Synaptic recruitment of gephyrin regulates surface GABA\(_A\) receptor dynamics for the expression of inhibitory LTP

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Postsynaptic long-term potentiation of inhibition (iLTP) can rely on increased GABA\(_A\) receptors (GABA\(_A\)Rs) at synapses by promoted exocytosis. However, the molecular mechanisms that enhance the clustering of postsynaptic GABA\(_A\)Rs during iLTP remain obscure. Here we demonstrate that during chemically induced iLTP (chem-iLTP), GABA\(_A\)Rs are immobilized and confined at synapses, as revealed by single-particle tracking of individual GABA\(_A\)Rs in cultured hippocampal neurons. Chem-iLTP expression requires synaptic recruitment of the scaffold protein gephyrin from extrasynaptic areas, which in turn is promoted by CaMKII-dependent phosphorylation of GABA\(_A\)-R-\(\beta3\)-Ser\(^{383}\). Impairment of gephyrin assembly prevents chem-iLTP and, in parallel, blocks the accumulation and immobilization of GABA\(_A\)Rs at synapses. Importantly, an increase of gephyrin and GABA\(_A\)R similar to those observed during chem-iLTP in cultures were found in the rat visual cortex following an experience-dependent plasticity protocol that potentiates inhibitory transmission \textit{in vivo}. Thus, phospho-GABA\(_A\)-R-\(\beta3\)-dependent accumulation of gephyrin at synapses and receptor immobilization are crucial for iLTP expression and are likely to modulate network excitability.
Inhibition in the central nervous system is involved in many processes, including dendritic computation, network oscillations and selection of cell assemblies. Changes in inhibitory synaptic strength are thus expected to profoundly affect network function. Over the last decade, accumulating evidence has shown that inhibitory synapses exhibit several forms of long-term plasticity, expressed at either the presynaptic or postsynaptic level. Although inhibitory plasticity at the presynaptic level has been extensively characterized, the detailed mechanisms of postsynaptic inhibitory plasticity remain elusive. Postsynaptic forms of long-term potentiation of inhibition (iLTP) have been shown to involve (i) changes in channel function; (ii) changes in GABAAR receptor (GABAAR) intracellular trafficking; or (iii) alterations in the intracellular chloride concentration. Interestingly, some of these types of synaptic plasticity depend on the activation of Ca²⁺/calmodulin-dependent kinase II (CaMKII). A key role of CaMKII has been shown in the cerebellum, when low-frequency stimulation of climbing fibres potentiates GABAergic synapses formed by stellate/basket cells onto cerebellar Purkinje neurons. Likewise, in hippocampal...
Results

CaMKII mediates synaptic GABA<sub>A</sub> receptor immobilization during iLTP.

To test postsynaptic potentiation of GABAergic synapses in cultured hippocampal neurons (16–18 days in vitro (DIV), see Methods), we adopted a chemical protocol based on moderate NMDAR activation (NMDA 20 μM, 6-cyano-7-nitroquinolin-2-3-dione (CNQX) 10 μM for 2 min). This protocol (Fig. 1a, upper panel) caused a persistent 41 ± 7% (n = 18; P < 0.001, one-way analysis of variance (ANOVA), followed by Dunnett’s post test, Fig. 1a,b) increase in the amplitude of miniature GABAergic synaptic currents (mIPSCs) and, similarly, the potentiation of the amplitude of spontaneous inhibitory postsynaptic currents (sIPSCs) (Supplementary Fig. 1a,b) and of evoked GABAergic currents (Supplementary Fig. 1c,d). Importantly, these forms of NMDA-induced potentiation of inhibition lasted up to 30 min, thus allowing referring to as chemically induced iLTP (chem-iLTP) (Supplementary Fig. 1a-e). The molecular modifications occurring during chem-iLTP were analysed 20 min after stimulation, when potentiation reached the steady state. The NMDA-mediated potentiation of mIPSCs amplitude was prevented by 10 min treatment with the CaMKII inhibitor KN-62 (3 μM) and by adding ICAPTA (1,2-bis(o-aminoophenoxo)ethane-N,N,N',N'-tetraacetic acid, 11 mM) in the recording pipette to avoid an increase of intracellular Ca<sup>2+</sup> (P < 0.001, one-way ANOVA, followed by Dunnett’s post test, Fig. 1a,b), thus suggesting a postsynaptic mechanism. In line with this, after NMDA stimulation, the total number of GABA<sub>A</sub>Rs and their postsynaptic accumulation were increased in a CaMKII-dependent manner, as revealed by immunolabelling for surface GABA<sub>A</sub>R<sub>z1</sub> subunit. Indeed, NMDA-treated neurons exhibited an increase in total surface GABA<sub>A</sub>Rs average immunoreactivity.
that was prevented by KN-62 (sham: 40.4 ± 1.4 a.u. per pixel, n = 23; NMDA: 48.4 ± 2.5 a.u. per pixel, n = 24; NMDA/KN-62: 39.7 ± 1.6 a.u. per pixel, n = 24, one-way ANOVA, followed by Newman–Keuls’ post test). Similarly, NMDA-induced enhancement of the density and intensity of synaptic GABA_ARs clusters was blocked by KN-62 treatment (clusters per 100 μm: sham = 65.2 ± 10.2 a.u., n = 23; NMDA = 122.5 ± 16.1 a.u., n = 24; NMDA/KN-62 = 90.5 ± 13.1 a.u., n = 24; one-way ANOVA, followed by Newman–Keuls’ post test). GABA_AR integrated fluorescence intensity: Sham = 1,701 ± 93 a.u.; NMDA = 2,200 ± 154 a.u.; NMDA/KN-62 = 1,449 ± 92 a.u.; n = 24 in each condition; one-way ANOVA, followed by Newman–Keuls’ post test) (Fig. 1c,d). Importantly, these results were accompanied by an unaffected density of vGAT-positive puncta after NMDA application (Supplementary Fig. 1g), thus arguing against changes in the number of GABAergic synapses during chem-iLTP. To further investigate the postsynaptic features of the NMDA-mediated potentiation of synaptic current amplitude, we next aimed at dissecting out the possibility that chem-iLTP was due to an actual increase of receptor number or to changes in receptor single-channel conductance. The non-stationary fluctuation analysis performed on evoked IPSCs (eIPSCs) recorded before and 20 min after the stimulation showed that after NMDA treatment GABA_ARs single-channel conductance was not altered, whereas the number of open receptors at the current peak significantly increased (Supplementary Fig. 1h). Taken together, these data demonstrate that NMDA-dependent iLTP is expressed at the postsynaptic level through an increased number of synaptic receptors and depends on CaMKII-mediated activity.

To understand whether the lateral diffusion of GABA_ARs contributes to changes in receptor number at inhibitory synapses during chem-iLTP, the mobility of individual QD-coupled GABA_ARs was monitored by SPT before and after NMDA stimulation (see Methods). These experiments describe the mobility of receptors present at the neuronal surface at the beginning of the experiment, as QD labelling was performed before the stimulation. The effects of NMDA treatment on the lateral mobility of GABA_ARs was first probed on cultured hippocampal neurons (DIV 16–18) transfected with GABA_AR β3 subunit at DIV 7. NMDA stimulation strongly immobilized recombinant β3-GABA_ARs at synapses (Fig. 1e) by reducing the diffusion coefficient of mobile receptors (median diffusion coefficient and interquartile range (IQR) before = 0.034 μm² s⁻¹, IQR: 0.012–0.073, n trajectories = 365; after = 0.013 μm² s⁻¹, IQR: 0.002–0.032; P < 0.001, Student’s t-test) and by promoting a massive increase in the fraction of immobile receptors (before: 0.29 ± 0.04; after NMDA: 0.50 ± 0.05; P < 0.001, Student’s t-test) (n trajectories before = 365, after = 351 from 26 neurons; Fig. 1f and Supplementary Movie 1). This result was reinforced by matched observations of the same GABA_ARs present at synapses before and 20 min after stimulation, showing that receptor diffusion coefficients were significantly reduced after NMDA application (n = 67 trajectories out of 351–365, P < 0.001, paired Wilcoxon test, Fig. 1g). Those effects were paralleled by increased GABA_AR confinement at synapses as indicated by the reduced steady state of the mean square displacement (MSD) curve (P < 0.001, paired t-test at steady state, Fig. 1h). In sham-treated controls, the diffusive properties of synaptic and extrasynaptic GABA_ARs remained stable over the duration of the experiment (Supplementary Fig. 2a,b). It is worth mentioning that NMDA stimulation did not alter the lateral mobility of extrasynaptic GABA_ARs (Supplementary Fig. 2c), suggesting that the immobilization observed during chem-iLTP was selective for synaptic receptors.

To assess the reliability of our SPT data on recombinant β3 subunits, the lateral mobility of endogenous z1 subunits was studied during chem-iLTP. The diffusion properties of native synaptic and extrasynaptic z1-containing GABA_ARs before and after NMDA stimulation were indistinguishable from those of transfected β3-containing GABA_ARs (Supplementary Fig. 2d–g).

Given the CaMKII dependence of chem-iLTP, we next examined the role of CaMKII in the immobilization of synaptic GABA_ARs during potentiation of inhibition. On treatment with KN-62 (3 μM), the mobility of all synaptic GABA_ARs was not affected by NMDA stimulation (n trajectories = 202–211 from 16 neurons; P > 0.05, Mann–Whitney U-test; Supplementary Fig. 2h). Consistently, matched observations of synaptic GABA_ARs before and after NMDA application revealed unchanged diffusion coefficient and confinement at synapses in the presence of KN-62 (n trajectories = 38 out of 202–211; P > 0.05, paired Wilcoxon test, Fig. 1i). As a control, we observed that under basal conditions, KN-62 treatment did not affect the mobility of synaptic GABA_ARs (Supplementary Fig. 2i). Similar to KN-62, KN-93 (5 μM), an alternative inhibitor of CaMKII activity, prevented the NMDA-mediated immobilization and confinement of GABA_ARs at synapses, leaving receptor lateral diffusion at synapses unaffected by NMDA (n trajectories = 258–265 from 19 neurons; Supplementary Fig. 2j; matched observations n trajectories = 53 out of 258–265, Fig. 1j). On the contrary, on treatment with KN-92 (5 μM), an inactive analogue of KN-93, the lateral mobility of synaptic GABA_ARs was significantly reduced and confined after NMDA stimulation (n trajectories = 216–222 from 18 neurons, Supplementary Fig. 2k; matched observations n trajectories = 51 out of 216–222, Fig. 1k).

The possibility that internalization of QD-receptor complexes during the experiment duration might affect the measurements of QD-receptor lateral mobility was assessed with the acid strip procedure to remove surface antibodies (see Methods). The lack of residual QDs when the acid strip was performed 25 min after QD labelling confirms that over this time span the SPT approach exclusively monitors surface receptors due to minimal QD receptor internalization (Supplementary Fig. 3). Altogether, these data indicate that during chem-iLTP, pre-existing surface GABA_ARs are immobilized at synapses via a CaMKII-dependent mechanism.

Catalytic activity of CaMKII is required for iLTP. To confirm that the catalytic activity of CaMKII is involved in NMDA-mediated iLTP, we overexpressed a CaMKII-K42R mutant, known to impair CaMKII-mediated phosphorylation. NMDA treatment failed to significantly potentiate mIPSCs amplitude in neurons transfected (at DIV 7) with CaMKII-K42R-mCherry (9.7 ± 8.7%, n = 12) as compared with neurons transfected with the control plasmid mCherry (43.3 ± 10.9%, n = 12; one-way ANOVA, followed by Dunnett’s post test, Fig. 2a,b). Next, the mobility of synaptic GABA_ARs on impairment of CaMKII kinase activity was studied during chem-iLTP induction with SPT experiments. In agreement with the electrophysiology results, the diffusion coefficients and the immobile fraction of β3-containing GABA_ARs in neurons expressing CaMKII-K42R-mCherry were not affected by the stimulation protocol and exhibited comparable values before and after NMDA treatment (n trajectories before = 246; after = 231 from 15 neurons; Fig. 2c).

On the contrary, β3-containing GABA_ARs tracked in control neurons expressing mCherry confirmed a significant reduction of the median diffusion coefficient and an increase in the immobile fraction after NMDA application (n trajectories before = 198; after = 184, from 14 neurons; Fig. 2c). Along the same lines, matched observations of GABA_AR-QD complexes before and 20 min after NMDA treatment demonstrated that CaMKII-K42R overexpression prevents the NMDA-induced drop of synaptic mobility.
Figure 2 | GABA_A receptor immobilization at GABAergic synapses during chem-iLTP depends on CaMKII kinase activity. (a) Example averaged traces of mIPSCs recorded before and after NMDA application from neurons overexpressing CaMKII-K42R-mCherry or mCherry alone as a control. (b) Quantification over time of the relative increase of mIPSCs amplitude after stimulation in control (mCherry) and in CaMKII-K42R-mCherry-expressing neurons ($n = 12$ in each condition). (c) Median diffusion coefficient (top, one-way ANOVA, followed by Bonferroni post test) and immobile fraction (bottom, one-way ANOVA, followed by Bonferroni post test) of synaptic GABA_A Rs tracked in neurons overexpressing the kinase dead mutant CaMKII-K42R ($n = 231–246$ from 15 neurons) or in control cultured hippocampal cells ($n = 184–198$ from 14 neurons). (d) Matched diffusion coefficients of individual synaptic GABA_A Rs before (black) and after NMDA treatment (red) in control neurons ($n_{\text{trajectories}} = 36$ out of $184–198$, $P < 0.01$, paired Wilcoxon test) and on overexpression of CaMKII-K42R. ($n_{\text{trajectories}} = 50$ out of $231–246$, $P < 0.05$, paired Wilcoxon test). (e) MSD over time plot of matched individual GABA_A Rs observed at synapses before and after NMDA in control ($n_{\text{trajectories}} = 36$, $P = 0.008$, paired t-test) and in CaMKII-expressing neurons ($n_{\text{trajectories}} = 50$, $P > 0.05$, paired t-test). Error bars represent s.e.m. “$P < 0.05$; “$P < 0.01$ and ns, nonsignificant.

receptor mobility and the increased receptor confinement at synapses observed in control neurons (matched observations $n_{\text{trajectories}}$: control $= 36$ out of $184–198$; CaMKII-K42R $= 50$ out of $231–246$; Fig. 2d,e).

In the attempt to additionally provide a more holistic description of the mobility of all surface GABA_A Rs during synaptic potentiation at inhibitory synapses and the contribution of CaMKII activity in this process, fluorescence recovery after photobleaching (FRAP) experiments were performed in control neurons and neurons exhibiting impaired CaMKII activity due to CaMKII-K42R overexpression at DIV 7. The mobility of GABA_A Rs was estimated from the recovery of super-ectoplasmic pHlueorin (SEP)-tagged-β3 fluorescence in photobleached synaptic areas visualized by live vGAT staining (see Methods) before and after NMDA stimulation on the same neuron. The pH sensitivity of SEP-β3 (Supplementary Fig. 4a–d) demonstrates that these FRAP experiments describe the mobility of only surface receptors. After NMDA treatment in control mCherry-expressing neurons, SEP-β3 fluorescence recovery at synapses was significantly reduced as compared with basal conditions (before: 45 ± 2 %, $n = 56$; after NMDA: 38 ± 2 %, $n = 69$; Supplementary Fig. 4e,f, Student’s t-test), suggesting a global lower GABA_A R mobility at synapses on plasticity induction. This effect was prevented by the overexpression of the inactive CaMKII, as the fluorescence recovery of SEP-β3 was comparable before (45 ± 2 %, $n = 61$) and after NMDA treatment in CaMKII-K42R-mCherry-transfected neurons (44 ± 3 %, $n = 70$ synapses from three neuronal preparations; Supplementary Fig. 4e,f). Overall, these experiments show that CaMKII activity is required for the immobilization of surface GABA_A Rs at inhibitory synapses during synaptic potentiation of inhibition.

Phosphorylation of GABA_A R-β3 Ser^{383} is essential for iLTP. In another set of experiments we examined whether CaMKII phosphorylation of GABA_A Rs could contribute to chem-iLTP expression. Among the potential phosphorylation sites identified on GABA_A R subunits, only the Ser^{383} residue of the β3 subunit is exclusively phosphorylated by CaMKII^{34} with direct functional implications on GABA_A R-mediated currents^{35,36}. The possible involvement of Ser^{383} phosphorylation by CaMKII in chem-iLTP expression was directly tested with a specific anti-phospho-β3Ser^{383} antibody^{36} in immunoblot assays before and at various time points (10 and 20 min) after the stimulation protocol (Fig. 3a). Although the levels of Ser^{383} phosphorylation remained
constant at all time points in sham-treated hippocampal cultures, in NMDA-treated neurons the phosphorylation of β3Ser⁴⁸³ progressively increased, becoming significant 20 min after stimulation (n = 8; P = 0.04, one-way ANOVA followed by Dunn’s post test; Fig. 3b).

To assess the functional role of β3-Ser⁴⁸³ phosphorylation on chem-iLTP, the effects of NMDA stimulation were tested in neurons expressing β3S³⁸³A, a mutation that prevents the phosphorylation by CaMKII on this residue²⁴. Experiments were performed on DIV 16–18 hippocampal neurons transfected with β3S³⁸³A or β3wt at DIV 7. GABAergic synaptic currents were recorded from β3S³⁸³A-expressing neurons were not significantly potentiated after NMDA treatment (10.9 ± 14.6%, n = 10; P > 0.05, one-way ANOVA, followed by Dunnett’s test post) in contrast to those recorded from β3-expressing neurons (44.6 ± 9.2 %, n = 10, from three independent neuronal preparations; P < 0.01, one-way ANOVA, followed by Dunnett’s post test; Fig. 3c,d). In parallel, NMDA stimulation on β3S³⁸³A-containing neurons left unchanged the total surface expression and synaptic clustering of GABAₐRs, assessed by live surface z₁ subunit immunostaining as compared with control neurons (n = 24 cells in each condition, from three independent neuronal preparations; P = 0.00 ns, paired t-test) and immobile fraction (P > 0.05, Student’s t-test). (f) Matched diffusion coefficients of individual β3S³⁸³A receptors present at synapses before and after NMDA (n observers = 61 out of 236-272; P > 0.05, paired Wilcoxon test). Unless otherwise stated, error bars represent s.e.m. *P < 0.05; **P < 0.01; ns, nonsignificant.
preparations; one-way ANOVA, followed by Newman–Keuls’ post test; Fig. 3c,f). In control experiments, we found that the transfection of the β3 subunit did not alter surface GABA_A receptors expression and synaptic clustering, as the basal amount of GABA_A receptors synaptic clusters immunoprobated for γ1 or β3 subunits in β3-expressing neurons was comparable to those in control (enhanced GFP (EGFP) expressing) neurons (Supplementary Fig. 5). SPT experiments revealed that surface GABA_A receptors carrying the β3S383A point mutation were not immobilized at synapses after chemical induction of iLTP (Fig. 3g and Supplementary Movie 2). In contrast to β3wt receptors (Fig. 1f–h), the confinement, diffusion coefficient and immobile fraction of synthetic β3S383A receptors were unaffected by NMDA treatment (ntrajectories: before = 272; NMDA = 263 from 17 neurons; matched observations – 61; Fig. 3h–j). These findings indicate that CaMKII phosphorylation at β3S383 plays a pivotal role in the accumulation and retention of GABA_A receptors at synapses during chem-iLTP.

Recruitment of extrasynaptic gephyrin to synapse during iLTP. We next investigated whether synaptic GABA_A receptor immobilization during chem-iLTP was associated with an increase in the scaffold protein gephyrin. Immunocytochemical experiments revealed that following NMDA stimulation, the fluorescence intensity, density and area of synaptic gephyrin clusters increased as compared with that of controls. These effects were completely abolished by KN-62 (3 μM) (n cells: sham = 22, NMDA = 28, NMDA + KN-62 = 34, from 4 different neuronal preparations; one-way ANOVA, followed by Newman–Keuls’ post test; Fig. 4a,b), indicating that the accumulation of gephyrin at synapses during potentiation of inhibition is CaMKII dependent. To corroborate this result and to exclude any concern about the specificity of the antibody used, we quantified gephyrin availability during chem-iLTP by immunocytochemistry with an alternative anti-gephyrin antibody (Supplementary Fig. 6) as well as by live fast-confocal imaging of recombinant gephyrin in an antibody-free assay (see below). Notably, the increase in synaptic gephyrin clustering detected with the mAb3B11 antibody after NMDA treatment was similar to that detected with the mAb7a antibody (synaptic cluster integrated intensity; sham = 861.0 ± 41.2 a.u., n = 53; NMDA = 1096.0 ± 45.5 a.u., n = 51; P < 0.001, Student’s t-test, Supplementary Fig. 6). To assess whether the NMDA-induced increase in synaptic gephyrin

**Figure 4 | During chem-iLTP gephyrin is accumulated at inhibitory synapses.** (a) Representative confocal images of hippocampal neurons immunoprobated for gephyrin (red), vGAT (blue) in EGFP-expressing neurons (green). Neurons treated with a sham solution (left), NMDA (middle) or NMDA + KN-62 (right) are compared. Scale bar, 10 μm. (b) Quantification of synaptic gephyrin integrated intensity, cluster density and cluster area in the indicated conditions (n: sham = 22, NMDA = 28, NMDA + KN-62 = 34; one-way ANOVA, followed by Newman–Keuls’ post test). (c) Left: representative immunoblotting of total protein levels of gephyrin 20 min after incubation with sham solution or NMDA, in control conditions and on application of CHX to inhibit protein synthesis. Right: quantification of gephyrin protein levels 20 min after NMDA stimulation normalized to sham treatment shows no significant gephyrin upregulation after 20 min (n = 10; one-way ANOVA followed by Dunn’s post test, P = 0.99). (d) Left: example immunoblotting of total gephyrin levels performed on neuronal lysates 45 min after sham or NMDA stimulation in control conditions and in the presence of CHX. Right: quantification of WB assays described on the left indicates that 45 min after NMDA application gephyrin protein levels are significantly increased (n = 10; one-way ANOVA followed by Dunn’s post test, P = 0.04). γ-Adaptin immunoreactivity is reported in c and d to check equal loading. Error bars represent s.e.m. **P < 0.01; ***P < 0.001 and ns, nonsignificant.
Figure 5 | Gephyrin redistribution to synapses during chem-iLTP. (a) Example image of presynaptic terminal live staining (with vGAT-Oyster 550) for the localization of inhibitory synapses. Dotted lines depict the profile of the dendrite. The yellow line indicates the position of the linescan reported in e, beginning at (o) and ending at (oo). Scale bar, 2 μm. (b) Pseudocolour images of mRFP-gephyrin fluorescence in the same dendrite as in a at different time points before and after NMDA stimulation (plain arrow). Arrows indicate gephyrin synaptic clusters that increase during chem-iLTP. (c) Normalized gephyrin average fluorescence (after NMDA/before NMDA) observed at soma and dendrites (n = 13 neurons; P = 0.30, Mann–Whitney U-test). (d) Gold pseudocolour representation of the same images shown in b with a different scale to highlight the decrease of extrasynaptic gephyrin (arrowhead) over time. Note that with such scale, synaptic gephyrin clusters are saturated. The arrowhead indicates extrasynaptic gephyrin. (e) Linescans (15 pixels scan width) of gephyrin fluorescence before and 22 min after NMDA stimulation along the line reported in a. The beginning (o) and the end (oo) of the linescans correspond to those in a. The arrows indicate gephyrin synaptic clusters as in b, the arrowhead indicates extrasynaptic gephyrin as in d. (f) Cumulative fluorescence intensity of extrasynaptic gephyrin in the portion of dendrite shown in d. Axis breaks indicate the exclusion of synaptic clusters. (g) NMDA-induced changes in gephyrin intensity shown in b and d at extrasynaptic (extrasyn) compartments are on par with those at synapses (syn). The bar plot represents the difference in normalized gephyrin fluorescence at t = 22 min and t = −3 min. (h) Quantification of normalized total and extrasynaptic gephyrin fluorescence intensity over time after NMDA application (plain arrow) in 13 neurons (total: P = 0.36; extrasynaptic: P = 0.006; one-way ANOVA followed by Dunn’s post test for each plot). In live fast-confocal microscopy experiments, photobleach over time was quantified in sham-treated controls (n = 11 neurons) and corrected accordingly. Data are expressed as mean ± s.e.m. **P < 0.01; and ns, nonsignificant.

involved de novo protein synthesis, western blot (WB) assays were conducted on stimulated neurons in the presence or absence of the protein synthesis inhibitor cycloheximide (CHX, 0.5 mg ml⁻¹). Quantitative analysis of gephyrin immunoblots revealed that within 20 min after stimulation, de novo synthesis was not significantly contributing to gephyrin increase (gephyrin intensity: ctrl = 100%; NMDA = 98.4 ± 14.0%; NMDA + CHX = 99.5 ± 18.4%; n = 10; P = 0.99, one-way ANOVA test followed by Dunn’s post test; Fig. 4c), whereas at 45 min the total gephyrin protein levels were significantly upregulated as compared with control (gephyrin intensity: ctrl = 100%; NMDA = 118.4 ± 9.9%; NMDA + CHX = 102.5 ± 8.6%; n = 10; P = 0.04, one-way ANOVA followed by Dunn’s post test; Fig. 4d).

Having excluded de novo gephyrin protein synthesis in the first 20 min after NMDA stimulation, the source of gephyrin synaptic increase in the early phase of chem-iLTP expression remains to be assessed. To this purpose, the distribution of mRFP-tagged gephyrin was studied over time before and after NMDA stimulation by live fast-confocal microscopy (see Methods). Hippocampal neurons were transfected with mRFP-gephyrin at DIV 7 and observed at DIV 16–18 after live labelling with an anti-vGAT Oyster650 antibody to visualize presynaptic terminals (Fig. 5a). Fluorescence images were acquired every 60 s for 6 min before and for 26 min after NMDA stimulation (see Methods). Data were corrected for fluorescence photobleaching estimated in control experiments by replacing NMDA with a sham solution (11 neurons from 4 independent neuronal preparations). In line with the immunocytochemistry results, we first observed that the fluorescence intensity of mRFP-gephyrin synaptic clusters (exhibiting a juxtaposed vGAT puncta) significantly increased over time after NMDA stimulation (Fig. 5b,e, see also Fig. 6 and text below). Next, to explore the possibility of gephyrin
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Figure 6 | Time course of GABA<sub>A</sub>R and gephyrin synaptic increase during chem-iLTP. (a) Example pseudocolour images of simultaneous live fast-confocal imaging of GABA<sub>A</sub>R (SEP-β3) and gephyrin-mRFP at different time points in neurons stimulated with NMDA (black arrow). White arrows indicate synaptic clusters. Scale bar, 2.5 μm. Note that, similar to Fig. 5b,d, the pseudocolour scale required to see changes in gephyrin cluster intensity does not allow visualization of extrasynaptic gephyrin. (b) Relative GABA<sub>A</sub>R and gephyrin fluorescence increase over time during chem-iLTP (n = 144 areas/time point in 13 different neurons; one-way ANOVA followed by Dunn’s post test, P < 0.001 for each curve). The time points include those indicated in a. (c) ΔF/F<sub>max</sub> plot over time of GABA<sub>A</sub>R and gephyrin fluorescence intensities shows that increase of GABA<sub>A</sub>R slightly, although not significantly, precedes that of gephyrin (n = 144 areas/time point; synapses; two-way ANOVA followed by Bonferroni post test). In the live fast-confocal imaging experiments, photobleach over time was quantified in sham-treated controls and corrected accordingly. Data are presented as mean ± s.e.m. ***P < 0.001 and ns, nonsignificant.

Gephyrin synaptic increase requires phosphorylated β<sub>3</sub>S<sub>383</sub>. To investigate the relative timing of NMDA-induced accumulation of GABA<sub>A</sub>R and gephyrin at synapses, we simultaneously monitored SEP-β3-GABA<sub>A</sub>R and mRFP-gephyrin synaptic fluorescence over time before and after iLTP induction (Fig. 6a) by using the live fast-confocal microscopy approach described above (see also Methods). On NMDA application, the fluorescence of SEP-β3 synaptic clusters increased over time (n = 144 areas/time point in 13 different neurons from 4 independent neuronal preparations; one-way ANOVA test followed by Dunn’s post test, P < 0.001; Fig. 6a,b) and was paralleled by a similar increase of mRFP-gephyrin synaptic cluster intensity (n = 144 areas/time point in 13 different neurons from 4 independent neuronal preparations; one-way ANOVA test followed by Dunn’s...
post test, $P<0.001$; Fig. 6a,b). Data were corrected for fluorescence photobleaching estimated in control experiments by replacing NMDA with a sham solution ($n=120$ areas/time point in 11 different neurons from 4 independent neuronal preparations). It is worth pointing out that the results of this antibody-free assay are consistent with those obtained by immunocytochemistry (see also Fig. 1c,d, Fig. 4a,b and Supplementary Fig. 6). Next, we calculated the normalized time course of live GABA$_A$Rs and gephyrin fluorescence increase (AF/Fmax) during chem-iLTP (Fig. 6c, $n=144$, 13 different neurons from 4 independent neuronal preparations). Unexpectedly, we found that gephyrin increase did not significantly precede GABA$_A$Rs accumulation at synapses ($P=0.49$, two-way ANOVA test followed by Bonferroni post test), thus challenging the consolidated idea that adjustments in scaffold availability precede changes in receptor number at synapses.

To clarify the functional link between CaMKII activity on β3 subunit and the concerted increase of GABA$_A$R/gephyrin at synapses, we next studied the role of β3S383 phosphorylation in the accumulation of synaptic gephyrin during chem-iLTP (Fig. 7a,b). In β3S383A-expressing neurons (transfected at DIV 7 observed at DIV 16–18), NMDA did not increase synaptic gephyrin as compared with sham treatment. On the contrary, same protocol applied to neurons transfected with β3wt induced a significant enhancement of synaptic gephyrin accumulation ($n=24$ cells in each condition; one-way ANOVA, followed by Newman–Keuls’ post test; Fig. 7c). Moreover, the increase in synaptic gephyrin induced by NMDA in β3wt-transfected neurons was comparable to that in EGFP-transfected neurons (see Fig. 4a,b), indicating that receptor overexpression does not alter gephyrin synaptic abundance under basal conditions and during chem-iLTP. In control experiments under basal conditions, gephyrin immunoreactivity levels were not affected by β3 overexpression, being indistinguishable among β3wt, β3S383A, and control EGFP-transfected neurons (Supplementary Fig. 7). These data suggest that CaMKII phosphorylation of β3S383 residue is essential to promote gephyrin accumulation at GABAergic synapses.

Figure 8 | Impaired gephyrin clustering affects GABA$_A$R mobility. (a) Representative confocal images of a control neuron expressing EGFP (green, left) and gephyrin 2-188-EGFP (DN) (green, right) immunoprobed for gephyrin (red) and vGAT (blue). Scale bar, 10 μm. Plain arrowheads indicate gephyrin synaptic clusters; empty arrowheads show vGAT puncta lacking juxtaposed gephyrin. (b) Quantification of the immunocytochemical assays comparing gephyrin synaptic cluster density (left), synaptic cluster integrated intensity (middle) and synaptic cluster area (right) in gephyrin 2-188-expressing neurons ($n=23$) and in control neurons ($n=33$, Student’s t-test). (c) Left: representative traces of sIPSCs recorded from control and gephyrin 2-188-expressing neurons in basal conditions. Right: plot bars of average sIPSCs amplitude and frequency in control ($n=11$) and 2-188-expressing neurons ($n=11$), Student’s t-test. (d) Representative confocal images of neurons immunoprobed for GABA$_A$R (α1-subunit, green) and for vGAT (red) under control conditions and on impairment of gephyrin assembly. Arrowheads indicate synaptic GABA$_A$R clusters. Scale bar, 1 μm. (e) Quantification of total surface GABA$_A$R average immunoreactivity (left), synaptic cluster density (middle) and integrated intensity of synaptic clusters (right) on gephyrin 2-188 overexpression. ($n=24$ cells in each condition from three independent hippocampal cultures, Student’s t-test). (f) Reconstructed trajectories of β3-containing receptors diffusing at synaptic (red) and extrasynaptic (blue) compartments in control neurons (left) and on gephyrin impairment (right). Scale bar, 1 μm. (g) Median diffusion coefficient (and IQR) of synaptic GABA$_A$Rs in control ($n_{\text{trajectories}}=186$ from 12 neurons) and in gephyrin 2-188-expressing neurons ($n_{\text{trajectories}}=121$ from 10 neurons, $P=0.005$, Mann–Whitney U-test) and the corresponding receptor immobile fraction ($P<0.005$, Student’s t-test). (h) Diffusion coefficient (median ± IQR) of the mobile receptor population in control and on gephyrin impairment ($P>0.05$, Mann–Whitney U-test). Unless otherwise stated data are expressed as mean and error bars represent s.e.m. *$P<0.05$; **$P<0.01$; ***$P<0.001$; ns, nonsignificant.
Gephyrin availability is essential for iLTP. Although the phospho-
lation of GABA<sub>AR</sub>-β3<sup>382</sup> induces gephyrin accumulation at
synapses, it remains to be established whether the synaptic
increase of gephyrin is necessary for chem-iLTP. To explore the
involvement of synaptic gephyrin in chem-iLTP expression,
NMDA stimulation was applied to neurons transfected at DIV 7
with a dominant-negative (DN) gephyrin polypeptide, gephyrin
2-188, that hampers gephyrin assembly<sup>38</sup>. In a first set of
experiments, we assessed the efficiency of this DN in disrupting
endogenous gephyrin clustering (Fig. 8a). Gephyrin 2-188
overexpression markedly reduced the density of gephyrin
synaptic clusters (ctrl: 112.8 ± 9.6 clusters per 100 μm, n = 35;
DN: 50.0 ± 6.3 clusters per 100 μm, n = 23; P < 0.001, Student’s t-test) and also decreased the integrated fluorescence intensity
(ctrl: 591.1 ± 36.9 a.u., DN: 280.8 ± 42.8 a.u.; P = 0.005, Student’s
test) and the area (ctrl: 0.97 ± 0.03 a.u., DN: 0.85 ± 0.03 a.u.;
P = 0.005, Student’s t-test) of gephyrin synaptic clusters (Fig. 8b).
It is worth mentioning that the anti-gephyrin antibody mAb7α,
raised using the amino terminus portion of gephyrin did not
recognize the gephyrin 2-188 polypeptide in immunocyto-
chemistry. Overexpression of the DN gephyrin 2-188 also
affected spontaneous GABAergic synaptic transmission as
compared with control. Indeed, in gephyrin 2-188-expressing
neurons the amplitude of sIPSCs as well as the frequency was
significantly reduced (n = 11 cells in each condition, Student’s
t-test; Fig. 8c). Nonetheless, the peak amplitude and frequency of
miniature GABAergic currents recorded in neurons expressing
gephyrin 2-188 did not differ with respect to control neurons
(Supplementary Fig. 8). Consistent with these observations,
gephyrin 2-188 moderately affected the surface expression of
GABA<sub>AR</sub>Rs. Indeed, in gephyrin 2-188-expressing neurons
the total surface receptor average immunoreactivity (ctrl: 26.4 ± 2.3 a.u. per pixel, n = 24; DN: 25.0 ± 1.4 a.u. per pixel,
n = 24; P = 0.05, Student’s t-test) and synaptic cluster density
of z1 subunit (ctrl: 42.8 ± 5.0 clusters per 100 μm, n = 24; DN:
32.4 ± 5.8 clusters per 100 μm, n = 24; P = 0.05, Student’s t-test;
Fig. 8d,e) were comparable to control neurons, although the
integrated intensity of GABA<sub>AR</sub> synaptic clusters was
significantly reduced (ctrl: 1875 ± 211 a.u., n = 24; DN:
1609 ± 154 a.u., n = 24; P = 0.04, Student’s t-test; Fig. 8d,e).
However, disruption of endogenous gephyrin assembly
markedly increased the overall GABA<sub>AR</sub> mobility with respect
to control (Fig. 8f). In particular, gephyrin 2-188 increased the
median diffusion coefficient of total synaptic GABA<sub>AR</sub>Rs (ctrl:
0.022 μm<sup>2</sup> s<sup>-1</sup>, IQR = 0.004–0.059, n = 186 from 12 neurons;
geph2-188: 0.040 μm<sup>2</sup> s<sup>-1</sup>, IQR = 0.016–0.089, n = 121 from 10
neurons from 6 independent hippocampal cultures; P = 0.005,
Mann–Whitney U-test; Fig. 8g) and reduced the fraction of
immobile receptors at synapses (ctrl: 0.31 ± 0.04, n<sub>trajectories</sub> = 186;
DN: 0.19 ± 0.02, n<sub>trajectories</sub> = 121; P < 0.005, Student’s t-test;
Fig. 8g). Interestingly, the diffusion coefficient of mobile
receptors was similar in control and gephyrin 2-188-expressing
neurons (ctrl: 0.049 μm<sup>2</sup> s<sup>-1</sup>, IQR = 0.022–0.099, n = 128;
DN: 0.050 μm<sup>2</sup> s<sup>-1</sup>, IQR = 0.024–0.010, n = 98; P > 0.05,
Mann–Whitney U-test; Fig. 8h), indicating that the main effect of
gephyrin impairment on GABA<sub>AR</sub> mobility is to reduce the
trapping of GABA<sub>AR</sub> at synapses, rather than to change the
intrinsically diffusion properties of GABA<sub>AR</sub>Rs.

Gephyrin and GABA<sub>AR</sub>Rs are upregulated during iLTP in vivo. To
test the physiological relevance of our observations, we
focused on monocular deprivation (MD), a classical in vivo
protocol of experience-dependent plasticity. By degrading the
spatial vision through the closed eye, brief MD causes loss of
responsiveness in principal neurons in the primary visual cortex
(V1) accompanied by the potentiation of inhibitory transmission
selectively onto pyramidal cells<sup>30–32</sup>. In pigmented rats, a brief
MD episode (2 days) at the peak of the critical period for ocular
dominance plasticity (P22–P24) has been reported to induce iLTP
at synapses between inhibitory fast-spiking interneurons and
pyramidal cells in the main thalamicoreipient lamina of the visual
cortex (layer 4)<sup>30–32</sup>. Here we investigated whether such sensory
immunolabelling of surface z1 subunits revealed that interfering
with gephyrin assembly prevented the accumulation of GABA<sub>AR</sub>Rs
at synapses after NMDA treatment (synaptic integrated intensity:
sham: 1.788 ± 228 a.u., n = 21; NMDA = 1.860 ± 192 a.u.,
n = 23; P > 0.05, Student’s t-test, Fig. 9b). However, despite
gephyrin impairment, an increase in the total GABA<sub>AR</sub> surface
expression was still observed after NMDA application in 2-188-
expressing neurons (total average intensity: sham: 21.6 ± 1.6 a.u.
per pixel, n = 21; NMDA = 27.3 ± 2.9 a.u. per pixel, n = 23;
P = 0.04, Student’s t-test, Fig. 9b), indicating that gephyrin
involvement in chem-iLTP is downstream of receptor exocytosis.
Consistent with this interpretation, gephyrin impairment com-
pletely abolished NMDA-induced GABA<sub>AR</sub> immobilization,
leaving GABA<sub>AR</sub> mobility similar to that under basal conditions
(Fig. 9c and Supplementary Movie 3). Indeed, no reduction was
observed in the confinement of synaptic GABA<sub>AR</sub>Rs during
matched observations before and after NMDA application in
gephyrin 2-188-expressing neurons (n<sub>trajectories</sub> = 26 out of 100–
118 from 8 neurons; P > 0.05, paired t-test at the steady state
of the MSD curve, Fig. 9d). Accordingly, the median diffusion
coefficient of all synaptic receptors was not affected after NMDA,
as compared with before (before: 0.0535 μm<sup>2</sup> s<sup>-1</sup>, IQR = 0.0301–
0.0895, n<sub>trajectories</sub> = 118; after: 0.0513 μm<sup>2</sup> s<sup>-1</sup>, IQR = 0.0161–
0.1168, n<sub>trajectories</sub> = 100 from 8 neurons; P > 0.05 Mann–Whitney
U-test, Fig. 9e). In addition, matched tracking of individual
synaptic receptors revealed unchanged diffusion coefficient before
and after NMDA treatment in gephyrin 2-188-expressing
neurons (n<sub>trajectories</sub> = 26; P > 0.05, paired Wilcoxon test, Fig. 9e).

To confirm these findings, we used chronic administration of
gephyrin antisense oligonucleotides (5 μM) from DIV 2 to DIV 16 as
an alternative approach to impair gephyrin function<sup>18,39</sup>. FRAP
experiments performed to assess GABA<sub>AR</sub> mobility at
synapses showed that gephyrin antisense oligonucleotides
abolished the NMDA-induced GABA<sub>AR</sub> immobilization
(Fig. 9f). Indeed, the SEP-β3 fluorescence recovery observed at
synapses in antisense oligonucleotides-treated neurons was
similar before and after NMDA treatment (before: 44.7 ± 2.1%,
n = 56; after: 44.2 ± 2.6, n = 55; P > 0.05, Student’s t-test). On
the contrary, control neurons treated with sense oligonucleotides
exhibited reduced FRAP of synaptic SEP-β3 fluorescence
after NMDA stimulation (before: 44.5 ± 1.7%, n = 93; after:
36.0 ± 1.6%, n = 89; P = 0.002, Student’s t-test, Fig. 9f,g).
It is worth emphasizing that NMDA-induced GABA<sub>AR</sub> immobilization
observed by FRAP in neurons treated with sense oligonucleotides
was transferred with mCherry was similar (compare Fig. 9f,g with
Supplementary Fig. 4e,f). These data reinforce the hypothesis that
collection of gephyrin to synapses and the consequent accumulation and immobilization of
GABA<sub>AR</sub>Rs at synapses are crucial for chem-iLTP expression
and suggest that the total population of surface GABA<sub>AR</sub>Rs is
involved.
manipulation also upregulates gephyrin and GABA$_A$R clustering in layer 4 pyramidal cells in V1. To this purpose two independent sets of immunohistochemical assays for gephyrin/GABA/DAPI and for GABA$_A$R/vGAT/GABA/DAPI were performed on brain slices from monocularly deprived pigmented rats, along with age-matched controls (Fig. 10a,b). We focused on layer 4 binocular visual cortex (V1b), particularly in the somatic area of pyramidal cells, the neuronal compartment where the vast majority of synapses between fast-spiking interneurons and pyramidal cells are located (Fig. 10c). Inhibitory neurons were identified by GABA-positive staining (Fig. 10a and Supplementary Fig. 9) and were excluded from further analysis. In a first set of immunohistochemical experiments the total gephyrin fluorescence intensity (see Methods) was quantified in the maximal projection of confocal stacks. We found that MD slices had higher gephyrin average immunoreactivity compared with control (MD: 17.3 ± 0.7 a.u. per pixel, n = 16 slices; ctrl: 13.8 ± 1.3 a.u. per pixel, n = 12 slices, P = 0.02, Student’s $t$-test;
Fig. 10a,d). This result was confirmed with a more detailed analysis focusing on the three-dimensional reconstruction of the perisomatic region of each GABA-negative principal cell, showing that the integrated fluorescence intensity of somatic gephyrin in MD slices was larger with respect to control slices (ctrl: 123,379 ± 5,059 a.u., n = 474 cells; MD: 152,782 ± 5,341 a.u., n = 606 cells; P < 0.0001, Student’s t-test, Fig. 10d). In a parallel set of experiments, the expression and synaptic localization of GABA_ARs was studied to assess whether, also in vivo, potentiation of synaptic inhibition is sustained by increased

Figure 10 | Gephyrin and GABA_ARs are upregulated in the rat visual cortex after brief monocular deprivation (MD). (a,b) Representative confocal images of IHC experiments on slices from control and MD rats immunoprobed for (a): gephyrin (red), GABA (green) and DAPI (blue) or for (b): GABA_ARs (green), vGAT (red), DAPI (blue) and GABA (see Supplementary Fig. 9). In both protocols, GABA staining was used to selectively analyse GABA-negative bona fide excitatory neurons. Top: representative maximal projections of 18 confocal z-stack images (7.5 μm depth) of the binocular visual cortex (V1b) acquired from control and MD samples. Scale bar, 15 μm. Bottom: magnification of the boxes above. Three equatorial planes of the cells indicated by arrowheads are merged. Arrows in b show GABA_AR synaptic clusters. Scale bar, 10 μm in all images. (c) Example of a low magnification (× 4) transmitted light image of the slice used for IHC. The framed box indicates the area acquired at higher magnification (× 60) corresponding to layer 4. vGAT. Scale bar, 200 μm. (d) Quantification of gephyrin immunostaining in slices from control (light grey) and MD (dark grey) animals (28 optical planes corresponding to 11.7 μm were considered) (n = 12–16, Student’s t-test). Total gephyrin average fluorescence (left) and gephyrin-integrated fluorescence detected in the somatic region of each principal cell (right) are shown. (e) Left: total GABAAR average fluorescence intensity in control and MD slices (n = 15 in each condition, Student’s t-test). Middle: integrated fluorescence intensity of GABA_AR clusters identified in the somatic region of principal cells in control and MD slices (n = 495 and 552 cells, respectively, Student’s t-test). Right: density of synaptic GABAAR clusters in slices from MD rats and age-matched controls (n = 495–552 cells, Student’s t-test). *P < 0.05; **P < 0.001; Student’s t-test. Data are expressed as means ± s.e.m.

Figure 9 | Gephyrin impairment prevents chem-ilTP and the associated GABA_AR accumulation and immobilization at synapses. (a) Left: representative traces of mIPSCs in control cultured 2188-expressing neurons before and after NMDA. Right: relative amplitude increase of mIPSCs after NMDA application on gephyrin 2-188 (green, n = 12) and control (black, n = 14) neurons (one-way ANOVA, followed by Dunnett’s post test). (b) Left: representative confocal images of surface GABA_AR immunoreactivity (α1-subunit, green) opposed to vGAT (red) in sham and MD-treated neurons overexpressing gephyrin 2-188. Scale bar, 10 μm. Right: quantification of the total surface GABA_AR average immunoreactivity and synaptic GABA_AR cluster intensity in gephyrin 2-188-expressing neurons treated with NMDA or with a sham solution (n = 21–23 cells, from three independent neuronal preparations; Student’s t-test) (c) Left: representative trajectories (yellow) of an individual GABA_AR (β3 subunit-QD) diffusing on a neuron transfected with EGFP-gephyrin 2-188 (green) before and after NMDA application. Presynaptic terminals are represented in red (vGAT). Scale bar, 1 μm. See Supplementary Movie 3. Right: diffusion coefficient versus time plots of the GABA_AR-QD complex shown on the left before and after NMDA treatment. Lines above the curves represent synaptic (red) and extrasynaptic (black) localization of the particle. (d) MSD versus time plot of matched individual synaptic GABA_AR clusters monitored before and after NMDA stimulation in gephyrin 2-188-expressing neurons (n trajectories = 26 from 8 neurons, P < 0.05, paired t-test at steady state). (e) Left: diffusion coefficient (median ± IQR) of total synaptic receptors after NMDA treatment (n trajectories = 100) as compared with before (n trajectories = 118, from 8 neurons from 3 independent hippocampal cultures, P > 0.05, Mann–Whitney U-test). Right: matched diffusion coefficients of the same receptors at synapses before and after NMDA (n trajectories = 26 out of 100–118, P > 0.05, paired Wilcoxon test). (f) FRAP over time curves of surface synaptic SEP-β3 receptors before and after NMDA treatment in neurons treated with gephyrin sense (left, n = 93–89) and antisense (right) oligonucleotides (left, n = 56–55). (g) FRAP recovery before (black) and after (violet) NMDA treatment of synaptic SEP-β3 fluorescence in neurons exposed to the control oligonucleotides (gephyrin sense, left, n = 93–89, Student’s t-test) or gephyrin antisense oligonucleotides (right, n = 56–55, Student’s t-test). Unless otherwise stated, data are expressed as means ± s.e.m. *P < 0.05; **P < 0.01; ns, nonsignificant.

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GABA<sub>A</sub>R synaptic clustering. Confocal images of sagittal brain slices immunoprobed for GABA<sub>A</sub>R, vGAT, GABA and DAPI (Fig. 10b and Supplementary Fig. 9) showed a significant increase in total GABA<sub>A</sub>R average immunoreactivity in the visual cortex of MD animals as compared with aged-matched controls (MD: 29.3 ± 1.6 a.u. per pixel, n = 15 slices; ctrl: 21.6 ± 1.3 a.u. per pixel, n = 15 slices, P = 0.001, Student’s t-test; Fig. 10e). A specific analysis focusing on the perisomatic region of 3D-reconstructed principal (GABA-negative, see Supplementary Fig. 9) cells revealed that the integrated fluorescence intensity of GABA<sub>A</sub>Rs clusters in the somatic area of principal cells in the V1b region was larger in slices from MD rats than in those from controls (ctrl: 369.8 ± 33.8, n = 495 cells; MD: 670.9 ± 48.4, n = 552 cells, Student’s t-test). Finally, we observed that the manipulation of the sensory environment of the animal associated with iLTP also significantly increased the density of synaptic GABA<sub>A</sub>Rs clusters, defined as those juxtaposed to vGAT immunoreactivity (ctrl: 0.57 ± 0.08, n = 495 cells; MD: 1.34 ± 0.13, n = 552 cells, Student’s t-test). Thus, a brief period of MD, that is sufficient to cause iLTP in layer 4 pyramidal neurons in rat V1, likewise leads to perisomatic upregulation of gephyrin and synaptic GABA<sub>A</sub>Rs.

Discussion

In this study, we addressed for the first time the detailed mechanisms leading to postsynaptic potentiation of GABAergic synapses. Our data highlight an important role for gephyrin and CaMKII activity in iLTP expression. In particular, we show that gephyrin recruitment at synapses is essential for iLTP, but is dependent on CaMKII phosphorylation of the GABA<sub>A</sub>R β3S383 residue. As a consequence, GABA<sub>A</sub>Rs are accumulated and retained at synapses, thus mediating increased inhibitory synaptic responses. In support of the physiological relevance of the model proposed here, we also report that gephyrin upregulation and increased GABA<sub>A</sub>R accumulation at synapses are observed in principal cells in the primary visual cortex of rodents after MD, a classical in vivo model of experience-dependent plasticity. These latter results suggest that the molecular mechanisms underlying chem-iLTP in culture also play a role in potentiation of inhibition in vivo.

Our finding that during chem-iLTP, gephyrin accumulation at synapses does not precede the increase in synaptic GABA<sub>A</sub>R number challenges the current view that synaptic receptor accumulation requires pre-available scaffold-docking sites at synapses. This evidence supports a model where GABA<sub>A</sub>R and gephyrin comitantly accumulate at synapses during iLTP expression. However, it is not clear whether these two proteins diffuse from the extrasynaptic to the synaptic compartment independently or after formation of GABA<sub>A</sub>R–gephyrin complexes pre-assembled in the extrasynaptic compartment. This latter possibility has been hypothesized for glycine receptor–gephyrin interactions. The occurrence of GABA<sub>A</sub>R–gephyrin extrasynaptic complexes could be demonstrated by the ‘parallel’ diffusion behaviour of GABA<sub>A</sub>R and gephyrin in simultaneous SPT experiments. Unfortunately, as gephyrin is an intracellular protein, these experiments would be technically very challenging. Furthermore, we cannot exclude that technological advances allowing live-imaging experiments with higher temporal resolution than those presented here may unmask a transient temporal mismatch between the relative synaptic accumulation of GABA<sub>A</sub>Rs and gephyrin.

Importantly, we show that the fine tuning of GABA<sub>A</sub>Rs number at synapses is crucially influenced by gephyrin accumulation during synaptic potentiation, while it is marginally affected by gephyrin availability under basal conditions, at least for the cell type and the GABA<sub>A</sub>R isoforms we studied. The slight reduction of GABA<sub>A</sub>R clusters after gephyrin impairment under basal conditions supports the hypothesis that protein(s) other than gephyrin could maintain GABA<sub>A</sub>R clustering, as previously demonstrated in gephyrin−/− mice. Accordingly, it has been shown that loss of γ2-containing GABA<sub>A</sub>Rs led to only a slight reduction of mIPSCs current amplitude. Consistently with this, recent findings emphasized that other proteins, including neuroglin-2, dystrophin and collybistin, are upstream to gephyrin in the hierarchy of the postsynaptic scaffold protein organization. Under basal conditions, mIPSCs amplitude was not affected after interfering with gephyrin assembly, despite reduced GABA<sub>A</sub>R cluster intensity and increased GABA<sub>A</sub>R mobility. This can be explained if unitary presynaptic release does not activate all postsynaptic receptors. As a consequence, a moderate decrease of postsynaptic receptor number would not result in a sizable current amplitude reduction. This hypothesis is substantiated by the fact that sIPSCs, typically evoked by larger GABA release with consequent larger receptor activation, are more sensitive to gephyrin impairment.

It is worth mentioning that in our experiments, NMDA could also affect GABAergic currents through the activation of presynaptic NMDARs. Although we cannot directly exclude this possibility, several lines of evidence point in favour of postsynaptic effect of NMDA on GABAergic currents. Indeed, despite the fact that we observed an increase in mIPSCs frequency during chem-iLTP (Supplementary Fig. 1f), the persistence of such increase argues against a presynaptic source of this phenomenon. In this concern, it is likely to be that small events previously buried in the noise become sizable after NMDA application and account for the apparent mIPSCs frequency increase. This possibility is also suggested by the fact that NMDA did not affect the frequency of sIPSCs (Supplementary Fig. 1b) that show larger signal-to-noise ratio compared with mIPSCs. Finally, the unchanged density of presynaptic inhibitory terminals further supports the lack of significant presynaptic effect following NMDA treatment.

The GABAergic system is crucial for neuronal maturation and network formation at early developmental stages. Several lines of evidence indicate that interfering with the early action of depolarizing GABA may lead to alterations of both glutamatergic and GABAergic synapse formation. Thus, it is possible that the lack of NMDA-induced potentiation of inhibition observed on impairment of endogenous gephyrin could be a consequence of deficits in neuronal development. However, this hypothesis is unlikely in the light of the lack of major changes in the amplitude and frequency of GABAergic synaptic currents under basal conditions on gephyrin impairment. In addition, as gephyrin 2-188 was transfected at 7 days in vitro (DIV), its major protein expression would be expected at DIV8-9, when the GABAergic system is relatively close to mature.

This study provides two converging lines of evidence indicating that β3S383 phosphorylation is a key determinant for chem-iLTP. First, phosphorylation of the β3 subunit residue S383 is enhanced during synaptic potentiation of inhibition. Second, β3S383A, containing GABA<sub>A</sub>Rs were not immobilized at synapses after NMDA stimulation and they prevented the recruitment of gephyrin to synapses. These findings show the importance of β3-S383 phosphorylation for the functional interaction of GABA<sub>A</sub>Rs with gephyrin (and/or with other proteins involved in the synaptic clustering of GABA<sub>A</sub>Rs) during chem-iLTP and are in accordance with a recent study reporting that overexpression of β3S383A prevents the potentiation of tonic currents induced by positive allosteric modulation of L-type calcium channels. Importantly, the similarity of the results obtained here with CaMKII inhibition (KN-62, KN-93 and the kinase-dead mutant CaMKII-K42R) and with the non-phosphorylatable β3S383A.
GABA AR suggests that CaMKII phosphorylation of potential substrates other than β3 S383, such as gephyrin or other scaffold/auxiliary proteins, would not be sufficient for chem-iLTP expression.

A study describing that the phosphorylation of Ser270 affects the epitope recognition of the mAb7a antibody39 raised the possibility that the higher immunoreactivity of synaptic gephyrin after NMDA treatment reported here might reflect an increase in gephyrin phosphorylation, rather than its accumulation at synapses. This possibility is ruled out by the consistency of synaptic gephyrin increase observed during chem-iLTP by immunocytochemistry with an alternative anti-gephyrin antibody (mAb3B11) and by the antibody-free live fast-confocal imaging of recombinant gephyrin.

According to our model, during iLTP, CaMKII phosphorylation of β3 S383 promotes the delivery of GABA ARs to the cell surface, enhances synaptic clustering of GABA AR and favours the assembly of gephyrin clusters at synapses. The documented interaction of gephyrin with several GABA AR subunits, including β3 (refs 21–24) allows speculation that phosphorylated β3 S383 might exhibit enhanced affinity for gephyrin, thus promoting synaptic gephyrin recruitment from extrasynaptic compartments. Additional modulation of receptor-scaffold interactions at synapses during chem-iLTP might involve distinct gephyrin splice variants46 and changes in synaptic receptor subunit composition47 as demonstrated for AMPARs48. Moreover, the phosphorylation state of gephyrin47,46,49–51 due to the activation of different kinases (GSK, CDK5, ERK, Akt2) could further influence synaptic GABA AR mobility by modulating scaffold assembly during chem-iLTP. The evidence that GABA AR β and γ subunits are directly phosphorylated by CaMKII3,35,52 and by other kinases, including PKA, PKC, Src6 suggests that the phosphorylation of these subunits could add further complexity in receptor–scaffold interactions during iLTP.

Importantly, our SPT data demonstrated that in addition to exocytosed receptors, pre-existing surface GABA ARs are involved in chem-iLTP: after stimulation, these receptors are immobilized at synapses and contribute to increased surface synaptic GABA AR number. This finding represents an important complement to other studies that previously highlighted the role of GABA AR exocytosis in the potentiation of inhibitory transmission5,10. Assuming that the molecular machinery that brings GABA ARs to the cell surface is the same under basal conditions and during synaptic plasticity, a phosphoβ3 S383-dependent modulation of GABA AR intracellular trafficking might be also hypothesized in light of the reduced receptor exocytosis and lack of potentiation in β3 S383A mutants. This would represent an additional mechanism coexisting with the CaMKII phosphorylation-dependent immobilization of pre-existing surface GABA AR identified here.

Although the present work and Marsden et al. report that NMDA treatment induces chem-iLTP, NMDAR activation has also been described to be causal for i-LTD3,13,15. This differential effect of NMDA on the induction of inhibitory synaptic plasticity can be explained in terms of the degree of NMDAR activation leading to different levels of intracellular Ca2+ increase. Indeed, moderate Ca2+ rise induces iLTP5,10, while higher intracellular Ca2+ levels (induced by high NMDA concentrations, sustained excitatory activity or high-frequency stimulation) elicit iLTD13,15,16,53. In analogy to excitatory synapses36, it can be hypothesized that depending on the extent of intracellular Ca2+ elevation, it is possible to induce either iLTP or iLTD due to differential activation of CaMKII and/or calcineurin, thereby potentiating/inhibiting diverse intracellular pathways10.

Potentiation of inhibitory synapses correlates with LTD at excitatory synapses triggered by moderate Ca2+ entry on brief NMDA application55. Indeed, similar to previous reports56, in our experiments NMDA-treated neurons exhibited significantly reduced spine density compared with sham-treated neurons (Supplementary Fig. 10). Conversely, the same postsynaptic stimulus that induces depression of inhibitory synapses (for example, massive Ca2+ entry on strong NMDAR activation or high-frequency stimulation) has been reported to concurrently trigger LTD at excitatory synapses53,57. Concomitant inhibitory potentiation and excitatory depression (or vice versa) in response to the same stimulus suggest that the convergence of signal transduction pathways allows a coordinated control of plasticity at excitatory and inhibitory synapses.

Assuming that the Ca2+ rise responsible for the induction of plasticity of inhibitory synapses occurs through the activation of NMDARs at glutamatergic synapses5, the occurrence of either iLTP or iLTD could be influenced by the relative location of excitatory and inhibitory synapses that determines the profile of Ca2+ concentration sensed by inhibitory synapses. The recent observation that Ca2+ rise mediated by L-type Ca2+ channels activates CaMKII and induces potentiation of tonic inhibition36 raises the intriguing possibility that other events besides NMDAR activation might lead to an adequate intracellular Ca2+ concentration for iLTP induction. Those events might include back propagation of action potentials58 and NMDA, Na+, or Ca2+ dendritic spikes59. In addition, it has been recently proposed that the induction of rebound potentiation (RP) obeys to specific temporal patterns of Ca2+ dynamics60.

We found that chem-iLTP is associated with GABA AR immobilization at synapses. The functional impact of receptor lateral mobility was first shown by Heine et al.14 who reported that rapid replacement of desensitized synaptic AMPAR with extrasynaptic receptors by lateral diffusion maintains synaptic responses in face of repetitive synaptic stimulation. This suggests that receptor lateral mobility acts as a frequency-dependent filter of synaptic currents by exerting a stronger reduction of high-frequency synaptic signals with respect to those occurring at lower frequency. As a consequence, the reduction of synaptic GABA AR mobility during iLTP might accelerate the fading of synaptic responses during high-frequency activity by decreasing desensitized receptor replacement at synapses. The evidence that chem-iLTP expression relies on GABA AR recruitment and immobilization at inhibitory synapses reminds of analogous mechanisms occurring at glutamatergic synapses, where LTD is associated with Ca2+–dependent immobilization of AMPAR14,17, likely to be mediated by postsynaptic activation and translocation of CaMKII61. Similarly, in the spinal cord, GlyR are immobilized at synapses during potentiation of glycinergic mIPSCs amplitude62. However, the same NMDA-based protocol that induces this glycinergic potentiation (which differs from the chemical protocol adopted in this study), fails to potentiate GABAergic transmission and to immobilize GABA ARs in the spinal cord62, whereas it elicits iLTD and dispersal of GABA ARs from synapses in the hippocampus13. As mentioned above, it is likely to be that depending on the level of NMDAR activation (NMDA concentration/exposure time), NMDA can lead to potentiation or inhibition of synaptic currents and modulation of receptor mobility by regulating the concentration profile of intracellular Ca2+ that may activate different intracellular pathways.

Validation of our model of iLTP is provided here by the comparable molecular modifications observed in vitro during NMDA-induced iLTP and those found after in vivo manipulations of the sensory environment known to induce potentiation of inhibition30–32. Indeed, the perisomatic upregulation of gephyrin in the principal cells of the rat visual cortex 48 h after brief MD is consistent with the significant increase of gephyrin total protein levels observed 45 min after NMDA treatment in vitro. Similarly,
the increased abundance and synaptic clustering of GABAARs observed in principal cells in the layer 4 of the binocular visual cortex of MD animals are reminiscent of the increased accumulation of synaptic GABAARs in cultured neurons after the chem-ILTP-inducing protocol. Within this framework, an increase in postsynaptic GABAARs or gephyrin upregulation have been observed during long-lasting potentiation of somatic inhibition in rat visual cortex during slow-wave sleep and during the extinction of conditioned fear in the basolateral amygdala.

The postsynaptic expression of the inhibitory plasticity addressed in the present work can have strong effects at the network level, as ILTP can, in principle, be extended to all inhibitory synapses impinging on the postsynaptic neuron. By clarifying the mechanisms of inhibitory postsynaptic plasticity, this work provides the molecular basis for future studies addressing the role of activity-driven modulation of inhibition in brain microcircuits both under physiological conditions and in neurological disorders such as autism and epilepsy.

Methods Animals. All experiments were carried out in accordance with the guidelines established by the European Communities Council (Directive 2010/63/EU of 22 September 2010) were permitted by the Italian Ministry of Health and followed the rules approved by the Italian Institute of Technology. All animal surgeries were done in agreement with the Italian Ministry of Health Regulation and Authorization and have been approved by the Italian Institute of Technology.

Plasmid constructs. SEP-tagged β3β4 was previously described. HA-tagged β3β4 was derived from SEP-tagged constructs by swapping SEP with HA sequence. The increased abundance and synaptic clustering of GABAARs and gephyrin upregulation have been observed during long-lasting potentiation of somatic inhibition in rat visual cortex during slow-wave sleep and during the extinction of conditioned fear in the basolateral amygdala.

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anti-vGAT-Oyster550 at 37 °C. Live-cell imaging and QD recording were performed by acquiring 1,200 consecutive frames at 20 Hz with a 512 × 512 pixel EM-CoolSNAP camera (Hamamatsu, Japan) for 10 min. In this condition, the fluorescence intensity is stable (ver. 7.5, Molecular Devices, USA). The highly diluted QD labelling resulted in < 20 QDs per field of view, so that individual QD receptor complexes did not overlap the trajectories of neighbouring complexes. When, occasionally, two QDs were too close to unambiguously reconstruct their individual trajectories, both QDs were discarded from the analysis. During the experiments, neurons were kept at 32 °C in an open chamber and continuously superfused with the recording solution (see below) at 12 ml h⁻¹. SEP and Oyster550 fluorescence was acquired with 100–200 ms integration time. Samples were illuminated in epifluorescence with a mercury lamp. EGFP, Oyster550 and QD655 fluorescence signals were observed with appropriate excitation (472/30, 543/22 and 435/40 nm, respectively) and emission filters (520/35, 593/40 and 655/15 nm, respectively) (Semrock, Italy) controlled by filter wheels mounted onto an inverted microscope (Eclipse Ti, Nikon, Japan) equipped with a × 60 oil-1.4 numerical aperture (NA) immersion objective.

Single-particle tracking. For tracking, single QDs, recognized by their diffraction-limited fluorescence spot shape and characteristic blinking, were followed with 50-ms time resolution. QD spatial coordinates were identified in each frame as sets of > 4 connected pixels using two-dimensional object wavelet-based localization at a sub-diffraction limited resolution (~40 nm) with MIA software based on simulated annealing algorithm 68. Continuous tracking between blinks was performed with a custom software originally written in MATLAB (The Mathworks Inc., Italy) in Dr Choquet’s lab. The method is based on a QD maximal allowable displacement (4 pixels) during a maximal allowable duration of the dark period (25 frames, corresponding to 1.25-s acquisition). This stringent reconnection of trajectories across QD blinking combined with the highly diluted QD solution allowed us to avoid any signal from a neighbouring QD in the same trajectory and to provide unambiguous observations of individual receptor QD complex trajectories. Receiver trajectories were defined as ‘synaptic’ (or ‘extrasynaptic’) when their spatial coordinates coincided (or not) with those of the localization of the postsynaptic compartment. As inhibitory synapses were identified by presynaptic vGAT fluorescence labelling (with vGAT-Oyster550), postsynaptic compartments were defined as a 2-pixel enlargement of vGAT staining. Although the definition of the compartments was diffraction limited, the sub-wavelength resolution of the single particle detection (~40 nm) allowed accurate description of receptor mobility within such small regions. In addition, diffusion coefficients D were calculated from the displacement S MSD according to the equation: 

\[ r^2 = \frac{1}{2} \left( \frac{1}{n} \sum_{i=1}^{n} (X_i - X_{i-1})^2 + (Y_i - Y_{i-1})^2 \right) / (N - n) dt \]

(1)

for reconstructed trajectories of >100 frames using a custom-made software developed by Dr Choquet (Bordeaux, France). To better characterize receptor mobility, receptor QDs have been distinguished into ‘mobile’ and ‘immobile’ populations by using a threshold the local minimum of the bimodal distribution of mobile receptor QD (mRFP-gephyrin) fluorescence observed in the total neuronal area excluding synaptic when they exhibited a juxtaposed vGAT puncta within a 2-pixel enlargement. Cluster density represents the number of clusters detected for 100 mm dendrite length.

Multicolour live fast-confocal scanning. SEP–β3 and mRFP-gephyrin fluorescence signals were simultaneously acquired in an Eclipse Ti microscope (Nikon, Italy) equipped with × 60 (1.4 NA) and with LiveScan Swept Field Confocal (Nikon, Italy). Samples were illuminated with 488 nm and 568 nm lasers with 500–600-nm temporal integration. Fluorescence was collected with 1.04 × 1.02 EC1. Mon DU858 (Andor Technology, UK) and Nikon Ti2. In addition to vGAT-Oyster650 signal. Synaptic gephyrin was quantified as the integrated fluorescence intensity yielding a gephyrin staining comparable to that obtained with the methanol/acetone surgical thread (Ethicon, 6–0) and a topical ointment containing cortisone and antibiotic was applied. Animals were transcardially perfused 48 h after MD using cold (4 °C) 0.1 M PBS followed by 2% PFA solution in the same buffer. Sagittal brain slices from MD and control brains were cut on a freezing microtome at 50 μm thickness. Slices were incubated with anti-GFP or anti-HA antibodies, respectively, in the surgical thread (Ethicon, 6–0) and a topical ointment containing cortisone and antibiotic was applied.

Monoclonal deprivation and IHC. Pigmented Long Evans rats aged P22 were subjected to MD by means of eyelid suture after averin anesthesia (1 ml kg⁻¹ intraperitoneal). Eyelids were sutured around a surgical thread (Ethicon, 6–0) and a topical ointment containing cortisone and antibiotic was applied. Images were acquired using a Leica TCS SP5 laser scanning confocal microscope (see above). Immersion x 63 (NA 1.4) Plan Apochromat objective following the confocal microscopy procedure described above. Confocal stacks of 28 optical sections corresponding to 11.7-μm-thick confocal sections. Integrated somatic gephyrin fluorescence intensity was quantified in the somatic region of each principal cell, identified in a 6-μm radial enlargement of DAPI staining in each plane. Integrated somatic GABAAR cluster fluorescence intensity refers to the fluorescence intensity of all GABAAR clusters identified in the three-dimensional-reconstructed perisomatic regions of each principal cell. Finally, synaptic GABAAR cluster density represents the number of GABAAR clusters juxtaposed to vGAT immunoreactivity found per μm². The same acquisitions and analysis parameters were applied to all samples. In all quantifications, data were corrected for background.
normalized to the fluorescence measured before the photobleaching. Residual fluorescence immediately after the photobleaching was set to zero. Recovery curves were corrected for constant photobleaching and background noise. In each neuron, FRAP was performed on several synapses before and 20 min after NMDA stimulation. Experiments were conducted at 32°C and at pH 7.4.

**Acid strip.** The acid strip procedure, performed on living QD-labelled neurons at the end of the iLT experiment session (∼25 min after QD labelling), consisted in 1 min incubation in pH 2.4 extracellular solution containing 0.5 M NaCl and 0.2 M acetic acid for 10°C. QDs were described above before and after the acid strip to assess the presence of internalized QD receptor complexes. The efficiency of the acid strip in removing surface antibodies was assessed in control QD-labelled neurons immediately after the labelling (1 min). To verify that internalized receptors could be detected, the acid strip was also performed on neurons kept at 37°C for 1 min before labelling to allow receptor endocytosis. Under these conditions, the acid strip was compared with a control treatment consisting in 1 min incubation in cold culture medium. Control treatment did not affect QD labelling.

**SDS-PAGE and WB assays.** Cultured hippocampal neurons stimulated with NMDA or with a sham solution were allowed to recover for different durations (10, 18 or 43 min). Samples for GABA(R) phosphorylation assays were lysed and harvested in RIPA buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Igepal, 0.5% Nonidet P-40, 0.1% SDS, 1 mM EDTA) supplemented with protease inhibitors (P8340–Protease inhibitor cocktail, Sigma) and phosphatase (2 mM Na3VO4, 10 mM NaF) inhibitors. After centrifugation (13,000 r.p.m., 15 min, 4°C), the protein supernatant was quantified using the micro BCA protein assay kit (Pierce). Total proteins (30 μg) were heated in SDS sample buffer at 50°C for 10 min and then resolved by SDS–PAGE. Phosphorylated S383 was detected by immunoblotting with rabbit anti phospho-S383 (ref. 36), followed by peroxidase-conjugated anti-rabbit IgGs and detection with ECL. Blots were then stripped (Thermo Scientific, P121059–Restore Western Blot Stripping Buffer) for subsequent probing of total GABA(R) β3 (with rabbit anti-β3 IgGs) and actin for equal loading check (with rabbit anti-actin antibody). Blots were re-probed with peroxidase-conjugated anti-rabbit IgGs and detection with ECL. All subsequent steps were normalized to the loading control.

In the experiments aimed at assessing de novo gephyrin protein synthesis, a set of cultured hippocampal neurons were supplemented with 0.5 mg ml–1 cycloheximide (Sigma) during recovery period. All subsequent steps were performed at 4°C. Cells were rinsed in PBS and harvested in lysis buffer (1% TritonX-100 (v/v) in PBS supplemented with Complete Mini Protease inhibitor cocktail (Roche)). The supernatant harvested after 30 min centrifugation at 1,000 g was boiled in SDS sample buffer, subjected to SDS–PAGE and analysed by WB assays. The following primary antibodies were used for protein detection: anti-gephyrin and anti-γ-adaptin. Signal intensities were analysed using ImageJ (version 1.38) analysis software. (National Institutes of Health). Signal intensities were then normalized to the loading control.

**Statistics.** Values are given as means ± s.e.m. or, in the case of non-normally distributed data, as medians ± IQR. Statistical significance was tested using Prism 5.0 Software (GraphPad, USA). Normally distributed data sets were compared using the unpaired two-tailed Student’s t-test, whereas non-Gaussian data sets were tested by the Mann–Whitney U-test. For in vitro experiments, when the paired non-parametric values, Wilcoxon paired test was used. Statistical significance between more than two normally distributed data sets was tested by one-way ANOVA variance test followed by a Dunnett’s test (to compare data to control) or Newman–Keuls’ test (to compare individual pairs of data) always applying a family-wise 95% confidence interval. In case of paired non-parametric multiple data sets the Kruskal–Wallis test followed by Dunn’s post-hoc test was adopted. Indications of significance correspond to P-values < 0.05 (**), 0.01 (***), P<0.001 (****) and nonsignificant (ns). For QD tracking, n is the total number of trajectories reconstructed after different experiments performed on multiple neurons per condition. Number of primary antibodies = n, represents the number of neurons observed. All statistical tests were two-tailed.

**References**

1. Klausberger, T. & Somogyi, P. Neuronal diversity and temporal dynamics: the unity of hippocampal circuit operations. Science 321, 53–57 (2008).

2. Castillo, P. E., Chiu, C. Q. & Carroll, R. C. Long-term plasticity at inhibitory synapses. Curr. Opin. Neurobiol. 21, 328–338 (2011).

3. Houston, C. M., He, Q. & Smart, T. G. CaMKII phosphorylation of the GABA(A) receptor: subunit- and synapse-specific modulation. J. Physiol. 587, 2115–2129 (2009).

4. Kichtler, J. T. & Moss, S. J. Modulation of GABA(A) receptor activity by phosphorylation and receptor trafficking: implications for the efficacy of synaptic inhibition. Curr. Opin. Neurobiol. 13, 341–347 (2003).

5. Maffei, A., Friscol, M. E. & Turrigiano, G. G. Potentiation of cortical inhibition by visual deprivation. Nature 443, 81–84 (2006).

6. Yamagata, Y. et al. Kinase-dead knock-in mice reveal an essential role of kinase activity of Ca2+ +calmodulin-dependent protein kinase I alpha in dendritic spine enlargement, long-term potentiation, and learning. J. Neurosci. 29, 7607–7618 (2009).

7. McDonald, D. B. & Moss, S. J. Conserved phosphorylation of the intracellular domains of GABA(A) receptor beta2 and beta3 subunits by CAM-dependent protein kinase, gGMP-dependent protein kinase protein C and Ca2+ +calmodulin type II-dependent protein kinase. Neuropharmacology 53, 1377–1385 (1997).

8. Vithlani, M., Terunuma, M. & Moss, S. J. The dynamic modulation of GABA(A) receptor trafficking and its role in regulating the plasticity of inhibitory synapses. Physiol. Rev. 91, 1009–1022 (2011).

9. Fuimudi, H. & Woodin, M. A. Role of activity-dependent regulation of neuronal chloride homeostasis in development. Curr. Opin. Neurobiol. 17, 81–86 (2007).

10. Kano, M., Fukunaga, K. & Konnerth, A. Ca2+ +induced rebound potentiation of gamma-aminobutyric acid-mediated currents requires activation of Ca2+ +calmodulin-dependent kinase II. Proc. Natl Acad. Sci. USA 93, 13351–13356 (1996).

11. Nusser, Z., Hjämsjö, N., Somogyi, P. & Mody, I. Increased number of synaptic GABA(A) receptors underlies potentiation at hippocampal inhibitory synapses. Nature 395, 172–178 (1998).

12. Marsden, K. C., Shemesh, A., Bayer, K. U. & Carroll, R. C. Selective translocation of Ca2+ +calmodulin protein kinase I alpha (CaMKIIalpha) to inhibitory synapses. Proc. Natl Acad. Sci. USA 107, 20559–20564 (2010).

13. Choquet, D. & Triller, A. The Dynamic Synapse. Neuron 80, 691–703 (2013).

14. Levi, S. T. in The Dynamic Synapse: Molecular Methods in Ionotropic Receptor Biology, (eds Kittler, J. T. & Moss, S. J.) (CRC Press, Boca Raton, FL), 2006.

15. Bannai, H. et al. Activity-dependent tuning of inhibitory neurotransmission based on GABAAr diffusion dynamics. Neuron 62, 670–682 (2009).

16. Heine, M. et al. Surface mobility of postsynaptic AMPARs tunes synaptic transmission. Science 320, 201–205 (2008).

17. Van der Vaart, A. J. & Somogyi, P. Neuronal diversity and temporal dynamics: the unity of hippocampal circuit operations. Science 321, 53–57 (2008).

18. Moss, S. J. et al. Long-term plasticity at inhibitory synapses. Curr. Opin. Neurobiol. 21, 328–338 (2011).

19. Houston, C. M., He, Q. & Smart, T. G. CaMKII phosphorylation of the GABA(A) receptor: subunit- and synapse-specific modulation. J. Physiol. 587, 2115–2129 (2009).

20. Kichtler, J. T. & Moss, S. J. Modulation of GABA(A) receptor activity by phosphorylation and receptor trafficking: implications for the efficacy of synaptic inhibition. Curr. Opin. Neurobiol. 13, 341–347 (2003).

21. Maffei, A., Friscol, M. E. & Turrigiano, G. G. Potentiation of cortical inhibition by visual deprivation. Nature 443, 81–84 (2006).

22. Yamagata, Y. et al. Kinase-dead knock-in mice reveal an essential role of kinase activity of Ca2+ +calmodulin-dependent protein kinase I alpha in dendritic spine enlargement, long-term potentiation, and learning. J. Neurosci. 29, 7607–7618 (2009).

23. McDonald, D. B. & Moss, S. J. Conserved phosphorylation of the intracellular domains of GABA(A) receptor beta2 and beta3 subunits by CAM-dependent protein kinase, gGMP-dependent protein kinase protein C and Ca2+ +calmodulin type II-dependent protein kinase. Neuropharmacology 52, 1377–1385 (1997).
35. Houston, C. M., Lee, H. H., Hosie, A. M., Moss, S. J., & Smart, T. G. Identification of the sites for CaMK-II-dependent phosphorylation of GABA(A) receptors. *J. Biol. Chem.* 282, 17554–17565 (2007).

36. Saliba, R. S., Kretschmannova, K. & Moss, S. J. Activity-dependent phosphorylation of GABA(A) receptors regulates receptor insertion and tonic current. *EMBO J.* 31, 2937–2951 (2012).

37. Kuhse, J. et al. Phosphorylation of gephyrin in hippocampal neurons by cyclin-dependent kinase CDK5 at Ser-270 is dependent on callybassin. *J. Biol. Chem.* 287, 30952–30966 (2012).

38. Maas, C. et al. Neuronal cotransfert of glycine receptor and the scaffold protein gephyrin. *J. Cell Biol.* 172, 441–451 (2006).

39. Maas, C. et al. Synaptic activation modifies microtubules underlying transport of postsynaptic cargo. *Proc. Natl Acad. Sci. USA* 106, 8731–8736 (2009).

40. Ehrenpreis, M. V., Hanus, C., Vannier, C., Triller, A. & Dahan, M. Multiple association states between glycine receptors and gephyrin identified by SPT analysis. *Biophys. J.* 92, 3706–3718 (2007).

41. Barberis, A., Petrinj, E. M. & Mozzymas, J. W. Impact of synaptic neurotransmitter concentration time course on the kinetics and pharmacological modulation of inhibitory synaptic currents. *Front Cell Neurosci.* 5, 6 (2011).

42. Duguid, I. C. & Smart, T. G. Retrograde activation of presynaptic NMDA receptors enhances GABA release at cerebellar interneuron-Purkinje cell synapses. *Nat. Neurosci.* 7, 525–533 (2004).

43. Ben-Ari, Y., Gaiarsa, J. L., Tyzio, R. & Khazipov, R. GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. *Physiol. Rev.* 87, 1215–1284 (2007).

44. Chudotvorova, I. et al. Early expression of KC2CC in rat hippocampal cultures augments expression of functional GABA A synapses. *J. Physiol.* 566, 671–679 (2005).

45. Wang, D. D. & Kriegstein, A. R. GABA regulates excitatory synapse formation in the neocortex via NMDA receptor activation. *J. Neurosci.* 28, 5547–5558 (2008).

46. Herweg, J. & Schwarz, G. Splice-specific glycine receptor binding, folding, and phosphorylation of the scaffolding protein gephyrin. *J. Biol. Chem.* 287, 12465–12466 (2012).

47. Renner, M., Schweizer, C., Bannai, H., Triller, A. & Levi, S. Diffusion barriers constrain receptors at synapses. *PLoS ONE* 7, e43032 (2012).

48. Derkach, V. A., Oh, M. C., Guire, E. S. & Soderling, T. R. Regulatory mechanisms of AMPA receptors in synaptic plasticity. *Nat. Rev. Neurosci.* 8, 101–113 (2007).

49. Tyagarajan, S. K. et al. ERK and GSK3beta regulate gephyrin postsynaptic aggregation and GABAergic synaptic function in a calpain-dependent mechanism. *J. Biol. Chem.* 287, 9634–9647 (2012).

50. Tyagarajan, S. K. et al. Regulation of GABAergic synapse formation and plasticity by GSK3beta-dependent phosphorylation of gephyrin. *Proc. Natl Acad. Sci. USA* 108, 379–384 (2011).

51. Wuchter, J. et al. A comprehensive small interfering RNA screen identifies signaling pathways required for gephyrin clustering. *J. Neurosci.* 32, 14821–14834 (2012).

52. McDonald, B. J. & Moss, S. J. Differential phosphorylation of intracellular domains of gamma-aminobutyric acid type A receptor subunits by calcium/calmodulin type 2-dependent protein kinase and cGMP-dependent protein kinase. *J. Biol. Chem.* 269, 18111–18117 (1994).

53. Lu, Y. M., Mansuy, I. M., Kandel, E. R. & Tugar, J. M. Activity-dependent phosphorylation of gephyrin at synapses. *Neuron* 290, 18111–18117 (2008).

54. Hausser, M., Spruston, N. & Stuart, G. J. Diversity and dynamics of dendritic signaling. *Science* 290, 739–744 (2000).

55. Kawaguchi, S. Y., Nagasaki, N. & Hirano, T. Dynamic impact of temporal context of Ca signals on inhibitory synaptic plasticity. *Sci. Rep.* 1, 143 (2011).

56. Opazo, P. et al. CaMKII triggers the diffusional trapping of surface AMPARs through phosphorylation of stargazin. *Neuron* 67, 239–252 (2010).

57. Levi, S. et al. Homeostatic regulation of synaptic GlyR numbers driven by lateral diffusion. *Neuron* 59, 261–273 (2008).

58. Kurotani, T., Yamada, K., Yoshiumura, Y., Grain, M. C. & Komatsu, Y. State-dependent bidirectional modification of somatic inhibition in neocortical pyramidal cells. *Neuron* 57, 905–916 (2008).

59. Chhatwal, J. P., Myers, K. M., Ressler, K. J. & Davis, M. Regulation of gephyrin and GABA receptor binding within the amygdala after fear acquisition and extinction. *J. Neurosci.* 25, 502–506 (2005).

60. Jacob, T. C. et al. GABA(A) receptor membrane trafficking regulates spine maturity. *Proc. Natl Acad. Sci. USA* 106, 12500–12505 (2009).

61. Clements, J. D. & Bekkers, J. M. Detection of spontaneous synaptic events with an optimally scaled template. *Biophys. J.* 73, 220–229 (1997).

62. Silver, R. A., Call-Candy, S. G. & Takahashi, T. Non-NMDA glutamate receptor occupancy and open probability at a rat cerebellar synapse with single and multiple release sites. *J. Physiol.* 494(PT 1): 231–250 (1996).

63. Izeddin, I. et al. Wavelet analysis for single molecule localization microscopy. *Opt. Express* 20, 2081–2095 (2012).

64. Racine, V. et al. Multiple-target tracking of 3D fluorescent objects based on simulated annealing. *IEEE Int. Symp. Biomed. Imaging* 1020–1023 (2006).

65. Tardin, C., Cognet, L., Bats, C., Lounis, B. & Choquet, D. Direct imaging of lateral movements of AMPA receptors inside synapses. *EMBO J.* 22, 4656–4665 (2003).

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Author contributions

E.M.F. and A.B. conceived and designed the experiments; E.M.P., T.R., T.I.H., G.L., U.O. and T.C.J. performed the experiments; E.M.P., T.R., T.I.H., T.C.J., M.K. and A.B. analysed the data; E.M.P. and A.B. wrote the manuscript; T.R., T.I.H., V.R., I.S., T.C.J., S.M., F.B., P.M. and M.K. critically read the manuscript; S.M. provided some molecular tools; V.R. and J.-B.S. developed some features of the analysis software.

Additional information

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