Visualization of NMDA receptor–dependent AMPA receptor synaptic plasticity in vivo

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Regulation of AMPA receptor (AMPAR) membrane trafficking is critical for synaptic plasticity, as well as for learning and memory. However, the mechanisms of AMPAR trafficking in vivo remain elusive. Using in vivo two-photon microscopy in the mouse somatosensory barrel cortex, we found that acute whisker stimulation led to a significant increase in the intensity of surface AMPAR GluA1 subunit (sGluA1) in both spines and dendritic shafts and a small increase in spine size relative to prestimulation values. Interestingly, the initial spine properties biased spine changes following whisker stimulation. Changes in spine sGluA1 intensity were positively correlated with changes in spine size and dendritic shaft sGluA1 intensity following whisker stimulation. The increase in spine sGluA1 intensity evoked by whisker stimulation was NMDA receptor dependent and long lasting, similar to major forms of synaptic plasticity in the brain. In this study we were able to observe experience-dependent AMPAR trafficking in real time and characterize, in vivo, a major form of synaptic plasticity in the brain.

AMPA receptors mediate the majority of fast excitatory synaptic transmission in the central nervous system and are therefore critical targets for experience-dependent regulation of information processing and storage in the brain. Long-term potentiation (LTP) and long-term depression (LTD) of excitatory synaptic transmission in the central nervous system are major forms of synaptic plasticity that are thought to be crucial for experience-dependent modification of brain functions such as learning and memory. AMPAR trafficking to and from synapses is a highly dynamic process that mediates certain forms of LTP and LTD: increases in AMPAR function at synapses result in LTP, whereas the removal of synaptic AMPARs leads to LTD1–3. Thus, it is crucial to understand the temporal and spatial dynamics and molecular processes governing experience-dependent AMPAR plasticity in vivo in order to understand how experience shapes brain function and behavior in health and disease.

Previous studies have shown that chronic sensory deprivation resulting from whisker trimming regulates spine turnover in mice in vivo4,5. However, because of the lack of functional synaptic markers, these studies did not address the effect of sensory manipulation on relative synaptic strength or AMPAR trafficking in preexisting stable spines. The manipulation of chronic whisker experience through stimulation or trimming has also been shown to regulate AMPAR content and subunit composition in the mouse barrel cortex in ex vivo slices6,7, but the ex vivo nature of these studies precludes real-time acute or longitudinal analysis of AMPAR dynamics. Here, we used in utero electroporation8 to transfect layer 2/3 pyramidal neurons in mouse barrel cortex with the following: the AMPAR GluA1 subunit tagged with a pH-sensitive form of GFP (Super Ecliptic pHluorin (SEP)); the AMPAR GluA2 subunit tagged with myc; and the morphological marker dsRed2. We then monitored AMPAR dynamics in anesthetized animals through a cranial window by means of two-photon microscopy. Our data show that acute whisker stimulation led to a significant increase in the intensity of spine sGluA1 and shaft sGluA1 in a subpopulation of dendrites. Whisker-stimulation-evoked changes in the intensity of spine sGluA1 were positively correlated with changes in spine size and the intensity of shaft sGluA1. Moreover, acute whisker-stimulation-induced increases in the intensity of spine sGluA1 were NMDA receptor dependent and long lasting, which suggests that acute whisker stimulation might lead to an LTP-like phenomenon in vivo. Our study indicates that analyzing surface postsynaptic AMPAR receptor dynamics is a new way of monitoring and dissecting the mechanisms of synaptic plasticity in real time in vivo.

RESULTS

In vivo imaging of AMPARs in layer 2/3 neurons in the barrel cortex

The mouse primary somatosensory cortex has an exquisite somatotopic map in which each individual whisker is represented as a discrete anatomical unit, the ‘barrel’, allowing precise delineation of functional organization, development and plasticity9. To monitor AMPAR dynamics and spine turnover in the mouse barrel cortex, we transfected layer 2/3 neurons with SEP-GluA1, myc-GluA2 and dsRed2 by in utero electroporation of mouse embryos aged 15 embryonic days (E15). We used low concentrations of DNA for electroporation to minimize the degree of AMPAR overexpression and to sparsely label a small population of neurons. Immunostaining of GluA1 in brain slices of electroporated animals showed that the transfected neurons had only modest overexpression of GluA1 (Supplementary Fig. 1). We then made a cranial window over the barrel cortex in 10-week-old mice that had previously undergone neuronal transfection by means of in utero electroporation10. After allowing the mice to recover for 2–3 weeks to allow inflammation to subside (Supplementary Fig. 2),

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we mapped individual barrel columns using optical intrinsic signal imaging (Fig. 1a,b) and acquired in vivo two-photon images of apical dendrites from layer 2/3 neurons both within and outside the mapped barrel columns in anesthetized animals.\textsuperscript{10-12} Transfected neurons had high expression of SEP-GluA1 in synaptic spines throughout the dendritic arbor, with relatively lower expression in dendritic shafts (Fig. 1c and Supplementary Videos 1 and 2). The basal expression of SEP-GluA1 in spines in vivo had a wide distribution and was correlated with spine size (Fig. 1d), consistent with previous findings that the number of postsynaptic AMPARs is strongly correlated with spine size\textsuperscript{13,14} and most likely is a determinant of synaptic strength\textsuperscript{15}. Interestingly, we observed dramatic differences in SEP-GluA1 expression in spines within a few micrometers of each other along the same dendrite (Fig. 1c). In extreme cases, some spines expressed high levels of SEP-GluA1 while neighboring spines had barely detectable levels.

To determine the basal stability of the SEP-GluA1 distribution over time, we imaged neurons repeatedly in the absence of sensory manipulation. We were able to detect stable expression of both dsRed2 and SEP-GluA1 in individual spines in the cortex for over 1 month, and the relative expression of SEP-GluA1 at specific synapses was also maintained over this period (Fig. 2a). Because AMPARs have a metabolic half-life of 30–40 h\textsuperscript{16}, the AMPARs imaged at 1 month were not the same proteins as those observed at the beginning of the imaging period. This indicates that GluA1 is targeted to specific spines on a dendrite and that there is an inherent mechanism to maintain the relative amount of surface AMPARs along dendrites throughout many rounds of receptor turnover.

**Acute whisker stimulation leads to an increase in spine and shaft sGluA1 intensity**

To examine whether acute sensory stimulation regulates AMPAR trafficking in the mouse barrel cortex, we deflected a single whisker at 10 Hz for 1 h and took images of the barrel column corresponding to the deflected whisker immediately before whisker stimulation and at 1, 2, and 3 h after whisker stimulation (Fig. 2b). Control animals were imaged at the same time points with no whisker stimulation. Studies in brain slices have shown that chronic sensory experience can drive GluA1 into synapses between layer 4 and layer 2/3 neurons through an LTP-like process\textsuperscript{7,17,18}. We monitored surface AMPAR dynamics in vivo and found that acute whisker stimulation led to an ~30% average increase in spine sGluA1 intensity in preexisting spines on dendrites in the stimulated barrel (Figs. 2 and 3 and Supplementary Fig. 3). In contrast, we did not observe a significant change in average spine sGluA1 intensity on dendrites of distant unstimulated barrels or in control (unstimulated) animals (Fig. 3e). This increase in spine sGluA1 intensity following whisker stimulation was rapid, occurring...
We calculated the Monte Carlo value by summing the number of spines showing changes in spine sGluA1 intensity in the same direction as their neighboring spines. (a) Spine sGluA1 intensity (SEP-GluA1 signal) in control and stimulated animals. (b) Shaft sGluA1 intensity in control and stimulated animals. (c) Shaft sGluA1 intensity in control and stimulated animals. (d) Categorization of spine sGluA1 responses at hour 1; same (>70% and <130%), up (≥130%) and down (≤70%). (e) Changes in spine structure intensity (left) and in spine sGluA1 intensity (right) at hour 1 in distant unstimulated barrels. (f) Average sGluA1 intensity in spines belonging to the same dendrites in control and stimulated animals. (g) Categorization of dendritic responses at hour 1 into the ‘same’, ‘up’ and ‘down’ categories (same criteria as in d). Numbers in bars are the number of dendrites in each category: 585 spines and 52 dendrites in six stimulated animals; 493 spines and 38 dendrites in five control animals. Forty spines and 6 dendrites in two animals for the distant unstimulated barrel in e. **P < 0.01, ***P < 0.001, two-way analysis of variance (ANOVA) with Bonferroni post-tests. n.s., not significant. Error bars represent s.e.m.

in the first hour after stimulation, and persisted for at least 3 h. To further characterize the change in spine sGluA1 intensity following whisker stimulation, we grouped spines into three categories (same, up and down) on the basis of their percent change in sGluA1 intensity at 1 h. Using the s.d. of the percent change at 1 h in control animals as a threshold (±30%), we grouped spines as follows: same (>70% and <130%), up (≥130%) and down (≤70%). At 1 h after the onset of whisker stimulation, there was a dramatic increase in the population of spines in the ‘up’ category (35% versus 10%), a substantial decrease in the population of spines in the ‘same’ category (55% versus 74%) and no change in the population of spines in the ‘down’ category (10% versus 16%) (Fig. 3d). We also investigated the dynamics of sGluA1 intensity in the dendritic shaft underlying each spine (Supplementary Fig. 4 and Online Methods). Interestingly, acute whisker stimulation also led to an ~30% average increase in shaft sGluA1 intensity relative to prestimulation levels (Fig. 3c and Supplementary Fig. 3c–f). These findings indicate that sensory experience can drive increases in surface AMPAR subunit expression in both dendritic spines and shafts.

Next we examined whether whisker-stimulation-induced changes in spine sGluA1 intensity are dendrite specific by studying groups of spines belonging to the same dendrite. At 1 h after whisker stimulation, spine sGluA1 intensity was increased by more than 30% in 11 out of 52 dendrites (Fig. 3f,g). Across these 11 dendrites, spine sGluA1 intensity increased approximately twofold, by an average of

Figure 3. Acute whisker stimulation led to an increase in intensity in spine sGluA1 and shaft sGluA1 but had little effect on spine size. (a) Spine structure intensity (dSRed2 signal) at 0, 1, 2 and 3 h following stimulation in control and stimulated animals. (b) Spine sGluA1 intensity (SEP-GluA1 signal) in control and stimulated animals. (c) Shaft sGluA1 intensity in control and stimulated animals. (d) Categorization of spine sGluA1 responses at hour 1; same (>70% and <130%), up (≥130%) and down (≤70%). (e) Changes in spine structure intensity (left) and in spine sGluA1 intensity (right) at hour 1 in distant unstimulated barrels. (f) Average sGluA1 intensity in spines belonging to the same dendrites in control and stimulated animals. (g) Categorization of dendritic responses at hour 1 into the ‘same’, ‘up’ and ‘down’ categories (same criteria as in d). Numbers in bars are the number of dendrites in each category: 585 spines and 52 dendrites in six stimulated animals; 493 spines and 38 dendrites in five control animals. Forty spines and 6 dendrites in two animals for the distant unstimulated barrel in e. **P < 0.01, ***P < 0.001, two-way analysis of variance (ANOVA) with Bonferroni post-tests. n.s., not significant. Error bars represent s.e.m.
202.2%. In control animals, the average spine sGluA1 intensity in all 38 dendrites studied remained unchanged. These data indicate that only a subpopulation of dendrites (∼20%) respond to sensory stimuli by modifying their surface AMPARs and are consistent with previous findings that layer 2/3 excitatory neurons of sensory cortex show high stimulus selectivity19–21. Interestingly, the 11 dendrites that showed increased intensity in the current study were evenly distributed across animals. However, because we could not unambiguously assign every dendrite to a specific neuron in this data set, we were not able to group the 52 examined dendrites further and investigate potential differences between neurons or effects of dendritic branch order.

Acute whisker stimulation has small effects on spine size and spine turnover

Previous imaging of cultured slices has shown that LTP induction leads to spine enlargement22 and LTD induction leads to spine shrinkage23, although Sdrulla and Linden found no association between LTD and dendritic spine shrinkage in cerebellar Purkinje cells in acute slices24. Studies using electron microscopy have also shown that a 24-h period of single-whisker stimulation in adult mice results in a significant increase in the density of synapses in the corresponding cortical barrel25. To our knowledge, our imaging approach is unique in its ability to measure the temporal dynamics of surface AMPARs and the mechanisms of their regulation in real time. We examined spine structure intensity at 0, 1, 2 and 3 h following 10-Hz whisker stimulation. Although there was a slight trend toward an increase in spine size, on average we did not detect a significant change in spine structure intensity following whisker stimulation compared to the control (Fig. 3a,c and Supplementary Fig. 3a,b). This result indicates that acute whisker stimulation for 1 h does not have a large effect on spine size in vivo.

Dendritic spines are dynamic and may provide a structural basis for information storage, and it has been suggested that spine turnover is important in synaptic plasticity26. Spine turnover has been characterized in response to chronic sensory deprivation and motor learning27–29. However, over the short period of time studied here (3 h beyond the end of whisker stimulation), we saw very little spine turnover in the apical dendrites of layer 2/3 neurons, and the rate of this turnover was not substantially affected by whisker stimulation (Supplementary Fig. 5). Thus, for the remainder of this study we focused on AMPAR dynamics in the preexisting stable spines during sensory manipulation.

Figure 5 Increases in spine sGluA1 intensity following whisker stimulation were NMDA receptor dependent. (a) Representative images of dendrites taken before (0 h) and after (1, 2 and 3 h) acute whisker stimulation in animals treated with CPP. Arrowheads mark spines, and arrows mark dendritic shafts (SEP-GluA1 in green, dsRed2 in purple, overlap in white). (b) Spine structure intensity following whisker stimulation with CPP treatment. P = 0.6806. (c) Spine sGluA1 intensity following whisker stimulation with CPP treatment. P = 0.0913. (d) Shaft sGluA1 intensity following whisker stimulation with CPP treatment. P = 0.1281. Data are from 30 spines and five dendrites in two animals for CPP treatments. Two-way ANOVA with Bonferroni post-tests. n.s., not significant. Error bars represent s.e.m.

Initial spine and shaft properties have effects on spine changes

Spines in vivo have heterogeneous sizes and AMPAR contents, which probably reflect differences in prior activity, and these differing basal states may affect the manner in which individual spines respond to future experiences30. Indeed, it has been shown that small spines are preferential sites for long-term synaptic potentiation in brain slices22. When we examined the correlation coefficient between the initial spine size and the spine size at 1 h after whisker stimulation, we found that there was a significant negative correlation (Supplementary Fig. 6a, r = −0.2546, P < 0.001), which showed that small spines increased in size more than large spines following whisker stimulation. We also observed a smaller but still significant negative correlation between initial spine size and changes in spine sGluA1 intensity at 1 h following whisker stimulation (Supplementary Fig. 6b, r = −0.1057, P = 0.012), indicating that the GluA1 content increased more in small spines than in large spines following whisker stimulation. In contrast, we found no significant trend in changes in shaft sGluA1 intensity between small and large spines (Supplementary Fig. 6c, r = 0.0685, P = 0.1052). Although there was a trend of positive correlation between changes in spine sGluA1 intensity and initial spine sGluA1 intensity or initial shaft sGluA1 intensity following whisker stimulation, it was not statistically significant (Supplementary Fig. 6d,e). Interestingly, a significant positive correlation was detected between initial shaft sGluA1 intensity and changes in shaft sGluA1 intensity following whisker stimulation (Supplementary Fig. 6f, r = 0.1608, P < 0.001). All together, these results suggest that the initial spine properties can affect spine changes following whisker stimulation.

Changes in spine sGluA1 intensity are positively correlated with changes in spine size and shaft sGluA1 intensity

The interesting relationships among spine size, spine sGluA1 intensity and shaft sGluA1 intensity prompted us to investigate the correlations among changes in these three parameters following whisker stimulation. Although we did not detect a significant stimulation-evoked increase in spine size on average, we observed a significant positive correlation between changes in spine sGluA1 intensity and changes in spine size at 1 h after whisker stimulation (Fig. 4a, r = 0.44, P < 0.001). A one-sample t-test revealed that the spine population in stimulated animals had a mean that was considerably above the y = x line. These results indicate that although changes in spine size and sGluA1 content are correlated, whisker stimulation produces a much larger increase in the amount of spine...
sGluA1 than in spine size, suggesting an increase in the density of sGluA1 in spines after whisker stimulation. This is consistent with our finding that the total average intensity of spine sGluA1 increased by ~30% following whisker stimulation, whereas the total average spine size showed only a 6% increase (Fig. 2a,b). Next, when we examined changes in spine size in spines that showed a >30% increase in GluA1 content 1 h after whisker stimulation, we observed a 24% increase in spine size, compared to a 93.5% increase in GluA1 content in these spines (Supplementary Fig. 7). Interestingly, the changes in GluA1 content and spine size were not well correlated in this population of spines (r = 0.12), which suggests that increases in GluA1 content and spine size can be uncoupled. In addition, we found a significant positive correlation between changes in spine sGluA1 intensity and changes in adjacent shaft sGluA1 intensity in stimulated animals (Fig. 4b, r = 0.67, P < 0.001). In contrast, changes in shaft sGluA1 intensity and spine size were not correlated (Fig. 4c, r = 0.03). Together, these findings indicate that acute whisker stimulation induces a coordinated increase in the intensity of spine sGluA1 and shaft sGluA1, whereas changes in spine size can be uncoupled from sGluA1 changes.

Ex vivo studies have demonstrated clustered synaptic potentiation following sensory deprivation by whisker trimming, and in vivo imaging has shown the induction of clustered spine turnover by motor learning. We noted that increases in spine sGluA1 intensity occurred in a subset of dendrites after acute whisker stimulation, and thus these increases were generally colocalized along dendritic segments. To address the distribution of increases in sGluA1 intensity on a finer spatial scale, we plotted the change in spine sGluA1 intensity for each spine versus that of its nearest neighbor at 1 h. We found a significant positive correlation between neighboring spines (r = 0.52, P < 0.001). In addition, when we grouped neighboring spines that changed in the same direction (Fig. 4d, x > 130% and y > 130%, x < 70% and y < 70% (group 1)) and in opposite directions (Fig. 4d, x > 130% and y < 70%, x < 70% and y > 130% (group 2)), we found that the number of neighboring spines changing in the same direction was significantly greater than would occur by chance (Fig. 4e), whereas the number of neighboring spines changing in the opposite direction was significantly less than would occur by chance (Fig. 4f). These results demonstrate that spine sGluA1 intensity changes cooperatively in neighboring spines following whisker stimulation. No such significant positive trend was found between neighboring spines in control animals (Fig. 4g).

Increased spine sGluA1 intensity is NMDA receptor dependent and is stable for 48 h

One of the hallmarks of the most common form of LTP is that its induction is dependent on the activation of NMDA receptors. To test whether the whisker-stimulation-induced increases in spine sGluA1 intensity were NMDA receptor dependent, we injected the NMDA receptor antagonist CPP (3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid) at 10 mg/kg 30–45 min before whisker stimulation. Remarkably, CPP application completely blocked the increase in both spine and shaft sGluA1 intensity following whisker stimulation (Fig. 5), which indicates that this form of AMPAR plasticity is an NMDA receptor–dependent process.

Another characteristic of LTP is that it is long lasting and is maintained for several hours in vitro as measured using extracellular field recordings, and for several days or even months in vivo as measured using chronic field recordings. To determine whether the increase in spine sGluA1 intensity was long lasting, we imaged neurons for up to 2 d after whisker stimulation. We found that the 30% increase in spine sGluA1 intensity following whisker stimulation was maintained for at least 48 h, and that changes in spine size were, on average, small and not significant (Fig. 6). Interestingly, the initial 30% increase in shaft sGluA1 intensity at 1 h after whisker stimulation (Fig. 2c) decreased to 10% by 6 h after stimulation and returned to unstimulated levels by 48 h after stimulation (Fig. 6d). Furthermore, the initial correlation between changes in the intensity of spine sGluA1 and of shaft sGluA1 became weaker over time, and no significant correlation remained at 48 h after stimulation (Supplementary Fig. 8). These results suggest that shaft sGluA1 insertion and spine sGluA1 incorporation are two related but distinct events such that initial extrasynaptic (shaft) insertion of sGluA1 may supply GluA1 for spine incorporation, but after stabilization of the additional spine sGluA1, the amount of shaft sGluA1 declines. Together, these results demonstrate that acute whisker stimulation leads to an NMDA receptor–dependent LTP-like phenomenon that results in long-lasting spine-surface incorporation of GluA1.

DISCUSSION

In our study we visualized AMPARs in vivo in live animals and observed the real-time dynamics of AMPARs in response to sensory stimulation. We demonstrate that acute whisker stimulation drove increases in surface GluA1 intensity in both spines and dendritic shafts in vivo. Interestingly, only a subset of dendrites in the stimulated
barrel column responded to sensory stimuli by modifying their surface GluA1 levels. The initial spine properties can affect future experience-dependent changes in spines: specifically, small spines increased more in size and sGluA1 content than did large spines following whisker stimulation. Absolute change and percent (relative) change in sGluA1 intensity are two different measures for quantifying spine changes following stimulation, and we used both types of change in this study to characterize the properties of synapse dynamics. However, percent changes may be more relevant physiologically, as synaptic plasticity, such as LTP, is usually quantified as a percent change from baseline synaptic transmission. Increases in spine sGluA1 intensity following whisker stimulation were well coordinated with and distinct from changes in shaft sGluA1 intensity. Shaft sGluA1 may serve as a pool of extrasynaptic AMPAR subunits used for synaptic recruitment after stimulation. Furthermore, sGluA1 intensity in neighboring spines tended to change in the same direction as that in stimulated spines following whisker stimulation, which suggests that neighboring spines cooperatively increase their AMPAR content. Finally, the increase in spine sGluA1 intensity evoked by whisker stimulation was NMDA receptor dependent and long lasting, indicating that in vivo whisker stimulation may induce an LTP-like phenomenon in layer 2/3 pyramidal neurons similar to what has recently been shown by in vivo electrophysiological techniques.

In most previous studies, spine structural dynamics have been used as a measure of synaptic plasticity. However, we saw no significant difference in spine turnover between stimulated and control animals during the same time frame in which we saw significant changes in amounts of sGluA1. These results indicate that one might miss key plasticity events occurring within existing spines in an investigation of spine turnover in isolation. Although the number of spine AMPARs does not directly indicate synaptic strength, spine AMPAR content is a well-known strong correlate of synaptic strength and, it has been established that synaptic connections can be strengthened or weakened by the addition or removal of synaptic AMPARs. AMPAR imaging allowed us to obtain information about the dynamic plasticity that is occurring in spines, in addition to our investigation of spine dynamics and spine-size changes. In most spine-imaging studies, the number of spines that change in response to activity is quite small. In contrast, our data show that plasticity at preexisting spines was much more widespread, and the ability to image receptors in vivo opens up a totally new way to image plasticity. To our knowledge, our study is the first to examine AMPARs in vivo and provide direct observations, in real time, of AMPAR dynamics in response to sensory experience. Future experiments examining AMPAR dynamics during plasticity in other cortical regions and in mouse lines in which various key synaptic proteins, such as PSD-95, SAP-97 and PICK1, have been deleted or altered will help identify the essential regulators of AMPAR trafficking in vivo and explain the molecular mechanisms underlying long-term synaptic plasticity in the brain.

METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

Y.Z., D.-T.L. and R.L.H. designed experiments. Y.Z. performed experiments and analyzed data. R.H.C. developed analytic tools and designed the software. Y.Z., R.H.C., D.J.L. and R.L.H. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

All experimental protocols were conducted according to the National Institutes of Health guidelines for animal research and were approved by the Animal Care and Use Committee at Johns Hopkins University School of Medicine.

**In utero electroporation.** Layer 2/3 progenitor cells were transfected by in utero electroporation of E15 embryos from timed pregnant C57BL/6j mice (Charles River) as previously described. Approximately 0.2 μl of DNA solution containing Fast Green as a marker was pressure injected into the lateral ventricle of each embryo through a pulled-glass pipette. Five pulses of 35 V (50 ms on, 95 ms off at 1 Hz) were delivered, targeting the barrel cortex, through 5-mm tweezer electrodes connected to a square wave electroporator (CUY21, π Protech). SEP-GluA1, myc-GluA2 and dsRed2 were used for in utero electroporation at a 4:4:1 ratio. GluA2 was included in an attempt to approximate the natural GluA1/GluA2 ratio in transfected cells.

**Craniotomy.** Pups born after in utero electroporation (males and females) were implanted with a cranial window overlying the barrel cortex region at the age of 10–12 weeks as previously described. The antibiotics sulfamethoxazole (1 mg/ml) and trimethoprim (0.2 mg/ml) were chronically administered in the drinking water, and the animals were housed individually after surgery.

**Whisker stimulation.** All whiskers were kept intact for all mice. A single whisker (B2, C2 or D2) was deflected at 10 Hz for 1 h, and images were taken in the corresponding barrel column in the contralateral hemisphere.

**Optical intrinsic signal imaging.** Two to three weeks after the cranial window surgery, optical intrinsic signal imaging was performed as described previously. Mice were anesthetized and maintained on 0.5% isoflurane supplemented by xylazine (13 mg/kg). Optical images of the barrel cortex were acquired at 30 Hz using a CCD (charge-coupled device) camera (Sony XC-ST70) under red LED light (630 nm) with a 2.5:0.0.075 numerical aperture (NA) Plan Neofluar Zeiss objective. Images were first converted to 32-bit depth to display results with high precision, and then the multiframe image stacks were averaged across 30 trials. Next, we collapsed images across time, separately averaging 15 baseline and 15 response images. Finally, these two images were Gaussian filtered (σ = 10 μm) and baseline subtracted.

**Two-photon imaging.** In vivo images were acquired of mice under isoflurane anesthesia (0.5% vol isoflurane/vol O2) with a custom-built, two-photon laser-scanning microscope controlled by ScanImage written in MATLAB. Apical dendrites of layer 2/3 pyramidal neurons of the mouse barrel cortex were imaged using a 20×/1.0 NA water-immersion objective lens (Zeiss). SEP-GluA1 and dsRed2 were excited at 910 nm with a Ti:sapphire laser (Coherent) with −100 mW of power delivered to the back aperture of the objective. Green and red fluorescence signals were separated by a set of dichroic mirrors (MOM system, Sutter Instrument) and filters (ET525/50m for green channel, ET605/70m for red channel, Chroma). Image stacks were acquired at 1,024 × 1,024 pixels with a voxel size of 0.18 μm in x and y and a z-step of 1 μm. Representative images shown in figures were median filtered, up-scaled and contrast enhanced.

**Spine analysis.** All spine dynamics and intensity analyses were performed using custom-written software in Igor Pro (WaveMetrics). Documentation for the software can be found at http://robertcudmore.org/.

**Spine dynamics.** Spines at each time point were visually identified and manually marked as 3D points at their tips using the raw image stacks from the structural dsRed2 channel. Each dendrite (~100 μm) was traced to produce a dendritic backbone and radius using a modified version of the Simple Neurite Tracer Plugin in FIJI. The same dendritic tracings and spine markings were used for analyses of both spine turnover and intensity. Manually marked spines were automatically connected to the dendritic backbone by following the brightest linear path. This connection point was used to determine each spine's distance (in micrometers) along the dendritic backbone from a manually identified fiduciary point (common to all image stacks) and to semi-automatically identify corresponding spines from one image stack to the next. Finally, the point at which each spine connected to the dendritic backbone and the correspondence of spines between time points was visually verified and manually edited for errors. For a spine to be included in the final analysis, it had to protrude from the dendritic backbone by more than four pixels (>0.72 μm) and be primarily parallel to the imaging plane. Spines that were present from one time point to the next were categorized as 'persistent'; spines that were present at a given time point but absent at the preceding time point were categorized as 'added' and spines that were present at a given time point but not in the next were categorized as 'subtracted'.

**Intensity analysis.** The same dendritic tracings and spine markings were used for analysis of spine turnover and intensity. Only spines that could be visually identified at all time points were included in the final intensity analysis. Each spine was assigned three different nonoverlapping regions of interest (ROIs): spineROI, shaftROI and backgroundROI (Supplementary Fig. 4). The spineROI enclosed the spine head and did not include pixels within the radius of the dendritic backbone. The shaftROI was constructed from the backbone line and radius of the dendrite, centered on each spine and expanded along the dendrite to match the number of pixels in the spineROI. The backgroundROI (same shape and number of pixels as the spineROI) was translated in x/y to a nearby region of the image that was representative of the background fluorescence. Each spineROI was defined by a width (1 μm), and all ROIs extended in three dimensions up and down from the best imaging plane (±1 plane). We parameterized the intensity analysis to check that our results remained consistent. Briefly, we varied both the spine width (0.8–1.2 μm) and the ROI ± plane and obtained similar results.

To measure the spine intensity for the SEP-GluA1 channel, we applied the same three ROIs for each spine to the green/SEP-GluA1 channel. The spineROI and backboneROI were in the same position, and the backgroundROI was again translated in x/y to minimize the background intensity in the green/SEP-GluA1 channel. All spine and shaft intensity measures were normalized to the shaft of the red channel as follows:

\[
\text{Spine structure intensity} = \frac{\sum \text{spineROI}_{\text{GluA1}} - \sum \text{backgroundROI}_{\text{GluA1}}}{\sum \text{shaftROI}_{\text{dsRed2}}}
\]

\[
\text{Spine sGluA1 intensity} = \frac{\sum \text{spineROI}_{\text{GluA1}} - \sum \text{backgroundROI}_{\text{GluA1}}}{\sum \text{shaftROI}_{\text{dsRed2}}}
\]

\[
\text{Shaft sGluA1 intensity} = \frac{\sum \text{shaftROI}_{\text{GluA1}} - \sum \text{backgroundROI}_{\text{GluA1}}}{\sum \text{shaftROI}_{\text{dsRed2}}}
\]

Here, the “dsRed2” and “GluA1” subscripts refer to the intensities obtained from the red/dsRed2 and green/SEP-GluA1 channels, respectively.

We carefully examined the z-planes above and below the spine and shaft ROIs to confirm that our intensity values were not contaminated by signal from spines protruding out of the imaging plane.

**Categorizing changes in spine intensity.** To group spines into ‘up’, ‘down’ and ‘same’ categories, we used the s.d. of the percent change in the control at hour 1 as a threshold (30% for spine sGluA1; Figs. 3d, g and 4d).

**Monte Carlo simulations.** We tested whether the number of spines observed in group 1 (upper right or lower left regions of Fig. 4e) occurred by chance using a Monte Carlo method by randomly shuffling the spine GluA1 intensity of each spine's nearest neighbor and counting the number of spines in group 1. A P value was calculated as (n + 1)/(r + 1), where n is the number of shuffles with the number of spines in group 1 greater than the number we observed, and r is the number of shuffles (10,000). The same Monte Carlo method was used for Figure 4f, where n is the number of shuffles with the number of spines in group 2 (upper left or lower right regions) less than the number we observed, and r is the number of shuffles (10,000).
Statistical analysis. The data distribution was assumed to be normal but was not formally tested. No statistical methods were used to predetermine sample sizes, but our sample sizes were similar to those generally employed in the field. When possible, we used Monte Carlo shuffling or the nonparametric Mann-Whitney test (noted in figure legends). Otherwise, we assumed the data points had a normal distribution and used ANOVA or t-test. All the tests were two-sided. Data collection and analysis were not performed blind to the conditions of the experiments. Animals were randomly assigned to control or whisker stimulation groups if they had a detectable signal in the barrel cortex for two-photon imaging two weeks after surgery.

A Supplementary Methods Checklist is available.

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