Gephyrin-Independent GABA<sub>A</sub>R Mobility and Clustering during Plasticity

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Abstract

The activity-dependent modulation of GABA-A receptor (GABA<sub>A</sub>R) clustering at synapses controls inhibitory synaptic transmission. Several lines of evidence suggest that gephyrin, an inhibitory synaptic scaffold protein, is a critical factor in the regulation of GABA<sub>A</sub>R clustering during inhibitory synaptic plasticity induced by neuronal excitation. In this study, we tested this hypothesis by studying relative gephyrin dynamics and GABA<sub>A</sub>R declustering during excitatory activity. Surprisingly, we found that gephyrin dispersal is not essential for GABA<sub>A</sub>R declustering during excitatory activity. In cultured hippocampal neurons, quantitative immunocytochemistry showed that the dispersal of synaptic GABA<sub>A</sub>Rs accompanied with neuronal excitation evoked by 4-aminopyridine (4AP) or N-methyl-D-aspartic acid (NMDA) precedes that of gephyrin. Single-particle tracking of quantum dot labeled-GABA<sub>A</sub>Rs revealed that excitation-induced enhancement of GABA<sub>A</sub>R lateral mobility also occurred before the shrinkage of gephyrin clusters. Physical inhibition of GABA<sub>A</sub>R lateral diffusion on the cell surface and inhibition of a Ca<sup>2+</sup> dependent phosphatase, calcineurin, completely eliminated the 4AP-induced decrease in gephyrin cluster size, but not the NMDA-induced decrease in cluster size, suggesting the existence of two different mechanisms of gephyrin declustering during activity-dependent plasticity, a GABA<sub>A</sub>R-dependent regulatory mechanism and a GABA<sub>A</sub>R-independent one. Our results also indicate that GABA<sub>A</sub>R mobility and clustering after sustained excitatory activity is independent of gephyrin.

Introduction

Inhibitory neurotransmission plays a critical role in the regulation of neuronal excitability and information processing in the brain. GABA-A receptors (GABA<sub>A</sub>Rs) are neurotransmitter receptors that mediate fast inhibitory neurotransmission in the central nervous system [1]. The number of GABA<sub>A</sub>Rs at the synapse is a factor that controls the efficacy of GABAergic transmission [2,3]. The number of synaptic GABA<sub>A</sub>Rs can be altered within a few minutes depending on neuronal inputs in the hippocampus. A brief application of N-methyl-D-aspartic acid (NMDA), which induces a chemical form of long-term depression [2,3]. The interaction between neurotransmitter receptors and postsynaptic density proteins is an important factor that determines synaptic receptor number and density [14,15]. Gephyrin is a scaffold protein that directly binds to the α1–α3 subunit of GABA<sub>A</sub>Rs [16,17,18] and multiple proteins including tubulin, forming clusters at the GABAergic synapse [19]. Gephyrin plays a critical role in the regulation of synaptic GABA<sub>A</sub>R stability because gene knockout, RNAi knockdown, and prevention of GABA<sub>A</sub>R–gephyrin interaction result in a decrease in the number and density of synaptic GABA<sub>A</sub>Rs and an increase in GABA<sub>A</sub>R mobility on the cell surface [16,20,21]. On the other hand, the formation and maintenance of synaptic gephyrin clusters also require synaptic localization of GABA<sub>A</sub>Rs [13,22,23,24,25,26,27].
A previous study revealed that the amount of postsynaptic gephyrin decreases when the number of synaptic GABA<sub>A</sub>Rs decreases as a result of excitatory activity [11]. In the present study, we tested the hypothesis that gephyrin declustering could be the starting point of this activity-induced regulation of GABA<sub>A</sub>R lateral mobility and the number of postsynaptic GABA<sub>A</sub>Rs. Contrary to this hypothesis, we found evidence suggesting that excitatory activity impacts clustering of GABA<sub>A</sub>Rs first and gephyrin later.

Results

Activity-dependent decrease in synaptic GABA<sub>A</sub>Rs precedes that in gephyrin

We have previously shown the decrease in synaptic GABA<sub>A</sub>Rs and gephyrin when excitatory activity is increased [11]. To examine the timing of this process, we tracked changes in the immunofluorescence of synaptic GABA<sub>A</sub>R, gephyrin, and the presynaptic marker protein synapsin after pharmacological neuronal stimulation every 2.5 min in cultured rat hippocampal neurons. For GABA<sub>A</sub>R labeling, we developed a custom-made antibody that recognizes the extracellular domain of rat GABA<sub>A</sub>R (amino acids 39–67). We confirmed that this antibody specifically recognized mouse GABA<sub>A</sub>R γ2 subunits expressed in HEK cells (Fig. S1A–C). The antibody labeled clusters on the dendrites and cell bodies of cultured hippocampal neurons (Fig. S1D), as visualized by immunocytochemical staining with the antibody against the GABA<sub>A</sub>R γ2 subunit (amino acids 39–53) used in a previous study [11] (Fig. S1E). We therefore concluded that the anti-GABA<sub>A</sub>R γ2 antibody selectively recognizes the rodent GABA<sub>A</sub>R γ2 subunit.

Excitatory neuronal activity was induced by incubating cells with the potassium channel blocker 4-aminopyridine (4AP; 50 μM) for 2.5, 5, 7.5, and 10 min before fixation. Treatment with 4AP did not affect the immunofluorescence intensity of synapsin (Fig. 1A and D), suggesting that the increase in neuronal activity has only a minor effect on the size of presynaptic terminals. By contrast, the immunoreactivity associated with total (synaptic and extrasynaptic) GABA<sub>A</sub>Rs significantly decreased to 75.3%±2.9% of non-treated control cells within 2.5 min (p<0.005, Kruskal–Wallis test), no further decrease was observed (0.05, Tukey’s t-test; Fig. 2A). However, synaptic gephyrin-associated immunofluorescence in the cells stimulated by 4AP for 60 s maintained the same intensity as observed in 4AP non-treated cells (106.6%±3.8% of control cells, p>0.05, Welch’s t-test; Fig. 2B). We then examined the timing of NMDA-induced changes in GABA<sub>A</sub>R- and gephyrin-associated immunoreactivities, as the activation of the NMDA receptor and subsequent Ca²⁺ influx is also involved in the neuronal excitatory activity-dependent decrease in GABA<sub>E</sub>ergic synaptic transmission and declustering of GABA<sub>A</sub>Rs at inhibitory synapses [7,12,28]. When neurons were stimulated by 50 μM NMDA with its co-agonist, glycine, and TTX for 60 s, synaptic GABA<sub>A</sub>R immunoreactivity declined to 76.1%±2.3% of control cells (p<0.005, Welch’s t-test; Fig. 2C). By contrast, synaptic gephyrin-associated immunofluorescence was unaffected by NMDA stimulation for 60 s (96.8%±4.7% of control cells, p>0.05, Welch’s t-test; Fig. 2D). Longer NMDA treatment (150 s) resulted in the reduction of synaptic gephyrin immunoreactivity, as similarly observed with 4AP treatment; synaptic gephyrin immunoreactivity was reduced to 77.9%±2.2% of control cells (p<0.005, Welch’s t-test; Fig. 2E). These results, together with the results of the time-course analysis of 4AP treatment, indicate that the excitatory activity-induced decrease in the number of synaptic GABA<sub>A</sub>Rs at postsynapses takes place before the shrinkage of synaptic gephyrin clusters.

Modulation of GABA<sub>A</sub>R diffusion is complete before that of gephyrin clustering

The increase in GABA<sub>A</sub>R lateral diffusion dynamics plays a key role in neuronal activity-dependent decrease in GABA<sub>A</sub>R clustering at inhibitory synapses [11,12]. Therefore, we conducted a time-course analysis of GABA<sub>A</sub>R lateral diffusion dynamics after 4AP stimulation using single-particle tracking with quantum dots (QD-SPT) [29]. Endogenous GABA<sub>A</sub>Rs were targeted with an antibody against the extracellular domain of the γ2 subunit (Fig. S1D) and subsequently labeled with an intermediate biotinylated Fab fragment and streptavidin-coated QDs. The lateral diffusion parameters after 4AP stimulation were calculated from the trajectories of GABA<sub>A</sub>Rs labeled with QDs (GABA<sub>A</sub>R-QDs) (Fig. 3A). The location of the active synapse was visualized by labeling with the amphiphilic dye FM4–64, induced after a burst of activity with 40 mM KCl. We confirmed that this FM4–64 labeling did not affect the GABA<sub>A</sub>R diffusion coefficient both in the absence and presence of 4AP treatment (Fig. S3A). The diffusion coefficient of GABA<sub>A</sub>R-QD at the synapse obtained by synaptic trajectories (red in Fig. 3A) was significantly increased within 2.5 min after the onset of 4AP stimulation (0–10 min, p<0.005, Kruskal–Wallis test; 0 vs. 2.5 min, p<0.005, Mann–Whitney U test; Fig. 3B and C). An additional increase in diffusion coefficient was not induced by longer incubation (2.5–10 min, p>0.05, Kruskal–Wallis test). In the absence of FM4–64 labeling, a 4AP-induced increase in diffusion coefficient was observed within 4 min (Fig. S3B), suggesting that the KCl-induced burst during FM4–64 labeling does not significantly impact the time course of 4AP-induced changes in the GABA<sub>A</sub>R diffusion coefficient. Forty to fifty percent of synaptic GABA<sub>A</sub>R-QD exhibited “confined diffusion,” i.e., lateral diffusion limited to a
Figure 1. Time-course analysis of 4AP-induced decrease in GABA\(_{\alpha}R\)- and gephyrin-associated immunofluorescence. A–C: Representative examples of immunoreactivity associated with synapsin (A), GABA\(_{\alpha}R\) (B), and gephyrin (C) on the dendrites of hippocampal neurons (21–27 DIV) treated with 50 \(\mu\)M 4AP for 0–10 min. Scale bars: 5 \(\mu\)m. D–F: Time-course plots of changes in normalized fluorescence intensities (averages \(\pm\) SEM) of total clusters of synapsin (D), GABA\(_{\alpha}R\) (E), and gephyrin (F) following 4AP treatment. G, H: Time-course plots of 4AP-induced reduction in the normalized fluorescence intensities of synaptic GABA\(_{\alpha}R\) (G) and gephyrin (H) clusters. NS: \(p > 0.05\), *: \(p < 0.05\), ***: \(p < 0.005\), Tukey’s range test in ANOVA, \(n = 40\) cells/condition (4 cultures).

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Figure 2. Activity-dependent decrease in synaptic clusters of GABA\(_{\alpha}R\) preceding that of gephyrin. Left: Representative examples of immunoreactivity associated with GABA\(_{\alpha}R\) (A, C) and gephyrin (B, D, E) in the presence (A, B: 4AP, C–E: NMDA) or absence (Con) of stimulation for the indicated times. Right: Normalized fluorescence intensities (averages \(\pm\) SEM) of synaptic GABA\(_{\alpha}R\) (A, C) and gephyrin (B, D, E) clusters following stimulation. Note that fluorescence intensity of gephyrin was unchanged 60 s after the onset of stimulation (4AP: B, NMDA: D), while that of GABA\(_{\alpha}R\) significantly decreased at 60 s (4AP: A, NMDA: C). Scale bars: 5 \(\mu\)m. NS: \(p > 0.05\), ***: \(p < 0.005\), Welch’s t-test, \(n = 30\) cells/condition (3 cultures).

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small surface area [30], as reported previously [11]. The size of confinement was calculated for this population (see Materials and Methods). The average confinement size was significantly increased to 131.4% ± 6.9% of control cells by 4AP treatment for 2.5 min (0 vs. 2.5 min, \( p < 0.005 \), Tukey’s range test in ANOVA) and then maintained during further incubation (2.5-10 min, \( p < 0.05 \), Tukey’s range test in ANOVA; Fig. 3D). Furthermore, the synaptic dwell time of GABA\(_A\)-R-QD decreased to 73.8% ± 3.8% of control cells at 2.5 min (0 vs. 2.5 min, \( p < 0.005 \), Tukey’s range test in ANOVA); however, no further decrease was observed after 2.5 min (2.5-10 min, \( p > 0.05 \), Tukey’s range test in ANOVA) (Fig. 3E). These results indicate that 4AP-dependent modification of GABA\(_A\)-R lateral diffusion reaches a steady state within 2.5 min, which probably leads to the decrease in the number of synaptic GABA\(_A\)-Rs (Fig. 1G). The time taken by gephyrin-associated immunofluorescence to reach a steady state was 7.5 min (Fig. 1H). This is 5 min longer than the time taken for GABA\(_A\)-R diffusion dynamics to reach a steady state. Therefore, our results indicate that the activity-dependent change in the lateral diffusion of GABA\(_A\)-Rs is completed before the dispersion of gephyrin clusters.

4AP-dependent modulation of gephyrin clusters depends on GABA\(_A\)-R lateral mobility

It is well established that synaptic gephyrin clustering also requires synaptic localization of GABA\(_A\)-Rs [13,22,23,24,25, 26,27]. Based on the finding that the excitatory activity-induced modulation of GABA\(_A\)-R lateral diffusion was accomplished before gephyrin declustering, we hypothesized that gephyrin clustering could be sensitive to GABA\(_A\)-R diffusion dynamics, in addition to its existence and localization. To confirm this hypothesis, we manipulated GABA\(_A\)-R diffusion dynamics by artificially cross-linking (XL) the GABA\(_A\)-R \( \gamma \)2 subunits using antibodies, as performed previously for AMPA receptors and metabotropic glutamate receptors [31,32]. Successful XL of GABA\(_A\)-Rs was confirmed by the appearance of fluorescent clusters labeled with the Alexa Fluor®-conjugated antibody used for XL of primary antibodies targeted to GABA\(_A\)-Rs (Fig. 4A). The fluorescence intensities of these cross-linked GABA\(_A\)-R clusters were not affected by 4AP treatment (Fig. 4B). Next GABA\(_A\)-R mobility was examined by QD-SPT. Trajectories revealed that the area explored by GABA\(_A\)-R-QDs were greatly reduced when surface GABA\(_A\)-Rs were cross-linked, both inside (red, Fig. 4C) and outside (blue, Fig. 4C) the synapses. In the absence of 4AP, XL induced an approximately 100-fold reduction in GABA\(_A\)-R-QD diffusion coefficients (Fig. 4D), an approximately 3.7-fold increase in the percentage of immobilized GABA\(_A\)-R-QD (Fig. 4E), a 13.6% decrease in the confinement size (Fig. 4F), and an approximately 3.4-fold increase in the synaptic dwell time (Fig. 4G), indicating that GABA\(_A\)-R-QD lateral diffusion is greatly inhibited by XL. Moreover, XL blocked the 4AP-induced significant increase in the diffusion coefficient, enlargement of confinement size, and decrease in the synaptic dwell time of GABA\(_A\)-R-QDs (Fig. 4D-G).

We also confirmed that 4AP-induced increase in intracellular Ca\(^{2+} \) remained unaffected under XL conditions, which is responsible for the increase in GABA\(_A\)-R lateral diffusion. Ca\(^{2+} \) imaging with fluo-4 at proximal dendrites revealed that increase in intracellular Ca\(^{2+} \) was successfully induced by 4AP treatment even under XL conditions (Fig. 5B) as observed in the absence of XL (Fig. 5A), and that there was no significant difference in the peak amplitudes (Fig. 5C) and levels of increase in intracellular Ca\(^{2+} \) as represented by the area under the curve (Fig. 5D) between control and XL cells. Taken together, these experiments indicate that XL could inhibit GABA\(_A\)-R lateral diffusion without affecting intracellular Ca\(^{2+} \) elevation. Next we examined 4AP-induced declustering of gephyrin under XL conditions (Fig. 5E). Although a previous study showed that a 12-h XL of GABA\(_A\)-R resulted in the formation of extrasynaptic gephyrin clusters [33], the total number of gephyrin clusters in GABA\(_A\)-R XL conditions was not different from that without XL (Fig. 5F), suggesting that extrasynaptic artificial gephyrin clusters are not formed under our XL conditions. In the cells without GABA\(_A\)-R XL, 4AP incubation for 15 min significantly decreased gephyrin-associated immunoreactivity [Fig. 5G (−XL)]. Conversely, the same 4AP stimulation failed to induce reduction in gephyrin immunofluorescence in the cells with GABA\(_A\)-R XL (Fig. 5G (+XL)).

XL of surface GABA\(_A\)-Rs is an extreme condition in which a large proportion of surface GABA\(_A\)-Rs are immobilized. Therefore, we also examined the effect of a calcineurin inhibitor, cyclosporin A (CysA), which does not immobilize surface GABA\(_A\)-Rs but suppresses the NMDA-induced increase in GABA\(_A\)-R mobility [11,12], on gephyrin clustering. We confirmed that the 4AP-driven increase in the synaptic diffusion coefficient (Fig. 6A) and reduction in the synaptic dwell time (Fig. 6B) were completely inhibited in the presence of 1 μM CysA (Fig. 6C and D), which is in agreement with previous studies of NMDA stimulation [11,12]. Ca\(^{2+} \) imaging with fluo-4 revealed that increase in intracellular Ca\(^{2+} \), sustained for at least 15 min, was normally induced by 4AP even in the presence of CysA (Fig. 6E). The peak amplitude (Fig. 6F) and Ca\(^{2+} \) influx level represented by the area under the curve (Fig. 6G) was not significantly affected by CysA (\( p > 0.05 \), Welch’s t-test). Under this condition, the size of synaptic clusters of GABA\(_A\)-R and gephyrin was quantified by immunocytochemistry. The shrinkage of synaptic GABA\(_A\)-R clusters induced by 4AP stimulation for 30 min (Fig. 6H) was blocked completely in the presence of CysA (Fig. 6I). Furthermore, 4AP-driven gephyrin declustering at the synapse (Fig. 6J) was also prevented by CysA treatment (Fig. 6K), despite the increase in cytosolic Ca\(^{2+} \).

In summary, the above results indicate that 4AP-driven gephyrin declustering is inhibited when there is no increase in GABA\(_A\)-R lateral diffusion in response to neuronal excitation. Our results also imply that synaptic gephyrin clustering is dependent on the mobility of GABA\(_A\)-Rs during sustained activity induced by 4AP.

NMDA-driven gephyrin declustering is independent of GABA\(_A\)-R mobility

The result of GABA\(_A\)-R XL and CysA experiments with 4AP stimulation suggested the existence of a mechanism, dependent on GABA\(_A\)-R surface mobility, which regulates gephyrin clustering. Finally, we examined whether gephyrin clustering is constantly subjected to this GABA\(_A\)-R-dependent regulation during sustained neuronal excitation. NMDA stimulation was applied to increase neuronal activity, and effects of CysA treatment on synaptic GABA\(_A\)-R and gephyrin clusters were examined. In agreement with previous reports that CysA inhibits NMDA-induced increase in GABA\(_A\)-R lateral diffusion [11,12] and declustering of GABA\(_A\)-Rs [12], the dispersal of synaptic GABA\(_A\)-R observed after 30 min of NMDA treatment (Fig. 7A) was completely blocked by the presence of CysA (Fig. 7B). NMDA stimulation significantly diminished the size of gephyrin clusters to 26.7% ± 0.9% of control cells (Fig. 7C). Unlike the GABA\(_A\)-R clusters, synaptic gephyrin clusters were reduced (31.0% ± 2.1% of control cells, Fig. 7D) even in the presence of CysA. XL of surface GABA\(_A\)-Rs also failed to inhibit NMDA-induced declustering of gephyrin (Fig. 7E). Interestingly, increase in Ca\(^{2+} \) induced by NMDA stimulation, which persisted for at least 15 min, was larger than that induced by 4AP.
The average peak amplitude of Ca\(^{2+}\) elevation evoked by NMDA was 1.2 times larger than that induced by 4AP (p < 0.005, Welch's t-test; Fig. 7G) and the level of increase in Ca\(^{2+}\) during NMDA stimulation was 1.3 times higher than that during 4AP stimulation (p < 0.005, Welch's t-test; Fig. 7H). Taken together, these results suggest that gephyrin clustering is not dependent on GABA\(_{\alpha}\)R mobility during sustained activity induced by NMDA, possibly at high levels of increase in Ca\(^{2+}\). More importantly, despite the loss of synaptic gephyrin clustering by NMDA stimulation (Fig. 7D), Cys A blocked NMDA-induced declustering of GABA\(_{\alpha}\)Rs (Fig. 7B) and the increase in lateral diffusion [11,12].

These results clearly indicate that lateral diffusion of GABA\(_{\alpha}\)Rs at the synapse and synaptic GABA\(_{\alpha}\)R clustering during inhibitory synaptic plasticity are independent of the amount of synaptic gephyrin present.

**Discussion**

The main finding of this study is that changes in lateral diffusion dynamics and number of synaptic GABA\(_{\alpha}\)Rs preceded gephyrin declustering during excitatory activity. In addition, our results indicate that synaptic GABA\(_{\alpha}\)R diffusion and clustering are...
independent of the status of gephyrin clusters during sustained excitatory activity.

Gephyrin is considered a key protein that controls GABAAR stability at the postsynapse [13,16,20,21]. In this study, we tested the hypothesis that the excitatory activity-dependent reduction in postsynaptic GABAARs [11,12], which could be involved in GABAergic synaptic plasticity, is initiated by the dispersion of gephyrin from clusters. If this hypothesis were correct, excitatory activity should have affected gephyrin first or at least at the same time when affecting GABAARs. Contrary to this expectation, a detailed time-course analysis indicated that the dispersal of GABAAR clusters induced by the enhancement of GABAAR...
lateral mobility preceded the dispersal of gephyrin. Our results suggest that neuronal activity-induced rapid decrease in GABAAR numbers at mature inhibitory synapses is not mediated by gephyrin declustering. This notion was further supported by the observation that synaptic GABAAR mobility and clustering were not affected by NMDA in the presence of CysA, while gephyrin cluster largely decreased under the same conditions. Our findings suggest that excitatory activity-induced plasticity in GABAergic synapses is induced independent of the status of gephyrin clusters. There was no remarkable difference in the recovery time course of GABAAR and gephyrin cluster size after 4AP removal, similar to the process of synaptogenesis in hippocampal neurons [34,35]. This suggests that the reaccumulation of GABAAR and gephyrin to the inhibitory synapse occurs simultaneously. It remains unclear whether gephyrin is critical for the recovery of GABAAR clusters. Furthermore, our results suggested that there are existence two regulatory mechanisms of gephyrin clustering during sustained activity: GABAAR-dependent and GABAAR-independent mechanisms. The amount of gephyrin in clusters was maintained even in the presence of 4AP, when surface GABAARs were immobilized by XL and when 4AP-induced increase in GABAAR diffusion was prevented by CysA-treatment. This finding indicates that GABAAR lateral diffusion dynamics can affect clustering of the scaffold protein gephyrin. Recent theoretical modeling of postsynaptic structures based on chemical potential proposed another concept which states that the stabilization of the postsynaptic structure is reciprocal. In other words, scaffold proteins stabilize receptors and receptors stabilize scaffold proteins [36]. Together with the fact that gephyrin is crucial for the stabilization of postsynaptic GABAARs [16,20,21], our data provide direct evidence of a reciprocal mechanism that stabilizes...
the structure of GABAergic synapses. Regulation of postsynaptic scaffolds by neurotransmitter receptors is involved in synaptogenesis and the maintenance of GABAergic synapses, as evidenced by the fact that the absence of some GABAAR subunits results in the disappearance of gephyrin clusters [22,23,24,25,26,27]. Our present results, which imply that activity-induced mobilization of surface GABAARs destabilizes gephyrin clusters, also raise the possibility that GABAAR lateral mobility, in addition to its existence and localization, could be a primary determinant of stability of mature GABAergic synaptic structures during synaptic

Figure 6. Prevention of 4AP-induced gephyrin declustering by calcineurin inhibitor CysA. A–D: Inhibition of 4AP-driven mobilization of GABAAR-QDs by CysA treatment. Diffusion coefficients in the synapse (A and C, median ± IQR) and synaptic dwell times (B and D, averages ± SEM) in the absence (A, B) or presence (C, D) of 1 μM CysA. NS: p > 0.05, ***: p < 0.005, Mann–Whitney U test for A, C (Con: n = 535 QDs, 4AP: n = 537, CysA: n = 478, CysA+4AP: n = 506), Welch’s t-test for B, D (Con: n = 2107 events, 4AP: n = 2505, CysA: n = 1930, CysA+4AP: n = 2094). Data were obtained from 3 cultures. E–G: Intact 4AP-induced increase in cytosolic Ca^{2+} concentration under CysA treatment. Changes in intracellular Ca^{2+} levels indicated as F/F0 of fluo-4 after the addition of 4AP (black) or CysA+4AP (red) (E). Drugs, i.e. 4AP or CysA+4AP, were applied at time = 0 as indicated by the gray horizontal bar in the traces. Peak amplitudes of F/F0 (F) and areas under the F/F0-time curve (G) during 90 s after the onset of stimulation. Values indicate averages ± SEM. NS: p > 0.05, Welch’s t-test. 4AP: n = 26 cells, CysA+4AP: n = 28 cells (3 cultures). H–K: The effect of CysA treatment on 4AP-driven declustering of GABAAR and gephyrin. Left: Example of immunoreactivity associated with GABAAR (H, I) and gephyrin (J, K) on the dendrites treated with 4AP for 30 min, in the absence (H, J) and presence (I, K) of CysA. Scale bars: 5 μm. Right: Normalized fluorescence intensities (averages ± SEM) of synaptic GABAAR (H, I) and gephyrin (J, K) clusters. NS: p > 0.05, ***: p < 0.005, Welch’s t-test. Con, CysA: n = 30 cells/condition, 4AP, CysA+4AP: n = 25 cells/condition from 3 cultures.

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Figure 7. GABA<sub>A</sub>R-independent gephyrin declustering during sustained activity induced by NMDA stimulation. A–D: Effect of CysA treatment on NMDA-driven dispersal of GABA<sub>A</sub>R and gephyrin clusters. Left: Examples of GABA<sub>A</sub>R (A, B) and gephyrin (C, D) immunoreactivity in neurons incubated with NMDA for 30 min, with (B, D) and without CysA (A, C). Scale bars, 5 μm. Right: Normalized fluorescence intensities (averages ± SEM) of synaptic GABA<sub>A</sub>R (A, B) and gephyrin (C, D) clusters. *: p<0.05, **: p<0.005, Welch’s t-test. Con, CysA, CysA+NMDA: n = 30 cells/condition, NMDA: n = 25 cells/condition (3 cultures). CysA suppressed NMDA-induced dispersal of GABA<sub>A</sub>R clusters, but not that of gephyrin clusters. E: NMDA-induced gephyrin dispersal under GABA<sub>A</sub>R XL. Top: Gephyrin immunoreactive clusters in neurons with (+XL) and without (−XL) surface GABA<sub>A</sub>R XL after NMDA stimulation. Bottom: Effects of GABA<sub>A</sub>R XL and NMDA treatment on the normalized fluorescence intensity of gephyrin clusters (averages ± SEM). **: p<0.005, Welch’s t-test. n = 30 cells/condition (3 cultures). F–H: Comparison of the Ca<sup>2+</sup> influx level induced by 4AP and NMDA. Increase in Ca<sup>2+</sup> after the addition of 4AP (black) or NMDA (red). (F) Gray horizontal bars in the traces indicate the presence of 4AP or NMDA. Peak amplitudes (G) and areas under the curve (H) for F/F<sub>0</sub>-time plots during 90 s after the onset of stimulation. Values indicate averages ± SEM. ***: p<0.005, Welch’s t-test. 4AP: n = 21 cells, NMDA: n = 23 cells (3 cultures).

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plasticity. Changes in the chemical potential associated with GABA\(_\text{A}\)Rs and gephyrin, which are induced by the enhancement of lateral diffusion and subsequent decrease in synaptic GABA\(_\text{A}\)R density, could lead to a new steady state of postsynaptic molecular assembly [36].

The observation that gephyrin dispersed after NMDA stimulation regardless of GABA\(_\text{A}\)R mobility suggested that another GABA\(_\text{A}\)R-independent regulatory mechanism may control gephyrin clustering. Considering that NMDA induced a 1.3 times larger Ca\(^{2+}\) elevation than 4AP, the Ca\(^{2+}\) influx level could be one of the factors determining whether gephyrin is subjected to GABA\(_\text{A}\)R-dependent regulation or independently destabilized in response to Ca\(^{2+}\) elevation. Gephyrin is a substrate of the Ca\(^{2+}\)-dependent non-lysosomal cysteine protease calpain-1, which is activated when NMDA receptors are stimulated [37], and turnover of gephyrin is regulated by calpain-1 activity [38]. Therefore, it is possible that gephyrin stability is also controlled by the activation of calpain-1 during NMDA stimulation [39]. Therefore, it is possible that gephyrin stability is also controlled by the activation of calpain-1 during NMDA stimulation [39]. However, it must be noted that the same NMDA stimulation activated when NMDA receptors are stimulated [37], and turnover of gephyrin is regulated by calpain-1 activity [38]. Therefore, it is possible that gephyrin stability is also controlled by the activation of calpain-1 during NMDA stimulation [39]. However, it must be noted that the same NMDA stimulation activated when NMDA receptors are stimulated [37], and turnover of gephyrin is regulated by calpain-1 activity [38].

Activity-dependent regulation of GABA\(_\text{A}\)R lateral diffusion and clustering at inhibitory synapses is mediated by Ca\(^{2+}\) influx and subsequent activation of calcineurin [11,12,13]. Our present findings provide several insights into the molecular mechanism of how Ca\(^{2+}\) signaling enhances GABA\(_\text{A}\)R lateral diffusion. In the present study, we found that GABA\(_\text{A}\)R diffusion and clustering were independent of gephyrin clustering during NMDA stimulation in the presence of CysA. This finding strongly suggests that calcineurin-dependent regulation of GABA\(_\text{A}\)R mobility does not require gephyrin. Because alterations in receptor–scaffold interactions can modulate the lateral diffusion of receptors [15], we propose the existence of other GABA\(_\text{A}\)R-interacting protein(s) that contribute to GABA\(_\text{A}\)R stabilization in a gephyrin-independent manner. GABA\(_\text{A}\)R accumulation at the inhibitory synapse occurs before gephyrin accumulation during synaptogenesis in spinal cord neurons [42], suggesting the existence of a gephyrin-independent stabilization mechanism of GABA\(_\text{A}\)Rs. This gephyrin-independent pathway may enhance GABA\(_\text{A}\)R lateral diffusion via the calcineurin-dependent dephosphorylation of Ser327 in the GABA\(_\text{A}\)R y2 subunit [12]. We speculate that the dephosphorylation of Ser327 upon neuronal excitation induces the dissociation of unidentified GABA\(_\text{A}\)R-associating protein(s) from GABA\(_\text{A}\)Rs, which leads to the observed increase in GABA\(_\text{A}\)R lateral mobility.

The Ca\(^{2+}\)-dependent increase in GABA\(_\text{A}\)R lateral mobility is involved in synaptic plasticity at inhibitory synapses that may underlie neuronal disorders resulting from pathological disinhibition [11,12]. Therefore, elucidating the detailed molecular mechanism of the gephyrin-independent regulation of GABA\(_\text{A}\)R lateral mobility might contribute not only to understanding the basis of learning and memory but also to discovering therapeutic targets for neuropathies such as epilepsy.

Materials and Methods

Ethics statement

All animal procedures in this study were performed in accordance with the guidelines issued by the Japanese Ministry of Education, Culture, Sports, Science and Technology. All animal procedures in this study were approved by the Animal Experiment Committee of the RIKEN (H23-2-204). All efforts were made to minimize animal suffering and reduce the number of animals used.

Anti-GABA\(_\text{A}\)R γ2 subunit antibody production

The rabbit anti-GABA\(_\text{A}\)R γ2 subunit antibody (anti-GABA\(_\text{A}\)Rγ2) was raised against the peptide “QKSDDDYEDYASNKITWVTPLKVPEDVTVC” corresponding to amino acid residues 39–67 of the rat GABA\(_\text{A}\)R γ2 subunit, as shown previously [43]. The peptide was synthesized by the Support Unit for Bio-material Analysis at the RIKEN BSI Research Resources Center (RRC) and was subsequently injected into rabbits to obtain the antibody by the Support Unit for Animal Resources Development at the RIKEN BSI RRC.

The specificity of the antibody was confirmed using HeLa cells (RIKEN BioResource Center, Ibaraki, Japan) expressing γ1, β3, and γ2 subunits of GABA\(_\text{A}\)R (Fig. 1A and C). HeLa cells were plated onto 18-mm diameter glass coverslips and cultured in DMEM (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum and antibiotics. For transfection, a coverslip in 1 ml culture medium was incubated with the transfection mixture containing 100 μl OPTI-MEM (Invitrogen, Tokyo, Japan), mixture of DNA (γ1, β3, γ2: 0.7 μg each), and 4.2 μl TransIT-LT1 (Mirus, WI, USA) for 24 h before observation. Plasmids encoding γ1, β3, and γ2 subunits of GABA\(_\text{A}\)R were generated by subcloning the coding region into the mammalian expression vector [pcDNA3.1(zeo+)/−]; Invitrogen] using FANTOM3 clones as PCR templates (γ1: C630037M06; β3: C630014N19; γ2: B930018F17 and C290065G02) [44].

Primary cultures

Primary cultures of hippocampal neurons co-cultured with astrocytes were prepared from E18–21 Wistar rat embryos as previously described [45] with some modifications. Hippocampal cells were dissociated in plating medium comprising minimum essential medium (MEM; Invitrogen) supplemented with B27 (Invitrogen), 2 mM L-glutamine, 1 mM sodium pyruvate (Invitrogen), and antibiotics, and were plated at a density of 1.4 × 10⁶ cells/ml onto 18-mm diameter glass coverslips precoated with 0.04% polyethyleneimine (Sigma, Tokyo, Japan). Three days after plating, the culture medium was replaced with maintenance medium comprising Neurobasal-A medium (Invitrogen) supplemented with B27, 2 mM L-glutamine, and antibiotics. Cells were cultured for 21–27 days in vitro before the experiments. At least three independent cultures were used for each experiment.

Drug treatment

To increase excitatory activity, cultured hippocampal neurons were incubated with 50 μM 4AP (Nacalai Tesque) or 50 μM NMDA (Tocris, MO, USA), glycine (5 μM), and TTX (1 μM; Tocris) at 37°C in the imaging medium comprising MEM without phenol red (Invitrogen), 20 mM HEPES, 33 mM glucose, 2 mM glutamine, 1 mM sodium pyruvate, and B27. For time-course analysis of cluster recovery, neurons were treated with 50 μM 4AP for 10 min and subsequently incubated with the imaging medium for 0–15 min before fixation. For QD-SPT experiments, 4AP (final concentration, 30 μM) was added to the imaging medium immediately before recording. For Ca\(^{2+}\) imaging, recording were done for 1 min in the absence of drugs, then drugs were bath applied to the cells during the recording.

Immunocytochemistry and quantitative analysis

For GABA\(_\text{A}\)R immunostaining of cultured neurons with drug treatment, endogenous GABA\(_\text{A}\)Rs on cultured hippocampal neurons were identified using anti-GABA\(_\text{A}\)R antibodies (anti-GABA\(_\text{A}\)Rγ2). GABA\(_\text{A}\)R immunostaining was performed as previously described [46].

Expression of the β3 subunit of GABA\(_\text{A}\)R was detected using anti-GABA\(_\text{A}\)R antibody (anti-GABA\(_\text{A}\)Rβ3) [47]. Both GABA\(_\text{A}\)R antibodies recognized the γ2 subunits of GABA\(_\text{A}\)R as previously described [47]. The specificity of the antibody was confirmed using HeLa cells expressing γ1, β3, and γ2 subunits of GABA\(_\text{A}\)R (Fig. 1A and C). HeLa cells were plated onto 18-mm diameter glass coverslips and cultured in DMEM (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum and antibiotics. For transfection, a coverslip in 1 ml culture medium was incubated with the transfection mixture containing 100 μl OPTI-MEM (Invitrogen, Tokyo, Japan), mixture of DNA (γ1, β3, γ2: 0.7 μg each), and 4.2 μl TransIT-LT1 (Mirus, WI, USA) for 24 h before observation. Plasmids encoding γ1, β3, and γ2 subunits of GABA\(_\text{A}\)R were generated by subcloning the coding region into the mammalian expression vector [pcDNA3.1(zeo+)/−]; Invitrogen] using FANTOM3 clones as PCR templates (γ1: C630037M06; β3: C630014N19; γ2: B930018F17 and C290065G02) [44].
neurons were labeled with our γ2 antibodies by incubating live cells with 2.0 μg/ml antibody diluted in imaging medium for 30 min at 37°C. Subsequently, cells were stimulated by IAP or NMBA and fixed with 4% (w/v) paraformaldehyde (PFA) in PBS-0.02% NaN3 at room temperature (24–26°C) for 15 min. After permeabilization with 0.1% Triton X-100 for 3 min and incubation with 5% (w/v) bovine serum albumin (BSA; Sigma) for 30 min to block nonspecific staining, cells were labeled with the mouse anti-synapsin I antibody (1:3000; Synaptic Systems, Goettingen, Germany) in 2.5% BSA for 60 min. After washes, the cells were incubated on slides with Vectashield (Vector Laboratories, CA, USA), cells were incubated with our γ2 antibodies (2.0 μg/ml) for 30 min in the presence of drug (i.e., 4AP, NMBA+TTX+Gly, CysA) and subsequently fixed by 4% PFA. After fixation, the procedures were the same as those of experiments without CysA treatment. In some experiments (Fig. S1E), GABAAR was labeled with commercially available rabbit anti-γ2 subunit antibodies (6.0 μg/ml; Alomone Labs, Jerusalem, Israel), which were used in a previous study [11]. GABAARs on the GABAAR-expressing HeLa cells were labeled with our custom-made anti-GABAARγ2 antibody (0.8 μg/ml) as described above, and nuclei of HeLa cells were stained with DAPI.

For labeling of gephrin, cells were fixed with 4% PFA after drug stimulation and permeabilized with 0.1% Triton X-100. After blocking with 5% BSA, cells were incubated with anti-gephyrin antibody (0.33 μg/ml, clone mAb7a; Synaptic Systems) and the rabbit polyclonal anti-synapsin I antibody (1:200; Millipore, MA, USA) in the presence of 2.5% BSA for 90 min, and subsequently labeled with Alexa Fluor 488 or Alexa Fluor 594 (5–10 μg/ml; Invitrogen).

Immunofluorescence from isolated neurons was acquired on an inverted microscope (IX-70; Olympus, Tokyo, Japan) equipped with a Plan Apo 60× oil immersion objective with a numerical aperture (NA) of 1.42 (Olympus), cooled CCD camera (Orca-II-ER; Hamamatsu Photonics, Shizuoka, Japan), and appropriate filter sets for Alexa Fluor 488 (ex: 480±10 nm, em: 530±20 nm) and Alexa Fluor 594 (ex: 535±15 nm; em: 580 nm long pass). All images from a given culture were acquired with the same saturation exposure time.

Quantification of GABAAR-, gephrin-, and synapsin-associated immunofluorescence was performed using “Integrated Morphometry Analysis” function of the MetaMorph software (Molecular Device Japan, Tokyo, Japan). GABAAR- and gephrin-immunoreactive clusters and synapsin-positive presynapses were defined by processing images with multidimensional image analysis (MIA) interface, i.e., a 2D object segmentation by wavelet transform [46] and “auto threshold for light object (isodata method)” function of MetaMorph. GABAAR or gephrin clusters were defined as clusters that overlapped at least 1 pixel with presynaptic terminals. For each culture, all cluster fluorescence intensity was normalized to the average value in control cells.

**QD-SPT experiments**

Neurons were incubated with the custom-made anti-GABAARγ2 antibody (2.0 μg/ml) for 5 min, washed, and incubated with the biotinylated anti-rabbit Fab antibody (2.2 μg/ml; Jackson ImmunoResearch, PA, USA) for 5 min. Following washes, the coverslips were incubated with 1.0 nM streptavidin-coated QDs emitting at 605 nm or 625 nm (Invitrogen) in borate buffer for 1 min [29]. After washes, functional presynaptic boutons were labeled with 2 μM FM4–64 (Invitrogen) in imaging medium containing 40 mM KCl for 15 s. Incubation with antibodies and washes were performed at 37°C in the imaging medium.

The diffusive behavior of GABAAR-QD and FM4–64 signals was recorded at 37°C in the imaging medium using an inverted microscope (IX-71, Olympus) equipped with an oil immersion objective (NA 1.45, 60×; Olympus) and an EM-CCD camera (C9100; Hamamatsu Photonics) or an inverted microscope (IX-70; Olympus) equipped with an oil immersion objective (NA 1.42, 60×; Olympus) and cooled CCD camera (Orca-II-ER; Hamamatsu Photonics). Fluorescent signals were detected using appropriate filter sets for QD (ex: 455±70 nm, em: 605±20 nm) and FM4–64 (ex: 335±15 nm, em: 500 nm long pass). GABAAR-QD lateral diffusion was recorded with an integration time of 76 ms with 512 consecutive frames (38.9 s). All recordings were taken within 30 min.

**Data analysis for QD-SPT experiments**

The trajectory of GABAAR-QD was obtained by cross-correlating images with a Gaussian model of the point spread function [47], and diffusion coefficients and confinements were calculated using TI workbench software written by Dr. T. Inoue (Waseda University), as described previously [11]. Only single QDs identified by intermittent fluorescence (i.e., blinking) were analyzed. The synaptic area was defined by processing FM4–64 images with wavelet decomposition [46]. GABAAR-QDs were classified as “synaptic” when overlapping with synaptic area+2 pixels (294 nm). For the calculation of diffusion parameters in the synapse except for synaptic dwell time, the longest sub-trajectories of single GABAAR-QDs with greater than or equal to 30 points in each compartment were taken into account.

To obtain the diffusion parameters, such as the diffusion coefficient and confinement size, values of the mean square displacement (MSD) plot versus time were calculated for each trajectory by applying the following equation:

$$\text{MSD}(n, \tau) = \frac{1}{N-n} \sum_{i=1}^{N-n} \left[ (x((i+n)\tau) - x(i\tau))^2 + (y((i+n)\tau) - y(i\tau))^2 \right]$$

[(48)], where τ is the acquisition time, N is the total number of frames, and n and i are positive integers with n representing the time increment. Diffusion coefficients (D) were calculated by fitting first four points of the MSD versus time curves with the following equation:

$$\text{MSD}(n, \tau) = 4Dn\tau + b,$$

where b is a constant reflecting the spot localization accuracy. In this system, GABAAR-QDs with a diffusion coefficient (D) less than 0.0002 μm²/s were defined as immobile.

The confinement domain size, in which the diffusion of GABAAR-QD was restricted, was obtained by fitting the MSD-τ plot to the following equation:

$$\text{MSD}(n, \tau) = \frac{L^2}{3} \left( 1 - \exp \left( -\frac{12Dn\tau}{L^2} \right) \right) + 4D_{\text{mac}}n\tau$$

[30], where L² is the confined area in which diffusion is restricted, and D_{mac} is the diffusion coefficient on a long time scale. The
diffusion of GABA$_A$Rs with MSD-$
abla$ plot that does not apply \(|D - D_{\text{max}}| < 0.1 \times D_0 \text{ or } L < 0.001\) was defined as restricted motion, and only GABA$_A$R-QDs meeting this criteria were considered for calculations of confinement domain sizes [49].

The GABA$_A$R-QD dwell time inside the synapse was defined as the duration of synaptic sub-trajectories.

### GABA$_A$R XL experiments

GABA$_A$Rs on the cell surface were cross-linked by incubating neurons with the anti-$\gamma_2$ subunit antibody (0.8 $\mu$g/ml; Alomone Labs) for 10 min, washing, and incubating with Alexa Fluor®-conjugated anti-rabbit antibodies (20 $\mu$g/ml; Invitrogen) for 5 min in the imaging medium. Cells were further incubated with the biotinylated anti-rabbit Fab antibody and streptavidin-coated QDs for QD-SPT, or fixed and subsequently immunolabeled with the gephyrin antibody for quantitative immunocytochemistry, as mentioned previously. In all experiments, it was confirmed that surface GABA$_A$Rs were successfully cross-linked by fluorescence from GABA$_A$R-associated clusters (Fig. 4A).

### Ca$^{2+}$ imaging

Neurons were loaded with 0.5 $\mu$M fluo-4 AM (Invitrogen) for 5 min at 37°C. Fluo-4 fluorescence was acquired at 0.2 Hz with a 200-ms exposure at room temperature (24–26°C), with an inverted microscope [IX-70; Olympus] equipped with a 40× objective (NA 0.85, UPlanApo; Olympus), a cooled CCD camera (Orca-II-ER; Hamamatsu Photonics), and appropriate filters (ex, 480 ± 10 nm; em, 530 ± 20 nm). For longer recording (Figs. 6E and 7E), images were further acquired at 0.1 Hz from 6 min to 15 min after drug application. Data were analyzed using a TI Workbench. The ratio of the fluorescence intensities $F/F_0$, where $F$ is a fluorescence measurement, was calculated after subtraction of the background fluorescence. To estimate the level of Ca$^{2+}$ elevation, the area under the curve was calculated using Igor Pro software (WaveMetrics, OR, USA).

### Statistical analysis and image preparation

Statistical differences of data in the time course were determined using the Kruskal–Wallis (for the diffusion coefficient) and one-way ANOVA ($\rho = 0.05$) tests, followed by Tukey’s post-hoc tests (for others). For comparisons between two groups, the Mann–Whitney $U$ test or Welch’s $t$-test were performed as indicated. All statistical analysis was performed using KaleidaGraph (Synergy Software, PA, USA). Images were prepared for printing using Meta Morph, Adobe Photoshop, and Adobe Illustrator.

### Supporting Information

**Figure S1** Specificity of the anti-GABAAR $\gamma_2$ subunit antibody. (PDF)

**Figure S2** Recovery of GABAAR and gephyrin immuno-fluorescence after 4AP washout. (PDF)

**Figure S3** Lateral diffusion of GABAAR with or without FM4–64 labeling. (PDF)

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### Author Contributions

Conceived and designed the experiments: HB AT KM. Performed the experiments: FN HB MA. Analyzed the data: FN HB. Contributed reagents/materials/analysis tools: FN HB KF. Wrote the paper: FN HB MA AT KM.

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