Connectivity Patterns of Crayfish Giant Interneurons: Visualization of Synaptic Regions with Cobalt Dye

Abstract. *Intracellular injection of cobalt dye was used to visualize electrical synapses between two pairs of central giant interneurons and giant motoneurons in the crayfish central nervous system. A pair of giant motoneurons in each ganglion contacts the interneurons, but not all contact points are functional synapses. Cobalt dye reveals numerous fine projections that are present at synaptic contact points and absent at nonsynaptic contacts; intracellular recording confirms this correlation. The different connectivity patterns of the two pairs of interneurons are consistent with the different behavior patterns which they evoke.*

An essential step in the analysis of the neural basis of a behavior is an understanding of the "wiring diagram" of the circuits responsible [for example, see (*1, 2*)]. Elucidation of circuitry has typically depended heavily or exclusively on electrophysiological methods, but the recently developed techniques of dye injection (*3, 4*) have aided correlation of structure and function. However, a major difficulty in the anatomical analysis of neural circuits has been that visualization of synaptic contacts between nerve cells generally requires examination by electron microscopy. In this report we describe structural features that are correlated with en passant electrical synaptic transmission in the crayfish and can be visualized by using the "cobalt dye" technique of Pitman *et al.* (*4*). These synaptic specializations are not present at all zones of contact between the neurons, but our results show that their presence is perfectly correlated with functional synaptic interaction.

The junctions we investigated are formed between two pairs of giant interneurons and giant motoneurons that are part of short-latency escape reflex circuits in the crayfish (*5, 6*). Four central giant axons run in parallel along the dorsal surface of the ventral nerve cord throughout the animal. The two medial giant axons originate in the supraesophageal ganglion and are excited by rostral inputs; the lateral giant axons have cell bodies in each abdominal ganglion and are excited by caudal inputs. Action potentials in these central command neurons are transmitted monosynaptically to motoneurons (*2, 7, 8*) and thence to the abdominal fast flexor muscles to produce the powerful tail flips that often initiate escape swimming (*6*). The largest of the motoneurons [the "giant motor fibre" (*9*), also designated F1 (*8*)] has processes in all branches of the third root and ramifies extensively in the periphery to contact all of the oblique fast muscles; in a rested preparation it produces muscle action potentials and so is presumed to produce a particularly complete and powerful tail flip (*10*). The nongiant fast flexor motoneurons extend long dendritic branches that contact the central giant axons in the ganglion (*8*). In the connective caudal to some ganglia, near the third roots, the F1's synapse en passant with lateral and medial giant axons. In these synapses an F1 extends fine short processes which penetrate the glial sheaths enveloping the F1 and the lateral or medial giant, to synapse with the central giant axon (*5, 8, 11, 12*). These synapses were investigated in a classic study by Furshpan and Potter (*9*), who demonstrated that they operate electrically.

In their investigation, the two sets of giant interneurons were assumed to synapse with the F1's in every abdominal ganglion: This assumption was based on prior anatomical (*5, 11*) and physiological (*5, 13*) studies. However, behavioral observations in restrained (*14*) and freely moving (*6*) crayfish demonstrated that different movement patterns were produced by the two neurons. Behavioral differences were explained, in part, by demonstrating differential connections between the command neurons and certain motoneurons in the sixth (last) abdominal ganglion by a combination of Procion Yellow injections and extracellular recording (*14*). The remaining differences in the behavior involve several segments anterior to the sixth, and we

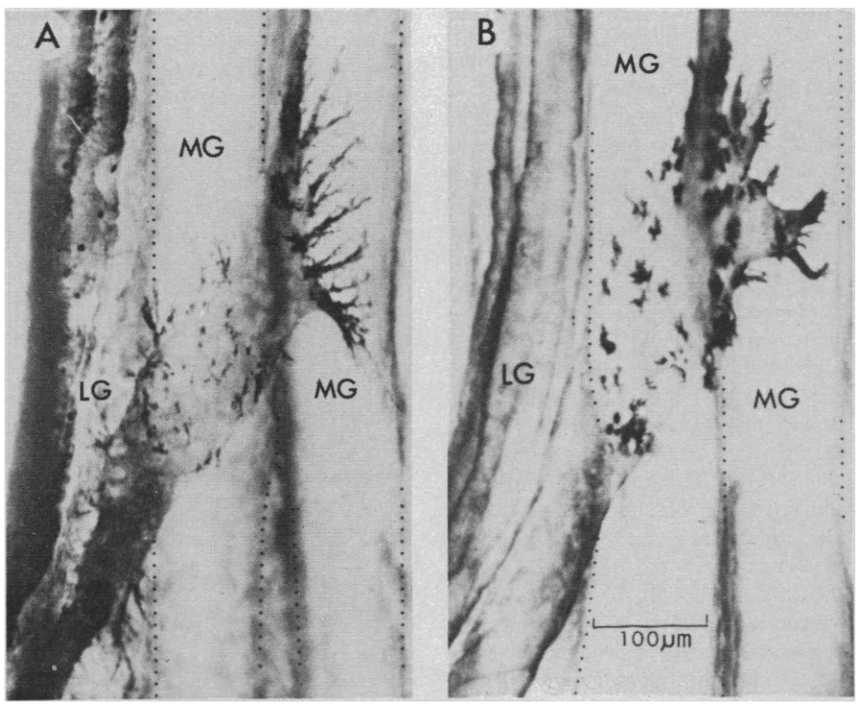

Fig. 1. Photographs of whole mounts showing cobalt-stained motor giant synapses in the second and fourth abdominal ganglia. In each part the top is rostral; the scale applies to both parts. (A) A dorsal view of the third root region of the connective between the second and third ganglia where the F1 neurite, seen as a dark line between the two medial giant (*MG*) axons, expands and extends numerous small projections that contact both medial giants and the left lateral giant (*LG*) axon before exiting in the left third root. The F1 passes ventral to the medial giants and dorsal to the lateral giant. The rostral portion of the left lateral giant is obscured by the tract of nongiant motoneuron axons on their course to the root. (B) The same view of an identically prepared fourth ganglion, showing well-developed branches of the F1 to the right medial giant and tufts to the left medial giant, but no specializations of any kind in the region of the lateral giant. The outlines of the cord giants are dotted in regions where they become indistinct. The line separating the two medial giants is the midline; most of the right lateral giant was cropped from each photograph.

were faced with the paradox that different behaviors were somehow produced by interneurons with supposedly identical connections to the F1's.

To test the idea that the connections might differ, we introduced cobalt dye into the F1's of each abdominal ganglion (G_1 through G_5), using a modification of iontophoretic introduction of the dye into the cut end of the axon (*15*). Physiological evidence for synaptic transmission was obtained by intracellular recordings from the F1 axons, while the giant interneurons were stimulated directly via suction electrodes. Standard procedures were used to prepare, maintain, and record from the abdominal nerve cord (*16*).

Typical anatomical results are illustrated in Fig. 1. The cobalt dye stained numerous tufts of small processes where the F1's contacted the medial giants (MG's). Similar staining was visible between the MG's and F1's in all ganglia (exemplified by G_2 and G_4, Fig. 1, A and B), but was seen between the lateral giants (LG's) and F1's only in the first three abdominal ganglia (Fig. 1A). In G_4 and G_5, no evidence of stained processes could be discerned in the region where the F1's cross over the LG's (Fig. 1B). A complete lack of connecting processes was observed in every one of 12 preparations.

Electrophysiological results are consistent with the anatomy. An impulse in a medial giant produces a short-latency (less than 0.2 msec) fast-rising excitatory postsynaptic potential (EPSP) in the F1 in all abdominal ganglia (Fig. 2A); in some preparations these exceeded the threshold, and one-to-one transmission resulted (for example, see Fig. 2A, G_1 and G_5). An impulse in the lateral giants always produced EPSP's and sometimes produced spikes in the three rostral ganglia, but never in G_4 and G_5 (Fig. 2, A and C). While the presence or absence of EPSP's in a ganglion was constant from animal to animal, the occurrence of spikes was variable. Sometimes F1 spikes recorded extracellularly in the third root were lost upon penetration, while in several preparations an initially small EPSP in F1 would gradually increase during the experiment and eventually result in a spike. For these reasons, one-to-one transmission is assumed to be the normal mode of operation for these junctions, and its absence is attributed to damage caused by the experimental procedures [see also (*9*)]. A slow, depolarizing potential occurs in the F1's of every abdominal ganglion following stimulation of lateral or medial giants (Fig. 2A). In agreement with Furshpan and Potter (*17*) we considered it to be an inhibitory postsynaptic potential (IPSP); this was demonstrated directly by showing that the slow potentials shunt the electrical EPSP's (Fig. $2B_2$) and can block F1 spikes in preparations that display them (Fig. $2B_1$). The available evidence suggests that the giant interneurons excite interganglionic interneurons to produce the IPSP in F1's (*18*).

The connectivity patterns that we found explain, at least in part, the behavioral differences between lateral giant and medial giant tail flips. The medial giants activate most or all of the fast flexor muscles and cause a complete abdominal flexion that propels the animal backward, whereas the lateral giants produce no flexion in the caudal segments, thereby pitching the animal upward (*6*, *14*). Since the medial and lateral giants are activated by rostral and caudal stimuli, respectively, activation of either of the escape circuits moves the animal away from the stimulus.

We do not yet know whether the cobalt dye technique will permit identification of synaptic junctions in general. Location by light microscopy of synaptic specializations, such as the tufts of motor giants or the dendritic spines of cortical neurons, allows the examination of spatial patterns of synaptic contacts [for example, see (*19*)]. Al-

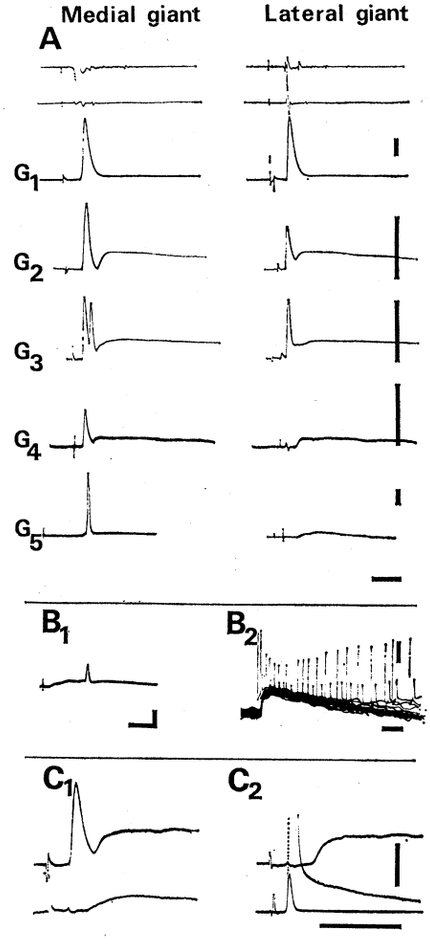

Fig. 2. Electrophysiological analysis of differential connectivity patterns of two sets of command interneurons with the giant motoneurons. (A) Intracellular records from the giant motoneuron in each abdominal ganglion, following stimulation of the medial giant and lateral giant axons. The top two traces in the record from G_1 are extracellular recordings of the medial giants (top) and lateral giants (middle). These were monitored in all experiments, but the traces have been cropped from the remaining records for clarity. Medial giant stimulation produced excitatory postsynaptic potentials (EPSP's) in all ganglia; in G_1 and G_5 these exceeded threshold and produced action potentials (note the different amplitude calibrations). In some cases differences in the velocity of conduction between the two medial giants led to two separate EPSP's (G_3 and B_2). Lateral giant stimulation produced EPSP's in only the first three ganglia, occasionally leading to action potentials (shown for G_1). The presence of EPSP's is constant from preparation to preparation, but spiking is variable and its absence is attributed to experimental damage (see text). Slow, depolarizing potentials are produced in all ganglia by either medial giant or lateral giant stimulation. Similar potentials have previously been interpreted as inhibitory postsynaptic potentials (IPSP's) (*17*); this was confirmed here. (B) Demonstration that slow depolarizations are IPSP's. (B_1) Combined stimulation of the lateral and medial giant axons results in the block of a spike produced by a medial giant in the F1 of the fifth ganglion by the IPSP produced by a lateral giant (same preparation as G_5). (B_2) The conductance change accompanying an IPSP produced by a lateral giant in the fifth ganglion shunts the EPSP's produced by the simultaneous activation of both medial giants. (The smaller EPSP was produced by the contralateral medial giant, which conducted less rapidly.) (C) Simultaneous intracellular records from an F1 and a lateral giant in the fourth ganglion. (C_1) A spike in a medial giant (not shown) produces an EPSP in the F1 and IPSP's in both the F1 and the lateral giant. (C_2) An action potential in the lateral giant (bottom, shown at two gains) produces only an IPSP in F1; the small deflection on the F1 trace was caused by coupling between the two closely spaced microelectrodes. The time calibration is 10 msec. The voltage calibrations (intracellular records only) are: A and B_1, 20 mv; B_2, C_1, and C_2 (upper traces), 5 mv; C_1 (lower trace) and C_2 (middle trace), 10 mv; C_2 (lower trace), 125 mv.

though such specializations are common in nervous systems, synapses in which they are absent cannot be visualized through this technique.

In summary, we found that, in the regions studied here, short projections or tufts of the postsynaptic cell membrane could be clearly visualized, and their presence was always associated with synaptic transmission verified electrophysiologically. When such processes were absent, even in cells that invariably make close anatomical contact, electrical signs of transmission were also absent.

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16. Details of electrophysiological procedures can be found in (*9*). Briefly, the nerve cord was prepared as if for dye iontophoresis, except that the sheath of the cord was removed at the exit point of the third roots. The preparation was transilluminated and FI's and occasionally the lateral giant axons were impaled under visual control with 15- to 25-megohm 3*M* KCl micropipettes. The preparation was maintained in oxygenated saline, cooled to 13° to 15°C.
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Paleozoic Seeds with Embryos

Abstract. *Seeds in a conifer cone from the Lower Permian of west Texas contain embryo tissue. These are the oldest plant embryos on record. Their development prior to seed dispersal shows that the sequence of embryo growth typical of most modern seed plants had evolved before the end of the Paleozoic Era.*

All seeds of Paleozoic plants recorded to date have one thing in common: they lack visible embryos (*1*). Reported here are the oldest known plant embryos. They occur in seeds of a new type of conifer cone (*2*) of the Voltziales or so-called "transition conifers" that comes from the Permian of west Texas.

The apical 2.5 cm of the cone preserved in a sandstone concretion was found loose in a steep gully at the Clay Slide locality (*3*, *4*) in the Glass Mountains, Brewster County, Texas. The Clay Slide is 4 km west of the summit of Iron Mountain (*5*) and is exposed on the south-southeast side of a cuesta constructed of Lower Permian rocks (*4*). The fossil was resting on rocks of the Cathedral Mountain Formation, having eroded from them or from the overlying Road Canyon Formation. Both formations are included in the Lower Permian Leonard Series (*4*).

Since the cone was preserved in a rounded concretion and was not found in place, it is remotely possible that it was not indigenous to the rocks now exposed at the Clay Slide. The Triassic Bissett Formation occurs 11 km northwest of the Clay Slide, and foliage of *Voltzia* was identified from an exposure of this formation 26 km from the Clay Slide (*6*). It could thus be argued that the Bissett Formation once covered the Clay Slide but eroded, leaving the fossil behind. However, there is no evidence that the Bissett Formation ever extended as far south as the Clay Slide and, considering the steepness of the collecting site, it is doubtful that a rounded concretion could remain on the slope for any great length of time. Furthermore, additional Permian rocks occur stratigraphically between the Road Canyon and Bissett Formations in the Glass Mountains and would have eroded after the latter rocks, obscuring traces left behind. The cone itself is more similar to those of the Voltziales from the Upper Carboniferous and Lower Permian of Europe than to the Upper Permian and Mesozoic forms. Finally, the rounded nature of the concretion could suggest as well that the cone was reworked into the Cathedral Mountain Formation or the Road Canyon Formation from still older rocks. Thus, it must be concluded that the cone is at least as old as Lower Permian.

There are three complete seeds in the fossil and apices of two others extend into the specimen from the missing cone base. Each seed occurs singly on the adaxial side of a flattened dwarf shoot that is axillary to a bract. The dwarf shoot also bears about 30 sterile scales. The seeds are dorsiventrally flattened and are 10 to 12 mm long, with a maximum width of 8 mm. The integument extends beyond the seed body, producing two broad flaps of tissue with a slit between them leading to the micropylar region (Fig. 1A). The internal structure of these seeds is basically similar to that of the Cordaitales and other Voltziales.

The best-preserved embryo appears