

Astrocytes mediate two forms of spike timing-dependent depression at entorhinal cortex-hippocampal synapses

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eLife Assessment

This **valuable** study reports the existence of specific spike-timing dependent synaptic plasticity processes at two excitatory synapses of the dentate gyrus granule cells. These synapses link the entorhinal cortex and the dentate gyrus but via different circuits. With state-of-the-art patch-clamp electrophysiological analysis, the authors provide **convincing** information on the molecular mechanisms underlying these 2 forms of synaptic plasticity showing a critical role for astrocytes in both alongside some features distinctive to each pathway. These results will be of interest to neuroscientists as they uncover detailed plasticity mechanisms involving the hippocampus.

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Abstract

The entorhinal cortex (EC) connects to the hippocampus sending different information from cortical areas that is first processed at the dentate gyrus (DG) including spatial, limbic, and sensory information. Excitatory afferents from lateral (LPP) and medial (MPP) perforant pathways of the EC connecting to granule cells of the DG play a role in memory encoding and information processing and are deeply affected in humans suffering Alzheimer's disease and temporal lobe epilepsy, contributing to the dysfunctions found in these pathologies. The plasticity of these synapses is not well known yet, as are not known the forms of long-term depression (LTD) existing at those connections. We investigated whether spike timing-dependent long-term depression (t-LTD) exists at these two different EC-DG synaptic connections in mice, and whether they have different action mechanisms. We have found two different forms of t-LTD, at LPP- and MPP-GC synapses and characterised their cellular and intracellular mechanistic requirements. We found that both forms of t-LTD are expressed presynaptically and that whereas t-LTD at LPP-GC synapses does not require NMDAR, t-LTD at MPP-GC synapses requires ionotropic NMDAR containing GluN2A subunits. The two forms of t-LTD require different group I mGluR, mGluR5 LPP-GC synapses and mGluR1 MPP-GC synapses. In addition, both forms of t-LTD require postsynaptic calcium, eCB synthesis, CB1R,

astrocyte activity, and glutamate released by astrocytes. Thus, we discovered two novel forms of t-LTD that require astrocytes at EC-GC synapses.

Introduction

The entorhinal cortex (EC) conveys spatial, limbic and sensory information to the hippocampus, a structure responsible for important activities in the brain including learning, memory and spatial information coding. Excitatory projections from the EC to the dentate gyrus (DG) play a role in memory encoding, and they are affected in humans suffering from Alzheimer Disease (AD) and temporal lobe epilepsy, contributing critically to these pathologies (Paré et al., 1992 [\[1\]](#); Llorens-Martín et al., 2014 [\[2\]](#)). In rodents, evidence suggests that the lateral perforant pathway (LPP) conveys more sensory related information, whereas the medial perforant pathway (MPP) conveys information related to spatial location and limbic signals that are involved in attention and motivation. Evidence suggests that LPP- and MPP-GC synapses have distinct electrophysiological properties, these differences potentially affecting the functional processing of information (Krueppel et al., 2011 [\[3\]](#); Petersen et al., 2013 [\[4\]](#); Kim et al., 2018 [\[5\]](#)). Indeed, these functional differences probably reflect their distinct inputs, as they contact the outer (LPP) or middle (MPP) third of the molecular layer of the DG (Paré et al., 1992 [\[1\]](#); Burgess et al., 2002 [\[6\]](#)). Significantly, MPP-GC synapses are associated with a higher probability of release (Min et al., 1998 [\[7\]](#)) and the existence and/or activity of presynaptic NMDARs, which differs between the two types of fibres (Savtchouk et al., 2019 [\[8\]](#)). Interestingly, LPP and MPP synapses appear to influence pathological changes distinctly, with the former showing changes in release probability in models of epilepsy (Scimemi et al., 2006 [\[9\]](#)), preferential β -amyloid deposition (Reilly et al., 2003 [\[10\]](#)), earlier susceptibility to AD (Khan et al., 2014 [\[11\]](#)) and reduced plasticity with age (Froc et al., 2003 [\[12\]](#)). The mechanistic bases of these differences are virtually unknown and they pose a challenge when attempting to understand circuit-specific biological computations, as well as the susceptibility to pathological insults. In addition, the forms of plasticity at these synapses remain unclear and the limited studies into plasticity available did not distinguish between these two pathways.

Plasticity drives the organization of cortical maps during development, and it underlies learning and memory (reviewed in Malenka and Bear, 2004 [\[13\]](#); Mateos-Aparicio and Rodríguez-Moreno, 2019 [\[14\]](#)). The most extensively studied forms of plasticity are long-term potentiation (LTP) and long-term depression (LTD) of synaptic transmission. Spike timing-dependent plasticity (STDP) is a Hebbian form of long-term synaptic plasticity found in all known nervous systems, from insects to humans. Indeed, STDP appears to be a synaptic mechanism that underlies circuit remodelling during development, and it is thought to be involved in learning and memory (Dan and Poo, 2004 [\[15\]](#); Feldman, 2012 [\[16\]](#)). In STDP, the relative order and millisecond timing of pre- and postsynaptic action potentials (spikes/APs) determine the direction and magnitude of the synaptic changes. Thus, spike timing-dependent LTP (t-LTP) occurs when a postsynaptic spike follows a presynaptic spike within ms, whereas spike timing-dependent LTD (t-LTD) is induced when this order is reversed (Feldman, 2012 [\[16\]](#)). In addition, *in vivo* studies have suggested modifications to LTP and LTD after mice undertake behavioural tasks (Kemp and Manahan-Vaughan, 2004 [\[17\]](#); Clarke et al., 2010 [\[18\]](#)). However, it is surprising that while plasticity of EC-DG granule cell (GC) synapses has been studied using classical protocols (Froc et al., 2003 [\[12\]](#); Harney et al., 2006 [\[19\]](#); Hayashi et al., 2014 [\[20\]](#), Savtchouk et al., 2019 [\[8\]](#)), no information exists as to whether t-LTP or t-LTD can be induced at these synapses.

Here we studied t-LTD at PP-GC synapses, differentiating the lateral and medial pathways, determining whether these individual synapses are subject to t-LTD and if this form of plasticity at these synapses influences learning, memory or information processing. As a result, we identified two different forms of presynaptic t-LTD at LPP- and MPP-GC synapses. Unlike LPP-GC synapses, the t-LTD at MPP-GC synapses requires ionotropic NMDARs that contain GluN2A subunits, and the t-LTD at each type of synapse require different group I mGluRs, with LPP-GC synapses dependent

on mGluR5 and MPP-GC t-LTD requiring mGluR1. In addition, we found that both forms of t-LTD require postsynaptic calcium, endocannabinoid (eCB) synthesis, CB₁ receptor (CB₁R) signalling, astrocytes and glutamate. Hence, we describe here two novel forms of presynaptic t-LTD in the brain at EC-GC synapses with different action mechanisms, probably implicated in the different aspects of information processing.

Materials and Methods

Animals and ethical approval

All animal procedures were conducted in accordance with the European Union Directive 2010/63/EU regarding the protection of animals used for scientific purposes, and they were approved by the Ethics Committee at the Universidad Pablo de Olavide and that of the Andalusian Government. C57BL/6 mice were purchased from Harlan Laboratories (Spain) and P13–21 (P, post-natal day) mice of either sex were used. Animals were kept at temperatures between 18 and 24 °C on a continuous 12 h light/dark cycle, and at 40–60% humidity, with ad libitum access to food and water. In some experiments, dominant-negative (dn) SNARE mice (Pascual et al., 2005 [\[1\]](#)) of the same age were used. These mice were not fed with doxycycline from birth and the transgenes were continuously expressed. In these mice, the human glial fibrillary acidic protein (hGFAP) promoter mediates the specific expression of the tetracycline transactivator (tTA) in astrocytes, which in turn activates the tetracycline responsive element operator and drives the cytosolic expression of VAMP2/synaptobrevin II in these cells, along with the enhanced green fluorescence protein (eGFP). Expression of the dnSNARE transgene interferes with the formation of the SNARE complex, blocking exocytosis and impairing vesicle release by astrocytes (Sultan et al., 2015 [\[2\]](#)).

Slice preparation

Hippocampal slices were prepared as described previously (Andrade-Talavera et al., 2016 [\[3\]](#); Pérez-Rodríguez et al., 2019 [\[4\]](#); Falcón-Moya et al., 2020 [\[5\]](#)). Briefly, mice were anesthetized with isoflurane (2%), decapitated, and their whole brain was removed and placed in an ice-cold ACSF solution (pH 7.2, 300 mOsm·L⁻¹) containing (in mM): 126 NaCl, 3 KCl, 1.25 NaH₂PO₄, 2 MgSO₄, 2 CaCl₂, 26 NaHCO₃, and 10 glucose. Slices (350 μm thick; Leica VT1000S Vibratome) were maintained oxygenated (95% O₂/5% CO₂) in the same solution for at least 1 h before use. Experiments were carried out at 33–34 °C and during the experiments; the slices were superfused continuously with the same solution indicated above.

Electrophysiological recordings

Whole-cell patch-clamp recordings of DG GCs were obtained under visual guidance by infrared differential interference contrast microscopy. The identity of the neurons was verified by their characteristic voltage response to a current step protocol applied in current-clamp configuration using a patch-clamp amplifier (Multiclamp 700B), and acquiring the data with pCLAMP 10.2 software (Molecular Devices). Patch-clamp electrodes were pulled from borosilicate glass tubes and had a resistance of 4–7 MΩ when filled with the intracellular solution consisting in (in mM, pH 7.2–7.3, 290 mOsm L⁻¹): potassium gluconate, 110; HEPES, 40; NaCl, 4; ATP-Mg, 4; and GTP, 0.3. Only cells with a stable resting membrane potential below −55 mV were assessed, and the cell recordings were excluded from the analysis if the series resistance changed by >15%. All recordings were low-pass filtered at 3 kHz and acquired at 10 kHz. For plasticity experiments, excitatory postsynaptic potentials (EPSPs) were evoked alternately at 0.2 Hz in two input pathways, test and control, by using two monopolar stimulating electrodes placed 200–400 μm from the cell soma. Stimulating electrodes were situated on fibres contacting the distal and medial third of the dendritic arbour of GCs, corresponding to LPP- and MPP-GC synapses, respectively. Stimulation was adjusted (200 μs, 0.1–0.2 mA) to obtain an EPSP peak amplitude of 3–5 mV in control conditions. Pathway independence was ensured by the lack of cross-facilitation when the

pathways were stimulated alternately at 50 ms intervals. Plasticity was assessed through the changes in the EPSP slope, measured in its rising phase as a linear fit between time points corresponding to 25-30% and 70-75% of the peak amplitude under control conditions. Miniature responses were recorded in the presence of tetrodotoxin (TTX, 300 nM). Astrocytes were identified by their morphology under differential interference contrast microscopy, and were characterized by low membrane potential (-81 ± 0.6 mV, $n = 55$), low membrane resistance (20 ± 0.3 M Ω , $n = 55$) and passive responses (they do not show action potentials) to both negative and positive current injection.

Plasticity protocols

After establishing a stable basal EPSP over 10 min, the test input was paired 100 times with a single postsynaptic spike. The single postsynaptic spike was evoked by a brief somatic current pulse (5 ms, 0.1–0.5 pA), whereas the control pathway was left unstimulated during the pairing period. To induce t-LTD, a postsynaptic AP was evoked 18 ms before the onset of the EPSP. EPSP slopes were monitored for at least 30 min after the pairing protocol, and the presynaptic stimulation frequency remained constant throughout the experiment. In some experiments, glutamate or WIN 55, 212-2 “puffs” were applied using a Picospritzer (Parker Hannifin), expelling glutamate dissolved in the external solution through a micropipette over an astrocyte in proximity to the LPP-GC or MPP-GC synapses at a pressure of 10 psi for 50–200 ms. In each experiment, 100 glutamate puffs were applied to the recording neuron at 0.2 Hz at baseline, 18 ms before the onset of the EPSP, which did not affect patch clamping. The EPSP slopes were then monitored for 30 min.

Drugs

The following agents were purchased from: Sigma-Aldrich - BAPTA, Zinc chloride, and the compounds used to prepare the ACSF and current clamp internal solutions; and Tocris Bioscience - MK-801 maleate, D-AP5, TTX, PPDA, Ro 25-6981 maleate, L-glutamic acid, MPEP, LY367385, LY341495, AM251, Orlistat (THL), Evans blue, WIN 55,212-2, FK506, Nimodipine, Thapsigargin, Ifenprodil, ruthenium red, bicuculline, SCH50911 and NBQX. All compounds were dissolved in distilled water except for Nimodipine, Thapsigargin, PPDA, FK506, AM251 and THL, which were dissolved in dimethyl sulfoxide (DMSO). Tetanus toxin light chain was acquired from Creative Diagnostics.

Data analysis

The data were analysed using the Clampfit 10.2 software (Molecular Devices) and the last 5 min of the recordings were used to estimate the changes in synaptic efficacy relative to the baseline. For the paired-pulse ratio (PPR) experiments, two EPSPs were evoked for 30 s at the baseline frequency, one at the beginning of the baseline recording (40 ms apart) and another 30 min after the end of the pairing protocol. The PPR was expressed as the slope of the second EPSP divided by the slope of the first EPSP. A Coefficient of Variation (CV) analysis was carried out on the EPSP slopes (Rodríguez-Moreno and Paulsen, 2008 [\[1\]](#); Falcón-Moya et al., 2020 [\[2\]](#)) and the noise-free CV of the EPSP slopes was calculated as:

$$CV = \sqrt{\frac{\sigma^2(EPSP) - \sigma^2(noise)}{EPSP\ slope}}$$

where σ^2 (EPSP) and σ^2 (noise) are the variance of the EPSP and baseline, respectively. The plot compares the variation in the mean EPSP slope (M) to the change in response variance of the EPSP slope ($1/CV^2$; see Brock et al., 2020 [\[3\]](#) for a comprehensive explanation). Graphs were prepared using SigmaPlot 14.0.

Statistical analysis

Before applying any statistical comparison, the data were subjected to Shapiro-Wilk normality and equal variance tests, applying a confidence interval (CI) of 95%. Unpaired or paired Student's *t*-tests were used for comparisons between two groups. For multiple comparisons of more than two groups, one-way ANOVA was used followed by a Holm–Sidak *post-hoc* test (when necessary). All the data are expressed as the mean $\pm$ standard error of the mean (SEM) and *P* values were considered significant when < 0.05 : **p* = 0.05, ***p* = 0.01, ****p* = 0.001.

Results

Pairing presynaptic activity with single postsynaptic action potentials at low frequency can induce t-LTD at mouse lateral and medial perforant pathway-dentate gyrus granule cell synapses

We initially determined whether spike timing-dependent LTD can be induced at perforant pathway-DG GC synapses by pairing presynaptic stimulation with single postsynaptic spikes at low frequency (0.2 Hz) in slices from P13–21 mice. We used a post-pre protocol, pairing a single postsynaptic spike followed 18 ms later by presynaptic stimulation 100 times, as described previously at cortical (L4–L2/3) and hippocampal (SC–CA1) synapses (Andrade-Talavera et al., 2016 [DOI](#); Martínez-Gallego et al., 2022a [DOI](#)). We monitored the EPSPs evoked by extracellular stimulation of the LPP- and MPP-GC synapses, which make synaptic contacts with the distal and medial third of the dendritic arbour of hippocampal DG GCs, respectively (Petersen et al., 2013 [DOI](#); Savtchouk et al., 2019 [DOI](#)). EPSPs were recorded from GCs using the current clamp mode of the whole-cell patch-clamp configuration (Fig. 1a, k [DOI](#)). A post-before-pre pairing protocol induced robust t-LTD at both LPP-GC ($63 \pm 5\%$, *n* = 14: Fig. 1b, c [DOI](#)) and MPP-GC synapses ($59 \pm 5\%$, *n* = 14: Fig. 1l, m [DOI](#)), while unpaired control pathways remained unaffected ($103 \pm 4\%$ and $105 \pm 7\%$, respectively: Fig. 1b, c, l, m [DOI](#)). We repeated the experiments at LPP-GC and MPP-GC synapses in the presence of GABA_A and GABA_B receptor blockers (bicuculline, 10 μ M and SCH50911, 20 μ M, respectively) to determine whether they have a role in this form of t-LTD. In these experimental conditions, t-LTD was not affected at these synapses, indicating that these receptors are not necessary for t-LTD induction (LPP-GC: Control t-LTD: $67 \pm 6\%$, *n* = 6; Bicuculline + SCH50911: $69 \pm 11\%$, *n* = 6; Fig. S1). MPP-GC synapses: Control t-LTD: $65 \pm 8\%$, *n* = 6; Bicuculline + SCH50911: $75 \pm 9\%$, *n* = 6; Fig. S1). Thus, we performed the rest of experiments in the absence of any GABA receptor blocker.

Presynaptic expression of t-LTD at lateral and medial perforant pathway-dentate gyrus granule cell synapses

We next determined the site where this t-LTD was expressed through several approaches. As we observed failures in synaptic transmission at both types of synapse, we analysed these and found that the number of failures increased consistently after t-LTD at both LPP-GC (from $43 \pm 7\%$ at baseline to $76 \pm 6\%$ after t-LTD, *n* = 8: Fig. 1d [DOI](#)) and MPP-GC synapses (from $40 \pm 5\%$ at baseline to $59 \pm 4\%$ after t-LTD, *n* = 6: Fig. 1n [DOI](#)), suggesting a presynaptic mechanism. We then analysed the PPR at baseline and 30 min after the pairing protocol, identifying a significant increase in the PPR after t-LTD at both LPP-GC (from 1.16 ± 0.11 at baseline to 1.67 ± 0.19 after t-LTD, *n* = 14: Fig. 1e [DOI](#)) and MPP-GC synapses (from 1.17 ± 0.11 at baseline to 1.81 ± 0.15 after t-LTD, *n* = 14: Fig. 1o [DOI](#)), again suggesting a presynaptic expression of these two forms of t-LTD. Third, we estimated the noise-subtracted CV of the synaptic responses before and after t-LTD induction. A plot of CV^{-2} versus the change in the mean evoked EPSP slope (*M*) before and after t-LTD mainly yielded points below the diagonal line at LPP-GC and MPP-GC synapses (LPP-GC: Mean = 0.607 ± 0.054 vs $1/CV^2 =$

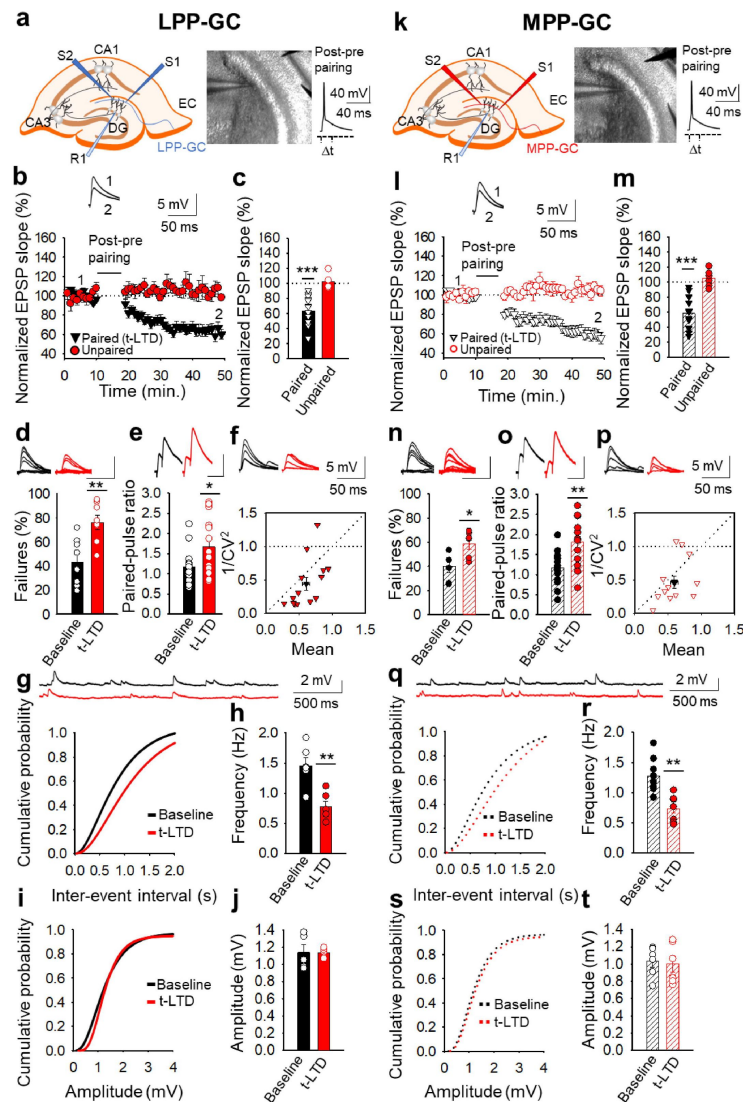


Figure 1.

Input-specific presynaptic spike timing-dependent LTD at lateral and medial perforant pathway-dentate gyrus granule cell synapses.

(a, k) Schemes and pictures show the general set-up for the electrophysiological recordings and pairing protocol ($\Delta t = -18$ ms, time between the peak of the postsynaptic spike and the onset of the EPSP) at both LPP- and MPP-GC synapses: CA, *cornu ammonis*; DG, dentate gyrus; EC, entorhinal cortex; S1 and S2, stimulating electrodes; R, recording electrode. A post-before-pre pairing protocol induced t-LTD at both LPP-GC (b) and MPP-GC (l) synapses. The EPSP slopes monitored in the paired (LPP-GC synapses black triangles, $n = 14$; MPP-GC synapses open black triangles, $n = 14$) and unpaired (LPP-GC synapses red symbols; MPP-GC synapses open red symbols) pathways are shown. The traces show the EPSP before (1) and 30 minutes after (2) pairing. Depression was only observed in the paired pathways. (c, m) Summary of the results. At the lateral (d-j) and medial (n-t) perforant pathway synapses onto dentate gyrus granule cells t-LTD is expressed presynaptically. The number of failures increases after t-LTD induction at both LPP- ($n = 8$, d) and MPP-GC ($n = 6$, n) synapses. Traces show the EPSPs before (black) and 30 min after pairing (red). PPR increases after t-LTD pairing protocol at LPP-GC (e) and MPP-GC (o) synapses. The traces show the EPSP before (black) and 30 min after t-LTD induction (red). (f, p) Normalized plots of CV^{-2} versus mean EPSP slopes suggest a presynaptic change in the release parameters at both LPP- ($n = 14$) and MPP-GC ($n = 13$) synapses. The traces show the EPSPs before (black) and 30 min after t-LTD induction (red). Miniature EPSPs (mEPSPs) were monitored at baseline and after t-LTD induction in the presence of TTX (300 nM) at LPP- (g-j, $n = 6$) and MPP-GC synapses (q-t, $n = 6$). Cumulative graphs and histograms show that after t-LTD induction, the frequency of mEPSPs decreases at both types of synapses, whereas the amplitude of the mEPSPs remains constant. Scale bar for the % failures - 5 mV, 50 ms; scale bar for PPR - 5 mV, 80 ms. Error bars indicate the S.E.M.: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-sided Student's t-test.

0.439 ± 0.096 , $R^2 = 0.337$; $n = 14$) and MPP-GC (Mean = 0.596 ± 0.056 vs $1/CV^2 = 0.461 \pm 0.090$, $R^2 = 0.168$; $n = 13$), respectively. (Fig. 1f, p), consistent with a modification of the release parameters (Rodríguez-Moreno and Paulsen, 2008; see Brock et al., 2020 for review). Finally, we recorded and analysed the miniature responses (mEPSP). We performed the experiments in the presence of tetrodotoxin (TTX, 300 nM) to avoid action potentials, before and after t-LTD, adding TTX at the baseline (we added TTX and started to record mEPSP after 10 min. of TTX in the bath for additional 10 min) and washing it out (for 30 min) before performing the t-LTD experiment (we recorded EPSP for 10 min as a baseline after 30 min TTX washout, then applied the t-LTD protocol and then recorded EPSP for additional 30 min), and then added TTX again after t-LTD to record mEPSP for other 10 min (Falcón-Moya et al., 2020). This experimental approach serves to determine whether the frequency and amplitude of mEPSP change because of the induction of t-LTD. Under these conditions, a t-LTD similar to previous experiments was evident at both types of synapse (LPP-GC synapses $55 \pm 11\%$, $n = 6$; MPP-GC synapses $67 \pm 8\%$, $n = 7$), and we found that the frequency of mEPSPs decreased at both types of synapse after t-LTD induction: LPP-GC - baseline 1.4 ± 0.13 Hz, after t-LTD induction 0.7 ± 0.09 Hz, $n = 6$ (Fig. 1g, h); and MPP-GC - baseline 1.2 ± 0.1 Hz, after t-LTD induction 0.7 ± 0.07 Hz, $n = 8$ (Fig. 1q, r). No effect of TTX on mEPSP amplitude was evident at any of these synapses: LPP-GC - baseline 1.13 ± 0.09 mV, after t-LTD 1.13 ± 0.02 mV, $n = 5$ (Fig. 1i, j); MPP-GC - baseline 1.03 ± 0.07 mV, after t-LTD 1.00 ± 0.09 mV, $n = 6$ (Fig. 1s, t). These results again suggest a presynaptic locus for t-LTD expression. Hence, together these results are consistent with a decrease in the probability of neurotransmitter release and they are indicative of a presynaptic locus for these two forms of t-LTD at both types of synapse.

Presynaptic t-LTD requires NMDARs at MPP-GC but not at LPP-GC synapses

Different forms of t-LTD have been shown to require NMDARs at cortical synapses and in the hippocampus (Bender et al., 2006; Nevian and Sakman, 2006; Rodríguez-Moreno and Paulsen, 2008; Rodríguez-Moreno et al., 2011, 2013; Andrade-Talavera et al., 2016). To determine whether the two forms of t-LTD studied here require NMDARs, we treated slices with the NMDAR antagonists D-AP5 (50 μ M) or MK-801 (500 μ M), completely blocking NMDARs and NMDAR-mediated currents (from 57 ± 8 to 6 ± 5 pA, $n = 5$). In these experimental conditions, t-LTD at LPP-GC synapses was not affected by these antagonists (D-AP5 $66 \pm 4\%$, $n = 8$; MK-801 $62 \pm 9\%$, $n = 6$; interleaved controls $60 \pm 6\%$, $n = 8$; Fig. 2a, b), whereas t-LTD at MPP-GC synapses was blocked in slices treated with either D-AP5 ($90 \pm 7\%$, $n = 10$) or MK-801 ($107 \pm 10\%$, $n = 6$) relative to the interleaved controls ($60 \pm 7\%$, $n = 8$; Fig. 2e, f). When the experiment was repeated by loading the postsynaptic cell with MK-801 (500 μ M-1 mM), t-LTD at MPP-GC synapses was not affected ($54 \pm 7\%$, $n = 9$) relative to the interleaved controls ($60 \pm 7\%$, $n = 8$; Fig. 2e, f). MK-801 (500 μ M-1 mM) loaded into the postsynaptic neuron blocked NMDAR-mediated currents recorded from the postsynaptic cell (from 58 ± 5 to 9 ± 5 pA, $n = 5$). These results indicate that while t-LTD at MPP-GC synapses requires NMDARs, t-LTD at LPP-GC synapses does not. Moreover, the NMDARs required for t-LTD at MPP-GC synapses are ionotropic and not postsynaptic, consistent with data from neocortical synapses where presynaptic NMDARs are required for t-LTD (Sjöström et al., 2003; Bender et al., 2006; Nevian and Sakmann, 2006; Rodríguez-Moreno and Paulsen, 2008).

Presynaptic NMDARs have been found at MPP-GC synapses where they modulate glutamate release. These receptors seem to contain GluN2B and GluN3A subunits (Jourdain et al., 2007; Savtchouk et al., 2019). To determine the subunit composition of the NMDARs involved in t-LTD at MPP-GC synapses, we first repeated the experiments in slices treated with Zn^{2+} (300 nM), an antagonist of NMDARs containing GluN2A subunits (Bidoret et al., 2009; Andrade-Talavera et al., 2016; Prius-Mengual et al., 2019) that prevented t-LTD ($95 \pm 5\%$, $n = 7$; Fig. 2g, h). We also repeated the experiments on slices in the presence of Ro25-6981 (0.5 μ M), an antagonist of NMDARs containing GluN2B subunits (Hu et al., 2009; Mares et al., 2021). This antagonist did not affect t-LTD ($55 \pm 11\%$, $n = 6$; Fig. 2g, h) and neither did an additional antagonist of NMDARs containing GluN2B subunits, ifenprodil (3 μ M, $62 \pm 11\%$, $n = 6$; Fig. 2g, h). Finally, t-LTD was not

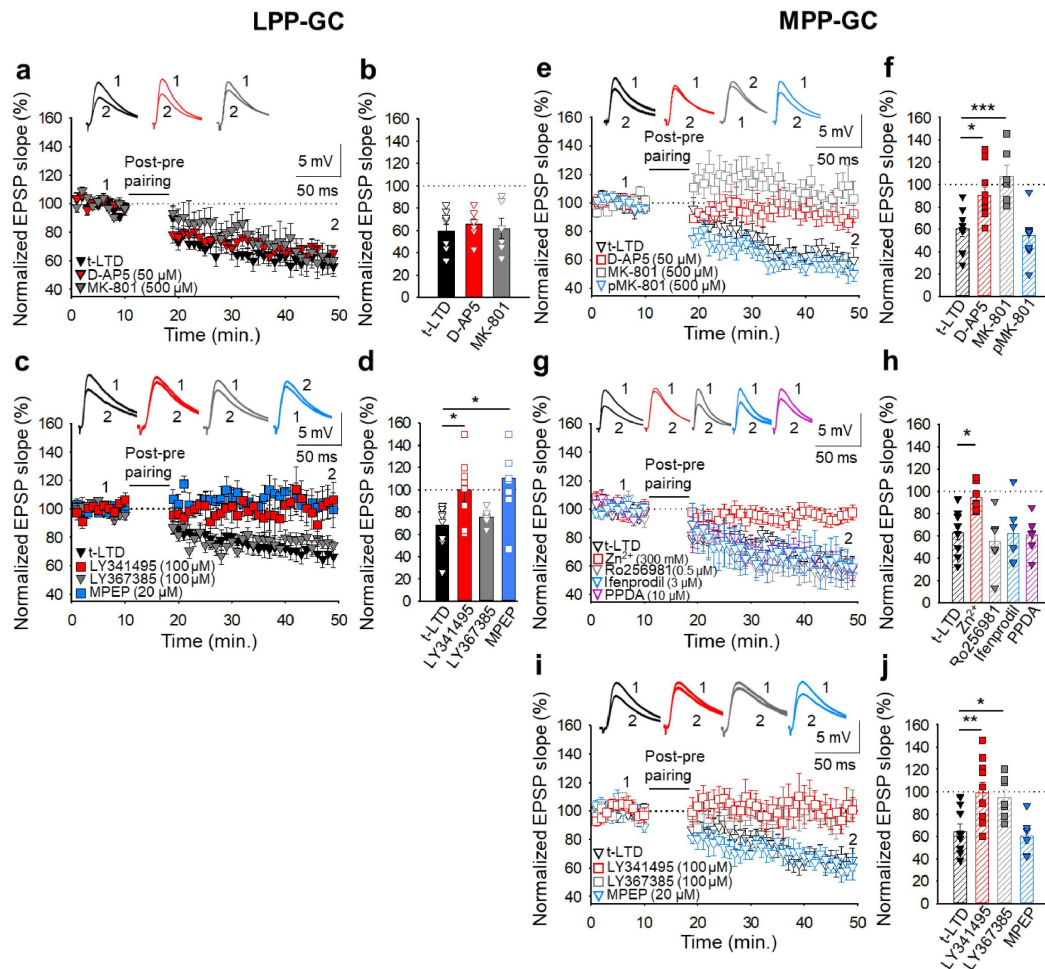


Figure 2.

The t-LTD at lateral and medial perforant pathway-dentate gyrus granule cell synapses has distinct requirements for glutamate receptors.

The t-LTD at LPP-GC synapses requires mGluR5. (a) The addition of D-AP5 (50 μ M) or MK-801 (500 μ M) to the superfusion fluid does not prevent the induction of t-LTD at LPP-GC synapses. The EPSP slopes shown are from D-AP5-treated (red triangles, $n = 8$), MK-801-treated (grey triangles, $n = 6$) and untreated cells (black triangles, $n = 8$). The traces show EPSPs before (1) and 30 min after (2) pairing. (b) Summary of the results. (c) The t-LTD at LPP-GC synapses requires mGluR5. The EPSP slopes are shown from control slices (black triangles, $n = 9$), and slices treated with the mGluR antagonist LY341495 (100 μ M, red squares, $n = 8$), the mGluR1 antagonist LY367385 (100 μ M, grey triangles, $n = 6$) or the mGluR5 antagonist MPEP (20 μ M, blue squares, $n = 8$). The traces show the EPSPs before (1) and 30 min after (2) pairing. (d) Summary of the results. (e) *The t-LTD at MPP-GC synapses requires NMDARs containing GluN2A subunits and mGluR1.* The addition of D-AP5 (50 μ M) or MK-801 (500 μ M) to the superfusion fluid prevented t-LTD induction at MPP-GC synapses, whereas postsynaptic loading of MK-801 (500 μ M) does not block t-LTD induction. The EPSP slopes are shown from D-AP5 (open red squares, $n = 10$) or MK-801 treated cells (batch, open grey squares, $n = 6$; loaded postsynaptic cell, open blue triangles, $n = 9$), and untreated cells (open black triangles, $n = 8$). The traces show the EPSPs before (1) and 30 min after (2) pairing. (f) Summary of the results. (g) *The NMDARs involved in t-LTD at MPP-GC synapses contain GluN2A subunits.* The t-LTD at MPP-GC synapses was completely blocked in slices exposed to Zn^{2+} (300 nM), while it remained unaffected in slices treated with Ro 25-6981 (0.5 μ M), ifenprodil (3 μ M) or PPDA. The EPSP slopes shown are from control slices (open black triangles, $n = 9$) and slices treated with Zn^{2+} (open red squares, $n = 7$), Ro-25-6981 (open grey triangles, $n = 6$), ifenprodil (open blue triangles, $n = 6$) or PPDA (open pink triangles, $n = 6$). The traces show the EPSPs before (1) and 30 min after (2) pairing. (h) Summary of the results. (i) *The t-LTD at MPP-GC synapses requires mGluR1.* The EPSP slopes shown are from control slices (open black triangles, $n = 9$), or slices treated with LY341495 (100 μ M, open red squares, $n = 10$), LY367385 (100 μ M, open grey squares, $n = 7$) or MPEP (20 μ M, open blue triangles, $n = 6$). The traces show the EPSPs before (1) and 30 min after (2) pairing. (j) Summary of the results. The error bars indicate the S.E.M.: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, One-way ANOVA + Holm-Sidak test.

affected in the presence of PPDA (10 μ M, 61 ± 7 %, $n = 6$: **Fig. 2g, h**) relative to the pooled interleaved controls (63 ± 6 %, $n = 9$), an antagonist of NMDARs that contain GluN2C/2D subunits (Chen et al., 2021; Wang et al., 2020). These results indicate that the presynaptic NMDARs involved in t-LTD induction at MPP-GC synapses probably contain GluN1/GluN2A subunits.

Presynaptic t-LTD at lateral and medial perforant pathway-dentate gyrus granule cell synapses requires mGluRs

As mGluRs have been implicated in plasticity and t-LTD in different brain regions, and at distinct synapses (Gladding et al., 2009), and they may modulate synaptic transmission at PP-DG synapses (Dietrich et al., 1997), we tested whether the presynaptic forms of t-LTD at LPP- and MPP-GC synapses require mGluRs. Exposure to the broad-spectrum mGluR antagonist LY341495 (100 μ M) completely blocked t-LTD at both LPP-GC (100 ± 10 %, $n = 8$: **Fig. 2c, d**) and MPP-GC synapses (99 ± 9 %, $n = 10$: **Fig. 2i, j**). Indeed, the mGluR5 antagonist MPEP (20 μ M) prevented t-LTD at LPP-GC (103 ± 10 %, $n = 8$: **Fig. 2c, d**) but not at MPP-GC synapses (61 ± 7 %, $n = 6$: **Fig. 2i, j**), whereas the specific mGluR1 antagonist LY367385 (100 μ M) did not affect t-LTD at LPP-GC synapses (76 ± 3 %, $n = 6$: **Fig. 2c, d**) but it did block t-LTD at MPP-GC synapses (95 ± 7 %, $n = 7$: **Fig. 2i, j**) relative to their respective interleaved controls (LPP-GC synapses 68 ± 7 %, $n = 9$; MPP-GC synapses 65 ± 7 %, $n = 9$: **Fig. 2c, d, i, j**). Hence, both LPP-GC and MPP-GC synapses require mGluR activity to induce these presynaptic forms of t-LTD, but whereas t-LTD at LPP-GC synapses requires mGluR5, mGluR1 receptors are required at MPP-GC synapses.

Postsynaptic Ca^{2+} is required for t-LTD at lateral and medial perforant pathway-dentate gyrus granule cell synapses

Both t-LTP and t-LTD require postsynaptic Ca^{2+} at neocortical and hippocampal synapses (Bender et al., 2006; Nevian and Sakmann, 2006; Rodríguez-Moreno et al., 2013; Andrade-Talavera et al., 2016; Inglebert et al., 2021; Martínez-Gallego et al., 2022a), which led us to investigate the postsynaptic Ca^{2+} requirements of t-LTD at PP-DG synapses. As L-type Ca^{2+} channels have previously been implicated in plasticity (Nevian and Sakmann, 2006; Andrade-Talavera et al., 2016; Wiera et al., 2017; Falcón-Moya et al., 2020; Martínez-Gallego et al., 2022a), we assessed whether they are involved in the t-LTD studied here by performing the pairing protocol after bath application of the L-type Ca^{2+} channel blocker, nimodipine (10 μ M). This channel blocker did not impede t-LTD induction at LPP-GC (79 ± 4 %, $n = 6$: **Fig. 3a, b**) or MPP-GC synapses (67 ± 6 %, $n = 6$: **Fig. 3e, f**), indicating that L-type Ca^{2+} channels are not required for these forms of t-LTD. The Ca^{2+} involved in synaptic plasticity may also be released from intracellular stores (Bender et al., 2006; Nevian and Sakmann, 2006; Andrade-Talavera et al., 2016; Martínez-Gallego et al., 2022a) and indeed, t-LTD was prevented at both LPP-GC and MPP-GC synapses when thapsigargin (10 μ M) was present in the superfusion fluid (LPP-GC synapses 108 ± 16 %, $n = 6$; MPP-GC synapses 108 ± 6 %, $n = 6$), a drug that avoids refilling of intracellular Ca^{2+} stores after Ca^{2+} depletion (Singh et al., 2021). A similar effect was witnessed when thapsigargin (1-10 μ M) was loaded into the postsynaptic neuron (LPP-GC synapses 107 ± 7 %, $n = 15$; MPP-GC synapses 98 ± 4 %, $n = 13$) relative to untreated interleaved controls (LPP-GC synapses 65 ± 5 %, $n = 11$; MPP-GC synapses 63 ± 6 %, $n = 11$: **Fig. 3a, b, e, f**). The presence of heparin (5 mg/ml), a blocker of IP_3 R-mediated Ca^{2+} release (Ghosh et al., 1988), in the recording pipette did not prevent t-LTD induction at LPP-GC (77 ± 8 %, $n = 6$; versus interleaved control slices, 65 ± 5 %, $n = 11$: **Fig. 3a, b**) or MPP-GC synapses (81 ± 10 %, $n = 6$; versus interleaved control slices, 63 ± 6 %, $n = 11$: **Fig. 3e, f**), suggesting that postsynaptic IP_3 R-mediated Ca^{2+} release is not required for t-LTD. In contrast, inclusion in the patch pipette of ruthenium red (a blocker of ryanodine receptors), prevented t-LTD at both, LPP-GC (98 ± 12 %, $n = 7$; versus interleaved control slices, 65 ± 5 %, $n = 11$, **Fig. 3a, b**) and MPP-GC synapses (98 ± 12 %, $n = 6$; versus interleaved control slices, 63 ± 6 %, $n = 11$, **Fig. 3e, f**), suggesting that Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores is required for this form of t-LTD. Hence, Ca^{2+} release from the intracellular stores appears to be necessary for t-LTD at both LPP and MPP-GC synapses.

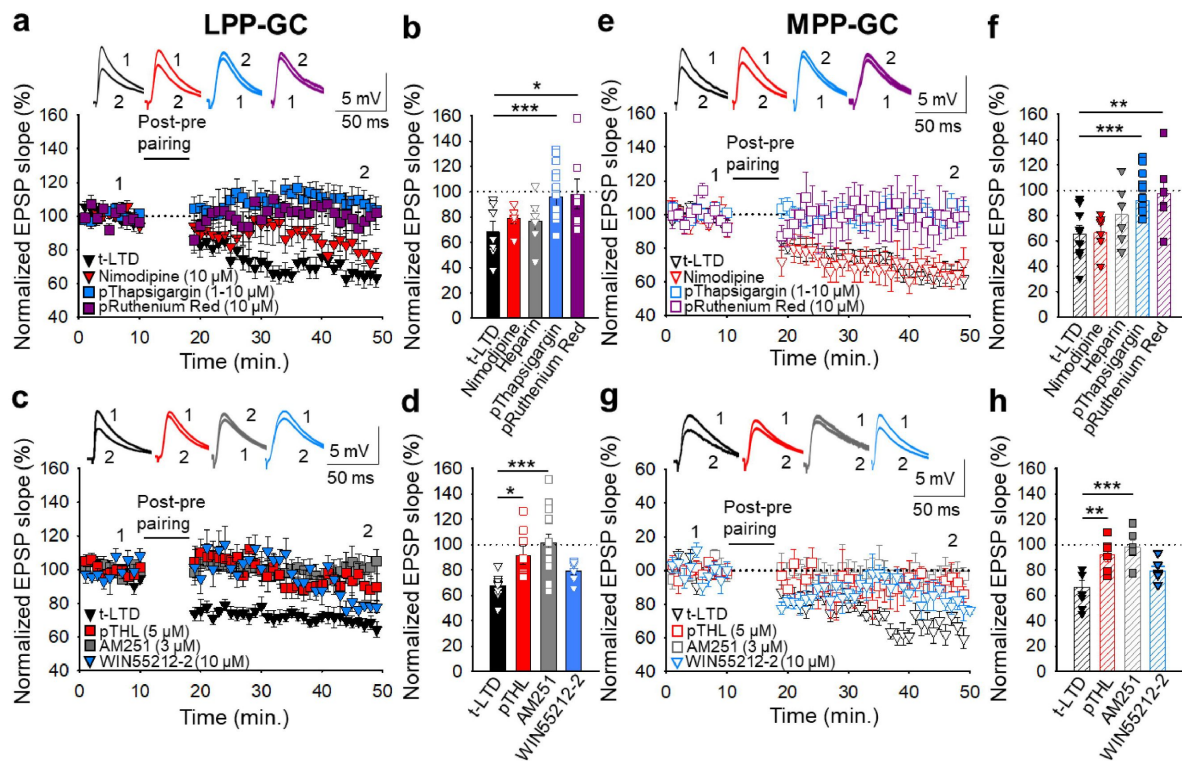


Figure 3.

The t-LTD at lateral and medial perforant pathway-dentate gyrus granule cell synapses requires postsynaptic calcium and eCB signalling.

(a) The induction of t-LTD at LPP-GC synapses in slices was unaffected by exposure to nimodipine (10 μ M) to the bath or to heparin (5 mg/ml) loaded into the postsynaptic neuron, but it was prevented by treatment with thapsigargin (1–10 μ M) or ruthenium red (10 μ M), loaded into the postsynaptic cell. The EPSP slopes shown are from control slices (black triangles, $n = 11$) and slices treated with nimodipine (red triangles, $n = 6$), thapsigargin (blue squares, $n = 15$) and ruthenium red (purple squares, $n = 7$). The traces show EPSPs before (1) and 30 min after (2) pairing. (b) Summary of the results. (c) The t-LTD at LPP-GC synapses requires endocannabinoids and CB₁ receptors, as it was blocked when THL was loaded into the postsynaptic neuron (pTHL, 5 μ M) or in slices treated with AM251 (3 μ M). In addition, LTD was induced by direct activation of CB₁R by treatment of the slices with WIN 55 512-2 (10 μ M). The EPSP slopes shown are from control slices (black triangles, $n = 9$), in slices with THL loaded into the postsynaptic neuron (red squares, $n = 8$), treated with AM251 (grey squares, $n = 14$) or treated with WIN 55 512-2 (blue triangles, $n = 6$). The traces show the EPSPs before (1) and 30 min after (2) pairing. (d) Summary of the results. (e) The induction of t-LTD at MPP-GC synapses was unaffected in nimodipine-treated slices (10 μ M) or heparin (5 mg/ml), but prevented in slices treated with thapsigargin (10 μ M) or ruthenium red (10 μ M), loaded into the postsynaptic cell. The EPSP slopes shown are from control slices (open black triangles, $n = 9$) and slices treated with nimodipine (open red triangles, $n = 6$), thapsigargin (open blue squares, $n = 13$) and ruthenium red (open purple squares, $n = 6$). The traces show EPSPs before (1) and 30 min after (2) pairing. (f) Summary of the results. (g) The t-LTD at MPP-GC synapses requires endocannabinoids and CB₁ receptors, and it was blocked when THL was loaded into the postsynaptic neuron (pTHL, 5 μ M) and in slices treated with AM251 (3 μ M). In addition, LTD was induced by direct activation of CB₁R by treatment of the slices with WIN 55 512-2 (10 μ M). The EPSP slopes shown are from control slices (open black triangles, $n = 8$) and slices with THL loaded into the postsynaptic neuron (open red squares, $n = 6$) or treated with AM251 (open grey squares, $n = 6$) or treated with WIN 55 512-2 (open blue triangles, $n = 6$). The traces show EPSPs before (1) and 30 min after (2) pairing. (h) Summary of the results. The error bars indicate S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, One-way ANOVA + Holm–Sidak test.

Postsynaptic calcineurin is required for t-LTD induction at LPP-GC but not MPP-GC synapses

To gain better insight into the mechanisms involved in t-LTD, we hypothesized that a Ca^{2+} -dependent enzyme might be involved. Since the Ca^{2+} -dependent protein phosphatase calcineurin has been implicated in synaptic plasticity in the hippocampus (Mulkey et al., 1994) and neocortex (Torii et al., 1995; Rodríguez-Moreno et al., 2013; Andrade-Talavera et al., 2016), we tested the effect of inhibiting calcineurin on t-LTD. Accordingly, t-LTD induction was prevented at LPP-GC ($110 \pm 7\%$, $n = 8$) but not MPP-GC synapses ($72 \pm 7\%$, $n = 6$; Fig. S2) when the calcineurin blocker FK506 (10 μM) was added to the superfusion fluid, relative to the untreated interleaved controls (LPP-GC: $68 \pm 6\%$, $n = 9$; MPP-GC: $71 \pm 7\%$, $n = 6$; Fig. S2). Subsequently, we loaded the postsynaptic neuron at LPP-GC synapses with FK506 (1–10 μM) and as t-LTD was no longer detected at these synapses ($109 \pm 8\%$, $n = 13$; Fig. S2), postsynaptic calcineurin appears to be involved in t-LTD induction at LPP-GC but not MPP-GC synapses.

Endocannabinoid signalling at lateral and medial perforant pathway-dentate gyrus granule cell synapses is required for t-LTD

Postsynaptic Ca^{2+} dynamics may affect the synthesis of eCBs like anandamide and 2-arachidonoylglycerol (2-AG), ligands of CB_1Rs (Laguerre et al., 2021) necessary for t-LTD at neocortical, striatal and hippocampal synapses in rodents (Bender et al., 2006; Andrade-Talavera et al., 2016; Cui et al., 2016; Hogrefe et al., 2022). These receptors may be expressed in neurons and/or astrocytes, where they can mediate the release of gliotransmitters like glutamate, ATP/adenosine and D-serine through exocytotic mechanisms (Covelo and Araque, 2018). The induction of t-LTD at hippocampal and neocortex synapses requires the eCB system and astrocyte signalling, which may in turn drive gliotransmitter release (Min and Nevian, 2012; Andrade-Talavera et al., 2016; Martínez-Gallego et al., 2022a). To investigate the role of eCB signalling in these forms of t-LTD, we loaded the postsynaptic neurons with tetrahydrolipstatin (THL, 5 μM) to inhibit diacylglycerol lipase activity and prevent eCB synthesis. THL prevented t-LTD induction at both LPP-GC ($92 \pm 7\%$, $n = 8$ versus interleaved controls $67 \pm 3\%$, $n = 8$; Fig. 3c, d) and MPP-GC synapses ($92 \pm 5\%$, $n = 6$ versus interleaved controls $66 \pm 5\%$, $n = 8$; Fig. 3g, h). Moreover, the presence of the CB_1R antagonist AM251 (3 μM) also blocked t-LTD at both LPP-GC ($102 \pm 7\%$, $n = 14$; Fig. 3c, d) and MPP-GC synapses ($98 \pm 5\%$, $n = 6$; Fig. 3g, h). In addition, the activation of CB_1R by puffs of the agonist WIN 55, 212-2 to the astrocyte, directly induced LTD at both LPP-GC ($83 \pm 9\%$, $n = 6$ versus interleaved controls $68 \pm 3\%$, $n = 9$; Fig. 3c, d) and MPP-GC synapses ($79 \pm 3\%$, $n = 6$ versus interleaved controls $67 \pm 5\%$, $n = 8$; Fig. 3g, h). Thus, eCB synthesis by postsynaptic cells and CB_1R activation of is required for presynaptic t-LTD at LPP- and MPP-DG GC synapses.

Astrocyte signalling and glutamate release at lateral and medial perforant pathway-dentate gyrus granule cell synapses is required for t-LTD

Astrocytes are involved in plasticity and synaptic transmission at hippocampal and cortical synapses (Min and Nevian, 2012; Araque et al., 2014; Andrade-Talavera et al., 2016; Covelo and Araque, 2018; Pérez-Rodríguez et al., 2019; Falcón-Moya et al., 2020; Durkee et al., 2021; Martínez-Gallego et al., 2022a), and at the synapses studied here (Jourdain et al., 2007; Savtchouk et al., 2019; Pérez-Otaño and Rodríguez-Moreno, 2019). To investigate the possible involvement of astrocytes (Fig. S3) in the induction of t-LTD at the EC-DG synapses studied here, different approaches were used. First, individual astrocytes were loaded with the Ca^{2+} chelator BAPTA (20 mM) through the patch pipette to inhibit vesicular and Ca^{2+} dependent gliotransmitter release (Parpura and Zorec, 2010), both at LPP-GC and MPP-GC synapses, and recorded in neurons situated at 50–100 μm from the loaded astrocytes (Fig. 4a, f). In these experimental

conditions, t-LTD was impaired at both LPP-GC ($103 \pm 9\%$, $n = 6$: **Fig. 4b, c**) and MPP-GC synapses ($94 \pm 6\%$, $n = 6$: **Fig. 4g, h**). Second, we assessed t-LTD in transgenic mice expressing a selective dnSNARE domain (dnSNARE) in astrocytes, preventing functional vesicular gliotransmitter release from these cells (Pascual et al., 2005; Sultan et al., 2015; Sardinha et al., 2017). In contrast to the typical t-LTD observed in WT mice (LPP-GC synapses $71 \pm 5\%$, $n = 7$; MPP-GC synapses $60 \pm 5\%$, $n = 6$: **Fig. 4b, c, g** and h), t-LTD could not be induced in these dnSNARE mice (LPP-GC synapses $102 \pm 9\%$, $n = 9$; MPP-GC synapses $112 \pm 6\%$, $n = 7$: **Fig. 4c, d, g** and h). In addition, we loaded astrocytes with the light chain of the tetanus toxin (TeTx_{LC}, $1\mu\text{M}$) which is known to block exocytosis by cleaving the vesicle-associated membrane protein, an important part of the SNARE complex (Schiavo et al., 1992; Min and Nevian, 2012). In these experimental conditions, t-LTD was impaired at both LPP-GC ($106 \pm 9\%$, $n = 6$: **Fig. 4b, c**) and MPP-GC synapses ($108 \pm 10\%$, $n = 6$: **Fig. 4g, h**). These results clearly indicate that astrocytes and vesicular release are required to induce t-LTD at both LPP-GC and MPP-GC synapses.

At these synapses it is thought that glutamate is released from astrocytes to modulate synaptic transmission and participate in plasticity (Jourdain et al., 2007; Savtchouk et al., 2019), as indicated for cortical synapses (Min and Nevian, 2012; Martínez-Gallego et al., 2022a). To determine whether glutamate, probably released by astrocytes, is required for t-LTD at LPP- and MPP-GC synapses, we repeated the experiments in slices from dnSNARE mutant mice but applying glutamate puffs ($100\mu\text{M}$) in the proximity of the astrocytes and neurons under study rather than pairing. In these experimental conditions, t-LTD, which was not evident in untreated dnSNARE mice (LPP-GC synapses $95 \pm 5\%$, $n = 7$; MPP-GC synapses $109 \pm 6\%$, $n = 6$) was fully recovered at both LPP-GC ($72 \pm 11\%$, $n = 6$: **Fig. 4d, e**) and MPP-GC synapses ($70 \pm 8\%$, $n = 6$: **Fig. 4i, j**) relative to the control interleaved slices from WT mice (LPP-GC synapses $62 \pm 8\%$, $n = 7$; MPP-GC synapses $64 \pm 5\%$, $n = 7$). In addition, to gain more insight into the fact that glutamate is released by astrocytes, we blocked glutamate release from astrocytes by loading the astrocytes with Evans blue ($5\mu\text{M}$), known to interfere with glutamate uptake into vesicles as it inhibits the vesicular glutamate transporter (VGLUT) (Harkani et al., 2004; Min and Nevian, 2012). In these experimental conditions, t-LTD was impaired at both LPP-GC ($106 \pm 6\%$, $n = 6$: **Fig. 4d, e**) and MPP-GC synapses ($106 \pm 11\%$, $n = 6$: **Fig. 4i, j**), indicating that t-LTD requires Ca^{2+} -dependent exocytosis of glutamate from astrocytes. These results indicate that glutamate released, involving SNARE-dependent exocytosis, by astrocytes as gliotransmitters, are necessary for the induction of t-LTD at LPP- and MPP-GC synapses.

Discussion

In this study, two new forms of t-LTD were seen to be elicited at LPP and MPP synapses onto DG GCs by pairing presynaptic activity with single postsynaptic APs at low frequency (0.2 Hz). Using different approaches, we found that these two forms of t-LTD are expressed presynaptically through a decrease in the glutamate release probability. Interestingly, t-LTD induction at MPP-GC synapses but not at LPP-GC synapses depends on presynaptic NMDARs containing GluN2A subunits. In addition, mGluRs are required for t-LTD at both types of PP-DG synapses, although LPP-GC synapses require mGluR5 activation and MPP-GC synapses require mGluR1 activity. These two forms of presynaptic t-LTD require postsynaptic Ca^{2+} released from postsynaptic intracellular stores and whereas t-LTD at LPP-GC synapses, requires calcineurin phosphatase activity; this is not the case at MPP-GC synapses. These two forms of t-LTD require eCB synthesis and CB₁R activation, and both require astrocytes and astrocyte signalling to promote gliotransmitter release, as well as glutamate that is probably released by astrocytes (**Figure 5**).

Pairing presynaptic activity with single postsynaptic action potentials at low frequency can induce

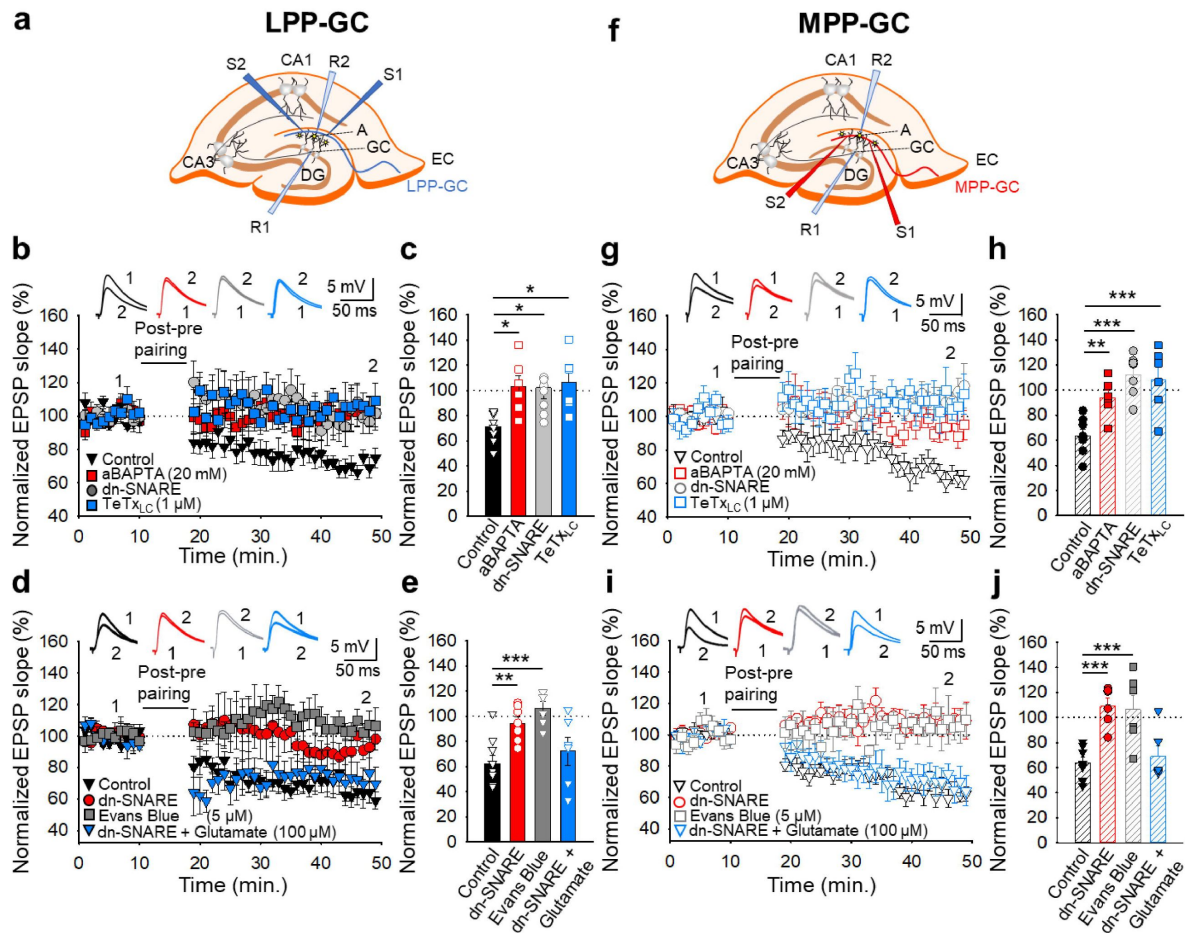


Figure 4.

The t-LTD at lateral and medial perforant pathway-dentate gyrus granule cell synapses requires astrocytes and glutamate.

(a, f) Schemes showing the general experimental set-up: A, astrocyte; CA, *cornu ammonis*; DG, dentate gyrus; EC, entorhinal cortex; GC, granule cell; S1 and S2, stimulating electrodes; R1 and R2, recording electrodes. (b) The t-LTD at LPP-GC synapses requires astrocytes as it was prevented by loading astrocytes with BAPTA (20 mM) and in slices from dnSNARE mice or when astrocytes are treated with the light chain of the tetanus toxin (TeTx_{LC}, 1 μM). The EPSP slopes shown are from control slices (black triangles, n = 7), slices with BAPTA-loaded astrocytes (red squares, n = 6), slices from dnSNARE mice (grey circles, n = 9) and in slices treated with TeTx_{LC} (blue squares, n = 6). The traces show the EPSPs before (1) and 30 min after (2) pairing. (c) Summary of the results. (d) The t-LTD at LPP-GC synapses requires glutamate as it is absent in slices from dnSNARE mice but it is restored by glutamate puffs (100 μM). Note that t-LTD is also absent in slices treated with Evans blue (5 μM). The EPSP slopes shown are from control slices (black triangles, n = 8), slices from dnSNARE mice (red circles, n = 7), slices from dnSNARE mice administered glutamate puffs (blue triangles, n = 6), and slices treated with Evans blue loaded into the astrocytes (grey squares, n = 6). The traces show the EPSPs before (1) and 30 min after (2) pairing. (e) Summary of the results. (g) The t-LTD at MPP-GC synapses requires astrocytes as t-LTD induction at MPP-GC synapses was prevented by loading astrocytes with BAPTA (20 mM), in slices from dnSNARE mice or when astrocytes are treated with the light chain of the tetanus toxin (TeTx_{LC}, 1 μM). The EPSP slopes shown are from control slices (open black triangles, n = 6), slices with BAPTA-loaded astrocytes (open red squares, n = 6), slices from dnSNARE mice (open grey circles, n = 7) and in slices treated with TeTx_{LC} (blue squares, n = 6). The traces show the EPSPs before (1) and 30 min after (2) pairing. (h) Summary of the results. (i) The t-LTD at MPP-GC synapses requires glutamate as it is absent in slices from dnSNARE mice but is restored by glutamate puffs (100 μM). Note that t-LTD is also absent in slices treated with Evans blue (5 μM). The EPSP slopes shown are from control slices (open black triangles, n = 8), slices from dnSNARE mice (open red circles, n = 6) slices from dnSNARE mice administered to glutamate puffs (open blue triangles, n = 6) and slices treated with Evans blue loaded into the astrocytes (grey squares, n = 6). The traces show the EPSPs before (1) and 30 min after (2) pairing. (j) Summary of the results. The error bars indicate the S.E.M.: *p < 0.05, **p < 0.01, ***p < 0.001, One-way ANOVA + Holm-Sidak test.

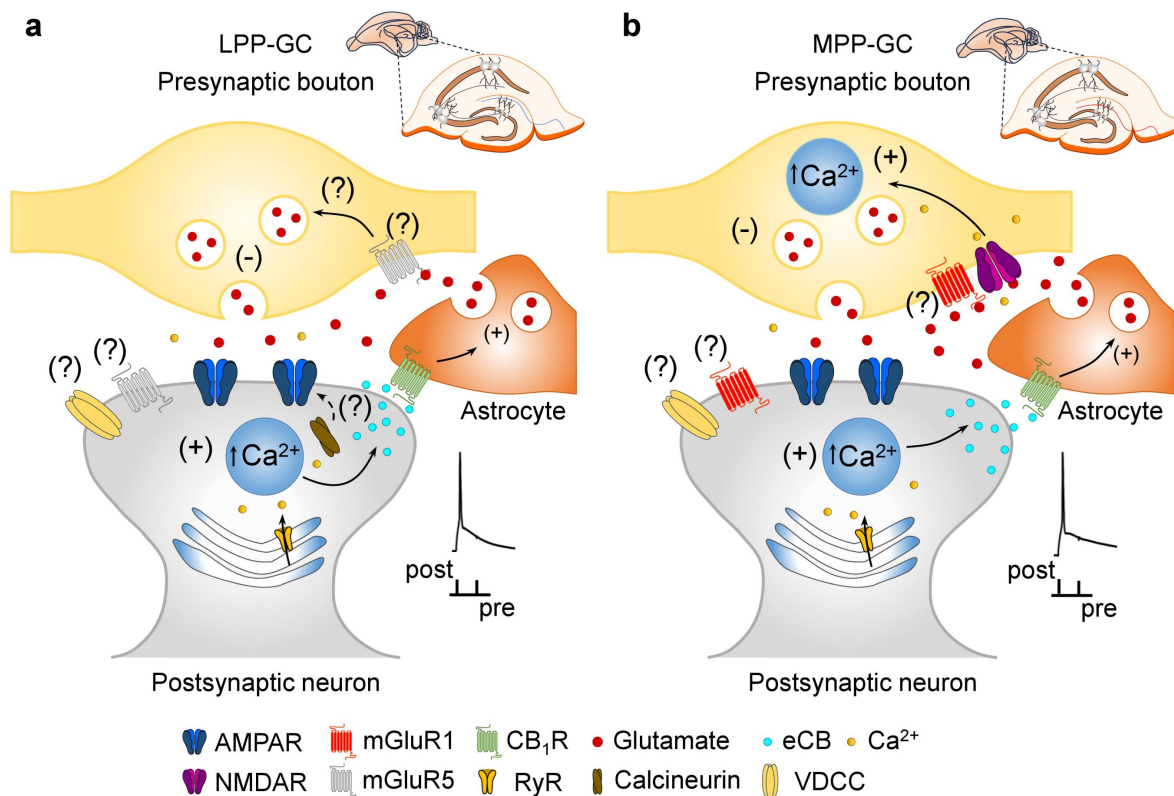


Figure 5.

Model of presynaptic t-LTD at LPP- and MPP-GC synapses.

t-LTD is induced by a post-before-pre, single-spike pairing protocol at P13-21. Postsynaptic action potentials depolarize the postsynaptic membrane that probably permeates Ca²⁺ into the postsynaptic neuron, causing calcineurin activation (at LPP-GC synapses) and Ca²⁺ release from internal stores in a Ca²⁺-dependent Ca²⁺ release manner at both, LPP- and MPP-GC synapses, driving eCBs synthesis and release. The eCB signal leads to the activation of CB₁ receptors, possibly situated in surrounding astrocytes, facilitating glutamate release from astrocytes, which, together with glutamate released from presynaptic neurons, that at LPP-GC synapses possibly activates mGluR5 and presynaptic NMDA receptors at MPP-GC synapses produce a decrease in glutamate release probability in both cases and t-LTD.

presynaptic t-LTD at mouse lateral and medial perforant pathway-dentate gyrus granule cell synapses

While different time intervals between the postsynaptic spike and presynaptic EPSP of a pairing protocol may induce t-LTD (Feldman, 2012 [DOI](#)), it is clear here that pairing EPSPs of 3-5 mV size 100 times with single postsynaptic spikes 18 ms later at 0.2 Hz induces robust LTD at both LPP- and MPP-GC synapses. Interestingly, a similar protocol induces t-LTD at hippocampal CA3-CA1 synapses (Andrade-Talavera et al., 2016 [DOI](#); Pérez-Rodríguez et al., 2019 [DOI](#); Falcón-Moya et al., 2020 [DOI](#)) and at L4-L2/3 synapses of the somatosensory (Rodríguez-Moreno and Paulsen, 2008 [DOI](#); Banerjee et al., 2009 [DOI](#); 2014 [DOI](#); Martínez-Gallego et al., 2022a [DOI](#)), visual (Larsen et al., 2014 [DOI](#)) and cingulate cortices (Hogrefe et al., 2022 [DOI](#)). As such, the results presented here extend the existence of t-LTD to LPP and MPP synapses onto hippocampal DG GCs. Although there are several uncertainties associated with quantal analysis in the CNS (see Brock et al., 2020 [DOI](#)), we used different approaches here to determine the locus of expression of these two forms of t-LTD (failure rate, PPRs, CV, and mEPSP frequency and amplitude analysis), all of which were consistent with presynaptic changes.

Presynaptic t-LTD requires NMDARs at MPP-GC but not at LPP-GC synapses

NMDARs are required for general synaptic plasticity and for STDP, and in different experimental models (cell cultures, rodent brain slices and human neurons) they are required for t-LTD and t-LTP at different synapses in the hippocampus, neocortex, cerebellum and striatum (Bi and Poo, 1998 [DOI](#); Bender et al., 2006 [DOI](#); Nevian and Sakmann, 2006 [DOI](#); Banerjee et al., 2009 [DOI](#), 2014 [DOI](#); Fino et al., 2010 [DOI](#); Verhoog and Mansvelder, 2011 [DOI](#); Kwag and Paulsen, 2012 [DOI](#); Andrade-Talavera et al., 2016 [DOI](#); Sgritta et al., 2017 [DOI](#); Louth et al., 2021 [DOI](#); Martínez-Gallego et al., 2022a [DOI](#)). NMDARs have been implicated in synaptic plasticity at MPP-GC but not LPP-GC synapses through classical protocols (Dahl et al., 1990 [DOI](#); Min et al., 1998 [DOI](#); Beck et al., 2000 [DOI](#); Hayashi et al., 2014 [DOI](#)). Our results are consistent with the presence of NMDARs at MPP-GC synapses, in our case participating in LTD. Indeed, t-LTD is not present at MPP-GC synapses in the presence of D-AP5, and the presence of presynaptic NMDARs at MPP-GC synapses was demonstrated previously by immunogold labelling (Jourdain et al., 2007 [DOI](#); Savtchouk et al., 2019 [DOI](#)). At these synapses t-LTD was also blocked when MK801 was present in the bath but not when it was loaded into the postsynaptic neuron, indicating that the NMDARs necessary for t-LTD are not postsynaptic, as seen in L4-L2/3 synapses of the somatosensory (Bender et al., 2006 [DOI](#); Nevian and Sakmann, 2006 [DOI](#); Rodríguez-Moreno and Paulsen, 2008 [DOI](#); Rodríguez-Moreno et al., 2011 [DOI](#), 2013 [DOI](#)) and visual cortex (Larsen et al., 2014 [DOI](#)). Since the NMDA channel blocker MK801 prevents t-LTD, the NMDARs required for t-LTD appear to be ionotropic, although an additional role for a postsynaptic metabotropic NMDARs cannot be ruled-out (Nabavi et al., 2013 [DOI](#)). Thus, the involvement of NMDARs is one important difference between LPP- and MPP-GC synapses that may account for at least some of the physiological differences described between these two pathways. By using antagonists with different NMDAR subunit specificities, we also found that the NMDARs required for t-LTD at MPP-GC synapses contain GluN2A but not GluN2B or GluN2C/D subunits. NMDARs that enhance glutamate release or that participate in LTP at PP-GC synapses are thought to contain GluN2B subunits in rats as their effect is blocked by ifenprodil (Jourdain et al., 2007 [DOI](#); Savtchouk et al., 2019 [DOI](#)). However, ifenprodil or Ro25698 had no effect on t-LTD here, such that the preNMDARs containing GluN2B subunits that participate in LTP and enhance glutamate release may differ from those participating in t-LTD, and there may even be differences between the species (i.e.: rat and mouse).

Presynaptic t-LTD at lateral and medial perforant pathway-dentate gyrus granule cell synapses requires mGluRs

While both forms of t-LTD described here appear to require group I mGluRs, they require different ones: mGluR5 at LPP-GC synapses and mGluR1 at MPP-GC contacts. Nevertheless, the exact location of these mGluRs is not clear at present, which may represent a further difference between LPP- and MPP-GC synapses, and with important behavioural and physiological consequences.

Postsynaptic Ca^{2+} is required for t-LTD at lateral and medial perforant pathway-dentate gyrus granule cell synapses

Both, t-LTD and t-LTP require postsynaptic Ca^{2+} at neocortical (Bender et al., 2006 [\[1\]](#); Nevian and Sakmann, 2006 [\[2\]](#); Rodríguez-Moreno et al., 2013 [\[3\]](#); Martínez-Gallego et al., 2022a [\[4\]](#)) and hippocampal synapses (Andrade-Talavera et al., 2016 [\[5\]](#); Falcón-Moya et al., 2020 [\[6\]](#); Mateos-Aparicio and Rodríguez-Moreno, 2020 [\[7\]](#); Ingebert et al., 2021). Moreover, like other forms of t-LTD, that at LPP- and MPP-GC synapses requires a rise in Ca^{2+} in the postsynaptic cell due to Ca^{2+} release from internal stores, as also seen in other situations of hippocampal, neocortical and cerebellar t-LTD (Bender et al., 2006 [\[1\]](#); Nevian and Sakmann, 2006 [\[2\]](#); Andrade-Talavera et al., 2016 [\[5\]](#); Sgritta et al., 2017 [\[8\]](#); Falcón-Moya et al., 2020 [\[6\]](#); Martínez-Gallego et al., 2022a [\[4\]](#)). This is similar to the LTD induced by LFS in DG GCs (Wang et al., 1997 [\[9\]](#)). Our data also implies an involvement of calcineurin in t-LTD induction at LPP-GC synapses but not at MPP-GC synapses, an additional striking difference between these two types of synapses. Protein phosphatases, including calcineurin, may be required for different forms of LTD, both in the hippocampus (Mulkey et al., 1994 [\[10\]](#)) and neocortex (Torii et al., 1995 [\[11\]](#)). Future experiments will determine the exact mechanism by which calcineurin mediates t-LTD at LPP but not at MPP synapses. What is clear for the moment is that while the requirements for Ca^{2+} seem to be similar at both LPP-GC and MPP-GC synapses, the Ca^{2+} -dependent protein phosphatase calcineurin is only involved in t-LTD at LPP-GC synapses. Significantly, the functional consequences of this difference remain to be determined.

Endocannabinoid signalling at lateral and medial perforant pathway-dentate gyrus granule cell synapses is required for t-LTD

Our results indicate that eCBs and CB_1Rs are involved in the induction of these two different forms of t-LTD, in agreement with previous field recordings showing that the eCB system is involved in synaptic plasticity at PP-DG synapses (Wang et al., 2016 [\[12\]](#); Peñasco et al., 2019 [\[13\]](#); Fontaine et al., 2020 [\[14\]](#)). Here we did not elucidate the location of the CB_1Rs mediating t-LTD, such that further work will be required to address this question. However, the demonstration that astrocytes are required for t-LTD and that WIN55, 212-2 puffs over astrocytes induce LTD are suggestive that astrocytic CB_1Rs might be involved.

Astrocyte signalling and glutamate release at lateral and medial perforant pathway-dentate gyrus granule cell synapses is required for t-LTD

Through four different approaches (introducing BAPTA, tetanus toxin light chain or Evans blue into astrocytes and using mutant dnSNARE mice) we demonstrated the involvement of astrocytes in presynaptic t-LTD, both at LPP- and MPP-GC synapses. Astrocytes participate in synaptic potentiation at PP-GC synapses (Jourdain et al., 2007 [\[15\]](#)), although little is known about the contribution of astrocytes to LTD. Thus, this is the first demonstration of the participation of astrocytes in LTD at these synapses. Interestingly, and as found in the somatosensory cortex (Min and Nevian, 2012 [\[16\]](#)), glutamate is required to mediate t-LTD induction at both types of PP-GC

synapses, and in fact, t-LTD is impaired when astrocytes were loaded with Evans blue and recovered by the local application of glutamate at both LPP- and MPP-GC synapses in slices from dnSNARE mice that do not develop t-LTD otherwise. These findings agree with previous studies showing that astrocytes can release glutamate that reaches the NMDARs located in the presynaptic boutons at PP-GC synapses (Bezzi et al., 2004 [DOI](#); Jourdain et al., 2007 [DOI](#)), influencing synaptic transmission at MPP-GC synapses (Savtchouk et al., 2019 [DOI](#)). It is interesting to note that the presynaptic targets of the glutamate, at least in part released by astrocytes, are not the same at LPP- and MPP-GC synapses, as they are mGluRs in the case of LPP-GC synapses, and both NMDARs and mGluRs in the case of MPP-GC synapses. While it is possible that glutamate from the presynaptic neuron also activates preNMDARs and mGluRs, the amount of glutamate released by presynaptic terminals may be insufficient to drive t-LTD, and hence, glutamate released from astrocytes may be required for t-LTD.

What is the physiological role of these forms of plasticity?

For the moment, the exact role of STDP in the perforant cortex or in the hippocampus is not known, and more work is necessary to specifically determine functions for t-LTP and t-LTD. STDP seems a good candidate to mediate spatial learning in the hippocampus (Bush et al., 2010) and the possible role of t-LTP and t-LTD in forms of learning involving the hippocampus and the perforant cortex will be addressed in future studies. Our studies were performed in developing P13-P21 animals indicating its relevance during maturation. The functions of t-LTP and t-LTD during development are most probably related to the refinement of synaptic connections and remodelling of neuronal circuits (Andrade-Talavera et al., 2023). As a Hebbian learning rule, t-LTP should occur when the spike order is pre-before-post, strengthening those connections in which the presynaptic neuron takes part in firing the postsynaptic cell, as predicted by Hebb, whereas t-LTD occurs when the spike order is reversed, so that non-causal spiking weakens the connections involved, possibly as a first step in the elimination of those connections during development as has been suggested (see Caporale and Dan, 2008). Indeed, further studies will be necessary to determine the true influence of STDP in the perforant cortex and in the hippocampus, and the specific developmental role of t-LTD and t-LTP in these circuits.

It is interesting to note that with these new results it is clear that both forms of t-LTD may require glutamate from astrocytes. Presynaptic plasticity may involve structural changes and may change the short-term properties of neurotransmitter release, participating in circuit computations, and changing the excitatory/inhibitory balance or sensory adaptations (Monday et al., 2018). Why some synapses, like L4-L2/3 synapses at somatosensory cortex (and as observed in the hippocampus at CA3-CA1 synapses), show pre- and/or post-synaptic plasticity requires further study. Interestingly, in the synapses studied here, t-LTD of LPP-GC and MPP-GC synapses have different requirements, indicating that the presynaptic expression of plasticity is fundamental for the correct functioning of brain circuits and it is possible that different presynaptic forms of t-LTD from synapses onto the same neuron as pre- and postsynaptic forms of LTD (Banerjee et al., 2009 [DOI](#), 2014 [DOI](#)) are regulated differently. Presynaptically expressed forms of t-LTD may in fact control the trial-to-trial reliability, and they may induce a larger change in signal-to-noise ratio than postsynaptic changes alone, as described in auditory cortex (Froemke et al., 2013). Broad-ranging input representations from the EC transmitted via the PP are sparse, and the DG processes this information and converts it into a less well-correlated output to become less similar and more specific (Borzello et al., 2023 [DOI](#)). Whether t-LTD at LPP and MPP synapses described here are involved in this important process require further research and the different cellular and molecular mechanisms involved in t-LTD at LPP- and MPP-GC synapses may account for some of the difference in their physiological influence. Notwithstanding this, the potential behavioural influence of the presynaptic forms of LTD studied here is still an emerging issue of particular interest in the near future.

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Author contributions

I. M-G. and H. C-C. performed the experiments and analysed the results. A. R-M. designed and supervised the research, and wrote the paper.

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Reviewer #1 (Public review):

Summary:

The study characterized the cellular and molecular mechanisms of spike timing-dependent long-term depression (t-LTD) at the synapses between excitatory afferents from lateral (LPP) and medial (MPP) perforant pathways to granule cells (GC) of the dentate gyrus (DG) in mice.

Strengths:

The electrophysiological experiments are thorough. The experiments are systematically reported and support the conclusions drawn. This study extends current knowledge by elucidating additional plasticity mechanisms at PP-GC synapses, complementing existing literature.

Comments on the revised version:

The revised study introduces two additional approaches to confirm astrocyte involvement in t-LTD: loading astrocytes with tetanus toxin light chain to inhibit exocytosis, and using Evans blue to block vesicular glutamate uptake. These new findings further reinforce the conclusion that t-LTD relies on Ca^{2+} -dependent glutamate exocytosis from astrocytes.

<https://doi.org/10.7554/eLife.98031.2.sa3>

Reviewer #2 (Public review):**Summary:**

This work reports the existence of spike timing-dependent long-term depression (t-LTD) of excitatory synaptic strength at two synapses of the dentate gyrus granule cell, which are differently connected to the entorhinal cortex via either the lateral or medial perforant pathways (LPP or MPP, respectively). Using patch-clamp electrophysiological recording of tLTD in combination with either pharmacology or a genetically modified mouse model, they provide information on the differences in the molecular mechanism underlying this t-LTD at the two synapses.

Strengths:

The two synapses analyzed in this study have been understudied. This new data thus provides interesting new information on a plasticity process at these synapses, and the authors demonstrate subtle differences in the underlying molecular mechanisms at play. Experiments are in general well controlled and provide robust data that are properly interpreted.

The data provided to demonstrate that glutamate release from astrocytes is necessary for these plasticity mechanisms are strong. This is particularly interesting as another example of how astrocytes regulate synapse plasticity.

Weaknesses:

This work was performed at young synapses and the highlighted mechanisms are therefore pertinent to this age, as acknowledged by the authors. We currently don't know if these mechanisms are still at play at the adult synapse.

Significance:

While this is the first report of t-LTD at these synapses, this plasticity process has been mechanistically well investigated at other synapses in the hippocampus and in the cortex. Nevertheless, this new data suggests that mechanistic differences in the induction of t-LTD at these two DG synapses could contribute to the differences in the physiological influence of the LPP and MPP pathways.

<https://doi.org/10.7554/eLife.98031.2.sa2>

Reviewer #3 (Public review):

Coatl et al. investigated the mechanisms of synaptic plasticity of two important hippocampal synapses, the excitatory afferents from lateral and medial perforant pathways (LPP and MPP, respectively) of the entorhinal cortex (EC) connecting to granule cells of the hippocampal dentate gyrus (DG). They find that these two different EC-DG synaptic connections in mice show a presynaptically expressed form of long-term depression (LTD) requiring postsynaptic calcium, eCB synthesis, CB1R activation, astrocyte activity, and metabotropic glutamate receptor activation. Interestingly, LTD at MPP-GC synapses requires ionotropic NMDAR activation whereas LTD at LPP-GC synapse is NMDAR independent. Thus, they discovered two novel forms of t-LTD that require astrocytes at EC-GC synapses. Although plasticity of EC-DG granule cell (GC) synapses has been studied using classical protocols, These are the first analyses of the synaptic plasticity induced by spike timing dependent protocols at these synapses. Interestingly, the data also indicate that t-LTD at each type of synapse require different group I mGluRs, with LPP-GC synapses dependent on mGluR5 and MPP-GC t-LTD requiring mGluR1.

The authors performed a detailed analysis of the coefficient of variation of the EPSP slopes, miniature responses and different approaches (failure rate, PPRs, CV, and mEPSP frequency and amplitude analysis) they demonstrate a decrease in the probability of neurotransmitter release and a presynaptic locus for these two forms of LTD at both types of synapses. By using elegant electrophysiological experiments and taking the advantage of the conditional dominant-negative (dn) SNARE mice in which doxycycline administration blocks exocytosis and impairs vesicle release by astrocytes, they demonstrate that both LTD forms require the release of gliotransmitters from astrocytes. These data add in an interesting way to the ongoing discussion on whether LTD induced by STDP participates in refining synapses potentially weakening excitatory synapses under the control of different astrocytic networks. The conclusions of this paper are well supported by data.

<https://doi.org/10.7554/eLife.98031.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public Review):

Summary:

The study characterized the cellular and molecular mechanisms of spike timing-dependent long-term depression (t-LTD) at the synapses between excitatory afferents from lateral (LPP) and medial (MPP) perforant pathways to granule cells (GC) of the dentate gyrus (DG) in mice.

Strengths:

The electrophysiological experiments are thorough. The experiments are systematically reported and support the conclusions drawn.

This study extends current knowledge by elucidating additional plasticity mechanisms at PP-GC synapses, complementing existing literature.

We thank the reviewer for the positive assessment of our work and the constructive suggestions to improve the manuscript.

Weaknesses:

To more conclusively define the pivotal role of astrocytes in modulating t-LTD at MPP and LPP GC synapses through SNARE protein-dependent glutamate release, as posited in this study, the authors could adopt additional methods, such as alternative mouse models designed to regulate SNARE-dependent exocytosis, as well as optogenetic or chemogenetic strategies for precise astrocyte manipulation during t-LTD induction. This would provide more direct evidence of the influence of astrocytic activity on synaptic plasticity.

We thank the reviewer for the suggestion. As stated in the manuscript and in figure 4, we already used two different approaches (aBAPTA to interfere with astrocyte calcium signalling and dnSNARE mice (that have vesicular release impaired) to determine the involvement of astrocytes in the discovered forms of LTD, and both approaches clearly indicated the requirement of astrocytes for t-LTD. In BAPTA-treated astrocytes and in dnSNARE mice, t-LTD was prevented. Notwithstanding this, and as suggested by the reviewer, we used two additional approaches to confirm astrocyte participation. We loaded astrocytes with the light chain of the tetanus toxin (TeTxLC), which is known to block exocytosis by cleaving the vesicle-associated membrane protein, an important part of the SNARE complex (Schiavo et al., 1992, Nature 359, 832-835). In this experimental condition, we observed a clear lack of t-LTD at both (lateral and medial) pathways, thus confirming the requirement of astrocytes and the SNARE complex and vesicular release for both types of t-LTD. In addition, to gain more insight into the fact that glutamate is released by astrocytes, we blocked glutamate release from astrocytes by loading the astrocytes with Evans blue, known to interfere with glutamate uptake into vesicles as it inhibits the vesicular glutamate transporter (VGLUT). In this experimental condition, again t-LTD was prevented, indicating that t-LTD requires Ca²⁺-dependent exocytosis of glutamate from astrocytes.

Reviewer #2 (Public Review):

Summary:

This work reports the existence of spike timing-dependent long-term depression (t-LTD) of excitatory synaptic strength at two synapses of the dentate gyrus granule cell, which are differently connected to the entorhinal cortex via either the lateral or medial perforant pathways (LPP or MPP, respectively). Using patch-clamp electrophysiological recording of tLTD in combination with either pharmacology or a genetically modified mouse model, they provide information on the differences in the molecular mechanism underlying this t-LTD at the two synapses.

Strengths:

The two synapses analyzed in this study have been understudied. This new data thus provides interesting new information on a plasticity process at these synapses, and the authors demonstrate subtle differences in the underlying molecular mechanisms at play. Experiments are in general well controlled and provide robust data that are properly interpreted.

We thank the reviewer for the positive assessment of our work and the constructive suggestions to improve the manuscript.

Weaknesses:

- *Caution should be taken in the interpretation of the results to extrapolate to adult brain as the data were obtained in P13-21 days old mice, a period during which synapses are still maturing and highly plastic.*

We thank the reviewer for noticing this. In fact, our experiments were intentionally performed in young animals (P13-21), just knowing that this is a critical period of plasticity. We indicate that in the methods, results, and discussion (where we discuss that in some detail) sections.

- *In experiments where the drug FK506 or thapsigargin are loaded intracellularly, the concentrations used are as high as for extracellular application. Could there be an error of interpretation when stating that the targeted actors are necessarily in the post-synaptic neuron? Is it not possible for the drug to diffuse out of the cell as it is evident that it can enter the cell when applied extracellularly?*

We thank the reviewer for rising this point. While it would be possible that these compounds cross the cell membranes, to do it and to pass to other cells, this would, in principle, require a relatively long time to occur. Additionally, to have any effect, the same concentration or a relatively high concentration of that we put into the pipette has to reach other cells. Furthermore, even if a compound is able to cross a cell membrane during the duration of an experiment, after this, it may be exposed to the extracellular fluid where will be diluted and most probably washed out. For all these reasons, we do not see this very plausible. Notwithstanding this, and as suggested, we have repeated the experiments using lower concentrations of thapsigargin (1 μ M) and FK506 (1 μ M), and have obtained the same results. These data are now included in the figure 3 and in the text.

- *The experiments implicating glutamate release from astrocytes in t-LTD would require additional controls to better support the conclusions made by the authors. As the data stand, it is not clear, how the authors identified astrocytes to load BAPTA and if dnSNARE expression in astrocytes does not indirectly perturb glutamate release in neurons.*

We thank the reviewer for rising this point. We now indicate how astrocytes have been identified to load BAPTA. We reply to this in detail in the “Recommendations for the authors” from reviewer 2.

Significance:

While this is the first report of t-LTD at these synapses, this plasticity process has been mechanistically well investigated at other synapses in the hippocampus and in the cortex. Nevertheless, this new data suggests that mechanistic differences in the induction of t-LTD at these two DG synapses could contribute to the differences in the physiological influence of the LPP and MPP pathways.

Reviewer #3 (Public Review):

Coatl et al. investigated the mechanisms of synaptic plasticity of two important hippocampal synapses, the excitatory afferents from lateral and medial perforant pathways (LPP and MPP, respectively) of the entorhinal cortex (EC) connecting to granule cells of the hippocampal dentate gyrus (DG). They find that these two different EC-DG synaptic connections in mice show a presynaptically expressed form of long-term depression (LTD) requiring postsynaptic calcium, eCB synthesis, CB1R activation, astrocyte activity, and metabotropic glutamate receptor activation. Interestingly, LTD at

MPP-GC synapses requires ionotropic NMDAR activation whereas LTD at LPP-GC synapse is NMDAR independent. Thus, they discovered two novel forms of t-LTD that require astrocytes at EC-GC synapses. Although plasticity of EC-DG granule cell (GC) synapses has been studied using classical protocols, These are the first analysis of the synaptic plasticity induced by spike timing dependent protocols at these synapses. Interestingly, the data also indicate that t-LTD at each type of synapse require different group I mGluRs, with LPP-GC synapses dependent on mGluR5 and MPP-GC t-LTD requiring mGluR1.

The authors performed a detailed analysis of the coefficient of variation of the EPSP slopes, miniature responses and different approaches (failure rate, PPRs, CV, and mEPSP frequency and amplitude analysis) they demonstrate a decrease in the probability of neurotransmitter release and a presynaptic locus for these two forms of LTD at both types of synapses. By using elegant electrophysiological experiments and taking advantage of the conditional dominant-negative (dn) SNARE mice in which doxycycline administration blocks exocytosis and impairs vesicle release by astrocytes, they demonstrate that both LTD forms require the release of gliotransmitters from astrocytes. These data add in an interesting way to the ongoing discussion on whether LTD induced by STDP participates in refining synapses potentially weakening excitatory synapses under the control of different astrocytic networks. The conclusions of this paper are mostly well supported by data, but some aspects the results must be clarified and extended.

We thank the reviewer for the positive assessment of our work and the constructive suggestions to improve the manuscript.

(1) It should be clarified whether present results are obtained with or without the functional inhibitory synapse activation. It is not clear if GABAergic synapses are blocked or not. If GABAergic synapses are not blocked authors must discuss whether the LTD of the EPSPs is due to a decrease in glutamatergic receptor activation or an increase in GABAergic receptor activation. Moreover, it should be recommended to analyze not only the EPSPs but also the EPSCs to address whether the decrease in synaptic transmission is caused by a decrease in the input resistance or by a decrease in the space constant (λ).

We thank the reviewer for rising these points. GABAergic inhibition was not blocked in our experiments. The observed forms of t-LTD seem to be due to a decrease in glutamate release probability as indicated in the manuscript, mediated by the mechanism we uncover and describe here. To determine and clarify whether GABA receptors have any role in these forms of t-LTD, we repeated the experiments in the presence of the GABAA and GABAB receptors antagonists bicuculline and SCH50911, respectively. Blocking GABA receptors do not prevent or affect t-LTD at LPP- or MPP-GC synapses, that is still present and with a similar magnitude that controls. These results indicating that these receptors are not involved in these forms of t-LTD. These results are now included in the text in the results section (page 8) and as a new figure S1. In our experiments, no changes in input resistance or space constant were observed, and importantly, no changes were observed in the amplitude/slopes of EPSP in the control pathway that does not undergo plasticity protocol that we routinely use in our experiments.

(2) Authors show that Thapsigargin loaded in the postsynaptic neuron prevents the induction of LTD at both synapses. Analyzing the effects of blocking postsynaptic IP3Rs (Heparin in the patch pipette) and Ryanodine receptors (Ruthenium red in the patch pipette) is recommended for a deeper analysis of the mechanism implicated in the induction of this novel forms of LTD in the hippocampus.

We thank the reviewer for this suggestion. We repeated the experiments loading the postsynaptic cell with heparin and ruthenium red using the patch pipette. In these experimental conditions, we observed that t-LTD was not affected by the heparin treatment (discharging a role of IP3Rs), but that it was prevented by the ruthenium red treatment (indicating the requirement of ryanodine receptors). We include now this data in the text (page 12) and in the Figure 3a, b, e, f.

(3) Authors nicely demonstrate that CB1R activation is required in these forms of LTD by blocking CB1Rs with AM251, however an interesting unanswered question is whether CB1R activation is sufficient to induce this synaptic plasticity. This reviewer suggests studying whether applying puffs of the CB1R agonist, WIN 55,212-2, could induce these forms of LTD.

We thank the reviewer for this suggestion. We repeated the experiments adding WIN55, 212-2 as suggested. The activation of CB1R by puffs of the agonist WIN 55, 212-2 to the astrocyte, directly induced LTD at both LPP- and MPP-GC synapses. We include now this data in the text (page 14) and in the Figure 3c, d, g, h.

(4) Finally, adding a last figure with a cartoon summarizing the proposed model of action in these novel forms of LTD would add a positive value and would help the reading of the manuscript, especially in those aspects related with the discussion of the results.

We thank the reviewer for the suggestion. We include now a figure showing the proposed mechanisms (Figure 5).

The extension of these results would improve the manuscript, which provides interesting results showing two novel forms of presynaptic t-LTD in the brain synapses with different action mechanisms probably implicated in the different aspects of information processing.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

There are just a few aspects that could be clarified to bolster the authors' conclusions.

The author centered the conclusion of their study on the role of astrocytic activity in regulating these two forms of plasticity (see title). To strengthen the evidence that astrocytes are key regulators of t-LTD at MPP and LPP GC synapses by regulating SNARE protein-dependent glutamate release, additional complementary approaches should be considered, such as other mouse models enabling the control of SNARE-dependent exocytosis and/or optogenetic/chemogenetic tools to selectively manipulate astrocytes during the induction of t-LTD, thereby directly assessing the impact of astrocytic activity on synaptic plasticity. Implementing calcium imaging or glutamate sensors to visualize the dynamics of astrocytic calcium signaling and glutamate release during t-LTD could be also considered.

We thank the reviewer for the suggestion. As stated in the manuscript and in figure 4, we already used two different approaches (aBAPTA to interfere with astrocyte calcium signalling and dnSNARE mice (that have vesicular release impaired) to determine the involvement of astrocytes in the discovered forms of LTD, and both approaches clearly indicated the requirement of astrocytes for t-LTD. In BAPTA-treated astrocytes and in dnSNARE, t-LTD was prevented. Notwithstanding this, and as suggested by the reviewer, we used two additional approaches to confirm astrocytes participation. We loaded astrocytes with the light chain of the tetanus toxin (TeTxLC), which is known to block exocytosis by cleaving the vesicle-associated membrane protein, an important part of the SNARE complex (Schiavo et al., 1992, Nature 359, 832-835). In this experimental condition, we observed a clear lack of t-LTD at both

(lateral and medial) pathways, thus confirming the requirement of astrocytes and the SNARE complex and vesicular release for both types of t-LTD. In addition, to gain more insight into the fact that glutamate is released by astrocytes, we blocked glutamate release from astrocytes by loading the astrocytes with Evans blue, known to interfere with glutamate uptake into vesicles as it inhibits the vesicular glutamate transporter (VGLUT). In this experimental condition, again t-LTD was prevented, indicating that t-LTD requires Ca²⁺-dependent exocytosis of glutamate from astrocytes. This information is now included in the text, pages 14 and 15 and in figure 4.

- *How were astrocytes identified to be loaded with BAPTA? The author should clarify this methodological aspect and provide confocal images of patched astrocytes situated 50-100 um from the recorded neuron.*

We thank the reviewer for the comment. We include now this information in the Methods section (page 6) and in figure S3. Astrocytes were identified by their rounded morphology under differential interference contrast microscopy, and were characterized by low membrane potential, low membrane resistance and passive responses (they do not show action potentials) to both negative and positive current injection.

- *Please provide confocal images of EGFP expression in the DG astrocytes of dnSNARE mice both on and off Dox, to verify transgene expression in astrocytes*

We thank the reviewer for this suggestion. We now include an image of GFP expression in the DG astrocytes of off Dox dnSNARE mice. We did not provide the animals with doxycycline since birth and thus the gene was constantly expressed. We now show this image in Fig. S3. All the pups and mice are not DOX fed, meaning that the transgenes are continuously being expressed and therefore the exocytosis should be blocked in astrocytes.

Minor points:

Lines 250-253: It is mentioned that TTX is added at baseline, washed out for the t-LTD experiment, and then reapplied post t-LTD. I suggest clarifying the timing and rationale for this application for a broad audience.

We thank the reviewer for the suggestion. We now include some information related to the timing and rationale of the experiment phases (page 9).

The discussion is quite detailed and provides a comprehensive overview of the study's findings. To enhance clarity and impact, the authors might consider to,

- *add subheadings and bullet points for key findings. This will improve readability.*
- *this section could benefit from streamlining to avoid redundancy.*
- *some sentences could be made more concise without losing meaning.*

We thank the reviewer for these suggestions. We now include subheadings in the discussion section to improve readability and have made some sentences more concise and simple without losing meaning.

*In figure legends, consistency with capitalization should be maintained, for example in the statistical significance notation, ***P < 0.001" or ***p < 0.001")*

We now include $p < 0.001$ in the figure legend 4 for consistency.

Reviewer #2 (Recommendations For The Authors):

Major:

- *All results were obtained in young still quite immature synapses. To strengthen the significance of the findings, the authors could repeat some of the main experiments in adult mice (8 weeks and beyond). If not, they should state clearly that these mechanisms were only evidenced in early post-natal conditions.*

We thank the reviewer for noticing this. In fact, our experiments were intentionally performed in young animals (P13-21), just knowing that this is a critical period of plasticity. As the reviewer suggests, we indicate that in the methods (page 5), results (page 8), and discussion (page 19) (where we discuss that in some detail) sections.

- *Lines 246-249 and fig 1f,p: Authors need to perform a statistical test on these two graphs to support their claim that 'A plot of CV-2 versus the change in the mean evoked EPSP 246 slope (M) before and after t-LTD mainly yielded points below the diagonal line at LPP-GC and MPP-GC synapses'.*

That could not be clear in the previous version. We observed an error in the points (with some points missing) of one of the graphs that we have corrected. In addition, and as suggested by the reviewer we performed a regression analysis that confirms the conclusions stated. This is now included in the text (page 9). Thus, we have added information about mean values $\pm$ SEM in the text and the linear regression of the data for LPP-GC (Mean = 0.607 ± 0.054 vs $1/CV2 = 0.439 \pm 0.096$, $R^2 = 0.337$; $n = 14$) and MPP-GC synapses (Mean = 0.596 ± 0.056 vs $1/CV2 = 0.461 \pm 0.090$, $R^2 = 0.168$; $n = 13$), respectively. Data yielded on the dotted horizontal line, $1/CV2 = 1$, indicates no change in the probability of release, in contrast, data yielded below the dotted diagonal line is suggestive of a change in the probability of release parameters (for review, see Brock et al., 2020, Front Synaptic Neurosci 12, 11).

- *We are not sure that the experiment with the MK801 provided in the patch pipet can be interpreted correctly (Figure 2 a,b and e,f). How sure are the authors that, when applying MK801 in the patch pipet, it can reach its binding site within the pore? The concentration of MK801 is also very high (500 microM) and used at the same concentration extracellularly and intracellularly. Why did the authors not use lower concentration when applied intracellularly?*

We thank the reviewer for rising this point. MK801 in the pipette is reaching the pore when loaded postsynaptically as when we record NMDA currents from postsynaptic neurons loaded with MK801, these currents are blocked. We include now a control experiment showing the effect of postsynaptic MK801 on NMDA current in the text (page 10). NMDA currents has been recorded at +40 mV, blocking AMPAR and GABAR with NBQX and bicuculline. Related to the concentration, it has been described that the affinity from the internal site is much lower (several orders of magnitude) than from the extracellular side (Sun et al., 2018 Neuropharmacology, 143, 122-129) and the concentrations used have been extensively used in previous studies. It is clear that the concentrations used in the present work blocked NMDAR currents but did not prevent LTD.

- *Linked to the point above, for the intracellular application of FK506 and thapsigargin, the concentrations used extracellularly and intracellularly are identical. The authors could have used lower concentrations for the intracellular*

application. Also, how can they be sure of the correct interpretation of these data as the drug essentially reaching a post-synaptic target when applied intracellularly? If the drug can enter the neuron, why could it not diffuse out of the neuron especially when loaded at a high concentration? Maybe using a lower concentration when applied intracellularly could at least partially address this issue.

It is evident that it can enter the cell when applied extracellularly?

We thank the reviewer for rising this point. While it would be possible that these compound cross the cell membranes, to do it and to pass to other cells, this would, in principle, require a relatively long time to occur. Additionally, to have any effect, the same concentration or a relatively high concentration of that we put into the pipette has to reach other cells. Furthermore, even if a compound is able to cross a cell membrane during the duration of an experiment, after this, it may be exposed to the extracellular fluid where it will be diluted and most probably washed out. For all these reasons, we do not see this very plausible. Notwithstanding this, we have repeated the experiments using lower concentrations of thapsigargin (1 μ M) and FK506 (1 μ M) and have obtained the same results. These data are now included in the figure 3 and the numbers in the text have been updated (pages 12-13).

- The data supporting the possibility of glutamate release by astrocytes as a main source of glutamate to promote t-LTD needs to be strengthened. In experiment Figure a-h, it is not clear how the authors recognize astrocytes to patch. No details are provided in the methods or in the main text. If we understand correctly, it is only by performing a current steps protocol to ensure that the patched cell did not produce action potentials. If this was the case, the authors need to be more specific and provide details of this protocol. More importantly, the one trace that was provided in Figures 4a and 4f suggests, albeit by a rough estimation that we made with a ruler, that the highest current step only depolarized the cell to about -40 mV. This is not sufficient to ensure that the recorded cell is not a neuron. The authors should increase their steps to high depolarizing currents to ensure that the patched cell is not a neuron. Better yet, they should load the cell with an dye to process the slice after the electrophysiological recording for immunohistochemistry to ensure that it was indeed an astrocyte. Alternatively, they can try to aspirate the cell content at the end of the recording to perform a qPCR for astrocyte markers eg. GFAP.*

We thank the reviewer for the comment. We include now information regarding how astrocytes were identified (also raised by reviewer 1) in the Methods section (page 6) and in figure S3. Astrocytes were identified by their rounded morphology under differential interference contrast microscopy, eGFP fluorescence (astrocytes from dnSNARE mice), and were characterized by low membrane potential, low membrane resistance and passive responses (they do not show action potentials) to both negative and positive current injection.

We agree with the reviewer that in figure 4a and 4f, the step protocol might not be completely clear. For this, we revised that and now include in a clearer way that we applied pulses that depolarized astrocytes beyond -20 mV, with no action potentials found at any point. We also include now this in figure S3.

- Related to the point above, the use of the model expressing dnSNARE in astrocytes is elegant. Yet, to really interpret the data obtained in these slices as a lack of vesicle release (and most importantly glutamate) we think that the authors should ensure that glutamate release from nearby neurons is not impacted. They could patch nearby neurons in dnSNARE slices and test PPR or synaptic fatigue*

when stimulating either the LPP or MPP. The authors should avoid overinterpretation of these results. As it stands, it is not evident that dnSNARE expression does not perturb other mechanisms within the astrocyte that in turn perturb pre-synaptic glutamate release. Adding back glutamate as puffs does not help to disentangle this issue.

To gain more insight into the fact that glutamate is released by astrocytes we blocked glutamate release from astrocytes by loading the astrocytes with Evans blue, known to interfere with glutamate uptake into vesicles as it inhibits the vesicular glutamate transporter (VGLUT). In this experimental condition, as indicated above, t-LTD was prevented, indicating that t-LTD requires Ca²⁺-dependent exocytosis of glutamate from astrocytes. This is included in the text (page 15) and in figure 4d,e, i, j.

In addition, we loaded astrocytes with the light chain of the tetanus toxin (TeTxLC) which is known to block exocytosis by cleaving the vesicle-associated membrane protein, an important part of the SNARE complex (Schiavo et al., 1992, Nature 359, 832-835). In this experimental condition, we observed a clear lack of t-LTD at both (lateral and medial) pathways, thus confirming the requirement of astrocytes and the SNARE complex and vesicular release for both types of t-LTD. These data indicate that t-LTD requires Ca²⁺-dependent exocytosis of glutamate from astrocytes. This information is now included in the text, page 14 and in figure 4.

Minor points:

- line 107, did the authors mean t-LTP and t-LTD? we don't understand STDP mentioned here.*

We meant to say t-LTP. This is now corrected.

- line 108: should STDP be replaced by t-LTD as the authors only focused on this plasticity mechanism.*

We agree, we indicate now t-LTD.

- line 131-132 : it is not clear when the animals were fed with doxycycline. If it was from birth, then the 'not' should be removed. Otherwise the authors should clearly state when the doxycycline was provided.*

DOX was not provided and that means that the transgene was continuously expressed and therefore the exocytosis should be blocked in astrocytes. We express that clearer in page 5, methods section.

- line 223 : which hippocampal synapses? needs to be stated*

As suggested this is now included in the text as for cortical synapses. Synapses are Schaffer collaterals SC-CA1 for hippocampus and layer L4-L2/3 for cortical synapses (page 8).

- line 273: what do the authors mean when writing 'from'? We don't understand the data provided on this line.*

We thank the reviewer for noticing this. That refers to the amplitude of NMDAR-mediated currents average before and after D-AP5 or MK801. We express this now in a clearer way (page 10, from 57±8 pA to 6±5 pA).

- line 286 : why do the authors point out work on GluN2B and GluN3A only here when they first investigate GluN2A contribution to t-LTD? what about previous data on GluN2A?

We have now expressed this in a different way to make it clear. We wanted to indicate that the available data for presynaptic NMDAR at MPP-GC synapses has been indicated to contain GluN2B and GluN3A subunits and to our knowledge, no data indicate that they contain GluN2A subunits.

- line 428 : what do the authors mean by 'not least' ?

This is a typo and we have removed that from the text.

Reviewer #3 (Recommendations For The Authors):

My only suggestion for improving data presentation in the manuscript would be to split some figures of the paper. In my opinion, the figures are too dense and therefore difficult to follow for the broad audience of eLife readers. In addition, a real image of the recorded dentate granule cells in the slice showing also the location of the real stimulation electrodes would significantly improve the presentation of Figure 1.

We thank the reviewer for the suggestion, but we would prefer to let the figures as they are organized, as while we agree in some cases they are a bit big, in this way it is easier to compare lateral and medial pathways. For this, it could be better to let information regarding the two pathways in the same figure. Nevertheless, we try now to make figures clearer to use a columnar organization of the figures for each pathway what we think, would make easier to compare pathways. As the reviewer suggests we include now a real image of the recorded dentate granule cells in the slice showing also the location of the real stimulation electrodes in Figure 1, that we agree will improve the presentation of this figure and thank the reviewer for the suggestion.

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