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Activity-dependent coordinated mobility of hippocampal inhibitory synapses visualized with presynaptic and postsynaptic tagged-molecular markers

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ABSTRACT

Axonal varicosities and dendritic spines at excitatory synapses are dynamic structures essential for synaptic plasticity, whereas the behavior of inhibitory synapses during development and plasticity remains largely unknown. To investigate the morphology and dynamics of inhibitory synapses, we used two distinct pre- and postsynaptic fluorescent probes: one is a yellow fluorescent protein, Venus, incorporated into vesicular GABA transporter (VGAT) gene as a specific marker of presynaptic inhibitory neurons and the other red fluorescent protein (mCherry)-tagged gephyrin, a postsynaptic scaffolding protein, as a postsynaptic marker. Using primary culture of mouse hippocampal neurons and confocal laser-scanning microscopy, we established a system by which close contacts of Venus-positive axonal varicosities with mCherry-labeled gephyrin clusters in the dendritic shafts of dissociated hippocampal pyramidal neurons could be clearly visualized. Time-lapse imaging revealed that: (1) the presynaptic varicosities actively moved with marked changes in their shapes, and the postsynaptic scaffolding protein gephyrin clusters underwent coordinated movements in a tight association with the presynaptic varicosities, (2) the extents of morphological changes and movements depended on the developmental stages, reaching a stable level as the inhibitory synaptic connections matured, and (3) the motility indexes of the varicosity and its counterpart gephyrin cluster were well correlated. Furthermore, action potential blockade with tetrodotoxin treatment reduced the varicosity size, gephyrin cluster mobility as well as the amplitude of GABAergic synaptic currents in pyramidal neurons. Such a neural activity-dependent dynamic change in GABAergic synaptic morphology is likely to play a critical role in the regulatory mechanism underlying the formation and plasticity of inhibitory synapses.

Introduction

Excitatory synapses on certain central neurons are formed at contact sites between presynaptic varicosities and postsynaptic spines (Harris and Kater, 1994; Megias et al., 2001). Both dendritic spines and presynaptic varicosities undergo morphological changes in development- and neural activity-dependent manners (Colicos et al., 2001; Fischer et al., 1998; Konur and Yuste, 2004a; Korkotian and Segal, 2001; Nikonenko et al., 2003). Such structural changes and postsynaptic receptor trafficking likely provide the mechanism for plasticity of excitatory synapses (Engert and Bonhoeffer, 1999; Kasai et al., 2010; Konur and Yuste, 2004b; Maletic-Savatic et al., 1999; Matsuzaki et al., 2004; Umeda et al., 2005). As compared to excitatory synapses, relatively little is known about how inhibitory synapses develop and undergo structural and functional modifications during development and synaptic plasticity.

To investigate the mechanisms underlying the regulation of formation and plasticity of inhibitory synapses, it is essential to devise an experimental system by which morphology and dynamics of both presynaptic and postsynaptic structures could be reliably monitored for a considerable time period. For this purpose, we adopted two strategies: one is to use a genetically manipulated mouse strain where inhibitory neurons are labeled with a yellow fluorescent protein, Venus (Nagai et al., 2002), and the other labeling of the scaffolding protein gephyrin with a red fluorescent protein, mCherry (Shaner et al., 2004), using transfection of dissociated neurons under culture with recombinant adenovirus. At central inhibitory synapses, gephyrin has been identified to play a critical role in clustering of postsynaptic $GABA_A$ receptors ($GABA_ARs$) and glycine receptors (GlyRs) (Fuhrmann et al., 2002; Moss and Smart, 2001; Prior et al., 1992; Yu et al., 2007). Gephyrin has been shown to display a cytoskeleton-controlled lateral movement along the dendritic shaft of postsynaptic neurons in a synaptic activity-dependent manner (Hanus et al., 2006), and it forms clusters highly colocalized with $GABA_AR$ clusters, thereby regulating the mobility of $GABA_ARs$ as well as GlyRs at inhibitory synapses (Bannai et al., 2009; Ehrensperger et al.,

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2007; Jacob et al., 2005; Maas et al., 2006; Meier et al., 2001; Yu et al., 2007).

By combined use of the double labeling technique, we found that dynamics of inhibitory synaptic connections could be clearly visualized in our hippocampal dissociated neurons in culture. Venus-positive presynaptic inhibitory varicosities exhibited dynamic changes in their morphology together with marked movements of postsynaptic mCherry-tagged gephyrin clusters in close association with presynaptic varicosities. More importantly, the mobility of contact sites between presynaptic varicosities and gephyrin clusters was dependent on development reflected by the culture period as well as the neural activity. Such dynamic changes of presynaptic and postsynaptic contact sites thus appear to underlie maturation and plasticity of inhibitory GABAergic synapses.

Results

Visualization of inhibitory presynaptic and postsynaptic sites in hippocampal neurons

To visualize axons derived from inhibitory GABAergic interneurons, we exploited a transgenic mouse strain called VGAT-Venus mouse which is genetically manipulated to express Venus, a brighter YFP variant, in inhibitory neurons under the control of vesicular GABA transporter (VGAT) promoter (Wang et al., 2009). VGAT takes up inhibitory amino acid transmitters into synaptic vesicles at GABAergic and glycinergic nerve terminals (McIntire et al., 1997; Sagne et al., 1997). Wang et al. has recently demonstrated that cell bodies and neurites of GABAergic and glycinergic neurons display bright fluorescent signals in various brain regions of the VGAT-Venus transgenic mice (Wang et al., 2009). When hippocampal neurons were dissociated under primary culture from these mice, we could observe in cell bodies and their neurites in a subpopulation of neurons prominent fluorescence Venus signals, which were exactly overlapped with the immunoreactivity for glutamate decarboxylase (GAD), a specific marker of GABAergic neurons (Fig. 1A). The finding is consistent with the notion that VGAT-expressing neurons in the hippocampus are mostly GABAergic, whereas glycinergic neurons occur in other brain areas including the spinal cord, brainstem and cerebellum (Esclapez et al., 1994; Legendre, 2001). It is also known that fast inhibitory transmission in the hippocampal neural circuits is predominantly mediated by $GABA_A$Rs (Mody et al., 1994). Therefore, the Venus-positive fluorescence detected in dissociated hippocampal neurons under primary culture derived from the VGAT-Venus transgenic mice appears to serve as a reliable marker for the varicosities of GABAergic axon terminals.

To label inhibitory postsynaptic sites, we then incorporated mCherry, a wavelength-shifted mRFP variant, into the scaffolding protein gephyrin cDNA using a recombinant adenovirus transfection system with an mCherry fusion construct. This method allowed us to visualize expression of mCherry-tagged gephyrin in dissociated hippocampal neurons derived from wild-type mice. Our low-density culture consisted of 80–90% of cells showing the morphology typical of pyramidal neurons (Okabe et al., 1999), and mCherry-tagged

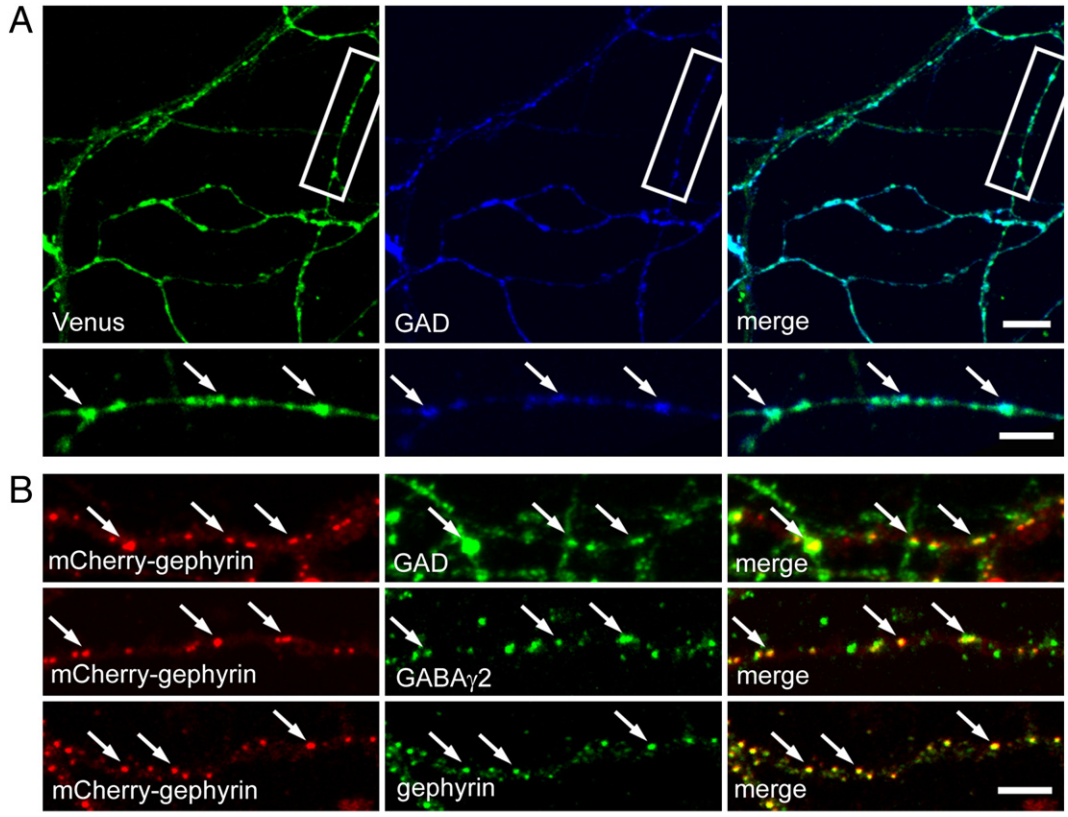

Fig. 1. Visualization of inhibitory GABAergic synaptic contact sites between Venus-positive presynaptic axon varicosities and postsynaptic mCherry-labeled gephyrin clusters on the dendritic shaft of dissociated hippocampal neurons under primary culture on 16–21 DIV. Fluorescence-positive interneurons were derived from VGAT-Venus mice, and mCherry-labeled gephyrin was transfected into cultured neurons by adenovirus. A, Maximal intensity projection images of Venus-expressing interneuron neurites in green (left), immunofluorescence staining for GAD in blue (middle), and green/blue overlay (right). Arrows indicate Venus-expressing axonal varicosities (left) that were also immunolabeled with an anti-GAD antibody (middle). Boxed areas in the upper images (scale bar, 10 μm) are arranged with higher magnification as lower images (scale bar, 5 μm). B, Maximal intensity projection images of mCherry-gephyrin spots in red in three distinct fields shown in the left column, immunofluorescence stainings for GAD (upper), $GABA_A$ receptor γ2 subunit (middle) as well as gephyrin in green (lower) in the middle column, and corresponding red/green overlays in the right column. Arrows in the horizontally aligned three images indicate the same positions, which show that the majority of mCherry-gephyrin clusters were colocalized with the immunoreactivities for inhibitory synaptic markers, GAD, and γ2 $GABA_A$ receptor subunit and authentic gephyrin (right, arrows).