Electrophysiological and Morphological Characterization of Identified Motor Neurons in the Drosophila Third Instar Larva Central Nervous System

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INTRODUCTION

Locomotion is a complex behavior that requires coordinated nervous system output to multiple muscles. This coordinated output has been shown to be the result of central pattern generator circuits in both vertebrate and invertebrate systems (Marder and Calabrese 1996). The output of most central pattern generators drives motor neuron firing and can be shaped by sensory inputs. Changes in locomotion usually involve alterations of synaptic strengths and/or the intrinsic properties of the constituent neurons. In both vertebrate spinal cord (Kiehn 1991), where the motor neurons are not part of the central pattern generator, and in lobster stomatogastric ganglion (Marder 1998), where they are, modulation of the properties of motor neurons is also important for adjusting output of the central pattern generator.

In Drosophila third instar larvae, the function of central pattern generators has been studied only by recording muscle output (Cattaert and Birman 2001; Gorczyca et al. 1991); circuit components have not yet been identified. The output synapse, the third instar larval neuromuscular junction (NMJ) has been studied intensely and is arguably the premier system for using genetics and electrophysiology to understand synaptic development, transmission and plasticity. In the traditional NMJ preparation, the synaptic response is measured by impaling the muscle or making a focal patch over a bouton. Typically, recordings are done with the CNS cut off so that the contribution of the presynaptic cell is limited to that of the distal axon and the terminal.

For understanding coordinated nervous system function, the NMJ preparation is not ideal. In the animal, the activation of synapses usually occurs as a consequence of an action potential generated by integration of synaptic inputs in the somatodendritic compartment. The firing properties of the neuron, e.g., spike frequency, amplitude, and duration, are determined by the balance of inward and outward currents in the cell. In principle, this allows individual cells to develop a unique response to inputs. Until recently (Rohrbough and Broadie 2002), third instar Drosophila motor neurons have been characterized primarily by their terminal morphology, position, and neurotransmitter content (Anderson et al. 1988; Hoang and Chiba 2001; Jia et al. 1993; Johansen et al. 1989; Kurdyak et al. 1994; Monastirioti et al. 1995). Differences in the release properties and plasticity of Ib and Is terminals have been documented indirectly with focal patch recordings over the two bouton types or low-level stimulation to distinguish terminal thresholds (Kurdyak et al. 1994; Lnenicka and Keshishian 2000), suggesting that these two neurons types are functionally different. Direct electrophysiological experiments in neurons of the larval stage were limited to cultured, dissociated cells (Scol and Aldrich 1988; Wu et al. 1983b) and boutons (Martinez-Padron and Ferrus 1997). In contrast, embryonic motor neurons have been studied electrophysiologically in vivo (Baines and Bate 1998; Baines et al. 1999, 2001) as have adult thoracic ganglion motor neurons (Trimarchi and Murphy 1997), suggesting that access to ventral ganglion motor neurons was technologically feasible. Recordings from unidentified ventral ganglion neurons confirmed this (Rohrbough and Broadie 2002).

In addition to answering functional questions, direct access to intact identified motor neurons in the third instar would also facilitate investigation of development of central circuits. Neurons of the ventral ganglion are arrayed in bilaterally symmetrical repeats that correlate with the segmental organization of

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the body wall, and the relative orientations of neuronal somata are stereotypic throughout segments A2–A7. During embryonic and larval development, the growth cone and varicosities of motor neurons are in a state of constant flux (Yoshihara et al. 1997). In the embryo, outgrowth, pathfinding, and target recognition of a group of dorsomedial neurons (RP1–4 and aCC) has been extensively studied (Bate and Broadie 1995; Broadie et al. 1993; Halpern et al. 1991; Sink and Whittington 1991b; Thomas et al. 1984). Filling of embryonic neurons allowed the identification of initial targets. Backfilling of third instar larval neurons from the terminal has suggested that the position and/or targets of these embryonic neurons may change during development of the larval CNS (Hoang and Chiba 2001; Sink and Whittington 1991a). The final targets, central connections, and relative positions in the ventral ganglion of these neurons need to be investigated by accessing the cell bodies.

To study the intact mature third instar CNS, we have morphologically and electrophysiologically characterized a subset of the motor neurons that innervate the body wall muscles. These neurons receive output from the central pattern generator for locomotion, and their modulation is likely to be important to plasticity of locomotor behavior. We find that significant structural reorganization occurs between the late embryo and the third instar. We also find that there is diversity in the intrinsic properties of larval motor neurons and that differences in potassium current regulation can control the cell-specific properties of identified neurons.

METHODS

Preparation

All experiments were performed on wandering, primarily female, third instar larvae. Animals were dissected and pinned in 0.2 mM Ca$^{2+}$-“A” solution (which contained, in mM, 128 NaCl, 2 KCl, 35 sucrose, 4 MgCl$_2$, and 5 HEPES, pH 7.1–7.2 (original solution contains 1.8 mM Ca$^{2+}$) (Jan and Jan 1976), with care taken to leave segmental nerves and the CNS intact. Calcium-free “A” solution was used as the external solution for all experiments with the exception of the confocal dye-fill experiments (see details in the following text). The preparation was visualized using an upright Olympus microscope equipped with differential interference contrast and epifluorescence. Under constant perfusion with laminar flow traversing, the larva toward the anterior direction, collagenase type I or protease XIV (1 and 2 mg/ml, respectively, Sigma-Aldrich, St. Louis, MO) was focally administered to the ventral ganglion using a glass pipette pulled to ~10 μm tip diameter. Debris was constantly cleared using the pipette with positive and negative pressure. With adequate exposure, the outer layer of the ganglion is disrupted, revealing neural tissue. Prominent somata with diameters of 10–15 μm could be seen at this point. The protease treatment was continued until an inner membrane layer enclosing the group of neurons was disrupted. A successful procedure rendered the soma clean, smooth and loose, but intact within its cluster. Recordings were performed mainly in segment A3.

Genetics, cell visualization, and identification

Motor neurons were visualized using GFP fluorescence. GFP expression was driven by C164-GAL4 (a generous gift of Vivian Budnik, University of Massachusetts, Amherst, MA), an enhancer trap line that expresses in all motor neurons (Packard et al. 2002). Crossed with UAS-mCD8-GFP, C164-GAL4 allows sharp, distinct visualization of cells, as mCD8 targets the GFP to the membrane (Lee and Luo 1999). After dissection and protease digestion, the targeted soma was approached with constant positive pressure until the pipette tip was apposed to the surface. After establishing a tight seal, the membrane was broken with instantaneous suction and/or current injection. The recording intracellular solution usually contained tetramethyl rhodamine dextran 3000 (10 mg/ml, Molecular Probes, Eugene, OR), either backfilled into the pipette or as part of the solution, which gave a slightly lower patch success rate but did not alter neuronal responses (data not shown). After ~15 min of filling, during which physiological recordings were obtained, the processes and terminal morphology for individual neurons were visualized on the same microscope. Images were taken with a Photometrics PXL liquid-cooled CCD camera and IPLab software package on a Macintosh computer. Combinations of brightfield, GFP, and dye images were rendered using NIH Image and Adobe Photoshop.

For every experiment, a specific soma within a stereotypical cluster in the dorsomedial surface was targeted, relying on its orientation relative to the midline and the other somata. Sh$^{K1353}$, a point mutation in the Sh gene (Lichtinghagen et al. 1990) that eliminates $I_h$ in muscle (Sole et al. 1987; Wu et al. 1983a) was used to dissect the $I_A$ potassium current. In this case, DIC and not GFP was used to target neurons for recording, and the identity of the neuron was confirmed with dye fill.

For detailed confocal visualization of axon and dendrites of individual motor neurons using confocal microscopy, larvae were dissected in HL3 (0 mM Ca$^{2+}$), and the recording intracellular solution contained a 10:2 mixture of tetramethyl rhodamine dextran 3000 (10 mg/ml) and AlexaFluor 568 hydrazide sodium salt (10 mg/ml, Molecular Probes). After 30–60 min of filling, larvae were fixed in phosphate-buffered 4% paraformaldehyde for 30 min at room temperature. The larval tissues were then washed in HL3, followed by 30, 60, and 100% glycerol incubations for 10 min prior to mounting in Vectashield (Vector Laboratories, Burlingame, CA). Confocal images were acquired using a Leica confocal system. Images were then further processed in Adobe Photoshop.

Electrophysiology

Filamented thin- and thick-walled capillary pipettes (WPI, Sarasota, FL) were pulled and fire polished to a resistance of 5–10 MΩ. Intracellular recording solution (adapted from O’Dowd 1995) contained (in mM) 120 potassium gluconate, 20 KCl, 10 HEPES, 1.1 EGTA, 2 MgCl$_2$, and 0.1 CaCl$_2$. The pH was adjusted to 7.2 and the osmolarity to 280 mmol/kg. Recordings were performed with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) and ITC-16 data acquisition board (National Instruments, Austin, TX) with Igor software (WaveMetrics, Lake Oswego, OR). Cell membrane potentials were recorded in whole cell current-clamp mode. Spikes were evoked by a series of current injections delivered via the recording pipette. The duration of current steps was 500 or 1,000 ms, and the amplitude ranged from ~20 to 140 pA in 20-pA increments. In prepulse experiments under current clamp, the external solution contained 0 mM Ca$^{2+}$. The duration of the prepulse was 1 s, and the amplitude was ≥20 pA below spike threshold, usually 20 pA. The average prepulse depolarization was to ~43 ± 1 mV (n = 8 cells). The prepulse was immediately followed by test current steps of 1-s duration. In experiments to pharmacologically eliminate transient potassium currents, 4-aminopyridine (4-AP; 1.5 mM, Calbiochem, San Diego, CA) was bath-applied. Recordings were obtained before and after application, and also 2 min after several washes of external solution. For recordings of spontaneous activity, 2.4 mM Ca$^{2+}$ was added to the external solution, and passive recordings were done in whole cell configuration.

In neuron-muscle dual recordings, EJP’s were recorded using sharp electrodes (~30 MΩ) filled with 3 M KCl for muscle. Having achieved whole cell access to the neuron, the preparation was visualized under lower magnification (×10), and the corresponding muscle was impaled with a recording electrode. The external Ca$^{2+}$ con-
centration was increased to 0.4 mM. Under current clamp, a stimulus protocol was performed on the neuron and recordings made simultaneously from neuron and muscle. EJPs were amplified with Axoclamp 2B (Axon Instruments), which was connected to the same acquisition board.

For voltage-clamp recordings, neurons were switched to voltage-clamp mode after initial current-clamp recordings. Sodium channels were blocked with tetrodotoxin (Sigma, 100 nM). Cells were held at either −80 or −100 mV. For trace subtractions, voltage was raised to +40 mV for 1 s, and the difference between the waveform obtained and one recorded from a holding potential of −40 mV was calculated. For current-voltage data, test pulses ranging from −120 to +40 mV (in 20-mV increments) were given in 15-s intervals. Waveforms obtained with the −20 mV pulse was used to subtract leakage currents. To obtain voltage-dependent inactivation plots, a 1-s prepulse of variable amplitude (−100 to +40 mV in 10-mV increments) was given, and a test pulse of +40 mV amplitude was given after a 10-ms interpulse interval. Perfusion was usually stopped for voltage-clamp recordings. Identity of neurons was subsequently verified by visualizing the dye fill. Capacitance normalization was done with values obtained directly from the parameter settings on the amplifier.

Series resistances of cells ranged from 20 to >100 MΩ, most likely due to clogging of the pipette tip by cellular constituents. As an acceptance criterion, only experiments where \( R_{\text{in}} > 1 \text{ M}\Omega \) and calculated maximum voltage error <25 mV were analyzed.

Data analysis

For injection of different levels of current, time to peak was measured as the time between the beginning of current injection to the peak of the first spike observed in the voltage trace. Peak amplitude was derived by averaging the amplitude of all the spikes in a given voltage trace. Spike frequency was calculated by averaging the instantaneous frequencies derived by measuring the time interval between adjacent spikes. Only the first 500 ms of each trace was analyzed. The inactivation time constant (\( \tau \)) was obtained by using a single exponential fit from peak to steady state for each waveform. For the inactivation plot, the peak was normalized with the maximum and minimum values, and plotted with respected to prepulse amplitude. A regular Boltzmann equation was used to fit the plot, and the half-maximal inactivation voltage was obtained.

Statistical analyses were done in Statview 4.5 (Abacus Concepts/SAS, Cary, NC) or the analysis tools package in Excel (Microsoft, Redmond, WA). \( P \) values reported for multiple comparisons were ascertained using ANOVA with Scheffe’s post hoc test for significance.

RESULTS

Identification of dorsomedial motor neuron projections and targets in the third instar larva

Using the standard NMJ dissection, neurons in the ventral ganglion can be readily visualized and particular groups of neurons identified using cell-specific GAL4 drivers. We have used C164-GAL4 (Packard et al. 2002) to drive expression of GFP in motor neurons to target our studies to this population. The motor neurons in the ventral ganglion are organized stereotypically (Sink and Whittington 1991a) with segmental arrays of somata repeating along the anterior-posterior axis. Axons project to the body-wall muscle through nerves that emanate from the ganglion in each hemisegment. We focused on the bilaterally symmetric dorsomedial clusters that are aligned parallel to the midline. Figure 1A shows GFP expression in the posterior end of a preparation after dissection. A set of five neurons that are GFP positive repeats in segments A2–A7 with all the neurons lying in the same plane of view. Figure 1, B and C, shows brightfield and GFP visualizations of the dorsomedial cluster at higher magnification after enzymatic digestion. The somata are typically in a cross formation, although shifts in relative orientation can cause some deviation from this pattern.

The identities of the neurons in the dorsomedial cluster were determined by filling GFP-positive cell bodies from the recording patch pipette, which contained rhodamine and/or Alexa 568. After recording, the body-wall muscles were imaged for GFP and dye. Each fill resulted in dye signal in a single axon and bouton arbor that could be visualized in the live preparation. Correlation of soma position, muscle target, and bouton morphology suggests that innervation type and target are invariant for each cell within the cluster. Each neuron has an elaborate projection pattern within the ventral ganglion that is always in the same relative position. Example fills of each of the five neurons are shown in Fig. 1, D–H, and a schematic of the arborization patterns is shown in I.

To name the neurons, we have adopted the nomenclature devised by Hoang and Chiba (2001), naming each neuron according to its target muscle and the morphology of its terminal boutons. The five neurons of the dorsomedial cluster were identified as MN1-Ib, MN6/7-Ib, MN14-Ib, MN30-Ib, and MN1-Ia, MNISN-Ia. Table 1 provides a summary of the cell’s morphological features. Bouton types were categorized primarily by relative size compared with the other boutons in the field of view, and their consistency with previously published accounts of muscle innervations (Hoang and Chiba 2001). Figure 2 shows the muscle termini of each of the five neurons. A schematic of the organization of the muscles and nerves in one hemisegment is shown in Fig. 2A.

Four of the five neurons in the dorsomedial cluster terminate with type Ib boutons. MN14-Ib and MN30-Ib are unipolar and project contralaterally through the segmental nerve (Fig. 1, E and G). Axons from these two neurons travel together until the terminal arborization point in SNb (segmental nerve branch b) where they diverge to innervate their respective neighboring muscle targets, 14 (Fig. 2D) and 30 (Fig. 2E). MN6/7-Ib projects bilaterally in the ventral ganglion, but a single process extends contralaterally past the midline and out through the segmental nerve, terminating exclusively with type Ib boutons on muscles 6 and 7 (Figs. 1F and 2C). MN1-Ia has prominent bilateral projections with two dendritic arborizations within the ventral ganglion (Fig. 1D); the motor axon projects ipsilaterally through the ISN (intersegmental nerve) to innervate the distal muscle 1 (Fig. 2B).

The anterior-most neuron in the cluster is MNISN-Ia, which projects ipsilaterally through the ISN. The axonal process climbs up and exits the nerve of the anterior segment, so that its muscle innervation is actually one segment anterior to those of other neurons within the cluster (Fig. 1H). This neuron targets multiple muscles on the distal (dorsal) body wall (Fig. 2F). The identification of this neuron as a Is neuron was supported by the fact that in muscle 4, in view of the slightly bigger boutons of Ib, the dye-filled innervating bouton type was clearly type Is and not type II (Fig. 2F). We unambiguously observed innervation of muscles 1, 2, 3, 4, 9, 10, 19, and 20, and muscle 18 may also have innervation that was less clear and is not seen in Fig. 2. Hoang and Chiba describe a neuron (MNISN-Ia) that innervates muscles 1, 2, 3, 4, 9, 10, 19, and 20 (Hoang and Chiba 2001). This is almost the exact target pattern of our...
observation of MNISN-Is. Hoang and Chiba (2001) note that all muscles that are innervated by the ISN most likely receive Is innervation from a single neuron. This is quite different from type Ib innervations which usually arise from neurons dedicated to a single muscle or pair of muscles.

On three occasions, in abdominal segment 2, we observed a type Is innervation of muscles 6/7. This neuron also innervated additional muscles with a type Is bouton (Table 1). The muscle pattern was consistent with Hoang and Chiba’s MNSNb/d-Is. The cell body of this neuron was located beneath the cluster of five dorsomedial neurons that we have more fully characterized but could not be consistently targeted as the cell body is not predictably in the superficial cluster.

Dye fills established anatomical connections between dorsomedial neurons and specific muscle targets. To demonstrate that these neuromuscular connections were functional and could be used to address questions of individual neuron function, neuron-muscle dual recordings were done. Figure 3 shows paired recordings from MN6/7-Ib and muscle 6. Each neuronal action potential reliably evoked an EJP in the target muscle. Spontaneous patterned activity within the ventral ganglion can also be observed when the extracellular solution has a high concentration of calcium. Figure 3 shows a current-clamp trace of MNISN-Is in 2.4 mM calcium. The frequency of bursting is similar to what has been previously identified for the locomotor pattern generator (Cattaert and Birman 2001).

### Table 1. Morphology of third instar dorsomedial motor neurons

<table>
<thead>
<tr>
<th>Third Instar Motor Neuron</th>
<th>Embryonic Identity</th>
<th>Nerve</th>
<th>Target Muscles</th>
<th>Projections</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN1-Ib</td>
<td>aCC</td>
<td>ISN</td>
<td>1, 2, 3, 4, 9, 10, (18), 19, 20</td>
<td>Ipsilateral axon, major contralateral process</td>
<td>6</td>
</tr>
<tr>
<td>MNISN-Is</td>
<td>ISN</td>
<td></td>
<td></td>
<td>Ipsilateral axon</td>
<td>17</td>
</tr>
<tr>
<td>MN14-Ib</td>
<td>RP1 or RP4?</td>
<td>SN</td>
<td>14</td>
<td>Contralateral axon</td>
<td>20</td>
</tr>
<tr>
<td>MN6/7-Ib</td>
<td>RP3</td>
<td>SN</td>
<td>6/7</td>
<td>Contralateral axon, major ipsilateral process</td>
<td>12</td>
</tr>
<tr>
<td>MN30-Ib</td>
<td>RP1 or RP4?</td>
<td>SN</td>
<td>30</td>
<td>Contralateral axon</td>
<td>14</td>
</tr>
<tr>
<td>MNSNb/d-Is</td>
<td>RP5?</td>
<td>SN</td>
<td>6, 7, 13, 14, 30, (15, 16)</td>
<td>Contralateral axon</td>
<td>3</td>
</tr>
</tbody>
</table>

The structure and targets of dorsomedial neurons were investigated in C164-GAL4; UAS-mCD8GFP animals by filling from the cell body with rhodamine. Nomenclature is adopted from Hoang and Chiba (2001) and indicates the muscle(s) innervated and the bouton type. Target muscles in parentheses were not examined but were seen by Hoang and Chiba (2001). For MNISN-Is, the target muscles listed are cumulative observations; not all muscles were observed in every preparation. Projections refers to the processes seen within the ventral ganglion. The number of preparations examined is indicated by n. ISN, intersegmental nerve; SN, segmental nerve.
Evoked spiking activity in dorsomedial larval neurons

The dorsomedial neurons show consistent position, target, and morphology from animal to animal. To determine if firing properties were also determined by cell identity, we recorded evoked action potentials from the somata of these neurons. Current injection readily caused repetitive spikes at frequencies that were dependent on stimulus intensity. Hyperpolarizing prepulses did not evoke significant activity (data not shown). Figure 3, A–F, shows representative traces from current-clamp recordings of the five neurons within the cluster and a recording from a putative MNISnb/d-Is cell. Data acquisition was typically done as the cells were being filled with dye; this allowed unequivocal identification of the neurons and correlation of morphology with physiology. Resting membrane potential, resting input resistance and current required for spiking are shown in Table 2. The amount of current required (in 20-pA increments) to initiate spiking was not different comparing the Ib neurons to each other, but MNIS-Is required significantly more current than any of the Ib cells (P < 0.001).

Data for time to first spike for each of the five cells are shown in Fig. 3H. The MNIS-Is neuron had a significant delay to spiking compared with the Ib neurons (Fig. 3H, P < 0.0001 for all current injections). Time to first spike was not different between Ib neurons at any current injection level (Fig. 3H, P > 0.35).

Mechanism of the MNIS-Is delay

Because the identity and properties of the larval motor neurons are stereotyped, the molecular basis of the behavior of each neuron can be addressed using pharmacological, biophysical, and genetic tools. A distinct structural feature of MNIS-Is and MNISnb/d-Is is that, unlike the type Ib neurons, they innervate multiple muscles. The striking delay in the time to first spike is likely to have effects on patterned muscle activity. In other neurons where such delays have been observed, they have often been due to the presence of an inactivating potassium current (Turrigiano et al. 1996; Zhao and Wu 1997). To determine if this was the mechanism underlying the MNIS-Is firing kinetics, we examined the effects of depolarizing prepulses and 4-AP on the delay. Both of these manipulations are known to decrease I_A-type currents. Figure 4A shows traces from an MNIS-Is cell before, during and after washout of 1.5 mM 4-AP. During exposure to the drug, there is a decrease in the time to first spike that is reversed on washout. 4-AP also affects spiking behavior; stereotyped, regular single spike activity is altered to give doublet spikes. In the example shown in Fig. 4A, the doublet spiking is also seen in the wash, but in other cells this effect of 4-AP did wash out (data not shown). Depolarizing prepulses delivered prior to suprathreshold test amplitudes had an effect similar to 4-AP (Fig. 4C), decreasing the time to spike onset. The mean level of depolarization achieved with prepulse was ~43 ± 1 mV (n = 8). Prepulse also caused doublets in the spike train, but the effect was less consistent, with multiple spike activity appearing sporadically during test pulse (data not shown). Subsequent experiments with longer test pulse protocols in control cells revealed that with long current injections (>2 s), even control cells could show doublet spiking.

The changes in delay with 4-AP and prepulse are quantified in Fig. 4, B and D, for different levels of current injection. The decrease in time to first spike was significant for comparison of control and washout with 4-AP for all current injections (P < 0.05). Control and washout were not significantly different, except at the lowest stimulus amplitude (P > 0.02). For prepulse experiments, control and prepulse delays were significantly different at all current spike-evoking injection levels (P < 0.01).

To determine the identity of the 4-AP- and prepulse-sensitive potassium current(s) responsible for the delay in onset of spiking and doublet spiking, we examined the firing behavior of MNIS-Is in Sh^{KS133} mutants. The Sh gene encodes one of the channel proteins known to produce I_A. In Sh mutants, the fast inactivating current of third instar larval muscles is selectively eliminated (Wu and Haugland 1985; Wu et al. 1983a). The other gene known to encode an I_A channel is shal. The Shal protein is thought to be responsible for the majority of I_A in embryonic neurons (Scholz et al. 1988; Tsunoda and Salkoff 1995). A comparison of the delay in MNIS-Is recordings from Sh^{KS133} (Fig. 4E) and control (Fig. 4A) neurons shows that the magnitude of delay is not significantly different for any spike-inducing current injection level (P > 0.1). In all of the recordings from Sh mutants, doublet spikes were seen in the spike train (Fig. 4E). These doublets were qualitatively similar to those seen with 4-AP treatment and prepulse. Prepulse of MNIS-Is in Sh mutants is still able to decrease the time to first spike, suggesting that there is a non-Shaker prepulse-sensitive current that causes the delay. A comparison of the delay before and after prepulse for different current levels is shown in Fig. 4F. For all levels of current injection, the decrease in the delay ranged from 80 to 95%, and Sh was significantly different from Sh with prepulse (P < 0.05 for all current injection levels). These results suggest that the role of Shaker in the MNIS-Is neuron does not involve setting the spike delay. Spike delay is mediated by another prepulse-sensitive current, likely carried by Shal. Unfortunately, be-

<table>
<thead>
<tr>
<th>Motor Neuron</th>
<th>Resting Input Resistance, MΩ</th>
<th>Resting Membrane Potential, mV</th>
<th>Average Current Injection to Evoke Spikes, pA</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN1-Ib</td>
<td>977 ± 89</td>
<td>−56 ± 1</td>
<td>27 ± 5</td>
<td>6</td>
</tr>
<tr>
<td>MN67-Ib</td>
<td>1204 ± 79</td>
<td>−58 ± 2</td>
<td>32 ± 4</td>
<td>12</td>
</tr>
<tr>
<td>MN14-Ib</td>
<td>1322 ± 127</td>
<td>−54 ± 1</td>
<td>26 ± 4</td>
<td>10</td>
</tr>
<tr>
<td>MN30-Ib</td>
<td>1435 ± 70</td>
<td>−58 ± 2</td>
<td>29 ± 7</td>
<td>7</td>
</tr>
<tr>
<td>MNIS-Is</td>
<td>1653 ± 172</td>
<td>−61 ± 2</td>
<td>74 ± 7**</td>
<td>10</td>
</tr>
</tbody>
</table>

Electrophysiological properties of dorsomedial neurons were measured in whole cell current-clamp mode. Resting input resistance was measured by potential difference with a −20-pA current pulse. MNIS-Is was had a significantly higher resting input resistance compared to MN1-Ib (*, P < 0.05). Spikes were induced by current injections from 20 to 140 pA in 20-pA increments. The level of current injection that 1st induced spiking was noted for each cell. There was no significant difference in the average current required to induce spiking in Ib neurons (P > 0.05). MNIS-Is required significantly more current than any of the Ib neurons (**, P < 0.001). There was no difference in resting membrane potential among all the neurons (P > 0.05). The number of cells of each type characterized is indicated by n. Values are means ± SE.
cause there are no shal mutants, the role of Shal cannot be directly approached genetically.

Mechanism of delay in Ib neurons

The Ib motor neurons have a very short delay to first spike. To determine if this delay was due to a Sh encoded \( I_{\text{K}} \), we measured the delay in wild-type and \( \text{Sh}^{\text{KS133}} \) neurons. Figure 4G shows the delay to first spike measured after a 60-pA current injection. None of the Ib neurons have a statistically significant decrease in delay when \( \text{Sh} \) is mutated (\( P > 0.05 \)). We also examined the effect of \( \text{Sh} \) mutation on spike rate and saw no significant differences in any of the neurons for current injections of 60–140 pA (\( P > 0.3 \)). These data suggest that in Ib neurons, as in MNISN-Is, the Shaker channel does not play a significant role in determination of spike rate or delay to first spike.

Isolation of potassium currents in larval motor neurons

To characterize the potassium currents present in larval motor neurons, we recorded outward currents from MNISN-Is and MN6/7-Ib in voltage clamp. Figure 5 shows example whole cell recordings and peak (solid line) and sustained (broken line) current \( I-V \) curves. Current densities were similar, as were current voltage relationships, indicating that the activation properties of the outward currents in Ib and Is neurons are similar.

The fast inactivating component of the whole cell outward current was isolated by subtracting currents obtained with a pulse to \(-40 \text{ mV} \) after holding at \(-100 \text{ mV} \) from total currents obtained after holding at \(-100 \text{ mV} \). Figure 6, A and B, shows example subtracted currents from MNISN-Is and MN6/7-Ib neurons. Figure 6C shows averaged subtracted currents for

FIG. 2. Identification of dorsomedial motor neuron targets. The targets of the dorsomedial neurons were identified by patching onto and filling GFP-positive neurons with rhodamine/Alexa 568 and following the axons onto the segmental muscles. A: a schematic of the relevant body-wall muscles of a single abdominal hemisegment. Muscles targeted by segmental nerve b/d (SNb/d) are shown in red, and those targeted by the intersegmental nerve (ISN) in blue. Muscles innervated by the dorsomedial motor neurons are shown in lighter tone. B–F: overlayed GFP and dye-fill images. B: MN1-Ib; C: MN6/7-Ib; D: MN14-Ib; E: MN30-Ib; F: MNISN-Is. Single arrow, bouton type Ib; double arrows, bouton type Is; and triple arrows, bouton type II. Scale bar = 25 \( \mu \text{m} \) for B–E, 10 \( \mu \text{m} \) for F.
multiple neurons ($n = 6$ for MNISN-Is and $n = 4$ for MN6/7-Ib). Although the amplitude of the prepulse-sensitive current is similar, the rate of decay was significantly different for the two neuron types, with a $\tau$ of $122 \pm 22$ ms for MNISN-Is and $54 \pm 12$ ms for MN6/7-Ib ($P < 0.05$).

Inactivation rate may contribute to the cell-specific difference in spike delay, but it is not likely to completely account for the magnitude. Using a range of prepulse amplitudes, we investigated the voltage dependence of prepulse inactivation in MN6/7-Ib and MNISN-Is. The normalized peak amplitudes of the resultant currents were plotted with respect to prepulse voltage amplitude, and a Boltzmann fit was applied. Figure 7 shows that there is a clear difference in the voltage dependence of inactivation. At a membrane potential of $-60$ mV, the amplitude of $I_A$ in MNISN-Is is at $\sim 90\%$, while for the MN6/7-Ib it is $<50\%$. At normal resting membrane potential, the activity of the transient current will therefore have a greater role in the MNISN-Is.

**DISCUSSION**

**Relationship of the larval dorsomedial cluster to the embryonic RP cluster**

While much is known about the proliferation and organization of specific neurons in the embryonic stage, only a few...
studies have detailed the subsequent developmental transformations that occur between the late embryonic stage and the third instar larval stage (Hoang and Chiba 2001; Landgraf et al. 2003; Schmid et al. 1999; Sink and Whitington 1991b). It is apparent that the organism undergoes significant morphological adjustments as the ventral ganglion retracts toward the anterior, and the larva grows exponentially in size and complexity. It is likely that considerable modification of the neural circuitry is also taking place. The dorsomedial cluster of neurons in the third instar ventral ganglion that we have observed bears a striking resemblance to the cluster of embryonic neurons containing aCC and RP1–4 with respect to spatial orientation. Like the larval cluster, the aCC and RP neurons are the most dorsal of the ganglion’s neurons, and the relative configurations of the somata and axonal processes seem to be generally consistent. Two of the embryonic neurons, aCC and RP3, appear to be wholly conserved in terms of projection and connectivity, corresponding to larval MN1-Ib and MN6/7-Ib, respectively. The identity of MN6/7-Ib as RP3 agrees with the findings of Hoang and Chiba (2001), who backfilled cell bodies from individual muscle terminals. In their study, there were no landmarks within the ventral ganglion to orient the cells with respect to each other, but the medial location and contralateral projection of MN 6/7-Ib, as well as its parallel dendritic arborizations, is well documented. The aCC neuron appears to be maintained as MN1-Ib, which uses an ipsilateral projection to connect to muscle 1. Both aCC and MN1-Ib have a major contralateral projection that terminates within the ventral ganglion as well as dendritic arborizations concentrated around the ipsilateral axonal tracts (Landgraf et al. 1997; Schmid et al. 1999). Single nerve backfills of embryonic and larval preparations showed that an embryonic neuron identified as aCC has a conserved larval counterpart that is directly lateral to the posterior of a group of cells resembling the RP cluster. MN14-Ib, MN30-Ib, and MNISN-Ib have muscle targets that do not match those of the embryonic RP neurons. RP1 and RP4 project contralaterally to innervate the proximal edge of muscle 13 in the embryo. MN14-Ib and MN30-Ib also have contralateral projections, but they go to different targets. If they are the

FIG. 5. Total outward currents in MNISN-Ib and MN6/7-Ib are equivalent. Outward currents were recorded in voltage clamp in the presence of TTX and 0 mM calcium. Cells were held at $V_m = -80$ mV and stepped to different potentials in 20-mV increments. A and B: sample traces of whole cell currents obtained from MN6/7-Ib and MNISN-Ib, respectively. Vertical scale bar is 50 pA; horizontal bar is 200 ms. C: I-V curves of average peak (solid lines) and steady-state (dashed lines) amplitudes of the outward currents in the MNISN-Ib and MN6/7-Ib neurons. Error bar indicates SE. $n = 5$ for MN6/7-Ib and 6 for MNISN-Ib.

FIG. 6. Isolated $I_h$ potassium currents in MNISN-Ib and MN6/7-Ib. A and B: sample traces of subtracted currents obtained in voltage clamp. A test pulse to $+40$ mV was given with the neuron held at $-40$ and $-100$ mV. Currents from the former were subtracted from the latter to isolate prepulse-sensitive currents. Vertical scale bar is 2 pA/pF; horizontal bar is 200 ms. C: averaged and normalized traces of the subtracted waveforms. $n = 4$ for MN6/7-Ib; $n = 6$ for MNISN-Ib. $t_{1/2}$ of inactivation is 27 ms for MN6/7-Ib and 71 ms for MNISN-Ib.
third instar incarnations of RP1 and RP4, it would imply that there has been retargeting between first and third instar. This possibility is supported by the fact that the arborization points for muscles 14 and 30 are in closest proximity to that of muscle 13; retraction of filopodia for RP1 and RP4 would place them at the appropriate branch point location. An alternate explanation would be that the MN14-Ib and MN30-Ib are new neurons and the cell bodies of RP1 and RP4 have shifted with respect to the location of RP3.

The third instar MNISN-Is is likely to correspond to the embryonic NB4–2-derived RP2 neuron. These two neurons both have somata that are one segment posterior to the rest of the dorsal cluster that project through the same nerve, and also have significant arborizations in the ipsilateral axonal fascicles. MNISN-Is innervates the majority of dorsal muscles that are targeted by the ISN (Table 1). In support of this identification, Landgraf et al. (2003) show comprehensive innervation of the dorsal muscles by RP2 in the third instar as identified by eve-Gal4RRK expression.

The ventral location of RP5 makes it an unlikely candidate to be any of the third instar dorsomedial cluster neurons (Sink and Whitington 1991a). However, on three occasions, we filled neurons with the MNSnb/d-Is innervation pattern (Table 1) that also had physiological characteristics similar to MNISN-Is (Fig. 3F; see following text). Although the organization of the intraganglionic projections were not recorded, observation of the ventral location of the soma and its medial position relative to the five neuron cluster makes it a possible RP5 derivative. RP5 has been observed on different occasions to innervate muscles 12 (Landgraf et al. 1997), 13, 15, 16, and 17 (Sink and Whitington 1991a) in earlier embryonic stages and muscles 6, 7, 12, 15, and 16 in late stage 17 (Schmid et al. 1999).

Our results support findings by others (Hoang and Chiba 2001; Sink and Whitington 1991b) that location of motor neurons and their axonal projections undergo major remodeling in the interval between the late embryonic and third instar stages. It may be significant that RP3 and aCC, the two neurons whose morphological character seem most conserved throughout development, are among the earliest motor neurons to differentiate, and that their targets are respectively the most proximal and distal longitudinal muscles to the midline. These two neurons may be conserved throughout development to be used as templates or guideposts in the formation and maintenance of the segmental organization of the neurons in the ventral ganglion and the axonal targeting of muscle innervation (Lin et al. 1995).

Intrinsic properties of ventral ganglion neurons are dictated by cell identity

The MNISN-Is and MNSNb/d-Is cells are outstanding among the dorsomedial motor neurons in threshold and spiking behavior, requiring more current to evoke spiking (Table 2) and having an appreciable delay between depolarization and the initiation of spiking (Fig. 3H). The delayed spiking phenomenon has been seen in other systems, where it has been shown that voltage-activated transient potassium currents ($I_A$) can cause a delay in depolarization toward threshold (Turrigiano et al. 1996; Zhao and Wu 1997). $I_A$ is also known to be important in the rendering of spike trains (Connor and Stevens 1971; Rogawski 1985) and has been shown to be the target of neuromodulators that affect motor neuron function (Harris-Warrick et al. 1998). The delay in MNISN-Is is prepulse (Fig. 4C) and 4-AP (Fig. 4A) sensitive; both of these manipulations are known to affect voltage-activated transient potassium currents $I_A$ (Socl and Aldrich 1988), suggesting a role for this current in the behavior of the type Is neurons.

Two fast-inactivating potassium channels are known to exist in Drosophila: Shaker and Shal. In studies of heterogeneous populations of cultured Drosophila embryonic neurons, a small
fraction were found to have both Shaker and Shal currents (Tsunoda and Salkoff 1995). Recordings from Sh mutants showed that the Shaker current does not have a significant role in soma-evoked spiking behavior (initial delay or rate) in any of the five motor neurons in this study. Thus the \(I_A\) current that controls the delay to first spike is likely to be Shal in both type Is and Ib neurons.

How then are the behaviors of type Is and Ib neurons differentiated? Distinct activation kinetics or amplitude differences are unlikely to underlie the cellular phenotypes. The \(I-V\) relationships for both the fast-inactivating and sustained outward currents were not statistically different (Fig. 5C). Using a prepulse subtraction protocol, we isolated the fast-inactivating potassium currents in MNISN-Is and MN6/7-Ib and found them to be similar in amplitude (Fig. 6, A and B). The major differences between the \(I_A\) currents in the two cell types was in their inactivation. \(I_A\) currents in MNISN-Is had a significantly longer time course of inactivation. This effect alone, while substantial, is unlikely to account for the magnitude of the difference in time to first spike in the two neuron types. An investigation of the voltage dependence of prepulse inactivation revealed a significant rightward shift for the MNISN-Is \(I_A\) as compared with that in MN6/7-Ib (Fig. 7). At the average resting potential (–61 mV) of MNISN-Is cells, the Shal current shows essentially no inactivation. In type Ib neurons, where resting membrane potential ranges from –58 to –54 mV, the current is already almost completely inactivated. Together, these differences in inactivation are likely to account for the disparity in the time to first spike that is seen between type Ib and Is motor neurons. There are also likely to be other currents (sodium, calcium, chloride) that are important determinants of the cell-specific properties of the type Ib and Is neurons, and their roles remain to be determined.

If both type Is and Ib neurons use Shal gene products to produce \(I_A\), the differences in inactivation are likely to involve alternative splicing, posttranslational modification of Shal channels or differences in auxiliary subunit expression. The Shal/Kv4 family is highly conserved across species (Baldwin et al. 1991; Baro et al. 1996; Pak et al. 1991), and all members of this gene family are alternatively spliced. Interestingly, there has been no demonstration of isoform-dependent variability in inactivation kinetics in heterologous expression systems (Baro et al. 2001; Po et al. 2001). Significant heterogeneity in Shal inactivation kinetics measured in situ, however, has been observed in cultured embryonic Drosophila neurons (Tsunoda and Salkoff 1995) and has also been seen in the lobster stomatogastric ganglion (Golowasch et al. 1999). In Drosophila, the shift between kinetic forms was very fast and seen at the single channel level, leading the authors to postulate phosphorylation as a mechanism of Shal modulation. Auxiliary subunit-independent changes in the voltage dependence of inactivation have been demonstrated for Kv4.3 and for lobster Shl (Decher et al. 2001; Zhang et al. 2003). Proteins of the KChip/Frequenin family were able to selectively modulate inactivation while minimally affecting activation. In Drosophila, a mutant that overexpresses Frequenin has alterations in \(I_A\) (Poulain et al. 1994). Differences in the Shal currents in third instar type Ib and Is motor neurons could be a consequence of differential expression of a member of the KChip/Frequenin family.

Implications of Ib/Is differences for locomotor activity

The similarities in firing behavior between MNISN-Is and MNSNb/d-Is and the similarities within the Ib neuron group suggests the possibility that there are inherent functional and molecular differences between these morphologically distinct neurons. Type Is neurons innervate groups of longitudinal and oblique muscles of a given hemisegment dorsally (MNISN-Is), ventrally (MNSNb/d-Is), and laterally (MNSNa-Is), allowing these neurons to control or modulate a large number of functionally related muscles in concert. This sharply contrasts with role of the Ib neurons which innervate only one or two muscles, and may act as muscle-specific “drivers.” Activation of (groups of) individual muscles by Ib neurons may provide a fine tuning of locomotion.

The delayed spiking mediated by Shal has been observed in other systems involving motor pattern generation. In Aplysia, L14 motor neurons, which control inking, fire action potentials only after a critical build-up of synaptic input that is able to inactivate transient potassium currents (Byrne et al. 1979). These neurons display strikingly similar spiking behavior to the Is neuron under current clamp. Whereas a sudden suprathreshold input will produce a delayed, slow spiking activity, a tonic subthreshold input could make instantaneous bursting activity more likely on suprathreshold input. This sort of activity dependence is particularly pertinent to the activity driving locomotor muscles as the particular sequence of driving input can affect contraction timing and amplitude. Also, the insensitivity of the neuron to short, nonsummating input may allow the motoneuron to act as a low-pass filter (Byrne et al. 1979). Further study of the differences between the two subtypes will elucidate how locomotor behavior is organized in the larval system.

Direct study of CNS neurons in the Drosophila third instar larva

The ventral ganglion preparation developed in this study has allowed direct access to identified central neurons in Drosophila. Previous studies using cultured larval neurons have documented the great diversity of morphological and electrophysiological properties in such preparations (Zhao and Wu 1997). A recent study by Rohrbough and Brodie in a similar ventral ganglion preparation has already shown great promise of this system as a means to study central synaptic transmission (Rohrbough and Brodie 2002). The stereotyped connectivity and functional properties of the motor neurons characterized in this study will allow genetics and pharmacology to be used to determine the molecular basis of modulation and cellular behavior. The function of specific motor neurons in multiply innervated muscle fibers as well as their role in the network of neurons effecting locomotor behavior can now be studied within the intact larval system.

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