

SHORT COMMUNICATIONS

IDENTIFICATION OF NEURONES IN THE LEECH THROUGH LOCAL IONIC MANIPULATIONS

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The identification of neurones which generate and control specific animal movements can typically be made with conventional intracellular recording techniques during the expression of the movement pattern. Neurones whose membrane potentials are highly correlated to the patterns under investigation are potentially part of the pattern-controlling system. Further searches may then be made to find the pre-synaptic and postsynaptic cells. Such neuronal circuits are now known for several invertebrates (Friesen & Stent, 1978; Delcomyn, 1980; Kristan, 1980). However, a serious drawback to these search procedures is that many time-consuming trial and error experiments are required, in which hundreds, or even thousands of candidate cells must be impaled with microelectrodes.

We have devised a method to screen neuronal preparations, both for neurones that control specific movements and for neurones that are presynaptic to previously identified neurones. Using this procedure we have discovered neurones in the suboesophageal ganglion (head brain) of the medicinal leech which appear to control leech swimming behaviour through their interactions with neurones of the ventral nerve cord.

It is well known that the membrane potentials of neurones are mainly dependent on the external K^+ and Cl^- concentrations (see Hodgkin, 1951). Whereas a rise in external K^+ level causes a depolarization, increase in extracellular Cl^- concentration causes a hyperpolarization. With our procedure, we use standard glass micropipettes filled with either 4 M potassium acetate (KAc) or 1 M Tris-HCl (Sigma, pH = 7.4). A range of useful tip diameters, from 0.2 μm to 1 μm , can be obtained by grasping the micropipette ends with a pair of fine forceps or by brushing them against a glass slide. Micropipettes with very small tip diameters can be used to excite or inhibit individual cells, while larger tipped micropipettes can be used to affect tens or hundreds of cells simultaneously. Micropipettes are mounted in electrode holders commonly used for pressure injection of materials into cells (WP Instruments). The pressure pulses applied to the micropipettes are controlled with a solenoid-activated valve (Clippard Instrument Lab.). Continuous perfusion of the preparation with physiological saline ensures that the ionic concentrations are changed only near the micropipette tip.

Experiments were performed on preparations consisting of the isolated leech central nervous system, including the head brain and most of the segmental ganglia

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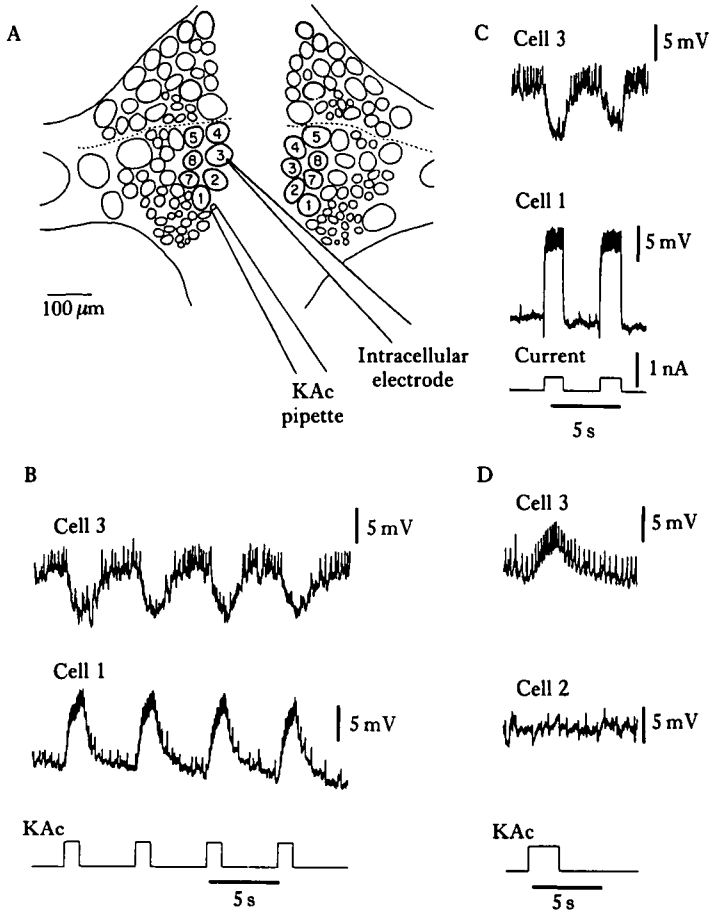


Fig. 1. Depolarization evoked by pulses of high K^+ solution. (A) Dorsal aspect of a segmental ganglion. The numbered circles indicate the approximate positions of the motor neurones. Micropipette tip diameters are not drawn to scale. (B) Intracellular recordings from two segmental neurones, cell 3 and cell 1. Cell 1 was tonically inhibited by 0.4 nA intracellular holding current. Brief pulses of 4 M potassium acetate (KAc) were released near cell 1 at times indicated in the bottom trace, causing rapid depolarizations in cell 1 which, in turn, hyperpolarized cell 3. (C) Direct depolarization of cell 1 by brief interruption of tonic holding current (see current monitor). Because the electrometer bridge was not well balanced, the cell 1 trace does not reflect potential changes accurately. (D) The effect of KAc application is local. A KAc pulse was released above cell 3 at the time indicated in the bottom trace, depolarizing cell 3 with no effect on cell 2, even though these cells are contiguous. Some of the traces in this and succeeding figures have been retouched slightly to improve clarity. Abbreviations: cell 3, dorsal excitator motor neurone; cell 1, dorsal inhibitor motor neurone; cell 2, ventral inhibitor motor neurone.

(Ort, Kristan & Stent, 1974; Yau, 1976). To expose the neuronal somata directly to the ionic solutions ejected from the micropipettes, and to facilitate cell impalement, the connective tissue sheath surrounding the ganglia was cut away with fine iridectomy scissors.

We found that depolarization of a leech neurone by a local increase in K^+ concentration is rapid, reversible and repeatable. For example, with the tip of the KAc-filled micropipette about 20 μm above the soma of cell 1 (Fig. 1A), pulses of KAc led to brief 10 mV depolarizations of cell 1, accompanied by an increased firing rate (Fig. 1B)

The excitation induced in cell 1, in turn, inhibited cell 3, a motor neurone which is postsynaptic to cell 1 (Ort *et al.* 1974). The effects produced with KAc pulses can be obtained many times without noticeable cell damage. The depolarization of cell 1, and the inhibition caused in cell 3, resemble the potentials obtained in these neurones by direct depolarization of cell 1 with intracellularly-injected current (Fig. 1C). Because of this resemblance, the ionic procedure can be used instead of intracellular current injection to identify presynaptic neurones. We also found that the effect of KAc application can be restricted spatially. As shown in Fig. 1D, KAc pulses ejected from a small-tipped micropipette (about $0.2\ \mu\text{m}$ diameter) placed about $20\ \mu\text{m}$ above the cell 3 soma produced no potential change in the contiguous neurone, cell 2 (Fig. 1A). From these and similar results, we conclude that individual neurones can be depolarized by KAc pulses without affecting neighbouring cells.

Neurones can be hyperpolarized by ejecting Tris-HCl instead of KAc from the micropipette (Fig. 2A). During this experiment, cell 2 was tonically excited by intracellular current injection, producing tonic inhibition in cell 4. This synaptic inhibition was reduced during the hyperpolarizations elicited in cell 2 by Tris-HCl pulses. The effects of Tris-HCl pulses, like those of KAc, are rapid, reversible and spatially restricted. These hyperpolarizations of cell 2, as well as the disinhibition of cell 4, are similar to the potential changes obtained by direct hyperpolarization of cell 2 with current pulses (Fig. 2B). Thus Tris-HCl pulses provide a means for

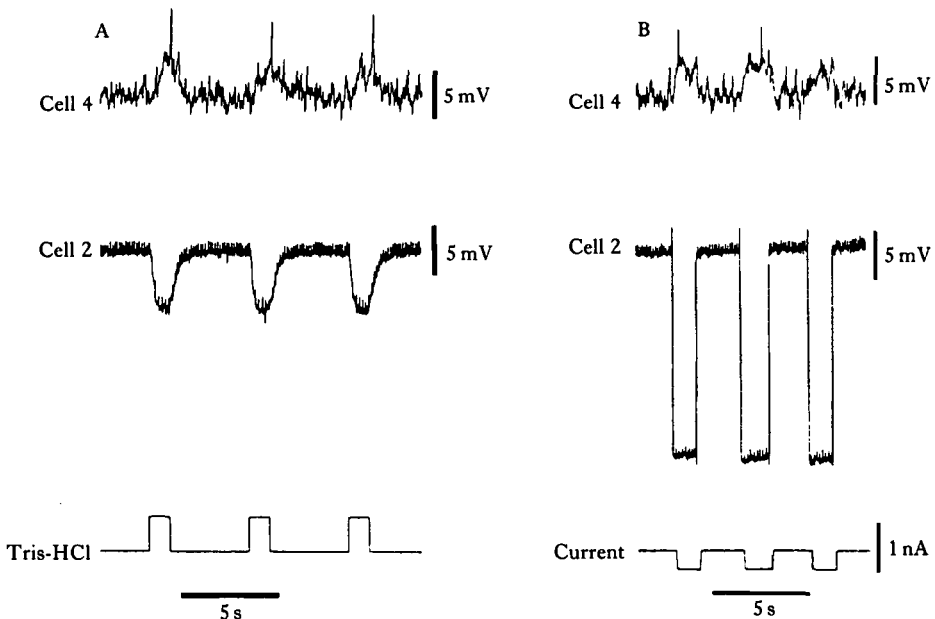


Fig. 2. Hyperpolarization evoked by Tris-HCl pulses. (A) Intracellular recordings from segmental neurones, cell 4 and cell 2. Cell 2 was tonically excited by $0.2\ \text{nA}$ intracellular current, thereby causing tonic inhibition in cell 4. Brief pulses of $1\ \text{M}$ Tris-HCl were ejected about $10\ \mu\text{m}$ above cell 2 at the times indicated in the bottom trace. Each Tris-HCl pulse hyperpolarized cell 2 which, in turn, released cell 4 from inhibition. (B) Direct inhibition of cell 2 by brief hyperpolarizing pulses (see bottom trace). These direct hyperpolarizations had the same effects as the Tris-HCl pulses. Abbreviations: cell 4, ventral excitor motor neurone; cell 2, contralateral ventral inhibitor motor neurone.

inhibiting neurones rapidly and selectively without the necessity for intracellular penetrations.

Our purpose in developing this technique was to identify neurones in the leech brain which control the central pattern generator (CPG) underlying swimming movements (Friesen, Poon & Stent, 1978). Since the head brain in *Hirudo*, which consists of four fused ganglia, contains more than one thousand tightly packed cells, finding neurones which control this behaviour is a formidable task. To search for swim-controlling neurones, we first used large-tipped micropipettes filled with KAc to depolarize ten to twenty neurones simultaneously. Then, in brain regions where such broadly applied excitation evoked swimming activity, we applied KAc more selectively until pulses again elicited swimming activity. The properties of candidate cells identified with these restricted KAc pulses were then examined with standard intracellular recording techniques.

The results of one such successful search are illustrated in Fig. 3. We first ejected a KAc pulse from a 1 μm diameter micropipette positioned near the anterior midline of the suboesophageal ganglion (Fig. 3A). This KAc pulse led to depolarization of a segmental Retzius cell (Fig. 3B) and, several seconds later, initiated a short episode of swimming activity, as shown by motor neurone bursting in the extracellular trace (DP). These effects are due to selective stimulation of about five neurones near the midline. Ejection of KAc 50 μm lateral to the midline evoked no responses, either in the Retzius cell or in the segmental nerve (Fig. 3C). We then explored neurones near the midline with intracellular electrodes and discovered a pair of bilaterally symmetrical neurones (BN) whose depolarization excited segmental Retzius cells and initiated swimming activity (Fig. 3D). A series of similar experiments have led to the discovery of eight neurones in the brain which influence the expression of leech swimming activity. In addition to these experiments, we also are using the ionic stimulation technique to find neurones presynaptic to identified swim interneurones.

Previously published reports describe several neuronal screening methods which do not require time-consuming intracellular recordings. With one simple method, cells are stimulated by extracellular current, which is applied to groups of cells *via* a suction electrode in contact with the nervous system (Willows, Dorsett & Hoyle, 1973). In contrast to this procedure, our ionic technique requires no contact between the nervous tissue and the electrode, thus minimizing the possibility of damage while permitting more rapid searching. Farber & Grinvald (1983) have recently described another promising search method, one which employs photostimulation. With this approach, the nervous tissue to be examined is first soaked in a dye solution. Candidate neurones are then stimulated by an intense laser beam, which can be directed at selected neuronal targets. We find that ionic stimulation has several advantages over photostimulation: first, excitation *and* inhibition can be obtained many times without apparent damage; second, no dyes, which may be toxic, are required; and third, the equipment required is much cheaper and is available already in many neurophysiological laboratories.

The ionic technique described here should be applicable to many diverse neuronal preparations. For example, we have used this technique to stimulate selected populations of molluscan photoreceptors without the use of light (G. D. Block & W. O. Friesen, unpublished). The technique should prove useful also for screening neuronal

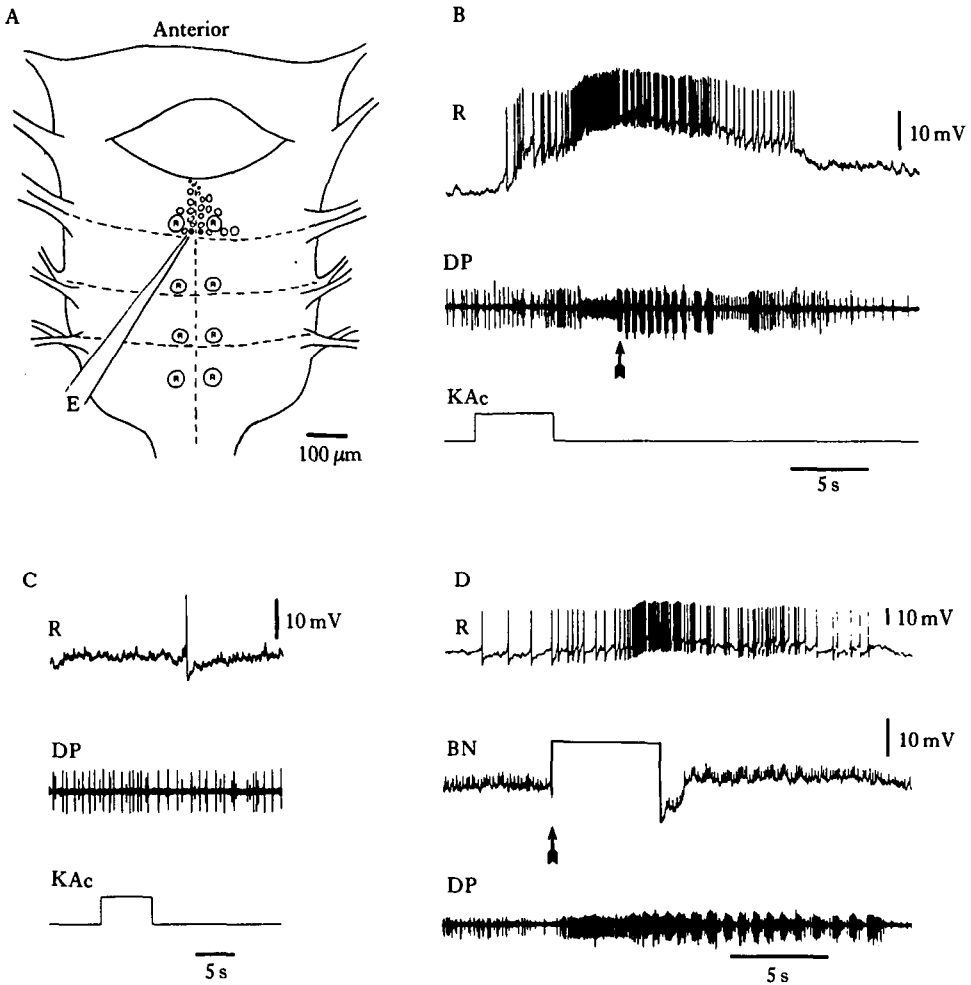


Fig. 3. Identification of a swim-initiating neurone in the leech brain. (A) Ventral aspect of the head brain. The open circles labelled 'R' represent the positions of the Retzius cells in the suboesophageal ganglion. The micropipette tip is positioned near a neurone BN (filled circle). Redrawn from Yau (1976). (B) KAc pulse released near the anterior midline of the suboesophageal ganglion depolarized the segmental Retzius cell and subsequently evoked swimming activity (DP nerve bursts beginning at the arrow). (C) The KAc effect is localized. The KAc pulse was directed 50 μm lateral to the midline. Neither the Retzius cell potential nor motor neurone activity were affected by this pulse. (D) Swim-initiation by direct current injection. The trace labelled 'BN' is an intracellular recording obtained from the neurone indicated in (A). Depolarization of BN by intracellular current injection (at arrow) mimicked the effects of the KAc pulse. Abbreviations: E, micropipette positioned for releasing KAc pulses or for recording intracellularly; R, Retzius cell; DP, dorsal posterior nerve, a peripheral nerve in which the largest rhythmically occurring impulse is that of cell 3; BN, brain neurone in the suboesophageal ganglion.

populations in vertebrate brain-slice preparations, provided that the target neurones are accessible to the ejected ionic solutions. With an appropriately sized micropipette, large neuronal ensembles or individual neurones can be stimulated or inhibited. The latter may prove particularly useful for locating tonically active neurones which serve to inhibit the expression of some behaviour patterns.

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