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Localization of stomatogastric IV neuron cell bodies in lobster brain

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Summary. The cell bodies of the inferior ventricular nerve (IVN) 'through-fibers' of the lobster stomatogastric nervous system were located using cobalt chloride backfills and intracellular recordings. Following backfills of the IVN, two cell bodies in the supraesophageal ganglion (or brain) were stained with cobalt. These cells, each approximately 30 μm in diameter, were located at the base of the IVN, just inside the connective tissue sheath surrounding the brain, and were identifiable on the basis of their close proximity to the IVN.

In order to record from the cells, an in vitro preparation was made which included the cell bodies, their axons in the IVN and the stomatogastric nervous system. Intracellular recordings showed that the axons projected to the stomatogastric ganglion and made synaptic connections onto identified neurons. The axon trajectories and synaptic connections correlated with those previously described for the IVN through-fibers using extracellular stimulation and recording techniques.

Introduction

Evidence now shows that groups of central neurons can produce rhythmic activity without peripheral feedback (Wilson 1966; Grillner and Zangger 1974; Kristan and Calabrese 1976; Getting et al. 1980), and that the activity of such 'central pattern generators' can be influenced by sensory or central neurons that are not themselves part of the pattern producing ensemble (Grillner 1977). The stomatogastric ganglion of the lobster is one central pattern generator whose thirty neurons are capable of producing rhythmic foregut movements without sensory feedback, but whose activity can be increased or interrupted by extrinsic, central neurons (Selverston et al. 1976; Russell 1976, 1979). Two of these extrinsic neurons are the through-fibers of the inferior ventricular nerve (IVN), which have also been called command interneurons. They make chemical synapses with identified neurons in the stomatogastric ganglion, and, depending on their firing frequency, can induce various alterations in the pattern of activity of stomatogastric neurons, including a complete cessation of normal rhythmic activity (Dando and Selverston 1972; Sigvardt and Mulloney 1982a, b). It has been suggested that this interruption causes a 'swallowing' reflex in the intact animal which serves to move food from one foregut region to another (Sigvardt and Mulloney 1982b).

Previously, because the positions of the through-fiber cell bodies were not known, they were characterized on the basis of extracellular recordings from the IVN. As part of a study aimed at identifying the transmitters used by these neurons, we attempted to locate their cell bodies. In this report, we present evidence from cobalt backfills and physiological recordings that their somata are located in the supraesophageal ganglion (or brain) of the spiny lobster, at the base of the IVN, and that they are identifiable on the basis of position. Further, we describe an in vitro preparation composed of these 'IV neurons' and the stomatogastric nervous system which allows physiological studies of these cells and their effects on the stomatogastric system.

Materials and methods

Animals and dissections: Lobsters (*Panulirus interruptus*) of both sexes weighing between 0.4 and 1.5 kg were obtained from local fishermen (San Diego, California) and kept in running seawater aquaria. The esophagus and stomach were removed as described by Mulloney and Selverston (1974) and, in addition, the brain (supraesophageal ganglion) was left attached

Abbreviations: *IVN* inferior ventricular nerve; *SN* stomatogastric nerve

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to the system by the IVN. The nerves and ganglia were pinned out in a Sylgard-coated dish for anatomical or physiological studies, and perfused with *Panulirus* saline. The composition of the saline was as follows (in mmol/l): 486.9 Na^+, 519 Cl^-, 12.7 K^+, 13.7 CA^{+2}, 10 Mg^{++}, 13.9 SO_4^{-2}, and 4.9 TES and 4.9 HEPES buffers (Sigma). The pH was adjusted to 7.45 by the addition of NaOH.

Cobalt backfills. Because the branching pattern of the IVN through-fibers suggested that the cell bodies might be located in the brain, the IVN was sectioned and backfilled with cobalt chloride toward the brain. Cobalt chloride backfills were done following the procedure of Kushner (1979). The IVN was sectioned approximately one centimeter from the supraesophageal ganglion: the cut end was placed in a well and isolated from the supraesophageal ganglion with vaseline. Distilled water was placed in the well for 2 min and the nerve resectioned. The water was removed and 300 mmol/l cobalt chloride (dissolved in distilled water) was added to the well. The supraesophageal ganglion was covered with normal *Panulirus* saline and the preparation was left at room temperature (21 °C) for 24 h. The entire preparation was washed in saline for 20 min, reacted with 0.2% ammonium sulphide and washed again in saline. It was then fixed for 20 min in Carnoy's solution, dehydrated in ascending concentrations of ethanol, and cleared with methyl salicylate.

Electrophysiology. The stomatogastric system was prepared for recording as described elsewhere (Mulloney and Selverston 1974; Russell 1976), and standard electrophysiological recording techniques were used. The putative IV neurons were included in the preparation as described below in the Results section. Extracellular electrodes were placed on the stomatogastric nerve (SN) and the IVN for recording and stimulation. Intracellular recordings were made from the putative IV neurons and from identified neurons in the stomatogastric ganglion using 3 mol/l KCl electrodes with resistances between 30 and 60 MOhm. Current was injected intracellularly through the bridge circuit of a WPI M4A electrometer.

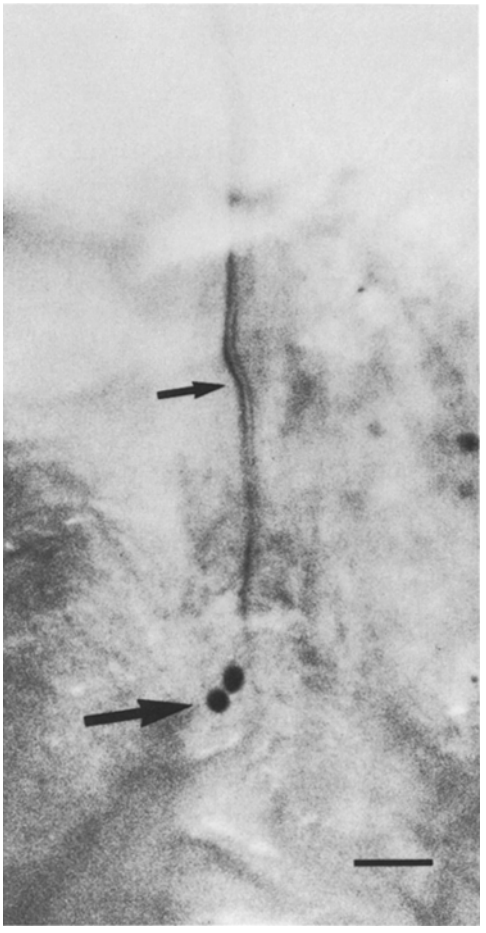

Fig. 1. Light micrograph of IV neuron cell bodies stained by backfilling the IVN with cobalt chloride toward the supraesophageal ganglion. Two stained cell bodies are visible (large arrow) as are their axons in the IVN (small arrow). Final magnification = ×100. Scale bar = 100 μm

Results

Location of cell bodies: Following cobalt backfills of the IVN toward the brain, two cell bodies were stained (Fig. 1). They were the only cells that stained and were located on the ventral edge of the brain, at the base of the IVN. Each soma was approximately 30 μm in diameter, was clearly visible through the connective tissue sheath in stained preparations, and did not appear to have any processes other than an axon in the IVN.

After noting the location of the cells in stained preparations, we were able to locate them in unstained ganglia, but only after an opening had been made in the connective tissue sheath. The sheath was slit several millimeters away from the base of the IVN and was gently pulled away from the brain. When this was done carefully, the IV cells remained attached to their axons and adhered to the *inside* of the sheath at the point where the IVN entered the sheath. Occasionally, clusters of other neurons would also pull away from the brain along with the sheath; however, the IV cells were distinguishable by their close proximity to the IVN.

Electrophysiological identification: In *Panulirus interruptus*, the IVN contains nine axons (Claiborne, unpublished observations) and therefore it was necessary to show that the two somata which stained with cobalt were the cell bodies of the IVN through-fibers. To do this, an in vitro preparation for electrophysiological recordings was devised which included the putative IV cells and the stomatogastric nervous system. The preparation was made by first dissecting out the entire stomatogastric nervous system with the brain attached, next pinning out the stomatogastric nervous system in a Sylgard dish for recording, and then exposing the IV cells in the brain as described above. Once the cells were visible, the connective tissue sheath surrounding them was cut away from the brain

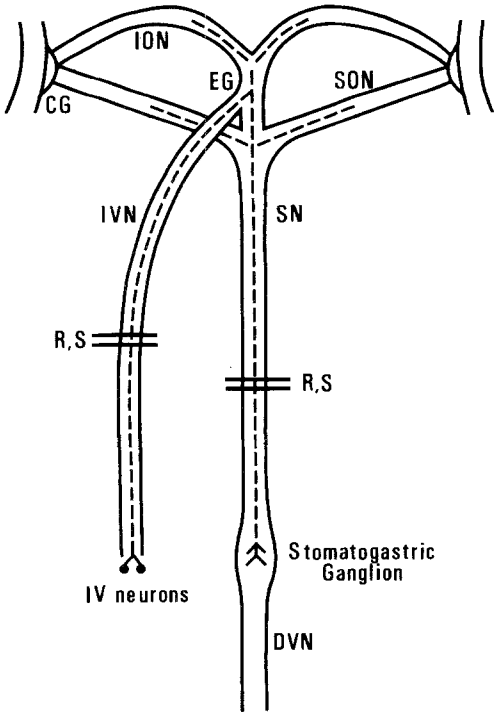

Fig. 2. Diagrammatic representation of the in vitro preparation used to physiologically identify the IV neuron cell bodies. A piece of connective tissue sheath surrounding the cell bodies was removed from the brain and pinned with the inside surface facing up in the recording dish, along with the stomatogastric nervous system. Intracellular recordings were made from the IV neuron cell bodies and from identified neurons in the stomatogastric ganglion. Extracellular electrodes were placed on the inferior ventricular nerve (*IVN*) and the stomatogastric nerve (*SN*) for recording and stimulation (*R*, *S*). The axons of the through-fibers are represented here by the dashed line. They send branches into the superior and inferior esophageal nerves (*SON*s and *ION*s), and recent evidence (E. Marder, personal communication) indicates that the ION branches reach the commissural ganglia (*CG*). Vedel and Moulins (1977) have shown that the axons do make synaptic connections in the esophageal ganglion (*EG*). The through-fibers terminate in the stomatogastric ganglion and do not enter the dorsal ventricular nerve (*DVN*)

and pinned out in the Sylgard-coated dish with the *inside* surface facing up. This exposed the cells for intracellular recordings, and left them attached to the stomatogastric nervous system via their axons in the IVN. A diagrammatic representation of this preparation is shown in Fig. 2.

Previously the through-fibers were shown to be the only axons in the IVN which traveled through the esophageal ganglion to the stomatogastric nerve (Dando and Selverston 1972; Kushner 1979; Russell and Hartline 1981). To discern whether the cell bodies we located were the somata of the identified through-fibers, we evoked action potentials in either the IVN or the SN, and looked for antidromic action potentials in the cell bodies. Extra-

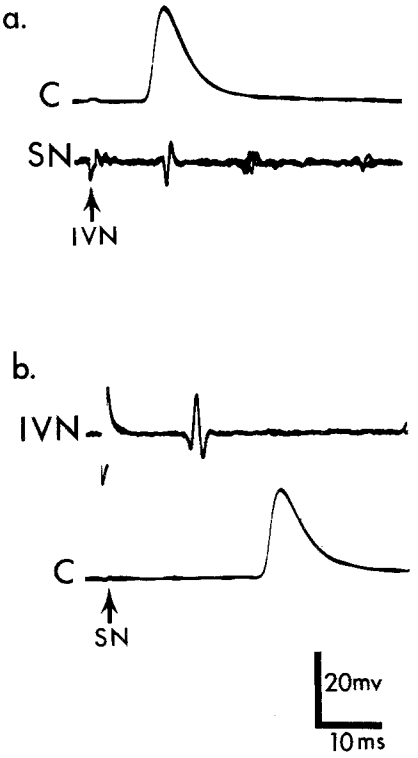

Fig. 3a , b. Two superimposed traces of intracellular recordings from IV cell bodies (*C*) showing time-locked action potentials following extracellular stimulation of either the *IVN* (panel a) or the *SN* (panel b). Resting potential of *C* was −50 mV. In **a** the *IVN* was stimulated at 10 Hz (arrow) and action potentials recorded in an IV cell body (*C*), and extracellularly from the stomatogastric nerve (*SN*). In **b** the SN was stimulated at 10 Hz (arrow) and action potentials recorded in an IV cell body (*C*) and from the *IVN*

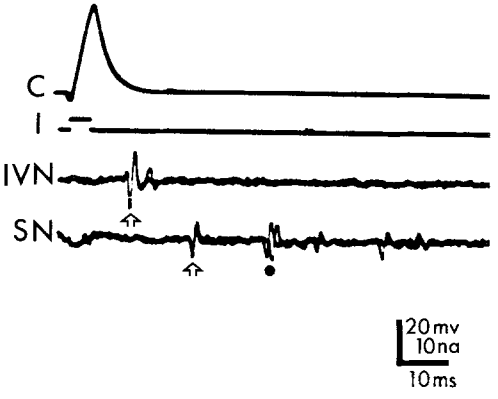

Fig. 4. Two superimposed oscillograph traces showing an intracellular recording from an IV cell body (*C*) and extracellular recordings from the *IVN* and *SN*. The resting potential in *C* was −50 mV. Current injected (I) into the cell body elicited an action potential in the cell which was followed by time-locked action potentials in the *IVN* and *SN* (arrows). A second action potential (dot) with a variable latency was seen on the SN recording trace. This may represent a spike elicited in *CDI* by the action potential in the IV cell body: *CDI* is an esophageal neuron which has an axon in the *SN* and which receives an excitatory synapse from the *IVN* through-fibers (Vedel and Moulins 1977)

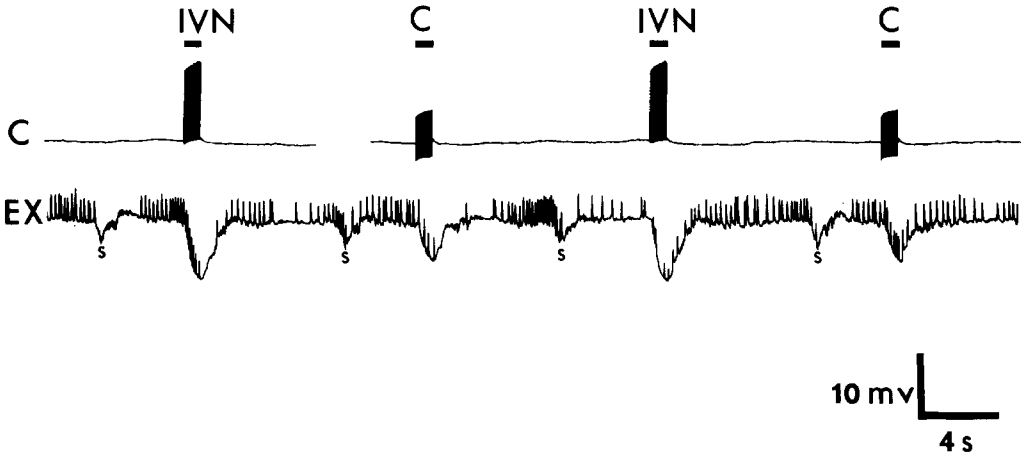

Fig. 5. Intracellular recordings from IV cell body (*C*) and an EX neuron (*EX*) located in the stomatogastric ganglion, showing the inhibitory synaptic potentials elicited in the EX neuron by both intracellular stimulation of the IV cell body and by extracellular stimulation of the IVN. Trains of stimuli (800 ms, 40 Hz) were applied as indicated by bars, either intracellularly (*C*) or extracellularly (*IVN*). Both forms of stimuli elicited action potentials in the IV neuron cell body (C, top trace) and summed inhibitory potentials in the EX neuron (EX, bottom trace). Note that the amplitude of the summed inhibitory potential is greater following extracellular IVN stimulation, presumably because both through-fibers were activated (see text). Spontaneous excitatory and inhibitory synaptic potentials are also visible in the EX recording

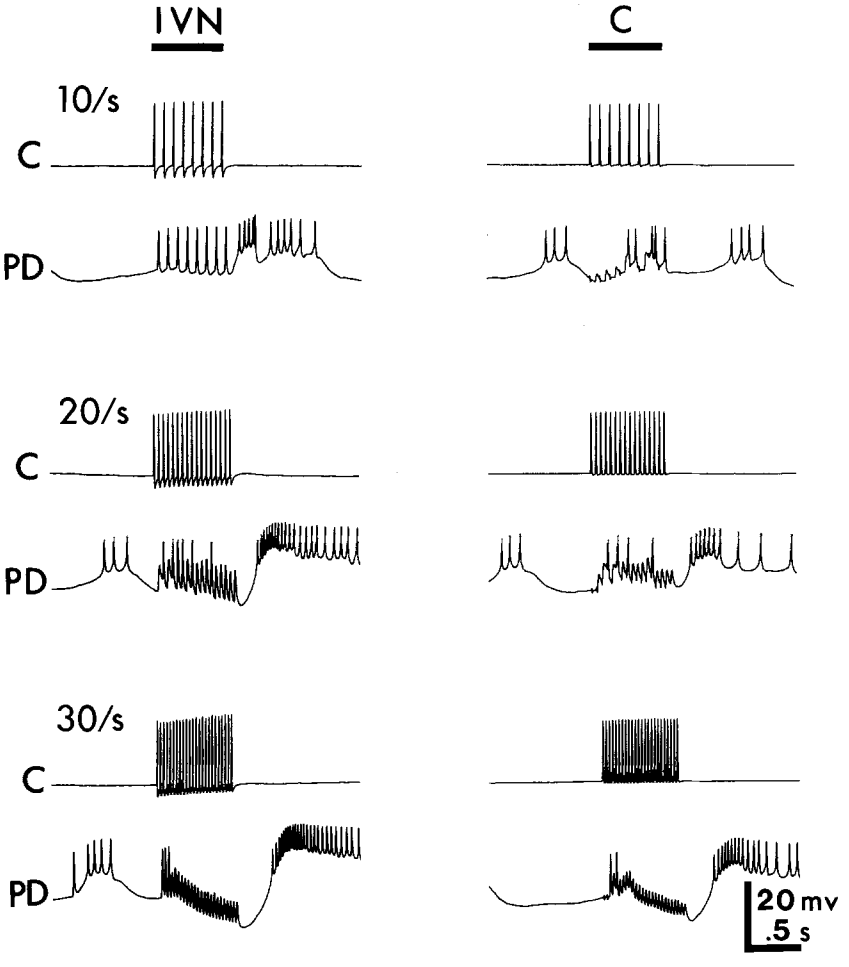

Fig. 6. Synaptic potentials in a *PD* neuron (located in the stomatogastric ganglion) are elicited by both extracellular stimulation of the *IVN* (first panel) and intracellular stimulation of an IV cell body (second panel). Simultaneous intracellular recordings were made from an IV cell (*C*) and a *PD* neuron (*PD*). Resting potential of the IV cell was −45 mV and resting potential of the *PD* neuron was −58 mV. Trains of stimuli at 10, 20 and 30 Hz (top, middle and bottom panels) were applied extracellularly to the through-fibers (*IVN*) or intracellularly to the IV cell body (*C*). At all stimulation frequencies, a biphasic potential was elicited in the *PD* neuron, but at 10 Hz the excitatory potential predominated whereas at 30 Hz the inhibitory potential predominated. Note that potentials in the *PD* neuron are larger following extracellular stimulation, presumably because both through-fibers were activated (see text)

cellular stimulation of either nerve elicited time-locked action potentials in the putative IV cell bodies (Fig. 3). It was also possible to elicit action potentials directly in the cell bodies by injecting brief depolarizing pulses through the recording electrode. When this was done, time-locked action potentials were recorded from the IVN and the SN (Fig. 4).

A second distinguishing feature of the IVN through-fibers is that they evoke discrete post-synaptic responses in certain neurons in the stomatogastric ganglion (Dando and Selverston 1972; Selverston et al. 1976; Claiborne 1981b; Sigvardt and Mulloney 1982a, b). In this study, the putative IV neurons were stimulated intracellularly and the post-synaptic responses in the PD, EX, GM and CD2 neurons were examined in detail. Examples of the potentials in the EX and PD neurons are shown in Figs. 5 and 6. Data indicated that action potentials in one IV cell body produced post-synaptic responses in these stomatogastric neurons that were identical to, but smaller in amplitude than, responses produced by extracellular stimulation of the through-fibers. This difference in amplitude is to be expected: extracellular stimulation of the IVN almost always produces action potentials in both through-fibers, whereas intracellular stimulation presumably elicits action potentials in only one cell body. Previous work has shown that when it is possible to stimulate the two fibers separately, their effects on stomatogastric neurons are additive (Dando and Selverston 1972).

Discussion

Using the technique of cobalt backfilling, the putative IV neuron cell bodies were located in the lobster brain. They are the only two cells apposed to the connective tissue sheath at the base of the IVN and hence are identifiable on the basis of position. Electrophysiological data show that the axons of these cells travel in the IVN and SN, as do the IVN through-fibers, and that action potentials in these cells elicit post-synaptic responses in certain stomatogastric neurons which correlate with the responses elicited by extracellular stimulation of the through-fibers. These data suggest that the cells are the somata of the through-fibers.

Several points remain to be addressed. First, all of the IV neuron connections onto stomatogastric cells were not examined in detail. Second, it is not yet known if the individual effects of both cells on stomatogastric neurons are identical. Although preliminary data from sequential penetrations suggest their effects are the same, simultaneous recordings have not been done. Similarly, it is not known if the two cells interact with one another.

The in vitro preparation described here should prove useful in addressing these cellular questions and in answering others. For example, neurotransmitter studies show that the cells have a high concentration of endogenous histamine and may use histamine at their inhibitory synapses in the stomatogastric ganglion (Claiborne 1980, 1981a, b; Claiborne and Selverston, in preparation). However, there is only preliminary evidence which suggests that they use histamine at their excitatory terminals (J. Eisen, personal communication). Further, Russell and Hartline (1981) have shown that extracellular stimulation of the through-fibers elicits bursting properties in some stomatogastric neurons which as yet cannot be accounted for by histamine. It is therefore of interest to investigate the possibility that a second transmitter is used by these cells.

It will also be of interest to determine if the IV neurons receive input directly onto their cell bodies, as their location in the brain opens the possibility that they may integrate the control of foregut movements with other behaviors. When the IV neurons fire a burst of action potentials, which often happens spontaneously in vitro, the patterned activity of the stomatogastric ganglion temporarily ceases, with some neurons firing tonically while others are silent. In the animal, this motor neuron activity presumably causes foregut muscles to be held in a motionless state, which Sigvardt and Mulloney (1982b) have proposed forces food from one region to another. They found that stretch of the pyloric stomach region can induce a burst of through-fiber action potentials in a semiintact preparation. It is not yet known how often this reflex occurs in the intact animal, or if it can be triggered by other sensory input. It is known, however, that rhythmic activity of the gastric mill region in the intact animal can be interrupted by tactile or visual stimuli. By recording gastric mill muscle activity in freely moving lobsters, Fleischer (1981) showed that gastric mill cycling stops abruptly when the animal is disturbed, either by touch or by turning on normal tank lighting. The location and synaptic connections of the IV neurons make them candidates for the cells mediating this reflex.

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