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REVIEW ARTICLE

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Inhibitory synaptic transmission tuned by Ca^{2+} and glutamate through the control of $GABA_AR$ lateral diffusion dynamics

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Abstract

The GABAergic synapses, a primary inhibitory synapse in the mammalian brain, is important for the normal development of brain circuits, and for the regulation of the excitation-inhibition balance critical for brain function from the developmental stage throughout life. However, the molecular mechanism underlying the formation, maintenance, and modulation of GABAergic synapses is less understood compared to that of excitatory synapses. Quantum dot-single particle tracking (QD-SPT), a super-resolution imaging technique that enables the analysis of membrane molecule dynamics at single-molecule resolution, is a powerful tool to analyze the behavior of proteins and lipids on the plasma membrane. In this review, we summarize the recent application of QD-SPT in understanding of GABAergic synaptic transmission. Here we introduce QD-SPT experiments that provide further insights into the molecular mechanism supporting GABAergic synapses. QD-SPT studies revealed that glutamate and Ca^{2+} signaling is involved in (a) the maintenance of GABAergic synapses, (b) GABAergic long-term depression, and GABAergic long-term potentiation, by specifically activating signaling pathways unique to each phenomenon. We also introduce a novel Ca^{2+} imaging technique to describe the diversity of Ca^{2+} signals that may activate the downstream signaling pathways that induce specific biological output.

1 | INTRODUCTION

In the central nervous system (CNS), the chemical synapse is a highly specialized structure for neuronal transmission. There are two types of synapses, i.e., the excitatory synapse that leads to depolarization of neurons, and the inhibitory synapse that balances neuronal excitation. The balance between the excitatory and inhibitory synapses is important, as it determines neuronal output and ultimately brain activity. In CNS, $GABA_A$ receptor ($GABA_AR$), a pentameric ion channel, is essential for inhibitory GABAergic synaptic inputs and plays a key role in maintaining this excitation-inhibition balance. $GABA_ARs$ are required for the onset of the critical period (Fagiolini et al., 2004) that is a fundamental process of visual development in newborn mammals, and "Synapse pruning" for normal development of the cerebral neural circuit (Nakayama et al., 2012). Furthermore, abnormalities in GABAergic synaptic transmission are known to cause various mental/neurological diseases such as epilepsy, anxiety disorder, Huntington's disease, Angelman syndrome, Fragile X syndrome, schizophrenia, alcoholism, and postpartum depression etc. (Jacob, Moss, & Jurd, 2008; Maguire & Mody, 2008) For this reason, elucidating the regulatory mechanism of GABAergic synaptic transmission is essential, not only for understanding the function of the brain, but also for determining the pathogenesis and diagnosing and developing treatments for mental/neurological diseases.

In this review, we discuss novel regulatory mechanisms of GABAergic synaptic transmission revealed using cutting-edge bioimaging techniques including single-molecule imaging and Ca^{2+} imaging at subcellular resolution. We introduce the ingenious regulation of GABAergic transmission by Ca^{2+} and glutamate, discovered for the first time using "Quantum dot-single particle tracking (QD-SPT) (Bannai, Levi, Schweizer, Dahan, & Triller, 2006)", i.e., a super-resolution imaging technique to analyze the dynamics of membrane molecules.

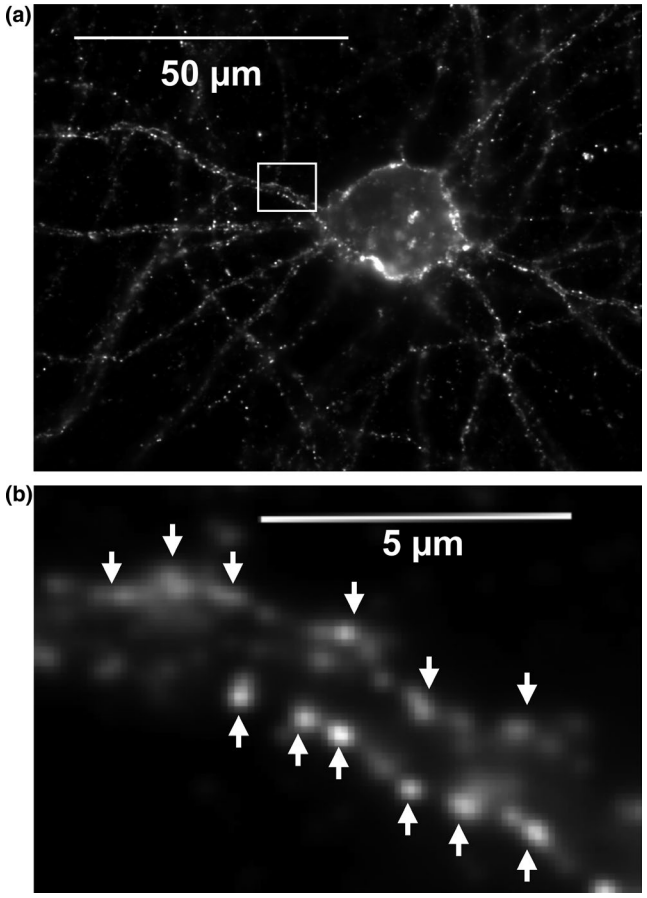


FIGURE 1 $GABA_ARs$ accumulate in the synapse. (a) Rat hippocampal primary neurons cultured for 3 weeks were immunostained with antibodies against the $GABA_AR$ γ2-subunit. The white dots are clusters of $GABA_AR$ that accumulated in the synapse. Scale bar: 50 μm. (b) Enlarged image of the region highlighted by a white square in a. The size of the cluster reflects the number of synaptic $GABA_ARs$ and determines GABAergic synaptic transmission efficacy

2 | EXCHANGE OF SYNAPTIC $GABA_AR$ BY LATERAL DIFFUSION IS A NOVEL FACTOR TO DETERMINE GABAERGIC SYNAPTIC TRANSMISSION

$GABA_AR$ is a Cl^- ion channel that plays an important role in rapid inhibitory synaptic transmission in the mammalian CNS. A typical $GABA_AR$ exists as a pentamer consisting of two α-subunits, two β-subunits, as well as a γ or δ subunit. There are six isoforms of the α-subunit, three of β, three of γ and one of δ, and when combined with the minor subunits ε, θ, and π, 19 types of $GABA_AR$ subunits have been identified.

In general, neurotransmitter receptors are accumulated at the postsynaptic membrane to efficiently receive the neurotransmitter released from the presynaptic terminal. Synaptic accumulation of $GABA_ARs$ can be visualized by immunocytochemistry as a cluster at the postsynaptic membrane (Figure 1). However, immunoelectron microscopy has shown that approximately 60% of all $GABA_AR$ are present outside the synapse (Kasugai et al., 2010), suggesting that a considerable number of $GABA_ARs$ are located outside the synapse, without forming clusters. The α1- and γ2-subunit containing $GABA_ARs$ localize primarily within the synapse, whereas the $GABA_ARs$ which contain the α4, α5, α6, and δ-subunits primarily localize outside the synapse (Farrant & Nusser, 2005). The number of $GABA_AR$ present in the synapse is the main factor that determines GABAergic synaptic transmission efficiency. The number of synaptic $GABA_AR$ depends on the total number of $GABA_AR$ present on the cell surface. The number of $GABA_ARs$ on the cell surface is a result of the dynamic equilibrium among $GABA_AR$ synthesis, exocytosis that presents $GABA_AR$ onto the cell membrane, endocytosis that takes $GABA_AR$ into the cell, and degradation of $GABA_AR$ in the cell (Figure 2). Furthermore, because the endocytosis and exocytosis of $GABA_AR$ occur outside the synapse (Bogdanov et al., 2006), the movement of $GABA_AR$ to the post-synaptic membrane by lateral diffusion has received much attention in recent years as another

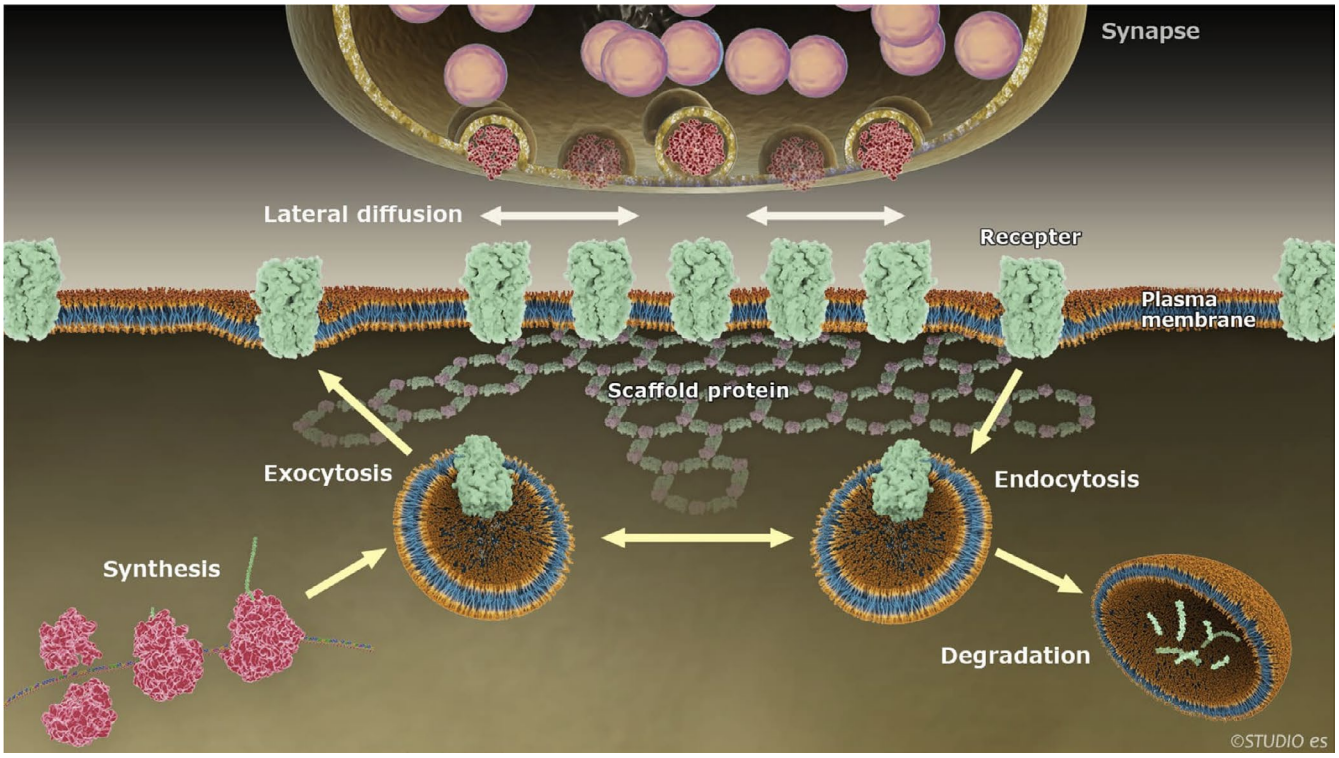


FIGURE 2 Dynamic equilibrium supports synaptic $GABA_AR$ clusters. Although $GABA_ARs$ accumulate at the synapse, each individual $GABA_AR$ undergoes dynamic turnover. The number of $GABA_AR$ is determined by the dynamic equilibrium between the following factors: (1) protein synthesis, (2) insertion into the membrane by exocytosis, (3) migration to and (4) from the synapse by lateral diffusion, (5) removal from the plasma membrane by endocytosis, and (6) degradation

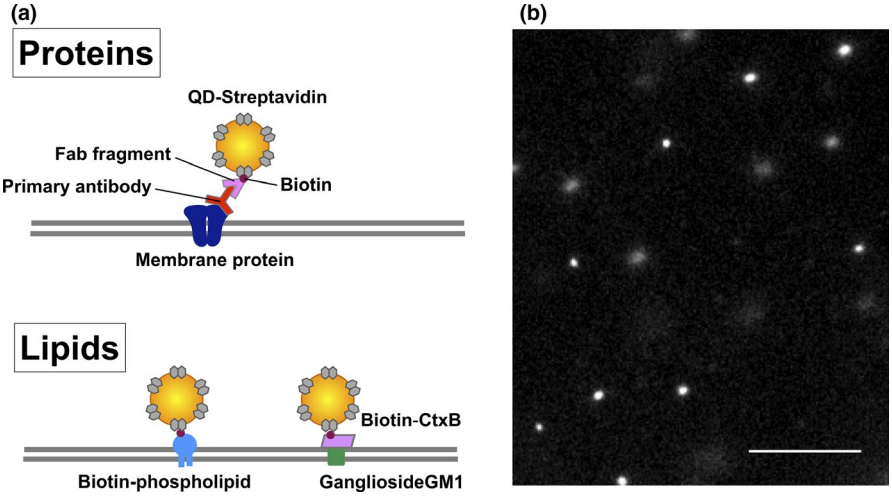


FIGURE 3 Labeling the membrane molecules with QDs. (a) Diagram showing the method to conjugate QDs to membrane proteins (top), lipid and GM1-ganglioside molecules (bottom). The membrane protein is bound by a QD through the primary antibody, biotinylated Fab fragment of a secondary antibody, and streptavidin on the QD surface (top). To label lipids, biotinylated phospholipids are incorporated into the membrane (bottom left). Endogenous GM1-ganglioside on the cell surface is targeted by biotinylated cholera toxin subunit B (CtxB) that has strong affinity to GM1-ganglioside (bottom right). Streptavidin-coated QDs are then bound to lipids through biotin. (b) Representative QD-labeling of $GABA_ARs$ on the primary culture neuron from mouse hippocampus. White spots are signals from single QDs. Scale bar: 10 μm

important factor that determines the number of $GABA_AR$ in the synapse.

The presence of a considerable proportion of $GABA_AR$ outside the synapse has important implications for neuron function. One important role of extrasynaptic $GABA_ARs$ is long-term inhibition of neural activity, called tonic inhibition. GABA spilled over from the inhibitory synaptic cleft binds to extrasynaptic $GABA_AR$ and induces sustained neural inhibition. Even $GABA_AR$ containing α1,

α2, and β3-subunit, which is considered as "synaptic" $GABA_AR$, carries out this tonic inhibition function when outside the synapse (Kasugai et al., 2010). The other role of extrasynaptic $GABA_ARs$ could be replaced with the inactivated synaptic $GABA_AR$ to constantly maintain the GABAergic synapse in its functional state. It has also been shown that "inactivated" $GABA_ARs$ translocate to another neighboring synapse by lateral diffusion and inhibits transmission in that synapse (de Luca et al., 2017). In light of these discoveries, understanding the mechanism for controlling the diffusion movement of $GABA_AR$ on the cell membrane has become an important challenge.

3 | QUANTUM DOT-SINGLE PARTICLE TRACKING (QD-SPT), AN IMAGING TECHNIQUE TO VISUALIZE THE BEHAVIOR OF MEMBRANE MOLECULES AT SINGLE-MOLECULE RESOLUTION

Lateral diffusion dynamics of $GABA_AR$ has been analyzed using quantum dot-single particle tracking (QD-SPT) techniques. In QD-SPT, quantum dots (QDs), i.e., semiconductor fluorescent nanocrystals, are used as a marker to track a molecule of interest (Bannai et al., 2006). QDs are targeted to membrane molecules through specific antibodies to the extracellular domain, biotin, and streptavidin, and observed under a fluorescent microscope (Figure 3). There are several advantages to use QD-SPT for the study of $GABA_AR$ dynamics in the neuron. Firstly, the QD-SPT technique allows the tracking of endogenous proteins, without overexpression of exogeneous recombinant proteins tagged with reporter (e.g., fluorescent proteins). Secondly, the fluorescence signals from a QD is brighter and more resistant to photo-bleach; therefore, QD-SPT enables to track the target molecule for a longer period with better signal to noise ratio, compared with chemical dyes and fluorescent proteins. Thirdly, QD has a broad absorption spectrum and a narrow emission spectrum (Michalet et al., 2005), facilitating multicolor imaging with other fluorescent markers (such as fluorescent Ca^{2+} indicators, fluorescent dyes, and marker proteins tagged with fluorescent proteins) (Bannai, Inoue, Hirose, Niwa, & Mikoshiba, 2020; Bannai et al., 2006). Finally, relatively small hydrodynamic radius of QDs (4–7 nm) (Swift & Cramb, 2008) enables QD-antibody to enter into the synaptic cleft (Dahan et al., 2003; Heine et al., 2008). However, QD-SPT has a few limitations that cannot be ignored, for instance, the hydrodynamic volume of QD-antibody complex is relatively large and affects the diffusion property of the target molecules in crowed region such as synaptic clefts (Groc et al., 2004, 2007), QD-SPT remains the most powerful technique to track membrane molecule movement for long periods to visualize the transition of receptors between synapses using a relatively simple imaging system. Although dissociated cell culture is one of the most suitable preparations for performing QD-SPT, while the acute brain slice and the organotypic slice culture are also available for QD-SPT (Al Awabdh et al., 2016; Biermann et al., 2014; Varela et al., 2016).

QD-SPT can also be used to analyze various membrane molecules: neurotransmitter receptors, ion channels, aquaporins, cell adhesion proteins, and lipids (Bannai et al., 2020). Moreover, QD-SPT is not limited in the neuroscience, but is now applicable to other cell biology studies. In addition to measuring the lateral diffusion of membrane molecules, QD-SPT can also be applied for monitoring of various biological events such as transient fusion and retrieval of synaptic vesicles (Zhang, Li, & Tsien, 2009), endocytosis and exocytosis of lectin (Liu et al., 2011), endocytosis of voltage-gated receptors (Wen et al., 2018), effect of amyloidogenic variant of β2-macroglobulin GM1 lateral diffusion (Leri et al., 2016), and internalization of viruses such as the influenza into the cell (Liu et al., 2012, 2020). However, QD-SPT has limitations that should be taken into account. Although QDs targeted to the surface molecules through the antibody can enter into the synaptic cleft (Dahan et al., 2003; Heine et al., 2008), the hydrodynamic volume QD-antibody-receptor complex affects the diffusion properties of membrane molecules.

Making full use of advantages of QD-SPT, fundamental questions in the field of neuroscience, such as these regarding synaptic structures and the molecular mechanisms for learning and memory, have been addressed by QD-SPT (Choquet, 2018; Petrini & Barberis, 2014). QD-SPT has also highlighted abnormal dynamics of membrane proteins in the model cells for epilepsy and Alzheimer's disease. In the next section, we will review the regulatory mechanisms for GABAergic synapses revealed by QD-SPT, together with other cell biological studies.

4 | SYNAPTIC SCAFFOLD PROTEIN THAT CONTROLS THE LATERAL DIFFUSION MOVEMENT OF $GABA_AR$

One of the most important elements that affects the lateral diffusion of the neurotransmitter receptor is the synaptic scaffold protein that clusters in the cytoplasm of the post-synaptic membrane (Choquet & Triller, 2013). In the GABAergic synapse, it has been discovered that the $GABA_AR$ α1-subunit interacts with the inhibitory synaptic scaffold protein gephyrin to inhibit the lateral diffusion movement of $GABA_AR$ in the synapse (Mukherjee et al., 2011). Accumulation of $GABA_ARs$ at the postsynaptic membrane is considered to result from the slow diffusion of $GABA_ARs$ in the synapse due to interaction with gephyrin. Inhibition of the lateral diffusion of $GABA_AR$ due to long-term potentiation (LTP) in the GABAergic synapse is reported to be dependent on the increase in gephyrin levels in the synapse (Petrini et al., 2014). These results suggest that gephyrin plays an important role in the regulation of GABAergic synaptic transmission by controlling the lateral diffusion of $GABA_AR$.

Interestingly, the change of synaptic $GABA_AR$ number induced by neuronal stimulation is known to occur faster than the change of synaptic gephyrin number (Niwa, Patrizio, Triller, & Specht, 2012; Petrini et al., 2014). These results indicate that lateral diffusion of $GABA_AR$ also affects the accumulation of its scaffold gephyrin, whereas gephyrin is necessary for the accumulation of $GABA_AR$ in

the synapse. Simultaneously, these findings imply the existence of other unknown molecular mechanisms underlying the lateral diffusion of $GABA_AR$ in the synapse, other than gephyrin. Indeed, radixin has been identified as another factor that controls the lateral diffusion of $GABA_AR$ outside the synapse in a gephyrin-independent manner. The actin filament-bound protein radixin controls the lateral movement of α5 subunit-containing $GABA_AR$ outside of the synapse (Hausrat et al., 2015). However, the factor that controls $GABA_AR$ lateral diffusion has not been identified yet. Unlike excitatory synaptic scaffold proteins that are easy to isolate as "synaptosomes" GABAergic post-synaptic density proteins are difficult to isolate biochemically. For this reason, the discovery of a new scaffolding protein that accumulates $GABA_AR$ in the synapse has been a difficult task for many years. Recently, proteomic studies have revealed over one hundred GABAergic post-synaptic proteins (Nakamura et al., 2016; Uezu et al., 2016). As an example, GIT1 and βPIX were reported to stabilize $GABA_AR$ with stabilization of actin filament at synapse (Smith et al., 2014). Furthermore, auxiliary subunits of neurotransmitter receptors (Yan & Tomita, 2012) also contribute to the regulation of $GABA_AR$ clustering and functions. $GABA_AR$ regulatory Lhfpl (GARLH) family protein that was discovered as an auxiliary subunit of the γ2-containing $GABA_AR$, was shown to play the role of accumulating $GABA_AR$ to the $GABA_AR$ synapse by neuroligin2 binding to $GABA_AR$ (Davenport et al., 2017; Yamasaki, Hoyos-Ramirez, Martenson, Morimoto-Tomita, & Tomita, 2017). Another auxiliary $GABA_AR$ subunit Shisa7 has been also shown to control receptor abundance at the synapse, in addition to the regulation channel deactivation kinetics (Han et al., 2019). Whether these newly found proteins are also involved in modulating the lateral diffusion of $GABA_AR$ needs to be clarified in the future.

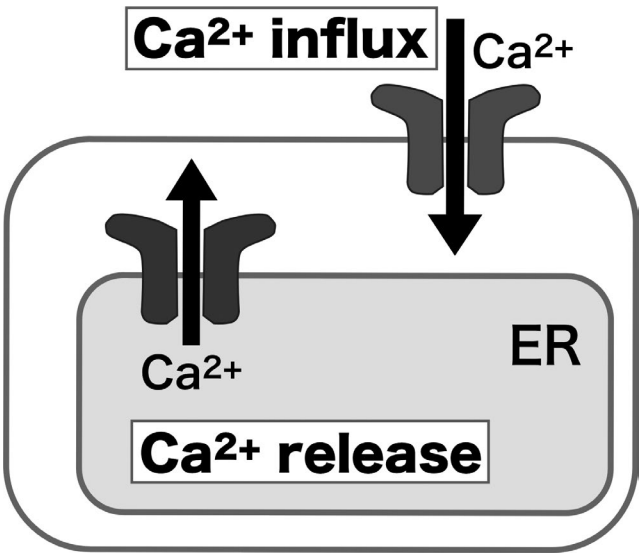


FIGURE 4 Two types of intracellular Ca^{2+} signaling pathways. "Ca^{2+} release" from the intracellular Ca^{2+} store in the endoplasmic reticulum (ER) and "Ca^{2+} influx" from the extracellular space

5 | CONTROL OF $GABA_AR$ LATERAL DIFFUSION THROUGH Ca^{2+} SIGNALING — AN INGENIOUS REGULATORY MECHANISM THAT MAKES FULL USE OF A LIMITED NUMBER OF SIGNALING MOLECULES

In addition to synaptic scaffolding proteins, the Ca^{2+} signal, i.e. "the increase or decrease in intracellular Ca^{2+} ion concentration", is another important factor that can control $GABA_AR$ lateral diffusion. Ca^{2+} signaling can be classified as "Ca^{2+} influx" where Ca^{2+} ions enter the cell from the extracellular space, and "Ca^{2+} release" from the intracellular Ca^{2+} storage endoplasmic reticulum (ER) (Figure 4). Intriguingly, mice lacking the ER Ca^{2+}-releasing channel "IP_3 receptor type 1 (IP_3R1)" exhibit epilepsy-like symptoms (Matsumoto et al., 1996), thereby suggesting the possibility that these animals may have abnormal inhibitory neurotransmission. Therefore, we examined whether Ca^{2+} release from the IP_3R1 controls the lateral diffusion of $GABA_AR$, using QD-SPT (Bannai et al., 2006). Hippocampal neurons from IP_3R1-deficient mice showed increased lateral diffusion of $GABA_AR$ in the synapse, compared to those in wild type mice. Through immunofluorescence staining, we found that the number of $GABA_AR$ accumulating in the synapse were decreased in IP_3R1-deficient neurons. Furthermore, inhibition of the Ca^{2+}-release pathway by IP_3R antagonist in wild type rat hippocampal neurons for 1 hr using an inhibitor showed similar $GABA_AR$ destabilization as that in IP_3R1-deficient neurons (Bannai et al., 2015) (Figure 5a). Furthermore, we showed that $GABA_AR$ lateral diffusion is suppressed by Ca^{2+}-releasing signal downstream of the metabotropic glutamate receptor (mGluR), followed by the activation of protein kinase C (PKC) (Figure 5b). This result indicates that the ER-mediated Ca^{2+}-releasing signal pathway suppresses lateral diffusion of synaptic $GABA_AR$, and finally causes accumulation of $GABA_AR$ inside the synapse. In other words, in addition to scaffolding proteins, Ca^{2+} release from the ER, which starts from constitutive activation of mGluRs and causes activation of PKC, is necessary for continuous $GABA_AR$ accumulation at the postsynaptic terminal.

On the contrary, Ca^{2+} influx also controls the lateral diffusion of $GABA_AR$, and is involved in GABAergic synapse plasticity, i.e. the ability of neurons to change GABAergic synaptic transmission efficacy in response to neuronal input. In the rodent hippocampus, GABAergic synaptic transmission efficacy is weakened by the application of high-frequency stimulation (Wang & Stelzer, 1996). This phenomenon is a form of synaptic plasticity called "long-term depression (LTD)" in GABAergic synapse, and is known to be involved in learning, memory, and pathogenesis of epilepsy. GABAergic LTD requires Ca^{2+} influx from the ionotropic glutamate receptor "NMDA receptors", activation of Ca^{2+}/calmodulin-dependent protein phosphatase "calcineurin" downstream of the Ca^{2+} influx, and calcineurin-induced dephosphorylation of Serine-327 in the γ2 subunit of $GABA_AR$ (Lu, Mansuy, Kandel, & Roder, 2000; Wang et al., 2003). However, it is unclear how calcineurin controls $GABA_AR$ accumulation at the postsynaptic terminal. Through investigation of $GABA_AR$ using QD-SPT, we found that Ca^{2+} influx from NMDA receptors, which is equivalent to LTD-inducing

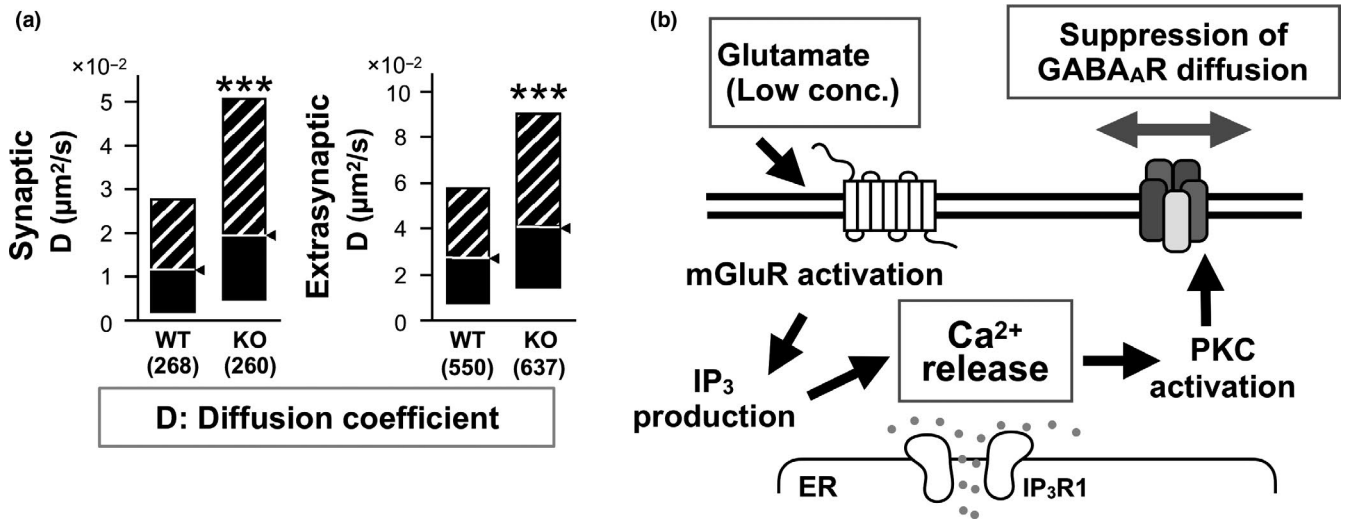
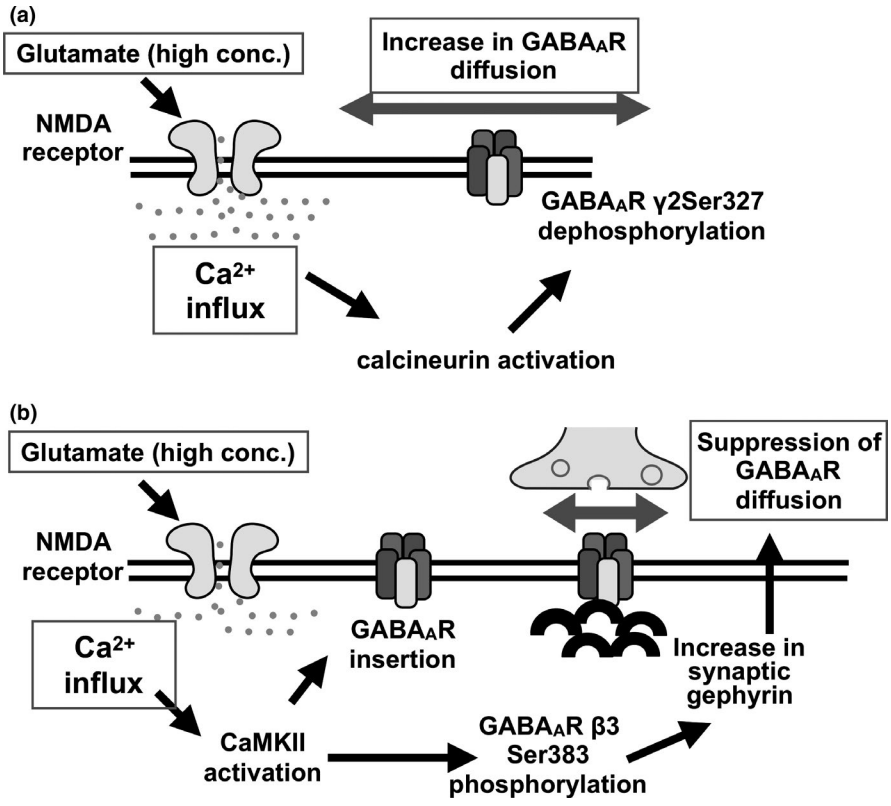


FIGURE 5 Ca^{2+} release from the ER suppresses $GABA_AR$ lateral diffusion. (a) Results of lateral diffusion analysis of $GABA_AR$ using QD-SPT. The $GABA_AR$-diffusion coefficient (D) in IP_3R1-deficient mice was greater than that in wild type mice (Bannai et al., 2015). The diffusion coefficient, D, is a physical parameter representing how fast the molecule moves in the plasma membrane, as calculated from its trajectory. For detailed description on this method, see Bannai et al., 2006; Bannai et al., 2020. Furthermore, these data indicate that the release of Ca^{2+} suppresses the lateral diffusion of $GABA_AR$ in the synaptic and extrasynaptic area. FM4-64 labeling was performed to identify the synaptic area. ***$p < .001$. Mann–Whitney *U*-test. The numbers in parentheses show the number of analyzed quantum dots. (b) Signaling mechanism to control lateral diffusion of $GABA_AR$ by Ca^{2+} release. Glutamate at low concentration activates mGluR to produce IP_3, and subsequently induces Ca^{2+} release from the IP_3R1. This pathway constitutively activates PKC, then suppresses the lateral diffusion of $GABA_ARs$

FIGURE 6 Molecular mechanism of GABAergic synaptic plasticity. (a) Molecular mechanism underlying GABAergic LTD. Ca^{2+} influx through NMDA receptor activates calcineurin, and dephosphorylates the $GABA_AR$ γ2-subunit, leading to increased $GABA_AR$ lateral diffusion. (b) Molecular mechanism for GABAergic LTP. CaMKII promotes $GABA_AR$ exocytosis and causes accumulation of gephyrin in the synapse through phosphorylation of the $GABA_AR$ β3-subunit



stimulation, increases the lateral diffusion of $GABA_AR$ through activation of calcineurin (Bannai et al., 2009) (Figure 6a). It has been also shown that the elevation of $GABA_AR$ lateral diffusion caused by Ca^{2+} influx was due to the dephosphorylation of Serine-327 in the γ2-subunit of $GABA_AR$ by calcineurin, similar to that in GABAergic LTD (Fricke et al., 2019; Muir et al., 2010).

Previously, synaptic plasticity was considered to have been induced by modification of the number of surface $GABA_AR$. However, we also showed that the expression level and the total number of $GABA_ARs$ on the cell surface did not change before and after LTD-inducing stimulation (Bannai et al., 2009). This discovery indicates for the first time that "changes in lateral diffusion of the neurotransmitter receptor" is a new molecular mechanism underlying synaptic plasticity.

Surprisingly, "GABAergic long-term potentiation (LTP)", another form of GABAergic synaptic plasticity, is also caused by the control of lateral diffusion of $GABA_AR$ depending on Ca^{2+} influx from the NMDA receptor (Petrini et al., 2014). When neurons receive LTP-inducing stimulus, the calcium calmodulin-dependent kinase II (CaMKII) that is activated by Ca^{2+} influx from NMDA receptors translocate to the GABAergic synapse, and promotes exocytosis of $GABA_AR$ (Marsden, Beattie, Friedenthal, & Carroll, 2007; Marsden, Shemesh, Bayer, & Carroll, 2010). Simultaneously, CaMKII phosphorylates Serine-383 in the β3-subunit of $GABA_AR$. Phosphorylation of the β3-subunit of $GABA_AR$ causes an increase in the accumulation of the scaffold protein gephyrin at the synapse. QD-SPT revealed that interaction between gephyrin and $GABA_AR$ selectively suppresses the lateral diffusion of synaptic $GABA_AR$ (Petrini et al., 2014). The increase in the level of $GABA_AR$ presented on cell surface due to CaMKII-dependent exocytosis and the increase in the number of synaptic $GABA_AR$ due to suppression of lateral diffusion in the synapse is therefore the molecular mechanism of GABAergic synaptic long-term potentiation (Figure 6b). It is notable that this NMDA receptor and CaMKII-dependent signaling pathway is also involved in the input-specific potentiation of GABAergic inhibition from somatostatin-expressing interneurons in cerebral cortex (Chiu et al., 2018).

As above, analysis of $GABA_AR$ lateral diffusion through QD-SPT showed that "Ca^{2+} signal" and "glutamate" are common signaling molecules involved in all of: (a) $GABA_AR$ accumulation in the synapse under the normal state (Figure 4), (b) GABAergic synaptic long-term depression (Figure 6a) and (c) long-term potentiation (Figure 6b). Interestingly, glutamate, an excitatory neurotransmitter, controls the inhibitory synaptic transmission efficiency. How can three different types of target molecules, i.e. PKC, Calcineurin, and CaMKII, be selectively activated using the same signaling molecules such as Ca^{2+} and glutamate? One possible mechanism that produces the specificity of the downstream signal molecule is thought to be the extracellular glutamate concentration. Under normal conditions, low concentration glutamate released from the surrounding excitatory synapses and glial cells is expected to preferentially activate mGluRs, which have relatively high binding affinity with glutamate, resulting in dominance of the IP_3R and PKC signaling pathways. On the contrary, synaptic plasticity that involves release of large amounts of glutamate from the excitatory synapse elevates the extracellular concentration of glutamate significantly and activates the NMDA receptors. Despite Ca^{2+} influx from the same NMDA receptor, the LTD stimulation reportedly migrates calcineurin to the inhibitory synapse, whereas LTP stimulation specifically migrates the CaMKII to the inhibitory synapse (Marsden et al., 2010). A model has been proposed where the difference in the level of Ca^{2+} entering from the NMDA receptors induces different types of synaptic plasticity (Petrini & Barberis, 2014). It should be noted that Ca^{2+} can activate not only phosphatase/kinase but also other proteins. For example, calpain is directly activated by Ca^{2+} and control gephyrin clustering (Tyagarajan et al., 2011). In addition, cAMP which produced with adenylate cyclase whose activity is controlled by Ca^{2+} also control the diffusion of inhibitory neurotransmitters (Niwa et al. 2019). Therefore, localization of Ca^{2+} signal itself is another important factor to be taken into consideration. Indeed, recent studies have discussed a possible relationship between compartmentalization of glutamate-induced Ca^{2+} signals and the specification of synapses that causes synaptic plasticity (Chiu et al., 2018). With all the combinations of these factors, i.e. Ca^{2+} signals and diverse enzymes, spatially and temporally complex regulation of GABAergic synapse via Ca^{2+} is processed. It will be a challenge to elucidate the detailed mechanism for $GABA_AR$ dynamics by local stimulation and compartmentalization of Ca^{2+} signals.

6 | DECODING OF Ca^{2+} SIGNALS

Analysis of $GABA_AR$ dynamics highlighted amazing aspects of neurons wherein the same signaling molecule, Ca^{2+}, can have a completely different effect on synaptic regulation depending on where it comes from. In neurons, Ca^{2+} release and Ca^{2+} influx also work in opposite directions to determine the direction of extension for the growth cone of axons, causing attraction and repulsion, respectively (Tojima, Hines, Henley, & Kamiguchi, 2011). Therefore, elucidating the source of the Ca^{2+} signal and analyzing the precise time and space patterns of the Ca^{2+} signal is an important challenge in the field of neuroscience.

To achieve this objective, we recently created a genetically encoded Ca^{2+} indicator that preferentially reports Ca^{2+} release from the ER (Niwa et al., 2016). By targeting GCaMP6f to the outer membrane of the ER, we succeeded in reporting the moment of Ca^{2+} release from the ER at a higher spatiotemporal resolution than the conventional cytosolic GCaMP6f. This outer ER-targeted-GCaMP6f (OER-GCaMP6f) was available for in vivo Ca^{2+} imaging of *C. elegans*. It was also found that the plasma membrane-targeted genetically encoded Ca^{2+} indicator Lck-GCaMP6f that detects Ca^{2+} influx with good sensitivity (Shigetomi, Kracun, Sofroniew, & Khakh, 2010), shows a different pattern of Ca^{2+} signal from that of OER-GCaMP6f. This result suggests that a Ca^{2+} signal can be diverse within a single cell. Furthermore, the aforementioned results indicate that the combination of the "ER-targeting sensor" and "cell membrane-targeting sensor" makes it possible to estimate the source of the Ca^{2+} signal (Bannai, 2018). The Ca^{2+} signal is a secondary messenger that induces diverse biological outputs in the nervous system as well as in various cells. Ca^{2+} imaging which elucidates the source of Ca^{2+} signal is expected to contribute to decode Ca^{2+} signals in various cells in the future.

7 | CONCLUSION

In this review, we have demonstrated that "$GABA_AR$ diffusion dynamics" is a new element that can control inhibitory neurotransmission. In recent years, the relationship between the abnormal diffusion dynamics of plasma membrane molecules including neurotransmitter receptors in Alzheimer's disease and other neurodegenerative diseases has been demonstrated (Bannai, 2018; Shrivastava, Aperia, Melki, & Triller, 2017). Elucidating the molecular mechanism that controls the lateral diffusion of membrane molecules may provide a new therapeutic strategy for cranial nerve diseases.

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