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Array tomography of physiologically-characterized CNS synapses

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HIGHLIGHTS

- We describe a novel combination of two established methods: paired cell electrophysiological recording and array tomography.
- The combination of these two techniques allows for the isolation and anatomical study of synapses that have been characterized physiologically.
- This technique can be used to find all of the synapses made between two individual neurons or a subset of those synapses.
- The Physiological history of these identified synapses is known.

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ABSTRACT

Background: The ability to correlate plastic changes in synaptic physiology with changes in synaptic anatomy has been very limited in the central nervous system because of shortcomings in existing methods for recording the activity of specific CNS synapses and then identifying and studying the same individual synapses on an anatomical level.
New method: We introduce here a novel approach that combines two existing methods: paired neuron electrophysiological recording and array tomography, allowing for the detailed molecular and anatomical study of synapses with known physiological properties.
Results: The complete mapping of a neuronal pair allows determining the exact number of synapses in the pair and their location. We have found that the majority of close appositions between the presynaptic axon and the postsynaptic dendrite in the pair contain synaptic specializations. The average release probability of the synapses between the two neurons in the pair is low, below 0.2, consistent with previous studies of these connections. Other questions, such as receptor distribution within synapses, can be addressed more efficiently by identifying only a subset of synapses using targeted partial reconstructions. In addition, time sensitive events can be captured with fast chemical fixation.
Comparison with existing methods: Compared to existing methods, the present approach is the only one that can provide detailed molecular and anatomical information of electrophysiologically-characterized individual synapses.
Conclusions: This method will allow for addressing specific questions about the properties of identified CNS synapses, even when they are buried within a cloud of millions of other brain circuit elements.

1. Introduction

Numerous attempts have been made over the years at developing methods to correlate the physiology and plasticity of synapses with their anatomy. While these attempts have allowed us to learn much about the relationship between synaptic physiology and synaptic anatomy, gaining a complete and comprehensive understanding has been elusive. This question has been particularly hard to address in the central nervous system of vertebrates because of the difficulty of recording the physiology of individual synapses and then finding those very same synapses within the cloud of millions of neighboring synapses in order to perform an anatomical analysis. One successful approach is to follow electrophysiological recordings from neuronal pairs by electron microscopy; such studies have revealed that the number of synaptic connections and their probabilities of release vary greatly depending on neuron type (reviewed in: Branco and Staras, 2009) (Buhl et al., 1997; Silver et al., 2003; Tamas et al., 1997; Deuchars and Thomson, 1995; Biro et al., 2006; Gulyas et al., 1993). However,

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electron microscopic reconstructions are very time-consuming, do not allow the investigation of large numbers of neuronal pairs, and, most importantly, cannot be used to study the molecular composition of the synapses in a pair. In this paper, we describe the development of a method combining paired-cell electrophysiological recording and array tomography that enables us to determine the physiological properties of a small population of synapses and then to study the anatomical and molecular characteristics of that same synapse population.

Simultaneous electrophysiology recordings from two synaptically-connected neurons have been used to study the detailed physiological properties of synaptic connections for a number of years (Pavlidis and Madison, 1999; Debanne et al., 1998; Malinow, 1991; Emond et al., 2010; Fourie et al., 2014; Montgomery and Madison, 2002; Montgomery and Madison, 2004). In this so-called 'cell pair recording', a current pulse-induced action potential travels down the axon of the presynaptic cell of the pair, and activates neurotransmitter release, inducing a synaptic current in the postsynaptic neuron of the pair. The main advantage of cell-pair recording is that it uniquely produces the minimal action potential-evoked synaptic response that can be unambiguously attributed to specific synapses: only those made between these individual pre- and postsynaptic neurons. Other types of minimal synaptic responses that have been employed in the past, are either not action potential evoked (Chen et al., 2004), or cannot be unambiguously attributed to specific members of the synapse population (e.g., "spontaneous" or "minimally stimulated") (Dobrunz and Stevens, 1997; Choi et al., 2000). With cell-pair recording however, it is known that the recorded responses are action potential-dependent, and that they arise from only the synapses made from one member of the cell pair to the other. A post-hoc analysis of those synapses with array tomography adds the ability to determine anatomical and molecular properties of the physiologically-characterized synapses. The main advantage of this combination of techniques is, thus, that function and structure can be directly correlated at the very same synapses.

In this paper, we describe the methodology of using array tomography to reconstruct neurons from pair recordings, locate points of contact between axons and dendrites, verify their identity as synapses using the presence of antibody markers for synaptic proteins, and visualize the distribution of receptor subunits. The technique as we have developed it can be used to image an entire filled pair of neurons, and thus to find all of the synapses between those two neurons, or to image just part of the interaction field of those neurons to sample the properties of synapses within that field, but limited to synapses made between those two neurons.

2. Materials and methods

2.1. Preparation of and recording from organotypic slices

Hippocampal organotypic slice cultures were prepared from 5 day old C57BL/6 mice or from P7 Sprague-Dawley rats, and maintained in vitro on a plastic membrane (Millicell culture insert) for 6–10 days before whole cell recordings were performed, as previously described (Stoppini et al., 1991). The physiological recording and array tomographic methods are identical when using rats or mice, with the only significant difference being that it is easier to fully reconstruct a cell pair from a mouse because the mouse neurons have smaller dendritic arbors. To prepare a cultured slice for recording, a patch of the plastic membrane containing a slice was carefully cut out of the insert, using the point of a scalpel. The slice, sitting on top of its membrane, was immersed in ACSF containing (mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 1 Na_2HPO_4, 26.2 $NaHCO_3$ and 11 glucose, and perfused at a rate of 2 mL/min. Recordings were made at room temperature (~21 °C). Pyramidal neurons in area CA3 were visualized with differential interference contrast microscopy (DIC) under infrared illumination for simultaneous whole cell recordings from two neurons. The use of infrared illumination improves the ability to visualize the live neurons, but the images must be viewed with a camera that can detect infrared (most commercial CCD cameras will detect infrared, as long as the filter that is commonly factory-installed in front of the lens is absent or removed). Presynaptic neurons were recorded in current clamp in whole-cell electrode configuration, using an internal solution consisting of (mM): 120 K gluconate, 40 HEPES, 5 $MgCl_2$, 0.3 NaGTP, 2 NaATP, and 5 QX-314; pH adjusted to 7.2 with KOH. Postsynaptic neurons were also recorded in whole-cell mode, in voltage clamp with an internal solution consisting of (mM): 120 Cs gluconate, 40 HEPES, 5 $MgCl_2$, 0.3 NaGTP, 2 NaATP, and 5 QX-314, with pH adjust to 7.2 with CsOH. To mark the neurons for later array tomography, either 0.2% Lucifer yellow (Sigma–Aldrich) or 0.1% Alexa 594 hydrazide and 0.5% neurobiotin (Vector Laboratories) was included in the internal solution. The Alexa 594 is present to allow us to see the neuron in the resin block (as in Figs. 1C or 4A), which facilitates block trimming. In earlier experiments, we put neurobiotin in the presynaptic and Lucifer yellow in the postsynaptic electrode, but later inverted this because we found that Lucifer yellow better filled the axonal processes. Neurons were recorded for 25 min before electrodes were carefully withdrawn to allow time to fill with the markers. The slice remained in the recording chamber for another 15 min before fixation.

2.2. Fixation and resin embedding

Slices were removed from the recording chamber by holding the edge of the millicell membrane with a pair of Dumont forceps. With the slice still on the membrane, it was placed in a solution of 4% formaldehyde (diluted from 8% formaldehyde, EM grade, Electron Microscopy Supplies) with 2.5% sucrose in PBS in a scintillation vial. To speed tissue fixation, the slice was microwaved in a PELCO BioWave Pro microwave equipped with a PELCO ColdSpot preset to 12 °C (Micheva et al., 2010a). The procedure was to irradiate the tissue samples once at 100–150 W 1 min on/1 min off/1 min on, and then 3 times at 350–400 W 20 s on/20 s off/20 s. The fixed slice was then left in fixative at room temperature for one hour, followed by a wash in PBS buffer. The slice was then carefully teased away from the plastic membrane using the tip of a 3/0 White Sable brand nylon artist's paintbrush. The CA3 region was cut from the slice under a dissection microscope using a #9 single edge razor blade, and then dehydrated serially in washes of 50%, 70%, 95% and 100% ethanol, each time microwaved at 350 W for 30 s. The dehydrated tissue was then infiltrated with LR White resin, medium grade (SPI Supplies), first with a mixture of ethanol and LR White (1:1) and then in three changes of 100% LR White, microwaved at 350 W for 30 s each time (Micheva et al., 2010a). The slice was then left in unpolimerized LR White resin overnight at 4 °C.

To ensure that the slice did not curl during embedding, a short stub of polymerized LR White resin was put into the bottom part of a 00 gelatin capsule to provide a flat surface. The resin stub was covered by a circle of Aclar plastic (Ted Pella), over which the slice was positioned, and then unpolymerized LR White resin was slowly added to fill the bottom part of the capsule. The top part was used to close the gelatin capsule, which was then polymerized at 55 °C for 24 h. After polymerization, the gelatin capsule was peeled off and the supporting resin stub snapped off at the Aclar border, leaving the flat polymerized slice on top of the remaining resin block (Fig. 1B) (Palmieri and Kiss, 2005; Migheli and Attanasio, 1991).