Electrophysiological Differences in the CPG Aerial Respiratory Behavior Between Juvenile and Adult Lymnaea

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INTRODUCTION

Aerial respiration is an important homeostatic behavior in Lymnaea and compliments cutaneous respiration in providing oxygen for the animal (Jones 1961; Taylor et al. 2003). The respiratory central pattern generator (CPG) of Lymnaea stagnalis was reconstructed in culture (Syed et al. 1990), demonstrating that three identified interneurons (RPeD1, VD4, and IP3) were sufficient to produce a rhythm, which in vivo drives aerial respiratory behavior (Fig. 1). The rhythm produced by this circuit is the result of emergent network properties; none of the three cells in isolation produces a rhythmic output (Barnes et al. 1994; Lukowiak and Syed 1999; Syed et al. 1990). Using cell “killing” and cell transplantation techniques, it was further shown that the three neurons were necessary for aerial respiratory behavior (Haque 1999; Lukowiak and Syed 1999; Scheibenstock et al. 2002; Syed et al. 1992). This preparation is a powerful model system in which to make observations and predictions about CPG activity, rhythm generation and neuronal mechanisms of behavior.

Because aerial respiration only occurs periodically, CPG activity is not always “on.” CPG activity is initiated when RPeD1 receives excitatory mechanosensory or chemosensory input from the pneumostome (the respiratory orifice) area, causing it to become more active (e.g., when the snail reaches the water’s surface and the pneumostome can open) (Inoue et al. 2001; Syed et al. 1990) (Fig. 1). RPeD1, in turn, synapses onto two interneurons, IP3 (the interneuron responsible for pneumostome opening) and VD4 (the interneuron responsible for pneumostome closing). RPeD1 makes a biphasic (inhibitory/excitatory) chemical synaptic connection to IP3 and makes an inhibitory synapse with VD4. VD4, in turn, makes an inhibitory chemical synapse back onto RPeD1 and also makes an inhibitory synaptic connection to IP3. IP3 makes an inhibitory synaptic connection back onto VD4, forming an antagonistic “half-center” (Brown 1911) (i.e., expiration-inspiration). IP3 also makes an excitatory synaptic connection to RPeD1. This is the only central excitatory connection known to exist to RPeD1 from within the central ring ganglia (Spencer et al. 1999, 2002; Syed 1988; Syed and Winlow 1991a, b), and it typically re-excites RPeD1 sufficiently to initiate further cycles of CPG activity (Lukowiak 1991a,b; Syed 1988; Syed et al. 1990). Because IP3 lies buried internally beneath the ventral surface of the left parietal ganglion, IP3 cannot be recorded from simultaneously with RPeD1, VD4, or any of its known follower motor neurons. Thus unless IP3 is isolated from the ganglion and cultured along with the other members of the CPG, it is impossible to record from all three neurons simultaneously (Syed et al. 1990). Recording from one of IP3’s follower motor neurons (e.g., VI/J) together with RPeD1 is the best indirect method for assaying IP3 activity in either semi-intact or isolated ganglionic preparations (Inoue et al. 2001; Spencer et al. 2002; Syed 1989; Syed et al. 1990; Syed and Winlow 1991a).

As in other CPGs, peripheral feedback and central neural modulation play important and necessary roles in sculpting the rhythmic output appropriate for the specific environmental situation (Le Feuvre et al. 1999; Pearson 2000). Aerial respiratory CPG activity is modified by inputs, both excitatory and inhibitory, from the periphery to ensure that the snail meets its oxygen requirements (Inoue et al. 2001; Syed 1988; Syed and Winlow 1991a; Taylor and Lukowiak 2000; Wedemeyer and Schild 1995). Peripheral excitatory mechanosensory input to RPeD1, initiated when the pneumostome breaks through the water’s surface, combined with excitatory chemosensory input from the pneumostome area and oesphradial ganglion, pro-
here that juvenile RPeD1s are significantly more excitable than adults, have a higher spontaneous firing rate, and yet drive aerial respiration significantly less often than adults.

METHODS

Animals

Specimens of *L. stagnalis* originally obtained from Vrije Universiteit were bred and raised in tanks in the snail facility at the University of Calgary. Snails were maintained in well-aerated pond water (de-chlorinated City of Calgary tap water) and had continuous access to food (Romaine leaf lettuce). *Lymnaea* were selected and classified on the basis of shell length, which is a useful index of age (McComb and Lukowiak, unpublished results). Snails were measured at the longest part of the shell, from the shell’s base to the apex of the spiral.

Juveniles and adults used in this study had average shell lengths of 1.5 cm (7.5 wk old) and 2.5 cm (13 wk old), respectively.

Isolated ganglionic preparation

Animals were anaesthetized for 7 min in a solution consisting of 40% Listerine and 60% normal *Lymnaea* saline [containing (in mM) 51.3 NaCl, 1.7 KCl, 4.1 CaCl₂, and MgCl₂] buffered to pH 7.9 using HEPES, as previously described (Spencer et al. 1999, 2002; Syed and Winlow 1991a). The CNS was dissected from the animal and pinned out dorsal-side up in a recording dish containing normal *Lymnaea* saline. The outer sheath surrounding the ganglia was removed using fine forceps.

Semi-intact preparation

The preparations were prepared as previously described (Inoue et al. 2001; Spencer et al. 1999, 2001). Briefly, after anesthesia, a dorsal incision was made before removing the buccal mass, esophagus, penis, stomach, and upper half of the body. The CNS, the pneumostome, and the nerves from the CNS to the pneumostome were left intact. Preparations were pinned down in individual recording dishes with their ventral sides uppermost. The central ring ganglia (CNS) were flipped over and pinned out dorsal-side up. The outer sheath surrounding the CNS was then removed using fine forceps. Following this procedure, semi-intact preparations exhibited pneumostome movements (opening and closing) only if the saline level in the recording dish was such that the pneumostome area was not submerged. We did not have to make the bathing solution more hypoxic by bubbling N₂ through the recording chamber to induce sufficient pneumostome openings for the various analyses performed.

Standard electrophysiological techniques were used as previously described in *Lymnaea* semi-intact preparations (Inoue et al. 2001; Spencer et al. 2002). Intracellular recordings were obtained using sharp glass microelectrodes filled with saturated K₂SO₄ solution. Tip resistances of the microelectrodes used for recordings ranged from 30 to 80 MΩ. Intracellular signals were amplified via a NeuroData amplifier and displayed simultaneously on a Macintosh PowerLab/4SP (AD instruments) and a Hitachi oscilloscope. Recordings were analyzed and stored using the PowerLab software.

Intrinsic membrane properties of RPeD1

Isolated ganglionic and semi-intact preparations were given 1 h to recover from surgical trauma following dissection and to allow the effects of the anaesthetic to wear off. RPeD1 cells were allowed 10 min to recover from minor damage to the membrane due to impaling before recordings of electrical activity were obtained for use in analyses. Spontaneous activity was recorded for a period of 10 min to obtain a value of the frequency of RPeD1 activity. Depolarizing current was injected into RPeD1 to determine a value for the minimum current required to elicit spiking (i.e. rheobase current). Square hyperpolarizing current pulses (10-s duration) were injected into the cell within the linear range of the membrane. Values for the time constant (the time to reach 67% of the cell’s maximum current carrying capacity) were acquired by measuring the time for the cell to reach 67% of its maximum voltage displacement. Square depolarizing and hyperpolarizing current pulses (10-s duration) were injected into the cell to acquire an IV curve. The slope of the IV curve for each cell was used to calculate the input resistance.

Resting membrane potential

The voltage displacement was observed both when the cell was impaled and when the electrode was removed at the end of the recording. In this study, values for the membrane potential were obtained at the end of the recording. However, in all successfully
completed experiments the membrane potential at the end of the experiment did not differ by >1 mV from its initial value 10 min after impalement.

**Action potential parameters**

Three action potentials per cell were measured during the 10-min recording of spontaneous activity and averaged to obtain values for the amplitude (mV), duration (ms), and undershoot (mV). Amplitude was measured from the cell’s baseline to the peak of the action potential. Duration was determined as the time between the depolarization and repolarization phases at half-amplitude. The undershoot was measured from the cell’s baseline to the lowest point of the repolarization phase.

**Peripheral suppression experiments**

Semi-intact preparations were given 1 h to recover from surgical trauma. RPeD1 was then impaled and allowed 10 min to recover from minor damage to the membrane. The activity of the cell was then recorded for a period of 10 min to determine the frequency of spontaneous activity. The nerves connecting the CNS to the periphery were subsequently cut, transforming the semi-intact preparation into an isolated ganglionic preparation. Preparations were given another 1-h period to recover from trauma due to cutting. A second 10-min recording of the cell’s spontaneous activity was obtained. In control preparations, two 10-min recordings of spontaneous activity were performed 1 h apart without cutting the nerve fibers.

**Rhythmic activity of the respiratory CPG**

Over a 10-min period, breathing behavior (pneumostome openings) and RPeD1 and VI/J activity were monitored simultaneously. Pneumostome openings were observed visually and marked directly on the electrophysiological traces using the PowerLab software. The number of IP3-induced bursts was determined for the 10-min recording period. IP3-induced bursts were classified into three types: situation 1, situation 2, and situation 3 (Fig. 7). Briefly, in situation 1 IP3-induced bursts, pneumostome opening and excitatory IP3-induced burst activity in both RPeD1 and a VI/J cell were observed. In situation 2 bursts, excitatory IP3-induced burst activity was observed in both RPeD1 and a VI/J cell but there was no pneumostome opening. In situation 3 bursts, pneumostome opening and excitatory IP3-induced burst activity in a VI/J cell were observed but there was no excitatory activity in RPeD1.

**Number of spikes per burst**

The number of action potentials occurring in an IP3-induced burst (situation 1, 2, or 3; see Fig. 7) in a VI/J cell was measured to obtain a value of the number of spikes per burst. The duration of an IP3-induced burst (situation 1, 2, or 3) in the J-Cell was also measured.

**Depolarization of RPeD1 and VI/J to elicit a pneumostome opening**

The ability of electrical stimulation (±1.5 nA, for 2 s) to induce a burst of APs in RPeD1 and VI/J cell to trigger pneumostome openings was tested in all preparations. Semi-intact preparations were only experimented on where direct depolarization of RPeD1 and VI/J caused a pneumostome opening.

**Statistics**

Data between groups were analyzed using the two-tailed Student’s t-test (independent groups). Data within groups were subjected to an analysis of variance (1-way ANOVA) followed by a post-hoc-protected t-test. Differences were considered to be significant if $P < 0.05$. Data are expressed as percentages or as means ± SE.

**RESULTS**

Juvenile *Lymnaea* perform aerial respiration significantly less often (i.e., fewer pneumostome openings) and for less time (i.e., total breathing time) than do adult snails under both eumoxic and hypoxic conditions (McComb 2002; McComb and Lukowiak, 2003). Therefore we wished to investigate whether there were also significant age-related differences in the intrinsic membrane properties of the CPG neurons, their synaptic connections, and the overall activity of this neuronal circuit. Based on the behavioral findings, the most parsimonious hypothesis was that RPeD1, which initiates rhythmogenesis, would exhibit less spontaneous activity in juveniles compared to adults.

We first determined whether RPeD1’s size changed with age. Cell size has a significant impact on the behavior mediated by that specific neuron and also on the cell’s intrinsic membrane properties (Edwards et al. 1994a,b; Henneman et al. 1965). RPeD1 cell size was measured both in isolated ganglionic preparations ($n = 12$ for juveniles and 12 for adults) using the method Klaasen et al. (1998) and following the removal of the somata for culture purposes. In both cases, similar data were obtained, and we show here the somata of adult and juvenile RPeD1 following their removal from the CNS. RPeD1 had a greater diameter in adults (93.33 ± 3.33 µm) compared to juveniles (59 ± 2.45 µm, $P < 0.005$; Fig. 2).

As neurons increase in size, they often become less excitable (i.e., the “size principle”) (Henneman et al. 1965), and this contributes to changes observed at the behavioral level (Edwards et al. 1994a,b). However, in some organisms, membrane properties are conserved despite significant neuronal growth (Hill et al. 1994). Therefore a number of the intrinsic membrane properties were measured to gain insight into the possible differences between adult and juvenile RPeD1 cells (Table 1).

In isolated brain preparations, the frequency of spontaneous
TABLE 1. Intrinsic membrane properties of RPeD1 neurons in adults compared to juveniles in both semi-intact and isolated brain preparations

<table>
<thead>
<tr>
<th>Intrinsic Membrane Properties</th>
<th>Semi-Intact</th>
<th>Isolated</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude, mV</td>
<td>Adult: 63.47 ± 1.59</td>
<td>Juvenile: 61.44 ± 1.4</td>
<td>NSD</td>
</tr>
<tr>
<td>Duration, ms</td>
<td>0.39 ± 0.02</td>
<td>0.336 ± 0.02</td>
<td>NSD</td>
</tr>
<tr>
<td>Rheobase, nA</td>
<td>11.44 ± 0.87</td>
<td>10.96 ± 0.96</td>
<td>NSD</td>
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<tr>
<td>Time constant, ms</td>
<td>15.86 ± 2.025</td>
<td>7.42 ± 0.88</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Membrane resistance, mΩ</td>
<td>48.06 ± 4.99</td>
<td>60.82 ± 8.46</td>
<td>NSD</td>
</tr>
<tr>
<td>Resting membrane potential, mV</td>
<td>58.32 ± 1.03</td>
<td>57.4 ± 1.08</td>
<td>NSD</td>
</tr>
<tr>
<td>Frequency, Hz</td>
<td>0.155 ± 0.03</td>
<td>0.338 ± 0.04</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Intrinsic membrane properties of RPeD1 cells in adults and juveniles. Summary of the intrinsic membrane properties of RPeD1 neurons in adults compared to juveniles in semi-intact and isolated ganglionic preparations. In the semi-intact preparation, the rheobase current and the membrane time constant were significantly higher in the RPeD1 cells of adults compared to juveniles (P < 0.05 and P < 0.01, respectively). The frequency of RPeD1 activity was significantly lower in the semi-intact preparations of adults compared to juveniles. In the isolated ganglionic preparation, the rheobase current and the time constant were also found to be significantly higher in the RPeD1 cells of adults compared to juveniles (P < 0.05 and P < 0.01, respectively). The membrane resistance was significantly lower in adult compared to juveniles RPeD1 neurons (P < 0.05). The frequency of RPeD1 activity was not significantly different in the isolated brain preparations of adults compared to juveniles (P > 0.05). All data are the means ± SE. n = 21, 20, 14, and 15 for semi-intact adults and juveniles and isolated adults and juveniles, respectively.

RPeD1 activity was not significantly different between adults (0.43 ± 0.05 Hz) and juveniles (0.55 ± 0.05 Hz, P > 0.05; Fig. 3A). In addition, the resting membrane potential and action potential (AP) amplitude, duration, and undershoot were also not significantly different in adults versus juveniles (P > 0.05). In contrast, significant age-related differences were detected in the intrinsic membrane properties that were directly related to the cell’s dimensions and excitability. First, the rheobase current, the minimum depolarizing current to elicit spiking, was significantly higher in the RPeD1 cells of adults (0.17 ± 0.04 nA) compared to juveniles (0.06 ± 0.01 nA; P < 0.05). Second, the membrane resistance was significantly lower in adult compared to juveniles RPeD1 neurons (P < 0.05). The frequency of RPeD1 activity was not significantly different between adults and juveniles (P > 0.05). The rheobase current and the time constant were significantly higher in adults (14.63 ± 1.79 ms) versus juveniles (8.47 ± 1.07 ms; P < 0.01). Third, the input resistance was found to be significantly lower in the RPeD1 neurons of adults (43.18 ± 5.85 MΩ) compared to juveniles (62.59 ± 5.00 MΩ; P < 0.05). Thus in isolated ganglionic preparations, there were significant differences in certain intrinsic membrane properties between juvenile and adult RPeD1’s, but overall the spontaneous level of RPeD1 activity, although higher in juveniles, was not statistically different from adults.

We next determined if there were differences in peripheral modulation of CPG activity between adults and juveniles (Table 1). In contrast to isolated ganglionic preparations, the frequency of spontaneous RPeD1 activity was significantly higher in juveniles (0.34 ± 0.04 Hz) compared to adults (0.16 ± 0.03 Hz; P < 0.01; Fig. 3B). As in isolated ganglionic preparations the resting membrane potential and AP amplitude, duration, and undershoot were not significantly different between adult and juvenile RPeD1 cells (P > 0.05). The rheobase current and the time constant were significantly higher in adults (0.21 ± 0.03 nA and 15.86 ± 2.03 ms, respectively) compared to juveniles (0.12 ± 0.02 nA and 7.42 ± 0.88 mV, respectively; P < 0.05 and P < 0.01). However, the input resistance was not found to be significantly different between adults (48.06 ± 4.99 MΩ) and juveniles (60.82 ± 8.46 MΩ; P > 0.05).

Contrary to our expectations, juvenile RPeD1 cells exhibited significantly higher spontaneous activity compared to adult RPeD1 cells in semi-intact preparations; yet freely behaving juveniles perform aerial respiration significantly less often than adults. We therefore hypothesized that there would be more suppressive input from the periphery in adults compared to juveniles. More suppressive input to the respiratory CPG in...
adults would explain the following two findings: significantly higher RPeD1 activity in juveniles compared to adults in the semi-intact preparation and no significant differences in RPeD1 activity in the isolated brain preparation. To test this hypothesis, experiments were designed in which recordings of RPeD1 activity were obtained in preparations before and after removing the peripheral input to the CNS (Fig. 4A). Initially, to determine if spontaneous RPeD1 activity changed (i.e. run-down or increased activity) over the course of the experiment, “run-down” controls were performed. When two 10-min recordings were obtained 1 h apart without cutting the connectives from the periphery, RPeD1 activity did not change significantly in either adult (P > 0.05) or juvenile (P > 0.05) preparations (Fig. 4B).

Recordings of RPeD1 spontaneous activity (10-min) were then obtained in semi-intact adult and juvenile preparations, and after this period, the nerves connecting the periphery to the CNS were severed, removing all peripheral input and transforming the previous semi-intact preparation into an “isolated brain preparation.” After a 1-h recovery period, a second 10-min recording of RPeD1 spontaneous activity was obtained (Fig. 4C). There was a 2.64-fold increase in RPeD1 activity in adults compared to a 1.64-fold increase in juveniles after the removal of the peripheral input to the CNS. Figure 5 shows representative examples (electrophysiological data) of RPeD1 activity before and after removing the peripheral input to the CNS. These data are consistent with the hypothesis that there is more suppression from the peripheral nervous system in adults compared to juveniles.

Next we tested whether aerial respiratory behavior (i.e. number of pneumostome openings and total breathing time) was different in juvenile versus adult in vitro semi-intact preparations as they are in intact snails (Fig. 6). We found that the juvenile in vitro semi-intact preparations exhibited significantly less aerial respiratory behavior compared to adult in vitro semi-intact preparations. Total breathing time and the mean number of breaths were both found to be significantly lower in juveniles compared to adults (P < 0.05). The average breathing time (data not plotted) was also found to be significantly lower in juveniles compared to adults (P < 0.05).
data show that as in freely moving, intact Lymnaea, adult semi-intact preparations perform aerial respiration significantly more often than do semi-intact preparations obtained from juveniles.

We next ascertained, in the in vitro semi-intact “behaving” preparations, the “effectiveness” of spontaneously occurring respiratory rhythmogenesis in the CPG network to cause pneumostome openings in juveniles and adults. Because we are unable to simultaneously record from IP3 and the other CPG neurons simultaneously, we made intracellular recordings from RPeD1 and VI/J (a pneumostome opener motor neuron) to monitor IP3-induced bursts. IP3 sends distinctive excitatory inputs to both RPeD1 and to VI/J motor neurons. We therefore characterized IP3-induced bursts as an index of synaptic connectivity within the CPG circuit. Three different IP3-induced burst situations were observed (Fig. 7): situation 1 (an IP3-induced burst of action potentials in both RPeD1 and a VI/J cell and a pneumostome opening), situation 2 (an IP3-induced burst of action potentials in both RPeD1 and a VI/J cell, in the absence of pneumostome opening), and situation 3 (a pneumostome opening and an IP3-induced burst of action potentials in a VI/J cell, but not in RPeD1). We found that adults exhibited significantly more situation 1 type IP3-induced bursts ($P < 0.05$) compared to juveniles. Adults had 50% less situation 2 events as juveniles (6 vs. 13%, respectively) and also had half as many situation 3 events as juveniles (16 vs. 34%, respectively). Thus in adults, the IP3-induced bursts were more effective in causing concomitant activity in RPeD1 and VI/J cells and a pneumostome opening even though juvenile RPeD1s are more easily excitable (Fig. 7).

These findings led us to ask whether there were age-related differences in the IP3-induced bursts parameters in RPeD1 (Table 2). The number of IP3-induced burst observed was not significantly different between adults and juveniles ($P > 0.05$). However, IP3-induced bursts in adults had significantly more spikes ($P < 0.01$) and were of significantly longer duration ($P < 0.05$) compared to juveniles. We also examined the interspike interval (ISI) for each IP3-induced burst. Adults displayed a significantly lower average ISI compared to juveniles ($P < 0.05$). However, the minimum and maximum ISIs were not significantly different between adult and juvenile preparations ($P > 0.05$).

Induced spiking activity in RPeD1 and VI/J, by the passage of depolarizing current through the recording electrode, elicits a pneumostome opening movement. Depolarizing current was injected alternately into RPeD1 and VI/J cell, and the level of current required to induce pneumostome opening was reduced by 10.22 ± 3.6% on June 22, 2017 http://jn.physiology.org/ Downloaded from

![Figure 6](http://jn.physiology.org/)

**FIG. 6.** Aerial respiratory behavior in the semi-intact preparation. There was significantly more aerial respiratory behavior in the semi-intact preparations of adults compared to juveniles. Breathing observations were performed over a 10-min period. **A**: total breathing time was significantly higher in adults compared to juveniles ($P < 0.05$). **B**: the mean number of breaths (pneumostome openings) was significantly higher in adults compared to juveniles ($P < 0.05$).

![Figure 7](http://jn.physiology.org/)

**FIG. 7.** IP3-induced burst activity in adults and juvenile semi-intact preparations. Adults displayed more situation 1 type IP3-induced bursts compared to juveniles. Adults had also exhibited ~50% less situation 2 and 3 type IP3-induced bursts than did juveniles ($P > 0.05$).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Adults</th>
<th>Juveniles</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of IP3 Bursts</td>
<td>9.7 ± 0.998</td>
<td>7.5 ± 1.522</td>
<td>NSD</td>
</tr>
<tr>
<td>No. of spikes per burst</td>
<td>50.43 ± 3.97</td>
<td>33.38 ± 2.91</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Duration of burst, s</td>
<td>5.74 ± 0.28</td>
<td>4.31 ± 0.31</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Minimum ISI, ms</td>
<td>76.46 ± 7.53</td>
<td>98.36 ± 10.03</td>
<td>NSD</td>
</tr>
<tr>
<td>Maximum ISI, ms</td>
<td>227.26 ± 10.81</td>
<td>257.04 ± 17.36</td>
<td>NSD</td>
</tr>
<tr>
<td>Average ISI</td>
<td>125.92 ± 8.12</td>
<td>152.82 ± 9.94</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

IP3-induced burst parameters in adults and juveniles. Summary of the differences in IP3-induced burst parameters in the semi-intact preparations of adults compared to juveniles. Adults exhibited significantly more action potentials per IP3-induced burst ($P < 0.01$) and significantly longer IP3-induced bursts ($P < 0.05$) compared to juveniles. The average interval between spikes in the IP3-induced burst was significantly higher in adults ($P < 0.05$). There was no significant difference between the minimum and maximum interval between spikes in the IP3-induced burst in adults compared to juveniles. ISI, inter-spike interval. All data are the means ± SE.
corded. Significantly more current was necessary in both RPeD1 ($P < 0.05$) and VI/J ($P < 0.01$) to cause pneumostome opening in adults compared to juveniles. Specifically, in adults $0.95 \pm 0.07$ and $0.91 \pm 0.08$ nA of current injected into RPeD1 and VI/J, respectively, was required to cause pneumostome opening. In juveniles, only $0.68 \pm 0.08$ and $0.47 \pm 0.08$ nA of depolarizing current injected into RPeD1 and VI/J, respectively, was required to induce a pneumostome opening movement.

Figure 8 shows representative data traces of rhythmic respiratory activity (neurophysiology and behavior) in adults and juveniles. Adults exhibited pneumostome opening (aerial respiration) during 70% of the 10-min observation period, whereas juveniles showed pneumostome opening only 30% of the time. Traces were selected to reflect this difference in respiratory behavior.

**DISCUSSION**

Since freely behaving juvenile *Lymnaea* show significantly reduced aerial respiratory behavior compared to adults (Mc-Comb 2002; McComb and Lukowiak, unpublished results), it seemed reasonable to suppose that these behavioral differences would be similarly reflected at the neuronal level. Because an increase in RPeD1 activity is necessary to initiate respiratory rhythmogenesis and aerial respiration, we hypothesized that there would be less spontaneous activity in RPeD1 of juvenile snails compared to adult snails. However, contrary to this hypothesis, spontaneous RPeD1 activity was found not to be significantly lower in juveniles than in adults in isolated ganglionic preparations and actually to be significantly higher in the semi-intact preparations of juveniles compared to adults. We therefore designed experiments to determine why RPeD1 cells of juveniles are more active (i.e., higher spontaneous activity) than those in adults and yet not result in increased aerial respiratory behavior.

Henneman et al. (1965) proposed the “size principle” to explain observed differences in neuronal excitability in the mammalian spinal cord, stating that smaller cells are more excitatory than larger cells. We first determined if there were age-related differences in RPeD1 cell size and found that the RPeD1 soma diameter was significantly smaller in juveniles compared to adults. Our data are complementary to earlier data of Klaasen et al. (1998), who examined RPeD1 cell size and excitability over an age range of 3–16 mo. Their 3-mo-old snails correspond to our adults and RPeD1 in their 3-mo-old snails was of a similar size and had similar electrophysiological properties as we report here. As their snails aged a further 6 mo, RPeD1 continued to significantly increase in size with a concomitant significant decrease in input resistance and a significant increase in current needed to produce APs. In their study, like ours, the RMP of RPeD1, which had a similar value to the value reported here, did not change with the size of the neuron. Together the two independent studies show that as RPeD1 becomes larger, its RMP does not alter but it becomes less excitabile. That is, the input resistance (greater in juveniles), time constant (smaller in juveniles), and rheobase current (lower in juveniles), make juvenile cells significantly more excitable than adult RPeD1s. The data presented here as well as the Klaasen et al. (1998) data are in general agreement with the Henneman size principle and confirm reports from other studies using both invertebrate (Atwood 1992; Edwards et al. 1994a,b; Hatakeyama and Ito 2000; Peretz and Lukowiak 1975; Pawson and Chase 1985; Zacharov and Balaban 1987) and vertebrate preparations (Bao et al. 1995; Cepeda et al. 1992; Martin-Caraballo and Greer 1999; Wu and Oertel 1987). Thus adult RPeD1 are less excitable than the smaller juvenile RPeD1 neurons.

While there was a significant age-related difference in the spontaneous activity of RPeD1 in semi-intact preparations, this difference was not observed in isolated brain preparations. Therefore differences in cell size and the intrinsic membrane properties of RPeD1 alone were not sufficient to account for all...
age-related differences in RPeD1 activity. In adult *Lymnaea*, the peripheral pneumostome area, including the oesphradial ganglion, exerts a suppressive regulatory control over the respiratory CPG (Inoue et al. 2001). Therefore we tested the hypothesis that the periphery in adults exerts more suppressive control over the CPG than it does in juveniles. When we examined RPeD1 activity before and after removing the peripheral input in the same preparation by cutting the nerves that innervate the pneumostome area, on average there was a 2.75-fold increase in RPeD1 activity in adults compared to a 1.64-fold increase in juveniles. There are, therefore, age-related differences in suppression exerted on the CPG by neurons in the pneumostome area. Thus another age-dependent factor leading to increased excitability of juvenile RPeD1 is less suppressive input from the periphery. It is unclear why there is this difference in peripheral input from the pneumostome area to the respiratory CPG. It is also unclear what peripheral elements are responsible for the suppression. Inoue et al. (2001) speculated that chemosensory neurons responsible for the detection of hypoxia located in or near the oesphradial ganglion were responsible for the suppression, but this has not yet been experimentally confirmed.

In addition to the significant difference in RPeD1 spontaneous activity in semi-intact preparations, less depolarizing current has to be injected into juvenile RPeD1 and VI/J neurons to both initiate spiking activity and pneumostome opening. Yet juvenile semi-intact preparations performed aerial respiratory less often than adults. A possible reason for this is that the CPG circuit that drives aerial respiration does not function optimally in juveniles. We examined this possibility. We first determined the properties of the spontaneous IP3-induced bursts in RPeD1 and VI/J in adults and juvenile semi-intact preparations and then assessed the effectiveness of the spontaneous IP3-induced bursts to elicit a pneumostome opening.

Spencer et al. (1999) previously used IP3-induced bursts of activity in RPeD1 and VI/J cells in isolated ganglionic preparations as a measure of aerial respiratory CPG activity in operantly trained versus naïve or yoked control preparations. They found that changes in these bursts correlated well with conditioned versus unconditioned snails. That is, in snails trained not to perform aerial respiration, the number and duration of IP3-induced bursts were significantly less than in controls. When we measured the properties of the IP3-induced bursts in adults and compared them to what we observed in juveniles, we found that IP3-induced bursts in adults had significantly more spikes and were of significantly longer duration compared to juveniles. In addition, while the number of IP3-induced bursts between adults and juveniles were statistically equivalent, there were numerically more IP3-induced bursts in adults. Thus even though juvenile RPeD1’s are more easily excitable than adult RPeD1’s, the excitatory input to RPeD1 from IP3 is more effective in adults than it is in juveniles to promote continued CPG activity.

We also assayed the effectiveness of the CPG to drive aerial respiration by monitoring the coincidence of IP3-induced bursts in RPeD1 and VI/J with a pneumostome opening and found that adults showed a much higher coincidence than juveniles (78 vs. 53%, respectively). On the other hand, there were twice as many instances in juveniles compared to adults (13 vs. 6%, respectively) when we detected IP3-induced bursts in RPeD1 and VI/J with no pneumostome opening. Even though it was unlikely that an IP3-induced burst would only elicit spiking activity in a VI/J cell and cause a pneumostome opening (a situation 3 event) if it occurred, it was most likely to happen in juveniles. This may be due to the fact that the VI/J cell is more easily excited in juveniles. Together the data suggest that there are differences in synaptic connectivity and orchestration of the respiratory neural network between adults and juveniles, such that it is easier for the respiratory network to produce an effective rhythmic output in adults, as judged by the occurrence of aerial respiratory behavior, compared to juveniles. We conclude that the respiratory network in adults operates more effectively to cause aerial respiratory behavior than it does in juveniles.

Why does the adult respiratory network operate more effectively than the respiratory network in juveniles? To answer that question, we first have to examine just how rhythmogenesis is produced. In culture or in isolated ganglionic preparations, respiratory rhythmogenesis is initiated by induced activity in RPeD1 (Syed et al. 1990). In semi-intact preparations, it is only when there is sufficient excitatory input (such as the depolarizing mechanosensory input from breaking through the water’s surface coupled with excitatory chemosensory input) leading to increased APs in RPeD1, that a biphasic (inhibition followed by excitation) response is recorded in IP3 (Syed 1988; Syed et al. 1990). APs are evoked in IP3 after the synaptic input to it from RPeD1, and this activity re-excites RPeD1 resulting in further APs in it, which ultimately leads to the initiation of APs in VD4 (i.e. rhythmogenesis of the respiratory CPG) (Lukowiak 1991a,b; Syed et al. 1990, 1991). However, when adult RPeD1s were made tonically hyperactive by the injection of depolarizing current, IP3-induced bursting activity was inhibited resulting in less rhythmicity of the respiratory CPG (Haque 1999; Syed 1988). As Turrigiano (1999) has reported, most neurons display a limited range of firing capabilities and maintain activity levels that fall within their functional boundaries. The cell’s firing rate is typically regulated to preserve the efficacy of synaptic transmission (Turrigiano 1999). Because the level of spontaneous activity displayed by juvenile RPeD1 cells is high, they may be operating above their “optimal” range, impeding their ability to send and receive inputs. Thus even though RPeD1 is more active it cannot as effectively orchestrate the production of the rhythm necessary to drive aerial respiratory behavior.

An experiment to directly test the possibility that RPeD1 cells of juveniles are operating above their optimal range, leading to reduced rhythmic activity of the CPG, would be to compare the “circuit properties” of the CPG network between adult and juvenile snails in reconstructed CPGs in culture (Syed et al. 1990). Thus a circuit made up of exclusively adult neurons could be compared to one made from only juvenile neurons. Such a direct examination of adult versus juvenile networks would provide a means of ascertaining whether there are intrinsic differences in the respective neural circuits. It might also be possible to directly determine how, or if, “emergent” properties of the network (Lukowiak 1991a) differ between adults and juveniles. It would also be possible in these experiments to compare the differences in properties of both IP3 and VD4
between adult and juvenile animals. We do not believe that it is only differences in RPeD1 that account for the differences in aperistalsis behavior between adult and juvenile *Lymnaea*. However, because RPeD1 is the neuron that initiates rhythmogenesis and because RPeD1 has been studied more often than the other CPG neurons, it seemed to be the logical place to start the investigation of age-related differences in a neuron that contribute to age-related differences in behavior.

A similar situation, as regards the age-dependent maturation of a CPG, is that of the somatogastric nervous system (STNS) in lobster. The embryonic STNS expresses a unique rhythmic output, which is dependent on specific neuromodulatory input, that is different from that expressed by adults (Le Feuvre et al. 2001). In the adult the single “embryonic network rhythm” is “split” into different functional networks (Casasnovas and Meyrand 1995); the expression of each is controlled by specific central modulatory systems (Richards and Marder 2000). However, the embryonic STNS has the capability of expressing adult-like patterns (Le Feuvre et al. 1999) with pharmacological manipulation. It appears that the adult network results not from a progressive ontogenetic change in the networks but rather from maturation of neuromodulatory systems and synaptic interactions already present in the embryonic STNS (Bem et al. 2002).

The maturation of modulatory input from the periphery to RPeD1 and age-related changes in CPG synaptic inputs to it combined with age-related changes in RPeD1’s intrinsic membrane together allow the aperistalsis CPG to function at a significantly higher rate in adults compared to juveniles. These age-dependent neuronal changes are necessary because adults rely significantly more on aeral respiration to obtain their required oxygen than do juveniles, whose size to volume ratio favors cutaneous respiration (McComb and Lukowiak, unpublished data).

REFERENCES


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