The Inhibitory Motoneurons of Crayfish Thoracic Limbs: Identification, Structures, and Homology With Insect Common Inhibitors

T.J. WIENS AND H. WOLF

Department of Zoology, University of Manitoba, Winnipeg R3T 2N2, Manitoba, Canada (T.J.W.), and Fakultät für Biologie, Universität Konstanz, W-7750 Konstanz, Germany (H.W.)

ABSTRACT

The three inhibitory motoneurons supplying crayfish thoracic limbs were identified, stained, and compared structurally. The inhibitors to the walking leg muscles (in *Orconectes*) were identified anatomically by a combination of immunocytochemical staining for gamma-aminobutyric acid (GABA) or glutamate decarboxylase and differential backfill staining with nickel and cobalt ions. The cheliped inhibitors were identified intracellularly and injected with Lucifer Yellow (*Pacifastacus*) or cobalt (*Procambarus*). The common inhibitor (CI) in each thoracic segment has a medial or slightly contralateral soma near the ganglion's posterior boundary, a gently curving primary neurite, an extensive ipsilateral dendritic tree, and an axon emerging through the anterior root. The stretcher-closer inhibitor (SI) has a soma slightly anterior and ipsilateral to the CI's, a sharply bent proximal neurite, a smaller dendritic tree, and an axon in the posterior root. The opener inhibitor (OI) lies more laterally and often posterior to the CI; its diagonally directed neurite enters the posterior root. The inhibitors' structures were related to major neuroanatomical landmarks within the ganglion, to soma positions of excitatory motoneurons revealed by backfilling, and to soma locations of inhibitory interneurons revealed by GABA-like immunoreactivity.

In their peripheral distributions to the leg muscles and in their central structures, these crayfish limb inhibitors show striking similarities with those of the locust. Crayfish and locust thoracic ganglia also show more general neuroanatomical similarities. These observations suggest that the crayfish CI, SI, and OI are, respectively, homologous with the locust CI₁, CI₂, and CI₃. The implications of such a homology for arthropod phylogeny are discussed. © 1993 Wiley-Liss, Inc.

Key words: arthropod, crustacean, evolution, GABA, immunocytochemistry

In decapod crustacean limbs, neuromuscular inhibition forms an integral and indispensable component of motor control strategy. Three inhibitory motoneurons exert different effects on the leg musculature (reviewed in Wiens, '89). A common inhibitor (CI) of all limb muscles is directed at the slowly responding tonic muscle fiber population in each muscle (Atwood, '65). It becomes active during locomotion, suppressing tonic tension and enhancing rapid, rhythmically alternating limb movements (Ballantyne and Rathmayer, '81). A specific inhibitor of the opener muscle (OI) acts synergistically with the closer muscle exciters to effect closing of the propodite-dactyl joint (Bush, '62). OI and the stretcher inhibitor (SI) are necessary to permit independent contraction of the opener and stretcher muscles because these muscles are activated solely by the same excitatory axon, the shared opener-stretcher excitor (OE–

SE). SI, however, has recently been shown in the crayfish claw and walking legs not to be restricted to the stretcher muscle as previously thought, but also to innervate the closer muscle (Wiens, '91, '93); thus, the crayfish SI is neither specific nor truly common in its output.

While the three inhibitors' peripheral distributions and effects have been reasonably well studied, their central structures and connections have received less attention. This has limited our understanding in at least two areas. First, little is yet known of the central circuitry that integrates inhibitory output with ongoing limb behavior. Synaptic inputs to the inhibitors from tactile and proprio-

Accepted April 7, 1993.

Address reprint requests to T.J. Wiens, Department of Zoology, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2.

ceptive sources (Wiens and Gerstein, '76), from other motoneurons (Wiens and Gerstein, '75), and from interneurons (Smith, '72; Cooke and MacMillan, '83) have been inferred from extracellular recordings. In some cases (Wiens and Atwood, '78; Lindsey and Gerstein, '79; Skorupski and Sillar, '88; Bevenгут and Clarac, '91), these have been confirmed by intracellular studies, but further progress in this direction will require detailed knowledge of these neurons' central structures. Second, systematic structural comparisons have so far not been possible between the set of crayfish limb inhibitors and those of other arthropods, in particular the three inhibitory motoneurons (CI_1 , CI_2 , CI_3) described for locusts (Hale and Burrows, '85; Watson et al., '85; Wolf, '90) and cockroaches (Pearson and Iles, '71). Some observations suggest similarities between decapods and insects in their limb inhibitors' peripheral distributions (Wiens, '91; '93) and central structures (Wiens, '76; Bevenгут et al., '83; Moffett and Yox, '86), but a more complete knowledge will be necessary to shed light on these groups' phylogenetic relationships.

In the study reported here, the CI , SI , and OI neurons of crayfish chelipeds and walking legs were identified centrally, and their structures were compared through differential back-filling, immunocytochemical staining, and intracellular dye injection. The results reveal a striking similarity in central layout of the inhibitors between the crayfish and the locust, consistent with their peripheral parallels. On this basis, we propose a one-for-one homology of the crayfish CI , SI , and OI with the locust CI_1 , CI_2 , and CI_3 , respectively, and discuss its phylogenetic implications. Some of these results have been published in abstract form (Wiens, '91; Wolf, '92).

MATERIALS AND METHODS

Animals

Backfilling and immunohistochemical studies were performed in Konstanz on walking leg inhibitors of adult *Orconectes limosus*, obtained from a commercial supplier and kept in aerated lake water at 16°C. They were anesthetized prior to experiments by immersion in tap water saturated with chloroform, after which their supraesophageal ganglia were destroyed. Comparative backfills and immunocytochemical stains were made on decapitated adult *Locusta migratoria* obtained from a breeding colony at the University of Konstanz. Intracellular staining and electrophysiological studies were performed in St. Andrews on cheliped inhibitors of adult *Pacifastacus leniusculus* obtained from Riversdale Farm (Dorset, England). These animals were anesthetized by chilling in ice-cold oxygenated fluid before and during dissection, and were decapitated immediately thereafter.

Backfill procedure

Differential backfill technique. To locate and identify the soma of a walking leg CI anatomically, a differential backfill technique was used (Quicke and Brace, '79; Sakai and Yamaguchi, '83). The method exploits the facts that: (1) all motoneurons except the common inhibitor travel in only one of the leg nerve branches proximal to the autotomy plane, while CI has branches in all except the posterior root (see below; Bevenгут et al., '83; Moffett et al., '87); and (2) rubenic acid forms a blue precipitate with Ni^{++} ions, a yellow one with Co^{++} ions, and a stain of mixed color in neurons backfilled with both ions. The animals were immobilized on a wax platform, ventral side up, and submerged

in cold (ca. 4°C) tap water up to the level of the leg bases. The anterior and posterior nerve roots and several leg muscle nerves were dissected free in the ischio- and coxopodite of a walking leg, usually a leg of the third thoracic segment. Care was taken to remove the hemolymph vessel attached to the nerve roots to prevent diffusion of heavy metal ions into the circulatory system. The operation site was kept moist by addition of small amounts of saline (195 mM NaCl, 5.4 mM KCl, 13.5 mM $CaCl_2$, 2.6 mM $MgCl_2$, 10 mM Tris buffer at pH 7.4; Erxleben, '89). The cut ends of the nerves were placed in two Vaseline wells in various configurations and immersed in distilled water for 5–10 minutes. The water in the wells was then replaced with 3–6% aqueous solutions of $NiCl_2$ and $CoCl_2$, respectively. With this concentration of metal ions, predominantly motoneurons were labelled along with only few sensory afferents. After diffusion for 50–80 hours at 4°C, the thoracic nerve cord was removed from the animal and the metal ions were precipitated with rubenic acid (five drops of a saturated solution of dithiooxamide in absolute ethanol per 10 ml saline). The ganglia were fixed for 4 hours at room temperature or overnight at 4°C (fixative: three parts saturated picric acid, one part 25% glutaraldehyde, acetic acid added to 1%), dehydrated in an ethanol series, and cleared in methyl salicylate for subsequent microscopic examination, drawing, and photography.

Co^{++} backfills. Backfills in *Locusta migratoria* were made as described for the differential backfill procedure but using only $CoCl_2$ (Altman and Tyrer, '80). Access to the body cavity was achieved by an incision in the dorsal midline. Tracheal sacs, the gut, fatty tissue and overlying muscles with their apodemes were removed to expose the thoracic nerve cord. The roots of nerves 3, 4, and 5 of the mesothoracic ganglion were dissected, backfilled with Co^{++} ions, and processed further as described above.

Intensification

Block intensification. After the identity of motoneuron somata as revealed by the differential backfill technique had been noted, some of the preparations were intensified with silver nitrate according to Obermayer and Strausfeld ('80). This revealed structural details that were otherwise not discernible, particularly in motoneurons filled with cobalt ions, which produced only a relatively low-contrast yellow stain.

Intensification of sections. Other ganglia were embedded in Paraplast Plus (Monoject Scientific, St. Louis, MO) and cut in 10 μ m slices in the transverse plane. Alternate sections were made for intensification and immunocytochemistry (see below), and both sets were mounted on glycerine-albumin-coated slides. The sections for intensification treatment were deparaffinized in xylene, rehydrated to distilled water, and silver-intensified according to Bacon and Altman ('77) but using 0.01% $AgNO_3$. After the intensification procedure, preparations were dehydrated in an ethanol series. Whole ganglia were cleared in methyl salicylate and embedded in Canada balsam; sections were mounted in Permunt (Fisher Scientific, Fair Lawn, NJ) under glass coverslips.

Immunohistochemistry

Antisera. The anti-gamma-aminobutyric acid (GABA) serum was provided by T.G. Kingan (University of Arizona, Tucson). It had been raised in rabbits, in response to conjugates of GABA coupled to keyhole limpet hemocyanin (Hoskins et al., '86). The serum had been characterized (Hoskins et al., '86) and previously tested for specificity

(Homberg et al., '93). The anti-glutamic acid decarboxylase (GAD) serum was a gift from J. Kopin (NIMH, Bethesda, MD). The serum was produced in sheep with GAD isolated from rat brain serving as immunogen (Oertel et al., '81, '83). Its specificity had been demonstrated on rat brain tissue (Oertel et al., '81) and crab nervous tissue (Homberg et al., '93).

Anti-GABA and anti-GAD immunocytochemistry. For immunocytochemistry, one set of the alternate sections (see above) was deparaffinized in xylene and rehydrated to a solution of 0.1 M Tris/HCl, 0.3 M NaCl, and 0.1% Triton-X (pH 7.4). The indirect peroxidase-antiperoxidase (PAP) technique developed by Sternberger ('79) was employed for anti-GABA immunocytochemistry. The antibody was applied at a dilution of 1:3000. Anti-GAD immunocytochemistry was carried out by the avidin-biotin (ABC) technique according to Hsu et al. ('81), the antibody being diluted 1:1750. Detailed descriptions of both techniques are given in Homberg et al. ('93). Corresponding procedures were used for anti-GABA immunocytochemistry of intact locust ganglia.

Electrophysiology and Lucifer Yellow injection

Preparations comprising only the two chelipeds and the first thoracic ganglion were made by the procedure of Wiens ('76). The chilled animal's hemolymph was first replaced with HEPES-buffered van Harreveld's ('36) saline to enhance visibility and minimize clotting; saline was slowly injected through the tip of one of the claw propodites while hemolymph was allowed to drain through a small incision in the soft cuticle of the abdomen. Preparations were studied at cool room temperature (15–20°C) and remained viable for about 12 hours. Cuticle was removed above muscles of interest to permit intracellular recording, usually near their distal apodemes. Conventional intracellular recordings were made from muscle fibers with 10–20 MΩ electrodes filled at the tips with 2 M KAcetate and backfilled with 3 M KCl. Central intracellular recordings from the inhibitory motoneurons were made with 30–40 MΩ micropipettes filled at the tip with a 5% solution of Lucifer Yellow (Stewart, '78) in 1 M LiCl and backfilled with 1 M LiCl (Heitler and Fraser, '89). Axons were stimulated by means of paired hook electrodes or en passant suction electrodes, either in the anterior (first, smaller) or posterior (second, larger) root leading from the thoracic ganglion to the limb or in the terminal nerve bundles to selected muscles (see Fig. 3, inset). Axon spike recordings were made through en passant suction electrodes placed on the nerves entering the stretcher, closer or opener muscles. Recorded data and stimulus information were stored on eight-track digital audio tape by means of a Biologic DTR-1800 recorder. Recorded traces were digitized and displayed through the Computerscope (RC Electronics) analysis system connected to a Hewlett-Packard Laserjet printer; signal averaging was performed through the same system. For intracellular staining, Lucifer Yellow was iontophoresed into the penetrated cell by negative current pulses, typically of 250 ms duration and 2 Hz frequency. Filled cells were fixed (5% formalin in van Harreveld's solution), dehydrated, cleared in methyl salicylate, and then viewed, drawn, and photographed in whole-mount through a Zeiss fluorescence microscope.

Histology

The anatomy of the *Orconectes* ventral nerve cord was surveyed by use of the osmium-ethylgallate technique

introduced by Wigglesworth ('59). Serial 15 μm sections of the third thoracic ganglion were examined and interpreted with reference to Skinner's ('85a,b) description of the abdominal nerve cord in *Procambarus*. Anatomical features of the three inhibitory motoneurons within the anatomical framework of the ganglion were reconstructed from serial sections processed for silver intensification and immunocytochemistry (see above). In a similar way, the distribution of somata with GABA-like immunoreactivity was reconstructed from serially sectioned ganglia subjected to anti-GABA immunocytochemistry.

RESULTS

Identification and central structure of the thoracic limbs' inhibitory motoneurons

Identification of the common inhibitor of *Orconectes* walking legs by differential backfilling. The CI motoneuron of the third thoracic ganglion was identified anatomically by the differential $\text{Co}^{++}/\text{Ni}^{++}$ staining technique (Quicke and Brace, '79; Sakai and Yamaguchi, '83). Anterior and posterior nerve roots and leg muscle nerves were dissected in the coxo- and ischiopodite and backfilled in various configurations from two wells containing cobalt and nickel salts, respectively. Only these proximal branches could be used because ion diffusion from more distal motor axons proved to be blocked almost completely in the autotomy plane. After precipitation with dithiooxamide, motoneurons were found to be stained either blue or yellow except for one which showed a stain of mixed color, reddish brown to magenta, indicating ion uptake from both wells through two separate nerves. This cell was inferred to be CI, and its putative inhibitory character was supported by subsequent immunocytochemistry. Silver intensification of cells so identified then permitted CI's central structure to be examined in greater detail.

Immunocytochemical identification of the three walking leg inhibitors. To confirm CI's identification and to locate OI and SI anatomically, antibodies against the inhibitory transmitter GABA or the enzyme glutamic acid decarboxylase (GAD, a key enzyme for GABA's synthesis) were used to stain the cells of the backfilled ganglia. After the locations of CI (as identified above) and other backfilled motoneurons had been noted, the ganglion was serially sectioned, usually in the transverse plane. Alternate sections were processed for immunocytochemistry with either anti-GABA or anti-GAD antibodies, and for the silver intensification procedure of Bacon and Altman ('77). Subsequent comparison of adjacent sections (Fig. 1) allowed somata which were labelled by both procedures to be identified; these were presumed to belong to inhibitory motoneurons.

Three such presumably inhibitory motoneurons were revealed in each of the eight ganglia examined in this way (Fig. 2), among them CI as identified before sectioning. The other two were concluded to be SI and OI; they could be distinguished on the basis of OI's more lateral soma position (Wiens, '76) and of electrophysiological results (see below). Antibodies against GABA and GAD were found to produce identical staining patterns with regard to the backfilled motoneurons, reinforcing the characterization of the labeled cells as inhibitory. Many interneurons also showed GABA-like immunoreactivity (see below).

Electrophysiological identification of the cheliped CI in *Pacifastacus*. The closer nerve containing the axons of CI, SI, and the two closer exciters was isolated in the carpopodite-propodite joint of the cheliped (see Fig. 3,

A silver-intensified

B anti-GABA immunocytochemistry

500µm

Fig. 1. Identification of cell bodies labeled by both $\text{Co}^{++}/\text{Ni}^{++}$ backfilling of motor axons and anti-GABA immunocytochemistry. Two adjacent serial sections through a third thoracic ganglion of *Orconectes* are shown, the upper (A) revealing $\text{Co}^{++}/\text{Ni}^{++}$ -stained motoneuron somata after silver intensification, and the lower (B) showing immunoreactive structures after immunocytochemistry with an anti-GABA antibody. Both prominent somata labeled by the backfill procedure also show GABA-like immunoreactivity. The cell body located close to the

midline had been identified previously by its mixed color stain as CI's, and the more lateral soma is OI's. CI's soma lies among a group of other cell bodies with pronounced anti-GABA immunoreactivity. CI's labeling is positive but weak when compared to these surrounding cells; this may reflect interference between the $\text{Co}^{++}/\text{Ni}^{++}$ and anti-GABA staining, observed several times in this study and others (Honegger et al., '90). Dorsal is upward in all figures in the transverse plane.

inset); the nerve was stimulated near the closer muscle while the spike activity in its axons was recorded more proximally. Alternatively, CI's axon could be activated more

easily though less selectively by a hook electrode on the anterior root (Fig. 3A,C). An intracellular micropipette was placed in a tonic fiber of the distal accessory flexor muscle or

Fig. 2. General morphology of the three inhibitory motoneurons in the third thoracic ganglion of *Orconectes*. Only the main dendritic processes are shown in the camera lucida drawings of CI, SI, and OI, and each represents a composite from several preparations. This was necessary since only part of a neuron's structure was discernible in any one backfill preparation because of superposition of several stained motoneurons. Dotted lines indicate neuropil areas. Anterior is upward in all figures in the horizontal plane.

one of the other muscles inhibited only by CI, to act as a monitor of CI's activation. CI's axon spike could then be identified as the one correlated one-for-one with inhibitory junctional potentials (IJPs) in this monitor muscle fiber, particularly when, as in Figure 3A, random failures of activation occurred during stimulus trains delivered barely at the threshold voltage for CI's activation.

While the CI axon was repeatedly stimulated, somata of neurons in the desheathed first thoracic ganglion were penetrated successively until that of CI was identified by the attenuated (2–5 mV) antidromic action potential recorded in it, similar in amplitude to such potentials observed in other crustacean motoneuron somata (e.g., Sandeman, '71; Wine and Mistick, '77; Wiens and Atwood, '78). Figure 3B demonstrates coupling among the axon spikes (second trace), antidromic soma potentials (bottom), and monitor muscle IJPs (top) that identify CI. Stimulation of CI's axon in the anterior root elicited a large antidromic soma potential correlated with a CI axon spike (Fig. 3C, left); with the stimulus voltage just below CI's threshold (Fig. 3C, right), some sensory afferents were still activated, causing reflex synaptic depolarization of CI, less abrupt in onset and not accompanied by an axon spike. In many preparations, CI fired spontaneously or in response to tactile stimulation of the claw (Fig. 3D); the impaled CI soma then exhibited attenuated action potentials, preceding the correlated CI axon spikes by a constant interval (10–20 ms, dependent on the size of the animal). Finally, injection of current into the impaled soma could trigger CI axon spikes: Figure 3E shows bursts of action potentials generated during injection of depolarizing current pulses (not shown for the soma because the amplifier was saturated); also, "rebound" spikes often occurred upon release from hyperpolarization (not shown).

Once identified as CI according to the criteria illustrated above, impaled cells were intracellularly stained with Lucifer Yellow, processed for fluorescence microscopy in whole-mount, drawn by use of a camera lucida (Fig. 3F), and photographed in several focal planes to preserve depth information.

Morphology of the CI cell. The description given below is based on six Lucifer-injected CI neurons of *Pacifastacus* chelipeds and on 18 cobalt-backfilled walking leg CIs of *Orconectes* (Figs. 2, 3, 6, and 7). The structures observed were very similar between the two samples despite their segmental and species differences.

CI's soma lies on or just contralateral to the midline of the ganglion near its posterior border and below the ventral surface; the cell's central structure is otherwise confined to the ipsilateral hemiganglion. The primary neurite emerges from the soma in a nearly rostral direction and gently curves laterally and dorsally to cross the lateral margin of the connective almost perpendicularly. It then curves rostrally again toward the anterior root, broadening into a large integrating segment (Sandeman, '69). This narrows to form the efferent axon which bends laterally to enter the anterior root; an axon branch is given off into the thin nerve to the basal musculature at the edge of the ganglion. The neurite gives off about a dozen dendrites at irregular intervals as it traverses the hemiganglion. The first two major dendrites emerging on the anterior side before the neurite broadens are respectively directed strongly ventrally and dorsally. Two further distinctly anterior dendrites are given off from the integrating segment and curve back toward the connective, roughly paralleling the neurite but a few hundred microns anterior and slightly dorsal to it. From the posterior side of the neurite, dendrites fan out across the posterior part of the hemiganglion; one in particular, originating near the beginning of the integrating segment in Figure 3F, extends to the posterior periphery of the ganglion giving off several major branches that angle laterally.

Comparison among the cheliped CI cells of Figures 3, 6, and 7 suggests that some of CI's dendrites may vary from preparation to preparation in the domain each occupies, although their aggregate arborization covers the same overall area in each. For example, the major posterior primary dendrite seen in Figure 3F appears through its extensive secondary and higher order branching to occupy a ganglionic area which, in the cells shown in Figures 6 and 7,

Fig. 3. Identification and central structure of the CI neuron to the right cheliped of *Pacifastacus leniusculus*. The inset shows the general relationships of the axons, nerves, nerve roots, and muscles observed in this study; recording and stimulation sites, and the usual arrangement of simultaneously recorded traces, are also indicated. Muscles: ext, extensor; daf, distal accessory flexor; str, stretcher; bnd, bender; opn, opener; cls, closer. Axons: FCE, SCE, fast and slow closer excitators; FEE, SEE, fast and slow extensor excitators; OE-SE, shared opener-stretcher excitator. **A:** Intracellular (ic) recording from a distal accessory flexor muscle fiber (top trace) and extracellular (ec) recording from an axon in the closer nerve (second trace) upon stimulation of the anterior root with stimulus voltage just at the threshold for CI's intermittent activation. The axon is identified as CI's since its spikes (smaller than the stimulus artifact) are coupled 1:1 with inhibitory junctional potentials (IJPs) in the accessory flexor muscle: some pulses (3rd, 5th; see

stimulus artifacts) fail to elicit either axon spike or IJP. **B:** As **A**, except that the stimulation was above threshold and delivered to the closer nerve. An intracellular recording (third trace) from the putative CI soma has been added, showing antidromic attenuated spikes associated 1:1 with axon spikes and IJPs. The traces shown represent an average over three responses. **C,D:** CI axon (top) and soma (bottom) recordings during (**C**) electrical stimulation of the anterior root with voltage suprathreshold (left) and subthreshold (right) for CI activation (20 sweeps averaged), and (**D**) during tactile stimulation of the claw. **E:** CI axon spikes (top) elicited by depolarizing current pulses (bottom) injected into the soma. **F:** Camera lucida drawing of the Lucifer-injected CI soma identified in **A-E**. Vertical calibration (right-hand bars): ic-2 mV, 100nA; ec-0.5 mV. Trace durations: **A,B,E**, 0.8 seconds; **C**, 40, 60 milliseconds; **D**, 1.6 seconds.

Fig. 4. Identification and central structure of the SI neuron to the right cheliped of *Pacifastacus*. A–C: Recordings from a stretcher muscle fiber (top traces), the stretcher nerve (middle traces), and the impaled putative SI soma (bottom traces). Stimulation was delivered to the closer nerve in A (10 responses averaged), to the posterior root in B, and to the soma in C, where the bottom trace monitors injected current instead of membrane voltage. D: Camera lucida drawing of the SI soma identified in A. Vertical calibration: 0.1 mV, 100 nA. Trace durations: A, 0.1 seconds; B, C, 0.8 seconds.

is shared among their two or three most proximal posterior primary dendrites.

Electrophysiological identification and intracellular staining of the cheliped SI in *Pacifastacus*. As recently shown (Wiens, '91, '93), SI's axon can be reliably distinguished from CI's because it innervates only the stretcher and closer muscles and because it exits the ganglion through the posterior root. Figure 4A–C shows recordings that identify the stained neuron of Figure 4D as SI. Antidromic stimulation of the closer nerve (Fig. 4A) or the posterior root (Fig. 4B) evokes axon spikes in the stretcher nerve (second trace), IJPs in the stretcher muscle (top), and antidromic attenuated spikes in the impaled soma (bottom), all coupled one-for-one. Stimulation of the anterior root fails to elicit the soma potential directly, showing that it is SI and not CI being activated. Depolarizing current injected into the impaled soma (Fig. 4C, bottom trace) triggers an irregular burst of axon spikes accompanied 1:1 by IJPs in stretcher muscle fibers. To confirm that this cell was not an aberrant CI, the actual CI axon was subsequently located in the anterior root and identified by its inhibitory effect on the distal accessory flexor muscle.

Morphology of the stretcher-closer inhibitor, SI. Figure 4D shows a drawing of the SI cell identified in Figure 4A–C, and Figures 6 and 7 show two further cheliped SI

neurons similarly identified and stained. Their central structure is similar to that of the walking leg SI in *Orconectes* as revealed by cobalt/silver staining in Figure 2. The soma of SI is located near the midline, but usually slightly ipsilateral to it and slightly more anterior than CI's soma. SI's neurite heads rostrally and in some cases even slightly medially from the soma before taking a distinctively sharp lateral turn that contrasts with CI's gradual arc. Dendrites are given off only in the ipsilateral hemiganglion, but seem less extensive than CI's. One or two of SI's more distal dendrites form a distinctive arch, leaving the neurite in an anteroventral direction, but curving posteriorly down and under the neurite along the ventral surface of the ganglion. A branch of one of these distal dendrites, like some of CI's, travels back toward the connective parallel to the neurite but anterior and ventral to it. Finally, the neurite deflects posterolaterally to form the axon heading out the posterior root.

Electrophysiological identification and intracellular staining of the cheliped OI in *Procambarus* and *Pacifastacus*. The third inhibitor of the cheliped, the opener inhibitor, has been studied by cobalt injection along with the opener and closer excitors in previous work on *Procambarus* (Wiens, '76). For reference, two OI cells stained in that study are drawn in Figures 5E and 7B. The soma lies on or

Fig. 5. Identification and structure of the cheliped OI soma. A–C: Recordings from cheliped opener muscle fibers (top trace), the OI axon (middle trace), and the impaled putative OI soma (bottom trace) in *Pacifastacus*. In A, OI's axon was stimulated in the opener nerve (16 responses averaged); in B, OI fired spontaneously; in C, depolarizing current (bottom trace) was passed into the soma to trigger axon spikes.

D: Camera lucida drawing showing the location of the Lucifer-stained OI soma identified in A–C, relative to that of SI which had also been identified and injected with Lucifer. E: Drawing of a cobalt sulfide-stained OI cell of *Procambarus clarkii*, identified electrophysiologically (Wiens, '76). Vertical calibration: 2 mV, 50 nA. Trace duration: A, 0.5 seconds; B, C, 0.8 seconds.

just medial to the lateral edge of the connective near the posterior edge of the ganglion, usually posterior to the CI and SI somata. Its neurite runs obliquely to about the middle of the hemiganglion, where it bends laterally and thickens into an integrating segment, giving off two or three major dendrites and a number of smaller ones. As in SI, one large dendrite leaves the integrating segment anteroventrally and then arches in a postero-ventro-medial direction to cross the integrating segment.

The cheliped OI soma of *Pacifastacus* was likewise impaled and identified as outlined in Figure 5A–C in two preparations. The recordings from the opener muscle, opener nerve, and OI soma show coupled muscle IJPs, OI axon spikes, and attenuated OI soma spikes during opener nerve stimulation (Fig. 5A), spontaneous activity (Fig. 5B), or depolarization of the soma by current injection (Fig. 5C). Lucifer Yellow injected into these somata failed to stain the cells' central processes, so their dendritic structure cannot be compared with that in *Procambarus*. However, the positions of the somata in the *Pacifastacus* preparations (Figs. 5D, 7A) were similar to those in *Procambarus* (Figs. 5E, 7B). The fast and slow closer exciters were also impaled

and injected in one *Pacifastacus* preparation; they too are located as in *Procambarus* (not shown).

General sources of synaptic activation of CI, SI, and OI. The somatic impalements made to identify and stain CI and SI yielded some recordings of attenuated synaptic potentials from various sources. Both cells could be depolarized and sometimes orthodromically fired by stimulation of either the anterior or posterior root, indicating that sensory input from the cheliped plays a role in determining their output (e.g., Fig. 3C). Manual stimulation of tactile receptors on the cheliped surface (Fig. 3D) or of proprioceptors in the propodite–dactyl joint could increase firing rates of these cells, and underlying EPSPs were sometimes visible. Some indications of motor–motor synaptic connections involving these inhibitors were also seen at this preliminary stage. In the experiment of Figure 6, for example, current pulses injected into the SI soma influenced CI's ongoing spontaneous firing (not shown): CI spikes occurred during and not between depolarizing pulses, and between but not during hyperpolarizing pulses to SI, even though SI itself did not fire during either treatment at that point in the experiment. The opener inhibitor has previously been

shown to receive synaptic excitation from the cheliped's tactile and proprioceptive afferents and from the opener excitator (Wiens, '76; Wiens and Atwood, '78); recordings (not shown) from the OI cells shown in Figures 5D and 7A confirmed these observations.

Structural comparisons among crayfish limb inhibitors

Intrasegmental comparison of CI and SI. The descriptions above show significant similarities as well as differences between the two midline inhibitors, CI and SI. To eliminate any doubt that these are distinct cells, a direct comparison of the two within the same preparation is shown in Figure 6. CI was electrophysiologically identified and stained first, and then SI was located, identified, and lightly stained. CI, activated from the anterior root or from the closer nerve, produces a distinctive axon spike in the stretcher nerve (Fig. 6A, left), moderately strong IJPs in a stretcher muscle fiber (Fig. 6B), strong IJPs in a distal accessory flexor fiber (Fig. 6C), an antidromic attenuated spike in CI's soma with latency 17 ms from the stimulus pulse delivered in the carpopodite-propodite joint (Fig. 6D), and no antidromic spike in the SI soma (Fig. 6E). SI's activation from the posterior root or the closer nerve, in contrast, produces larger stretcher nerve spikes (Fig. 6A, right) and larger IJPs in the stretcher muscle fiber (Fig. 6B) than does CI, and an attenuated spike in the SI soma about 14 ms after the stimulus in the carpopodite-propodite joint (Fig. 6E); it does not affect CI's soma or the distal accessory flexor muscle (recordings not shown). The drawing of the stained neurons (Fig. 6F) makes clear the difference in soma location and the course of the neurites near the soma. The inadvertent cracking of the ganglion along the midline that occurred during the wholemounting process confirms the respective contralateral and ipsilateral locations of CI's and SI's somata in this preparation, but exaggerates their lateral separation. The staining of SI was not intense enough to reveal dendritic or axonal structure, but the double fill does show an apparent contact between the neurites of the two cells within or near their integrating segments. Preliminary electrophysiological evidence for coupling between these cells (see above) may lend significance to this observation (Sandeman, '71; Burrows and Hoyle, '72).

Comparisons among crayfish species. Figure 7A displays the structures of another set of CI, SI, and OI neurons identified and stained in first thoracic ganglia of *Pacifastacus*. Though staining was not complete for SI or OI, these drawings together with Figures 4, 5, and 7 illustrate the variation of each cell's main structural features and permit further comparison among them. Figure 7B shows a similar compilation obtained by cobalt staining of the cheliped CI, SI, and OI of *Procambarus*. The differences demonstrated between CI and SI with regard to soma position and neurite shape in *Pacifastacus* also hold for *Procambarus*. The superior staining of the *Procambarus* OI cells shown here and in Figure 5E corroborates the soma position of OI in *Pacifastacus*, and serves to complete the picture of the crayfish cheliped inhibitors' central structures.

Comparison between cheliped and walking leg segments. Comparison of the data in Figures 3–7 with those in Figure 2 demonstrates a very close resemblance between the cheliped inhibitors of *Pacifastacus* or *Procambarus* and the walking leg inhibitors of the third species, *Orconectes*. The comparison cannot be critical because of differences in

Fig. 6. Identification and central structures of the CI and SI neurons innervating the right cheliped of the same specimen of *Pacifastacus*. A–E: Recordings from the stretcher nerve (A), the stretcher muscle (B), the distal accessory flexor muscle (C), the CI soma (D), and the SI soma (E) identify the stained CI and SI somata shown in F (see text). F: Camera lucida drawing of the well-stained CI neuron and lightly stained SI neuron identified in A–E. Vertical calibration: 0.5 mV. Trace durations: A, 16 milliseconds; B,C, 1 seconds; D,E, 40 milliseconds. All recordings represent averages over four to eight responses.

Fig. 7. Drawings comparing Lucifer-stained CI, SI, and OI neurons of *Pacifastacus* chelipeds (A) with the same cells, cobalt-stained without intensification, in *Procambarus* (B). The complete dendritic structure is not revealed by the stain in all cases, but the soma positions and neurite shapes (except for OI in A) are discernible.

methodology and species, but the inhibitory motoneurons to these differently utilized but serially homologous thoracic limbs appear very similar in structure.

Neuroanatomy of crayfish thoracic ganglia and the limb inhibitors; comparison with the locust

Reconstruction of *Orconectes*' inhibitory motoneurons in the framework of ganglionic structure. To establish a ganglionic frame of reference for the inhibitors' structures and to facilitate interspecific comparisons, thoracic ganglia of *Orconectes* were examined by serial sectioning and staining with osmium-ethylgallate. This revealed a ganglionic structure (Fig. 8) which is basically similar to that of the abdominal ganglia described previously by Skinner ('85a,b) and Leise et al. ('86). Therefore, this report utilizes Skinner's nomenclature, which in itself implies the existence of strong anatomical parallels in the basic plans of insect and crustacean ventral ganglia, and hence a certain degree of homology. Basic anatomical features of the crustacean inhibitory motoneurons are shown in Figure 8 in the context of ganglionic structure. The locations of the motoneuron somata and the courses of their primary neurites are indicated in thick sections (Fig. 8, inset) reconstructed from a set of serial sections (see Fig. 1). As described above, CI's soma lies on the ganglion midline or just contralateral to it (Fig. 8A). As its primary neurite curves anteriorly and laterally, it enters the lateral neuropil between the ventral intermediate (VIT) and median ventral (MVT) tracts, and runs toward a region dorsal to the ventral lateral tract

(VLT). Arborizations of this neuron occupy about the dorsal lateral half of the neuropil region (as do those of virtually all the other motoneurons—hatched areas). SI has a structure resembling that of CI (Fig. 8B), in particular with regard to the course of its primary neurite. SI's soma, however, is located more anteriorly and always ipsilaterally (the latter feature is obscured in the preparation shown by a crack in the ganglion just lateral to the SI soma). The OI soma shown in Figure 8C is slightly more anterior than in most other preparations, just posterior to CI but more lateral (compare Fig. 9B). The primary neurite of OI also runs anteriorly and laterally, entering the neuropil quite laterally, below the lateral ventral tract (LVT), and continuing toward a region dorsal to the ventral lateral tract. As in the cases of the other inhibitors, branches of OI occupy approximately the dorsal lateral half of the lateral neuropil.

Arrangement and clusters of motoneuron somata. In all 18 backfill preparations used for CI's identification, a characteristic pattern of motoneuron somata was observed (Fig. 9A): a prominent anterior lateral cluster contained between 12 and 14 somata of excitatory motoneurons; a smaller posterior lateral group comprised three or occasionally four cell bodies, of which OI was the most medial; and three somata were located close to the ganglion midline—CI on or slightly contralateral to the midline, SI usually just anterior to CI but ipsilateral, and a third soma located more anteriorly and dorsally. There was some variability but the overall arrangement of motoneuron cell bodies was very similar in all the preparations examined.

Fig. 8. Anatomical features of CI, SI, and OI motoneurons in the third thoracic ganglion of *Orconectes*. From serial sections in the transverse plane, such as shown in Fig. 1, the anatomy of CI (A), SI (B), and OI (C) neurons was reconstructed with reference to the framework provided by longitudinal tracts and neuropil. Thick sections are shown (see **inset** for sampled area), illustrating soma position, trajectory of the primary neurite, and neuropil area occupied by the neurons' branches (hatched areas). Nomenclature after Skinner (1985a): lg, lateral giant axon; mg, medial giant axon; mv, median hemolymph vessel; LDT, lateral dorsal tract; MDT, median dorsal tract; DIT, dorsal intermediate tract; DMT, dorsal median tract; DLT, dorsal lateral tract; VIT, ventral intermediate tract; VMT, ventral median tract; VLT, ventral lateral tract; MVT, median ventral tract; LVT, lateral ventral tract; LN, lateral neuropil. The two main commissural levels—one between LDT/MDT and DIT/DMT, the other between DIT/DMT and VIT/VMT—are also discernible. Dotted lines indicate lateral neuropil area.

The variation is illustrated for the somata of the second walking leg's inhibitory motoneurons in Figure 9B. Among the three inhibitors, the soma position of OI appeared to be the most variable. A few backfills (six in all) were made in other thoracic ganglia, including the cheliped segment. The location of CI's soma and the pattern of motoneuronal cell bodies appeared to be very similar in all these segmental ganglia.

The arrangement of motoneuron somata in thoracic ganglia of *Orconectes* bears a clear resemblance to that in *Locusta* (compare Watson et al., '85). Figure 9C shows the typical result of a backfill of nerve roots 3, 4, and 5 in a locust mesothoracic ganglion. The anterior and posterior lateral clusters of excitatory motoneuron somata are apparent. CI₃ is located near the medial border of the posterior lateral group. The CI₁ and CI₂ somata lie in posterior positions and close to the ganglion midline, with the CI₁ soma usually just contralateral.

Pattern of somata with GABA-like immunoreactivity. Figure 10 shows the distribution of cell bodies with GABA-like immunoreactivity in the thoracic ganglia of *Orconectes* and *Locusta*. In *Orconectes*, most clusters of immunoreactive somata are not clearly demarcated, and immunoreactive cell bodies are mixed with others showing no label. Nevertheless, a rough grouping of immunoreactive somata is possible (compare Mulloney and Hall, '90). The groups are labeled in correspondence with the nomenclature established by Watson ('86) for *Schistocerca*, in order to indicate possible correspondences. The most prominent cluster is located posteriorly on both sides of the ventral midline (MPG). CI and SI somata are clearly associated with this cluster, and OI's cell body, although located more laterally, is usually still near its boundary. The apparently dense packing of somata near the midline, as discernible in the figure, reflects the fact that this cluster extends deep into the ganglion between the neuropil regions of the two hemispheres. In fact, this group of cell bodies often connects the ventral and dorsal surfaces of the ganglion in its more posterior part. The anterior portion of this cluster may correspond to the locust MVG. The posterior and lateral extensions of the cluster may constitute separate groups of immunoreactive cell bodies, a few of which are located on the lateral and dorsal surface of the ganglion. A pair of smaller lateral clusters is associated with the nerve roots (LVG). Most of these somata are located on the ventral surface of the ganglion. Finally, there is a pair of anterior lateral clusters, extending from the ventral cortex around the base of the anterior connectives to the dorsal surface of the ganglion (LAG).

In *Locusta*, most clusters of immunoreactive somata are more clearly delimited than in *Orconectes*. Their arrangement within the ganglion cortex (compare Watson, '86) does show several similarities to the pattern in the crayfish. (It should be borne in mind during such comparisons that the ventrally located neuron cell bodies in *Locusta* form a

Fig. 9. Soma locations of inhibitory motoneurons, compared in *Orconectes limosus* and *Locusta migratoria*. **A:** Camera lucida drawing of a typical backfill preparation in *Orconectes*. **B:** The soma locations of CI (filled), SI (open), and OI (open with schematic nucleus) are superimposed as they were observed in seven backfill preparations subsequently processed for anti-GABA or anti-GAD immunohistochemistry. **C:** Camera lucida drawing of a backfill of nerve roots 3, 4, and 5 (N3, N4, N5) in a locust mesothoracic ganglion. Note the characteristic arrangement of motoneuron clusters, comparable in A and C. Dotted lines indicate neuropil areas.

Fig. 10. Pattern of cell bodies with GABA-like immunoreactivity in thoracic ganglia of *Orconectes limosus* (A) and *Locusta migratoria* (B). Labeling of immunoreactive soma groups follows Watson (1986) in order to indicate possible correspondences between locust and crayfish (LAG, lateral anterior group; LVG, lateral ventral group; MVG, medial ventral group; MPG, medial posterior group; LDG, lateral dorsal group). A: Reconstruction from serial sections of a third thoracic ganglion subjected to anti-GABA immunocytochemistry (see Materials and Methods). Circles indicate positions and approximate sizes of

immunoreactive somata, as projected onto the ganglion's horizontal plane. However, the number of cell bodies shown is not accurate since it was often difficult, in particular for the smaller somata, to establish whether cells appearing in adjacent sections were identical. Dotted lines indicate borders of area occupied by stained somata as viewed from the ventral surface. B: Camera lucida drawing of a wholemount preparation of a mesothoracic ganglion of *Locusta*. Dotted line indicates neuropil area.

continuous layer while in *Orconectes* the cortex of somata has been displaced to the margins of the prominent and bulging neuropil hemispheres.) There is a posterior median cluster with poorly defined posterior lateral boundaries (MPG), merging anteriorly into a dense arrangement of immunoreactive cell bodies on both sides of the midline (MVG). The three common inhibitory motoneurons of the locust are associated with the MPG. Close to the posterior lateral margins of the MPG is a dense group of somata located on the lateral and dorsal surface of the ganglion (LDG). Another pair of smaller clusters is positioned more anteriorly, lateral to the dense median cluster (LVG). It may correspond to the group of somata located near the nerve roots in *Orconectes*. Finally, there is a paired anterior lateral cluster of immunoreactive cell bodies (LAG), lying near the root of nerve 1 and the base of the anterior connective.

DISCUSSION

In this study, anatomical, immunocytochemical, and electrophysiological methods have been used to identify and compare structurally the inhibitory motoneurons of walking legs and chelipeds in three species of crayfish. Differential backfilling (*Orconectes* walking legs) and electrophysiology (*Pacifastacus* and *Procambarus* chelipeds) apparently identify the same cell as CI. Thus, this anatomical technique, used previously to identify the CI of *Carcinus* (Moffett et al., '87), is likely to prove useful in more widespread studies of common inhibition in arthropods. Similarly, the various approaches have yielded consistent descriptions of the opener and stretcher inhibitors, although distinguishing these two must ultimately rely on electrophysiology.

The results have implications both for the physiology of motor control of crustacean limbs and for the general evolutionary relationships of arthropods. First, our findings help to complete a basic picture of the central structures and innervation patterns of the inhibitory motoneurons of crayfish limbs. We find that chelipeds and walking legs have a very similar inhibitory supply, even when compared between different species. This is not a trivial result, since the two limb types are used very differently. Our results also distinguish further between the two inhibitors whose identities and effects have been confused in the past, SI and CI. Operationally useful distinctions between SI and CI (see Fig. 11) include soma position, neurite shape, roots of exit, and peripheral distribution; SI also has a greater conduction velocity and hence axon diameter (compare data for the crab in Homberg et al., '93). Dendritic trees differ in extent and shape to some degree, but show considerable overall similarity. OI's dendritic structure is much more similar to that of the closer exciters (its synergists) than to those of either SI or CI; this is consistent with common synaptic inputs to the closing synergists (Wiens and Gerstein, '75, '76), possibly involving local signal processing in specialized neuropil areas (e.g., Murphey et al., '85). However, SI does also possess the hoop-shaped dendrites characteristic of the three closing synergists (Wiens, '76), and it will be interesting to compare SI's arborization with those of its own synergists, the bender exciters. The crayfish CI also resembles structurally the backfilled common efferent of *Carcinus* identified as CI (Bevengut et al., '83; Moffett and Yox, '86; Moffett et al., '87), particularly in regard to the slightly contralateral

soma position and the axon's exit via the more anterior root.

The new findings have also made it possible to identify SI and CI with two small closer nerve efferents previously reported in *Procambarus* ("CI" and "CX" of Wiens and Gerstein, '75), permitting the interspecific structural comparisons of Figure 7. The new information will now permit intracellular study of SI's and CI's central synaptic outputs and inputs, including a possible reciprocal inhibition between SI and OI, a possible inhibition of the opener excitor by SI (both suggested by Wiens and Gerstein's spike-train cross-correlation studies), and possible interactions between SI and CI (this study). Another open question is how the thoracic limbs' inhibitory supply is related to that of the swimmerets in the abdomen. Davis ('71) identified one common inhibitor of many swimmeret powerstroke muscles. Mulloney and Hall ('90) found a total of five axons with GABA-like immunoreactivity in the two swimmeret nerves, and tentatively inferred that five inhibitory motoneurons serve the swimmeret muscles. It is clear, however, that two or more of these axons could branch from the same neuron (Fig. 3F; see below), or could serve other purposes.

Of more general zoological interest is the comparison (Fig. 11) that our findings permit between the set of three crustacean thoracic limb inhibitors and the corresponding set supplying legs of a more distantly related arthropod group, the neopteran insects. Locusts (Orthoptera; Hale and Burrows, '85) and cockroaches (Dictyoptera; Pearson and Fournier, '73) have three inhibitors per limb, each shared among several muscles. All three are termed common inhibitors (CI₁₋₃), although none is common to all leg muscles as the crustacean CI appears to be. The following considerations suggest that specific homologies exist between the limb inhibitors of the crayfish and the locust, namely of CI with the locust CI₁, SI with CI₂, and OI with CI₃.

First, Figure 11A compares the peripheral distributions of the two sets of inhibitors. In the locust (Hale and Burrows, '85; Watson et al., '85), CI₁'s axon branches within the ganglion and sends branches via nerves 3, 4, and 5a (the more anterior nerve roots) to innervate the extensor of the tibia and seven more proximal functional muscle groups. The crayfish CI also branches within the ganglion and exits via the anterior root to innervate all muscles except, effectively, the most distal opener muscle in the claw. The locust CI₂ and CI₃ are considerably more specific and more distal in their distribution than CI₁; both innervate the flexor of the tibia and the three muscles distal to it, and both exit the ganglion as single axons within the more posterior nerve 5b. Correspondingly, in the crayfish, SI and OI are also more specific than CI, also exit via the more posterior root, and innervate three of the four most distal muscles, with SI innervating a pair of muscles in the two most distal segments.

Comparison of central structures strengthens the case for homology and identifies the putative homologues of OI and SI specifically. Figure 11B (left) summarizes the soma locations and neurite courses found for the three inhibitors to the cheliped of *Pacifastacus* (the inhibitors of *Procambarus* chelipeds and *Orconectes* walking legs are similar). For comparison, the corresponding features of the locust mesothoracic limb inhibitors as described by Watson et al. ('85) are shown on the right. In keeping with the relationships just outlined, the location of the CI₁ soma (posteriorly on the ganglion midline, often contralateral) is similar to that of the crayfish CI. The location of the more distally

Fig. 11. A structural comparison of the inhibitory motoneurons supplying crustacean and insect limbs. **A:** Diagrams comparing the peripheral distributions of the three limb inhibitors to the muscles acting at the various leg joints of the crayfish (Cooke and MacMillan, '83; Wiens, '85, '91) and the locust (Hale and Burrows, '85). Coil symbols represent elastic forces opposing muscles that lack antagonists. The dashed connection of CI to the crayfish opener muscle represents the claw opener's probably vestigial innervation by CI

(although CI inhibits the walking leg's opener muscle effectively). Note that the muscles shown often represent functional muscle groups, each (especially in the locust) comprising several separate muscles. **B:** Maps comparing the envelopes of all identified soma locations and the roots of exit of the three cheliped inhibitors of the crayfish *Pacifastacus* (this study), and of the three mesothoracic limb inhibitors of the locust *Schistocerca* (Watson et al., '85).

projecting midline inhibitor CI₂ closely resembles that of SI in the crayfish, while CI₃'s soma location is more variable and lateral, like OI's. Further, CI₁ and CI₂ resemble each other in their central dendritic structure more than they resemble CI₃, as do CI and SI vis a vis OI in the crayfish.

The anatomy of the three crayfish inhibitory motoneurons in relation to ganglion structure (Fig. 8) is also reminiscent of the locust common inhibitors (Watson et al., '85). This holds true not only for soma positions, but also for the trajectories of the primary neurites (those of CI and CI₁ and of SI and CI₂ entering the neuropil region rather medially, those of OI and CI₃ distinctly more laterally), and for the neuropil area occupied—dorsolateral in the case of all six neurons. In detail, there appear to be some differences if the major tracts labeled in Figure 8 are assumed to

correspond respectively to the homonymous tracts described in the locust ventral ganglia (see above, Skinner, '85a,b; Leise et al., '86; Tyrer & Gregory, '82). The primary neurites of the locust CI₁ and CI₂ enter the neuropil between the median ventral (MVT) and lateral ventral (LVT) tracts, whereas the neurites of CI and SI in *Orconectes* follow a different path (Fig. 8A, B). Also, the integrative neurite segment in CI₃ of the locust is ventral to the ventral lateral tract (VLT) while in OI of *Orconectes* the neurite runs dorsal to this conspicuous structure. However, if the VLT in insects corresponds to the DLT of crustaceans as Skinner ('85a) assumes, this difference may be only an apparent one.

The proposed homology of inhibitory motoneurons fits into a larger pattern of similarity between the crayfish and

locust with respect to central nervous system organization and neuromuscular endowment. The layout of excitatory motoneurons within the ganglion, the clustering of interneuron somata with GABA-like immunoreactivity, and the general structure of the ganglion tracts and neuropil areas all appear to conform to a common general plan (Figs. 9–11; Skinner, '85a,b). Motoneurons to the axial musculature may display specific homologies among crayfish and insects (Mittenthal and Wine, '78). Importantly for comparisons between arthropods, leg musculature in the crayfish and the locust corresponds closely (Snodgrass, '29), with an unpaired reductor muscle occurring at corresponding proximal joints in both groups (Fig. 11A). The main differences in musculature are the presence of three (rather than one) pairs of muscles at the more maneuverable thoracicocoxal joint in the locust, and of a distal opener muscle opposing the claw closer muscle in the crayfish, in place of mainly elastic forces opposing the locust's claw retractor muscle (Radnikow and Bässler, '91). It seems significant that it is this crustacean opener muscle, lacking an insect homologue, that shares its single excitatory axon with the proximally neighboring stretcher muscle, thus necessitating specific inhibition of these two muscles by OI and SI.

Another intriguing neuroanatomical oddity shared by crayfish and locust limbs occurs in the excitatory innervation of their main extensor muscles, namely, the tibial extensor of the locust pro- and mesothoracic legs and the carpopodite extensor of the crayfish limbs. In both taxa, these probably homologous muscles (Snodgrass, '29) receive two excitatory motoneurons, one fast and one slow. However, rather than emerging from the ganglion together via the same root as such axon pairs do for other muscles with dual excitatory innervation (e.g., Burrows and Hoyle, '72; Wiens, '76), the fast and slow extensor exciters in both groups exit separately through the anterior and posterior roots respectively before converging on their target muscle (Burrows and Hoyle, '72; Wilson and Hoyle, '78; Wiens, '93; Figure 3, inset, compare closer and extensor exciters). Since this unusual axon routing has no functional importance or selective value in itself, its correspondence in the crayfish and the locust is not likely the result of convergent evolution, and therefore has particular significance for the phylogenetic relationship of the crustaceans and insects. The same can be said of other correspondences noted above in soma positions, in the association of cell bodies into particular clusters, and in the occurrence of exactly three, similarly arranged limb inhibitory motoneurons. The last-mentioned seems particularly significant in view of the apparent redundancy of the locust CI₂ and CI₃, which innervate the same muscles and even the same fibers within a given muscle (e.g., Theophilidis and Burns, '83) and are activated similarly during walking (Wolf, '90); put simply, three neurons are being used in the locust to do the work of two.

Although the detailed comparisons we have drawn are restricted to locusts and crayfish and cannot by themselves support general phylogenetic conclusions, they are consistent with observations in other insect and crustacean taxa. The cockroach *Periplaneta* (Order Dictyoptera) possesses three limb inhibitors laid out centrally much as in the orthopteran locust (Pearson and Fournier, '73) although somewhat modified in their peripheral output (all three inhibiting coxal depressor muscles, for example see Pearson and Iles, '71). Among other crustaceans, Homberg et al. ('93) described a repeated grouping of three neurons with

GABA-like immunoreactivity in the fused thoracic ganglion of *Eriphia* (Brachyura). Peripheral limb innervation by exactly three inhibitors is well documented in *Anomura* and *Palinura* (Wiersma and Ripley, '52) and *Brachyura* (Wiens and Rathmayer, '85), as well as *Astacura* (Wiens, '85, '90).

The foregoing suggests that the three limb inhibitors may represent a symplesiomorphic character set (Hennig, '66) shared by crustaceans and insects. We propose that this set was inherited as a synapomorphy from a common arthropod ancestor of both groups, together with the articulated leg and its distinctive musculature (Fig. 11), which these inhibitors would have evolved to serve. This inherited synapomorphy underwent differing transformations under differing evolutionary pressures, resulting, for example, in redundant utilization of the two distal locust inhibitors. This mechanism explains the neuromuscular coincidences and variations adduced in the preceding paragraphs. Recent cladistic analysis of 12S ribosomal RNA sequences indeed identifies "a monophyletic crustacean plus hexapod assemblage," and finds in a broader perspective that "arthropods include onychophorans and are monophyletic relative to annelids and mollusks" (Ballard et al., '92). These findings are fully consistent with our data and with one classical view (Snodgrass, '29; Lauterbach, '73, '80). They also raise the possibility that this basic pattern of limb inhibition may have evolved early and may occur in some variation among all arthropods. Studies in this regard on Chelicerata, Pycnogonida, Onychophora, and Myriapoda would thus be of obvious interest.

Our results and the RNA sequence data are difficult to reconcile with the opposing view that crustaceans and insects evolved their arthropod characteristics convergently from separate non-arthropod ancestries (e.g., Manton, '77; see review in Willmer, '90). A key argument for this view, namely that the biramous appendages of crustaceans set them fundamentally apart from the putatively monophyletic Uniramia (insects and myriapods), has lost force recently. Fossil evidence (Emerson and Schram, '90) suggests that biramous appendages could have evolved from uniramous ones by fusion of adjacent body segments and of the two adjacent limbs thus brought together. Lake's ('90) 18S ribosomal RNA sequence comparisons are consistent with such a progression. The phylogenetic validity of the Uniramian assemblage is also in doubt: the 12S rRNA analyses of Ballard et al. ('92) identify the uniramous myriapods as the oldest arthropod branch, widely separated from the uniramous insects; further, fossil evidence (Kukalova-Peck, '92) suggests that modern insect uniramy may itself be derived from a more primitive polyramy among ancestral insects (see Shear, '92). Thus, new insights from molecular biology, paleontology, and neurology all support a close relationship of the insects and crustaceans among the arthropods.

In conclusion, our results suggest a detailed homology of the three limb inhibitors of the crayfish with those of the locust. Together with other structural, physiological, and molecular similarities, this supports the hypothesis that crustaceans and insects evolved from a common ancestor that possessed jointed limbs innervated by three inhibitory motoneurons with distinctive structures and outputs. This hypothesis predicts a widespread distribution of the shared features demonstrated in this study, among at least crustaceans and insects, and perhaps all arthropods. Our understanding of arthropod evolution might well be advanced by a broadened comparative study of neuromuscular inhibi-

tion and excitation among the arthropods and related groups.

ACKNOWLEDGMENTS

This work was supported by a grant from NSERC (Canada) to T.J. Wiens, by a Heisenberg fellowship to H. Wolf, and by the Deutsche Forschungsgemeinschaft (SFB 156). We thank W. Rathmayer, U. Homberg, W.J. Heitler, R.M. Pitman, K.T. Sillar, and A.J. Simmers for valuable discussions during the course of the experiments. We are also grateful to Ch. Dittrich for excellent technical assistance, particularly with immunocytochemistry. S. Bartmann and Ch. Musal helped with the initial backfill experiments in *Orconectes*. T.J.W. wishes to thank Dr. W.J. Heitler and the Gatty Marine Laboratory for their generous provision of facilities.

LITERATURE CITED

- Altman, J.S., and N.M. Tyrer (1980) Filling selected neurons with cobalt through cut axons. In N.J. Strausfeld and T.A. Miller (eds): *Neuroanatomical Techniques*. Berlin: Springer, pp. 373-402.
- Atwood, H.L. (1965) Excitation and inhibition in crab muscle fibres. *Comp. Biochem. Physiol.* 16:409-426.
- Bacon, J.P., and J.S. Altman (1977) A silver intensification method for cobalt-filled neurones in wholemount preparations. *Brain Res.* 138:359-363.
- Ballantyne, D., and W. Rathmayer (1981) On the function of the common inhibitory neurone in the walking legs of the crab, *Eriphia spinifrons*. *J. Comp. Physiol.* 143:111-122.
- Ballard, J.W.O., G.J. Olsen, D.P. Faith, W.A. Odgers, D.M. Rowell, and P.W. Atkinson (1992) Evidence from 12S ribosomal RNA sequences that onychophorans are modified arthropods. *Science* 258:1345-1348.
- Bevengut, M., and F. Clarac (1991) Spiking activity of the common inhibitory motoneurone in crustacean leg. Synaptic connections with excitatory motoneurons and sensory afferences. *Soc. Neurosci. Abstr.* 17:1389 (Abstract).
- Bevengut, M., A.J. Simmers, and F. Clarac (1983) Central neuronal projections and neuromuscular organization of the basal region of the shore crab leg. *J. Comp. Neurol.* 221:185-198.
- Burrows, M., and G. Hoyle (1972) Neural mechanisms underlying behavior in the locust *Schistocerca gregaria*. III. Topography of limb motoneurons in the metathoracic ganglion. *J. Neurobiol.* 4:167-186.
- Bush, B.M.H. (1962) A comparative study of certain limb reflexes in decapod crustaceans. *Comp. Biochem. Physiol.* 10:273-290.
- Cooke, I.R.C., and D.L. MacMillan (1983) The common inhibitory innervation of the pereopods of the crayfish *Cherax destructor*. *J. Exp. Zool.* 225:513-517.
- Davis, W.J. (1971) Functional significance of motoneuron size and soma position in swimmeret system of the lobster. *J. Neurophysiol.* 34:274-288.
- Emerson, M.J., and F.R. Schram (1990) The origin of crustacean biramous appendages and the evolution of Arthropoda. *Science* 250:667-669.
- Erxleben, C. (1989) Stretch-activated current through single ion channels in the abdominal stretch receptor organ of the crayfish. *J. Gen. Physiol.* 94:1071-1083.
- Hale, J.P., and M. Burrows (1985) Innervation patterns of inhibitory motor neurones in the thorax of the locust. *J. Exp. Biol.* 117:401-413.
- Heitler, W.J., and K. Fraser (1989) Thoracic output of crayfish giant fibres. I. Pereiopod promotor motor neurones. *J. Comp. Physiol.* 166:117-124.
- Hennig, W. (1966) *Phylogenetic Systematics*. Urbana: University of Illinois Press.
- Homberg, U., A. Bleick, and W. Rathmayer ('93) Immunocytochemistry of GABA and glutamic acid decarboxylase in the thoracic ganglion of the crab *Eriphia spinifrons*. *Cell Tissue Res.* 271:279-288.
- Honegger, H.-W., B. Brunniger, P. Bräunig, and K. Elekes (1990) GABA-like immunoreactivity in a common inhibitory neuron of the antennal motor system of crickets. *Cell Tissue Res.* 260:349-354.
- Hoskins, S.G., U. Homberg, T.G. Kingan, T.A. Christensen, and J.G. Hildebrand (1986) Immunocytochemistry of GABA in the antennal lobes of the sphinx moth *Manduca sexta*. *Cell Tissue Res.* 244:243-252.
- Hsu, S.M., L. Raine, and H. Fanger (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques. A comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.* 29:577-580.
- Kukalova-Peck, J. (1992) The "Uniramia" do not exist: The ground plan of the Pterygota as revealed by Permian Diaphanopteroidea from Russia (Insecta: Paleodictyopteroidea). *Can. J. Zool.* 70:236-255.
- Lake, J.A. (1990) Origin of the Metazoa. *Proc. Nat. Acad. Sci.* 87:763-766.
- Lauterbach, K.-E. (1973) Schlüsselereignisse in der Evolution der Stammgruppe der Euarthropoda. *Zool. Anz. N.F.* 19:251-299.
- Lauterbach, K.-E. (1980) Schlüsselereignisse in der Evolution des Grundbauplans der Mandibulata (Arthropoda). *Abh. Naturwiss. Ver. Hamburg. (N.F.)* 23:105-161.
- Leise, E.M., W.M. Hall, and B. Mulloney (1986) Functional organization of crayfish abdominal ganglia: I. The flexor systems. *J. Comp. Neurol.* 253:25-45.
- Lindsey, B.G., and G.L. Gerstein (1979) Interactions among an ensemble of chordotonal organ receptors and motor neurons of the crayfish claw. *J. Neurophysiol.* 42:383-399.
- Manton, S.M. (1977) *The Arthropoda*. Oxford: Oxford University Press.
- Mittenthal, J.E., and J.J. Wine (1978) Segmental homology and variation in flexor motoneurons of the crayfish abdomen. *J. Comp. Neurol.* 177:311-334.
- Moffett, S.B., and D.P. Yox (1986) The common inhibitor innervates muscles proximal to the autotomy fracture plane in *Carcinus maenas*. *Brain Res.* 366:388-391.
- Moffet, S.B., D.P. Yox, L.B. Kahan, and R.L. Ridgway (1987) Innervation of the anterior and posterior levator muscles of the fifth leg of the crab, *Carcinus maenas*. *J. Exp. Biol.* 127:229-248.
- Mulloney, B., and W.M. Hall (1990) GABA-ergic neurons in the crayfish nervous system: An immunocytochemical census of the segmental ganglia and stomatogastric system. *J. Comp. Neurol.* 291:383-394.
- Murphey, R.K., J.P. Bacon, and S.E. Johnson (1985) Ectopic neurons and the organization of insect sensory systems. *J. Comp. Physiol. A.* 156:381-389.
- Obermayer, M., and N.J. Strausfeld (1980) Silver-staining cobalt sulfide deposits within neurons of intact ganglia. In N.J. Strausfeld and T.A. Miller (eds): *Neuroanatomical Techniques*. Berlin: Springer, pp. 403-427.
- Oertel, W.H., D.E. Schmechel, M.L. Tappaz, and I.J. Kopin (1981) Production of a specific antiserum to rat brain glutamic acid decarboxylase by injection of an antigen-antibody complex. *Neurosci.* 6:2689-2700.
- Oertel, W.H., D.E. Schmechel, and E. Mugnani (1983) Glutamic acid decarboxylase (GAD): Purification, antiserum production, immunocytochemistry. In J.L. Barker and J.F. McKelvy (eds): *Current Methods in Cellular Neurobiology*, Vol. 1. Anatomical techniques. New York: Wiley, pp. 63-109.
- Pearson, K.G., and C.R. Fournier (1973) Identification of the somata of common inhibitory motoneurons in the metathoracic ganglion of the cockroach. *Can. J. Zool.* 51:859-866.
- Pearson, K.G., and J.F. Iles (1971) Innervation of coxal depressor muscles in the cockroach, *Periplaneta americana*. *J. Exp. Biol.* 54:215-232.
- Quicke, D.L.J., and R.C. Brace (1979) Differential staining of cobalt- and nickel-filled neurones using rubeanic acid. *J. Microsc.* 115:161.
- Radnikow, G., and U. Bässler (1991) Function of a muscle whose apodeme travels through a joint moved by other muscles: Why the retractor unguis muscle in stick insects is tripartite and has no antagonist. *J. Exp. Biol.* 157:87-99.
- Sakai, M., and T. Yamaguchi (1983) Differential staining of insect neurons with nickel and cobalt. *J. Insect Physiol.* 29:393-397.
- Sandeman, D. (1969) Integrative properties of a motoneuron in the brain of the crab *Carcinus*. *Z. Vergl. Physiol.* 64:450-464.
- Sandeman, D. (1971) The excitation and electrical coupling of four identified motoneurons in the brain of the Australian mud crab, *Scylla serrata*. *Z. Vergl. Physiol.* 72:111-130.
- Shear, W.A. (1992) End of the 'Uniramia' taxon. *Nature* 359:477-478.
- Skinner, K. (1985a) The structure of the fourth abdominal ganglion of the crayfish, *Procambarus clarkii* (Girard). I. Tracts in the ganglionic core. *J. Comp. Neurol.* 234:168-181.
- Skinner, K. (1985b) The structure of the fourth abdominal ganglion of the crayfish, *Procambarus clarkii* (Girard). II. Synaptic neuropils. *J. Comp. Neurol.* 234:182-191.
- Skorupski, P., and K.T. Sillar (1988) Central synaptic coupling of walking leg motor neurones in the crayfish: Implications for sensorimotor integration. *J. Exp. Biol.* 140:355-379.

- Smith, D.O. (1972) Central nervous control of presynaptic inhibition in the crayfish claw. *J. Neurophysiol.* 35:333-343.
- Snodgrass, R.E. (1929) The thoracic mechanism of a grasshopper, and its antecedents. *Smithsonian Misc. Coll.* 82:1-111.
- Sternberger, L.A. (1979) *Immunocytochemistry*. New York: Wiley.
- Stewart, W.W. (1978) Functional connections between cells as revealed by dye-coupling with a highly fluorescent naphthalimide tracer. *Cell* 14:741-749.
- Theophilidis, G., and M.D. Burns (1983) The innervation of the mesothoracic flexor tibiae muscle of the locust. *J. Exp. Biol.* 105:373-388.
- Tyrer, N.M., and G.E. Gregory (1982) A guide to the neuroanatomy of the locust subesophageal and thoracic ganglia. *Philos. Trans. R. Soc. Lond. (Biol.)* 297:91-123.
- Van Harreveld, A. (1936) A physiological solution for freshwater crustaceans. *Proc. Soc. Exp. Biol. Med.* 34:428-432.
- Watson, A.H.D. (1986) The distribution of GABA-like immunoreactivity in the thoracic nervous system of the locust *Schistocerca gregaria*. *Cell Tissue Res.* 246:331-341.
- Watson, A.H.D., M. Burrows, and J.P. Hale (1985) The morphology and ultrastructure of common inhibitory motor neurones in the thorax of the locust. *J. Comp. Neurol.* 239:341-359.
- Wiens, T.J. (1976) Electrical and structural properties of crayfish claw motoneurons in an isolated claw-ganglion preparation. *J. Comp. Physiol.* 112:213-233.
- Wiens, T.J. (1985) Triple innervation of the crayfish opener muscle: the astacuran common inhibitor. *J. Neurobiol.* 16:183-191.
- Wiens, T.J. (1989) Common and specific inhibition in leg muscles of decapods: sharpened distinctions. *J. Neurobiol.* 20:458-469.
- Wiens, T.J. (1990) The inhibitory innervation of the walking leg of the lobster *Homarus americanus*. *J. Comp. Physiol.* 167:43-50.
- Wiens, T.J. (1991) Dual inhibition of the crayfish's closer muscle by the stretcher and common inhibitors. *Soc. Neurosci. Abstr.* 17:1389 (Abstract).
- Wiens, T.J. (1993) The closer muscle is a second target for the stretcher inhibitor motoneuron of the crayfish's thoracic limbs. *J. Comp. Physiol.* (in press).
- Wiens, T.J., and H.L. Atwood (1978) Motoneuron interactions in crayfish claw control: Evidence from intracellular recording. *J. Comp. Physiol.* 124:237-247.
- Wiens, T.J., and G.L. Gerstein (1975) Cross connections among crayfish claw efferents. *J. Neurophysiol.* 38:909-921.
- Wiens, T.J., and G.L. Gerstein (1976) Reflex pathways of the crayfish claw. *J. Comp. Physiol.* 107:309-326.
- Wiens, T.J., and W. Rathmayer (1985) The distribution of the common inhibitory neuron in brachyuran limb musculature. I. Target muscles. *J. Comp. Physiol.* 156:305-313.
- Wiersma, C.A.G. and S.H. Ripley (1952) Innervation patterns of crustacean limbs. *Physiol. Comp. Oecol.* 2:391-405.
- Wigglesworth, V.B. (1959) The histology of the nervous system of an insect, *Rhodnius prolixus* (Hemiptera). II. The central ganglia. *Q. J. Microsc. Sci.* 100:299-313.
- Willmer, P. (1990) Arthropod phylogeny. In *Invertebrate Relationships*. Cambridge: Cambridge University Press, pp. 271-299.
- Wilson, J.A., and G. Hoyle (1978) Serially homologous neurones as concomitants of functional specialization. *Nature* 274:377-379.
- Wine, J.J., and D.C. Mistick (1977) Temporal organization of crayfish escape behavior: Delayed recruitment of peripheral inhibition. *J. Neurophysiol.* 40:904-925.
- Wolf, H. (1990) Activity patterns of inhibitory motoneurons and their impact on leg movement in tethered walking locusts. *J. Exp. Biol.* 152:281-304.
- Wolf, H. (1992) Identification of inhibitory motoneurons in crustacean thoracic ganglia and homology with insect common inhibitors. *Verh. Dtsch. Zool. Ges.* 85:229 (Abstract).