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Benzodiazepine-dependent stabilization of $GABA_A$ receptors at synapses

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ABSTRACT

$GABA_A$ receptors constitutively enter and exit synapses by lateral diffusion in the plane of the neuronal membrane. They are trapped at synapses through their interactions with gephyrin, the main scaffolding protein at inhibitory post-synaptic densities. Previous work has shown that the synaptic accumulation and diffusion dynamics of $GABA_A$Rs are controlled via excitatory synaptic activity. However, it remains unknown whether $GABA_A$R activity can itself impact the surface trafficking of the receptors. Here we report the effects of $GABA_A$R agonists, antagonists and allosteric modulators on the receptor's surface dynamics. Using immunocytochemistry and single particle tracking experiments on mouse hippocampal neurons, we show that the agonist muscimol decreases $GABA_A$R and gephyrin levels at synapses and accelerates the receptor's lateral diffusion within 30–120 min of treatment. In contrast, the $GABA_A$R antagonist gabazine increased $GABA_A$R amounts and slowed down $GABA_A$R diffusion at synapses. The response to $GABA_A$R activation or inhibition appears to be an adaptative regulation of GABAergic synapses. Surprisingly, the positive allosteric modulator diazepam abolished the regulation induced by muscimol, and this effect was observed on α1, α2, α5 and γ2 $GABA_A$R subunits. Altogether these results indicate that diazepam stabilizes synaptic $GABA_A$Rs and thus prevents the agonist-induced regulation of $GABA_A$R levels at synapses. This occurred independently of neuronal activity and intracellular calcium and involved $GABA_A$R–gephyrin interactions, suggesting that the changes in $GABA_A$R diffusion depend on conformational changes of the receptor. Our study provides a new molecular mechanism involved in the adaptative response to changes in $GABA_A$R activity and benzodiazepine treatments.

1. Introduction

$GABA_A$ receptors ($GABA_A$Rs) mediate most fast inhibitory transmission in the brain. These chloride-selective ligand-gated ion channels are pentameric complexes typically composed of two α, two β and one γ subunit. $GABA_A$Rs form clusters in the post-synaptic membrane in apposition to pre-synaptic GABA release sites. They are activated by the binding of two agonists on the GABA binding sites at the interface of the α and β subunits. $GABA_A$Rs are molecular targets for benzodiazepines (BZ), allosteric positive modulators that are widely used for their anti-convulsant, anxiolytic, sedative and myorelaxant effects.

Neurotransmitter receptors randomly explore the surface of neurons and can be transiently trapped at synapses via their interaction with underlying scaffolding proteins, as shown for $GABA_A$Rs, glycine receptors, and glutamate receptors (Gerrow and Triller, 2010; Tretter et al., 2012). Together, diffusion and trapping determine the number of receptors present at synapses and thus the synaptic strength (Triller and Choquet, 2008). Receptor stabilization depends on both the number of post-synaptic partners as well as the affinity between receptors and these partners (Gerrow and Triller, 2010). It is well established that the scaffold protein gephyrin plays a crucial role in $GABA_A$R accumulation and $GABA_A$R surface dynamics at inhibitory synapses (Tretter et al., 2012). Recent studies have shown that gephyrin directly interacts with $GABA_A$Rα1, α2 and α3 subunits and the binding affinities of these interactions have been characterized (Tretter et al., 2008; Saiepour et al., 2010; Mukherjee et al., 2011).

Importantly, studies in cultured neurons have correlated changes in the lateral diffusion of the receptor with rapid (<1 h) remodeling of excitatory and inhibitory neurotransmission, suggesting that lateral diffusion provides a rapid mechanism to adapt receptor localization at synapses. In particular, the level of neuronal activity tunes the dynamics and accumulation of excitatory receptors as well as that of inhibitory receptors (Gerrow and Triller, 2010). More precisely, enhanced excitatory activity regulates $GABA_A$R diffusion in an anti-homeostatic manner, resulting in a loss of $GABA_A$Rs at inhibitory synapses (Bannai et al., 2009; Niwa et al., 2012). Such regulation has important implications for neuronal activity and could be part of the mechanisms involved in the onset of long term potentiation (Lu et al., 2000).

However, it remains unknown whether $GABA_A$R activity itself could directly impact the surface trafficking of the receptors at inhibitory synapses. Using a pharmacological approach in hippocampal cultures, we

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investigated the rapid effects (30–120 min) of $GABA_AR$ activity on the lateral diffusion and synaptic accumulation of $GABA_ARs$. We show that $GABA_AR$ agonists and antagonists have opposite effects on the lateral diffusion of $GABA_ARs$ containing the α1, α2, α5 and γ2 subunits and on the accumulation of these receptors at synapses. Moreover, benzodiazepines (BZs) had an unexpected stabilizing effect on synaptic $GABA_ARs$ in the presence of the agonist. We provide evidence for a receptor-autonomous regulation of $GABA_AR$ diffusion by BZs. We propose that the action of BZs is due to changes in receptor–scaffold interactions, and is mediated by conformational changes induced by the binding of BZs to $GABA_ARs$.

2. Results

The goal of this study was to determine whether $GABA_AR$ activity and allosteric modulation could alter the dynamics and the synaptic localization of the receptor itself. To this aim, we combined single particle tracking (SPT) of $GABA_AR$ diffusion with immunocytochemical analysis of the synaptic receptor levels in dissociated hippocampal neurons. Hippocampal cultures were derived from knock-in mice expressing endogenous mRFP–gephyrin (Machado et al., 2011). Experiments were performed at 21–27 days in vitro (DIV), by which time the cultures exhibit mature GABAergic synapses and inhibitory GABAergic currents (Khirug et al., 2005). All experiments were performed in the presence of tetrodotoxin (TTX) to block action potentials and minimize network activity.

2.1. $GABA_AR$ activity modulates the levels of $GABA_ARs$ and gephyrin at inhibitory synapses

We investigated the effect of receptor activity on the synaptic accumulation of $GABA_ARs$ using the agonist muscimol (10 μM) and the competitive antagonist gabazine (1 μM). Both drugs are specific ligands of the GABA binding site of the receptor (Sieghart, 1995). Previous FRAP experiments on $GABA_ARs$ have shown that only ~20% of synaptic $GABA_ARs$ are renewed within 1 h (Jacob et al., 2005). The fact that the flux of receptors between synaptic and extrasynaptic compartments is low at steady state means that small changes in this balance will take time to impact the concentration of receptors at synapses (Lévi et al., 2008). Therefore, changes in $GABA_AR$ levels will be more pronounced after longer incubation times (>1 h) and thus more easily detected by immunolabeling. The $GABA_AR$ levels were quantified after 2 h of drug treatment by immunocytochemistry using antibodies against $GABA_AR\alpha2$, one of the most prominent α subunit in hippocampal pyramidal neurons (Sperk et al., 1997). Inhibitory synapses were identified using the inhibitory presynaptic marker VGAT (Fig. 1A). We found that muscimol treatment reduced $GABA_AR$ immunoreactivity at synapses (Fig. 1A, B). When compared with untreated neurons, $GABA_AR$ immunoreactivity was decreased to 84.8 ± 2.1% (Fig. 1C). In contrast, gabazine treatment increased $GABA_AR$ cluster immunoreactivity to 130.6 ± 4.8% (Fig. 1C). Muscimol or gabazine treatments did not alter the density of $GABA_AR$ clusters (values normalized to control, Mu, 104 ± 5%, Gbz, 105 ± 4%, Fig. 1D). Thus, $GABA_AR$ activity appears to control the accumulation of $GABA_ARs$ at inhibitory synapses.

Since $GABA_AR\alpha2$ containing receptors are stabilized at synapses through a direct interaction with the scaffold protein gephyrin (Tretter et al., 2008) we investigated whether the changes in $GABA_AR$ clustering were associated with changes in the synaptic gephyrin levels. The intensity of mRFP–gephyrin clusters that colocalized with the synaptic marker VGAT was quantified in the above set of experiments (Fig. 1D–F). We found that muscimol reduced the intensity of gephyrin clusters to 85.7 ± 2.6%, whereas gabazine increased the intensity of gephyrin clusters to 120.5 ± 5.9% (Fig. 1F). Consistent with previous reports (Vlachos et al., 2013), the treatments had no effect on the density of gephyrin clusters (Fig. 1H), demonstrating that the number of inhibitory synapses remained unchanged (values normalized to control, Mu, 102 ± 4%, Gbz, 88 ± 6%). Taken together, these data indicate that $GABA_AR$ agonists and antagonists regulate the receptor numbers at inhibitory synapses and equally alter the synaptic levels of gephyrin.

2.2. Short-term modulation of $GABA_AR$ activity alters receptor diffusion and confinement at synapses

To test whether the changes of receptor numbers at synapses involve a regulation of $GABA_AR$ expression at the plasma membrane, we carried out surface biotinylation experiments on hippocampal cultures treated for 2 h with muscimol or gabazine in the presence of TTX (Fig. 2). The ratio of surface to total receptor was unchanged by muscimol or gabazine treatments. In contrast, long-term treatment (24 h) with TTX reduced the surface/total receptor ratio (Fig. 2), in agreement with previous reports (Saliba et al., 2007). These data suggest that $GABA_AR$ agonists and antagonists do not affect the levels of $GABA_AR$ at the plasma membrane within short time scales.

An alternative mechanism for the regulation of synaptic $GABA_AR$ levels is the dynamic exchange between synaptic and extrasynaptic receptors (Thomas et al., 2005; Bannai et al., 2009). Agonist binding and receptor activation may constitute a fast and efficient mechanism by which the diffusion and local trapping of receptors by scaffolding proteins could be regulated. In this model, changes in the diffusion properties and exchange rates necessarily precede and have a cumulative effect on the levels of $GABA_ARs$ at synapses seen in immunofluorescence experiments. We have therefore studied the consequences of short-term (30 min) enhancement or blockade of $GABA_AR$ activity on the diffusion properties of the receptor. The movement of $GABA_AR\alpha2$ subunits was tracked using quantum dots (QD) and single particle tracking (SPT). In neurons derived from mRFP–gephyrin knock-in mice, the majority of mRFP–gephyrin clusters were apposed to inhibitory presynaptic boutons (90.4 ± 1% apposed to VGAT, n = 15 neurons). Clusters of mRFP–gephyrin were therefore used for the identification of inhibitory synapses in SPT experiments. The movement outside synapses was typically Brownian, whereas a confined type of motion was observed within synapses (Fig. 3A). Because the diffusion rates of QD-receptors span over four orders of magnitude and vary between cultures, treated conditions were systematically compared to internal control experiments done under identical conditions and on the same day. We compared the diffusion properties of $GABA_ARs$ in the presence of specific agonists and antagonists by measuring the diffusion coefficients D of the synaptic and extrasynaptic receptor populations. This analysis revealed that at synapses, the agonist muscimol (Mu) accelerated the diffusion of $GABA_AR\alpha2$ (control, $D_{med} = 9.6 \times 10^{-3}$ μm²/s, Mu, $D_{med} = 15.5 \times 10^{-3}$ μm²/s, Fig. 3B1). No significant effect was seen in the extrasynaptic compartment (control, $D_{med} = 2.3 \times 10^{-2}$ μm²/s, n = 1616; Mu, $D_{med} = 2.5 \times 10^{-2}$ μm²/s, n = 1582), suggesting that muscimol specifically affected the receptor's displacements at synapses. In contrast, the antagonist gabazine (Gbz) significantly decreased $GABA_AR\alpha2$ diffusion coefficients at synapses (control, $D_{med} = 3.7 \times 10^{-3}$ μm²/s, Gbz, $D_{med} = 2.1 \times 10^{-3}$ μm²/s, Fig. 3B2). This treatment also reduced the diffusion coefficients of extrasynaptic $GABA_AR\alpha2$ (control, $D_{med} = 6.8 \times 10^{-3}$ μm²/s, n = 954; Gbz, $D_{med} = 3.3 \times 10^{-3}$ μm²/s, n = 1155). Several studies have shown concomitant changes in synaptic and extrasynaptic diffusion velocity following the induction of different forms of synaptic plasticity (Lévi et al., 2008; Bannai et al., 2009; Charrier et al., 2010). Whether extrasynaptic gephyrin and/or other partners of $GABA_AR$ are involved in the slower diffusion in extrasynaptic membranes remains to be explored.

We further analyzed the synaptic trajectories using the mean squared displacement (MSD) as a function of time (Fig. 3C). The confined diffusion observed for synaptic receptors is characterized by a curved MSD versus time plot, where the MSD values rise slower at higher intervals (i.e. greater confinement). In the presence of muscimol the confinement of $GABA_ARs$ was notably decreased (greater MSD values, Fig. 3C1), whereas the confinement was increased by gabazine