Supplementary Information

A small number of open Ca²⁺ channels trigger transmitter release at a central GABAergic synapse

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METHODS

Simultaneous paired recording and presynaptic Ca²⁺ imaging

Transverse hippocampal slices (300 µm thickness) were cut from brains of 18- to 21-day-old Wistar rats using a VT1200 vibratome (Leica). Animals were killed by decapitation, in agreement with national and institutional guidelines. Patch pipettes were pulled from thick-walled borosilicate glass tubing (2 mm outer diameter, 1 mm inner diameter); when filled with intracellular solution, the resistance was 2 – 3.5 MΩ. Simultaneous recordings from monosynaptically connected BCs and GCs in the dentate gyrus were obtained under visual control using infrared differential interference contrast videomicroscopy. Tight-seal whole-cell recordings were first established in a putative BC and subsequently in an adjacent GC. BCs were identified during the experiment on the basis of the fast-spiking AP phenotype (> 50 action potentials during 1-s depolarizing current pulses; 600 pA - 1 nA) and the location of the axon in the granule cell layer as revealed by confocal imaging. Furthermore, BC morphology was confirmed by post-hoc biocytin labeling, using 3,3’ diaminobenzidine as chromogen. The recording temperature was 22 ± 2°C.

Two Axopatch 200A amplifiers (Axon Instruments) were used for electrophysiological recordings. The presynaptic neuron was held in the current-clamp mode (I-clamp fast or normal, holding potential -70 mV) and stimulated every 10 s. Action potentials were elicited by a single current pulse (duration 1 ms, amplitude 1.4 - 2.4 nA). The postsynaptic cell was held in the voltage-clamp mode at a holding potential of -80 mV. Postsynaptic series resistance (mean: 11.1 ± 0.8 MΩ; range: 8 - 17 MΩ) was not compensated, but continuously monitored using a 4-mV test pulse; only pairs with < 2 MΩ change were analyzed. Signals were filtered at 5 kHz (4-pole low-pass Bessel filter) and digitized at 20 kHz using a CED 1401plus interface (Cambridge Electronic Design). Pulse generation and data
acquisition were performed using FPulse (U. Fröbe, Physiological Institute Freiburg) running under Igor (version 5.03) on a PC.

To measure presynaptic Ca\(^{2+}\) transients with adequate signal-to-noise ratio, the high-affinity Ca\(^{2+}\) indicator dye Oregon green 1,2-bis(2-aminophenoxy)ethane-
\(N,N,N',N'\)-tetraacetic acid 1 (OGB-1) was used\(^3,4\). 200 µM OGB-1 and 100 µM of the morphological tracer Alexa Fluor 594 were added to the presynaptic pipette solution\(^5\). Putative BC terminals were visualized 20 – 60 min after establishing the whole-cell configuration using a confocal LSM 510 microscope (Zeiss; equipped with an Olympus LumPlan Fl/IR; 60 x, NA 0.9 objective). For OGB-1 fluorescence, excitation wavelength was 488 nm (Argon laser), laser intensity was set to 0.1 – 1 % (corresponding to a power of 0.15 – 1.5 µW at the specimen), and detection range was 500 - 550 nm. For Alexa Fluor 594 fluorescence, excitation wavelength was 543 nm (He-Ne laser), laser intensity was set to 10 % (corresponding to a power of 4 µW), and detection range was 560 - 700 nm. Using the Alexa Fluor 594 fluorescence, boutons were identified as varicosities with diameter > 2 times that of the adjacent axon\(^6\). For measuring Ca\(^{2+}\) transients, areas containing putative synaptic boutons were scanned with ~20 ms sample interval (pixel size 0.3 x 0.3 µm). Ca\(^{2+}\) transients were measured from multiple (2 – 10) boutons ~20 – 60 µm from the surface, each presumably containing multiple release sites\(^1\). To enable simultaneous measurements from boutons at slightly different focal planes and to minimize the effects of drifts in focal plane during long-term recording, the pinhole size was increased to ~2 – 6 Airy units. Fluorescence changes were quantified as ΔF/\(F_0\) = (\(F - F_{baseline}\))/(\(F_{baseline} - F_{background}\))\(^5\), where \(F\) is the fluorescence in a region of interest (ROI) containing putative boutons at any given time, \(F_{baseline}\) is the mean fluorescence from a 700-ms period preceding the stimulus, and \(F_{background}\) is the background fluorescence from an adjacent ROI without any indicator-containing cellular processes. Experiments in which the resting fluorescence \(F_0\) increased by > 20 % after 20 min, presumably as a result of phototoxic damage\(^7\), were excluded from the analysis.

To test whether the OGB-1 fluorescence signals linearly reported the presynaptic Ca\(^{2+}\) concentration, we compared the peak amplitudes of \(\Delta F / F_0\) traces evoked by brief bursts of 2, 4, and 8 APs (20 ms interpulse interval) with the peak amplitudes of the algebraic sum of the corresponding number of identical \(\Delta F / F_0\)
traces evoked by single APs, shifted in time by 20 ms with respect to each other. Measured peak amplitudes were 97.8 ± 5.0 %, 82.1 ± 7.0 %, and 67.0 ± 8.7 % of computed peak amplitudes for 2, 4, and 8 APs respectively (n = 9 BCs), showing that the responses to single APs were accurately monitored by the Ca^{2+} indicator dye.

Previous analysis revealed that transmitter release at the BC-GC synapse is exclusively mediated by P/Q-type Ca^{2+} channels. Likewise, pharmacological analysis of presynaptic Ca^{2+} transients suggested that Ca^{2+} inflow is mediated by a relatively homogenous channel population. On average, 250 nM ω-agatoxin IVa reduced the presynaptic Ca^{2+} transient to 20.4 % (n = 12) of control, while 1.5 µM ω-agatoxin IVa reduced the Ca^{2+} transient to 9.1 ± 1.2 % (n = 4). As the IC_{50} value of P-type channels for ω-agatoxin IVa is 1 − 3 nM, whereas that for Q-type channels is 90 nM, these results are consistent with the hypothesis that a relatively pure population of Q-type Ca^{2+} channels mediates Ca^{2+} transients in BC terminals. This simplifies the analysis of the effects of ω-agatoxin IVa on the IPSC-[Ca^{2+}]_{i} relation.

**Solutions**

For storage of slices, a solution containing 87 mM NaCl, 25 mM NaHCO_{3}, 10 mM glucose, 75 mM sucrose, 2.5 mM KCl, 1.25 mM NaH_{2}PO_{4}, 0.5 mM CaCl_{2}, and 7 mM MgCl_{2} (equilibrated with 95 % O_{2} / 5 % CO_{2} gas mixture) was used. For the experiments, the slices were superfused with physiological saline containing 125 mM NaCl, 25 mM NaHCO_{3}, 25 mM glucose, 2.5 mM KCl, 1.25 mM NaH_{2}PO_{4}, 2 mM CaCl_{2} (or 0.2 or 0.5 mM CaCl_{2} in a subset of experiments with replacement by sucrose as required), and 1 mM MgCl_{2} (95 % O_{2} / 5 % CO_{2}). The intracellular solution for the presynaptic neuron contained 125 mM Kgluconate, 20 mM KCl, 10 mM HEPES, 4 mM MgCl_{2}, 4 mM K_{2}ATP, 10 mM Na_{2}phosphocreatine, 0.2 mg ml^{-1} biocytin (Mobitec), 200 µM OGB-1, and 100 µM Alexa Fluor 594 (both Invitrogen). The intracellular solution for the postsynaptic neuron contained 110 mM KCl, 35 mM Kgluconate, 10 mM EGTA, 2 mM MgCl_{2}, 2 mM Na_{2}ATP, 10 mM HEPES, 1 - 4 mM QX-314 (Biotrend), and 0.2 mg ml^{-1} biocytin; the pH was adjusted to 7.2 with KOH in both solutions. ω-Agatoxin IVa (Bachem; Product # H-1544) was applied using a recirculation system with a peristaltic pump (Ismatec). The total volume of the system was ~10 ml, and the solution was equilibrated with 95 % O_{2} / 5 % CO_{2}. Bovine serum albumin was added at a concentration of 1 mg ml^{-1} to prevent adsorption of the toxin.
Chemicals were from Merck, Sigma, and Riedel-de Haën, unless specified differently.

Data analysis
Data analysis was performed using Mathematica (Wolfram Research). The peak amplitude of the unitary IPSC was measured as the difference between baseline and the peak inward current < 8 ms after the presynaptic AP. The peak amplitude of the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) transient was measured as the mean of the first four data points after the presynaptic AP.

IPSC-[Ca\(^{2+}\)]\(_i\) relations (peak IPSC versus peak [Ca\(^{2+}\)]\(_i\)) were fitted with a power relation \(y(x) = x^m\), where \(m\) is the power coefficient. The power coefficient of the IPSC-[Ca\(^{2+}\)]\(_i\) relation during a reduction from 2 mM to 0.2 or 0.5 mM Ca\(^{2+}\) was slightly higher than that previously determined from the relation between IPSC and extracellular Ca\(^{2+}\) concentration\(^1\), presumably due to saturation of inflow through presynaptic Ca\(^{2+}\) channels with increasing Ca\(^{2+}\) concentration\(^10,11\). Mean values of \(m\) were obtained by fitting pooled data from multiple pairs. SEM values and coefficients of variation (CV) of \(m\) among pairs were calculated by fitting IPSC-[Ca\(^{2+}\)]\(_i\) relations separately for each pair. Finally, CVs of \(m\) among boutons of the same pair were obtained by fitting IPSC-[Ca\(^{2+}\)]\(_i\) relations separately for each putative bouton.

Values are given as means ± SEMs. Error bars in Figures also indicate SEMs whenever they exceed the size of the symbols. Confidence intervals and significance of differences were assessed by bootstrap analysis\(^12\) at the significance level (\(P\)) indicated.

Modeling the number of open Ca\(^{2+}\) channels triggering release
To determine the number of open Ca\(^{2+}\) channels required for transmitter release, we modeled the IPSC-[Ca\(^{2+}\)]\(_i\) relation during ω-agatoxin IVa application as follows. We assumed that a cluster of \(N\) Ca\(^{2+}\) channels were coupled to the Ca\(^{2+}\) sensors with either uniform or non-uniform coupling distance. Furthermore, we assumed that the toxin blocked the Ca\(^{2+}\) channels randomly and irreversibly\(^13,14\). In the uniform model, the probability of observing exactly \(k\) unblocked channels \(p_k\) (\(k = 0 \ldots N\)) was computed according to a binomial distribution as \(p_k = \binom{N}{k} p_{ub}^k p_b^{N-k}\), where \(p_{ub}\) or \(p_b\)
represent the probabilities that an individual channel is either unblocked or blocked ($p_{ub} + p_b = 1$). The Ca$^{2+}$ transients and the corresponding release probability for $k$ unblocked channels were calculated as $[\text{Ca}^{2+}]_k = (k / N) [\text{Ca}^{2+}]_{\text{max}}$ and $p_{\text{release, k}} = p_{R}[k / N] [\text{Ca}^{2+}]_{\text{max}}$, where $p_R[x]$ is the IPSC-[$\text{Ca}^{2+}$]$_i$ relation (approximated from experiments in which the extracellular Ca$^{2+}$ concentration was reduced; Fig. 1e) and $[\text{Ca}^{2+}]_{\text{max}}$ is the amplitude of the Ca$^{2+}$ transient under control conditions. Subsequently, the mean Ca$^{2+}$ concentration $<[\text{Ca}^{2+}]>$ and the mean release probability $<p_{\text{release}}>$ were computed as $\sum_{k=0}^{N} p_k [\text{Ca}^{2+}]_k$ and $\sum_{k=0}^{N} p_k p_{\text{release, k}}$, respectively, and the $<p_{\text{release}}>$ versus $<[\text{Ca}^{2+}]>$ curve was obtained by plotting the two parameters against each other for a wide range of blocker concentrations.

In the non-uniform model, the probability of observing a particular combination of $k$ unblocked channels $p_k$ ($k = 0 .. N$) was calculated as $p_k = p_{ub}^k p_b^{N-k}$. The Ca$^{2+}$ transient for such a particular combination was computed from the contributions from individual unblocked channels as $[\text{Ca}^{2+}] \propto \sum_{i=1}^{k} (1 / r_i \exp(-r_i / \lambda_o))$, where $r_i$ is the coupling distance of the $i^{th}$ open channel, $\lambda_o$ is the length constant $\lambda_o = \sqrt{D_{\text{Ca}} / (k_{\text{on}} [B]^o)}$, $D_{\text{Ca}}$ is the diffusion coefficient of free Ca$^{2+}$ (220 µm$^2$ s$^{-1}$), and $k_{\text{on}} [B]^o$ is the buffer product of endogenous buffer (1010 s$^{-1}$)$^{15}$. The Ca$^{2+}$ transients and the corresponding release probabilities for $k$ unblocked channels were calculated by summing over all possible combinations of unblocked channels. According to previous experimental data, channel density was assumed to be normally distributed, with a minimum at 1 nm, a peak at 12 nm, and a standard deviation of 8 nm$^{15}$.

The number of open Ca$^{2+}$ channels necessary for release was determined by fitting the $<p_{\text{release}}>$ versus $<[\text{Ca}^{2+}]>$ function of the model to the experimental data, with extrapolation for $<p_{\text{release}}> > 1$ and $<[\text{Ca}^{2+}]> > 1$ based on a power function as required (Fig. 2b). The sum of squared differences was minimized using FindMinimum of Mathematica (Wolfram Research).

A stochastic model of Ca$^{2+}$ channel – exocytosis coupling

A stochastic model of synaptic transmission in which a single Ca$^{2+}$ channel was coupled to a Ca$^{2+}$ sensor was implemented as follows. First, stepwise openings and closings of a single Ca$^{2+}$ channel during an overshooting AP were simulated, using a
Hodgkin-Huxley type Ca\textsuperscript{2+} channel model with two ‘gating particles’\textsuperscript{16}. The single-channel conductance was assumed as 2.5 pS\textsuperscript{17}. Second, the Ca\textsuperscript{2+} concentration 12 nm away from the source was calculated by solving the partial differential equation of the full reaction diffusion problem using NDSolve of Mathematica 7.0; the spatial grid resolution was 0.1 nm\textsuperscript{15,18}. Finally, the release rate was computed using an allosteric 6-state model of transmitter release\textsuperscript{19}. As experimental data from hippocampal inhibitory terminals are currently unavailable, AP waveform, Ca\textsuperscript{2+} channel model\textsuperscript{16} (their Fig. 8), and release model\textsuperscript{19} were based on data from the calyx of Held. An equivalent deterministic model corresponding to an infinite number of Ca\textsuperscript{2+} channels was implemented similarly, except that a smooth Ca\textsuperscript{2+} channel open probability waveform with a maximum scaled to 1 was used. Transmitter release rate was calculated by solving the corresponding differential equation using a Q matrix approach.

Both mobile and immobile buffers were considered in the model. The affinities of mobile buffers (ATP) and endogenous fixed buffers were assumed as 200 µM and 2 µM, respectively, and the binding rates for Ca\textsuperscript{2+} were chosen as 5 \textcdot 10\textsuperscript{8} M\textsuperscript{-1} s\textsuperscript{-1} in both cases\textsuperscript{20}. The concentration of mobile buffers was assumed as 290 µM\textsuperscript{15,20}. The concentration of the fixed buffers was assumed as 160 µM, twofold higher than at the calyx\textsuperscript{20} to account for the higher endogenous Ca\textsuperscript{2+} binding ratio of BCs\textsuperscript{21}. The resting Ca\textsuperscript{2+} concentration was set to 71 nM, as previously measured in BCs using 100 µM fura-2\textsuperscript{21}. The diffusion coefficients were chosen as D\textsubscript{Ca} = D\textsubscript{B} = 220 µm\textsuperscript{2} s\textsuperscript{-1}, where D\textsubscript{B} is the diffusion coefficient for the mobile buffer.

REFERENCES
**Supplementary Figure 1.** Probing the number of open Ca$^{2+}$ channels required for transmitter release at the BC-GC synapse.

(a, b) Schematic illustration of the effects of a slow Ca$^{2+}$ channel blocker for several Ca$^{2+}$ channels (a) and a single Ca$^{2+}$ channel per release site (b). Sequential Ca$^{2+}$ channel block is indicated by crosses of different color (red, channels blocked first; green, channels blocked second; blue, channels affected last). The predictions for the shape of the IPSC-[Ca$^{2+}$]$_i$ relation are shown on the right. For changes in extracellular Ca$^{2+}$ concentration (not illustrated), the prediction would be identical to that for the coupling scenario with slow blocker and several channels (a).
Supplementary Figure 2. IPSC-[Ca^{2+}]_{i} relations in single experiments.

(a, b) Plot of peak amplitudes of IPSCs (filled squares) and ∆F / F₀ (open squares) evoked by single APs against time during reduction of extracellular Ca^{2+} concentration from 2 mM to 0.5 mM (a) and application of 250 nM of the P/Q-type Ca^{2+} channel blocker ω-agatoxin IVa (b). Both IPSCs and ∆F / F₀ values were normalized to the values in a 3–8 min control period (absolute values: 24 %, 372 pA in a; 24 %, 149 pA in b). Data points connected by red lines for clarity. (c, d) Normalized IPSC - ∆F / F₀ relation obtained during reduction of extracellular Ca^{2+} concentration from 2 mM to 0.5 mM (c) and application of 250 nM ω-agatoxin IVa (d). Red curves represent a power function fitted to the data points. Note the very different power coefficients (m = 3.49 in c, m = 1.28 in d). Data from two different experiments; same pairs as those from which the traces in Fig. 1c, d, top are taken.