Five types of nonspiking interneurons in local pattern-generating circuits of the crayfish swimmeret system

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Smarandache-Wellmann C, Weller C, Wright TM Jr, Mulloney B. Five types of nonspiking interneurons in local pattern-generating circuits of the crayfish swimmeret system. J Neurophysiol 110: 344–357, 2013. First published April 24, 2013; doi:10.1152/jn.00079.2013.—We conducted a quantitative analysis of the different nonspiking interneurons in the local pattern-generating circuits of the crayfish swimmeret system. Within each local circuit, these interneurons control the firing of the power-stroke and return-stroke motor neurons that drive swimmeret movements. Fifty-four of these interneurons were identified during physiological experiments with sharp microelectrodes and filled with dextran Texas red. Neurobiotin, or both. Five types of neurons were identified on the basis of combinations of physiological and anatomical characteristics. Anatomical categories were based on 16 anatomical parameters measured from stacks of confocal images obtained from each neuron. The results support the recognition of two functional classes: inhibitors of power stroke (IPS) and inhibitors of return stroke (IRS). The IPS class of interneuron has three morphological types with similar physiological properties. The IRS class has two morphological types with physiological properties and anatomical features different from the IPS neurons but similar within the class. Three of these five types have not been previously identified. Reviewing the evidence for dye coupling within each type, we conclude that each type of IPS neuron and one type of IRS neuron occur as a single copy in each local pattern-generating circuit. The last IRS type includes neurons that might occur as a dye-coupled pair in each local circuit. Recognition of these different interneurons in the swimmeret pattern-generating circuits leads to a refined model of the local pattern-generating circuit that includes synaptic connections that encode and decode information required for intersegmental coordination of swimmeret movements.

graded transmission; motor pattern generation; nonspiking neurons; local circuit; quantitative morphology

ALL OVERT BEHAVIOR RESULTS from movements controlled by coordinated firing of motor neurons, and explaining the neural control of behavior requires explaining this motor coordination in neural terms. In segmented animals like vertebrates and arthropods, causal explanations of this coordinated firing necessarily require knowledge of the structure and distribution of segmental premotor circuits that control different groups of motor neurons. The interneurons that compose these segmental circuits and the intersegmental projection neurons that coordinate them must also be identified and included in the explanation.

One motor system whose intersegmental coordinating circuits have been described in cellular detail is the crayfish swimmeret system (Smarandache et al. 2009), a chain of local neural modules distributed in different body segments that control precisely coordinated periodic cycles of limb movements (Mulloney and Smarandache-Wellmann 2012). The kernel of each of these modules is composed of nonspiking local interneurons that organize periodic alternating bursts of spikes in different pools of motor neurons (Heitler and Pearson 1980; Mulloney 2003; Sherff and Mulloney 1996). Unlike the local commissural neurons that integrate information from other segments, these axonless interneurons have dendritic arbors restricted to just one side of their ganglion. Recently, we have observed how projection neurons and commissural neurons in the intersegmental coordinating circuit encode and decode information (Smarandache-Wellmann, Weller, Mulloney, unpublished observations). During this research, we realized that previous descriptions of the unilateral nonspiking interneurons in these local pattern-generating circuits were incomplete and needed to be revisited with new methods in order to complete the description of the intersegmental circuit that coordinates them. Here we expand upon previous descriptions of these nonspiking neurons (Heitler 1981; Heitler and Pearson 1980; Paul and Mulloney 1985a, 1985b) and lay the groundwork for new models of their synaptic organization. We used a combination of electrophysiology and confocal microscopy to study 54 individual interneurons recorded in preparations that were continuously producing coordinated motor output from the interneuron’s local circuit. We tested each neuron’s influence on this output and used differences in these influences as the basis for identifying two separate functional classes. For each neuron, we measured 16 morphological parameters and used these parameters to test alternative classifications of anatomical types within each functional class. We describe five types of unilateral local neurons, three of which are previously unidentified. We think all five types play roles in organizing the motor output from each local circuit. From consideration of dye coupling evident in neurons filled with Neurobiotin or Lucifer yellow (Mills and Massey 2000; Stewart 1978), we conclude that four of these five kinds probably occur as single copies in each local circuit.

Glossary

D-V depth Full length of the cell along the dorso-ventral axis of the ganglion (µm)

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Morphological parameters in bold in Glossary differed significantly in one or more comparisons of different types of neurons. See Tables 1 and 2.
experiments, microelectrodes contained a solution of 1 M KCl and either 1% dextran Texas red (dTR; dextran Texas red mol wt 3,000 lysine fixable, Life Technologies, Grand Island, NY) or 5% Neurobiotin (NB; Vector Labs, Burlingame CA). These electrodes had tip resistances of 30–50 MΩ. In one experiment, the microelectrode contained 1% dTR + 5% NB. In a second experiment, it contained 1% dextran-FITC (dFITC; biotinylated dextran FITC mol wt 10,000 fixable, SP-1130, Vector Labs) + 5% NB. Neurons were filled iontophoretically with +1.0 nA current steps 0.25 s long at 2 Hz for 20 min, with either bridge circuits or discontinuous current clamp.

Extracellular and intracellular recordings were digitized with an Axon Instruments Digidata 1322A or 1440A digitizer and pCLAMP software (Molecular Devices, Union City, CA) and saved as computer files for later analysis.

Isolated ventral nerve cord preparations sometimes express spontaneously the normal swimmeret motor pattern. In other preparations that were either intermittently active or silent, a 1.5–3 μM solution of the cholinergic agonist carbachol (Sigma) in crayfish saline was perfused over the preparation to elicit continuous expression of a stable motor output (Mulloney 1997).

Confocal Microscopy and Imaging Procedures

At the end of each experiment, the preparation was fixed overnight in 4% paraformaldehyde. Those preparations that contained neurons filled with NB were then washed three times for 10 min in 0.1 M glycine, dehydrated, and then rehydrated in an ascending and descending ethanol series (25%, 50%, 70%, 80%, 95%, and 100%). This was followed by six 30-min washes in 0.3% Triton X-100 in phosphate-buffered saline (PBS; Sigma) and an overnight incubation in 1:250 streptavidin conjugated to Alexa Fluor 488 or to Alexa Fluor 568 (Life Technologies) in 0.3% Triton in PBS on the dark side of a rotator at 2–4°C. Both dTR and NB preparations were then rinsed with PBS four times for 10 min each, pinned out dorsal-side up, and cleared in an ascending ethanol series to methyl salicylate. Cleared whole mounts were mounted in methyl salicylate in a Permanox dish with a coverslip base. A small, thick slip of glass was placed on top of the nerve cord to prevent movement during imaging.

Both dTR and NB preparations were examined as whole mounts oriented for frontal view, dorsal side up (Fig. 1A). The structure of each labeled neuron was captured as a stack of confocal images that extended from the most dorsal to the most ventral part of the cell. Images were captured with an Olympus Fluoview 300 confocal microscope (Olympus America, Center Valley, PA) equipped with a krypton (488 nm) and argon (568 nm) lasers and an Olympus 20x NA UPlanApo lens. Step size was 0.75 μm. The images were converted to 24-bit TIF images in Fluoview software, where the gamma and intensity were adjusted to enhance the background intensity. The resulting images were then transferred to Adobe Photoshop for further adjustment of brightness, contrast, and sharpness. All images in each stack were adjusted uniformly in the same way. To facilitate comparisons, images of neurons that had been filled in a ganglion’s left side were flipped so that all images in this report appear on the right side of the ganglia. This reversal did not bias the measurements of morphological features of individual neurons.

The core of each segmental ganglion that innervates swimmerets has an orderly array of tracts and commissures (Skinner 1985a) that can be used as a context to describe a neuron’s position (Fig. 1C). For this report, the anterior ventral commissure (AVC), the dorsal commissures (DC1–4, DC5–7), the ventral tracts (VTs; VIT, VMT), and the first layer of dorsal tracts (DIT, DMT) proved particularly useful and have been used to anchor the depth-coded images.

Depth-Coding Confocal z Stacks

To illustrate the vertical projection of individual neurons within the ganglion, we used ImageJ and a set of plug-ins provided by the McMaster Biophotonics Facility (McMaster University, Hamilton, ON, Canada) to create depth-coded images of the stack of confocal images. Each stack was opened in FluoView, and the ventral and dorsal limits of the filled neuron were determined. In every case, the AVC was situated ventral to the neuron’s ventral limit, and DC1–4 were dorsal to its upper limit. The stacks were then opened in ImageJ,
Measurements of Neuronal Anatomy

To measure specific dimensions of these neurons, we imposed a hemispherical coordinate system (Fig. 1A) on the stack of confocal images. In this coordinate system, the longitudinal midline of the ganglion formed the y-axis (Fig. 1B) and an orthogonal line from the y-axis precisely through the center of N1 formed the x-axis (Fig. 1D). In instances where N1 had been bent during fixation, the base of N1 served as the point of reference. These coordinates establish an x-y plane quite ventral in each ganglion at the level of the AVC (Fig. 1C), a reliable landmark (Skinner 1985a). A perpendicular line through the 0,0 point of the x, y plane then formed the z-axis (Fig. 1D) and defined the x, y, z origin (0,0,0). These axes were scaled in micrometers. In this system (Fig. 1A), both y and z values could be positive or negative but x values only positive because none of these interneurons crossed the midline of the ganglion.

To map the positions of a neuron’s features within this coordinate system, the distance from the origin to the feature was measured with ImageJ (imagej.nih.gov/ij/). ImageJ measurements are made in pixels, which we converted to micrometers. Depth of an object along the z-axis was calculated from the number of optical sections between the origin and the object. The radial distance from the origin to the feature, \( r \), was then calculated as \( r^2 = x^2 + y^2 + z^2 \). The polar angle between the radial vector and the z-axis, \( \theta \), was calculated as \( \theta = \arccos(z/r) \). The azimuthal angle between the radial vector and the x-axis, \( \varphi \), was calculated as \( \varphi = \arctan(y/x) \).
(Fig. 1A). This conversion from Cartesian to spherical coordinates allowed us to describe the positions of different parts of each neuron in three dimensions. It also simplified later analysis of variance (ANOVA) because distances and angles were expressed as single numbers, not as $x$, $y$ pairs.

**Statistics and Analysis of Variance**

Sixteen morphological parameters of each neuron were calculated from the ImageJ data; see Glossary and Fig. 1A. Descriptive statistics of these parameters for each type of neuron were calculated with SigmaPlot 11.2 (Systat, San Jose CA) and are given here as means ± standard deviations. To test the probability that a measured parameter was not really different in different types of neurons, we calculated single-factor ANOVA, using the routines in SigmaPlot. Probabilities ($P$) < 0.05 that a parameter measured in different types of neurons came from the same population were taken to be significantly different. For those parameters where $P$ < 0.05, we used Holm-Sidak post hoc multiple pairwise comparisons to attribute significant differences to particular pairs of types of neurons because the Holm-Sidak procedure is more likely to identify differences where they occur than other commonly used post hoc tests.

**Generating Phase-Response Curves**

The effects of perturbations of an individual neuron’s membrane potential on the timing of the system’s periodic output were mapped by injecting pulses of current 250 ms long at low frequencies, <0.1 Hz, with discontinuous current clamp during continuous production of the swimmeret motor pattern. Since the periods of these motor patterns ranged between 0.5 and 1 s (Mulloney et al. 2006) and the timing of these pulses was independent of the motor output, individual pulses occurred at different points in the cycle. We used power-stroke excitor (PSE) bursts from the module in which we perturbed the interneuron to monitor each cycle’s period (Mulloney et al. 2006). Period was defined as the time from the start of one PSE burst to the start of the next burst. To describe changes in periods caused by current pulses, we measured the periods of the four cycles that immediately preceded the start of the pulse, the period of the cycle in which the pulse occurred, and the time lag between the start of that cycle and the start of the pulse. The phase of the pulse was calculated as the ratio of that time lag to the period of that cycle and could range between 0 and 1. For current pulse $i$ in cycle $j$, the mean of the four periods preceding $j$, $X_{ij}$, made a good predictor of the expected period following the start of the pulse. For each pulse, we calculated the phase difference, $Df_{ij}$, between each experimental period, $X_{ij}$, and the mean period just preceding it, $X_{i-4j}$, as $Df_{ij} = (X_{ij} - X_{i-4j})$. Plotting these $Df_{ij}$ as functions of the phase of the pulse gives the phase-response curve (PRC). We fitted a sine curve to each PRC, using the nonlinear regression function in SigmaPlot.

**RESULTS**

The swimmeret system is modular in the sense that each swimmeret is controlled by its own set of ~70 motor neurons, its own sensory neurons, and a local pattern-generating circuit that organizes the firing of these motor neurons (Murchison et al. 1993). During forward swimming, each swimmeret moves through cycles of PS and RS that generate forward thrust. These movements are driven by bursts of spikes in four sets of motor neurons: power-stroke excitors (PSE), return-stroke exciters (RSE), power-stroke inhibitors (PSI), and return-stroke inhibitors (RSI) (Davis 1968b, 1971; Mulloney and Hall 1990, 2000).

Each module of the swimmeret system includes a set of unilateral nonspiking local interneurons that are components of the local pattern-generating circuit (Heitler and Pearson 1980; Mulloney 2003; Paul and Mulloney 1985a, 1985b). These monopolar interneurons occur as bilateral pairs, one in each of the two modules in each ganglion. Each neuron has an arbor of branches that is largely restricted to one of the ganglion’s pair of LNs (Skinner 1985b) (Fig. 1A).

The effects of depolarizing individual nonspiking local neurons fall into two distinct groups: inhibitor of power stroke (IPS) neurons inhibit PS motor output, while inhibitor of return stroke (IRS) neurons inhibit RS motor output. This distinction is also clearly evident in the PRCs of IPS and IRS neurons (Fig. 2). Depolarizing perturbations of IPS neurons that occur when the PS neurons are firing lengthen the period, a phase delay, but those that occur later in the cycle shorten the period. The opposite is true of IRS neurons: perturbations during the PS burst shorten the period, a phase advance, but later perturbations lengthen it. To classify and describe these pattern-generating neurons more thoroughly, we first separated them into two groups, using these functional characteristics.

**Three Types of IPS Neurons**

All IPS neurons have cell bodies located in the ventral part of their ganglion’s posterior-lateral quadrant, between nerves N1 and N2 (Figs. 3A, 4A, 5A). A thin neurite extends anteriorly from the cell body into the LN. Experimental depolarization of an IPS neuron inhibits the population of PSE motor neurons in the same module (Figs. 3B, 4B, 5B). In some quiet preparations, slight hyperpolarization of an IPS neuron would start rhythmic motor output in that segment. Critical comparison of the structures of 26 IPS neurons revealed three types that differed in the anatomy of their arbors within the LN.

IPS tangent neurons ($n = 7$ fills). The main process of an IPS tangent (IPSi) neuron extends from above the base of N1 anteriorly toward the interganglionic connective, seemingly tangent to the curving edge of the LN (Fig. 3A). This main

![Phase of Stimulus (+1 nA)](phase différence) ![](phase différence)
process is quite thick and gives rise to many thin processes that permeate the LN. Their somas are found at the level of the VTs, the center of their main process lies at the level of DC5, and their arbors extend from below the VTs to above DC1 (Fig. 1C). These neurons do not have processes that extend into the base of N1.

Experimental depolarization of an IPSt neuron with injected current usually inhibited PSE and RSI firing but excited RSE and PSI neurons (Fig. 3B). These effects were graded with the strength of the applied currents. Hyperpolarization often mildly excited PSE and RSI firing but inhibited RSE activity. In those experiments where we failed to see these effects on motor neurons, the preparations were not producing well-structured motor output.

In active preparations, the membrane potentials of IPSt neurons oscillated with the same period as the swimmeret motor pattern.

Fig. 3. A: 5 examples of IPS tangent neurons, shown as whole mounts in frontal view in the ganglion’s right side. Top center: line parallel to the filled neuron’s main process lies tangent to the curve of the ganglion’s core. Neurons were filled with either dTR or Neurobiotin (NB). The ganglion in which each neuron was filled is indicated as A3 or A4. Left: color spectrum that originates at the AVC (Fig. 1C) encodes the positions of the neuron’s different parts on the dorso-ventral z stack of confocal images. The positions of other major tracts and commissures (see Fig. 1C) in this depth scale are marked on the spectrum. VT: VIT and VMT tracts; DT: DIT and DMT tracts. B: recording of an IPS tangent neuron during expression of the swimmeret motor pattern. Depolarization of this neuron inhibited firing of PSE units and excited continuous firing of return-stroke excitor (RSE) and power-stroke inhibitor (PSI) units. Hyperpolarization of the same cell increased the durations of PSE bursts and return-stroke inhibitor (RSI) bursts. Horizontal bars (+2 nA) mark the periods of current injection. C: simultaneous intracellular recording from 1 IPS tangent neuron and extracellular recordings from the PS and return stroke (RS) branches of the swimmeret nerve from the same module during 2 cycles of the swimmeret motor pattern.
system’s motor output. They were maximally depolarized during each burst of spikes in RSE neurons (Fig. 3C) and were repolarized during each PSE burst. Their maximum hyperpolarization occurred late in each PS burst. IPSt neurons received a barrage of medium-sized inhibitory postsynaptic potentials (IPSPs) during each PS burst, but as they depolarized again between PS bursts, discrete postsynaptic potentials were largely absent (Fig. 3C).

IPS orthogonal neurons (n = 13 fills). The neurite of an IPS orthogonal (IPSo) neuron extends anteriorly from the cell body toward the base of the swimmeret nerve and then turns toward the midline, orthogonal to the ganglion’s longitudinal axis, before turning again toward the anterior reaches of the LN (Fig. 4A). Their somas lie at or ventral to the VTs, the center of their main process lies at the level of the VTs, and their arbors extend from ventral to the VTs dorsally up to DC1. The

Fig. 4. A: 5 examples of IPS orthogonal neurons, shown as whole mounts in frontal view in the ganglion’s right side. Each neuron was filled with dTR in either ganglion A3 or A4. Left: spectrum of colors encodes the position of its different parts on the dorso-ventral z-axis (see Fig. 3A). Top center: line parallel to the lateral part of the neuron’s major process is orthogonal to the longitudinal and then turns diagonally to parallel the rest of the main process. B: intracellular recording of an IPS orthogonal neuron during expression of the swimmeret motor pattern, recorded simultaneously on the extracellular PS and RS traces. Depolarization of the IPS neuron inhibited PSE bursts and allowed continuous firing of RSE and PSI units. Hyperpolarizing the same cell caused somewhat stronger PSE bursts and RSI bursts and weakened RSE bursts. Horizontal bars (±1 nA) mark the periods of current injection. C: simultaneous intracellular recording from an IPS orthogonal neuron and extracellular recordings from the PS and RS branches of N1 from the same module during 2 cycles of the swimmeret motor pattern.
many thin branches that arise from their main process permeate the LN to the same extent as those of IPSt neurons, and they extend a branch into DC1 or DC2 toward the midline.

Experimental depolarization of an IPSo neuron effectively inhibited PSE and RSI firing but strengthened RSE firing (Fig. 4B). Hyperpolarization usually had opposite effects: PSE and RSI bursts were somewhat stronger and RSE bursts weaker during the hyperpolarization (Fig. 4B).

The membrane potentials of IPSo neurons oscillated with the same period as the system’s motor output. They reached a maximum depolarization during each RSE burst and began to repolarize shortly before the next PSE burst began. In each cycle, most of these neurons (11 of 13) received a cluster of discrete excitatory postsynaptic potentials (EPSPs) while their membrane potentials were depolarized (Fig. 4C). During each PSE burst they received a barrage of medium-sized IPSPs similar to those arriving at the same time in IPSt neurons (Fig. 3C).

**IPS wedge neurons** (n = 6 fills). The anatomy of IPS wedge (IPSw) neurons is quite different from that of the other IPS neurons. The neurite extends anteriorly from the cell body...
toward the middle of the LN, where it extends a process toward the base of N1 (Fig. 5A). This process forms a tuft of branches with many varicosities that is oriented along the posterior side of N1. Viewed from the dorsal side of the ganglion, this oriented tuft resembles a wedge extending into N1. The somas of these neurons lie below the level of the VTs, the center of their main process lies at DC5, and their arbor extends from near the VTs upward to the level of the D Ts (Fig. 1C). IPSw neurons do not have as many branches in the central parts of the LN as other IPS neurons, and they extend at most a few sparse branches toward the anterior reaches of the LN (Fig. 5A).

Depolarization of an IPSw neuron inhibited PSE firing and excited RSE firing (Fig. 5B). Hyperpolarization excited PSE and RSI units but inhibited RSE firing (Fig. 5B). These effects were graded with the magnitude of the injected currents, but the strengths of these effects seemed weaker than those of the other IPS neurons.

The physiology of these neurons is also different from that of other IPS neurons. Their membrane potentials oscillated with the same period as the system’s motor output, but the phase at which they were maximally depolarized varied both between preparations and at different times during recording from one cell in the same preparation. IPSw neurons received a barrage of large discrete IPSPs during each PSE burst, but EPSPs were absent (Fig. 5C).

The anatomies of these different IPS neurons differ quantitatively. Visual comparison of different filled neurons first led us to think that there might be three distinctly different types of neurons with qualitatively similar physiological properties. If this idea is correct, then quantitative analysis of these neurons’ structures should reveal systematic differences between different types. To measure the structures of individual IPS neurons, we imposed a coordinate system on each whole mount (Fig. 1A) and measured specific features of the neurons’ structures. We measured 16 parameters of each of 26 IPS neurons, and used single-factor ANOVA (Zar 1996) to see whether any of these parameter values were probably different between these three types. Ten parameters differed significantly between two or more types (Table 1). The dorso-ventral extent of the IPSw neurons’ structure along the z-axis was greater than that of the other two types. The lengths of the IPSo neurons’ neurites were shorter but the lengths of their main process and ventral to the VTs (Fig. 1A). Their neurite first extends from the cell body toward the midline and then curves posteriorly before branching to form the main process. The main process lies at the level of DC5, their arbor of secondary branches extends from below the VTs upward to the level of DC1, and they extend a branch toward the midline in DC1 or DC2. This arbor is dense and can reach the base of N1.

Depolarization of an IRS hook neuron by injected currents effectively inhibited RSE firing and mildly excited PSE activity (Fig. 6B). In preparations that were producing well-defined PS-RS alternation, hyperpolarization of an IRS hook neuron excited RSE units and inhibited PSE and RSI units (Fig. 6B).

The membrane potentials of IRS hook neurons oscillate with the same period as the system’s motor output (Fig. 6C). It is surprising that they are maximally depolarized during the RSE burst. At the beginning of each PSE burst they receive a cluster of small discrete IPSPs that contribute to their repolarization.

**IRS hook neurons (n = 7).** All IRS hook (IRSh) neurons have a distinctive curve in their main process within the LN, a "hook" that turns posteriorly as the process extends toward the midline of the ganglion (Fig. 6A). Their cell bodies are relatively large, located anterior to the midpoint of their main process and ventral to the VTs (Fig. 1C). Their neurite first extends from the cell body toward the midline and then curves posteriorly before branching to form the main process. The main process lies at the level of DC5, their arbor of secondary branches extends from below the VTs upward to the level of DC1, and they extend a branch toward the midline in DC1 or DC2. This arbor is dense and can reach the base of N1.

**IRS nohook neurons (n = 11).** IRS nohook (IRSn) neurons lack the curved main process characteristic of the IRSh neurons (Fig. 6A). Their arbor is also less dense and are more restricted to the anterior edge of the LN (Fig. 7A). The sizes and positions of the cell bodies of IRSn neurons are also more variable than those of IRSh neurons. The cell bodies of several of these neurons are nearly as large as those of IRSh neurons but lie closer to the ganglion’s midline, medial to their main process and ventral to the VTs (Fig. 7A). Their neurites project

Table 1. **Morphological parameters that differed between three types of IPS neurons**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>IPS Tangents (n = 7)</th>
<th>IPS Orthogonals (n = 13)</th>
<th>IPS Wedges (n = 6)</th>
<th>ANOVA Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-V depth, μm</td>
<td>115.1 ± 33.1*</td>
<td>86.3 ± 16.7*</td>
<td>86.2 ± 15.9</td>
<td>P = 0.024</td>
</tr>
<tr>
<td>Neurite length, μm</td>
<td>238.4 ± 48.7</td>
<td>195.8 ± 42.2*</td>
<td>262.3 ± 48.7*</td>
<td>P = 0.039</td>
</tr>
<tr>
<td>Soma area, μm²</td>
<td>525.4 ± 239.0*</td>
<td>417.1 ± 132.0</td>
<td>263.8 ± 62.3*</td>
<td>P = 0.023</td>
</tr>
<tr>
<td>Soma vector length, μm</td>
<td>424.9 ± 40.1</td>
<td>380.5 ± 44.3</td>
<td>431.8 ± 32.9</td>
<td>P = 0.024</td>
</tr>
<tr>
<td>Soma θ, °</td>
<td>8.8 ± 3.2</td>
<td>88.6 ± 3.1</td>
<td>82.1 ± 4.6*</td>
<td>P = 0.002</td>
</tr>
<tr>
<td>MP vector length, μm</td>
<td>293.1 ± 51.7</td>
<td>435.8 ± 39.3*</td>
<td>293.8 ± 36.4</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>MP θ, °</td>
<td>85.1 ± 2.7</td>
<td>85.0 ± 1.6</td>
<td>78.0 ± 3.0*</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>MP φ, °</td>
<td>8.9 ± 7.6*</td>
<td>-0.5 ± 1.1*</td>
<td>-2.2 ± 4.1*</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Arbor θ, °</td>
<td>86.0 ± 3.6*</td>
<td>80.8 ± 2.9</td>
<td>80.1 ± 2.7</td>
<td>P = 0.002</td>
</tr>
<tr>
<td>Arbor φ, °</td>
<td>14.2 ± 8.2*</td>
<td>7.2 ± 3.0*</td>
<td>-4.3 ± 1.7*</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

Values are means ± SD. *Measurements identified by Holm-Sidak post hoc analysis as significantly different from the others. See definitions in MATERIALS AND METHODS and Glossary.
laterally before turning posteriorly to branch to form the main process, which lies at the level of DC5. The rest have smaller cell bodies located laterally, anterior to the base of N1 at the level of the VTs. The neurites of these other neurons project diagonally and branch to form their main process. The arbors of both types extend from ventral to the VTs upward to the DTs (Fig. 1C).

The physiology of individual IRSn neurons also varied more than that of the other types of nonspiking local neurons. Experimental depolarization of these neurons inhibited RSE firing, and in some cases excited PSE (Fig. 7B). The membrane potentials of all of these neurons oscillated with the same period as the system’s motor output. Seven of the eleven cells were maximally depolarized during the RSE burst (Fig. 7C), the rest during the PSE burst.
Quantitative differences in the structures of IRS neurons. Visual comparison of IRS neurons suggested that there were distinct anatomical types. We measured 16 anatomical features of each neuron (see Glossary) and used single-factor ANOVA to see whether any parameters differed between IRS\textsubscript{h} and IRS\textsubscript{n} neurons. Four parameters were significantly different (Table 2). The cell bodies of IRS\textsubscript{h} were larger and were farther away from the origin than those of IRS\textsubscript{n} neurons. IRS\textsubscript{h} neurons also had longer main process vectors and arbor vectors. Twelve other parameters were probably not different.

There is residual morphological heterogeneity in our sample of IRS\textsubscript{n} neurons (Fig. 7A). In particular, NB fills of IRS\textsubscript{n} neurons have sometimes revealed two very similar neurons with neighboring cell bodies and neurites running closely together (e.g., Fig. 7A, left). We recognize that these might be a dye-coupled pair of similar or identical neurons. dTR fills of what might be the same neuron (e.g., Fig. 7A, bottom right) are not relevant to this question because dTR does not cross gap junctions. Our attempts to further divide this group into neurons with cell bodies medial, anterior, and lateral to their main processes yielded ANOVAs for the 16 parameters that said only that their cell bodies were in different positions, which we knew from the start. No other consistent differences came from these distinctions. Still, we cannot rule out the possibility that...
IRSn neurons with laterally located cell bodies form a distinct type that occurs as an electrically coupled pair in each module.

The Ten Remaining Neurons Did Not Fit Readily into Any Category

For one reason or another, the 10 remaining neurons in this sample could not be classified. Four had physiological characteristics of IRS neurons but had been filled with NB. In those four NB preparations there were several labeled cell bodies, and we could not be sure which of them was connected to the filled arbor. Two more neurons with IRS-like anatomy were filled in preparations that did not express rhythmic swimmeret motor output, so we could not demonstrate the physiological properties needed to classify them. The last four had cell bodies located posterior to N1, and so were IPS-like in that feature, but either had arbors shaped unlike any other neurons in the sample or had no demonstrable physiological effects on their local module.

Dye Coupling Does Not Reveal Multiple Copies of IPSw or IRSh Neurons

How many neurons of each type occur in each local circuit? The question of copy number is important for our understanding of the roles these neurons play in controlling different sets of swimmeret motor neurons and is one that can be answered by evidence of dye coupling when they are filled with low-molecular-weight markers like NB or Lucifer yellow (Stewart 1978). In two experiments, we used a microelectrode that contained both NB and a dextran-conjugated fluorescent marker to fill one neuron simultaneously with both markers (Fig. 8). One filled neuron was an IPSw and the second an IRSh. In both cases, the NB images show cell bodies of other neurons that were weakly dye-coupled to the filled neuron, but none of these other neurons had shapes like the filled neuron. From this evidence, IRSh and IPSw neurons occur as single copies in each hemiganglion and are weakly dye-coupled to a small number of other neurons of different types.

Dye coupling between an IPSt neuron (Interneuron 1A in Paul and Mulloney 1985a) and one RSE motor neuron is also evident if the RSE neuron is filled using hyperpolarizing current, as was standard with Lucifer yellow electrodes (Paul and Mulloney 1985b). They found that just one IPSt neuron in each module was dye-coupled to this RSE neuron, evidence that IPSt neurons also occur as single copies in each module. This dye coupling is unidirectional; we did not observe dye coupling of this RSE neuron and any IPSt neurons that were filled using depolarizing currents. Some electrical synapses in crayfish are strongly rectifying because they are gated by differences in the potential across the synapse. When these synapses are nonconducting, dye coupling does not occur. When the potential difference is reversed, the synapse becomes conducting, and dye coupling is evident (Giaume and Korn 1984). Different electrical synapses, even synapses made by the same neuron but with different postsynaptic partners, can also show different dye-coupling characteristics due to heterologous gap junctions that differ in ionic or charge selectivity (Antonsen and Edwards 2003; Dykes et al. 2004; Phelan et al. 2008).

DISCUSSION

The principal goal of this study was to refine the identification of unilateral nonspiking interneurons found in the local pattern-generating circuits of the swimmeret system. New experimental results on the structure of the intersegmental circuit that coordinates these local circuits required a new standard of accuracy in identification of these interneurons (Smarandache-Wellmann, Weller, Mulloney, unpublished observations). Confocal microscopy and superior fluorescent markers allowed us to image filled neurons far better than the methods used in pioneering studies (Heitler and Pearson 1980; Paul and Mulloney 1985a, 1985b) that were based on neurons recorded in irregularly active preparations, filled with Lucifer yellow (Stewart 1978), and drawn with a camera lucida. The combination of better physiological characterization and quantitative morphological analysis has allowed us to identify five types of unilateral nonspiking local interneurons that we think are components of the pattern-generating circuit in each swimmeret module (Table 3). Three of these five types are described here for the first time.

Table 2. Morphological parameters that differed between two types of IRS neurons

<table>
<thead>
<tr>
<th>Parameter</th>
<th>IRS Hooks (n = 7)</th>
<th>IRS Nohooks (n = 11)</th>
<th>ANOVA Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soma area, ( \mu m^2 )</td>
<td>579.5 ± 135.9</td>
<td>367.6 ± 143.0</td>
<td>( P = 0.007 )</td>
</tr>
<tr>
<td>Soma vector length, ( \mu m )</td>
<td>347.2 ± 41.1</td>
<td>284.5 ± 48.1</td>
<td>( P = 0.012 )</td>
</tr>
<tr>
<td>MP vector length, ( \mu m )</td>
<td>294.1 ± 26.7</td>
<td>246.6 ± 43.1</td>
<td>( P = 0.019 )</td>
</tr>
<tr>
<td>Arbor vector length, ( \mu m )</td>
<td>281.7 ± 13.3</td>
<td>240.4 ± 39.9</td>
<td>( P = 0.019 )</td>
</tr>
</tbody>
</table>

Values are means ± SD. See definitions in MATERIALS AND METHODS and Glossary.
The evidence we have presented supports recognition of three distinct types of IPS interneurons, all with different anatomical features and with subtle physiological differences. IPS interneurons are synonymous with Paul and Mulloney’s Interneuron 1A (1985a, 1985b). IPSh and IPSw neurons have not been recognized previously. The several morphological differences between these three types (Table 1) and the absence of numerous intermediate forms argues to us that these are really different types of neurons, not developmental variants of one functional type. We think that each of the three types occurs as a single copy in each module.

The morphological evidence also supports two distinct types of IRS neurons distinguished by the shapes of their major process and the positions of their cell bodies relative to that process. IRSh neurons are distinctly different from IRSn neurons, not only in their morphological properties (Table 2) but also in at least one feature of their synaptic connections; each IRSh makes an electrical synapse with a commissural neuron, ComInt 1 (Mulloney and Hall 2003), that is part of the intersegmental coordinating circuit (Smarandache-Wellmann, Weller, Mulloney, unpublished observations). When an IRSh is filled with NB, no other unilateral local neurons with similar shapes are filled (Fig. 8), so we think that IRSh neurons occur as a single copy in each swimmeret module. This synapse has the odd property that dye coupling between the neurons is apparent only when the IRSh neuron is injected with NB, not when the ComInt 1 is injected, a feature that has been attributed in other systems to heterologous gap junctions or to voltage-gating of the synapse. We have never seen dye coupling between ComInt 1 and IRSn neurons.

The morphologies of IRSn neurons in our sample were more heterogeneous than any other type. In particular, IRSn neurons with laterally located cell bodies might be a distinct type that occurs as an electrically coupled pair in each module. NB fills of IRSn neurons have sometimes revealed two very similar neurons with neighboring cell bodies and neurites running closely together (e.g., Fig. 7A, left). NB readily passes through electrical synapses and can reveal electrically coupled circuits of seemingly identical neurons (Antonsen and Edwards 2003; Fan et al. 2005; Huang et al. 1992; Mills and Massey 2000; Vaney 1993). The original Lucifer yellow fills of Paul and Mulloney’s Interneuron 2 (1985a), an RS inhibitor, were illustrated as a dye-coupled pair with shapes like those in Fig. 7A. Despite this, ANOVA of the morphological properties of three groups of IRSn neurons partitioned by cell body location did not identify other probable differences between the three groups, so we have no evidence other than dye coupling to support recognizing these “paired” IRSn neurons as distinct from other IRSn neurons. We suspect that, as new evidence comes in, they will prove to be a sixth type of nonspiking neuron in these circuits.

Distributions of Unilateral Local Interneurons in Different Abdominal Ganglia

The neural modules in which these interneurons are found occur as bilateral pairs in each ganglion that innervates swimmerets (Murchison et al. 1993). Each swimmeret has its own module, anatomically separated from the module that innervates the contralateral swimmeret (Mulloney and Hall 2000). We think that these local interneurons (Table 3) occur as bilateral pairs in each swimmeret ganglion, with one member of the pair in each module. We have filled what appeared to be different types of IRS neurons in the same module, but probably because of microelectrode damage the module’s pattern-generating performance immediately collapsed, so we could not characterize the neurons’ physiological properties. Without the combination of anatomy and physiology, we cannot claim to have filled different types of one class in the same module.

Most of the neurons illustrated here were recorded in ganglia A3 and A4, but Fig. 8 shows an IPSw in A5, and IPSn neurons have been filled in A3, A4, and A5 (Paul and Mulloney 1985b). Extensive evidence of serial repetition of other components of the swimmeret systems—coordinating neurons, commissural neurons, and motor neurons—in ganglia A2, A3, A4, and A5 supports this idea (Mulloney and Hall 2000; Mulloney et al. 2006; Smarandache et al. 2009). Accordingly, we expect to encounter a pair of each type of interneuron in each swimmeret ganglion, and we predict that the synaptic organization of these interneurons into each module’s pattern-generating circuit will be the same.

Why Are There Multiple Types of IPS and IRS Neurons in Each Module?

If we naively think of swimmeret movements as simple alternations of PS and RS movements driven by a motor pattern in which all PS and RS motor neurons are equivalent, finding more than one type of IPS and one type of IRS neuron in each module is puzzling. We think that this perception of the swimmeret system is too simple because it fails to consider differential changes in swimmeret movements in response to proprioceptive input from individual swimmerets or in response to descending signals from the statocysts or from the walking system in the thoracic ganglia. Proprioceptive input to a module from receptors in a swimmeret can alter the module’s output and its phase relative to its neighbors (Hall et al. 2008; Heitler 1982, 1986). Roll and pitch of the body detected by the statocysts cause differential twisting of left and right swimmerets that generate corrective thrusts during each cycle of movements (Davis 1968a; Mulloney and Smarandache-Wellmann 2012; Neil and Miyan 1986). There are nonspiking unilateral local interneurons in the thoracic ganglia that inhibit specific subsets of leg motor neurons (Chrachri and Clarac

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Table 3. Summary of different types of nonspiking unilateral interneurons in swimmeret local pattern-generating circuits

<table>
<thead>
<tr>
<th>IPS neurons</th>
<th>Soma Location</th>
<th>Number in Each Circuit</th>
<th>Motor Neurons Inhibited</th>
<th>Dye-Coupled to</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPSi</td>
<td>Posterior</td>
<td>1</td>
<td>PSE, RSI</td>
<td>One RSE motor neuron*</td>
</tr>
<tr>
<td>IPSo</td>
<td>Posterior</td>
<td>1</td>
<td>PSE, RSI</td>
<td>uk</td>
</tr>
<tr>
<td>IPSw</td>
<td>Posterior</td>
<td>1</td>
<td>PSE, RSI</td>
<td>uk</td>
</tr>
<tr>
<td>IRS neurons</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRSh</td>
<td>Anterior</td>
<td>1 or 2</td>
<td>RSE, PSI</td>
<td>ComInt 1 neuron*†</td>
</tr>
<tr>
<td>IRSn</td>
<td>Anterior</td>
<td>1</td>
<td>RSE, PSI</td>
<td>uk, or paired neuron</td>
</tr>
</tbody>
</table>

IPSi, IPS tangent; IPSo, IPS orthogonal; IPSw, IPS wedge; IRSh, IRS hook; IRSn, IRS nohook; PSE, power-stroke excitor; PSL, power-stroke inhibitor; RSE, return-stroke excitor; RSI, return-stroke inhibitor; uk, unknown. *Paul and Mulloney (1985b); †Smarandache-Wellmann, Weller, Mulloney, unpublished observation.
1989), and it is likely that those thoracic interneurons are homologs of the IPS and IRS neurons we describe. We postulate that these different IPS and IRS interneurons will prove to be a system that allows differential control of subsets of PS and RS motor neurons innervating muscles that operate across different joints, a control system that permits much more subtle adjustments of the system's biomechanical performance than our naive model would encompass. Now that we can identify these different types of IPS and IRS neurons, experimental analysis of functional differences between types and their synaptic organization within the local circuit becomes a realistic goal.

Nonspiking Locals in Other Motor Systems

In locusts, cockroaches, and stick insects, both spiking and nonspiking unilateral local neurons integrate proprioceptive information from the legs and shape movements elicited by touching a leg (Burrows and Siegler 1978; Laurent and Burrows 1989; Pearson and Fourtner 1975; Siegler and Burrows 1979; Wilson 1981). The morphology and physiology of nonspiking interneurons that organize stepping of individual walking legs have been studied extensively (Büschges 1990, 1995; Büschges and Wolf 1995; von Uckermann and Büschges 2009; Wolf and Büschges 1995), and it is likely that some of these same neurons are also components of local circuits active during walking (Büschges 2005; Wolf and Büschges 1995). In crustaceans, the local circuits in each thoracic ganglion that control movements of walking legs are serial homologs of the swimmeret modules in abdominal ganglia (Mulloney et al. 2003). Nonspiking unilateral local interneurons in these thoracic circuits inhibit specific subsets of leg motor neurons (Chracri and Clarac 1989). More anteriorly, movements of the scaphognathites that drive the water currents to ventilate the gills are also controlled by nonspiking interneurons that influence the period and strength of those movements (DiCaprio 1989; DiCaprio and Fourtner 1988; Simmers and Bush 1980). In all of these other motor systems, however, the synaptic organization of nonspiking local interneurons into the pattern-generating circuits remains undescribed.

The long-term goal of this research is to explain in terms of its neuronal components and their synaptic organization how the CNS generates motor patterns that enable effective behaviors. Our recent description of the intersegmental coordinating circuit (Smarandache et al. 2009) and the mechanisms that enable neurons in this circuit to encode and decode information essential for effective swimmeret movements (Smarandache-Wellmann, Weller, Mulloney, unpublished observations) has transformed our understanding of swimmeret coordination. Together with the description of the intersegmental circuit that coordinates these local circuits, this will provide a neuron-by-neuron, synapse-by-synapse explanation of how the CNS controls this set of limbs.

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