Supplementary Methods

Immunocytochemistry. Hippocampal slice cultures (> 40 days in vitro, Thy1-mGFP\(^{e}\) and Thy1-mGFP/spRFP\(^{e}\)) in 6-well plates were fixed for 10 min at RT with 3.5% PFA- containing culture medium. Tissues were rinsed in PBS, solubilized in 1% Triton X-100 in PBS (2ml) overnight (ON, 4°C), blocked in 50 mM NH\(_4\)Cl and 20% horse serum, rinsed with PBS containing 1% horse serum (PBS + HS), and incubated ON (4°C) in PBS+HS, with 2 \(\mu\)g/ml affinity-purified anti-NR-1 mouse monoclonal antibody (Pharmingen) (Thy1-mGFP\(^{e}\) and Thy1-mGFP/spRFP\(^{e}\)), plus rabbit-anti-synaptophysin (DAKO, Denmark) (Thy1-mGFP\(^{e}\)). Tissue slices were then rinsed with PBS + HS, incubated for 10–16 hours (4°C) in Alexa-Fluor-546 conjugated goat-anti-rabbit and Oregon green-438 conjugated goat-anti-mouse (Molecular Probes, Eugene, Oregon), rinsed and analyzed. Argon and He, Ne-G lasers (488/543 excitation), with Rhodamine and GFP filter sets (560-600 and 505-525 barrier filters) were used to simultaneously visualize both synaptic markers in the same samples. Step sizes were 0.2 \(\mu\)m, for stacks of 9-30 \(\mu\)m. A 100X water immersion objective (NA = 0.1, Zeiss) was used for image acquisition. Synaptophysin and NR1 contents in MF terminals were scored in single optical planes and in 3D reconstructions.

In situ hybridization was carried out with digoxigenin-labeled cRNA probes corresponding to the entire EGFP gene.

Propidium iodide assay for cell death. Cell death in the slices was assessed by Propidium Iodide (PI, Sigma) incorporation into the cells\(^1\). PI was added to the slices in tyrode’s at a final concentration of 1 \(\mu\)g/ml for 30 min at RT. After a brief (5 min) wash in tyrode’s, PI fluorescence images were acquired with an optimized filter set (530-585 filter; 2.5X objective
connected to a Hamamatsu digital CCD camera). Images (512×512 pixels) were opened in Image software after the same mask to visualize the nuclei was applied in Photoshop (Suppl. Fig. 7). Dead cells were counted with the function “analyze particles”. For each experimental condition, data were compared (ANOVA) to those from untreated control slices, which exhibited a low level of spontaneously degenerating cells (Suppl. Fig. 7).

**Live imaging in hippocampal slice cultures.** The method of (ref. 13) was used to culture hippocampal slices because of its convenience for long term imaging in comparison to the “roller tubes” technique, which presented problems in preserving sterile conditions. Briefly, P8-9 pups were killed by decapitation, and hippocampi rapidly dissected out and placed in a MEM (Gibco) based ice-chilled medium. Usually, six to eight 400 µm-thick slices were selected from one hippocampus. They were then incubated in 5% CO$_2$ at 35 °C. For time lapse imaging, slices were taken out of the incubator in a physiological solution at RT (Gibco based). An Olympus set up (Bx61 LSM Fluoview) was used for confocal microscopy (see below). For conventional microscopy images were acquired with a Zeiss Axioskop equipped with a HBO 50 mercury lamp for fluorescence illumination, water immersion objectives (Achroplan 40X/0.75W, 63X/0.9W and 100X/1.0W), NDF (Neutral-Density Filters) (0.06, 0.25, 0.5, d = 18), and a Hamamatsu digital CCD camera (C4742-95) controlled by the QED Camera Plug-in for Power Mac G4 (QED Imaging Inc., Pittsburgh, PA). Due to the high expression levels in Thy1-transgenic mice, fluorescent images exhibited high signal-to-noise ratios (Suppl. Fig. 8), and where either not, or only slightly contrasted, if not stated otherwise. Each imaging session, from the time the slice was taken out of the incubator to the time it was put back, lasted approximately 10 minutes. The following imaging conditions were used for *Thy1-mGFP* and
*Thy1-mGFP/spRFP* slices: 40X objective, 0.25+0.5 NDF for up to 2 min to identify the proper axon (MF), 100X objective 0.25+0.5 NDF for 10-30 sec to identify the LMT (landmark); 100X objective, 0.25 NDF for 5-7 min to acquire images (20-70/session, 512×512 pixels). Live imaging was carried out in region CA3b\(^{18}\); imaging of CA3c was excluded to avoid photo damaging of sensitive neuronal cell bodies. Under these conditions no photo damaging was detected following up to five separate imaging sessions (see later). For time lapse recordings data set consisted of 36 different MF regions (N=4 MF) from >40 day-old hippocampal slice cultures, imaged for 3-6 minutes (10 sec frames). Measurements were performed with Image software, using a threshold function, by an observer blind to the experimental conditions. Where indicated, Sp-cAMPS (Biolog), Glutamate (RBI), Cytochalasin D (Sigma), TTX (Allergen AG, Switzerland), AMPA (RBI, 400 nM), AP5 (RBI, 100 µM), cycloheximide (Sigma, 60 µM), SYM2206 (Tocris, Bristol, 100 µM), and BDNF (serum starvation over night, followed by serum free conditions with the neurotrophin\(^2\)) were added to the culture medium after a first imaging session. The treatment with Sp-cAMPS was limited to 20 min, when cultures were washed and then imaged at the intervals indicated in the figures. Glutamate was applied at concentrations of 10-100 µM directly into the culture medium after the first two time points were acquired (To and T6h). AMPA (with or without AP5) and cycloheximide were applied for 6h (24 h treatments were toxic). Basal values of LMTfil dynamics at 1d were not affected by a 6h treatment with cycloheximide (MI = 0.48 ± 0.05, unpaired t-test \(P = 0.9\)).

When cycloheximide was combined with AMPA + AP5, the protein synthesis inhibitor was applied 1h before the Glutamate receptor agonists.

Hippocampal slice cultures were stimulated in the CA3 stratum lucidum region with monopolar glass electrodes. The electrodes were placed in the proximity of mGFP-positive MF axons,
which were subsequently analyzed for LMTfil dynamics. Reliable stimulation of MF is achieved by this protocol (not shown). Two stimulation patterns were used: 1) High Frequency Stimulation (4 times 100 Hz for 1s each; interstimulation interval: 30 sec; single stimulus duration: 100 µs); 2) Low Frequency Stimulation (1 Hz for 15 min; single stimulus: 100 µs).

**Hippocampal whole mount preparation**. Following decapitation, the brain was dissected in ice-cold medium in the presence of TTX and Mg²⁺ to prevent transmitter release-dependent toxicity. Cerebellum and frontal lobes were removed by coronal cuts, and brain stem, midbrain and striatum were separated from the hippocampus, using 2 spatulas. The entire procedure, from sacrificing the mouse to the whole-mount preparation, took less than 6 minutes. The right and left hippocampi, connected ventrally through the septum, were then exposed for direct imaging (continuous oxygenation with 95% O₂, 5% CO₂) under a fluorescent upright microscope. Only terminals belonging to granule cells in the free blade of the hippocampus could be imaged with this procedure.

**Analysis of epV dynamics**. Typically, axonal segments in CA3 (400-500 µm) were selected based on the presence of easily identifiable landmarks, such as LMT or characteristic axonal curvatures. Segments were then scored for the presence of swellings and/or filopodia. To be considered as epV, mossy fiber axonal swellings had to fulfill the following criteria: 1) The maximal diameter (perpendicular to long axis if epV is elliptical) had to be at least twice the width of the corresponding axon (the focal plane where a varicosity was most evident was used for size measurements); 2) swellings had to be visible in at least 2 focal planes to avoid potential image acquisition artifacts; 3) to exclude swellings reflecting axonal transport, epV had to be detectable at the same position along the axon throughout at least two separate
observations. Data from 400-700 \( \mu \text{m} \) long continuous stretches of axons were acquired for all experimental conditions, and all terminal structures of a given type (epV, LMT, LMTfil) were included in the analysis. An experimenter blind to the experimental conditions performed all size measurements with Image software at 8:1 magnification, using a threshold function to a single level throughout each session. To control for possible artifacts that may have been introduced through image analysis, the experimenter blind to the experimental conditions also scored the raw data. No significant difference was detected between the two data sets (9.4% versus 8.6% dynamic epV). Values were averages of three measurements.

**Quantification of LMTfil dynamics.** LMTfil were defined as any axonal extension unequivocally traced back from a swelling larger than 3 \( \mu \text{m} \) (LMT) in diameter. Individual images were superimposed in Adobe Photoshop, and rotated to correct for x-y shifts. Single layers were then opened with Image software, "Threshold" and "Make Binary" tools were used to standardize measurements within one imaging session, and filopodial areas were calculated. Measurements were carried out by an observer blind to the experimental conditions. A motility index (MI) based on accumulated filopodial areas while the filopodia move over time was derived as described\(^4\,^5\). Two filopodium configurations exhibiting equal length, and a 50% overlap in area lead to an MI value of 0.5. By only considering as dynamic LMTfil with MI values larger than 0.6, we excluded the possibility of including variations due to local tissue distortions. This possibility was further excluded by analyzing 3D reconstructions of LMTfil configurations. Ranges of LMTfil retraction/elongation rates were as follows: untreated slice, average value: 2 \( \mu \text{m}/4 \text{ h} \); max. value: 4 \( \mu \text{m}/4 \text{ h} \); BDNF treated slices: average value 4 \( \mu \text{m}/4 \text{ h} \);
max. value 8 μm/4 h; NCAM−/− slices: average 5 μm/4 h; max. value 10 μm/4 h. Stat view (for Mac) was used for all the statistical analysis.

**Time-lapse imaging and analysis of slices from Thy1-spGFP<sup>mu</sup> mice.** To visualize the dynamics of synaptic puncta, imaging fields were about 80 μm<sup>2</sup>. To maximize the discrimination of puncta from background, parameters were set as follows: argon laser 488 nm (GFP imaging); barrier filter 505-525; intensity minimized to 5% with C.A. 100μm; PMT 800; gain 5.3; offset 0%. Initial scanning of the slice was carried out using neutral density filters (fluorescent mode). Stacks included all puncta within a given range of z-axis, and were projected onto one plane for analysis (horizontal view). Background was subtracted using an option of the Fluoview software, and "Metamorph 4.6r7" was used for the subsequent steps. All images acquired during the same session were thresholded equally, and superimposed correcting for x-y shifts. To try to standardize fluorescent signals among different confocal sessions, at the beginning of each session we set the gain and offset to ensure that the same saturation levels were obtained (i.e. most intense signal just below saturation, and background adjusted just above detection limit). Sizes of puncta were calculated with an Integrated Morphometric Analysis tool. Only puncta between 1.5-3 μm and/or 50-300 pixel area (0.14667 pixel/ μm) were included in the analysis. This range was chosen to exclude transport packets and LMT. To exclude ambiguities due to sub-micron (0.1-0.5 μm) shifts in the position and shape of puncta, only gain/loss events with no detectable fluorescence in the surrounding 0.5 μm were included in the analysis. Changes in the size and/or shape of puncta were not scored as gain/loss events. The size of boutons in the CA3 region was in accordance to previous in vivo data (1-10 μm), and subsequent immunocytochemistry for synaptic vesicle markers
yielded labeled structures undistinguishable from those previously detected in the living cultures (data not shown).

**Controls for photo damage.** All slices and structures included in the analysis were assessed for signs of photo damage one day after the last imaging session. Characteristic signs of damage (mGFP marker) include: drastic weakening of fluorescent signals; swelling and breakdown of axons into a chain of beads; blurred GFP signals around membranes due to degradation and leakage; formation of large blebs on cell bodies and dendrites; loss of dendritic spines. Using Neutral Density Filters, objectives with high NA, laser intensity <5%, and minimizing exposure and picture acquisition times (one imaging session lasted around 10 min overall), axonal segments of up to 500 µm could be imaged repeatedly (at least 4 different time points), without inducing photo damage. These imaging conditions did not influence dendritic motility and short term axonal dynamics or axonal transport (data not shown). In a further set of control experiments, we determined the possible influence of fluorescent light on the translocation rates of transport packets (spGFP marker). Translocation rates were 0.08 ± 0.05 µm/sec (in the vicinity of stable clusters; N = 24, 6 slices), and 0.3 ± 0.1µm/sec (>10 µm away from stable clusters; N = 37, 9 slices). These values are in good agreement with those reported from dispersed culture studies, and were not affected by the imaging conditions used in this study (no detectable changes between sessions, or up to 2 days after the last session, when up to 3 fields of ca. 150 µm² were imaged repeatedly).

**References**


