

Optogenetic Mapping of Cerebellar Inhibitory Circuitry Reveals Spatially Biased Coordination of Interneurons via Electrical Synapses

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SUMMARY

We used high-speed optogenetic mapping technology to examine the spatial organization of local inhibitory circuits formed by cerebellar interneurons. Transgenic mice expressing channelrhodopsin-2 exclusively in molecular layer interneurons allowed us to focally photostimulate these neurons, while measuring resulting responses in postsynaptic Purkinje cells. This approach revealed that interneurons converge upon Purkinje cells over a broad area and that at least seven interneurons form functional synapses with a single Purkinje cell. The number of converging interneurons was reduced by treatment with gap junction blockers, revealing that electrical synapses between interneurons contribute substantially to the spatial convergence. Remarkably, gap junction blockers affected convergence in sagittal slices, but not in coronal slices, indicating a sagittal bias in electrical coupling between interneurons. We conclude that electrical synapse networks spatially coordinate interneurons in the cerebellum and may also serve this function in other brain regions.

INTRODUCTION

γ -Aminobutyric acid (GABA)ergic interneurons located in the molecular layer provide inhibitory inputs to Purkinje cells (PCs),

the sole output neurons of the cerebellar cortex (Eccles et al., 1967; Palay and Chan-Palay, 1974). Molecular layer interneurons (MLIs) traditionally are divided into two types, basket and stellate cells, although these may be a single type of neuron that differ in their target location: stellate cells innervating PC dendrites and basket cells innervating PC somata and forming “pinneau” structures at the PC axon initial segment (King et al., 1993; Sultan and Bower, 1998; Bower, 2010).

MLIs cause lateral inhibition via sagittal extensions of their axons (Palay and Chan-Palay, 1974). This lateral inhibition is thought to play an important role in coordination of motor behavior by shaping the temporal and spatial pattern of PC activation (Cohen and Yarom, 2000; Dunbar et al., 2004; Gao et al., 2006). The actions of MLIs can also provide feedforward inhibition (Mittmann et al., 2005) that regulates PC activation in response to granule cell input (Gao et al., 2006; Santamaria et al., 2007; Bower, 2010; Dizon and Khodakhah, 2011).

The functional organization of the circuits formed by MLIs is not clear. MLIs outnumber PCs by a factor of ten, suggesting a high degree of convergence (Korbo et al., 1993). Anatomical studies indicate that a single PC is contacted by three to seven basket cell axons, though only one to two of these form synapses within the pinneau structure (Palay and Chan-Palay, 1974; Somogyi and Hámori, 1976). In addition, MLIs are often connected to each other through chemical and/or electrical synapses that could influence information flow between MLIs and PCs (Mann-Metzer and Yarom, 1999; Mittmann et al., 2005; Alcamí and Marty, 2013). Although traditional electrophysiological recordings can reveal the function of each of these connections, such measurements provide limited information about the spatial organization of these circuits. As a result, it remains

unclear precisely how many MLIs functionally converge upon a postsynaptic PC and how this convergence is spatially organized within the molecular layer.

Here, we have visualized the functional organization of these inhibitory circuits by an optimized optogenetic mapping technique (Wang et al., 2007; Petreanu et al., 2007). With this approach, we could quantify the spatial range and degree of convergence between MLIs and PCs. We also identified a role for electrical synapses in organizing MLIs into spatially structured clusters that amplify the degree of convergence between MLIs and PCs and thereby increase the amount of lateral and feedforward inhibition. Remarkably, these clusters are spatially biased and are oriented in the sagittal plane. These findings provide features for future computational models of these local circuits (Medina and Mauk, 2000; Santamaria et al., 2007) and suggest a general role for electrical synapses in spatial coordination of interneurons.

RESULTS

Selective Expression of Channelrhodopsin in Cerebellar Interneurons

To selectively photostimulate MLIs, we engineered bacterial artificial chromosome (BAC) transgenic mice expressing channelrhodopsin-2 (ChR2; Zhao et al., 2011) under the control of the neuronal nitric oxide synthase (nNOS) promoter (Figure S1A). These transgenic mice were viable and had no obvious anatomical or behavioral deficits. To determine the location of ChR2 expression, we imaged yellow fluorescent protein (YFP) fused to the ChR2 (Figure S1B). There was high expression of ChR2-YFP in the molecular layer of the cerebellum, but not in the granule cell layer (Figure S1C). At higher magnification, ChR2-YFP was evident in the plasma membrane of somata of individual MLIs (Figure S1D, arrows) and was prominent in both axonal pericellular baskets around PC somas (Figure S1D) and pinceau terminals that envelop the axon hillock of PCs (Figure S1D, arrowheads).

Within the cerebellar cortex, both MLIs and PCs were labeled by antibodies directed against GAD67, a marker for GABAergic neurons (Figures S1E–S1G). GAD67-positive MLIs were readily identified by their smaller cell bodies and their location within the molecular layer. At postnatal day 31 (P31), $87.2\% \pm 1.9\%$ ($n = 3$) of MLIs exhibiting cytoplasmic GAD67 staining also were positive for ChR2-YFP, and a similar value (88.7% ; $n = 1$) was determined in an adult (P150) mouse. Conversely, ChR2-YFP was never observed in the PCs.

To examine whether the ChR2-YFP was functional, we made electrophysiological recordings from 52 MLIs in cerebellar slices from transgenic mice (P23–P33). Among these MLIs, 45 fired action potentials in response to the light spots used in our optogenetic circuit-mapping experiments (see below). This corresponds to 87% photostimulation efficacy and matches the 87%–89% of MLIs expressing ChR2 determined from histology. There was no difference between the electrical properties of ChR2-positive and ChR2-negative MLIs (Table S1). We also made recordings from five granule cells and found that even light spots 10-fold brighter than those used for mapping (see below) did not evoke action potentials in these cells (see also Heiney

et al., 2014). Thus, ChR2 was preferentially expressed in the MLIs of these mice, and this allowed us to exclusively photostimulate MLIs.

Photostimulation of Cerebellar Interneurons

We further assessed optogenetic control of MLIs by illuminating sagittal cerebellar slices over a broad area ($\sim 0.23 \text{ mm}^2$) while recording MLI responses. Illumination (465–495 nm) reliably evoked action potentials in MLIs from mice 2–4 weeks old or older (Figure 1A), due to light-induced currents associated with ChR2 activation (Figure S2A; Wang et al., 2007). Increasing light intensity increased action potential frequency (Figure 1A), with half-maximal responses observed at a luminance of $0.75 \pm 0.08 \text{ mW/mm}^2$ in 2- to 4-week-old mice (Figure 1B). Photostimulation evoked more action potentials in older animals (Figure 1B), due at least in part to increased ChR2 expression (Figure S2B). Brief (5 ms), low-frequency flashes reliably evoked action potentials in MLIs from mice of all ages (Figure S2C). At higher frequencies of photostimulation, the roll-off frequency was 45 Hz in 2- to 4-week-old mice, whereas MLIs from 12-week-old mice responded to even higher-frequency flashes (Figure S2D).

ChR2-mediated circuit mapping requires focal photostimulation of presynaptic neurons. For this purpose, small (approximately $1 \mu\text{m}$ diameter in the focal plane) spots of laser light (405 nm, 4 ms) were scanned throughout the slice. The dye-filled MLIs in Figure 1C can be identified as a basket cell because its axons have terminal basket structures (arrows). When the laser spot was positioned over its cell body, an action potential was elicited (position 2, Figure 1D), whereas light-induced depolarizations were smaller at other locations (such as positions 1 or 3). By correlating the amplitude of responses with the location of the light spot that elicited them, we could map the light sensitivity of the MLIs (Wang et al., 2007; Schoenenberger et al., 2008). In Figure 1D, locations that elicited an action potential are shown in red, whereas locations eliciting subthreshold (or no) responses are not color coded. Superimposing this map upon the structure of the basket cell (Figure 1E) makes clear that action potentials were evoked only when the light spot was near the soma.

We refer to the area over which light was capable of evoking action potentials as the “optical footprint.” MLI optical footprint size depended upon laser power: higher power increased the optical footprint (Figure S3A), due to the brighter light spots activating more ChR2 (Schoenenberger et al., 2008). To optimize the spatial resolution, we used the lowest laser power (0.54 mW) that reliably elicited action potentials when the light spot was focused on the cell bodies of ChR2-expressing MLIs. Under these “minimal photostimulation” conditions, the median area of MLI optical footprints ($n = 59$) was $5.5 \times 10^3 \mu\text{m}^2$ (Figure 1F). This median area can be approximated by a circle with a radius of $42 \mu\text{m}$, indicating that the spatial resolution of MLI photostimulation was approximately $42 \mu\text{m}$ under our conditions.

In the example shown in Figure 1E, light spots only evoked action potentials when positioned over the cell body or proximal dendrite of the basket cell. To determine whether this was a general feature of “minimally photostimulated” MLIs, we examined the spatial overlap between optical footprints and axons in a subset of 15 dye-filled MLIs whose axons could be clearly resolved

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