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Research report

Live imaging of inhibitory axons: Synapse formation as a dynamic trial-and-error process

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ABSTRACT

In this review I discuss recent live imaging studies that demonstrate that synapses, and in particular inhibitory synapses, are highly dynamic structures. The ongoing changes of presynaptic boutons within axons emphasize the stochastic aspect of inhibitory synapse formation and paint a picture of a dynamic trial-and-error process. Furthermore, I discuss recent and previous insights in the molecular and mechanistic pathways that underlie synapse formation, with a specific focus on the formation of inhibitory presynaptic boutons.

1. Introduction

Recent developments in microscopy have had a great impact in neuroscience. Live imaging in brain tissue has revealed a dynamic world of ongoing changes of molecular complexes and subcellular structures in dendrites and axons within the brain. In particular, live imaging studies have demonstrated that synapses are incredibly dynamic structures. Both pre- and postsynaptic sites undergo structural changes in both shape and size spontaneously, and in response to environmental signals (Bonhoeffer and Yuste, 2002; Bury and Sabo, 2015; Frias and Wierenga, 2013). At the molecular level synaptic proteins show fast turnover and movement in and out of synapses (Alvarez-Castelao and Schuman, 2015; Matz et al., 2010; Ziv and Fisher-Lavie, 2014). Synaptic dynamics presumably reflect ongoing adjustments of synaptic strength and connectivity of the neuronal circuitry and are thought to be very important for experience-dependent circuit adaptations. Inhibitory axons appear particularly dynamic (Dobie and Craig, 2011; Kuriu et al., 2012; Schuemann et al., 2013; Villa et al., 2016; Wierenga et al., 2008). Rapid adaptation of inhibitory synapses has been suggested to serve as a gating mechanism for plasticity at nearby excitatory synapses, occurring at a slower time scale (Chen et al., 2015; Froemke et al., 2007; Keck et al., 2011; Villa et al., 2016), and may be a general feature during circuit development and adaptation (Froemke, 2015; Hensch, 2005). In this review I will focus on dynamic changes in presynaptic structures during synapse formation, and specifically focus on bouton formation in inhibitory axons which reflects the initial stage of inhibitory synapse formation.

2. Live imaging of inhibitory axons in brain slices

In labeled presynaptic axons, synaptic terminals forming *en passant* boutons along the axon are visible under a microscope as local swellings of the axonal shaft. In our lab, we are using time-lapse two-photon microscopy to visualize presynaptic structures of inhibitory synapses in organotypic slices of mouse hippocampus. We make use of a transgenic mouse line, the GAD65-GFP line, in which a known subset of inhibitory neurons express GFP (López-Bendito et al., 2004; Wierenga et al., 2010). Only ~20% of inhibitory neurons are labeled and the bright labeling of all processes, including axons, makes this mouse line ideally suited to follow changes in individual GFP-labeled inhibitory axons over time. We study inhibitory synapse formation and plasticity by monitoring individual inhibitory boutons by repeated imaging (typically every 10 min) using two-photon microscopy (Fig. 1). Using this method we generally distinguish two classes of inhibitory boutons, defined by their dynamics. *Persistent* boutons (blue arrow heads in Fig. 1) are generally large and continuously present at a fixed location throughout the imaging period (2–6 h). They represent inhibitory synapses, and contain pre-and postsynaptic structural specializations such as synaptic vesicles and scaffolding proteins, which can be revealed by post-hoc immunostaining (Schuemann et al., 2013; Wierenga et al., 2008), or by electron microscopy (Müllner et al., 2015). Even though these boutons are stable in the sense that they are

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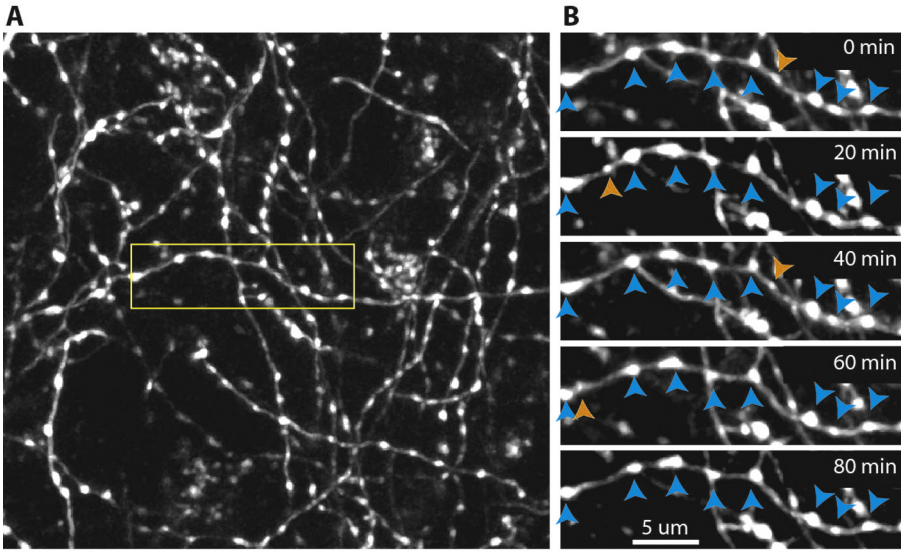

Fig. 1. Time-lapse two-photon microscopy of inhibitory axons.
A. Overview of a large field of view showing multiple GFP-labeled inhibitory axons in an organotypic slice from a GAD65-GFP mouse. B. Individual axon imaged repeatedly every 10 min (only every 2nd image is shown here). The majority of inhibitory boutons are present at all time points. These persistent boutons (blue arrow heads) reflect inhibitory synapses (see main text for references). Their shape and size can change substantially between time points. In addition, some boutons are only present at some, but not all time points. These non-persistent boutons (orange arrow heads) reflect ongoing processes of inhibitory synapse formation and disassembly. The axon in B is indicated with the box in A. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

continuously present during the imaging period, their shape and size varies significantly over time, presumably reflecting ongoing changes in their molecular content (Matz et al., 2010; Minerbi et al., 2009). In addition to persistent boutons, there are many boutons which appear, disappear and reappear during the imaging period (orange arrow heads in Fig. 1). In addition, neighboring boutons occasionally merge or split. We generally refer to these boutons as *non-persistent* boutons. They are usually smaller than persistent boutons and most likely reflect incomplete synapses that are in the process of being formed or disassembled (Schuemann et al., 2013; Wierenga et al., 2008).

Similar bouton dynamics have been observed in many studies by others in excitatory (Becker et al., 2008; Bury and Sabo, 2011; Sabo et al., 2006) and inhibitory (Fu et al., 2012; Kuriu et al., 2012) axons. These dynamics are not limited to *in vitro* preparations, but also occur *in vivo* (Chen et al., 2015; Grillo et al., 2013; Keck et al., 2011), indicating that dynamic boutons are a general feature of many, if not all, axons.

3. Dynamic boutons

The formation of inhibitory synapses along the axon is not random, but boutons appear, disappear and reappear repeatedly at specific axonal locations (Schuemann et al., 2013; Wierenga et al., 2008), suggesting multiple attempts to form new synapses at these spots. Indeed, when we performed post-hoc immunostaining at locations where boutons had appeared and reappeared, we noticed that these locations often contained pre- and/or postsynaptic proteins, suggesting that synapses were being built or being disassembled at these locations (Schuemann et al., 2013). We also found that newly formed boutons which stabilize, acquire pre- and postsynaptic markers over the course of several hours (Wierenga et al., 2008), with the presynaptic marker VGAT generally arriving before the postsynaptic gephyrin (Dobie and Craig, 2011; Schuemann et al., 2013). This indicates that persistent and non-persistent boutons are not separate categories of inhibitory boutons, but reflect inhibitory synapses at different stages of their life cycle. Indeed, non-persistent boutons that appeared *de novo* and then stabilized at an axon-dendrite crossings during an imaging session were found back at the same position on the next day, suggesting they had transformed into persistent boutons over time (Wierenga et al., 2008). Vice versa, some persistent boutons may destabilize and become non-persistent when imaged longer.

Our observations of inhibitory bouton dynamics build upon previous studies of axons in primary cultures, which showed that new boutons emerge not randomly along an axon, but at specific, apparently predefined, locations (Sabo et al., 2006). It was shown that clusters of synaptic vesicles continuously travel up and down the axonal shaft (Bury and Sabo, 2011; Darcy et al., 2006; Staras et al., 2010; Wu et al., 2013), and that new boutons are formed by clusters of synaptic vesicles stopping and pausing at these specific locations. Although it is not clear how these pausing sites are determined or 'marked' within the axonal shaft, these observations suggest that some structural change in the axon precedes or even induces subsequent molecular specialization. The formation of new boutons followed by subsequent recruitment of pre- and postsynaptic proteins is reminiscent of bouton formation at *Drosophila* neuromuscular junction (NMJ). During development or in response to elevated activity levels, the NMJ undergoes a rapid growth of many new presynaptic boutons. These newly formed boutons are called ghost boutons, as they do not contain pre- or postsynaptic membrane specializations (Ataman et al., 2008). Ghost boutons are small in size and their active zones are immature, but they already contain synaptic vesicles (Ataman et al., 2008). One of the first proteins to be transported into the ghost boutons is synapsin (Vasin et al., 2014), which is a known presynaptic organizer of synaptic vesicles (Chi et al., 2003). After their initial formation, the new boutons rapidly grow in size and acquire the pre- and postsynaptic molecular specializations of mature synapses. It was shown that ankyrin is required for this second growth phase (Koch et al., 2008; Pielage et al., 2008), providing structural stability as well as stabilization of microtubules, which guide the transport of building blocks for further bouton growth. This demonstrates that a structural change in the axon may be an early event during the formation of new synapses.

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