Presynaptic $GABA_A$ receptors enhance transmission and LTP induction at hippocampal mossy fiber synapses

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Supplementary Material

Methods

Tissue preparation

All procedures conformed to the Animals (Scientific procedures) Act 1986. Transverse hippocampal slices were prepared from 3-4 week-old Sprague Dawley rats using slightly different methods for the different experiments. For antidromic current threshold experiments (Supplementary Fig. 1 online), and for orthodromic transmission experiments (Fig. 5), both hippocampi were removed from the brain and placed in a block of agar, prior to slicing (Leica VT1200S) in ice-cold sucrose-based solution containing (in mM): sucrose (75), glucose (10), NaCl (87), KCl (2.5), NaH_2PO_4 (1.25), $MgCl_2$ (7), $CaCl_2$ (0.5), and $NaHCO_3$ (25) (bubbled with 95% O2 and 5% CO2). Slices (250-300 μm thick) were then stored in an interface chamber prior to use. For mossy fiber bouton recordings (Figs. 2-4) slices were cut through the entire brain glued on the vibratome stage[16,17]. The slicing method for the Ca^{2+} imaging experiments (Fig. 5), which allows optimal preservation of mossy fibers connected to their parent granule cells, has been described earlier[21]. Slices were transferred to the perfusion stage of an upright microscope (Olympus BX50WI or BX51WI) and recordings were obtained at 22-24ºC unless indicated. Drugs, chemicals and fluorophores were obtained from Tocris Cookson, Sigma or Invitrogen.

Antidromic action current recordings

Whole-cell pipettes used to record antidromic action currents contained (in mM): K-gluconate (135), NaCl (8), HEPES (10), MgATP (2), Na_3GTP (0.3), EGTA (0.2) (pH 7.2, osmolarity 295 mOsm). Extracellular stimuli were delivered via a bipolar electrode

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positioned in stratum lucidum, between 500 and 1500 μm from the granule cell (stimulus duration: 500 μs; interval 10 s), and granule cells were clamped at -60 mV. Recordings were only accepted if the action current latency was >2 ms. The access and input resistances were monitored using a –5 mV voltage step command. The access resistance was <20 MΩ, and results were discarded if it changed by more than 20 %. Junction potentials were not corrected. The stimulus was cycled through a saw-tooth intensity pattern ranging from 100% failures to 100% success for evoking an action current. THDOC and GBX application was associated with a small decrease in somatic holding current (THDOC: 18.7 ± 5.8 pA; n = 8; GBX: 61.1 ± 19.3 pA; n = 4), consistent with tonically active somatodendritic $GABA_A$ receptors. For perforated-patch recordings, gramicidin was dissolved in dimethylsulfoxide (DMSO). This stock solution was mixed with K-gluconate-based internal pipette solution just before use to a final concentration of 100 μg/ml. An access resistance of 40 – 60 MΩ was obtained 20 – 40 min after formation of a gigaseal.

Currents were acquired with an Axopatch 1D amplifier (Axon Instruments), and records were filtered at 2 kHz, digitized at 10-20 kHz, and stored on a personal computer. AC success rates were calculated from 10-15 trials per plotted point. The stimulus intensity required to obtain a 50% success rate, $Stim_{50}$, was obtained by fitting the relationship of the AC success rate A to stimulus intensity s with a logistic equation, $A = 1/[1 + (Stim_{50}/s)^h]$, with h analogous to a Hill coefficient. Because the minimal and maximal stimulus intensities and increment amplitudes were set empirically for each cell, these input-output curves were only used to obtain a qualitative insight into changes in axon excitability.

Mossy fiber bouton recordings

The perfusion solution for mossy fiber bouton recordings contained, in (mM): NaCl (125), KCl (2.5), NaH_2PO_4 (1.25), $MgCl_2$ (1), $CaCl_2$ (2), $NaHCO_3$ (25), glucose (11). For single channel recordings pipettes were fabricated from thick-walled borosilicate glass (OD: 2 mm; ID: 0.6 mm; Hilgenberg, Germany), pulled and fire-polished to obtain a final resistance of 8-15 MΩ. Pipettes used for bouton-attached or whole-bouton recordings

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were pulled from standard tubing (OD: 1.5 mm; ID: 0.75). Mossy fiber boutons were visualized in stratum lucidum using infra-red differential interference contrast (DIC) optics as small spherical structures (4-6 μm). A DIC image of the recorded mossy fiber bouton was taken routinely before each recording attempt. We confirmed that boutons recorded in cell-attached mode were able to follow extracellular stimulation at 200 Hz. The series resistance was typically 15-35 MΩ. For 'high [Cl^-]' whole-bouton recordings in current-clamp, the pipette solution contained: KCl (155), HEPES (10), Na_2ATP (2), Na_3GTP (0.3), EGTA (0.2). For 'normal [Cl^-]' recordings, the KCl concentration was reduced to 20 mM, and 135 mM $KMeSO_4$ was added. Whole-bouton voltage-clamp recordings were performed with a pipette solution containing: CsCl (145), NaCl (8), $MgCl_2$ (2), HEPES (10), Na_2ATP (2), Na_3GTP (0.5), EGTA (2). In some experiments the pipette solution also contained 0.4% biocytin for post-hoc visualization with confocal imaging. In 5 out of 7 slices where visualization was attempted, the basic anatomy of a mossy fiber bouton and parent axon could be recovered, confirming that the recordings originated from presynaptic elements (see Supplementary Fig. 2 online). Local pressure application of THDOC (50 nM in control perfusion solution) or gabazine (10 μM) was delivered via a patch pipette connected to a Picospritzer (General Valve Corporation, 5-50 ms, 5-20 PSI).

Outside-out patches from mossy fiber boutons were obtained with a symmetric Cl^--based internal pipette solution containing (in mM): CsCl (145), NaCl (8), $MgCl_2$ (2), HEPES (10), Na_2ATP (2), Na_3GTP (0.5), EGTA (2) and Na_2-phosphocreatine (5). Patches were pulled from mossy fiber boutons after checking high-frequency spike fidelity in response to 5 extracellular stimuli at 200 Hz in cell-attached mode (see Supplementary Fig. 2 online), and held in voltage-clamp at -60 mV. Channel behavior was measured by applying voltage pulses (-120 mv to +40 mV, step 20 mV). Patches with spontaneous channel openings in the absence of muscimol, or exhibiting more than one modal conductance level, were not retained for further analysis. Signals were recorded with an Axopatch 200A sampled at 20 kHz, low-pass filtered at 2 kHz (-3 dB, 8-pole Bessel) and stored on a personal computer for off-line analysis with PClamp 9.0 (Axon Instruments). Analysis of single-channel activity was performed on 20-40 s recording epochs at each holding potential. Single-channel open period duration and amplitude distributions were

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fitted with a sum of multiple Gaussian components using Levenberg-Marquardt least-square minimization (Microcal Origin 6.0).

To estimate the membrane potential of unperturbed mossy fiber boutons we used terminal-attached recordings as described for cell-attached recordings[19]. The pipette solution contained: KCl (155 mM), EGTA (10), NaCl (8), $MgCl_2$ (4), MgATP (0.3), Na_3GTP (0.3), HEPES (10) and Na_2-Phosphocreatine (10). Patches were held in tight seal configuration at -60mV (pipette potential, Vp = +60 mV) and ramp depolarization applied from -100 mV to +200 mV (3 mV/ms) to elicit K^+ currents. The reversal potential for K^+-mediated currents was calculated by fitting a straight line to the average macroscopic current amplitude as a function of potential, this typically corresponded to the interval between 1 and 5 ms after ramp onset. The intercept between the linear fit and the average of 10 successive current traces was used to determine the reversal of K^+ current, and therefore the Em of individual terminals. The seal conductance is irrelevant in these measurements, because it affects the holding current value recorded at each potential but not the voltage for which reversal occurs[18,19].

For visualization of biocytin-filled boutons, slices were fixed overnight in 4% paraformaldehyde at 4 °C. After permeabilization in 0.1% Triton X-100, slices were incubated in 0.1% streptavidin−Alexa 488 conjugate, mounted in DABCO anti-fading medium and imaged with a Zeiss LSM 510 meta-confocal microscope.

Postsynaptic recordings

Recordings from CA3 pyramidal neurons were obtained with one of two pipette solutions. For the experiments in Fig. 6, the solution contained: CsF (120), DIDS (1), HEPES (10), $MgCl_2$ (2), MgATP (2), Na_3GTP (0.3), EGTA (0.2), QX314 Br (5). For the experiments in Fig. 7, 112 mM Csgluconate and 8 mM NaCl were substituted for CsF, DIDS was omitted, the EGTA concentration was increased to 5 mM, and the cell was held at -60 mV, close to the estimated reversal potential for $GABA_A$ receptors. The pressure-application pipette was positioned 50 - 200 μm from the apical dendrite of the recorded CA3 pyramidal neuron, thus allowing selective manipulation of presynaptic $GABA_A$ receptors by puffing THDOC (50 nM, Fig. 6) or gabazine (10 μM, Figs. 6 and

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7). $GABA_B$ and NMDA receptors were blocked throughout. EPSCs were evoked via a stimulus electrode positioned in the dentate granule cell layer. Mossy fiber EPSCs were only accepted if they exhibited marked facilitation when the stimulation frequency was switched from 0.05 to 1 Hz, and were profoundly depressed by DCG-IV (1 μM) at the end of the experiment. Stabilization of EPSC amplitude was usually observed within 15 – 20 min of break-in. LTP was induced with three 100 Hz trains, lasting 1 s, with a 10 s interval between trains. Gabazine was pressure-applied before each train, with a 50 ms interval between the end of the puff and the start of the train.

Ca^{2+} imaging

For Ca^{2+} imaging experiments we used a Radiance 2000 confocal microscope (BioRad - Zeiss) optically linked to a MaiTai fs pulsed infrared laser (SpectraPhysics - Newport) and integrated with patch-clamp electrophysiology. Granule cells or CA3 pyramidal neurons were recorded in whole-cell mode with a pipette solution containing: $KMeSO_4$ (150), KCl (5), $MgCl_2$ (2), HEPES (10), Na_2-Phosphocreatine (10), Na_2ATP (4), Na_3GTP (0.4), Alexa Fluor 594 (20-40 μM) and Fluo-4 (200 μM). Both fluorophores were excited at λ_x = 800 nm. Giant boutons synapsing on CA3 pyramidal cells were traced 400-800 μm from the soma, and recordings started at least 1 hour after obtaining a whole-cell configuration, at which time dye concentrations have equilibrated[21]. For Schaffer collateral recordings boutons (200-400 mm from the soma, in the CA3 region) were either selected on the main axon or on side-branches (see Supplementary Fig. 6 online), and results were pooled together because no differences were found in their response to GABAA receptor manipulation.

Fluorescence line-scans (500 or 1000 ms long) were recorded at 500 Hz, with an inter-sweep interval 30 s or 1 min, and analyzed off-line. Action potentials were evoked using 1 ms depolarizing voltage command pulses delivered via the somatic pipette. To minimize the impact of focus drift, optical noise, extracellular fluorescence changes or fluctuations in resting Ca^{2+}, the amplitude of the Ca^{2+}-dependent fluorescence transient was calculated as $\Delta F/R = (F_{post} - F_o)/R$; F_o and F_{post} denote the fluorescence intensity in the 'green' Fluo-4 channel averaged over the visible bouton during 100 ms windows

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before and after the spike, respectively; and *R* denotes the Ca^{2+}-independent fluorescence intensity in the 'red' Alexa channel sampled in the same region of interest. In these imaging conditions Fluo-4 is far from saturation and can provide an accurate gauge of AP-evoked presynaptic Ca^{2+} concentration changes[21-23]. Image analysis was performed on stacks of stored images using NIH Image macros. False color tables and averaged images were used for illustration purposes but the quantitative analyses always applied to the original gray level images of each individual line-scan.

For epifluorescence wide-field imaging of HFS-evoked Ca^{2+} transients, 5-15 µl of 30-40 µM Fura2-AM was injected close to the site of stimulation in the dentate hilus. Field EPSPs were initially recorded to verify that the stimulation electrode activated mossy fibers, and ionotropic glutamate receptors were blocked with NBQX and APV prior to fluorescence image acquisition. Fura2 was alternately excited at 340 and 340 nm for 200 ms (25 image pairs), with a Polychrome IV monochromator under the control of TillVision software (Till Photonics), and images were acquired with an Imago CCD camera. Gabazine (10 µM) or zolpidem (20 µM) was pressure-applied (5-10 PSI) for 250 ms, terminating 50 ms prior to HFS. Three successive HFS trains were applied with or without drug application, with 4 minute intervals. Pressure application of vehicle (ACSF) was without effect.

Statistics

Statistical comparisons were made using Student's paired *t*-test or the Mann-Whitney U-test for unpaired data (Fig. 2b). Data are reported as mean ± S.E.M.

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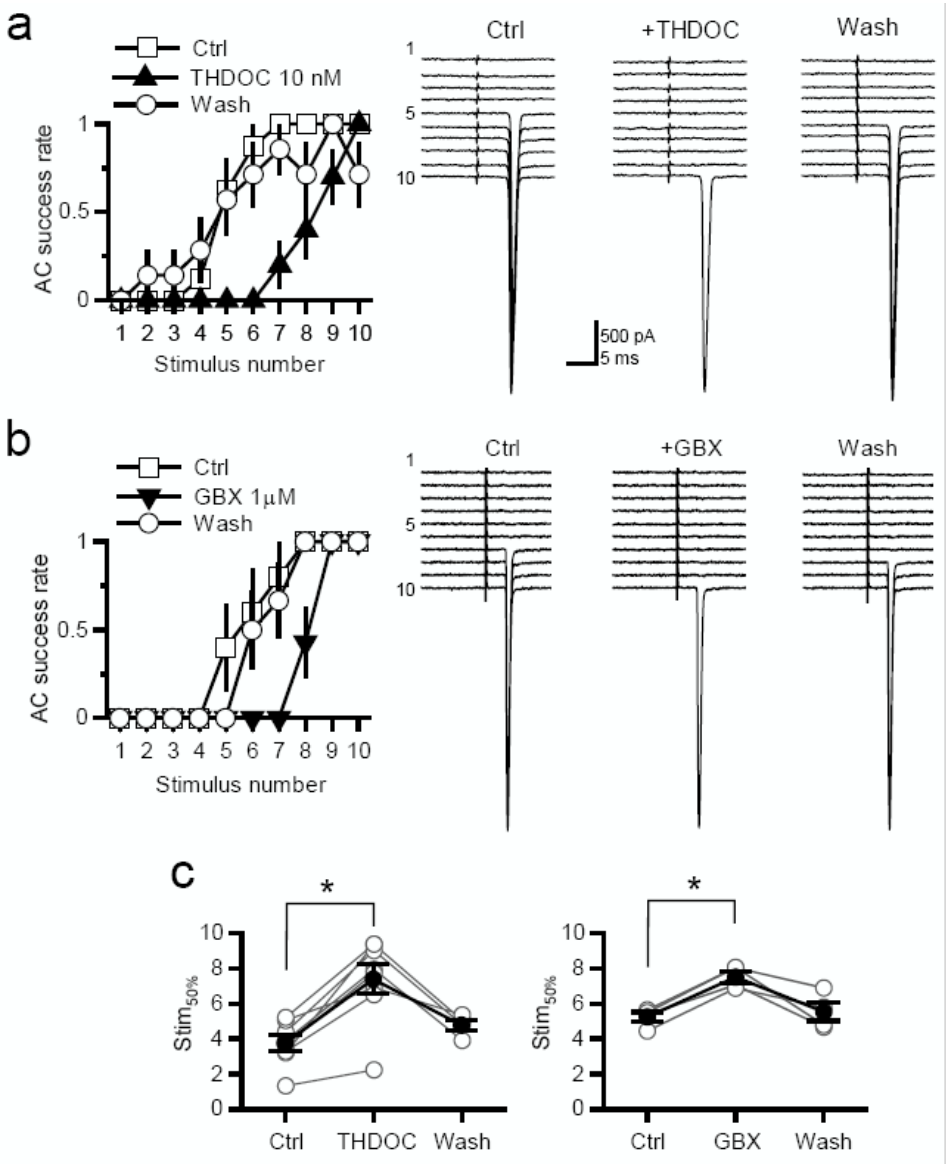

Supplementary Fig. 1. Tonically active neurosteroid-sensitive $GABA_A$ receptors modulate mossy fiber excitability. (a) Input-output curves obtained in a granule cell showing the probability of evoking antidromic action currents (ACs) in response to increasing stimulus intensities numbered from 1 to 10. THDOC (10 nM) reversibly decreased the probability for evoking ACs. Right, sample traces showing whole-cell currents in response to increasing stimulus intensity (from top to bottom), for each condition. **(b)** Input-output curve obtained in a different granule cell, showing the effect of gaboxadol (GBX, 1 μM) on AC success rate. Right, sample traces from the same cell. **(c)** Summary plots of the effect of THDOC and GBX on AC success rate in granule cells (THDOC, $n = 12$; GBX, $n = 4$). Input-output curves were fitted with a logistic function to infer the stimulus intensity yielding 50% success rate for each condition ($Stim_{50\%}$). *, $p < 0.05$, paired *t*-test.

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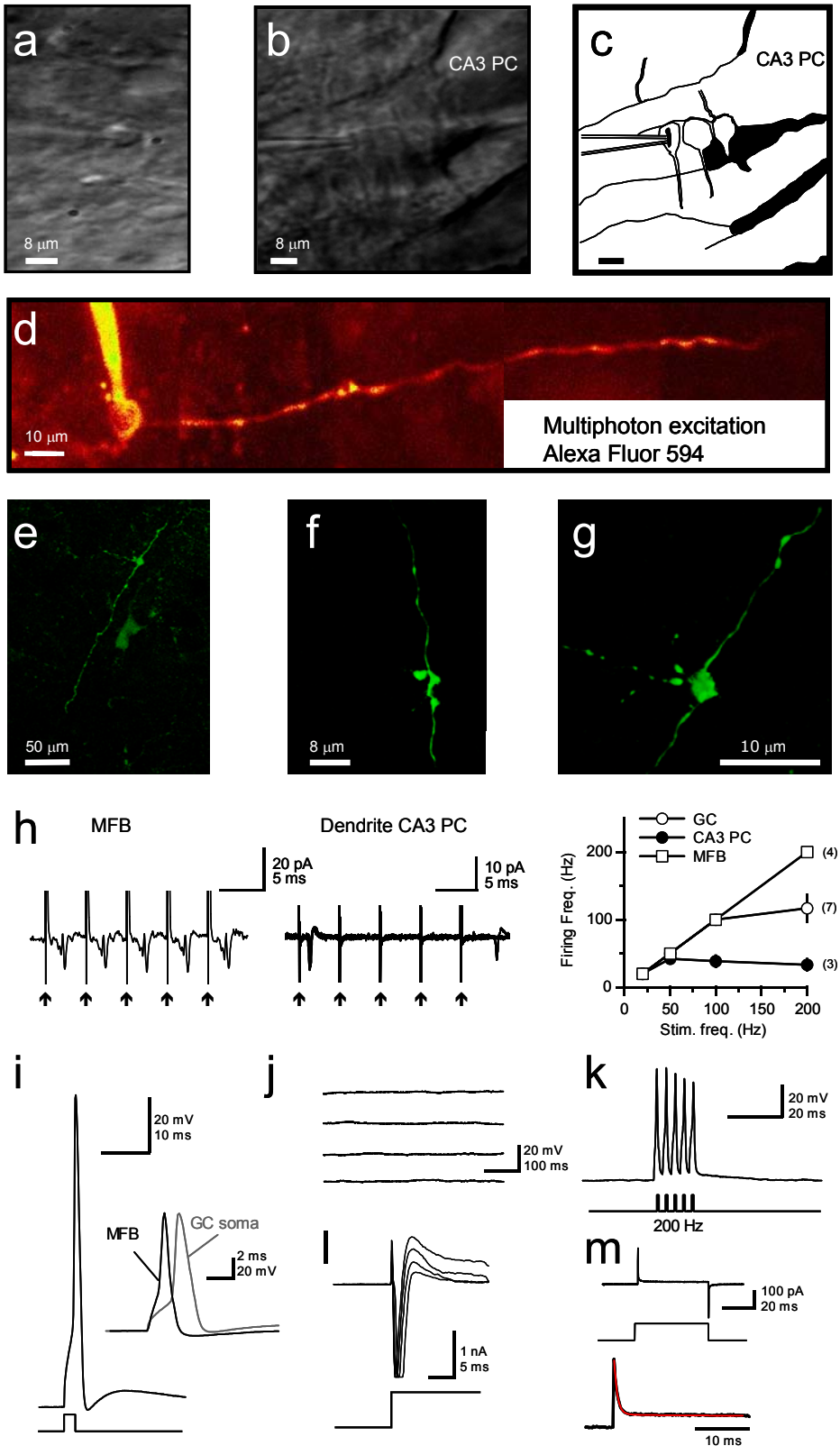

Supplementary Fig. 2

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Supplementary Fig. 2. Identification of mossy fiber boutons. (a - b) Infrared differential interference contrast images in two slices showing mossy fiber boutons identified as small ovoid structures with a long axis ~4–6 μm. **(c)** Schematic corresponding to image in (b), with putative identification of three mossy fiber boutons, one of which is in contact with the patch pipette, adjacent to the apical dendrite of a CA3 pyramidal cell (PC). **(d)** Mossy fiber bouton filled with AlexaFluor 594 imaged with two-photon laser scanning microscopy. **(e - g)** Confocal images of mossy fiber boutons and parent axons in 2 fixed slices processed for biocytin staining. The bouton in (f) was disrupted by the pipette tip. Panel g is a magnification of the bouton shown in panel e to highlight the filopodial protrusions emerging from the recorded bouton. **(h)** Cell-attached recordings showing action currents in a mossy fiber bouton (left) and in the apical dendrite of a CA3 pyramidal neuron (middle), in response to 200 Hz extracellular stimulation (arrows). Right, mossy fiber boutons (MFB), but not pyramidal cells (CA3 PC) or granule cells (GC) followed the stimulation frequency up to 200 Hz. **(i)** Action potential (average of 20 traces) recorded in a mossy fiber bouton in response to direct current injection, at room temperature. Note the after-depolarization. Inset, superimposed averages of 10 action potentials in a mossy fiber bouton and in a granule cell. **(j)** Absence of spontaneous synaptic activity during 2 s of recording. **(k)** Mossy fibers are able to fire at 200 Hz in response to current pulses injected via a whole-bouton pipette. **(l)** Voltage-clamp traces showing the Na^+ action current in response to supra-threshold depolarizing voltage steps from -70 mV. **(m)** Capacitive transients (average of 10 consecutive trials) in response to sub-threshold depolarizing voltage pulses (5mV, 100 ms). Bottom, expanded portion of the trace with superimposed double-exponential fit (τ_{fast} = 440 μs and τ_{slow} = 1170 μs).

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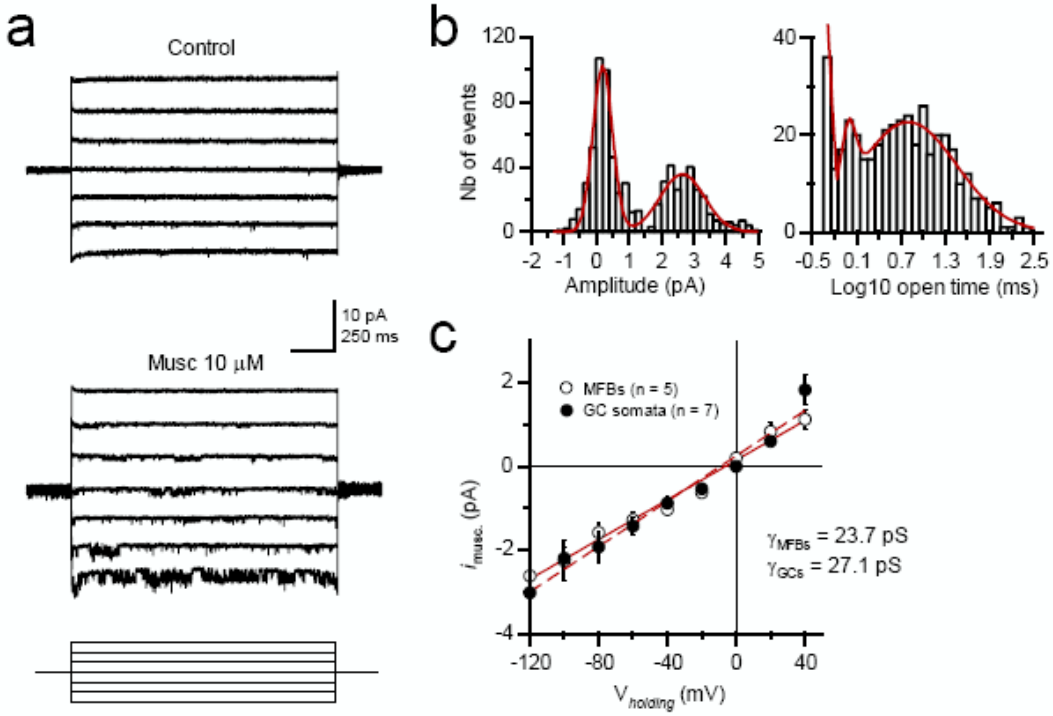

Supplementary Fig. 3. $GABA_A$ receptor-mediated apparent single-channel currents in outside-out patches from mossy fiber boutons and granule cell bodies. (a) Ensemble of traces obtained in an outside-out patch excised from a mossy fiber bouton showing single-channel currents evoked by muscimol (10 µM). **(b)** Distributions of muscimol-evoked current amplitude and open time at -120 mV (same patch as in a). A sum of Gaussians was used to fit the data both for amplitude and open time. **(c)** Current-voltage relationship for muscimol-evoked single-channel openings in mossy fiber boutons and granule cell bodies. The average modal single-channel conductance in patches from mossy fiber boutons was not significantly different from that measured in patches from granule cell bodies. All recordings were obtained in the presence of TTX (1 µM), TEA (5 mM), $CdCl_2$ (1 µM), D-APV (50 µM), NBQX (20 µM) and CGP-52432 (5 µM).

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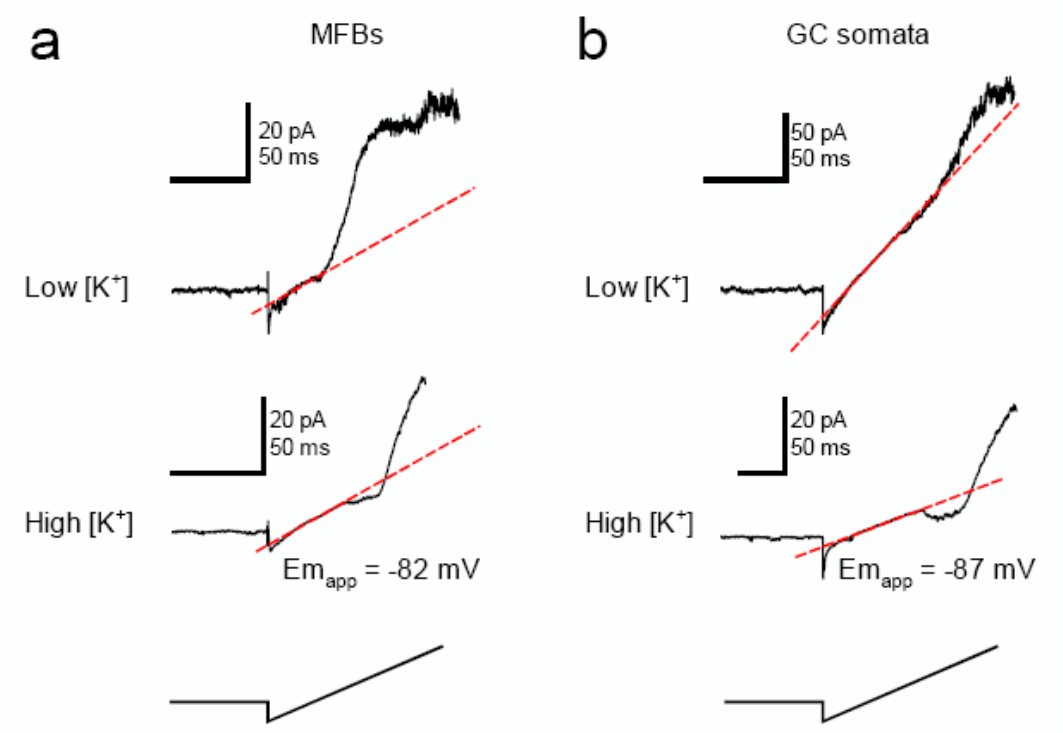

Supplementary Fig. 4. Ramp-evoked currents are carried by K^+ ions. (a) Example of mossy fiber bouton-attached (MFBs) recordings obtained with low (top) and high (bottom) [K^+] pipette solutions. The current was mainly outward (flowing from cell to pipette) with low [K^+] but had inward and outward components with high [K^+]. **(b)** cell-attached recordings from a granule cell body with low (top) and high (bottom) [K^+] pipette solutions.

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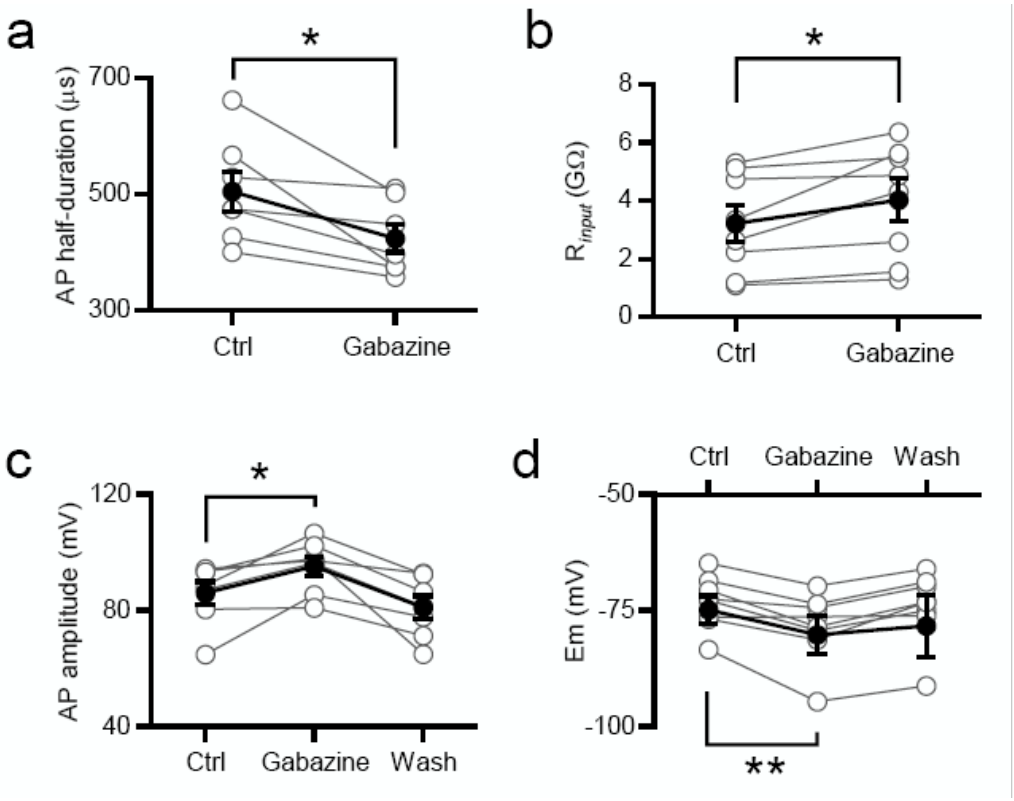

Supplementary Fig. 5. $GABA_A$ receptors modulate action potential parameters and input resistance in mossy fiber boutons (155 mM $[Cl^-]_i$). (a – d) Summary plots showing the effects of gabazine on action potential half-duration (a), input resistance (b), action potential amplitude (c) and membrane potential (d) of mossy fiber boutons. Bar charts represent the mean ± s.e.m., gray symbols show individual experiments. *, $p <$ 0.05; ** p, < 0.01, paired *t*-test.

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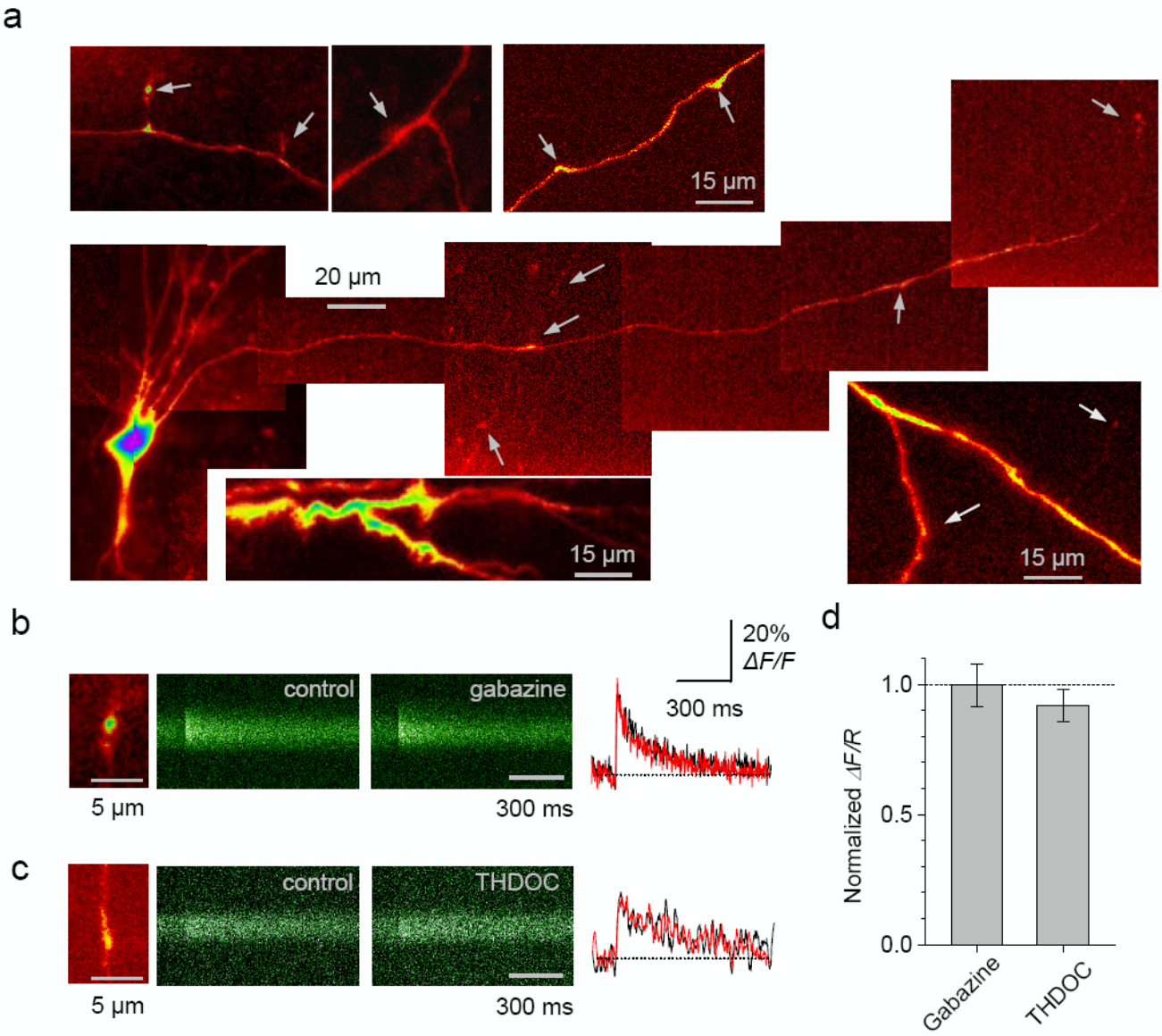

Suplementary Fig. 6. **Gabazine and THDOC do not affect Ca^{2+} transients in Schaffer collateral boutons. (a)** Reconstruction of a CA3 pyramidal cell (Alexa Fluor 594 channel, λx = 800 nm) and axon. Arrows indicate boutons, shown at higher resolution in separate panels. The lower panel (left) shows a dendritic fragment for comparison. The collage was obtained from Kalman-filtered averages of 10-15 µm stacks. **(b)** Gabazine did not significantly change spike-dependent presynaptic Ca^{2+} transients. Left panel: representative Schaffer collateral bouton ('red' Alexa channel). Line-scans and traces: Ca^{2+} transients in the same bouton ('green' Fluo-4 channel, 10-trace average) following a single action potential evoked at the soma, in control conditions and in 1 µM gabazine. **(c)** THDOC (10 nM) also failed to affect presynaptic Ca^{2+} entry; notation as in (b). **(d)** Summary of the effects of gabazine ($\Delta F/R$: 100 ± 8 % of baseline; n = 8; p = 0.98) and THDOC ($\Delta F/R$: 92 ± 6 % of baseline; n = 6; p = 0.25).

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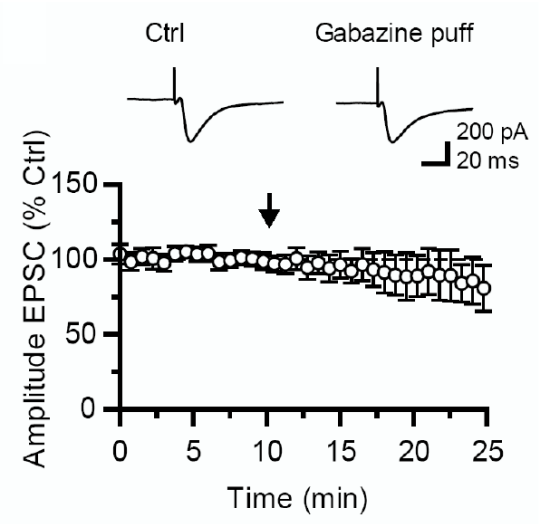

Supplementary Fig. 7. **Gabazine had non-significant effects on associational-collateral EPSCs recorded in CA3 pyramidal neurons.** Effect of pressure-application of gabazine plotted as in Fig. 6b.

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