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Supplemental Information

The Extracellular Matrix Molecule Hyaluronic Acid Regulates Hippocampal Synaptic Plasticity by Modulating Postsynaptic L-Type Ca^{2+} Channels

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Table S1. Passive Cell and Action Potential Properties (AP) after Sham and Hyaluronidase Treatment Recorded at Room Temperature (Related to Fig. 7)

	Sham	Hyaluronidase
Membrane potential (mV)	-62.5 ± 1.4 (14)	-61.3 ± 1.2 (18)
Input resistance (MΩ)	176.0 ± 12.8 (12)	166.4 ± 21.3 (12)
Membrane time constant (ms)	24.8 ± 2.5 (12)	27.0 ± 3.2 (12)
Rheobase (pA)	57.0 ± 10.8 (10)	47.3 ± 9.5 (11)
AP voltage threshold (mV)	-44.2 ± 1.9 (11)	-44.7 ± 2.1 (12)
AP amplitude (mV)	91.5 ± 2.8 (11)	94.4 ± 2.7 (12)
AP half-width (ms)	1.82 ± 0.09 (11)	1.78 ± 0.06 (12)

All data are shown as mean ± SEM (the number of cells). No significant differences could be detected (unpaired t-test, $p > 0.4$ for all parameters).

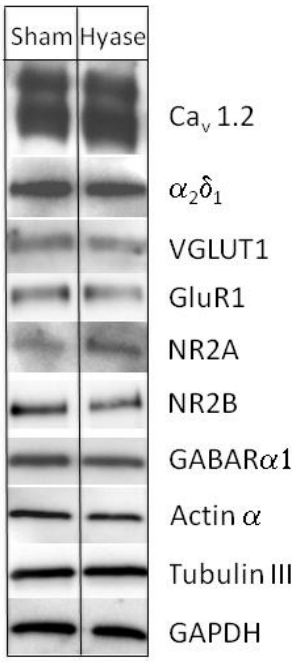

Figure S1. Hyaluronidase Treatment Does Not Change Protein Expression (Related to Fig. 1)

15 or 30 μg of brain homogenate from hippocampus slices were used to assay the expression levels of $Ca_v1.2$ α_1, $\alpha_2\delta_1$, VGLUT1, GluR1, NR2A, NR2B, GABARα1, actin α, tubulin III, and GAPDH. Two bands in $Ca_v1.2$ α_1 blots presumably represent the full length and C-terminal cleaved forms as previously reported (Hell et al., 1996). As shown by this representative blot from three independent experiments, no differences between sham- and Hyase-treated samples were detected.

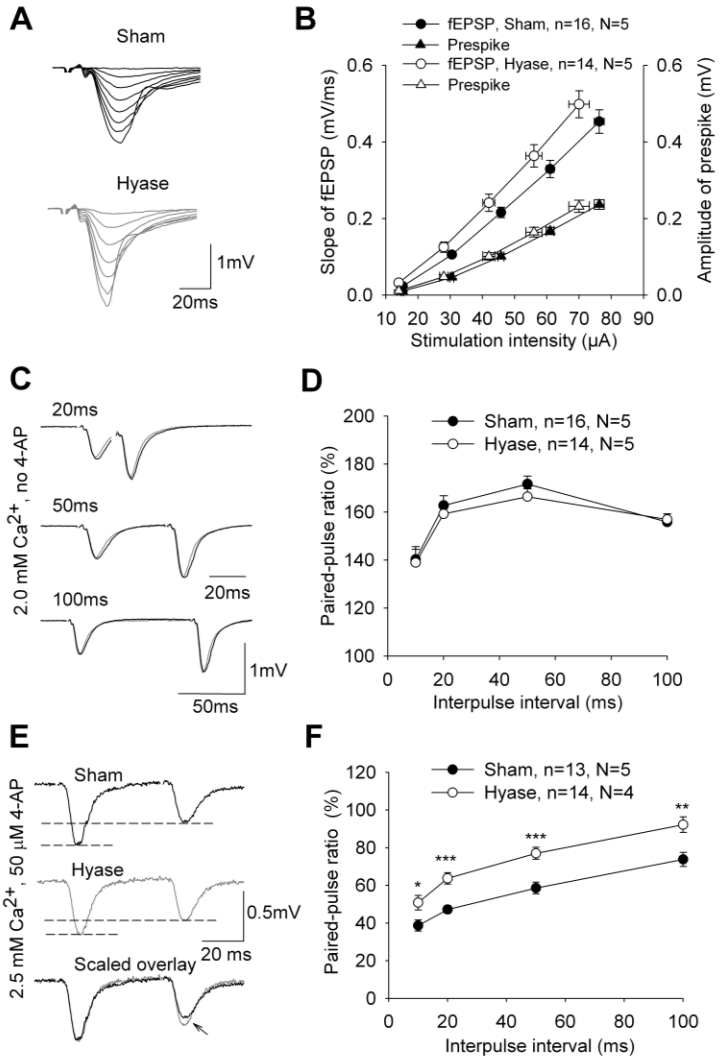

Figure S2. Hyaluronidase Treatment Has No Effect on Basal Synaptic Transmission and Paired-Pulse Facilitation, but Reduces Paired-Pulse Depression in Conditions of Increased Release Probability in Acute Hippocampal Slices (Related to Fig. 2)

(A) Representative examples of fEPSPs recorded with increasing stimulation intensities from 10 to 80 or 70 μA in sham (black) and Hyase-treated (grey) slices, respectively. (B) The relationships between the stimulus intensity and the slope of fEPSPs and between stimulus intensity and the amplitude of presynaptic volleys (prespikes). No effect of Hyase treatment was detected. (C) Examples of paired-pulse facilitation in sham- (black) and Hyase-treated (grey) slices. Interpulse intervals are indicated above traces. (D) The relationship between the interpulse interval and paired-pulse facilitation, measured as a ratio between the slopes of second and first responses and expressed in %. Mean + SEM values are shown. For short intervals (10 and 20 ms), the first fEPSP was digitally subtracted before measurements of the second fEPSP. No effect of Hyase treatment was detected by two-way ANOVA with repeated measures. (E,F) Recordings of paired-pulse depression in extracellular solution containing 2.5 mM Ca^{2+} and 50 μM 4-aminopyridine (4-AP) (instead of normal 2 mM Ca^{2+} and no 4-AP) to boost release probability in slices. (E) Representative examples of paired-pulse depression in sham- (black) and Hyase-treated (grey) slices. Interpulse interval is 50 ms. Scaled overlay shows scaled sweeps with equalized amplitudes of the first response. (F) The relationship between the interpulse interval and paired-pulse depression, measured as a ratio between the slopes of second and first responses and expressed in %. Mean + SEM values are shown. For short intervals (10 and 20 ms), the first fEPSP was digitally subtracted before measurements of the second fEPSP. Two-way ANOVA revealed an effect of Hyase ($p<0.001$). *$p<0.05$, **$p<0.01$, ***$p<0.001$, significant difference between sham and Hyase-treated slices, Bonferroni t-test. (B,D,F) Data represent means + SEMs; n and N, the numbers of tested slices and mice, respectively.

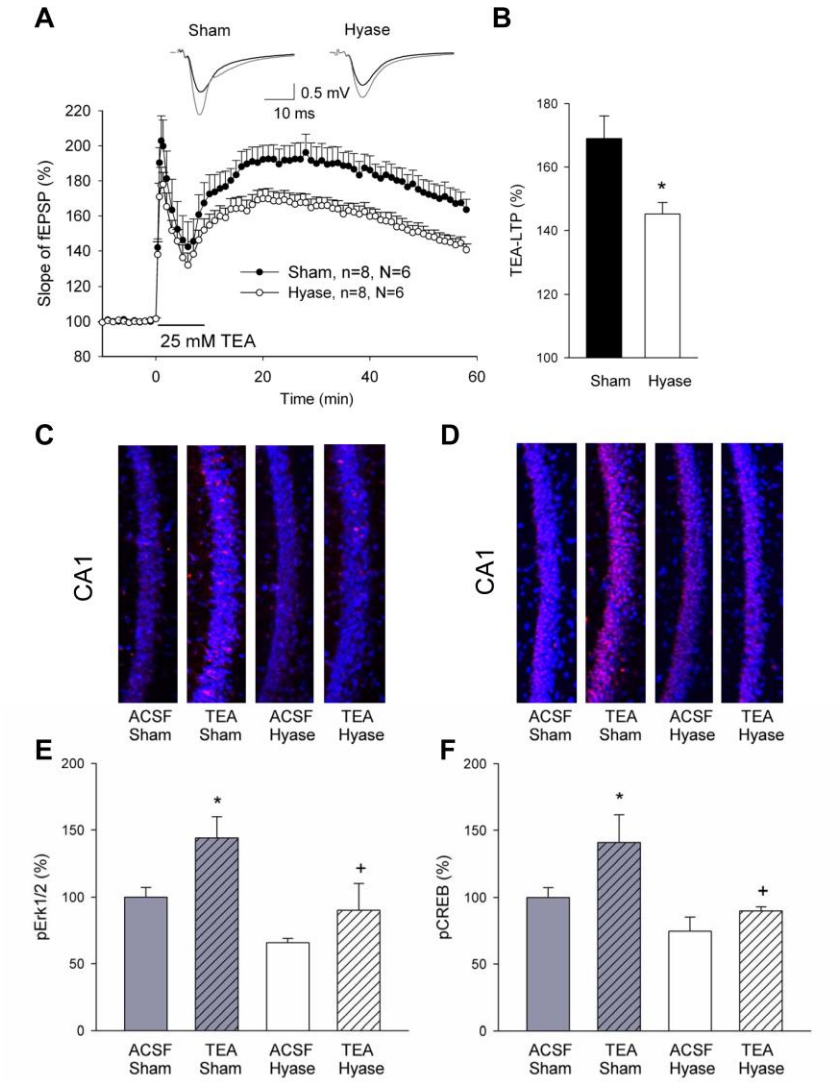

Figure S3. Hyaluronidase Treatment Impairs Tetraethyammonium (TEA)-Induced LTP and Activation of Erk1/2 and CREB (Related to Fig. 3).

(A) The level of TEA-induced LTP is reduced in Hyase-treated slices. The mean slope of fEPSPs recorded 10 min before application of TEA was taken as 100%. Data represent means + SEM, n and N provide the numbers of tested slices and mice, respectively. Each trace is the average of 30 fEPSPs recorded 10 min before or 50-60 min after TEA application. (B) Mean+SEM of LTP levels 50-60 min after the beginning of TEA application in sham and Hyase-treated slices. *p=0.01, t-test. (C,D) Immunolabeling of phospho-Erk1/2, pErk1/2 (C), and phospho-CREB, pCREB (D), in the CA1 stratum pyramidale 20 min after the end of TEA application is shown in red and nuclear DAPI labeling in blue. Upregulation in pErk1/2 and pCREB is seen after application of TEA in sham- but not in Hyase-treated slices. (E,F) Mean+SEM of pErk1/2 (E) and pCREB (F) signals. Two-way ANOVA revealed a significant effects of Hyase ($p<0.01$ in E and F) and TEA ($p<0.05$ in E and F). *$p<0.05$, significant TEA effect in sham-treated slices; +$p<0.05$, significant effect of Hyase in TEA-treated slices (Bonferroni t-test, number of animals N=4 for all groups).

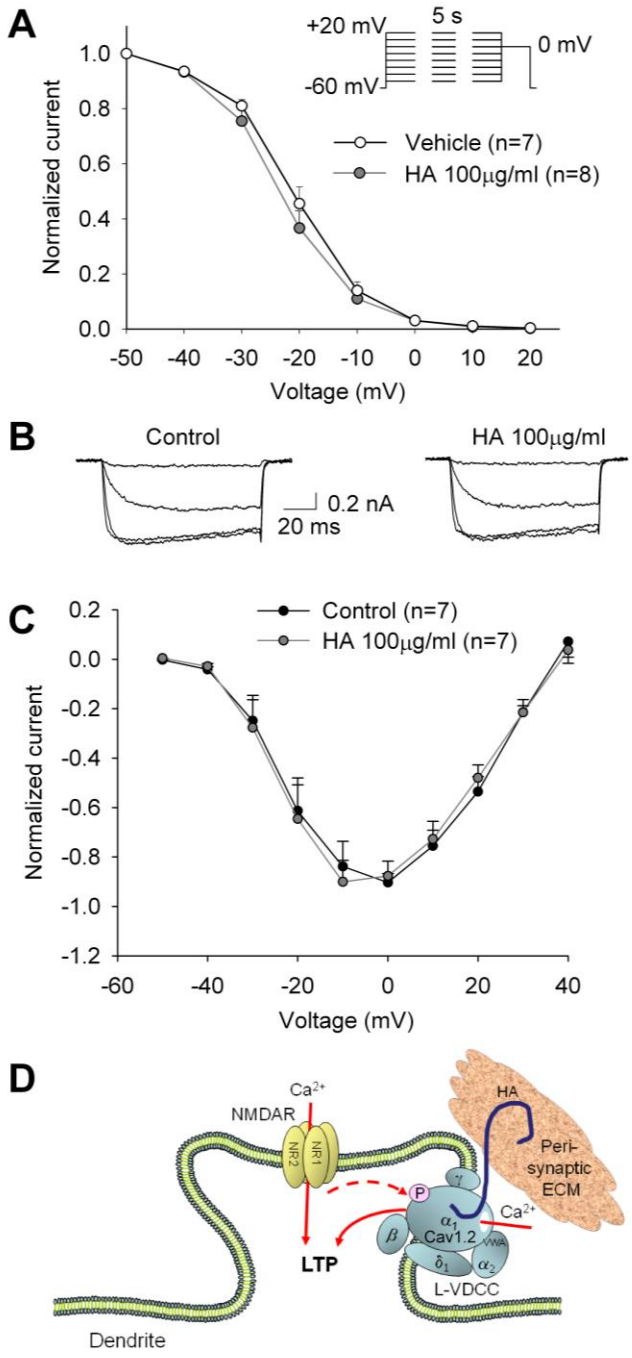

Figure S4. Voltage-Dependent Inactivation of $Ca_v1.2$ Channels and Activation of $Ca_v1.3$ Channels Are Not Affected by HA Application (Related to Fig. 4).

(A) Analysis of inactivation of $Ca_v1.2$ channels. $Ca_v1.2$ channel currents were recorded from transfected CHO cells for 100 ms at 0 mV after 5 s-long voltage pulses applied in 10-mV increments from -50 to +20 mV, as shown on the insert. Recordings of inactivation were made after 5 min-application of HA or vehicle. No difference between curves was detected by two-way ANOVA ($p>0.1$). (B) Representative recordings of currents in CHO cells transfected with $Ca_v1.3$ L-VDCCs 2 min after formation of whole cell configuration (control) and 5 min after application of 100 μg/ml HA. The currents shown were activated by voltage steps from -60 mV to -30, -20, -10 and 0 mV. (C) The mean voltage-current curves under control conditions and after application of 100 μg/ml HA. No difference between curves was detected by two-way ANOVA ($p>0.1$). (D) Hypothetical mechanisms by which hyaluronic acid may regulate synaptic plasticity via $Ca_v1.2$ L-VDCCs. Neuronal L-VDCCs are composed of α_1, β, γ and $\alpha_2\delta_1$ subunits. Signaling via NMDA receptors induces activity-dependent post-translational (P) modifications of L-VDCCs channels (dotted line), such as phosphorylation and proteolytic removal of the α_1 subunit C-terminus. Both modifications increase the activity of $Ca_v1.2$ L-VDCCs and thus Ca^{2+} influx through the postsynaptic membrane during repetitive theta-burst stimulation in the presence of extracellular matrix (ECM)-associated hyaluronic acid (HA). HA may interact with cluster(s) of basic amino acids on $\alpha1$ subunit of L-VDCCs and change activity of channels.

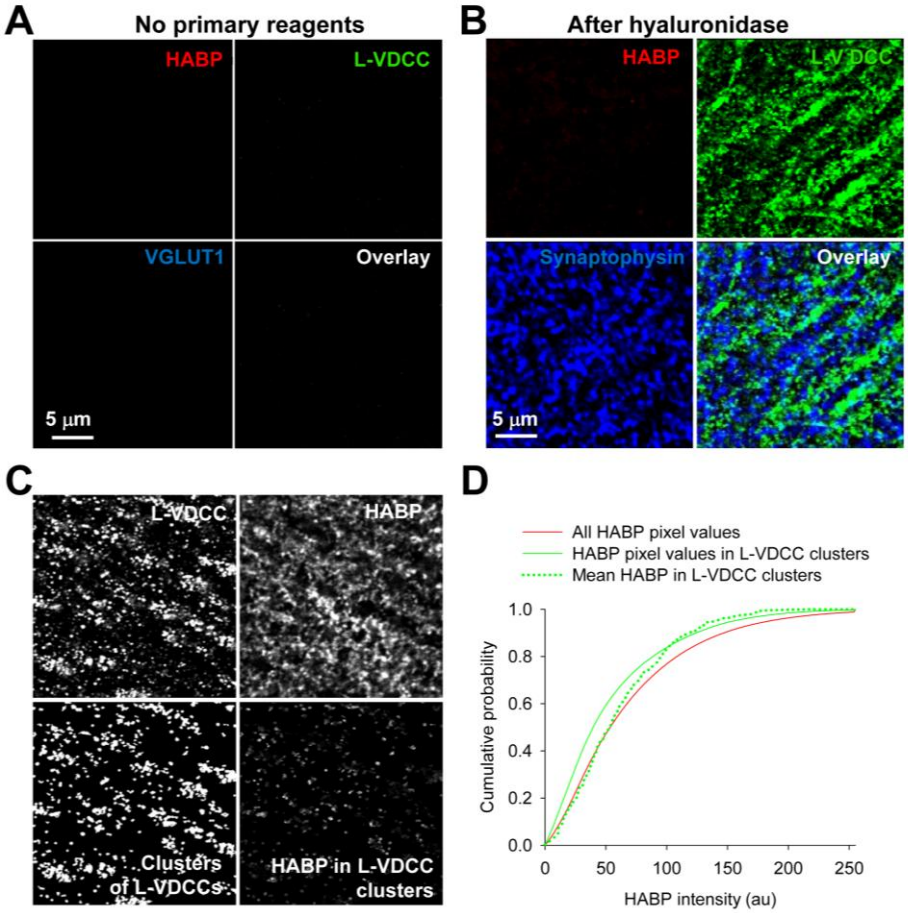

Figure S5. Labeling of HA, L-VDCCs and Synaptic Markers in Hippocampal Sections (related to Fig. 5). (A) Negative control for staining with HABP, and L-VDCC and VGLUT1 antibodies. The staining was performed on the same sections as for Fig. 5 but omitting primary reagents. No labeling is visible (fluorescence imaging settings were exactly the same as in Fig. 5). (B) The pattern of L-VDCC expression is not affected by Hyase treatment. Visualization of HA using biotinylated HA binding protein (HABP) shows the efficient removal of HA. L-VDCC immunoreactivity is visible as green dotted stripes surrounded by synaptic contacts, as in untreated or sham-treated slices. The images represent 0.8 μm-thick optical sections. (C) Characteristic labeling with anti-L-VDCC antibodies and HABP (upper panels). Clusters of L-VDCC and the co-localized areas of the discernable HABP signal could be identified (lower panels). The HABP signal (which is zero in 99% of all background pixels corresponding to the intracellular lumen in the same slice) inside L-VDCC clusters appears lower than outside. (D) Cumulative distributions of non-zero HABP signal in arbitrary units (au) inside of L-VDCC-immunopositive clusters versus all pixels, indicating a significant reduction of the HABP signal within L-VDCC-positive clusters ($p<0.001$, Kolmogorov-Smirnov test).

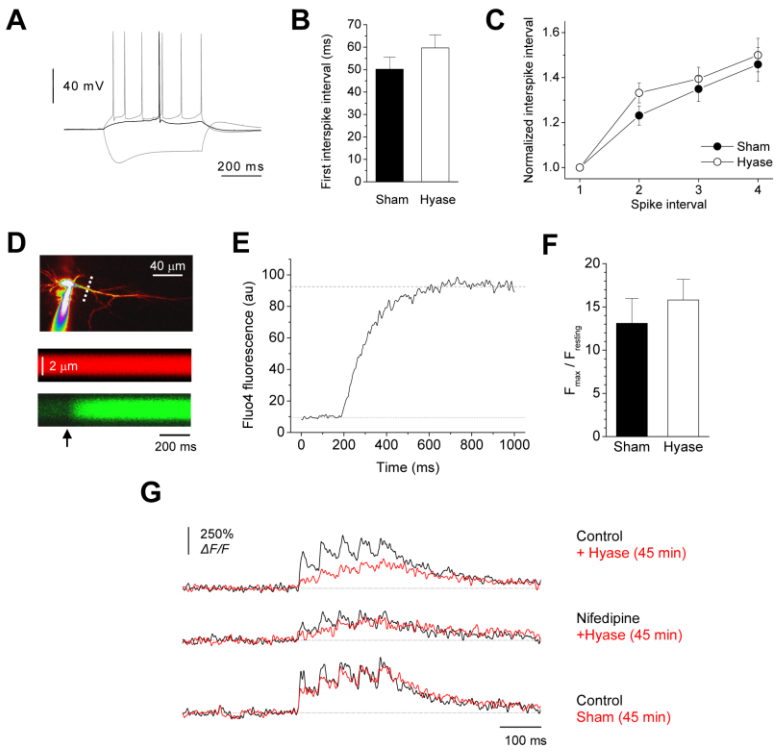

Figure S6. Removal of HA Does Not Affect the Spike Interval Adaptation in CA1 Pyramidal Cells and Resting Ca^{2+} Concentration, but Reduces Ca^{2+} Transients Evoked by the Backpropagating Action Potentials in Dendrites and Spines of CA1 Pyramidal Neurons (Related to Fig. 6).

(A) Example of voltage responses to current injections at rheobase and double rheobase. (B) The initial interspike interval in a spike train evoked by a double rheobase current injection is not affected by Hyase treatment (sham 50.2 ± 5.4 ms, n = 8; Hyase 59.7 ± 5.9, n = 10, p = 0.26). (C) Spike adaptation quantified by normalizing interspike intervals to the 1st interval is not significantly modified when HA is removed (p > 0.1, two-way ANOVA). (D) The Ca^{2+} concentration was determined in the apical dendrite 20-40 µm way from the cell body and the dialyzing patch pipette (upper panel). A line through the apical dendrite was scanned at 500 Hz in the Alexa 594 (middle panel) and the Fluo-4 channel (lower panel) while eliciting a train of action currents in the cell (arrow, 200-400 ms delay, 60-100 action currents at 100 Hz). (E) The time course of the background-corrected Ca^{2+}-dependent Fluo-4 fluorescence evoked by the train indicates that Fluo-4 is saturated by the high frequency stimulus (au, arbitrary units). (F) To circumvent any errors in defining the exact K_D for Fluo-4 in situ, we measured the ratio of the maximum and the resting (background-corrected) fluorescence (F_{max}/F_{rest}) as an unbiased gauge of the resting Ca^{2+} concentration (Scott and Rusakov, 2006). No difference between control conditions and Hyase treatment was detected (sham 13.1 ± 2.9, n = 6; Hyase 15.8 ± 2.4, n = 7, p = 0.49, t-test). (G) Traces show the global average Ca^{2+}-dependent fluorescence signals (200 µM Fluo-4, λ_x^{2P} = 800 nm) recorded in individual dendrites and spines of CA1 pyramidal cells in response to a brief train. Three experimental protocols are illustrated, as indicated: just before and 45 min after application of Hyase (top traces), a similar protocol but in the presence of 10 µM Nifedipine (middle trace), and sham control recording with the experimental timing matching that of test recordings (lower trace). Slight alterations in the fastest components of fluorescence responses (monitored over 45 min) may reflect minor redistribution of the postsynaptic Ca^{2+} indicator / endogenous buffers with time. We therefore focused on the most robust measure, total amount of Ca^{2+} entry (represented by the total fluorescence increment), which remained stable in control conditions throughout the experiment (Fig. 6G).

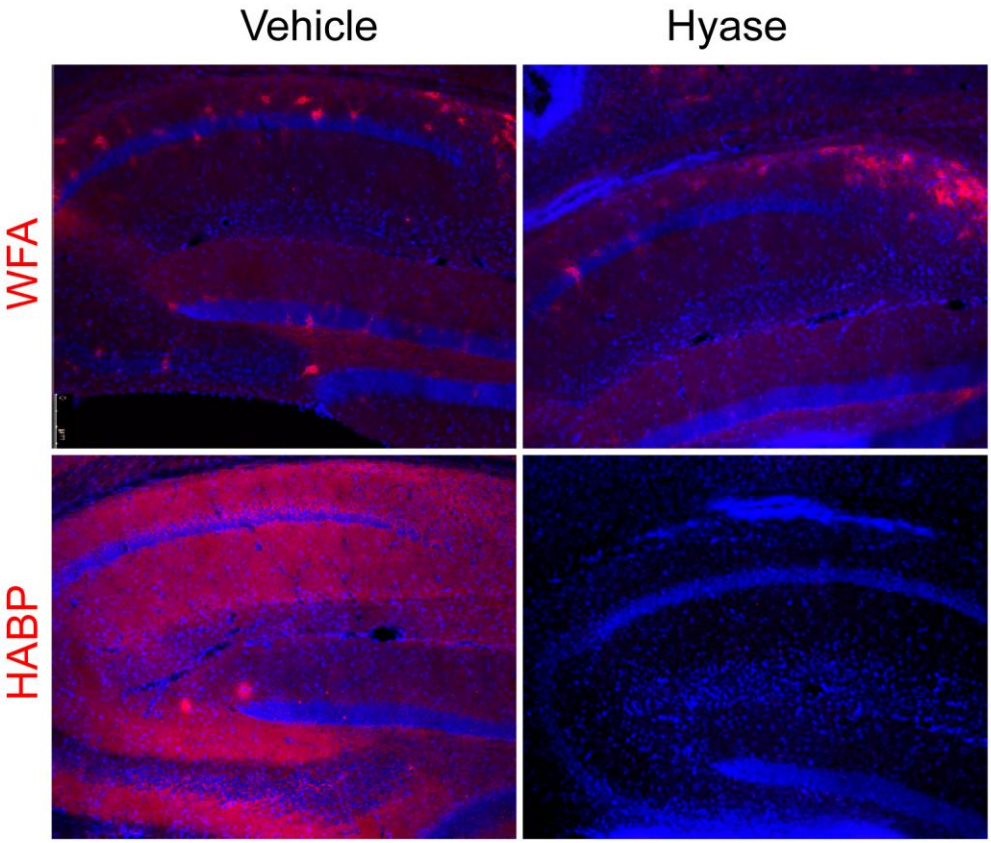

Figure S7. The Effects of Intrahippocampal Hyaluronidase Injection on Expression of HA and the Wisteria Floribunda Agglutinin (WFA)-Positive Perineuronal Nets In Vivo (Related to Fig. 8). In vivo injection of Hyase resulted in 24 hours in a removal of labeling for HA with biotinylated HA binding protein (HABP), but significant number of perineuronal nets remained to be labeled with Wisteria Floribunda Agglutinin (WFA).

Supplemental Experimental Procedures

Hippocampal Slice Preparation and Extracellular Recordings

Mice were decapitated after a brief CO_2 sedation. All treatments of animals were approved by the Committees on Animal Health and Care of the local governmental bodies. After removal of the brain, transverse hippocampal sections were cut with a VT 1000M vibratome (Leica, Nussloch, Germany) in ice-cold artificial cerebrospinal fluid (ACSF) infused with 95% O_2 / 5% CO_2 and containing (in mM): 250 sucrose, 24 $NaHCO_3$, 25 glucose, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1.5 $MgSO_4$ (pH 7.4). The slices were then kept for at least two hours before the start of recordings at room temperature in carbogen-infused ACSF, containing 120 mM NaCl instead of sucrose. Recordings were performed in the same solution, in a submerged chamber, at room temperature (22-24^oC). To elicit paired-pulse depression, the extracellular concentration of Ca^{2+} was increased to 2.5 mM and 50 μM 4-aminopyridine (4-AP) was added. Recordings of field excitatory postsynaptic potential (fEPSP) were performed in the stratum radiatum of the CA1b subfield with glass pipettes filled with ACSF and having a resistance of 1-2 MΩ. Schaffer collaterals were stimulated with a glass electrode placed approximately 300 μm closer to the CA3 subfield than the recording electrode. Basal synaptic transmission was monitored at 0.05 Hz. The inter-theta-train interval was 20 s and one or five trains were applied to induce LTP. Each train consisted of 8 bursts delivered at 5 Hz. Each burst consisted of 4 pulses delivered at 100 Hz. Duration of pulses was 0.2 ms and stimulation strength was set to provide baseline fEPSPs with amplitudes of approximately 50% from the subthreshold maximum.

The following drugs were bath applied to modulate induction of LTP: 50 μM picrotoxin, 10 μM nifedipine, or 15 μM Bay K 8644 (Sigma). Bay K 8644 was applied 15 min before till 5 min after induction of LTP. Two other drugs were present during the whole recording period or nifedipine was applied 10 min after induction of LTP, as indicated in the Results. For experiments with reintroduction of HA by bath application, the slices were treated with Hyase as described above, washed for 30 min in ACSF, incubated for 1 hour with HA (250 μg/ml, Calbiochem) before the beginning of recordings, and superfused with ACSF containing 250 μg/ml HA during recordings. In parallel to recordings in the presence of these drugs, control recordings in sham- and Hyase-treated slices without drugs were performed.

Histochemistry

To verify removal of HA by Hyase, some treated slices were fixed overnight in 4% formaldehyde in phosphate buffered saline (PBS; pH 7.3) and cut in 30μm-thick subslices. Before staining, slices were rinsed three times in 0.25% goat serum in PBS for 20 min each. After blocking in PBS containing 5% goat serum and 0.2% Triton-X100 for 1 hour, slices were incubated with cocktails of 2-3 primary reagents, including biotinylated hyaluronic acid-binding protein (HABP; Seikagaku, 10μg/ml) and the following antibodies: DDi-OS antibody (reacting with the unsulfated chondroitin stub of chondroitin sulfate proteoglycans, clone 1-B-5, Seikagaku, 1:300; Köppe et al., 1997);

rabbit polyclonal antibody against aggrecan (Chemicon, AB1031, 1:300); biotinylated WFA (Sigma, 1:400), $Ca_v1.2$ α_1 subunit of L-VDCCs (Hell et al., 1996); mouse monoclonal antibody against tenascin-R (clone 596, 1:1, Pesheva et al., 1989), or vesicular glutamate transporter 1 (VGLUT1; Synaptic Systems, 1:500). For visualization of astrocytes, a mouse expressing green fluorescent protein under the glial fibrillary acidic protein promoter (FVB/N-Tg(GFAPGFP)14Mes/J, The Jackson Laboratory) has been used. The primary reagents were diluted in PBS containing 2.5% normal goat serum. After incubation overnight at room temperature, slices were rinsed three times with PBS and reacted with a cocktail containing the carbocyanine-tagged secondary reagents (Cy3 streptavidin, Cy2 goat anti-mouse IgG and Cy5 goat anti-rabbit IgG, Dianova, 1:200) or corresponding Alexa dye-conjugated secondary reagents from Invitrogen for 1 hour at room temperature. Slices were washed with PBS and embedded using ProLong Gold antifade reagent (Invitrogen). Visualization of labeled structures was performed using a confocal laser scanning microscope (LSM510, Zeiss or SP5, Leica). The specificity of labeling was confirmed by staining with omitted primary reagents, in which no signal was detected (Fig. S5A).

Western Blot Analysis

A 10% (w/v) homogenate of hippocampus brain slices was prepared in 150 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate and 50 mM Tris (pH 7.5) supplemented with protease and phosphatase inhibitors (2.5 mM EDTA, 0.5 mM PMSF, 1μg/ml pepstatin A, 1μg/ml leupeptin, 3μg/ml Aprotinin, 1mM Na_3VO_4, 50 mM NaF, 8 μg/ml of both Calpain I and Calpain II inhibitors). All the protease and phosphatase inhibitors were purchased from Sigma. Five hippocampus slices were dissected from brain sections (sham- or Hyase-treated as described in the section “Recordings of LTP and immunohistochemistry”) to prepare each homogenate. After centrifugation at 1500 *g* for 10 min at 4°C the protein concentration in the supernatant was determined by Lowry assay (BioRad). 15 or 30 μg of protein extract were loaded into 6% or 10% gels to assay by Western blot analysis the expression profile of $Ca_v1.2$ $\alpha1$ (Hell et al., 1996), VGLUT1 (Synaptic Systems), NR2B, NR2A, GluR1 and $\alpha_2\delta_1$ (all four from Alomone Labs), tubulin III and actin α (both from Sigma). After electrophoresis the proteins were transferred to PVDF membranes (Millipore) and probed with the different antibodies following the manufacturer’s instructions. As a control for protein concentration, the same homogenate was tested by Western blot assay with anti-GAPDH antibodies (Millipore). Secondary antibodies and ECL detection kit were purchased from GE Healthcare.

Chemical LTP and Immunostaining for Active Erk1/2 and CREB

To elicit chemical L-VDCC-dependent LTP, 25 mM tetraethylammonium (TEA) was bath applied to hippocampal slices for 7 min. Twenty min after the end of TEA application, slices were fixed overnight in 4% formaldehyde in phosphate buffered saline (PBS; pH 7.3) and cut in 30μm-thick subslices. Before staining, slices were rinsed three times in 0.25% goat serum in PBS for 20 min each. After blocking in PBS containing 5% goat serum and 0.2% Triton-X100 for 1 hour, slices were incubated with primary rabbit

antibodies to phospho-Erk1/2 (Thr202/Tyr204; 1:100, Cell Signalling Technology) or phospho-CREB (Ser133; 1:100, Millipore). The primary reagents were diluted in PBS containing 2.5% normal goat serum. After incubation overnight at 4 ^{o}C, slices were rinsed three times with 0.25% goat serum in PBS and reacted with anti-rabbit Alexa 546-conjugated secondary antibodies for 1 hour at room temperature. Slices were washed in 0.25% goat serum in PBS and embedded using the ProLong Gold antifade reagent with DAPI (Invitrogen). Visualization and analysis of labeled structures was performed using SP5 (Leica) confocal laser scanning microscope. The specificity of labeling was confirmed by staining with omitted primary reagents, in which no signal was detected. For analysis, CA1 stratum pyramidale was outlined and mean intensities of Alexa 546 and DAPI were measured. The ratio between these measures was used for comparison between sham-, sham plus TEA-, Hyase-, and Hyase plus TEA-treated slices (2 slices per mouse, 4 mice per group).

Two-Photon Excitation Fluorescence Imaging

CA1 pyramidal cells were held either in voltage clamp at −70 mV or in current clamp and dye diffusion was allowed for 15-20 min before the recording started. To monitor theta-burst induced Ca^{2+} transients, Schaffer collaterals were stimulated with a bipolar electrode. The stimulus intensity was set at ten-fold the minimal current required to induce an action potential during a five-pulse 100 Hz train. Backpropagating action potentials were elicited by five 2-3 ms command voltage pulses at a frequency of 20 Hz. Fluo-4 fluorescence was collected by line-scanning at 500 Hz. In experiments involving backpropagating action potentials, four to six scans of an individual spine or dendrite were averaged for analyses, the background-corrected average response was normalized to the resting fluorescence and quantified by integrating the recorded fluorescence signal. Changes in the resting Ca^{2+} concentration were estimated by calculating the ratio of the resting Fluo-4 fluorescence and the Alexa Fluor 594 signal (both background-corrected) and used as a correction factor of the dynamic Ca^{2+} signal as detailed earlier (Scott and Rusakov, 2006). When evaluating Ca^{2+} responses to five back-propagating spikes during a relatively long-term experiment (Fig. 6F-H), the Ca^{2+}-dependent signal was calculated as the total Ca^{2+}-sensitive fluorescence (Fluo-4 channel) integrated over the recording time window and corrected for fluctuations in the baseline Ca^{2+} fluorescence, shown as $\Delta F/F_0$ (in arbitrary units of brightness, au).

For the analysis of TBS-induced fluorescence transients, fluorescence signals in both channels were first corrected for background (average fluorescence outside the stained structures). Next the Fluo-4 signal ($\Delta G=G-G_b$ where G and G_b stand for the emission intensities averaged over the post-stimulus and the baseline time windows, respectively) was normalized to the Alexa 594 channel fluorescence (R) to account for focus fluctuations during TBS, thus giving a standard Ca^{2+}-sensitive signal measure $\Delta G/R$.

In experiments with local injection, Hyase was dissolved in the bath solution to a final activity of 200 U/ml and delivered close to the dendrite/spine studied by constant local pressure application (PV830, WPI). The puffing solution was supplemented with 3 μM

Alexa Fluor 594 to visualize the area of drug delivery.

Analysis of Passive and Spiking Properties of Neurons

The recordings were performed in the same extracellular solution as used for Ca^{2+} imaging. The spiking phenotype of CA1 pyramidal cells was determined in current clamp recordings at room temperature using the following intracellular solution (in mM) 130 K-gluconate, 5 KCl, 10 HEPES, 10 di-tris-phosphocreatine, 2 $MgCl_2$, 4 Na_2-ATP, 0.4 Na-GTP. The input resistance was determined from the steady state voltage response to a small hyperpolarizing current injection (-5 to -20 pA). The membrane time constant was obtained by mono-exponential approximation of the rising phase of this voltage response. The rheobase was defined as the smallest depolarizing current that elicited an action potential (AP) when injected (current threshold). AP kinetics were determined at rheobase. Spike adaptation was characterized in spike trains evoked by injecting double the rheobase.

Mice for Behavioral Studies

30 wild type C57Bl/6J male adult (2-3 month old) mice from Charles River Laboratories (Kisslegg, Germany) were used in this study. 24 mice were used for behavior tests, and six mice for pilot experiments to verify effectiveness of Hyase injection *in vivo*. At least one week before the experiments, mice were transferred to a small vivarium where they were housed individually with food and water *ad libitum* on a reverse 12 h light/dark cycle (lights on 11:30 P.M.) under constant temperature (22±1°C) and humidity (55±5%). Since mice are nocturnal animals, all behavioral experiments were conducted during the dark part of the cycle, when mice are active. All surgical and behavioral procedures were approved by the Committee on Animal Health and Care of the government body in Hamburg.

Surgical Procedure

Implantation of bilateral cannulas was performed as described previously (Senkov et al., 2006). Briefly, mice were anesthetized with a freshly-made ketamine-xylazine cocktail containing ketamine (100 mg/kg; Albrecht, Aulendorf, Germany) and xylazine (Rompun, 16 mg/kg; Bayer, Leverkusen, Germany) injected i.p. with a dose (0.01 ml/g body weight) to provide a surgical level of anesthesia lasting about 1.5 h without a need in additional dose. The mouse skull was shaved and cleaned with 75% ethanol. Mice were placed in a stereotaxic frame (Narishige, Tokyo, Japan) with their eyes protected from drying by an eye gel. Before the skin of scalp was incised and retracted, a local analgesic was used. The skull was leveled to have lambda and bregma positions in the same horizontal plane. Three small holes (two for cannulas and one for a mounting screw) were drilled using a microsurgery hand bore (FST, Heidelberg, Germany). The surface of the cranium bone was carefully cleaned with 75% ethanol and dried with a hot air gun (HG 2000E; Steinel, Herzebrock-Clarholz, Germany) at a temperature not higher than 40°C. Sterile stainless steel 23 gauge guide cannulas closed with dummy cannulas (Plastic One, Roanoke, VA) were stereotaxically implanted bilaterally reaching

the surface layer of the cortex (from the bregma, AP: -2 mm, ML: ±1.5 mm, DV: 0.5mm from the skull) according to Paxinos and Franklin (2001). Guide cannulas were fixed on the skull bone by dental Kallocryl cement (CPGM rot; Speiko, Munster, Germany) and a small mounting screw. After implantation, the incision was closed and the wound was edged by Histoacryl tissue glue (Braun-Aesculap, Tuttlingen, Germany). The body temperature of mice was maintained at +37°C with a regulated homeothermic blanket control unit (HSEHarvard, March-Hugstetten, Germany) throughout the surgery. After the operation, mice were placed back to their home cages and kept under a warm (+37°C) red lamp until awakening. Metamizol (Novaminsulfon; Ratiopharm, Ulm, Germany; 50 mg/kg body weight, i.p.) was used as a postoperative analgesic. All subsequent behavioral investigations were performed after mice had fully recovered during 7 days after surgery.

Injections

Freshly defrosted Hyase from *Streptomyces hyalurolyticus* (Hyase) (#H1136, Sigma Aldrich, Germany), which exclusively cleaves hyaluronic acid and inactive for chondroitin sulfates, was diluted in sterile 0.9% NaCl and used always once in the experiments. The amount of the enzyme delivered into each hippocampi was nominally 2 Units. To evaluate the effects of Hyase, mice were randomly divided into two groups (Vehicle and Hyase) and injections made 24 h before fear conditioning (see Fig 8A). The time interval of 24 h between injections and training was chosen to let the enzyme diffuse in the brain and efficiently remove HA from the hippocampus (N=6; Fig. S7). There was no detectable reexpression of HA in the hippocampus and amygdala 48-72 hours after Hyase injection (N=2). Injections were performed while mice were sedated by CO_2 (Senkov et al., 2006), with the same injection volume (0.5 µl per side) and infusion rate (0.5 µl/min) for all animals. The injection cannula (Ø250 µm sharp needle) was connected to a pump (TSE Systems, Bad Homburg, Germany) through a Tygon tubing system. The injection cannula was gently inserted into the guide cannula before injection, extending the tip of the guide cannula by 2 mm. The site of the injection was aimed to the hilus and CA1 area of both dorsal hippocampi of the mouse brain. The injection cannula was left within the guiding cannula after injection for at least 1 min before removing for better diffusion of the enzyme. Then, the guide cannula was closed by a sterile dummy cannula. All animals recovered from this procedure within 2–3 min after transfer to their home cage without any visible side effects.

Fear Conditioning

Mice were allowed to recover after implantation for at least 7 days. 2-3 days before fear conditioning, all animals were gently handled and habituated to experimental conditions. The procedure of contextual fear conditioning was as follows: (1) a naive mouse was placed into the conditioning chamber for 180 s to record its baseline behavior before delivering the first US, abbreviated in the Fig. 8 as a trial “B” (baseline); (2) then, the animal received two foot shocks (1 s, 0.75 mA) served as an US, spaced apart by 60 s interval (“S” in Fig. 8); 1 min after the last US, the mouse was returned to its home cage. The conditioning chamber was an aluminum cage (20x20x40 cm) with a shocking

stainless-still grid on its floor cleaned every time with 75% ethanol. On day 1 (d1 in Fig 8) after fear conditioning, mice were randomly reintroduced again in conditioned context for 3 min to test contextual memory. The behavior of animals was recorded for each session digitally with a video camera. Analysis of freezing was done off-line by a trained observer blind to the identity of mice and to the injected group. Freezing, which served as a measure of fear-related memory, was quantified as described previously (Senkov et al., 2006; Tang et al., 2001). At the end of behavioral experiments, the animals were injected with methylene blue to verify positions of injection cannulas. We confirm that in all mice, cannulas were located in the dorsal hippocampus.

Supplemental References

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