Supporting Information

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SI Text

The Parallel Two-Pool Model. For a simple quantitative description of our results we assume that resting synapses have a pool of SV_{ss}, which is similar in size among synapses. SV_{ss} are released during an AP with release probability \( p_n \). The SV_{ss} pool, when fully loaded, has the size \( N_n \). In addition, individual synapses have a pool of SV_{f} of variable size, which has a size \( \alpha \times N_n \). Here, \( \alpha \) is a constant for a given synapse, representing the abundance of sites for SV_{f}. \( \alpha \) increases in response to Pdbu application or during induction of PTP. SV_{f} are released with higher release probability, which is \( \beta \times p_n \). Thus, the total quantal content \( m_1 \) of the first EPSC in response to a stimulus train is

\[
m_1 = N_n \times p_n \times (1 + \alpha' ),
\]

where \( \alpha' \) was substituted for \( \alpha \times \beta \). During stimulus trains release probability may change and pools get partially depleted. For simplicity, we assume that relative changes in release probability are the same for SV_{ss} and SV_{f}. They are characterized by the factor \( \varepsilon(f) \), which is defined as the ratio of \( p_n \) of the first stimulus over that at steady state. \( \varepsilon(f) \) is a function of stimulation frequency \( f \), which most likely is close to 1, except for the highest frequencies (100 Hz and 200 Hz). We include \( \varepsilon(f) \) in the equations here for completeness, but make the simplifying assumption \( \varepsilon(f) = 1 \) in most instances, except when discussing the time course of the SV_{ss} and SV_{f} components of EPSCs. Steady-state depletion of pools is also a function of frequency, which we denote with \( \gamma(f) \) for SV_{ss} and \( \delta(f) \) for SV_{f}. The functions \( \varepsilon(f), \gamma(f) \), and \( \delta(f) \) thus represent facilitation and short-term depression of SVs. With these definitions the steady-state quantal content \( m_{ss} \) is

\[
m_{ss} = N_n \times p_n \times \varepsilon(f) \times [\gamma(f) + \alpha' \times \delta(f)].
\]

In plots like that of Fig. 3C different synapses are assumed to differ only in \( \alpha' \). For the discussion of such plots it is convenient to introduce their \( y \) and \( x \) coordinates as \( x_i = q \times m_{si} \) and \( y_i = q \times m_{ss} \), where \( q \) is the quantal size, the subscript \( i \) denotes a given synapse, and \( ss \) refers to steady state. Also, we define the coordinates \( x_n \) and \( y_n \) for (hypothetical!) synapses with \( \alpha' = 0 \) as

\[
y_n = q \times N_n \times p_n \times \varepsilon(f) \times \gamma(f)
\]

\[
x_n = q \times N_n \times p_n.
\]

With these definitions, using [S1] and [S2] we obtain

\[
y - y_n = \varepsilon(f) \times \delta(f) \times (x - x_n).
\]

For a given frequency this is a straight line, passing through \( (y_n, x_n) \), with slope \( \varepsilon(f) \times \delta(f) \). Thus, the slope of line fits in Fig. 3C provide the pool depletion factors of SV_{ss}, multiplied by a facilitation factor. Likewise, considering [S3] and [S4], the ratio \( y_n/x_n \) is the product \( \varepsilon(f) \times \gamma(f) \), providing the pool depletion factors of SV_{f}, again multiplied by possible influence of facilitation. In the main text we assumed \( \varepsilon(f) \times \gamma(f) \times \delta(f) = 1 \) for simplicity, which should be valid for low frequencies. However, for frequencies \( \geq 50 \) Hz, there are clear indications for \( \varepsilon(f) \times \gamma(f) \times \delta(f) > 1 \). (see Results for the time course of the SV_{ss} component.) The \( y \)-axis intercept, \( y_0 \), of the line fit according to Eqs. [S3]–[S5] is

\[
y_0 = \varepsilon(f) \times \gamma(f) - \varepsilon(f) \times \delta(f).
\]

Kinetiks of Priming. For a quantitative analysis of priming processes (normal priming and superpriming) we make the simplest possible assumption, namely that both SV pools are consumed and refilled independently according to a first-order reaction scheme

\[
\frac{\text{Pool}_{ss}}{\text{Pool}_{i,0}} = \frac{\frac{k_{s,i} - p_i}{f}}{k_{+i} + p_i}
\]

where the subscript \( ss \) refers to steady state. Writing this equation for the two pools considered here, with the definition of \( p_n \), including its frequency dependence \( \varepsilon(f) \) (Eq. S1), and introducing the definitions

\[
\frac{f_n}{p_n \times \varepsilon(f)} = \frac{k_{s,i}}{k_{+i} + p_i}
\]

we obtain from [S3], [S4], [S9], and [S10]

\[
y_n/x_n = \varepsilon(f) \times \gamma(f) = \frac{f}{1 + f/f_n}
\]

and for the slope \( s(f) \) of Fig. 3C

\[
s(f) = \varepsilon(f) \times \delta(f) = \frac{f}{1 + f/f_s}
\]

The fit of Fig. 4B was calculated according to Eqs. [S10] and [S12], once for a fixed value of \( k_{+i,0} \) of 0.8 s\(^{-1}\) and once for a frequency-dependent one:

\[
k_{+i} = 0.5 \text{ s}^{-1} + 1.9 \frac{f}{f + 15 \text{ s}^{-1}}.
\]

\( \varepsilon(f) \) was assumed to be 1, which is expected to hold, except for the highest frequencies of 100 Hz and 200 Hz.

SI Materials and Methods

Slice Preparation. Juvenile, posthearing (P13–16) Wistar rats of either sex were used for most experiments. Brainstem slices were
prepared as previously described (35). In short, after decapitation, the whole brain was immediately immersed into ice-cold low Ca\(^{2+}\) artificial CSF (aCSF) containing 125 mM NaCl, 2.5 mM KCl, 3 mM MgCl\(_2\), 0.1 mM CaCl\(_2\), 10 mM glucose, 25 mM NaHCO\(_3\), 1.25 mM NaH\(_2\)PO\(_4\), 0.4 mM ascorbic acid, 3 mM myo-inositol, and 2 mM Na-pyruvate, pH 7.3, when bubbled with carbogen (95% O\(_2\), 5% CO\(_2\)). The brainstem was glued onto the stage of a VT1000S vibratome (Leica) and 200-μm thick coronal slices containing the MNTB were cut. Slices were incubated for 30–40 min at 35 °C in an incubation chamber containing normal aCSF and kept at room temperature (22–24 °C) for up to 4 h thereafter. The composition of normal aCSF was identical to that of low Ca\(^{2+}\) aCSF except that 1.0 mM MgCl\(_2\) and 2.0 mM CaCl\(_2\) were used.

**Electrophysiology.** Whole-cell patch-clamp recordings were made from calyx of Held terminals and principal neurons of the MNTB, using an EPC-10 amplifier controlled by Pulse software (HEKA Elektronik). Sampling intervals and filter settings were 20 μs and 5.0 kHz, respectively. Cells were visualized by infrared-differential interference contrast microscopy through a 40× water-immersion objective, using an upright microscope (Olympus BX51WI). During experiments, slices were continuously perfused with normal aCSF solution. All experiments were carried out at room temperature (22–24 °C). For postsynaptic recordings, patch pipettes were pulled from thin-walled glass (World Precision Instruments) on a PIP-5 puller (HEKA Elektronik). Open tip resistance was 2.5–3.5 MΩ. R\(_s\) ranged from 4 MΩ to 7 MΩ. R\(_f\) compensation was set to >84% (2 μs delay). The holding potential was ~70 mV. Pipettes were filled with a solution consisting of the following: 140 mM Cs-glucurate, 20 mM TEA-Cl, 10 mM Hepes, 5 mM EGTA, 5 mM Na\(_2\)-phosphocreatine, 5 mM ATP-Mg, 0.3 mM GTP, pH 7.3, with CsOH. No corrections were made for liquid junction potentials.

EPSCs were elicited by afferent fiber stimulation via a bipolar stimulation electrode placed halfway between the brainstem midline and the MNTB. Stimulation pulses (100 μs duration) were applied using a stimulus isolator unit (AMPI), with the output voltage set to 1–2 V above threshold for AP generation (~25 V) to exclude stimulation failures at higher frequencies. For each AP-evoked EPSC the series resistance (R\(_s\)) value was updated and stored with the data, using the automated R\(_f\) compensation routine implemented in Pulse. Postsynaptic recordings with a leak current >300 pA were excluded from the analysis. For clarity, stimulation artifacts were blanked for the EPSCs displayed in Figs. 1 and 5.

A total of 1 mM kynurenic acid was added to the bath solution to minimize AMPAR desensitization and saturation in the experiments illustrated in Figs. 1–5. Nevertheless, indications of residual desensitization were apparent in some recordings, particularly in those with large EPSCs. For instance, in Fig. 5D the line fits to data from PTP experiments intersect at smaller initial EPSC amplitudes than those in Fig. 3C. This result is most likely due to differences in slope of the line fits to the 100-Hz scatter plots. Desensitization in strongly potentiated synapses may be the cause for a negative slope of the plot in Fig. 5D. (For another indication of desensitization see Fig. 4A legend.)

In Fig. 5A and C data are presented for four time intervals, relative to the induction of PTP. For EPSC trains without preconditioning these time intervals were selected as follows: before, average of five trains immediately before induction of PTP; early, average of two trains at 45 s and 75 s after tetanus onset; medium, average of three trains at 105 s, 135 s, and 165 s after tetanus onset; and late, average of three trains at 255 s, 285 s, and 315 s after tetanus onset.

Preconditioned 100-Hz trains were acquired 15 s earlier than the respective values given above.

Offline analysis was performed using Igor Pro (Wavemetrics). Remaining series-resistance errors were corrected (67) using the R\(_f\) values stored in the data files (assuming a linear IV relationship with a reversal potential of +10 mV). EPSCs were subsequently offset corrected and low-pass filtered (f\(_{\text{cutoff}}\) = 5 kHz), using a 10-pole software Bessel filter. Original data are presented as mean ± SEM. SDs of fitting parameters are given, as supplied by Igor Pro curve-fitting algorithms. For derived quantities (products, ratios, etc.) SEM was calculated assuming Gaussian error propagation.

**Glutamate Sensor and Optical Recordings.** Primary hippocampal cultures were prepared from 1-d-old Wistar rats according to the regulations of the Max Planck Society and plated on poly-d-lysine–coated coverslips. Calcium phosphate-mediated transfection of neurons with iGluSnFR (Addgene plasmid 41732) was performed at 3 DIV. All imaging experiments were performed at DIV 14–21 at room temperature (22–24 °C). During measurements neurons were perfused with modified Tyrode's solution (140 mM NaCl, 2.5 mM KCl, 3 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM glucose, 10 mM Hepes, pH 7.4). A total of 10 μM 6-cyano-7-nitroquinolinaxline-2,3-dione (CNQX) and 50 μM d,l-2-amino-5-phosphonvaleric acid (AP5) were added to prevent recurrent activity.

Coverslips were mounted in a custom imaging chamber on the stage of a Nikon TE2000 inverted microscope. iGluSnFR fluorescence was excited with a 488-nm diode laser (Coherent) and emission was imaged with a Zyla 5.5 sCMOS camera (Andor Technology) at 100 Hz through a 60×, 1.2 NA Plan Apo objective, using a 510-nm dichroic and 515–560-nm emission filter. Action potentials were evoked by 1-ms current pulses (WPI A 385; World Precision Instruments), yielding a 10 V/cm field between two platinum electrodes. Timing of stimuli was controlled by a Master 8 pulse generator and triggered by computer-controlled TTL output. Image acquisition and hardware synchronization were controlled by Andor IQ2. Image analysis was performed in Matlab.
Fig. S1. Spatial correlation of PPR between neighboring boutons. Release site pairs were grouped into 2.5-μm bins and cross-correlation of corresponding PPR values was performed. The mean ± SEM of correlation coefficients recovered from all measurements (n = 9) is plotted for pairs up to 50 μm apart. The decay of correlation with increasing distance was fitted with a single exponential (inset) and a PPR correlation length constant of 4.1 μm (95% confidence interval: 2.6, 5.7) was determined. Error bars indicate SD.