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Research Report

GABA and glutamate are not colocalized in mossy fiber terminals of developing rodent hippocampus

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ABSTRACT

It has been hypothesized that, in the developing rodent hippocampus, mossy fiber terminals release GABA together with glutamate. Here, we used transgenic glutamic acid decarboxylase-67 (GAD67)-GFP expressing mice and multi-label immunohistochemistry to address whether glutamatergic and GABAergic markers are colocalized. We demonstrate that in the dentate gyrus, interneurons positive for GABA/GAD are sparsely distributed along the edge of the hilus, in a different pattern from that of the densely packed granule cells. Co-staining for synaptophysin and vesicular glutamate transporter1 (VGLUT1) in postnatal day 14 brain sections from both mice and rats showed mossy fiber terminals as a group of large (2–5 μm in diameter) VGLUT1-positive excitatory presynaptic terminals in the *stratum lucidum* of area CA3a/b. Furthermore, co-staining for synaptophysin and vesicular GABA transporter (VGAT) revealed a group of small-sized (~0.5 μm in diameter) inhibitory presynaptic terminals in the same area where identified mossy fiber terminals were present. The two types of terminals appeared to be mutually exclusive, and showed no colocalization. Thus, our results do not support the hypothesis that GABA is released as a neurotransmitter from mossy fiber terminals during development.

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1. Introduction

Mossy fibers, the axons from hippocampal dentate granule cells, terminate onto area CA3 pyramidal neurons, in a terminal field called the stratum lucidum (*sl*). These terminals are large, approximately 3–6 μm in diameter in adult rats (Amaral and Witter, 2000). As they are extensions arising from hippocampal principal cells, mossy fibers have long been thought to release glutamate as their primary neurotransmitter.

A hypothesis positing that mossy fiber terminals co-release GABA together with glutamate in the developing rodent brain (Gutiérrez, 2003, 2005; Gutiérrez and Heinemann, 2006; Jaffe

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and Gutiérrez, 2007) has attracted considerable attention, since excitatory glutamatergic and inhibitory GABAergic transmissions oppose each other in their induced neuronal actions. Electrophysiological recordings (Gutiérrez et al., 2003) demonstrated that in sections from neonatal rats up to postnatal day 22 (P22), stimulation of the granule cell layer in the presence of ionotropic glutamate receptor antagonists resulted in bicuculline-sensitive monosynaptic IPSPs recorded in area CA3 pyramidal cells. It has been hypothesized that these IPSPs are a result of GABA release from mossy fiber terminals onto CA3 pyramidal cells (Gutiérrez et al., 2003).

Conversely, other physiological experiments have lent credence to the hypothesis that mossy fibers exclusively release glutamate during development. Uchigashima et al. (2007) reported electrophysiological data in developing mice and rats (P14–P20) demonstrating that strong stimulation of the dentate granule cell layer in the presence of ionotropic glutamate receptor antagonists elicited picrotoxin-sensitive monosynaptic GABAergic IPSCs from CA3 neurons. Blocking mossy fiber-CA3 transmission abolished almost all postsynaptic CA3 responses elicited by weak stimulation of granule cell layer. These findings suggest that monosynaptic IPSCs previously reported might be evoked by co-stimulation of nearby inhibitory interneurons (Uchigashima et al., 2007; Gutiérrez, 2009). It was suggested that the source of co-stimulated interneurons might be mossy fiber associated interneurons (Uchigashima et al., 2007). These mossy fiber associated interneurons are located in *sl* of area CA3 and generate axonal collaterals to the hilus of the dentate gyrus (Vida and Frotscher, 2000; Losonczy et al., 2004).

Anatomical evidences supporting glutamate–GABA co-release hypothesis from previous studies are controversial. Immunohistochemical staining demonstrated immunoreactivity of GABA and glutamic acid decarboxylase-67 (GAD67, an isoform for the GABA synthesizing enzyme usually detected in GABAergic cell bodies and neuropiles) in granule cells and mossy fiber terminals from developing rats (Ramírez and Gutiérrez, 2001; Gutiérrez et al., 2003; Maqueda et al., 2003; Uchigashima et al., 2007; Gutiérrez, 2009) and mice (Uchigashima et al., 2007). Conversely, GAD65, which preferentially resides in axonal terminals (Kaufman et al., 1991; Esclapez et al., 1994; Castaneda et al., 2005) has not been shown in granule cells or mossy fiber terminals (Gutiérrez et al., 2003; Maqueda et al., 2003). While it has been suggested that the GABAergic phenotype of granule cells/mossy fiber terminals is transient, disappearing by adulthood (Gutiérrez, 2005, 2009), other studies reported that GAD and/or GABA were normally detected in granule cells and/or mossy fiber terminals from normal adult animals, including the mouse (Sloviter et al., 1996), rat (Sloviter et al., 1996; Lehmann et al., 1996; Bergersen et al., 2003; Zander et al., 2010) and monkey/human (Sandler and Smith, 1991; Lehmann et al., 1996). To classify a synaptic terminal as GABAergic the existence of vesicular GABA transporter (VGAT) is more convincing than labeling with other markers. A high expression level of mRNA for VGAT has been detected in granule cells from developing rats (Gutiérrez et al., 2003; Gómez-Lira et al., 2005; Zander et al., 2010) and adult rats (Zander et al., 2010). Although VGAT protein has been shown in mossy fiber terminals in developing (Safiulina et al., 2006) and normal adult rats (Zander et al., 2010), other studies reported a lack of VGAT staining (see Gutiérrez, 2009 for a review; Uchigashima et al., 2007). The inconsistency in anatomical findings from previous studies might be at least partially due to the lack of specific neuronal or synaptic markers to identify granule cells/mossy fiber terminals.

Using specific neuronal (GAD67-GFP to highlight GABAergic inhibitory neurons combined with EAAC1 to label hippocampal principal neurons including granule cells) and synaptic markers [type 1 vesicular glutamate transporter (VGLUT1) in conjunction with synaptophysin to label mossy fiber terminals], we re-examined if GABA/GAD positive structures are indeed granule cells or mossy fiber terminals. To further investigate whether GABA co-localizes with glutamate in developing mossy fiber terminals, we performed a series of double immunofluorescent staining experiments in fixed hippocampal sections from P14 rodents. We used both developing mice and rats in order to eliminate species dependence as the possible source of the reported experimental discrepancy. Using transgenic GAD67-GFP expressing mice (Tamamaki et al., 2003), we demonstrate that GAD67-GFP expressing interneurons are a discrete cellular population from dentate granule neurons. Large mossy fiber terminals were specifically positive for VGLUT1 in both mice and rats and could not be co-labeled with markers for GABAergic synapses, thus supporting the idea that mossy fiber terminals do not contain GABA during rodent development.

2. Results

2.1. *Immunoblot detection of GABA and other synaptic markers*

To ensure that the primary antibodies are specific for their targets we performed a dot blot for GABA and Western blots for the other primary antibodies. The sampling dot of the GABA conjugate could be clearly seen, whereas the dots of the other amino acids, including glutamate, aspartate and glutamine were not recognized (Fig. S1a).

Western blots conducted on whole brain lysates from P14 mice, confirmed that each antibody recognized a single protein band at the appropriate molecular weight of the target proteins (Fig. S1b). The synaptophysin antibody recognized a band between 37 and 40 kDa, while the GAD65/67, VGLUT1 and VGAT antibodies recognized bands between 50 and 60 kDa.

2.2. *Identity of granule cells using P14 GAD67-GFP mice and rats*

To determine whether granule cells are GABAergic at an early postnatal stage, we first evaluated the distribution pattern of inhibitory neurons in the hippocampus using P14 GAD67-GFP expressing mice. Since the transgenic mice were engineered with GFP tagged to the locus encoding GAD67, all GAD67 expressing inhibitory neurons are presumed to be GFP positive (Tamamaki et al., 2003). In general, GFP positive neurons were distributed throughout the hippocampus (Fig. 1a). A majority of them were immunoreactive to GABA (Fig. 1b,