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Technical note

New techniques for making whole-cell recordings from CNS neurons in vivo

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Abstract

Patch-type pipettes increasingly are being used to obtain intracellular 'whole-cell' recordings from neurons. Here we describe our methods for making whole-cell recordings in vivo from midbrain neurons in an electric fish. Novel elements in the procedure are: A device for micropositioning the pipette when near a cell, use of a 'Picospritzer' for cleaning the pipette tip and cell surface, and an electroporetic method for perforating the patch following seal formation. In addition, we show that extracellular and intracellular recordings can be made from the same neuron. Stable intracellular recordings can be made from neurons at least as small as 10 μm.

Keywords: Whole-cell; Patch pipette; Intracellular; Electrosensory; Technique; Piezoelectric

1. Introduction

Recording subthreshold intracellular potentials of neurons in vivo is vital to the understanding of how information is integrated and processed. Historically, intracellular recordings have been made from large neurons in invertebrate nervous systems and in the CNS of vertebrates. The conventional methodology for making intracellular recordings employs micropipettes with small tip diameters and high impedances. Recording from medium-sized or small neurons in vivo is often problematic with such electrodes. In addition, manipulation of the internal chemical or electrical environment of neurons is very difficult with conventional micropipettes.

These limitations can be overcome by using low-resistance 'patch-clamp' pipettes (Hamill et al., 1981; Marty and Neher, 1983) and methods similar to those used to make 'whole-cell' recordings from neurons in brain slices (Blanton et al., 1989; Edwards et al., 1989). In the latter studies, patch pipettes, approximately 2 μm tip O.D., were advanced into the tissue while applying positive pressure to clean membrane surfaces; in some cases separate pipettes were used for recording and cleaning. Upon contacting a cell membrane, the positive pressure was released and a slight negative pressure applied to form a GΩ seal. Additional suction was then used to rupture the patch and obtain a 'whole-cell' recording. Whole-cell recordings from central neurons in vivo have been made using patch-type pipettes of 1.0–1.5 μm (Ferster and Jagadeesh, 1992; Rose et al., 1994). With this methodology, small, subthreshold synaptic activity can be recorded for many minutes and chemical agents can easily be introduced into a neuron, either for labeling or manipulation of the internal chemical environment (Marty and Neher, 1983; Nelson et al., 1994).

Obstacles to performing whole-cell recording in vivo include not being able to visualize (in most cases) neurons and having to traverse hundreds of micra of tissue to get to neurons of interest. Here we extend our earlier description of whole-cell recording in vivo and describe methods for circumventing these problems.

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2. Materials and methods

2.1. Preparation for in vivo recording

The general procedures for preparing electric fish for neural recordings have been described in detail elsewhere (Heiligenberg and Rose, 1985; Rose and Call, 1992). Fish of the genus *Eigenmannia* were used. Their electric organ discharge (EOD) was measured and then attenuated by intramuscular injection of Flaxedil (4 μg/g fish). The fish's own EOD was substituted by a sinusoidal mimic applied through an electrode in the mouth and an external electrode at the tail. The amplitude and frequency of this field were adjusted to be similar to the animal's EOD prior to administration of Flaxedil.

2.2. Electrodes and solutions

Patch-type pipettes were pulled in three stages using a P-97 Flaming/Brown puller (Sutter Instrument Co.) equipped with a 3 mm trough-type filament. Aluminosilicate (1.0 mm OD, 0.75 mm ID, with filament) and borosilicate glass (1.0 mm OD, 0.58 mm ID, with filament) were used. Pipettes were pulled in three stages; setting values for borosilicate (thick-walled) and aluminosilicate (thin-walled) glass, respectively, were: Stage 1, heat = ramp value (minimum needed to melt the glass); pull = 5, 5; delay = 250, 200; velocity = 90, 90; Stage 2, heat, pull, delay = as in stage 1; velocity = 35, 25; Stage 3, heat = 70, 50 above ramp value; pull = 50, 50; delay = 30, 5; velocity = 50, 35. Air pressure was 600, 325. In 'delay' mode, the air jet used to cool the glass began a specified time after initiation of the pull. The third stage settings largely determined the dimensions of the patch pipette tip. These pipettes had outer tip diameters of approximately 1 μm.

The tips of the patch-type pipettes were filled with an osmotically-balanced (285–290 mOsmol) solution containing (in mM): potassium acetate (100), KCl (2), $MgCl_2$ (1), EGTA (5), HEPES (10), biocytin (43), KOH (20). Biocytin was replaced by mannitol in the solution used to fill the pipette shanks. A second solution was similar except that KCl and KAc were 10 mM and 92 mM, respectively. The higher chloride concentration of the latter solution was used to unmask possible inhibitory conductances. These pipettes had resistances of 20–30 MΩ (thick-walled) and 14–20 MΩ (thin-walled).

2.3. Special equipment

Several pieces of equipment are particularly useful for whole-cell recording with patch pipettes. First, a 'picospritzer II' (General Valve Co., Fairfield, NJ) is used to maintain a clean pipette tip (pressure pulses of 70 ms, 40 PSI). A stopcock valve is used to select either pressure pulses or suction (via 30 cc syringe). Second, an electrode holder with a 'perfusion' port (model A089, E.W. Wright Co., Guilford, CT) permitted negative or positive pressure to be applied to the pipette. Third, a custom-built micropositioning device (Fig. 1) is interposed between the microdrive (model 6100 1-1-1-3 Burleigh Instruments Inc., Fishers, NY) and the electrometer head stage. This device consists of a piezoelectric tube (Fig. 1b) cemented on either end to Plexiglas

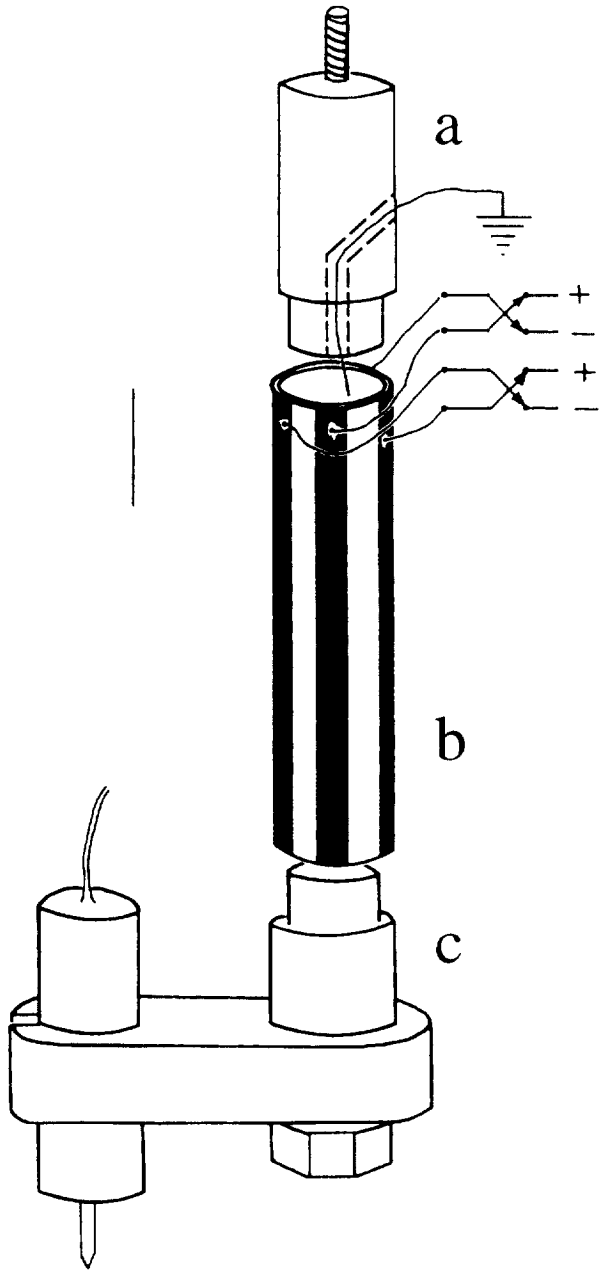

Fig. 1. Drawing of the piezoelectric micropositioning device used to make small changes in the medio-lateral and rostro-caudal position of the recording pipette. Components (a, c) are Plexiglas, (b) is a commercially available 'scan tube'. Scale is 1/4 inch for these components.

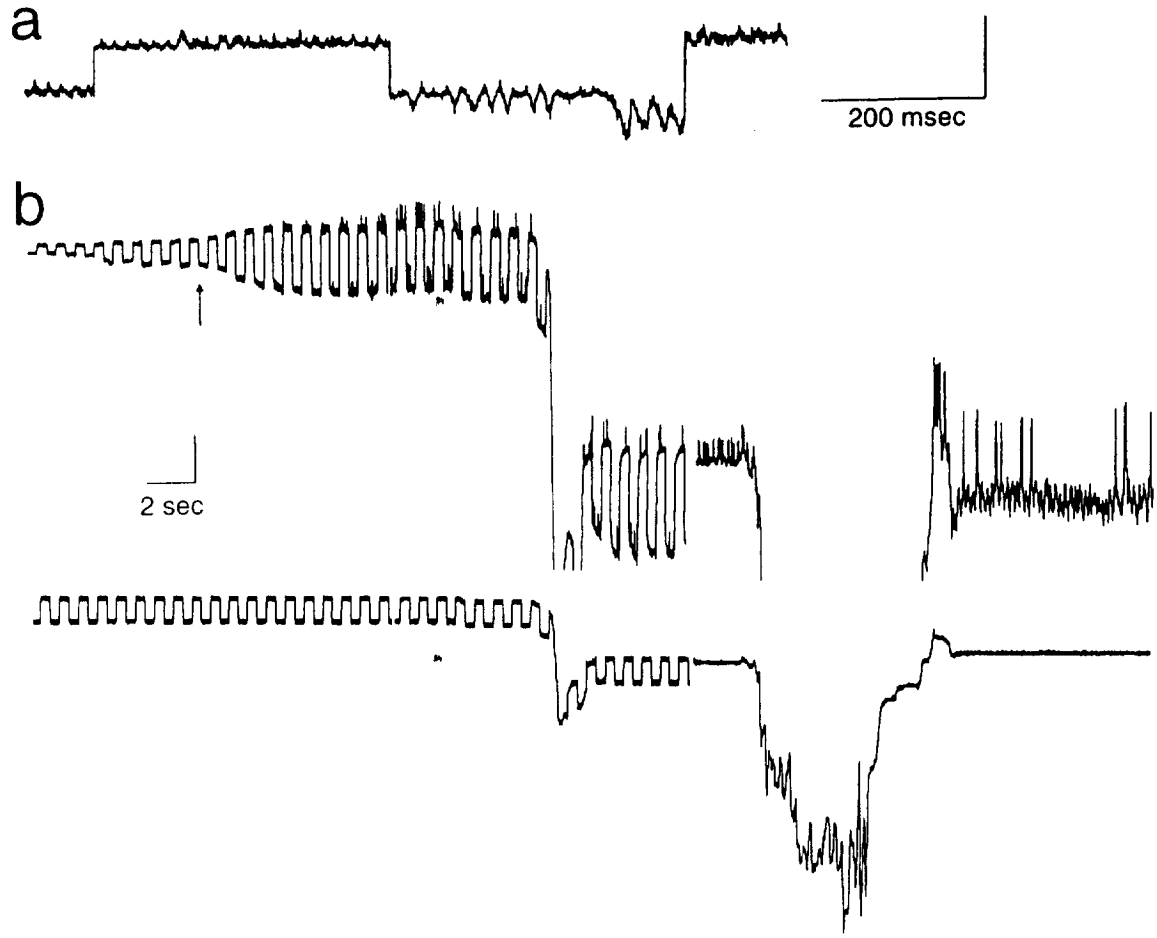

Fig. 2. Recording while approaching a neuron. Negative current pulses of 0.1 nA are being delivered while advancing the pipette in 1.5 μm steps. (a) 'Ripple' episodes in the trace are associated with small increases in resistance following advance of the pipette by 1.5 μm and reflect contact with a cell membrane. Scale = 25 mV. (b) Voltage trace from recording pipette (upper) and current monitor trace (lower). Approach of the neuron is signaled by a slight increase in resistance. Light suction is applied (arrow) resulting in a seal of approximately 300 MΩ and appearance of spikes. The seal resistance increases to at least 0.5 GΩ following delivery of −0.2 nA DC. An initial attempt (not shown) at rupturing the membrane patch was not successful. Vertical scale bar is 0.2 nA for current trace and 20 mV for the recording trace.

adapters. A ground wire is attached using conductive epoxy to the inside of the tube and travels through a channel in the upper Plexiglas component (Fig. 1a). Other wires are attached to the conductive strips on the piezoelectric tube (PZT-5A, ×0.25″ diameter, 1.0″ long; purchased from Burleigh Inst. Inc.). Voltages of up to ±90 V d.c. are supplied by a battery and applied through a rotary switch to wires connected to antipodal conductive strips. For this setup and a pipette approximately 3 cm long, the electrode tip moves approximately 1 μm for a difference of 2 V between two antipodal conductive strips; the exact value depends on the length of the pipette. The microelectrode headstage is held by a Plexiglas arm that attaches to the lower Plexiglas piece (Fig. 1c) via a rubber washer and Teflon nut (bottom).

2.4. Obtaining whole-cell recordings

To aid in keeping the pipette tip clean, a small amount of positive pressure (1–2 cc) was applied to the micropipette while advancing through the midbrain in 1.5 μm steps. During this searching phase, current pulses (0.1 nA, approximately 2 Hz square wave) were delivered and the output of the electrometer was displayed on an oscilloscope. When a small increase in resistance (20–50 MΩ) and a 'ripple' (Fig. 2a) in the trace was observed, 2–5 cc of suction was applied. In most cases, suction failed to reveal neuronal activity. Such episodes, however, often resulted in obstruction of the pipette tip. In these cases, the pipette was 'cleared' by backing up several micra and applying pressure pulses (70 ms, 40 PSI) from the Picospritzer. If this procedure failed to reduce the

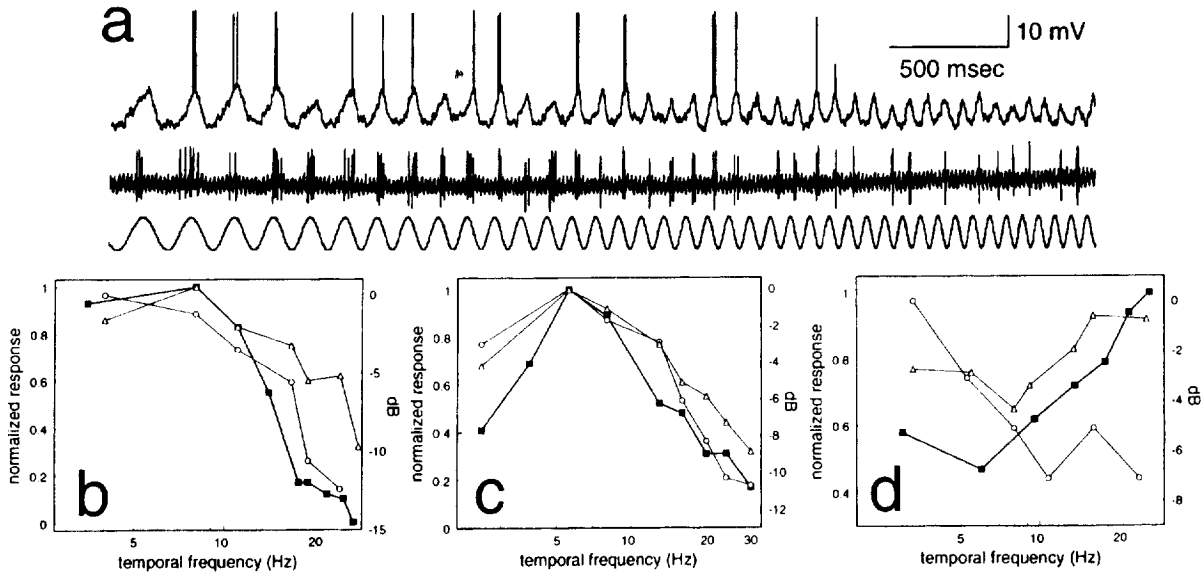

Fig. 3. Comparisons of extracellular and intracellular responses of neurons to electrosensory stimuli. (a) Intracellular (top trace) and extracellular (middle trace) recordings from a neuron in the torus semicircularis in response to a frequency sweep (bottom trace); the extracellular recording was made prior to establishing a full seal onto the neuron. Data from three neurons are presented in the panels (b, c, d). Spike rate, recorded extracellularly (bold lines and squares), and the mean value of the membrane potential (triangles) both were normalized by dividing by the maximum value; decibel (dB) values (circles) represent the heights of peaks in the power spectra of intracellular recordings at the temporal frequency of the stimulus. The data shown in (a) and (c) are from the same neuron.

electrode resistance to its original value, approximately 2 nA of negative current also was applied while delivering pressure pulses. Pipettes were rarely used for more than two penetrations.

When approaching a neuron, the 'cues' in the recording trace were very similar to those described above (small increase in resistance and 'ripple'), however spikes usually were seen following less than 2 cc of suction (Fig. 2b). The resistance generally increased dramatically to 300 MΩ or more in response to this light suction. The best recordings were obtained in cases where less than 3 cc suction gave rise to a large increase in resistance and the appearance of positive-going, monophasic spikes. If spikes were biphasic and/or high resistance seals could not be made easily, positive pressure (2–3 cc) was applied, the electrode was retracted 10–30 μm and small displacements of the electrode in the medial, lateral, rostral or caudal directions were made by applying voltages of ± 10 to 30 V to the piezoelectric tube. In approximately 10 to 20% of these situations such micropositioning improved the seal resistance and recording.

In cases where GΩ seal resistances were not obtained with suction alone, 0.1–0.2 nA negative current was applied, usually resulting in an increase of the seal resistance to 0.5 GΩ or more (Fig. 2b, upper). In all cases, additional suction was avoided because the membrane patch was often ruptured unpredictably, leading to injury of the neuron. To obtain a low access resistance to the interior of the neuron, the membrane patch was ruptured by holding the negative pressure and manually applying negative current to the electrode (Fig. 2b); generally, less than 1 nA was required. The amplitude of the current was manually oscillated while gradually increasing the negative offset of these oscillations. In the example shown in Fig. 2b (lower trace), the initial attempt (not shown) to rupture the patch using a maximum of −0.6 nA failed. After gaining access to the cell, psps as large as 20 mV and spikes of approximately 35 mV were elicited by the ongoing stimulus regime.

2.5. Labeling and tissue processing

Following recording, neurons were labeled by passing 1–2 nA positive d.c. for approximately 1 min. The avidin-biotin technique was used to produce a dark reaction product (Rose et al., 1994). In many cases, even just 1/2 min of iontophoresis was sufficient to label even the finest processes of the neuron. Best anatomical results were obtained by perfusing the fish (ringer, followed by 4% paraformaldehyde in 0.1 M phosphate buffer) 5–10 mins after filling the neuron. In many cases, however, neurons labeled 3–4 h prior to perfusion showed no obvious signs of degradation.

3. Results

Resting membrane potentials of −40 to −65 mV ($X = 50$, S.D. = 8.7) were measured for 14 neurons that exhibited physiology comparable to that shown in Fig.

2b and Fig. 3a. Best results were achieved with access resistances less than approximately 50 MΩ. Soma diameters for these neurons ranged from 9.4 to 16.9 μm ($X = 11.7$, S.D. = 1.8). Stable intracellular recordings were maintained from these neurons for 2.5–17 mins ($X = 7.4$, S.D. = 3.8).

A primary advantage of the whole-cell method is that extracellular and intracellular recordings can be made from the same neuron (Fig. 3). Extracellular recordings (middle trace, Fig. 3a) show that this cell responded best to the lower frequency section of the sinusoidal electrosensory stimulus (frequency sweep shown in bottom trace). Intracellular recordings (top trace) reveal fluctuations of the membrane potential that reflect the sinusoidal nature of the stimulus. The decline in the amplitude of these stimulus-related psps at the higher stimulus frequencies mirrors the decline in spike rate observed in the extracellular recordings; the lower overall spike rate in the intracellular recording is a result of the small (0.1 nA) negative current injected into the cell. Comparisons between extracellular activity (spike rate) and measures of intracellular responses are shown for three representative neurons (Fig. 3b, c, d) that were selective for low, mid and high stimulus frequencies, respectively. Two measures of intracellular responses were made. Spikes were digitally removed and the resulting traces were Fourier analyzed and the mean values of the membrane potential were computed. The mean membrane potential and the amplitude of the peak in the power spectrum at the stimulus frequency were plotted along with the extracellular spike rate; all values plotted were with respect to the maximum value. Secondary peaks in the power spectra were minimal and, therefore, not considered. There was close correspondence between the stimulus-response curves derived from the extracellular and intracellular measures for two of these neurons (Fig. 3b, c). For the neuron shown in Fig. 3c, however, the stimulus selectivity derived from extracellular recording of spikes was reflected in the relative mean membrane potential measurements, but not in the values obtained from the power spectra.

4. Discussion

Study of the integrative mechanisms underlying the function of neurons in the central nervous system requires the intracellular recording of subthreshold potentials and currents. Whole-cell recording with patch-type pipettes appears to be generally superior to conventional methods for intracellular recording, particularly for small neurons. Importantly, in contrast to conventional intracellular recording, patch pipettes permit extracellular recording of spikes while stimulus regimes are tested. Comparison of extracellular and intracellular data has at least two uses. First, poor correspondence between extracellular and intracellular data may indicate that the neuron has been damaged. Second, this comparison may reveal the parameters of the intracellular responses that best correlate with the responses (spikes) measured extracellularly. For example, in the neurons shown in Fig. 3b and c, the two intracellular parameters largely coincide with the extracellular measurements. In the third neuron (Fig. 3d), however, only the mean value of the membrane potential correlated with the response profile measured extracellularly.

Obstacles to performing whole-cell recording in vivo include not being able to visualize (in most cases) neurons and having to traverse hundreds of micra of tissue to get to neurons of interest. Although central neurons in vivo usually cannot be visualized, proximity to a neuron is revealed by the extracellular recording of spikes, particularly when passing by a cell. In fact, it was the frustration of advancing past neurons without being able to seal onto them that motivated the construction of the x–y micropositioning device shown in Fig. 1. Thus, although these recordings are 'blind', the probability of making a seal onto a neuron when near its soma can be improved by making small x–y movements. Additional experiments are required to determine the utility of this device when recording from neurons farther beneath the brain surface. Pipette tips can be kept clean while traversing hundreds of micra below the brain surface by maintaining positive pressure to the pipette during each penetration, as is done in vitro (Edwards et al., 1989), and by applying short, high pressure pulses occasionally to clean the pipette tip and cell membranes in its immediate environment.

As has been observed by investigators studying brain slices (Stuart et al., 1993), seal formation between the pipettes and cell membranes were best for young animals. Because the fish were wild caught, their exact age is uncertain, however best results were obtained with animals that were less than one year old.

The seal resistance can be measured prior to rupturing the membrane patch, but subsequent increases in seal resistance are difficult to quantify. Seals of greater than 0.5 GΩ, together with access resistances of less than 50 MΩ insure that measured magnitudes of intracellular potentials will be at least 90% of their true values. This calculation is based on the fact that the access resistance and seal resistance act as a voltage divider (Stuhmer et al., 1983). Thus with higher access resistances, lower values for resting membrane potential, spike and psp amplitude are measured.

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