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Visual patch clamp recording of neurons in thick portions of the adult spinal cord

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ABSTRACT

The study of visually identified neurons in slice preparations from the central nervous system offers considerable advantages over *in vivo* preparations including high mechanical stability in the absence of anaesthesia and full control of the extracellular medium. However, because of their relative thinness, slices are not appropriate for investigating how individual neurons integrate synaptic inputs generated by large numbers of neurons. Here we took advantage of the exceptional resistance of the turtle to anoxia to make slices of increasing thicknesses (from 300 to 3000 μm) from the lumbar enlargement of the spinal cord. With a conventional upright microscope in which the light condenser was carefully adjusted, we could visualize neurons present at the surface of the slice and record them with the whole-cell patch clamp technique. We show that neurons present in the middle of the preparation remain alive and capable of generating action potentials. By stimulating the lateral funiculus we can evoke intense synaptic activity associated with large increases in conductance of the recorded neurons. The conductance increases substantially more in neurons recorded in thick slices suggesting that the size of the network recruited with the stimulation increases with the thickness of the slices. We also find that that the number of spontaneous excitatory postsynaptic currents (EPSCs) is higher in thick slices compared with thin slices while the number of spontaneous inhibitory postsynaptic currents (IPSCs) remains constant. These preliminary data suggest that inhibitory and excitatory synaptic connections are balanced locally while excitation dominates long-range connections in the spinal cord.

1. Introduction

The advent of slice preparations from central nervous system during the 1970s followed by the development of techniques allowing visual patch clamp recordings of single neurons (Edwards et al., 1989) was a revolution in the field of neurophysiology. The visual patch recording of neurons in slices has several advantages compared to *in vivo* recordings. First, the electrical activity of neurons can be monitored with high mechanical stability. Second, recordings are obtained in the absence of anesthetics that might distort neuronal response properties. Third, the slice technique allows neurons to be visually identified (Stuart et al., 1993). Fourth, the pharmacological characterization of intrinsic properties of neurons and synaptic transmission is aided by the control of the composition of extracellular medium (e.g. Stuart and Sakmann, 1994). Fifth, it allows the study of synaptic transmission between pairs of neurons (Edwards et al., 1989; Gulyas et al., 1993; Buhl et al., 1994; Jonas et al., 1998). In spite of these benefits, the slice preparations also impose limitations. Transmitted light through the slice is required for imaging. A decent image of superficial neurons is thought to require thin slices (typically 300–500 μm), so that light scattering remains low. Thin slices also ensure oxygen supply to cells in the center strata of the tissue. However, neurons in thin slices are lacking most of their normal synaptic network, especially from neurons with long-range connections. This feature becomes particularly problematic if one wants to characterize the properties of a given network. Neurons *in vivo*, often receive intense synaptic bombardment that change their membrane potential and increases their conductance (Destexhe et al., 2003; Alaburda et al., 2005). The characteristics of the reduced network in slices cannot be scaled up for modeling bigger networks. In thin slices, some local circuitry remains. However, long-range neurons that project to the neurons present in the slice are systematically destroyed. In the neocortex, the number of intact synaptic connections from excitatory neurons is much more reduced in thin slices cut perpendicularly to the surface than the number of synapses from inhibitory neurons (Stepanyants et al., 2009). This uneven reduction prevents the experimental assessment of the relative importance of excitation and inhibition from slice experiments. A preparation that overcomes this problem would therefore be a significant improvement for studying network properties.

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Here we present a method for making visual patch clamp recording of different types of neurons in portions of the spinal cord as thick as one segment. We took advantage of the exceptional resistance to anoxia of the adult turtle to make slices one order of magnitude thicker than conventional slices from the nervous system of mammals. We demonstrate that neurons from the surface of the slices can be visualized and patched and that neurons in the deep portions of the preparation remain functional. We show that we can evoke a neuronal activity that changes qualitatively with the size of the network. Our preliminary results suggest that spinal inhibitory neurons preferentially project locally while excitatory neurons also have long-range connections.

2. Methods

2.1. Slice preparation

Experiments were performed on slices from the lumbar enlargement of the spinal cord of the adult turtle (*Chrysemys scripta elegans*). The surgical procedures complied with Danish legislation and were approved by the controlling body under The Ministry of Justice. The turtles were anesthetized by intravenous injection of propofol (30 μg/kg). To decrease the excitability of neurons during the dissection procedure, the animals were perfused with a high magnesium Ringer solution containing (mM): 20 NaCl; 5 KCl; 15 $NaHCO_3$; 20 $MgCl_2$; 3 $CaCl_2$; and 20 glucose; saturated with 98% O_2 and 2% CO_2 to obtain pH 7.6. The spinal cord was removed and kept in this Ringer. Slices of different thicknesses (300, 700, 1500 and 3000 μm) were cut by means of a vibratome (HM 650, Microm, Germany). Experiments were performed at room temperature (20–22 °C) in a solution containing (in mM): 120 NaCl; 5 KCl; 15 $NaHCO_3$; 2 $MgCl_2$; 3 $CaCl_2$; and 20 glucose; saturated with 98% O_2 and 2% CO_2 to obtain pH 7.6.

2.2. Recording

Slices were placed in a recording chamber under the X40 water immersion objective (LUMPLFL40XW/IR/0.80; Olympus, Japan) of a microscope (Olympus BX51WI, Japan). Neurons were visually patched in whole-cell configuration and recorded in voltage clamp mode. The micromanipulators holding the recording pipettes and the microscope were mounted on a motorized working station (Luigs and Neumann, Germany). The recording pipettes were filled with a solution containing (in mM): K-gluconate, 122; Na_2-ATP, 5; $MgCl_2$, 2.5; Mg-gluconate hemi Mg salt, 5.6; K-Hepes, 5; H-Hepes, 5; Biocytin, 10; Alexa Fluor® 488 hydrazide, sodium salt, 1. The pH was adjusted to 7.4 by adding KOH. All compounds are from Sigma, except Alexa (Invitrogen). The recordings were amplified by a Multiclamp 700B (MDS Analytical Technologies) and digitized by a Digidata 1332A (10–20 kHz). The reported data are not corrected for the liquid junction potential.

2.3. Stimulation

Deep stimulation was performed with Michigan electrodes (A1×16–3mm 50-413; Neuronexus Technologies; Ann Arbor, USA) activated with iridium oxide. A 16-stimulation probe was mounted on a motorized micromanipulator (Luigs and Neumann, Germany). An incision was made on the dorsal side of the spinal cord, of 1.5–2 mm below the surface of a 3000 μm thick preparation. The tip of the electrode was inserted transversally until all the stimulation points were in the grey matter (Fig. 3(A)). The location of the electrode was controlled after the experiment by removing it and repositioning it above the slice at the same horizontal coordinates. The electrode was connected to an isolation unit (Isolator 11; Axon Instruments, USA). Stimulation was obtained by passing current between points separated by a distance of 50 μm.

Stimulation of the lateral funiculus was performed by means of a broken patch electrode filled with NaCl (1 M) or with a bipolar electrode (TM33CCNON; World Precision Instruments, Sarasota, FL, USA) connected to an isolation unit (Isolator 11; Axon Instruments, USA).

2.4. Pharmacology

The following drugs were bath applied: ([R-(R*,S*)]-5-(6,8-dihydro-8-oxofuro[3,4-e]-1,3-benzodioxol-6-yl)-5,6,7,8-tetrahydro-6,6-dimethyl-1,3-dioxolo[4,5-g]isoquinolinium chloride (bicuculline; Tocris); strychnine hydrochloride (Sigma–Aldrich); SR 95531 hydrobromide (gabazine; Tocris); DL-2-amino-5-phosphonopentanoic acid (AP5; Tocris); 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Tocris); tetra ethyl ammonium chloride (TEA; Sigma–Aldrich); paxilline (Sigma–Aldrich).

2.5. Detection of synaptic currents

Spontaneously occurring postsynaptic currents (PSCs) were counted in voltage clamp recordings at different holding potentials. PSCs were automatically detected by means of a custom-made MATLAB program (MathWorks; Natick, USA). The detection was carried out in four steps. (1) The turns slower than 1 KHz in the raw signal were detected (Fig. 1(A)). For this purpose, a sliding differential filter algorithm was used. (2) The non-biological noise was detected. The distribution of the amplitude of the turns was plotted (Fig. 1(B)). A first order Gaussian function was fitted via an expectation-maximization algorithm. (3) Only turns with an amplitude larger than a threshold value fixed by the user, were considered as PSCs. (4) Turns that did not start from the baseline were detected (Fig. 1(C)). Finally events with a rise time slower that their decay were eliminated. When the membrane potential was held at −40 mV, the positive events were considered as IPSCs and the negative events were considered as EPSCs. This was confirmed by pharmacological tests (see Fig. 2). The detection of synaptic events was optimized by adjusting the threshold (ranging from 2 to 6 times the standard deviation of the first Gaussian function (Fig. 1(B)). For each recording, a 5 s frame was visually inspected. If more than 10% of the synaptic events were not detected by the program, or, if more than 10% of the detected events did not correspond to visually identified synaptic events, the whole recording was rejected.

For a fraction of neurons, the outward events were insensitive to classical blockers for fast inhibitory synaptic transmission. They persisted in the presence of a glycine receptor antagonist (strychnine, 10 μM) and $GABA_A$ receptor antagonist (gabazine, 10 μM) ($n = 5$). These events were removed by extracellular addition of potassium channel blockers (TEA, 1 mM; $n = 1$; and paxilline, 10 μM; $n = 2$; not illustrated). This suggests that they were not synaptic but instead mediated by an intrinsic property of the neuron, presumably the big K^+ activated Ca^{2+} channels (BK). Interestingly, the number of the gabazine/strychnine resistant events was strongly voltage sensitive. This feature allowed us to recognize neurons expressing the non-synaptic events. In all, 13 interneurons out of 44 (i.e. 30%) had such a non-linearity. For these neurons, we found a significant increase of the number of outward events between the two holding potentials (Mann–Whitney test ($p = 0.002$); Wilcoxon signed-rank test on paired data ($p = 0.004$)). Since the goal of the present project was to study the organization of the spinal network, we removed all neurons that displayed such non-synaptic outward events from our sample.