does not significantly alter that ($Fit_{2Ca} = -0.00002 \times t + 0.22$ and $Fit_{10Ca} = 0.00002 \times t + 0.19$; onset; $Fit_{2Ca} = -0.0001 \times t + 0.14$ and $Fit_{10Ca} = 0.0001 \times t + 0.15$; recovery). **b₁₋₂** Higher $[Ca^{++}]_o$ does not affect the decline of the replenishment coupling during stimulation [$Fit_{2Ca} = 0.21 - 0.0002 \times \exp(-t/11.52)$ and $Fit_{10Ca} = 0.32 - 0.004 \times \exp(-t/17.37)$], but its recovery is slower and to a higher steady-state [$Fit_{2Ca} = 2.15 - 1.01 \times \exp(-t/17.23)$ and $Fit_{10Ca} = 3.03 - 2.15 \times \exp(-t/30.45)$]. **c₁₋₂** The size of the RRP is lower at high $[Ca^{++}]_o$, but remains unaltered during stimulation ($Fit_{2Ca} = -0.009 \times t + 3.81$ and $Fit_{10Ca} = 0.004 \times t + 2.56$) or during recovery ($Fit_{2Ca} = -0.0007 \times t + 3.07$ and $Fit_{10Ca} = 0.003 \times t + 1.63$). Each circle represents the mean of parameter fits, and bars represent SE ($n \geq 9$)

by many investigators in terms of two-pool (or sometimes three-pool) models, because the decay of the synaptic output during tetanic stimulation is two-exponential (or three-exponential). We find that the same two-pool model fits adequately, not only the decay of the synaptic output during brief high frequency train, but also the recovery during brief low frequency train that follows. The same model, but with different parameters, fits well the decay and recovery of all brief high-low frequency trains during long patterned stimulation, as well as during subsequent recovery. The fits are also equally good for the small or large amplitudes, and the parameters estimated using patterned and random stimulation (this is a very rigorous test because random stimulation contains not one or several frequencies, but all frequencies) are very similar, and moreover change very similarly during stimulation (Bui and Glavinovic 2013). Finally, the estimated parameters are the same as those evaluated using two-pool, but rate based model (Heinemann et al. 1993). This is as expected, because in both cases the kinetics of the synaptic output are

structurally the same (the sum of two exponentials plus a baseline). Taken together it is clear that the model is appropriate and that the model-fitting is reliable.

The fractional release is typically evaluated from the tetanic rundown, but this assumes that the replenishment is negligible (Christensen and Martin 1970). Using model-fitting (this is more reliable method) we find that overall (though not necessarily in individual synapses) the fractional release does not change significantly during or after long high frequency stimulation.

Estimating the replenishment rate is more challenging and led to more controversial findings. Previous attempts to estimate the replenishment rate of the RRP from the time course of recovery of synaptic output following long, high frequency stimulation, provided values that ranged from 0.02 to 2 s^{-1} , i.e. they varied by approximately two orders of magnitude (Ryan et al. 1993; Stevens and Tsujimoto 1995; Stevens and Wesseling 1998; Wang and Manis 2008; Wesseling and Lo 2002). In this study we try to determine possible reasons causing such an uncertainty in the evaluation

Fig. 7 Longer stimulation causes the replenishment rate to recover faster, and to a higher steady-state. **a** EPSC amplitudes with best model fits, using 5 or 10 initial trains of stimulation. **b**_{1–2} Fractional release does not change during onset ($\text{Fit}_{5\text{Trains}} = -0.02 \times t + 4.88$ and $\text{Fit}_{10\text{Trains}} = 0.01 \times t + 5.56$) or recovery ($\text{Fit}_{5\text{Trains}} = -0.01 \times t + 5.87$ and $\text{Fit}_{10\text{Trains}} = -0.01 \times t + 5.79$) regardless of the duration of onset stimulation. **c**_{1–2} The replenishment rate

decreases to the similar steady-state level during onset stimulation [$\text{Fit}_{5\text{Trains}} = 0.31 + 0.0001 \times \exp(-t/5.66)$ and $\text{Fit}_{10\text{Trains}} = 0.34 + 0.000002 \times \exp(-t/7.82)$]. However, longer onset stimulation causes the replenishment to recover faster, and to a higher steady-state [$\text{Fit}_{5\text{Trains}} = 0.59 - 0.38 \times \exp(-t/100.25)$ and $\text{Fit}_{10\text{Trains}} = 1.54 - 0.99 \times \exp(-t/14.88)$]. Each circle represents the mean of parameter fits, and bars represent SE ($n \geq 5$)

of the replenishment rate. We find that several factors influence the replenishment rate estimated from the recovery following the long high frequency stimulation, and that at the excitatory synapses of rat hippocampus they are important.

If the replenishment rate does not remain constant during long, high frequency stimulation, the estimates will be those at the end, not at the beginning of stimulation, and at the excitatory synapses of rat hippocampus they are much lower at the end of stimulation. The replenishment rate, which is comparatively high at the beginning of stimulation decreases to low value at the end of stimulation (the rate changes from (0.72/s to 0.18/s; i.e. the time constant changes from 1.4 s to 5.6 s), and the decrease is rapid (8.3 s). The replenishment rate recovers quickly following the stimulation (time constant is 10.6 s). Interestingly, it recovers to a new steady-state value that is approximately twice as high. Both changes render the recovery of the

synaptic efficacy significantly faster and thus increase the discrepancy between the replenishment rate at the end of long, high frequency stimulation and the replenishment rate evaluated from the recovery of the synaptic output.

Even when the replenishment rate reaches the lower steady-state value during prolonged stimulation, the additional stimulation affects the speed of the subsequent recovery and the new steady-state level of the replenishment rate. Longer stimulation renders the replenishment rate recovery faster and its steady-state level higher, which renders the estimates of the replenishment rate even higher. This is in contrast to the previous report that the long stimulation leads to a ‘form of short-term depression that persists for a much longer time’ (Stevens and Wesseling 1999). The difference is striking, and is possibly due to the fact that we used rats and they used mice. Stimulation is not only needed to induce the synaptic depression, but also

Fig. 8 Replenishment coupling is responsible for the stimulation-induced changes to the recovery of the replenishment rate. **a**_{1–2} Release coupling is not significantly altered by stimulation during onset ($\text{Fit}_{5\text{Trains}} = -0.0004 \times t + 0.15$ and $\text{Fit}_{10\text{Trains}} = 0.0002 \times t + 0.19$) or recovery ($\text{Fit}_{5\text{Trains}} = -0.001 \times t + 0.13$ and $\text{Fit}_{10\text{Trains}} = 0.0001 \times t + 0.15$). **b**_{1–2} The replenishment coupling decreases similarly during the onset stimulation [$\text{Fit}_{5\text{Trains}} = 0.14 + 0.11 \times \exp(-t/23.00)$ and $\text{Fit}_{10\text{Trains}} = 0.3 + 0.004 \times$

$\exp(-t/17.37)$]. However, shorter onset stimulation causes it to recover slower, to a lower steady-state [$\text{Fit}_{5\text{Trains}} = 1.01 - 0.6 \times \exp(-t/57.37)$ and $\text{Fit}_{10\text{Trains}} = 3.23 - 2.12 \times \exp(-t/30.45)$]. **c**_{1–2} RRP size is not significantly dependent on stimulation during onset ($\text{Fit}_{5\text{Trains}} = -0.00008 \times t + 4.72$ and $\text{Fit}_{10\text{Trains}} = 0.009 \times t + 4.15$) or recovery ($\text{Fit}_{5\text{Trains}} = -0.005 \times t + 3.35$ and $\text{Fit}_{10\text{Trains}} = -0.0007 \times t + 3.12$). Each circle represents the mean of parameter fits, and bars represent SE ($n \geq 5$)

to test the subsequent recovery, but this also changes the parameters of the system and their recovery. It is interesting to note that the synaptic output recovers faster, but to a lower steady state if the stimulation frequency used during recovery is higher. In conclusion several factors act in different directions, and could render the values of the replenishment rate artificially significantly higher or lower from the true values. Based on the above it is clear that the replenishment rates of the secretory system previously reported need to be re-evaluated.

It is also worth pointing out that if a secretory system is time dependent, and we believe that this is generally true, the estimates of all parameters will be erroneous, and in

many cases they will be probably grossly so, if their estimation was based on the assumption that the system is time independent. More importantly, such approaches may suggest the models whose structure is invalid. Briefly, the change of parameters during stimulation will appear as ‘new parameters’ leading to the models that are artificially complex. Even if one tries to assess the change of one parameter of the secretory system (such as the replenishment rate of the RRP), but considers other parameters (fractional release, open system contribution, ...) constant, the problems will remain though they may be less severe (Birks and MacIntosh 1961; Kavalali 2007; Zucker and Regehr 2002; Gabriel et al. 2011).

Fig. 9 Okadaic acid does not affect the recovery of parameters. **a**_{1–2} Fractional release does not significantly change during onset ($\text{Fit}_{\text{Cont}} = -0.01 \times t + 5.56$ and $\text{Fit}_{\text{OA}} = -0.01 \times t + 2.64$) or recovery ($\text{Fit}_{\text{Cont}} = -0.01 \times t + 5.87$ and $\text{Fit}_{\text{OA}} = 0.01 \times t + 3.71$) stimulation. **b**_{1–2} The replenishment rate decreases similarly during

the onset [$\text{Fit}_{\text{Cont}} = 0.12 + 0.000006 \times \exp(-t/8.64)$ and $\text{Fit}_{\text{OA}} = 0.15 + 0.02 \times \exp(-t/38.94)$] and recovery [$\text{Fit}_{\text{Cont}} = 0.85 - 0.34 \times \exp(-t/6.54)$ and $\text{Fit}_{\text{OA}} = 0.78 - 0.30 \times \exp(-t/0.72)$] stimulation. Each circle represents the mean of parameter fits, and bars represent SE ($n \geq 6$)

The ‘mobilization by liberation’ model (Morales et al. 2000; Shakiryanova et al. 2007; Shtrahman et al. 2005) argues that $[\text{Ca}^{2+}]_i$ accumulation during stimulation leads to the higher replenishment rate of the RRP (Dittman and Regehr 1998; Weis et al. 1999). Although the progressive decrease of the replenishment rate during stimulation rules out such an explanation for the change of the replenishment rate during stimulation, the question still remains whether such a mechanism may be important during recovery. We indeed find that both higher $[\text{Ca}^{2+}]_o$ and longer stimulation cause the replenishment rate to increase to a higher steady-state during recovery, but their time courses of recovery are qualitatively different. The recovery becomes slower if $[\text{Ca}^{2+}]_o$ is higher, but it is faster after longer stimulation. The stimulation thus does not affect the replenishment rate solely through $[\text{Ca}^{2+}]_i$ accumulation, and this is supported by the changes of the underlying ‘basic’ parameters. $[\text{Ca}^{2+}]_o$ affects the replenishment rate because the size of the RRP is $[\text{Ca}^{++}]_o$ -dependent, whereas stimulation

modulates the replenishment rate via the replenishment coupling.

What is thus the mechanism controlling the recovery of the replenishment rate during recovery? This study does not provide definitive answer to that question. As stated above both $[\text{Ca}^{++}]_o$ and longer stimulation (which should lead to greater $[\text{Ca}^{++}]_i$ accumulation) lead to higher state-state values of the replenishment rate. However, the recovery time of the replenishment rate rises as $[\text{Ca}^{++}]_o$ increases, but becomes shorter following stimulation of longer duration. The stimulation thus does not influence the recovery of the replenishment rate just through $[\text{Ca}^{++}]_i$ accumulation. Finally, okadaic acid—a drug known to increase vesicular mobility (Betz and Henkel 1994; Gaffield et al. 2006), does not influence the recovery of the replenishment rate after long, high frequency stimulation, although high concentrations of drug were used (1 μM for 30 min prior to stimulation), much higher than those used in cultured rat hippocampal neurons (Malchiodi-Albedi et al. 1997).

This argues against the idea that the vesicular replenishment of the RRP during recovery is associated with significant vesicular movement, but this question requires further examination.

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