LTP Induction Boosts Glutamate Spillover by Driving Withdrawal of Perisynaptic Astroglia

Highlights

- Induction of synaptic LTP prompts withdrawal of perisynaptic astroglia
- The underlying mechanisms involve NKCC1 transporter and cofilin
- Reduced synaptic astroglial coverage boosts extrasynaptic glutamate escape
- LTP induction thus enhances NMDAR-dependent inter-synaptic cross-talk

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In Brief

Central synapses are often surrounded by thin astroglial processes that confine chemical neurotransmission to the synaptic cleft. Henneberger et al. find that memory trace formation at synaptic connections prompts withdrawal of these processes, thus boosting extrasynaptic neurotransmitter actions. Such actions can alter signal integration rules among neighboring synapses.
SUMMARY

Extrasynaptic actions of glutamate are limited by high-affinity transporters expressed by perisynaptic astroglial processes (PAPs): this helps maintain point-to-point transmission in excitatory circuits. Memory formation in the brain is associated with synaptic remodeling, but how this affects PAPs and therefore extrasynaptic glutamate actions is poorly understood. Here, we used advanced imaging methods, in situ and in vivo, to find that a classical synaptic memory mechanism, long-term potentiation (LTP), triggers withdrawal of PAPs from potentiated synapses. Optical glutamate sensors combined with patch-clamp and 3D molecular localization reveal that LTP induction thus prompts spatial retreat of astroglial glutamate transporters, boosting glutamate spillover and NMDA-receptor-mediated inter-synaptic cross-talk. The LTP-triggered PAP withdrawal involves NKCC1 transporters and the actin-controlling protein cofilin but does not depend on major Ca²⁺-dependent cascades in astrocytes. We have therefore uncovered a mechanism by which a memory trace at one synapse could alter signal handling by multiple neighboring connections.

INTRODUCTION

The surface of brain astroglia is packed with high-affinity GLT1 transporters that rapidly take up glutamate released by excitatory synapses (Danbolt, 2001; Verkhratsky and Nedergaard, 2018). GLT1-enriched perisynaptic astroglial processes (PAPs) that often surround synaptic connections (Grosche et al., 1999; Heller and Rusakov, 2015; Ventura and Harris, 1999) thus confine glutamate actions largely to the synaptic cleft. However, extrasynaptic glutamate escape, or “spillover,” can have a significant physiological impact. In the hippocampus, glutamate spillover has been caused to a co-operative action of dendritic NMDA receptors (NMDARs) (Challiff and Carter, 2011; Hires et al., 2008), functional inter-synaptic cross-talk (Arnth-Jensen et al., 2002; Asztely et al., 1997; Lozoya et al., 1999; Scimemi et al., 2004), heterosynaptic potentiation and depression (Vogt and Nicoll, 1999), and remote activation of metabotropic glutamate receptors (mGluRs) (Min et al., 1998; Scanziani et al., 1997). Glutamate escape underlies signaling between mitral cells in the olfactory bulb (Isaacson, 1999), and in the cerebellum between climbing fibers and interneurons (Coddington et al., 2013; Szapiro and Barbour, 2007) and between parallel fibers and stellate cells (Carter and Regehr, 2000). Changes in extrasynaptic glutamate signaling have also been related to cognitive decline (Pereira et al., 2014), fear conditioning (Tanaka et al., 2013; Tsvetkov et al., 2004), and heroin...
Figure 1. Reduced PAP Presence after LTP Induction at CA3-CA1 Synapses
(A) Left: 2PE point-spread function (PSF) excites dye-filled PAPs (yellow, 3D EM fragment) within an ~1 μm focal plane (dotted lines; bottom). Right: fluorescence within ROI (FROI) scales with PAP VF, reaching ~100% VF inside the 5–7 μm wide soma (FS).
(B) Astrocyte filled with AF 594 (single focal section; λex = 800 nm); dashed cone, extracellular recording pipette. FROI and FS, areas of VF readout; see Video S1 for extended dynamic range.
(C) Traces, s. radiatum fEPSPs, before (pre) and ~25 min after LTP induction (post); graph, relative fEPSP slope (mean ± SEM; arrow, induction onset); ***p < 0.001 (25–30 min post-induction: 151.0% ± 6.7% compared to baseline, n = 18).
(D) Relative change in PAP VF (%, mean ± 95% confidence interval [CI]) in control (green; n = 24 cells) and during LTP induction (orange; n = 29); red line, best-fit exponential decay to steady stateVF(t) = VFss + (1 − VFss)exp(−t/τ); VFss = 0.77 ± 0.04, τ = 14 ± 5 min.
(E) Relative change in PAP VF (%, mean ± SEM) plotted against initial PAP VF, in control (n = 8 cells) and ~25 min after LTP induction (orange; n = 13; *p < 0.05, **p < 0.01, compared to control, df = 19).
(F) Grey, PAP VF change (% sample size n shown) in hypo-osmotic (220 mOsm/L) and hyper-osmotic (420 mOsm/L) solutions, as shown. Green and orange, PAP VF change 25–30 min after LTP induction in control (LTP, mean ± SEM: 25% ± 7%), in 50 μM APV (+APV, 3.1% ± 9.9%), with no HFS (−0.8% ± 7.3%), (legend continued on next page)
and cocaine relapse (Shen et al., 2014; Smith et al., 2017). However, whether the PAP-controlled glutamate spillover can be adaptively regulated by neural activity has remained unknown.

Astrocytes can generate molecular signals that regulate excitatory transmission (Araque et al., 2014; Bazzargani and Attwell, 2016) and synaptic modifications associated with a memory trace (Adamsky et al., 2018; Henneberger et al., 2010; Min and Nevian, 2012; Shigetomi et al., 2013). Whether PAPs can also undergo activity-dependent remodeling has therefore been a longstanding question. Electron microscopy (EM) studies have reported increased astroglial coverage of synaptic samples that underwent induction of long-term potentiation (LTP) (Bernardinelli et al., 2014; Lushnikova et al., 2009; Wenzel et al., 1991) or in animals reared in complex environment (Jones and Greenough, 1996). In contrast, synaptic coverage by PAPs decreased following memory consolidation (Ostroff et al., 2014) or during lactation (Oliet et al., 2001). Nevertheless, EM cannot follow morphogenetic events in time and might be prone to distortions of PAP morphology during tissue fixation (Korogod et al., 2015).

These factors necessitate evidence in live cells, which has been a challenge because of the nanoscopic size of PAPs. Several studies have elegantly used confocal or two-photon excitation (2PE) fluorescence microscopy to monitor fine changes in PAP shapes seen in a light microscope are subject to interpretation. First, PAPs and inter-PAP distances are beyond the light diffraction (2PE) limit, potentially giving rise to spurious shapes (Rusakov, 2015). Second, cell-permeable fluorescent tracers appear to under-report astroglial structure (Reeves et al., 2011). Finally, subtle re-distribution of the fluorescent label could be mistaken for changes in PAP shape or motility.

To avoid such uncertainties, we set out to monitor PAPs with microscopy methods that are not limited by diffraction of light, under several LTP induction protocols, in hippocampal slices and in the barrel cortex in vivo. We employed optical glutamate sensors to relate LTP-associated changes in PAPs to extrasympathetic glutamate escape. We identified key players in cell signaling cascades that could underpin such changes. The results thus unveil how a plasticity-inducing pattern of neural activity could trigger local PAP remodeling thus altering local rules of synaptic signal integration.

**RESULTS**

**LTP Induction Reduces PAP Volume**

First, we visualized astrocytes loaded whole-cell with Alexa Fluor 594 (AF 594), in CA1 s. radiatum of acute hippocampal slices, using 2PE. Here, fluorescence intensity $F_{ROI}$ inside an ~1 μm focal plane over an x-y region of interest (ROI), scales with the tissue volume fraction (VF) occupied by the dye-filled PAPs (Figure 1A, left). Because astrocyte territories do not overlap (Bushong et al., 2002), $F_{ROI}$ represents all astroglia within the ROI. Thus, relating $F_{ROI}$ to the fluorescence intensity $F_{OG}$ over the somatic region representing 100% VF (Figures 1A, right, S1A, and S1B) provides the local PAP VF readout, as detailed previously (Medvedev et al., 2014; Savtchenko et al., 2018). This readout gave average PAP VF of 6%–7% (cell bodies excluded), with or without gap junctions blocked (Figures S1B and S1C). A similar value was reported previously in area CA1 (Savtchenko et al., 2018) or dentate gyrus (Medvedev et al., 2014) and was in line with earlier EM data (Lehre and Rusakov, 2002; Patrushev et al., 2013).

Next, we induced LTP at CA3-CA1 synapses, with the classical protocol of high-frequency stimulation (HFS) applied to Schaffer collaterals, while monitoring PAP VF and local fEPSPs (Henneberger et al., 2010) (Figures 1B and 1C; STAR Methods). LTP induction prompted a gradual PAP VF decrease, with a time constant of ~14 min and a projected steady-state value of ~23% (Figure 1D; Video S1). No VF change occurred in control conditions (Figure 1D), ruling out confounding effects, such as dye photobleaching. Interestingly, ROIs with smaller initial PAP VF showed a stronger VF reduction (Figure 1E). Similar tests using EGFP-expressing astroglia showed a smaller effect, most likely due to restricted diffusion of EGFP compared to small AF 594 molecules, and no VF changes were detected after the induction of long-term depression (Figures S1D and S1E).

The VF decrease was blocked when LTP was suppressed, either by the NMDAR antagonist APV, or by clamping Ca$^{2+}$ in the recorded astrocyte (Figure 1F) that inhibits astroglia-dependent release of the NMDAR co-agonist D-serine (Henneberger et al., 2010). In the latter test, LTP and VF reduction could be rescued by washing in 10 μM D-serine (Figure 1F), consistent with earlier reports (Adamsky et al., 2018; Henneberger et al., 2010). These results related VF reduction specifically to LTP induction rather than to HFS per se.

**LTP Induction Reduces Diffusion Coupling among Astroglial Processes**

Fluorescence recovery after photobleaching (FRAP) of AF 594 can report diffusion coupling among astrocyte processes when the bleaching line-scan is applied in their midst (Figure 1G), as shown earlier (Anders et al., 2014; Savtchenko et al., 2018). In these tests, LTP induction slowed down FRAP kinetics, with no changes in control conditions (Figures 1H and 1I), suggesting reduced coupling among PAPs, possibly due to their partial shrinkage. At the same time, LTP induction had no effect on

under Ca$^{2+}$ clamp (Ca-clamp, 6.8% ± 9.5%), under Ca$^{2+}$ clamp with 10 μM D-serine added (Ca-clamp+ D-ser, –24% ± 7%); **p < 0.01; *p < 0.05; dots, individual cells.

(G) Evaluating diffusion coupling inside astroglia using FRAP of dialyzed AF 594 (STAR Methods; single focal section; ~80 μm depth); arrow, example line scan position.

(H) Top: line scan as in (G) (baseline conditions; gray segment, shutter closed). Bottom: the corresponding fluorescence time course, before (Cntl) and ~20 min after LTP induction (LTP); F$_{ROI}$, initial intensity; arrows, FRAP during shutter-on period (full recovery takes 39–40 s).

(I) Summary of FRAP tests (G and H); diagram, LTP induction may taper PAPs lowering diffusion coupling. Graph (mean ± SEM), FRAP rate relative to baseline (left ordinate): ~25 min after LTP induction (LTP, 62% ± 12%, n = 11; *p < 0.05; in 50 μM APV (108% ± 32%, n = 7); no-HFS control (87% ± 15%, n = 7), Grey (right ordinate), change in extracellular diffusivity ~25 min post-induction (LTP-ECS; 104% ± 7%, n = 5; Figures S1F–S1H); dots, individual tests.

Please cite this article in press as: Henneberger et al., LTP Induction Boosts Glutamate Spillover by Driving Withdrawal of Perisynaptic Astroglia, Neuron (2020), https://doi.org/10.1016/j.neuron.2020.08.030
extracellular diffusion (Figure 1I) assessed with a fluorescence point-source method (Figures S1F–S1H) (Zheng et al., 2008). This was not surprising because CA1 astroglia, soma excluded, only occupy 6%–7% of the tissue volume (Figure S1C) (Savtchenko et al., 2018) of which 15%–20% is taken by the extracellular space (Sykova and Nicholson, 2008). Thus, a 20%–30% decrease in PAP VF would increase local extracellular space by only 5%–10%.

Stimulated Emission Depletion (STED) Imaging Reveals Decreased PAP Presence Near Spines upon LTP Induction

STED microscopy enables monitoring live astroglia beyond the optical diffraction limit (Arizono et al., 2020; Panatier et al., 2014). We therefore turned to two-color STED (Tønnesen et al., 2018) combined with patch-clamp (Figures 2A and S2A) in organotypic slices. We used the Thy1-YFP transgenic mice and whole-cell AF 488 dialysis to image, respectively, CA1 pyramidal neurons and PAPs, in separate channels before and ~25 min after LTP induction, as indicated; circles, ROIs centered at spine heads.

(B) LTP induction reduces the green/red (astroglia/neuron) pixel ratio within ROIs (G/R; mean ± SEM; 31% ± 10%, n = 22; *p < 0.01), with no effect on red pixel count (R; −31% ± 3.8%; *p < 0.02 compared to the G/R change, df = 42); dots, individual ROIs.

(C) Proportion of dendritic spines that adjacent to (green) and away from (gray) PAPs, in control (Cntrl), 20–25 min post-induction (LTP), and the latter with 50 μM APV (+APV); spine numbers shown (Figures S2A–S2D).

(D) Patched astrocyte loaded with biocytin (arrow, local astroglia stained through gap junctions), shown in the fluorescence (left) and DIC channel post-DAB conversion (right).

(E) Electron micrograph showing PAPs of the patched astrocyte (arrow in D) filled with precipitate (blue), and adjacent dendritic spines (yellow) featuring PSDs.

(F) Astrocyte fragment (cyan) reconstructed in 3D, including adjacent thin (white) and mushroom (yellow) dendritic spines with PSDs (red; Figure S2E).

(G) Volumetric measure of synaptic astroglial coverage: PAP VF is calculated within 100 nm-thick concentric 3D shells (circles, not to scale) centered at the PSD (red).

(H) PAP VF around PSDs (mean ± SEM) of thin and mushroom spines, in control and ~30 min after LTP induction, as indicated; sample sizes shown; ***p < 0.001 (df = 86 for mushroom and df = 241 for thin spines).
after LTP induction, followed by rapid slice submersion into fixative and DAB conversion for EM (Figures 2D and 2E; STAR Methods). The embedded slices were cut into 60–70 nm serial sections, the patched astrocyte was located (Figures 2D and 2E), and its fragment with the adjacent synapses were 3D-reconstructed from 200–300 sections (Figures 2F and S2E), as detailed earlier (Medvedev et al., 2014; Savtchenko et al., 2018).

To evaluate synaptic PAP coverage volumetrically, we calculated PAP VF inside 100 nm thick concentric spherical shells centered at individual postsynaptic densities (PSDs) (Figure 2G; STAR Methods), up to ~0.5 μm, the average nearest-neighbor distance between CA3-CA1 synapses. Although “thin” and “mushroom” spines have distinct identities (Matsuzaki et al., 2001), we found that LTP induction reduced local PAP VF for both types (Figure 2H). Here, VF values agreed with the earlier EM data (Lehre and Rusakow, 2002; Patrushev et al., 2013) and 2PE data (Medvedev et al., 2014; Savtchenko et al., 2018) (Figure S1C), arguing that our EM results are unlikely to be biased by fixation (Korogod et al., 2015) (see Discussion).

LTP-Induced PAP Withdrawal Depends on Activation of NKCC1

To explore cellular mechanisms underlying PAP withdrawal, we first examined major astroglial Ca2+–signaling cascades that engage mGlURs and IP3 receptors (Porter and McCarthy, 1997; Volterra et al., 2014) and can alter PAP motility (Perez-Alvarez et al., 2014). We spot-uncaged IP3 inside cell branches: this evoked local Ca2+ rises (Figures 3A and 3B) but had no effect on PAP VF (Figure 3C). Puff application of the mGlUR agonist DHPG had a similar outcome (Figure 3C), and PAP VF remained unaffected by WIN55, an agonist of the cannabinoid CB1 receptor that is active in astroglia (Navarrete and Araque, 2010). Similarly, the GABA A receptor agonist muscimol, which triggers slight shrinkage of sulforhodamine-101 stained astroglia (Florence et al., 2012), had no effect on PAP VF (Figure 3C).

We next tested morphogenic agents associated with synaptic remodeling. However, removing the extracellular matrix (ECM) chondroitin sulfate (Dityatev and Schachner, 2003) and chondroitin AABC (Kochlamazashvili et al., 2010), or blocking the ephrin/EphA4 cascade (Filosa et al., 2009; Murai et al., 2003; Nishida and Okabe, 2007) with EphA4-Fc had no effect on the LTP-induced PAP VF reduction (Figure 3D).

We next turned to ion and water exchange mechanisms, in which aquaporin-4 (AQP4) plays a prominent role (Nagelhus and Ottersen, 2013). However, the LTP-associated reduction in PAP VF remained intact in the AQP4 knockout (KO) mice (Thrane et al., 2011). Another key player in cell volume regulation is the cell-specific action of the Ca2+ pump NKCC1 (Navarrete and Araque, 2010). mGlUR- and IP3-dependent Ca2+ entry reported by OGB-1 (Figures S4A and S4B). Next, we switched to current clamp maintaining Vm at ~−60 to ~−65 mV, the range for CA1 pyramids in freely moving animals (Epsstein et al., 2010). Here, applying the spot-uncaging sequence that mimics the HFS protocol generated postsynaptic depolarization sufficient to trigger Ca2+ entry reported by OGB-1 (Figures S4A and S4B). Switching back to voltage clamp revealed potentiation of single-pulse EPSCs (Figure 4B), which was induced robustly at every recorded synapse (7 out of 7 cells) (Figure 4B and 4C).

Because CA3-CA1 synapses are only ~0.5 μm apart (Rusakov and Kullmann, 1998), spot-uncaging HFS should potentiate at least one synapse nearby, whether or not the unclamped postsynaptic cell is visualized. We therefore loaded an astrocyte with AF 594 and OGB-1 and applied spot-uncaging while monitoring VF and Ca2+ in the adjacent PAPs (Figure 4D). The HFS uncaging sequence in most cases evoked local Ca2+ rise in PAPs (Figures 4D, 4E, and 4D), indicating robust glutamate release. In some cases, we detected PAP VF reduction near the spot (Figures 4D, 4G, and S4C–S4E; Video S2), but no changes either in remote ROIs (~3 μm away, Figure 4D) or without MNI-glutamate in the bath (Figures 4G and 4H). Unsurprisingly, the VF change was smaller than under bulk LTP induction (Figures 1, 2, and 3) where a co-operative effect was likely. Blocking NKCC1 with

Activating Cofilin Cascade Occludes LTP-Induced Changes in PAPs

In glioblastoma cells, NKCC1 provides a protein scaffold regulating the phosphorylation of cofilin-1 (Schiapparelli et al., 2017), and in neurons, transporter KCC2 plays a similar role (Liao et al., 2015). Cofilin-1 is a pH-dependent regulator of actin filament polymerization, which in turn controls remodeling of thin cell protrusions (Bravo-Cordero et al., 2013; Ethel and Pasquale, 2005). To probe this cascade, we diazylated astroglia with peptide S3, a specific inhibitor of cofilin-1 phosphorylation (Aizawa et al., 2001; Liu et al., 2016). Unlike bumetanide, this preserved both LTP induction and the PAP VF decrease (Figures 3F, S3B, and S3C; STAR Methods). However, peptide S3 dialysis reduced PAP VF by 20%–25% in baseline conditions, too, similar to the LTP case (Figure 3G): here, astrocytes connected to the patched cell via gap junctions (permeable to S3, MW ~1.5 kDa) showed no PAP changes, confirming a cell-specific action (Figure 3G). Furthermore, when we combined LTP induction with S3 dialysis (Figures S3B and S3C), the kinetics of PAP shrinkage were the same as under LTP induction alone or under S3 dialysis alone (Figure 3H). Thus, peptide S3 action occluded the effect of LTP induction on PAP VF, suggesting a shared mechanism (see Discussion).

Single-Synapse LTP Induction Prompts Local PAP Retraction

Although HFS in the bulk of tissue potentiates multiple synapses, memory trace formation is likely to involve changes at individual connections. We therefore set out to test how LTP at individual synapses affects PAPs. First, we modified an established protocol in which LTP at a CA3-CA1 synapse is induced by local glutamate spot-uncaging (Harvey and Svoboda, 2007; Matsuzaki et al., 2004; Yasuda et al., 2003). We held a CA1 pyramidal cell in voltage clamp and spot-uncaged glutamate (1-ms pulse) near its dendritic spine (Figure 4A) achieving a typical unitary EPSC (Figure 4B; STAR Methods). Next, we switched to current clamp maintaining Vm at ~−60 to ~−65 mV, the range for CA1 pyramids in freely moving animals (Epsstein et al., 2010). Here, applying the spot-uncaging sequence that mimics the HFS protocol generated postsynaptic depolarization sufficient to trigger strong Ca2+ entry reported by OGB-1 (Figures S4A and S4B).
whole-cell loaded bumetanide suppressed the LTP-associated PAP change (Figure 4H). A complementary strategy, in which astrocytes were imaged using the membrane-bound GFP (AAV5.GfasABC1D.Pi.ick-GFP.SV40) produced a qualitatively identical result, with the PAP withdrawal lasting for up to 100–120 min post-induction (Figures S4F–S4H).

**LTP Induction Increases Glutamate Traveling Distance**

We next hypothesized that PAP withdrawal alters perisynaptic occurrence of GLT1. To test this, we turned to dSTORM, a super-resolution technique that we adapted previously (Heller et al., 2020), aiming to map 3D co-ordinates of the presynaptic protein bassoon, the PSD protein Homer1, and local GLT1 (Figure 5A). To potentiate synapses in bulk, we employed the classical chemically induced LTP (cLTP) protocol in acute hippocampal slices (Otmakhov et al., 2004) (Figure S5A).

Three-color dSTORM revealed 3D perisynaptic patterns of GLT1 molecules (Figures 5A and S5B). In potentiated slices, Released Glutamate Induction of LTP Extends Extracellular Exposure of

**Induction of LTP Extends Extracellular Exposure of Released Glutamate**

To test whether the withdrawal of GLT1-enriched PAPs indeed prompts increased extracellular travel of released glutamate, we employed the optical glutamate sensor FLIPE600n (Okumoto 2016).
Figure 4. LTP Induction at Individual CA3-CA1 Synapses Reduces Local PAP Presence

(A) Dendritic fragment, CA1 pyramidal cell (AF 594 channel), showing glutamate uncaging spot (red dot; 2.5 mM bath-applied MNI-glutamate) before (pre) and ~20 min after spot-uncaging LTP induction (post).

(B) One-spine example. Traces, EPSCs ($I_{syn}$, voltage-clamp) during baseline (black) and ~30 min after LTP induction (red; see Figures S4 A and S4B for Ca$^{2+}$ dynamics). Graph, relative EPSC amplitude ($I_{syn}$; black and red circles) and cell access resistance ($R_a$, green) time course; arrow, LTP induction onset.

(C) Statistical summary of experiments in (A) and (B) (mean ± SEM; n = 7, ***p < 0.005); notations as in (B); dots, individual tests.

(D) Example, astrocyte fragment (whole-cell AF 594, single focal section); red dot, glutamate uncaging spot; circles, ROIs for PAP VF monitoring near the spot and away, as shown.

(E) Time-lapse frames (area shown in D): astrocyte Ca$^{2+}$ response (Fluo-4, $\lambda_{ex}^{2P} = 840$ nm) to the spot-uncaging LTP protocol ($\lambda_{em}^{2P} = 720$ nm).

(F) Astrocyte fragment near the uncaging spot (as in D; arrow) immediately after (0 min), at 15 min and 25 min after LTP induction (~9 µm z stack average); PAP retraction seen at 15–25 min (Figures S4 C–S4E; Video S2).

(G) PAP VF change (%), mean ± SEM) in tests shown in (D) and (E) (Glu, n = 11), and with no MNI-glutamate (no Glu, n = 11; arrow, uncaging onset).

(H) Summary: PAP VF change (%), mean ± SEM) ~25 min post-induction (LTP, ~13% ± 4%; ***p < 0.005, n = 16), with no MNI-glutamate (no Glu, 1.3% ± 3.0%, n = 9), in remote ROI (as in D; 2.0% ± 3.5%, n = 11), and with 20 µM bumetanide whole-cell (~1.4% ± 3.3%, n = 9); *p < 0.05 (df = 15); ***p < 0.005 (df = 23).
et al., 2005) immobilized in the extracellular space (Okubo et al., 2010), as described previously (Zhang et al., 2018) (Figures 5C and S5D; STAR Methods). The sensor showed high glutamate sensitivity (Figure S5E) and could be delivered with a patch-pipette (Figures 5C and 5D). Burst stimulation of Schaffer collaterals induced a robust optical response (Figures 5E and S5F), as described previously (Zhang et al., 2018).

Figure 5. LTP Induction Triggers Withdrawal of Glial Glutamate Transporters Boosting Extracellular Glutamate Transient

(A) Perisynaptic patterns of bassoon (red cluster), Homer 1 (green cluster), and GLT1 (magenta dots) molecules localized with 3D dSTORM; one-synapse example, three viewing angles shown; x-y-z scale bars, 500 nm (STAR Methods).

(B) Nearest-neighbor distances (probability density, mean ± SEM) between GLT1 and bassoon, in control tissue and ~30 min after cLTP induction (Figures S5A and S5B; STAR Methods); sample size: $N_{\text{m}}$, inter-molecular distances; $N_{\text{syn}}$, synapses; $N_{\text{pre}}$, slices; SEM relates to $N_{\text{pre}} = 5$; *p < 0.05 (gray segments, significant difference).

(C) Diagram, extracellular immobilization of bFLIPE600n (Venus and ECFP attachments shown) via biotinylation and attachment to streptavidin (SA) (Figure S5D; STAR Methods) in s. radiatum (delivery pipette shown).

(D) Experimental design: sensor-injecting pipette (field) records fEPSPs evoked by Schaffer collateral stimulation (stim) while bFLIPE600n signal is monitored within an adjacent ROI (rectangle).

(E) Example, glutamate signal reported by bFLIPE600n ($\Delta R$, ECFP/Venus signal ratio) in response to Schaffer collateral HFS (100 Hz for 1 s, red arrow; 10 $\mu$M NBQX, 50 $\mu$M D-APV) in s. radiatum (also Figures S5E and S5F).

(F) Relative fEPSP slope (% mean ± SEM) in control (green, n = 8 slices), during LTP induction (n = 14, orange), and with 50 $\mu$M APV present (n = 7, orange empty); ***p < 0.005, difference over 20-25 min post-induction.

(G) Traces, bFLIPE600n response to paired-pulses (20 Hz, arrows; mean ± SEM) in control (green) and ~25 min after LTP induction (orange). Plot, summary (notations as in F); **p < 0.01, difference between LTP and either control or APV datasets.
Figure 6. LTP Induction Broadens Evoked Extracellular Glutamate Transients.
(A) Dendritic fragment, CA1 pyramidal cell (AF 594 channel); red dot, glutamate uncaging spot; yellow arrow, line scan position for iGluSnFR monitoring (Figures S6A–S6C).

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which was significantly increased after LTP induction (Figures 5F and 5G). Because in similar settings, LTP induction has no effect on the overall amount of released glutamate (Diamond et al., 1998; Lüscher et al., 1998), the increased bFlIPE600n response suggests a greater sensor exposure to the extrasynaptic glutamate transient. To test this at the synaptic level, we carried out two further experiments, as described below.

**LTP Induction Widens Spatial Transients of Released Glutamate**

In the first experiment, we expressed the glutamate sensor iGluSnFR (Marvin et al., 2013) in area CA1, in either neurons or astroglia of the mouse hippocampus (STAR Methods). Optical iGluSnFR response to paired-pulse stimuli faithfully reflected Ca²⁺-dependent changes in fEPSPs (Figures S6A and S6B) and also their preserved paired-pulse ratio after LTP induction (Figure S6C) (Diamond et al., 1998; Lüscher et al., 1998).

We next monitored the spatial spread (FWHM) of the iGluSnFR response to a 1-ms glutamate spot-uncaging pulse, either near a postsynaptic dendrite (Figure 6A) or within an astrocyte ROI (Figure S6A) using line scans (Figures 6B and S6F), before and 10–30 min after the spot-uncaging LTP protocol (as in Figures 4A and 4B). LTP induction appeared to widen the iGluSnFR signal (Figures 6C and 6D; n = 12) but not when the iGluSnFR-expressing astrocyte was dialyzed with bumetanide to block NKCC1 (Figures 6D, S6F, and S6G).

The second test aimed at detecting PAP changes near active axonal boutons visualized using relatively sparse iGluSnFR expression in S. radiatum. We focused on boutons that responded optically to Schaffer collateral stimulation (five pulses at 20 Hz; Figure 6E) and recorded iGluSnFR signal landscapes before and up to 90–120 min after LTP induction (Figures 6F and S6H). Again, LTP induction increased the signal FWHM, for up to 120 min (Figure 6H), although some boutons showed no change (Figure 6H), probably reflecting non-potentiated connections. The average effect was larger than that under spot-uncaging (Figure 6D), likely because burst stimulation amplifies glutamate spillover (Lozovaya et al., 1999).

**Cofilin-Dependent PAP Shrinkage Boosts Activation of Extrasynaptic NMDARs**

Because astrocyte dialysis with peptide S3 reduces PAP VF in baseline conditions (Figures 3G and 3H), we asked if this would, on its own, boost glutamate spillover. We noted that extrasynaptic NMDARs are predominantly GluN2B subunit-containing so that their contribution to NMDAR EPSCs/EPSPs in area CA1 varies with the extent of glutamate spillover, in particular during stimulus bursts (Lozovaya et al., 2004; Papouin et al., 2012; Scimemi et al., 2004). To confine ourselves to one astrocyte and its “territorial” synapses, we recorded local fEPSPs (7 pulses at 5 Hz) through the astrocyte patch pipette, previously termed as a-fEPSPs (Henneberger et al., 2010; Henneberger and Rusakov, 2012). In baseline conditions, blocking GluN2B-containing NMDARs with Ro 25-6981 unveiled their 8% ± 2% (n = 7) contribution to the 7th NMDAR a-fEPSP. However, if the astrocyte was dialyzed with S3, this contribution was 17% ± 3% (n = 7) (Figures 6l, 6J, and S6l). Thus, withdrawal of PAPs per se was capable of boosting glutamate escape.

**Whisker-Stimulation-Induced LTP Reduces PAP Presence Near Firing Axons**

To assess physiological relevance of our observations, we turned to tests in vivo. We focused on the established protocol of LTP induction at the thalamocortical synapses in the barrel cortex (layer II/III) by contralateral rhythmic whisker stimulation (RWS) (Gambino et al., 2014; Mégevand et al., 2009).

Building upon our previous in vivo imaging protocols (Reynolds et al., 2019; Savtchenko et al., 2018; Zheng et al., 2015), we expressed the green Ca²⁺ indicator GCaMP6f in the ventral posterial nucleus (VPM) that sends axonal projections to the barrel cortex (Figure 7A). In parallel, we sparsely expressed the red-shifted, cytosol-soluble indicator (GfaABC1D) tdTomato in the barrel cortex astroglia (Figure 7B). Thus, we could monitor, through an implanted cranial window, fine astroglial morphology together with presynaptic Ca²⁺ dynamics in individual thalamocortical projections (Figures 7C and 7D).

First, we confirmed that PAP VF readout with tdTomato was similar to that with AF 594 (Figures S7A and S7B). Next, within...
Figure 7. Whisker-Stimulation LTP Protocol in the Barrel Cortex In Vivo Triggers PAP VF Reduction in Astroglia Trespassed by Stimulated Axons

(A) Expression of GCaMP6f 3 weeks post-transfection (STAR Methods) into the mouse ventral posteromedial nucleus (VPM), coronal section; LV, lateral ventricle; CPu caudate putamen; wide-field image, fixed tissue.

(B) Composite post hoc image, barrel cortex area (coronal section), with astroglia expressing Gfabc1D tdTomato (magenta; STAR Methods) and neuronal structures expressing GCaMP6f (green); dotted rectangle (inset, arrow) highlights astrocytes with axonal boutons occurring nearby.

(C) Experiment diagram: 2PE imaging of the barrel cortex (S1BF) through a cranial window, with two fs lasers. LTP induction protocol uses RWS (5 Hz air-puffs for 120 s) on the contralateral side.

(D) Live barrel cortex view (S1BF) through the cranial window (l<sub>2PE</sub> = 1,040 and 910 nm, single focal section). Green (GCaMP6f), heatmap of axonal signals firing in response to RWS; magenta (tdTomato), local astroglia; circles, examples of ROIs for PAP VF readout in proximity to RWS-responding thalamocortical axons (green; Figures S7 A and S7B).

(E) Example, a thalamocortical axon in S1BF (GCaMP6f, green) crossing astroglial territory (tdTomato, magenta), with boutons responding to an RWS test (3 Hz, 5 s) with Ca<sup>2+</sup> elevations (middle panel).

(F) Time course of Ca<sup>2+</sup> signal (GCaMP6f) at five axonal boutons (green traces) shown in (E); black line, average.

(G) Ipsilateral RWS

(H) ContraLateral RWS

Legend continued on next page
the tdTomato-expressing astrocyte domains, we found axonal boutons that showed Ca\(^{2+}\) elevations in response to an RWS test (3 Hz air puffs for 5 s) (Figures 7E and 7F). This enabled us to monitor PAP VF in within ~3 \(\mu\)m of active boutons, before and after LTP induction by RWS (3 Hz air 100 ms stimuli for 120 s) (Figure 7C). LTP induction by contralateral RWS triggered PAP VF reduction (5 cells, 3 animals) whereas the same protocol applied ipsilaterally had no effect (12 cells, 4 animals) (Figures 7G and 7H).

We used a similar imaging design in a complementary test in acute hippocampal slices. We loaded a CA3 pyramidal cell with OGB-1 and traced its axon into area CA1, which was populated with tdTomato-expressing astroglia (Figures S7C and S7D). We then paired presynaptic spikes (triggered by somatic depolarization pulses) with postsynaptic CA1 pyramidal cell depolarization induced by periodic extracellular stimuli (Figures S7E and S7F). This LTP-inducing pairing protocol reduced PAP VF near activated axonal boutons by 12% ± 2% \((n = 5)\) whereas no such reduction occurred away from the firing axon (change 3.4% ± 1%, \(n = 10\); difference at \(p < 0.01\), degrees of freedom \([df] = 13\)) (Figure S7E; Video S3).

LTP Induction Prompts NMDAR-Mediated Cross-Talk among Synapses

To test if the LTP-associated increase in glutamate escape promotes activation of high-affinity NMDARs at neighboring, non-active connections, we used a protocol established specifically to monitor NMDAR-mediated cross-talk among independent CA3-CA1 synapses (Scimemi et al., 2004). It takes advantage of the use-dependent NMDAR inhibitor MK801, which blocks the receptor channel upon its opening. Thus, if NMDARs at non-active (silent) synapses get blocked by MK801 they must have been activated by glutamate molecules escaping from nearby active synapses.

First, we used paired-pulse stimuli to confirm independence of two Schaffer collateral pathways converging to a CA1 pyramidal cell (Figure S8A). Second, we recorded AMPA receptor-mediated EPSCs (AMPAR EPSCs), then NMDAR EPSCs, elicited in either pathway (Figure 8A). Third, we applied MK801 and recorded declining NMDAR EPSC responses in one (active) pathway only (Figure 8A). When stimulation resumed in the other, silent pathway, its NMDAR EPSC amplitude was close to its baseline value (Figures 8A, top dotted line, and S8B, no-LTP, silent). Thus, the silent pathway had little cross-activation of its NMDARs by synaptic dials in the active pathway.

The outcome was different when we induced LTP of AMPAR EPSCs in the active pathway (Figure 8B). Here, resuming stimulation of the silent pathway revealed reduced NMDAR EPSCs (Figure 8B, cross-talk). Thus, a proportion of NMDARs here must have been activated by glutamate escaping from synapses in the active pathway (see Discussion for quantitative estimates). LTP induction in the silent pathway, or in both pathways, produced similar outcome (Figures 8C and S8C). We confirmed that the trial-to-trial time decay of NMDAR EPSCs was similar among potentiated and non-potentiated pathways, suggesting no effects of LTP induction on the overall release probability (Figure S8D), as reported here (Figures S6A–S6C) and earlier (Diamond et al., 1998; Lüscher et al., 1998; Manabe and Nicoll, 1994).

**DISCUSSION**

**Biophysical Plausibility**

Our results suggest that LTP induction prompts nanoscopic withdrawal of PAPs, which boosts extrasynaptic glutamate escape, thus enhancing NMDAR activation away from the release site, potentially at nearby synapses (Figure 8D). To assess biophysical plausibility of these events, we modeled CA3-CA1 synaptic environment (Figure S8E) (Zheng et al., 2008) and simulated three scenarios that might reflect our observations: GLT1-enriched PAPs (1) withdraw without losing any GLT1, (2) withdraw while losing some GLT1, or (3) re-arrange laterally with the same GLT1 numbers (Figure S8F), which partly exposes extrasynaptic NMDARs. After multiple runs (example in Video S4), scenario (1) appeared most likely in boosting remote NMDAR activation (Figure S8G).

**Cellular Mechanics of LTP-Dependent PAP Withdrawal**

We found that the LTP-associated PAP withdrawal depends on NKCC1, a key morphology regulator in brain cell migration (Garzon-Muvdi et al., 2012; Haas and Sontheimer, 2010). In glioma cells, NKCC1 mediates dramatic hydrodynamic volume changes that enable invasion of intact tissue (Watkins and Sontheimer, 2011), probably by boosting intracellular chloride up to 140 mM (Habela et al., 2009). The NKCC1-regulated phosphorylation of cofilin-1 has been revealed in glioblastoma (Schiapparelli et al., 2017), and we found that inhibiting cofilin-1 phosphorylation with peptide S3 occluded LTP-induced PAP shrinkage, suggesting a shared molecular pathway. An alternative interpretation is that both mechanisms simply reduce PAP VF to a maximal degree. Yet, astrocyte dialysis with S3 does boost glutamate spillover, similar to the LTP case.

What activates NKCC1 upon LTP induction remains to be ascertained. One possibility is that intense activation of local NMDARs and GLT1 leads to a hotspot of K\(^+\) efflux (Shih et al., 2013). Classically, NKCC1 is activated by excess of external K\(^+\) (Russell, 2000) whereas proton transport by GLT1 could help boost cofilin-dependent actin assembly. Although this appears plausible (Figure 8E), a better understanding of the mechanisms relating PAP plasticity to NKCC1 and cofilin, and probably to other morphogenic agents of astroglia such as neurotrogelines (Stogsdill et al., 2017), requires a separate study.

**3D EM: Faithful Representation of Live Tissue?**

The relevance of fixed-tissue EM has recently been questioned: chemical fixation in vivo can cause linear tissue contraction by...
18% resulting in ~2% VF for the extracellular space and distorted PAP morphology (Korogod et al., 2015). However, different fixation protocols produce different outcomes. Here, we used rapid slice fixation by submersion: our earlier studies reported 5%–6% linear hippocampal shrinkage under a similar protocol (Rusakov et al., 1998), whereas in vivo fixation gave ~12% extracellular space VF in area CA1 (Rusakov and Kullmann, 1998). In chemically fixed CA1 tissue, PAPs occupied ~9% of tissue volume (Lehre and Rusakov, 2002), which falls within the range estimated here with live 2PE imaging. A similar correspondence was observed in other studies (Medvedev et al., 2014; Savtchenko et al., 2018).

We made no attempt to assess PAP shapes or exact position, which, in addition to protocol differences, might explain an apparent discrepancy with some previous results. For instance, smaller PAPs that occur closer to synapses might well count as an increased PAP occurrence (Lushnikova et al., 2009; Wenzel et al., 1991) even though their overall VF decreases. Similarly, we do not dispute previous findings reporting high PAP mobility or morphological plasticity detected with fluorescence imaging (Bernardinelli et al., 2014; Haber et al., 2006; Hirrlinger et al., 2004; Perez-Alvarez et al., 2014) but note that mobility of the fluorescent label, or fluctuations in focus, laser power, or tissue optical properties, might add to the perceived motility.

PAP Withdrawal and Extrasynaptic Glutamate Actions

Remodeling of GLT1-enriched PAPs on the nanoscale will not affect total glutamate uptake by astroglia because all released molecules will still be bound by local GLT1 and taken up by the same astrocyte. Thus, LTP induction should have little effect on the astrocyte uptake currents measured by a somatic pipette (Diamond et al., 1998; Lüscher et al., 1998) but note that mobility of the fluorescent label, or fluctuations in focus, laser power, or tissue optical properties, might add to the perceived motility.

Figure 8. LTP Induction Boosts NMDAR-Mediated Inter-synaptic Cross-Talk

(A) Inset diagram, experiment design to test NMDAR-mediated cross-talk between two afferent pathways (green and orange lightning) (Scimemi et al., 2004) (Figure S8A; STAR Methods). Plot, relative EPSC amplitude (mean ± SEM, n = 13), with single stimuli, 20 s apart, applied alternately to the two pathways (green and orange). AMPAR EPSCs are recorded for 12–15 min (V_m = −70 mV; left ordinate), then NMDAR EPSCs for ~5 min (10 μM NBOX, V_m = −20 mV; right ordinate). Once MK801 is added, NMDAR EPSCs are recorded in active (green) pathway only. Resuming stimulation in the silent (orange) pathway reveals little change in the NMDAR EPSC amplitude compared to baseline (dotted line).

(B) Experiment as in (A) but with LTP induced in the active pathway (red arrow; n = 7). Reduced NMDAR EPSCs in the silent (orange) pathway upon resumed stimulation (arrow, cross-talk) point to NMDAR activation by glutamate escaping from the active (green) pathway.

(C) Summary of experiments in (A) and (B). The degree of cross-talk (percentage of one-pathway NMDARs activated by glutamate discharges at the other pathway; mean ± SEM), in control (Cntr, n = 13), with LTP induced either in one (LTP-one, n = 10) or both (LTP-both, n = 11; Figures S8C and S8D) pathways, prior to NMDAR EPSC recordings; *p < 0.05 (df = 21 for Cntr versus LTP-one), **p < 0.01 (df = 22), ***p < 0.005.

(D) Proposed changes in PAPs after LTP induction. In baseline conditions (left), PAPs restrict glutamate action to the synaptic cleft and some extrasynaptic NMDARs (red dots). After LTP induction (right), some PAPs withdraw, widening the pool of activated extrasynaptic NMDARs, including neighboring synapses.

(E) Diagram, candidate cellular mechanisms of LTP-driven PAP withdrawal. LTP induction activates postsynaptic NMDARs and engages GLT1 transporters. This generates an extracellular K⁺ hotspot, activating the NKCC1-cofilin-1 pathway that engages, in a pH-sensitive manner, actin polymerization responsible for morphogenesis.
high-affinity optical sensors to compete more successfully with GLT1 (Armbruster et al., 2020; Kopach et al., 2020). Thus, the optical glutamate signal is enhanced after LTP induction.

We examined NMDAR-mediated cross-talk between two independent pools of CA3-CA1 synapses and found that, following LTP induction, ~120 discharges in the active pool activated ~40% NMDARs in the silent pool. Although this suggests only ~0.4% per discharge, this protocol activates only 2%–3% of CA3-CA1 connections (Scimemi et al., 2004). With the synaptic nearest-neighbor distance in CA1 of ~0.5 μm (Rusakov and Kullmann, 1998), 2% synapses will be separated by 0.5 × (0.02 m)1.8 ~1.8 μm. The travel distance increase from 0.5 to 1.8 μm corresponds to a >100-fold drop in the glutamate concentration transient post-release (Rusakov, 2001; Zheng et al., 2008). Thus, cross-talk among 2%–3% synapses accumulated over ~120 discharges could underestimate cross-talk between nearest neighbors per discharge.

The increased exposure of glutamate to the extracellular space after LTP induction might explain why some earlier studies reported increased extracellular glutamate transients detected with micro-dialysis (Bliss et al., 1986; Errington et al., 2003). It might also explain the reduced NMDAR EPSC variability at CA3-CA1 synapses (Kullmann et al., 1996), an enhanced local excitability of pyramidal cell dendrites (Frick et al., 2004), and why LTP at one synapse could lower the LTP induction threshold at its neighbors (Harvey and Svoboda, 2007). Other important consequences could be a boost in NMDAR-driven dendritic spikes (Chalifoux and Carter, 2011), facilitated plasticity at silent connections nearby (Tsvetkov et al., 2004), or increased heterosynaptic depression (Vogt and Nicoll, 1995).

### PAP Remodeling on Longer Timescales

Our observations in slices were necessarily limited to 30–90 min after LTP induction, and to 30–35 min in vivo (to avoid concomitants of animal stress in 2- to 3-h long experiments). This does not preclude the possibility for PAP coverage to re-establish itself on a longer timescale. Indeed, unlimited accumulation of PAP on a longer timescale. Indeed, unlimited accumulation of PAP on a longer timescale, which means an important and intriguing question to be addressed in chronic experiments.

### STAR Methods

Detailed methods are provided in the online version of this paper and include the following:

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### SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.neuron.2020.08.030.

### ACKNOWLEDGMENTS

We thank J. Angibaud for organotypic cultures and R. Chereau and J. Tonnensen for help with the STED microscope, also D. Gonzales and the Neurocentre Magendie INSERM U1215 Genotyping Platform, for breeding management and genotyping. This work was supported by the Welcome Trust Principal Fellowships 101896 and 212251, ERC Advanced Grant 323113, ERC Proof-of-Concept Grant 763732, EC FP7 ITN 606950, and EU CSA 811011 (D.A.R.); NRW-Ruckkehrprogramm, UCL Excellence Fellowship, German Research Foundation (DFG) SPP1757 and SFB1089 (C.H.); Human Frontiers Science Program (C.H., C.J.J., and H.J.); EMBO Long-Term Fellowship (L.B.); Marie Curie FP7, PIRG08-GA-2010-276995 (A.P.); ASTROMODULATION (S.R.); Equipe FRM DEQ 201 303 26519, Conseil Régional d’Aquitaine R12056GG, INSERM (S.H.R.O.); ANR SUPERHY, ANR Castro (ANR-17-CE16-0002), R-13-BSV4-0007-01, Université de Bordeaux, labex BRAIN (S.H.R.O. and U.V.N.); CNRS (A.P., S.H.R.O., and U.V.N.); HFSP, ANR CEC, and France-BioImaging ANR-10-INSB-04 (U.V.N.); and TP7 MemStick Project No. 201600 (M.G.S.).

### AUTHOR CONTRIBUTIONS

D.A.R. and C.H. conceived the study; C.H., L.B., O.K., D.M., M.K.H., S.A., and T.P.J. designed and carried out patch-clamp, morphometry, imaging experiments, and analyses in slices. A.P., S.H.R.O., and U.V.N. designed and carried out STED tests. J.P.R. designed and carried out in vivo tests and analyses. N.I.M., L.K., and M.G.S. designed and carried out 3D EM studies. I.S.-R., C.J.J., and H.J. designed the modified glutamate sensor bFLIPE600n. S.R. and D.M. performed S3 peptide tests. J.H. designed and carried out dSTORM studies. K.Z. performed biophysical modeling and dSTORM analyses. O.P.O. and E.A.N. provided expertise and materials pertinent to AQP4 tests. D.A.R. designed experiments, analyzed the data, and wrote the manuscript, which was subsequently contributed to by all the authors.
DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES


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