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Supplemental Information

Fast, Temperature-Sensitive and Clathrin-Independent Endocytosis at Central Synapses

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Supplemental Information

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Figure S1 (related to Figure 1). Associated $R_s$ changes and tetanustoxin-sensitivity of exocytosis

(A) Example $C_m$ recording and associated $R_s$ of a cMFB. The AP voltage command evoked a small increase in $C_m$ (top), associated with a small decrease in $R_s$ (bottom).

(B) Double-logarithmic plot of change in $C_m$ ($\Delta C_m$) vs. change in $R_s$ ($-\Delta R_s$), which displays a clear correlation (Hallermann et al., 2003). Data are from 39 cMFBs, $\Delta C_m$ was evoked using a single AP. The linear regression line has a slope of 1.15.
(C) Impact of sine-wave frequency on lock-in measurements. *Top:* Example recordings of membrane capacitance ($C_m$) from a cMFB using sine-wave frequencies of 1 kHz (left) and 2 kHz (right). *Middle and Bottom:* Associated series resistance ($R_s$) and membrane resistance ($R_m$). We used brief depolarizing pulses from $-80$ mV to 0 mV (duration, 1 ms) to evoke exocytosis.

(D) A higher sine-wave frequency (2 kHz vs. 1 kHz) gives a slightly smaller estimate of resting $C_m$ (left, $p = 0.03$, Wilcoxon signed-rank test), but the increase in $C_m$ evoked by 1-ms depolarizations was independent of sine-wave frequency (right, $p = 0.67$; Balakrishnan et al., 2015). Data are represented as mean ± SEM.

(E) Ca$^{2+}$ currents and exocytosis elicited by 1-ms voltage steps from $-80$ mV to 0 mV are stable over the recording time under control conditions. *Top:* Example traces of Ca$^{2+}$ currents under control condition immediately after break-in (dark gray) and at four points in time during whole-cell recording (shades of gray). *Bottom:* Corresponding $C_m$ increase reflecting exocytosis, which was stable over more than 8 minutes.

(F) *Top:* Example traces of Ca$^{2+}$ currents in a recording with 5 µM TeNT-LC in the pipette solution immediately after break-in (dark blue) and at four points in time during whole-cell recording (shades of blue). *Bottom:* Corresponding $C_m$ increase reflecting exocytosis, which was rapidly blocked by TeNT-LC.

(G) *Top:* Normalized $\Delta C_m$ vs. recording time in the presence of 5 µM TeNT-LC. $T_{1/2}$ for inhibition by TeNT-LC was 1:35 minutes. *Middle:* Normalized Ca$^{2+}$ currents ($I_{Ca}$) vs. recording time. *Bottom:* Normalized $C_m$ artifact (cf. Figure 1) vs. recording time. Colors represent individual cells; dashed lines indicate unity.
Figure S2 (related to Figure 2). Temperature dependence of exocytosis, endocytosis, and presynaptic Ca\(^{2+}\) currents

(A) Grand average data of C\(_m\) traces in response to a 3-ms depolarization from −80 mV to 0 mV in cMFBs at room- (blue) and physiological-temperature (red). The decay in the C\(_m\) traces was fit with a single exponential function with time constants of 8.9 s for 24°C and 1.7 s for 36°C. Inset depicts corresponding example Ca\(^{2+}\) currents at the two temperatures (scale bars, 1 ms and 500 pA).

(B) C\(_m\) increase (∆C\(_m\)) at 23°C and 36°C in cMFBs. Data include the experiments shown in panel A, and additional experiments with shorter C\(_m\) recording duration. The resulting Q\(_{10}\) temperature coefficient for exocytosis is indicated (bootstrap SEM, corresponding to a 16–84% confidence interval).

(C) Ca\(^{2+}\) charge at 23°C and 36°C. The resulting Q\(_{10}\) is indicated. Relating ∆C\(_m\) to Ca\(^{2+}\) charge revealed an exocytosis efficiency of 32 and 65 fF/pC (at 23 and 36°C, respectively) at cMFBs.

(D) Ca\(^{2+}\) current activation rate at 23°C and 36°C (i.e. inverse of the time constant of the exponential fit to the Ca\(^{2+}\) current activation; voltage protocol: 3-ms voltage step from −80 mV to +20 mV). Data are from Figure S3D (36°C) and from similar experiments at 23°C (data not shown). The resulting Q\(_{10}\) is indicated.

(E) Grand average data of C\(_m\) traces in response to ten 1-ms depolarizations from −80 mV to +20 mV at 50 Hz in hMFBs at room- (blue) and physiological-temperature (red). Inset depicts grand average of Ca\(^{2+}\) current elicited by a corresponding 1-ms voltage step (scale bars, 1 ms and 200 pA).
(F) $C_m$ increase ($\Delta C_m$) elicited by ten 1-ms depolarizations at 50 Hz at 24°C (blue) and 36°C (red) in hMFBs. The resulting $Q_{10}$ is indicated.

(G) $Ca^{2+}$ charge at 23°C and 36°C. The resulting $Q_{10}$ is indicated. Note that the higher $Q_{10}$ compared to cMFBs is likely related to the shorter pulse duration (1 ms vs. 3 ms), for which the temperature dependence of $Ca^{2+}$ channel activation is more relevant. The exocytosis efficiency of hMFBs was 18 and 21 fF/pC at 23 and 36°C, respectively.

(H) $Ca^{2+}$ current activation rate at 23°C and 36°C. The resulting $Q_{10}$ is indicated.

Throughout the figure, data are represented as mean ± SEM, and n represents number of cMFBs or hMFBs, respectively.
Figure S3 (related to Figures 3 and 4). Ca\(^{2+}\) current kinetics in cMFBs is unaltered by endocytosis inhibitors

(A) Top: Voltage-clamp protocol. Bottom: Example traces of pharmacologically isolated Ca\(^{2+}\) currents evoked by voltage steps from −80 mV to 40 mV with 10-mV increments.

(B) Top: Voltage-clamp protocol. Bottom: Example traces of pharmacologically isolated Ca\(^{2+}\) currents evoked by a voltage step to 40 mV followed by test potentials from 0 mV to −80 mV with 10-mV decrements. Same experiment as in A.

(C) Current-voltage relation of presynaptic Ca\(^{2+}\) currents. Steady-state amplitude of Ca\(^{2+}\) currents was unchanged by TeNT-LC (5 µM, blue), dynasore (100 µM, red), latrunculin A (25 µM, green), and pitstop 2 (25 µM, orange). Solid lines are spline interpolations. For all data points, n = 34–83.

(D) Exponential time constants of Ca\(^{2+}\) current activation (solid circles; n = 12–58) and deactivation (open circles; n = 4–16).

(E) Tail current integral of activation traces. Data are normalized to the maximum integral and superimposed with a sigmoidal fit (solid lines). For all data points, n = 35–79.

Throughout the figure, data are represented as mean ± SEM and n represents number of cMFBs.
Figure S4 (related to Figure 4). Biexponential endocytosis time course of step depolarizations and comparison of AP train and step depolarization in cMFBs

(A) Grand average $C_m$ traces for step depolarizations to 0 mV for 1 ms (light gray), 3 ms (medium gray), and 30 ms (dark gray). Magenta lines are biexponential fits to the $C_m$ decay.

(B) Double-logarithmic plot of endocytosis time constants versus amount of exocytosis ($\Delta C_m$). In the biexponential fits, the average contribution of the fast component was comparable for the three stimulus durations (1 ms: 57%; 3 ms: 47%; 30 ms: 55%). For all data points, $n = 5–8$.

(C) Grand average $C_m$ traces for stimulation at 300 Hz with 50 APs (red) and for 1-ms step depolarization (blue).

(D) Endocytosis time constants (left) and $\Delta C_m$ (right) for 50 APs and 1-ms depolarization. Endocytosis was significantly faster with 50 AP train stimulation ($p = 0.005; n = 12$; Wilcoxon signed-rank test), but evoked similar amount of exocytosis ($p = 0.88$).

Throughout the figure, data are represented as mean ± SEM and $n$ represents number of cMFBs.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Brain slice preparation

For recordings from cerebellar mossy fiber boutons, parasagittal 300-µm thick cerebellar slices were prepared from P35–55 C57BL/6 mice of either sex. Animals were treated in accordance with national and institutional guidelines. Mice were anesthetized with isoflurane and killed by rapid decapitation; the cerebellar vermis was quickly removed and mounted in a chamber filled with chilled extracellular solution. Slices were cut using a Leica VT1200 vibratome (Leica Microsystems), transferred to an incubation chamber at ~35°C for 30 minutes and then stored at room temperature until experiments. The extracellular solution for slice cutting and storage, contained (in mM): NaCl 125, NaHCO₃ 25, Glucose 20, KCl 2.5, CaCl₂ 2, NaH₂PO₄ 1.25, MgCl₂ 1, equilibrated with 95% O₂ and 5% CO₂, pH 7.3, ~310 mOsm.

For recordings from hippocampal mossy fiber boutons, transverse 350-µm thick hippocampal slices were prepared as described previously (Bischofberger et al., 2006). Animals were killed by rapid decapitation, in accordance with national and institutional guidelines. The left hemisphere was removed from P22–P30 rats (Charles River Laboratories) of either sex and placed in an ice-cold cutting solution. Slices were cut with a Leica VT1200S vibratome. Cutting and storage solution contained (in mM): NaCl 87, sucrose 75, NaHCO₃ 25, glucose 10, MgCl₂ 7, KCl 2.5, NaH₂PO₄ 1.25, and CaCl₂ 0.5, equilibrated with 95% O₂ and 5% CO₂, ~325 mOsm. Slices were kept at ~34°C for 60 minutes before recording.

Recordings from cerebellar mossy fiber boutons

cMFBs were visualized with oblique illumination and infrared optics and identified as described previously (Delvendahl et al., 2015; Ritzau-Jost et al., 2014). Recordings were performed in lobules III–VI of the cerebellar vermis. Slices were superfused with artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 105, NaHCO₃ 25, glucose 25, TEA 20, 4-AP 5, KCl 2.5, CaCl₂ 2, NaH₂PO₄ 1.25, MgCl₂ 1, and tetrodotoxin (TTX) 0.001, equilibrated with 95% O₂ and 5% CO₂. In subsets of experiments, one of the following endocytosis inhibitors was added: 100 µM dynasore to block dynamin-dependent synaptic vesicle endocytosis (Macia et al., 2006); dynasore was dissolved in dimethylsulfoxide (DMSO), and final DMSO concentration was 0.1% (v/v). Slices were incubated with dynasore for at least 15 minutes before experiments. 25 µM pitstop 2 to inhibit clathrin-
dependent endocytosis (von Kleist et al., 2011) was also dissolved in DMSO, final DMSO concentration being 0.2% (v/v). Because pitstop 2 may also have unspecific effects (Willox et al., 2014), care was taken to limit incubation time to 30 minutes in these experiments.

Presynaptic patch pipettes were pulled to open-tip resistances of 3–6 MΩ (when filled with intracellular solution) from thick-walled 2 mm/1 mm (OD/ID) quartz glass (Heraeus) using a hydrogen-flame powered DMZ Quartz-Puller (Zeitz-Instruments) (Dudel et al., 2000). The intracellular solution contained (in mM): CsCl 135, TEA-Cl 20, MgATP 4, NaGTP 0.3, Na₂phosphocreatine 5, HEPES 10, ethyleneglycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) 0.2. In some experiments, tetanus neurotoxin light chain (TeNT-LC, 5 µM), which cleaves synaptobrevin, was added to the intracellular solution to block SNARE-mediated synaptic vesicle exocytosis. In another subset of experiments, the actin-polymerization inhibitor latrunculin A (Coué et al., 1987) was added to the intracellular solution at a concentration of 25 µM. Latrunculin A was dissolved in DMSO; final DMSO concentration was 0.1% (v/v).

Experiments were performed at 35–37°C except where otherwise stated. Temperature was controlled by an inline heating system (Warner Instruments) of the bath perfusion. TeNT-LC was kindly provided by T. Sakaba and E. Neher, Pitstop 2 was from Abcam, and all other chemicals were from Sigma-Aldrich.

Whole-cell patch-clamp recordings were made using a HEKA EPC10/2 amplifier (HEKA Elektronik). Series resistance was typically <25 MΩ (median 18.9, range 6–80) and compensated online by 40–60% with 10 µs delay. We pharmacologically isolated presynaptic Ca²⁺ currents as previously described (Ritzau-Jost et al., 2014). Ca²⁺ currents were filtered with the internal low-pass filter of the amplifier at 10 kHz and digitized at 200 kHz. Ca²⁺ currents were elicited by square pulses of varying duration, and were corrected for leak and capacitance currents using the P/4 method. To investigate activation kinetics of presynaptic Ca²⁺ currents (Figure S3), steps of 3 ms duration to varying potentials from a holding potential of −80 mV were used. Steps to varying potentials after full activation (0 mV for 3 ms) were used to study deactivation of Ca²⁺ currents. Activation and deactivation kinetics were fit and analyzed as previously described (Ritzau-Jost et al., 2014). For AP or 300 Hz AP train voltage commands, APs were previously recorded from a cMFB, elicited by electrical stimulation of the axon as described (Ritzau-Jost et al., 2014). For recording of the AP waveform that was subsequently used as voltage command, quartz-pipettes were used. In Figure 1A, the voltage command was shifted by 20 µs due to the lag by the stimulus filter of the patch-clamp amplifier (Ritzau-Jost et al., 2014).
**Recordings from hippocampal mossy fiber boutons**

Patch-clamp recordings from hMFBs in CA3b,c regions of the hippocampus were performed under visual control as described previously (Geiger and Jonas, 2000; Vyleta and Jonas, 2014). Slices were superfused with ACSF containing (in mM): NaCl 125, NaHCO₃ 25, glucose 25, KCl 2.5, CaCl₂ 2, NaH₂PO₄ 1.25, and MgCl₂ 1, equilibrated with 95% O₂ and 5% CO₂, ~320 mOsm. All recordings were made in the presence of TTX (1 µM) to suppress voltage-activated sodium channel currents.

Presynaptic recording pipettes were fabricated from 1.5 mm/0.84 mm (OD/ID) borosilicate glass tubing and had open-tip resistance values of 10–17 MΩ. For presynaptic whole-cell voltage clamp experiments and capacitance measurements, a caesium-based intracellular solution was used. The presynaptic pipette solution contained (in mM): Cs-gluconate 100, CsCl 30, TEA-Cl 10, HEPES 10, Na₂-phosphocreatine 5, MgATP 4, NaGTP 1, and EGTA 0.2, (pH adjusted to 7.28 with CsOH, 305–310 mOsm). The presynaptic series resistance ranged from 26–74 MΩ (median 56 MΩ) and was compensated online (50% with 10 µs delay) during all experiments. Pipette tips were coated with dental wax to reduce stray capacitance. Resting hMFB capacitance was estimated to be 1.7 ± 0.1 pF (range 0.8–2.4 pF, median 1.72 pF, n = 19). Calcium channel currents were elicited by square pulses (1-ms duration to +20 mV from a holding potential of −80 mV), were filtered at 7.4 kHz and digitized at 100 kHz, and were corrected for leak and capacitance currents using the P/5 method.

**Capacitance recordings**

Membrane capacitance (Cₑ) measurements were performed using the „sine + DC“ mode (Lindau and Neher, 1988) of the software lock-in extension essentially as described previously (Hallermann et al., 2003; Ritzau-Jost et al., 2014).

For Cₑ recordings in cMFBs, the sine-wave frequency was 1 or 2 kHz and the peak amplitude was ±50 mV superimposed on a holding potential of −100 mV. Resting cMFB capacitance was estimated to be 3.6 ± 0.2 pF (control condition; n = 162; median 3.3 pF). In between sine-wave stimulation, the presynaptic terminal was depolarized from −80 mV to 0 mV for 1–100 ms. Alternatively, previously recorded APs were used as voltage commands in-between sine-wave stimulation. The increase in Cₑ elicited by 1-ms depolarizations was independent of sine-wave frequency (Figure S1C and D). Hydrostatic pipette pressure during Cₑ recordings was kept to a low and constant level (Heidelberger et al., 2002). The Cₑ increase was determined as the difference between the mean capacitance 50–100 ms after the
depolarizing pulse and the baseline during 200 ms before onset of the depolarizing pulse. For AP-evoked capacitance increase, 10–40 traces were averaged per cell to increase the signal-to-noise ratio. Longer capacitance measurements were made by sampling Cm for 20 ms every 250 ms as described (Renden and von Gersdorff, 2007). Because these longer Cm recordings were not feasible for AP- and AP-train-stimulations, a small second component of Cm decay with AP stimuli could not be detected by our experiments. In most experiments, the AP-evoked Cm increase was associated with a transient reduction in Rs (Figure S1A), which has previously been predicted for “sine + DC”-measurements at en passant synapses depending on the relative contribution of the axon capacitance (Hallermann et al., 2003). A linear regression of normalized −ΔRs vs. ΔCm revealed a slope of 1.15 (Figure S1B). Comparable values were obtained from data using 3-ms depolarizing pulses (slope = 0.92; data not shown) and in previous experiments at hMFBs (Hallermann et al., 2003), arguing that the AP-evoked ΔCm reflects a real increase in capacitance of the bouton and not an artifact caused by, e.g., transient membrane conductances (Wu et al., 2005; Yamashita et al., 2005).

For Cm recordings from hMFBs, a sine-wave of 2 kHz was used with peak amplitude of ±20 mV superimposed on a holding potential of −80 mV. Currents were filtered at 7.4 kHz and sampled at 50 kHz. A value for each Cm, Gm, and Gs was computed for each sine wave cycle. Identical sine-waves but with different durations were applied before and after stimulus trains. Trains of brief depolarizing stimuli (1-ms pulses to +20 mV, delivered at 50 Hz) were used to evoke Ca²⁺ influx and exocytosis of synaptic vesicles. Consistent with previous reports, the increase in Cm was associated with a transient reduction of Rm and a reduction of apparent Rs (Hallermann et al., 2003; see also Figure S1A and B). For each recording, data were fit only after Rm had decayed back to baseline following a stimulus train. Two to thirteen traces were averaged per cell for Cm measurements. Cm measurements were performed at room temperature (~24°C), 30°C, and at 36°C. Resting Cm at the three temperatures was 1.4 ± 0.2 pF (24°C), 2.1 ± 0.2 pF (30°C), and 1.9 ± 0.1 pF (36°C); series resistance (Rs) accounted to 61 ± 6 MΩ (24°C), 42 ± 5 MΩ (30°C), and 53 ± 3 MΩ (36°C). Corresponding membrane resistance (Rm) was 9.1 ± 1.4 GΩ (24°C), 5.0 ± 1.2 GΩ (30°C), and 3.5 ± 0.7 GΩ (36°C).

**Analysis of temperature coefficient**

To quantify the temperature dependence of endocytosis, the temperature coefficient of endocytosis (Q10; Běhrádek, 1930) was calculated from experiments performed at room temperature (~23 and ~24°C), ~30°C, and physiological temperature (~36°C). In both
hMFBs and cMFBs, the exocytosis, the $\text{Ca}^{2+}$ influx, and the $\text{Ca}^{2+}$ channel activation rate were increased at physiological compared with room temperature (Figure S2). The corresponding $Q_{10}$ was calculated according to:

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{10/(T_2-T_1)},$$

where $R_1$ and $R_2$ are $\Delta C_m$, the steady-state $\text{Ca}^{2+}$ current, and the $\text{Ca}^{2+}$ channel activation rate at room and physiological temperature ($T_1$ and $T_2$), respectively. To calculate the $Q_{10}$ of the endocytosis kinetics measured at up to three temperatures (Figure 2), the time constants of mono-exponential fits and the amplitude-weighted time constants of bi-exponential fits ($\tau_w$) were plotted against recording temperature and fit using the following function:

$$\tau(T) = a Q_{10}^{-T/10},$$

where $\tau$ is the time constant, $T$ the temperature, and $a$ is a constant. This equation is obtained by solving equation 1 for $R_2$ (note that $R_i$ and $T_i$ are combined in the constant $a$) and inverting $Q_{10}$ to account for the use of time constants instead of rate constants. Similar results were obtained by using endocytosis rate constants of the individual experiments (data not shown). Confidence intervals of $Q_{10}$ estimates were determined by bootstrap procedures implemented in Mathematica 10 as described previously (Delvendahl et al., 2015). Ten thousand artificial datasets were generated from the original data (by taking individual experiments with replacement) and analyzed as the original dataset.

**Data analysis**

Data were analyzed using Fitmaster (HEKA) and Igor Pro (Wavemetrics) software. A linear regression line obtained from the baseline before stimulation was subtracted from the $C_m$ trace to correct for linear baseline drifts when necessary. The $C_m$ decay after stimulation was fit with either single or double exponential functions with zero offset. For Figure 2A–B and Figure S4A–B, the amplitude-weighted time constant of bi-exponential fits ($\tau_w$) was calculated as $(a_1 \tau_1 + a_2 \tau_2)/(a_1 + a_2)$.

For Figure 4F, some experiments with 1–30 ms depolarizations and endocytosis inhibitors did not show a decay in $C_m$ during the first 2 s, which would correspond to a decay time constant of infinity. To nevertheless calculate an average time constant, the inverse of the average of the inverted time constants was used. Correspondingly, the error bars for the 1–30 ms depolarizations represent the inverse of the mean–SEM and the mean+SEM rate. Qualitatively similar results were obtained when these experiments were fit during the first
30 s with a mono-exponential function, in which case no infinite time constants were obtained and all time constants could simply be averaged (data not shown).

The presynaptic \( \text{Ca}^{2+} \) charge was calculated as integral of the \( \text{Ca}^{2+} \) current from the beginning of the current until at least three times the time constant of the decay of the \( \text{Ca}^{2+} \) current. For illustration purposes, some \( C_m \) traces were digitally filtered offline using 3–5 box averaging in Igor Pro. Statistical comparisons were performed via Kruskal-Wallis tests (Figure 4), Mann-Whitney U tests, or Wilcoxon signed-rank tests. \( p < 0.05 \) was considered significant; level of significance for multiple comparisons was adjusted using the Bonferroni-Holm method. Data are represented as mean ± SEM unless otherwise indicated.

SUPPLEMENTAL REFERENCES


