
Stefan R. Pulver, Dirk Bucher, David J. Simon,* Eve Marder

Volen Center and Biology Department, Brandeis University, Waltham, Massachusetts 02454

Received 10 March 2004; accepted 12 April 2004

**ABSTRACT:** As lobsters grow from early juveniles to adults their body size increases more than 20-fold, raising the question of how function is maintained during these ongoing changes in size. To address this question we studied the pyloric 1 (p1) muscle of the stomach of the lobster, *Homarus americanus*. The p1 muscle receives multiterminal innervation from one motor neuron, the lateral pyloric neuron of the stomatogastric ganglion. Staining with antibodies raised against synaptotagmin showed that as the muscle fibers increased in length, the spacing between the terminal innervation increased proportionally, so the number of synaptic contact regions/muscle fiber did not change. Muscle fibers were electrically coupled in both juveniles and adults. The amplitude of single intracellularly recorded excitatory junctional potentials evoked by motor nerve stimulation was the same in both juveniles and adults. Nonetheless, the peak depolarizations reached in response to ongoing pyloric rhythm activity or in response to high-frequency trains of stimuli similar to those produced during the pyloric rhythm were approximately twofold larger in juveniles than in adults. This suggests that homeostatic regulation of synaptic connections may operate at the level of the amplitude of the single synaptic potential rather than on the summed depolarization evoked during strong rhythmic activity.


**Keywords:** central pattern generators; electrical coupling; summation; homeostasis; stomatogastric ganglion; crustaceans

---

**INTRODUCTION**

Animals such as lobsters, turtles, and rats grow significantly after they reach adult form. Lobsters be-
muscle action potentials as the signal for contraction, most crustacean muscles are not electrically excitable. Instead, depolarization of the entire muscle fiber is achieved by multiterminal innervation, in which the motor neuron axon branches and makes multiple synaptic contacts with the same muscle fiber (Atwood, 1976). In multiterminally innervated muscle, the final level of depolarization depends on a number of physiological and anatomical properties. These include the length and diameter of the fibers, the synaptic conductance at each terminal site, the number of terminal sites, the distance between the terminal sites, the conductance of the nonsynaptic membrane, and the extent of membrane invagination. If the muscle fibers are electrically coupled to neighboring muscle fibers, the extent of the coupling will also contribute. Finally, if the release of neurotransmitter is influenced by patterns of activity in the presynaptic terminal that result in facilitation and/or depression (Atwood and Wojtowicz, 1986; Sen et al., 1996), the pattern of motor neuron discharge will influence the amplitude and duration of the depolarization that the fiber undergoes. This raises the question of how physiologically appropriate levels of muscle depolarization, and consequently, muscle contraction, are achieved during growth.

In this article we study the growth of the pyloric 1 (p1) muscle of the lobster stomach from postlarval (juvenile) to adult size. This muscle is innervated by only one motor neuron, the lateral pyloric (LP) motor neuron of the stomatogastric ganglion (STG). The LP neuron fires rhythmically in bursts as part of the pyloric rhythm throughout development. We wished to determine whether the LP neuron-evoked depolarizations were constant regardless of massive changes in size of the muscles innervated by the LP neuron during growth, and if so, how this is achieved. Accordingly, we employed anatomical and physiological methods to measure the properties of large and small muscles, and determine how many of the physiological parameters that account for overall muscle depolarization scale with size during growth. We compared in young and old animals the patterns of innervation, synaptic strength, and other features of the neuromuscular junctions that contribute to the amplitude of the evoked depolarizations.

METHODS

Animals and Saline
Adult lobsters, Homarus americanus, were purchased from Yankee lobster (Boston, MA), and maintained at 10–13°C in recirculating artificial seawater tanks. All adult animals were the smallest commercially available (0.57 kg) and of very similar size (83.5 ± 3.5 mm thorax length (TL), mean ± S.D.). Juvenile lobsters (TL = 5–40 mm) were obtained from the Lobster Rearing and Research Facility at the New England Aquarium and maintained in floating trays in the same tanks as adults. All dissections were carried out in saline containing (in mM) 479.12 NaCl, 12.74 KCl, 13.67 CaCl₂, 20 Mg₂SO₄, 3.91 Na₂SO₄, 5 HEPES, pH = 7.4–7.5. All animals were either anaesthetized on ice (adults) or in beakers containing artificial seawater at 4°C (juveniles) for 20 min prior to dissection.

Measurement of Muscle Length and Fiber Diameter
p1 muscle lengths (juveniles: N = 11; adults: N = 9) were measured at rest in situ when the muscle was still attached to the stomach lining. For these measurements, we used either an ocular micrometer on a dissecting microscope or a hand held micrometer. For antibody staining and physiology, care was taken to pin the muscles to rest length as measured in situ. Fiber diameters (juveniles: N = 11; adults: N = 8) were measured with the ocular micrometer in the physiological preparations, and are therefore called “approximate diameters,” as they were not measured in histological cross-section.

Immunocytochemistry
Juvenile (N = 11) and adult (N = 10) stomachs were removed as is customary for this system (Selverston and Moulins, 1987). The p1 muscle and a section of the lateral ventricular nerve (lvn) were dissected according to Richards and Marder (2000). The lvn contains the axon of the LP motor neuron that innervates the p1 muscle. Dissected preparations were pinned in a Sylgard-lined petri dish (Dow Corning, Midland, MI) and fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4. In some juvenile preparations (N = 4), the lvn was backfilled with 5–10% neurobiotin tracer (Vector, Burlingame, CA) before fixation. In these preparations, a well was made around the cut end of the lvn; the saline within the well was then replaced with distilled water for 5–10 min before adding neurobiotin to the well. The tracer was allowed to run for 48 h at 4°C, then the tissues were fixed as above. The day following fixation, preparations were washed five times over the course of 7–8 h in PTA (0.1 M sodium phosphate buffer, 0.3% Triton X-100, and 0.1% NaN₃, pH 7.4). The muscles along with small blocks of Sylgard (to which the muscles remained pinned) were then transferred to a 0.5 mL vial. These preparations were then incubated for 2 days in a rabbit polyclonal antibody raised against the Drosophila synaptotagmin I protein at a dilution of 1:1000 with 10% goat normal serum (GNS) in PTA. The anti-synaptotagmin I antibody was a gift of Hugo Bellen (Baylor College of Medicine, Houston, TX) and has been used in previous studies to map presynaptic release sites in Drosophila mela-
Preparations were then washed as above in PTA and incubated overnight in 1:400 anti-rabbit IgG coupled to Alexa Fluor 488 (Molecular Probes, Eugene, OR) and 1:50 phalloidin coupled to Alexa Fluor 660 with 10% GNS in PTA. Preparations in which the lvn had been backfilled were also incubated in 1:400 streptavidin coupled to Cy3 (Jackson Immunoresearch, West Grove, PA). After secondary incubation, the preparations were washed five to six times over the course of 12–48 h in 0.1 M phosphate buffer and mounted on glass slides in 80% glycerol and 20% 20 mM Na2CO3, pH 9.5. To avoid squashing muscle preparations during mounting, we glued glass coverslips (100 μm thickness) on either side of the preparation before placing a coverslip over the muscle. All processing was carried out at 4°C.

Imaging

Mounted preparations were viewed with the appropriate laser and filter settings on a Leica TCS confocal microscope. Optical sections were taken every 1–2 μm. Distance measurements and maximum projections were obtained from three-dimensional reconstructions in Amira (TGS, San Diego, CA), final figure construction was done with Canvas 8 (Deneba, Miami, FL).

In some preparations we injected p1 fibers with the fluorescent dye, Alexa Fluor 568 hydrazide (10 mM in 200 mM KCl, shanks of electrodes filled with 0.6 M K2SO4, with 0.02 M KCl). To image these preparations, we held a Nikon “Cool Pics” digital camera on the eyepiece of a Leica MZFLIII dissecting scope equipped with a 100 W mercury vapor lamp. The resulting images were then processed with Canvas 8.

Electrophysiology

Electrophysiological experiments were performed on juvenile (TL = 10–16 mm; N = 27) and adult (N = 20) animals. The p1 muscle along with either the entire anterior stomatogastric nervous system (STNS) or a section of the lvn was dissected out and pinned dorsal side down in a sylgard-lined dish and the stomach lining covering the p1 muscle removed. This configuration provided maximum stability during STNS intact rhythms, high frequency stimulation trains, and voltage clamp experiments. Preparations were superfused with chilled saline at a rate of approximately 10 mL/min and cooled to 12 ± 1°C using a peltier cooling system. Bath temperature was monitored constantly using a thermoelectric probe.

In adults, a vaseline well was made around the end of the lvn and the nerve stimulated with bipolar stainless steel pin electrodes. In juveniles, the lvn was stimulated with a suction electrode. Evoked Excitatory Junctional Potentials (EJPs) were recorded in bridge mode using an Axoclamp 2A amplifier (Axon instruments, Foster City, CA) and digitized using a Digidata 1200A board and pClamp 8.0 suite of software (Axon Instruments). Recording electrodes used for impaling muscle fibers were filled with 0.6 M K2SO4 with 0.02 M KCl. Electrodes used in adult preparations had resistances of 10–20 MΩ; electrodes used in juvenile preparations were 20–30 MΩ. Evoked Excitatory Junctional Currents (EJCs) were obtained in two electrode voltage clamp (Axoclamp 2A), and the input resistance of muscle fibers was measured in two electrode current clamp. In both voltage clamp and current clamp experiments, the distance between the two electrodes was minimized to reduce voltage decay along the length of the muscle fiber.

Nerves were stimulated with various spike trains using an A-M Systems (Carlsborg, WA) isolated pulse stimulator (model 2100). Reported single evoked EJP and EJC parameters for each preparation are the average of 10–15 events stimulated at 0.05 Hz. At this stimulus frequency, no depression or facilitation of the postsynaptic response was observed (data not shown). The coefficient of variation of the EJP amplitude was calculated by dividing the standard deviation by the mean EJP amplitude. The time to decay to half amplitude of EJPs or EJCs was used as a measure of the decay time constant. The evoked EJPs in response to trains of ten stimuli at 0.1, 1.0, 2.5, 5.0, 10.0 Hz were also recorded. The amplitude of depolarization in these experiments is an average of two to three trials at every stimulus frequency. To avoid depression of postsynaptic responses to repeated train stimulation, every train of events was sepa-
rated by at least 4 min. To mimic the type of input usually received by p1 during a pyloric rhythm, we stimulated the lvn with repetitive 350 ms bursts of 20, 25, 30, and 35 Hz at a 1.5 s cycle period. We based this protocol on data obtained from preparations in which we recorded EJPs in p1 in response to ongoing pyloric rhythms. To ensure that the response to rhythmic stimulation was at a steady state level, we analyzed the last 30 s of a 60 s long stimulation episode. All episodes of rhythmic stimulation were separated by 7–10 min.

Scripts written in Spike 2 (version 5, Cambridge Electronic Design, Cambridge, England) were used to analyze postsynaptic, response parameters. Statistical analysis and data plots were performed in Statview (SAS institute Inc., Cary, NC) and all data unless otherwise noted are reported as means \( \pm \) S.E.M (standard error of the mean). Unless otherwise noted, all p-values reported are the results of unpaired t tests. All figures were prepared in Canvas 8.

RESULTS

Morphometrics

By the time that lobsters undergo the last larval molt and become “juvenile” animals, the gross morphology of their stomachs has become typical of that seen in adult animals. Figure 1(A) shows a schematic representation of the pyloric muscles and STNS in an adult *H. americanus*. Figure 1(B) is a picture of the cardio pyloric valve 6 (cpv6), pyloric 1 (p1), and pyloric 2 (p2) muscles in a juvenile animal. The p1 muscle is clearly visible and can easily be distinguished from other pyloric muscles. We measured the length of the p1 muscle in 97 animals of a variety of sizes [Fig. 1(C)]. As lobsters grew from early juvenile (thorax length, TL = 5–40 mm) to adult (TL = 83.5 mm), p1 length increased sixfold [Fig. 1(C)]. The change in p1 length in juveniles was also accompanied by a change in individual fiber diameter. Approximate fiber diameter in juveniles with a TL of 10–16 mm was 21.1 \( \pm \) 1.2 \( \mu \)m \( (N = 11) \). Approximate fiber diameter in adults with a TL of 83.5 mm was 31.4 \( \pm \) 1.3 \( \mu \)m \( (N = 8) \).

Spatial Arrangement of Presynaptic Release Sites during Growth

We used an antibody raised against synaptotagmin to label presynaptic structures in juvenile and adult p1 muscles. To confirm that the antibody was co-localized with motor axon terminals on p1, we performed anterograde fills of the lvn in six juvenile preparations with neurobiotin. In four of these preparations, we costained the muscles for synaptotagmin (Fig. 2). Figure 2(A) shows a low magnification view of a typical lvn filled with neurobiotin as the nerve branches over the pyloric region in a juvenile animal. Neurobiotin coupled to Cy3 is shown in green, and muscles stained with phalloidin coupled to Alexa Fluor® 660 are shown in red. Figure 2(B) is a schematic representation of the branching patterns shown in Figure 2(A). In all 6 lvn fills, fine processes originating from multiple sites along the lower lvn and lateral pyloric nerve (lpn) converged to innervate the p1 muscle. Figure 2(C) shows a high magnification view of a neurobiotin fill over a juvenile p1 muscle. Synaptotagmin staining in the same region is shown in Figure 2(D). When these two images are overlaid [Fig. 2(E)], synaptotagmin staining clearly colocalizes with neurobiotin-filled varicosities (yellow stained varicosities indicate colocalized red and green staining).

Figures 2(F,G) show synaptotagmin staining on a juvenile p1 muscle and adult p1 muscle, respectively. In both the juvenile and adult, lines of varicosities are clearly visible running roughly perpendicular to p1 fibers. To quantify the changes in spatial organization of the synaptotagmin staining as the animals grew, we measured the distance between synaptic contact regions along bundles of p1 muscle fibers in juveniles \( (N = 11) \) and adults \( (N = 9) \). The distance between synaptic contact regions scaled linearly with the increase in p1 muscle length (Fig. 3). Furthermore, the y-intercept of the linear fit was not significantly different from zero. These results suggest that the number of synaptic contact regions along the p1 fiber remains constant as the lobster grows.
Muscle Fibers Are Dye and Electrically Coupled

Intracellular dye injections of individual muscles fibers in both juvenile \((N = 5)\) and adult muscles \((N = 5)\) revealed that the fibers were dye-coupled with other neighboring fibers [Fig. 4(A)]. In this adult preparation, the fluorescent dye Alexa Fluor® 568 was injected into a single fiber and traveled to four adjacent fibers. Dye coupled bundles of fibers were typically three to six fibers wide in both juvenile and adult preparations.

The presence of dye coupling among muscle cells predicted that individual fibers were likely to be electrically coupled. To determine the extent of electrical coupling and measure the apparent input resistance of \(p1\) fibers, we measured the voltage response to injected current in two electrode current clamp in single fibers while simultaneously measuring the voltage deflection in neighboring fibers. We then calculated the coupling coefficient by dividing the voltage deflection in the neighboring fiber by the voltage deflection in the same fiber in response to current injection. Figure 4(B) shows the steady state voltage response to hyperpolarizing current steps in a juvenile \(p1\) fiber (black circles) and the voltage response in a neighboring fiber (open triangles). A comparable experiment from an adult preparation is shown in Figure 4(C). In each preparation, the current voltage relationship in a single fiber was linear in the range tested and a substantial voltage deflection was recorded in response to current injection in an adjacent fiber. These results were typical of 18 experiments in juveniles (three of which included coupling measurements) and 13 experiments in adults (seven of which included coupling measurements). As seen in Figure 4(D), the mean apparent input resistance of juvenile fibers was approximately ninefold larger than that seen in adult fibers. In addition, the mean coupling coefficients seen in juveniles were more than twice as large as those measured in adults [Fig. 4(E)]. In these experiments and in those that follow, the resting membrane potential in juveniles \((-62.7 \pm 0.9 \text{ mV}, N = 27\) \) was not significantly different from that in adults \((-63.8 \pm 0.8 \text{ mV}, N = 20; \ p = 0.38\) \).

Estimating Length Constants

Obviously, the distance between synaptic contact regions would be electrically unimportant if the length constant, \(\lambda\), were longer than the actual length of the muscle fiber. Conventionally, \(\lambda\) would be measured by injecting current at one site along a fiber and measuring the voltage deflection both at the site of current injection and at known distances down the fiber. However, the electrical coupling that we have previously described demonstrates that traditional \(\lambda\) measurements in these fibers would underestimate \(\lambda\) for single fibers. The reason for this is that during real synaptic input all fibers get simultaneous depolarizing input that should keep the current flow between fibers small. In contrast, any experimental test that requires current injection will be confined to a single fiber and current will be lost via electrical junctions. Nonetheless, measuring an “apparent \(\lambda\)” provides a functional estimate of how far current will flow down the fiber in response to a local current injection, even with the confound of the electrical coupling. Figure 5(A) shows the voltage deflection in response to \(-2\) nA of injected current both at the site of current injection and 0.7 mm along the fiber. Figure 5(B) shows a similar experiment in an adult preparation. In this experiment the voltage deflection in response to \(-30\) nA of injected current was measured at multiple distances along the fiber. Measurements such as these show an “apparent \(\lambda\)” of 1.00 \pm 0.14 mm in juveniles \((N = 4)\) and 1.71 \pm 0.37 mm \((N = 3)\) in adult fibers [Fig. 5(C)]. In both cases this is less than the length of the muscle fibers and longer than the mean distance between synaptic contact regions. With these apparent values for \(\xi\) the electrotonic length of the muscle fibers (fiber length/\(\lambda\)) would be substantially larger in adults \((\sim 3.5)\) than in juvenile animals \((\sim 1.8)\).
Size and passive properties of the p1 muscle change as the lobster grows. Do these changes translate into a difference in p1 postsynaptic responses? To address this question, we measured single EJPs and EJCs in juvenile and adult p1 muscles. Figure 6(A) shows traces from experiments in which stimulus-evoked EJPs were first recorded in current clamp; afterwards, the fibers were voltage-clamped to record the stimulus-evoked EJCs. This allows comparison of the amplitude and time course of the EJP and EJC recorded in the same fiber. The raw traces from the juveniles in Figure 6(A) show that the EJP amplitudes in the juvenile and adult preparations were similar, but that the recorded EJC amplitudes in the juvenile were significantly smaller than in the adult. Pooled data comparing the EJP and EJC amplitudes in juveniles (EJPs: \( N = 20; \) EJCs: \( N = 8 \)) and adults (EJPs: \( N = 18; \) EJCs: \( N = 7 \)) are shown in Figure 6(B) and confirm these findings.

In juveniles, the EJC was much more rapid than the EJP [Fig. 6(A)], while in the adult the EJP and the EJC were approximately the same duration. To look more carefully at these relationships, Figure 6(C) shows the EJP and EJC traces from Figure 6(A) scaled to the same amplitude. This reveals that the EJP rise times in adult and juvenile were quite similar, but that the EJP decay was significantly slower in the juvenile [Fig. 6(D)], while the decay time of the EJC was slower in the adult [Fig. 6(D)]. We did not attempt to draw any functional conclusion from the observed changes in EJC time course and the absolute EJC amplitude values. Electrical coupling and changes in fiber length constants are serious confounds for EJC (but not EJP) measurements. In both juveniles and adults, synapses along a fiber and in

Figure 4 Dye coupling, apparent input resistance and electrical coupling measurements in juvenile and adult p1 muscles. (A) Dye coupling in an adult preparation. Fiber 1 was filled with Alexa Fluor 568; dye spread to fibers 2–5. A second bundle was in the process of being filled by the same electrode (arrow) as the picture was taken. (B,C) Current versus voltage plots obtained in two electrode current clamp in the same fiber as the current injecting electrode (black circles) and in an adjacent fiber (open triangles) in a juvenile (B) and an adult (C) preparation. D. Mean ± S.E. p1 apparent input resistance in juveniles and adults (\( p < 0.0001 \)). (E) Mean ± S.E. coupling coefficients (change voltage adjacent fiber/change voltage same fiber) in juveniles and adults (\( p < 0.05 \)).
neighboring fibers could be contributing to the total synaptic current measured at a given site. We did not attempt to determine how much of the measured synaptic current was due to distant synaptic input, as we had no way of evaluating the difference in space clamp errors between juveniles and adults. However, even though the absolute values for changes in EJC amplitude and input resistance measurements are not precise (and actually do not match), it is clear that the input resistance decreases with increasing muscle fiber size, and that the EJC amplitude increases to keep the EJP amplitude constant during growth.

**Evoked EJP Amplitudes Are More Variable in Juveniles Than Adults**

To obtain a measure of the reliability of juvenile and adult neuromuscular junctions, we analyzed the vari-

---

*Figure 5* Voltage decay along single fibers and estimates of “apparent” $\lambda$ (see text) in juveniles and adults. (A) Voltage response to $-2$ nA of injected current both at the site of current injection (0 mm) and 0.7 mm along the fiber. (B) Voltage response to $-30$ nA of injected current at the site of injection and at five distances along the fiber. Resting potentials: juvenile ($-63$ mV); adult ($-65$ mV). (C) Mean $\pm$ S.E. apparent $\lambda$ in juveniles and adults ($p < 0.05$).

*Figure 6* Single EJP and EJC parameters in juvenile and adult preparations. (A) Sample EJPs and EJCs from a juvenile and an adult preparation. EJCs were recorded in two electrode voltage clamp. Fibers were clamped at rest. (B) Mean $\pm$ S.E. EJP and EJC amplitudes ($p = 0.99$ for EJPs), $p < 0.01$ for EJCs). (C) Juvenile and adult EJPs and EJCs scaled to the same amplitude. (D) Mean $\pm$ S.E. EJP and EJC time to decay to half amplitudes ($p < 0.01$ for EJPs), and $p < 0.001$ for EJCs).
ability of evoked EJP amplitude within single preparations. Figure 7(A) shows 10 consecutive overlaid evoked EJPs in a juvenile and an adult preparation. Although the average amplitude in juveniles and adults was similar, the coefficient of variation of EJP amplitude was significantly higher in juveniles ($N = 20$) than in adults ($N = 18$) [Fig. 7(B)].

**How Do Juvenile and Adult Muscles Integrate Low Frequency Trains of Evoked EJPs?**

The amplitude of single EJPs at the p1 neuromuscular junction remained constant during growth. However, during the pyloric rhythm, the LP motor neuron does not fire single action potentials; instead, it fires rhythmically in bursts. To determine how juvenile and adult p1 muscles respond to low frequency motor neuron input, we stimulated the lvn with trains of 10 impulses at 0.1, 1.0, 2.5, 5.0, and 10.0 Hz and recorded the resulting evoked membrane potential depolarizations. Figure 8(A) shows sample traces of the evoked responses to 10 stimulus pulses at 10.0 Hz in a juvenile and an adult preparation. Even though the initial EJP amplitude is similar in both preparations, the envelope of depolarization is markedly smaller in the adult. Figure 8(B,C) shows plots of the total membrane depolarization reached at the peak of each event during the trains at each frequency. At 0.1 Hz, EJP amplitudes depressed during the stimulus train in both juveniles ($N = 11$) and adults ($N = 7$). The ratio of the second EJP amplitude to the first EJP amplitude was not significantly different between juveniles (0.95 ± 0.02) and adults (0.93 ± 0.04) ($p = 0.60$); however, the ratio of the tenth EJP amplitude to the first EJP amplitude was significantly higher in juveniles (0.87 ± 0.02) than in adults (0.76 ± 0.04) ($p < 0.02$).

At 1.0 Hz in juveniles ($N = 15$), EJP amplitude initially showed facilitation followed by depression during the last half of the stimulus train. In adults ($N = 13$) at 1.0 Hz, the initial facilitation seen in juveniles was absent but the depression was still evident. At this higher stimulus frequency, both the ratio of the second EJP amplitude to the first EJP amplitude (2/1) and the ratio of the tenth EJP amplitude to the first EJP amplitude were significantly different between juveniles and adults.

---

**Figure 7** EJP amplitude coefficient of variation in juvenile and adult preparations. (A) Ten consecutive EJPs overlaid in a juvenile and an adult p1 muscle. Resting potentials: juvenile, −62 mV; adult, −60 mV; (B) Mean ± S.E. coefficient of variation in juvenile and adults ($p < 0.05$).

**Figure 8** p1 EJP responses to low frequency stimulus trains in juveniles and adults. (A) Sample traces of 10 EJPs evoked at 10 Hz in a juvenile and an adult preparation. Resting potentials: juvenile, −62 mV; adult, −67 mV. (B,C) Mean ± S.E. level of peak depolarization in response to each stimulation during 0.1, 1.0, 2.5, 5.0, and 10.0 Hz trains in juveniles (B) and adults (C). In all preparations, trains of EJPs were separated by 4–6 min.
The area above baseline during LP bursts in juveniles during a pyloric rhythm in a juvenile and an adult. Figure 9(A) shows sample traces of p1 recordings neuromodulatory inputs to the STG were left intact. preparations in which the STG and all descending neuromuscular junctions respond to LP input during Marder, 2002). To determine how juvenile and adult ms duration bursts every 1–2 s (Thirumalai and rhythm, the LP motor neuron fires in regular 250–500 trains did not mimic perfectly the rhythmic input muscular junctions respond to low frequency stimuli, the ex-

Although the stimulus trains used in the previous experiments helped to illustrate how the p1 neuromuscular junctions respond to LP input during ongoing pyloric rhythms, we recorded from p1 in preparations in which the STG and all descending neuromodulatory inputs to the STG were left intact. Figure 9(A) shows sample traces of p1 recordings during a pyloric rhythm in a juvenile and an adult. The area above baseline during LP bursts in juveniles (N = 11) was significantly higher than in adults (N = 7) in multiple preparations (p < 0.05) [Fig. 9(B)]. Due to high spike frequencies and spike frequency adaptation within LP bursts, EJPs were difficult to detect by eye in these experiments. However, by high pass filtering our traces, we were able extract LP spike times and burst parameters. In juvenile preparations, the LP motor neuron burst duration was 360 ± 22 ms, the cycle period was 1.26 ± 0.05 s, and the spike frequency within bursts was 36.1 ± 2 Hz. In adults, the burst duration was 380 ± 31 ms, the cycle period was 1.44 ± 0.062 s, and the spike frequency within bursts was 26.7 ± 1.2 Hz. In this data set, the burst duration was not statistically different between juveniles and adults (p = 0.6), but both the spike frequency within bursts and the cycle period were significantly different (p < 0.01; p < 0.05, respectively).

**How Do Juvenile and Adult Muscles Integrate Bursting Input during Rhythmic Stimulus Trains with a Constant Cycle Period and Burst Duration?**

Figure 9 shows that juvenile p1 muscles depolarize more than adult muscles during ongoing pyloric rhythms. This difference could be due to growth related changes in the integrative properties of the p1 muscle or it could be caused by the small but significant changes in LP neuron burst parameters described above. Therefore, in the next set of experiments, we compared juvenile and adult p1 responses to rhythmic nerve stimulations at a constant cycle period (1.5 s), constant burst duration (350 ms), and varying intraburst spike frequencies. All measurements were made at steady state after 30 s of ongoing rhythmic stimulation. Figure 10(A) shows sample traces of p1 recordings in a juvenile and an adult during rhythmic 20 Hz bursts of nerve stimulation at steady state. Even though the amplitude of the first EJP is similar in both preparations, the juvenile p1 muscle shows a larger envelope of depolarization. In multiple preparations, the area above baseline during 20 Hz bursts in juveniles (N = 5) was not significantly different from that in adults (N = 5). However, at higher intraburst spike frequencies (25, 30, and 35 Hz) juvenile p1 depolarizations were significantly larger than those seen in adults (N = 5 for each burst frequency in both juveniles and adults) [Fig. 10(B)]. To ensure that the larger depolarizations seen in juveniles were not just an artifact of larger single EJP amplitudes, we also compared the mean amplitudes of the first EJP in a burst at each stimulus frequency [Fig. 10(C)]. The amplitude of the first EJP in a burst...
was dependent on the stimulus frequency, but was not different between juveniles and adults at a given stimulus frequency (ANOVA repeated measures: effect of frequency, \( p < 0.001 \); effect of frequency vs. age, \( p < 0.864 \)).

**DISCUSSION**

Lobsters and crabs continue to increase their body size over their entire lifetimes. While growing, these animals must maintain their ability to move, eat, and otherwise produce meaningful behaviors. Because most crustacean muscle fibers do not generate muscle action potentials, but are multiterminally innervated, muscle growth poses a series of problems for neuromuscular function. Here we describe some of the processes by which the p1 neuromuscular junction compensates for physical growth, and some ways in which growth is accompanied by alterations in how the muscle responds to its patterned neural inputs.

In vertebrate muscles, growth is seen as expansion of endplate regions (Balice-Gordon and Lichtman, 1990), associated with enhanced release of transmitter (Letinsky, 1974). Because the muscle fiber generates an action potential, the endplate must generate enough current to bring a larger muscle fiber to threshold, but beyond that, the size of the muscle fiber does not pose a significant problem.

Numerous studies have looked at the regulation of bouton structure and number in the body-wall muscles of *Drosophila* larvae (Budnik et al., 1990; Schuster et al., 1996a,b; Davis and Goodman, 1998; Aberle et al., 2002; Marqués et al., 2002; Sigrist et al., 2003). The body wall muscles grow 100-fold in surface area and four to sixfold in diameter and length (Schuster et al., 1996a; Li et al., 2002). As the muscles grow, nerve terminal length and the number of boutons along terminals increases (Atwood et al., 1993; Schuster et al., 1996a; Zito et al., 1999; Li et al., 2002). However, because these muscle fibers are isopotential during

![Figure 10](image-url)  
**Figure 10**  
\( p1 \) EJP responses to rhythmic stimulation with constant cycle period and burst duration. Cycle period = 1.5 s; burst duration = 350 ms. (A) EJPs in a juvenile and adult \( p1 \) muscle in response to rhythmic trains of 20 Hz stimulation. Resting potentials: juvenile, \(-65 \text{ mV}\); adult \(-63 \text{ mV}\). Black bars beneath traces indicate time of stimulation. (B) Mean ± S.E. areas above baseline during 20, 25, 30, and 35 Hz bursts in juveniles and adults. Unpaired \( t \) tests: 20 Hz, \( p = 0.078 \); 25 Hz, \( p < 0.01 \); 30 Hz, \( p < 0.01 \); 35 Hz, \( p < 0.001 \). ANOVA repeated measures: effect of frequency, \( p < 0.001 \); effect of frequency versus age, \( p < 0.0001 \). (C) Mean ± S.E. amplitudes of the first EJPs within bursts in juveniles and adults. At each stimulation frequency, there were no significant differences between the amplitude of leading EJPs. ANOVA repeated measures: effect of frequency, \( p < 0.001 \); effect of frequency versus age, \( p = 0.87 \). Sample traces and data plotted come from rhythmic trains at steady state.
larval times (Jan and Jan, 1976), the problem, as in vertebrate preparations, is only to match the total synaptic strength to the total input resistance of the growing muscle fiber. The specific location and spacing of the terminals is not functionally critical.

Because the crustacean muscles studied here do not produce action potentials and are not isopotential, the problem posed by growth has a degree of freedom in addition to those seen at the vertebrate and Droso-
pha neumuscular junction. Maintaining a uniform response to motor neuron input during growth in the p1 muscle is not just a question of matching synaptic strength to input resistance. In addition, the neuromuscular junction must compensate for voltage decay along the length of a muscle fiber. Obviously, compensating for voltage decay along a fiber is critical for maintenance of a homogeneous depolarization level along the fiber. However, it is also key for maintenance of the shape of a postsynaptic response, because integrating synaptic input over different electrotonic distances can change the time course of the postsynaptic response. This problem is similar to that posed by growing neurons in the central nervous system, where synapse spacing along dendrites and branches is critical for synaptic integration (Rall, 1967; Rall et al., 1967; Rall, 1969a,b).

We show here that the number of synaptic contact regions along p1 muscle fibers stayed constant during growth. To keep spatial integration of synaptic input constant, the muscle fibers would therefore also have to maintain the same amount of electrical attenuation along their entire length. According to the cable equations, this could be achieved with constant specific membrane properties and a growth pattern that scales the diameter with a factor that equals the square of the scale factor for the length (“isoelectrotonic growth”) (Hochner and Spira, 1987; Bekkers and Stevens, 1990; Hill et al., 1994). However, we found that p1 length increased sixfold during juvenile life, accompanied by a 1.5-fold increase in fiber diameter. Even when taking into account that our measurements only approximate fiber diameters, this pattern clearly does not represent isoelectrotonic growth but would predict an increase in electrotonic length with age. Our physiological measurements of the “apparent” λ, albeit somewhat confounded by electrical coupling between fibers, show the same tendency.

Electrical coupling among muscle fibers adds yet another variable to the problem posed by growth. Widespread electrical coupling is present early in development in both vertebrate and invertebrate sys-
tems (Goodman and Spitzer, 1979; Janse et al., 1986; Peinado et al., 1993a,b; Kandler and Katz, 1995, Naus and Bani-Yaghoub, 1998; Bem et al., 2002). In many cases, coupling does not persist later in life (Goodman and Spitzer, 1979; Spitzer, 1982; Connors et al., 1983; Walton and Navarrete, 1991). At the Droso-
pha neuromuscular junction, dye and electrical coupling among muscle fibers decrease abruptly after embry-
onic development (Broadie and Bate, 1993; Gho, 1994; Todman et al., 1999); however, some muscles do remain electrically coupled across segmental boundaries during larval life (Ueda and Kidokoro, 1994). In lobsters, p1 muscle fibers are strongly electrical coupled throughout post larval and adult life. During growth, a depolarization caused by synaptic input spreads both along the length of a p1 fiber and across bundles of coupled fibers. Although this makes accurate measurements of the true length constant of individual fibers nearly impossible, the arrangement does make functional sense for the animal. Extensive electrical coupling within a sheet of muscles would tend to dampen any local differences in voltage within the sheet and help ensure uniform depolarization in response to motor neuron input. This would add to other mechanisms that ensure reliability of muscle contraction amplitude to a given input pattern, despite the stochastic nature of synaptic input (Hoover et al., 2002).

Surprisingly, variability in EJP amplitude was highest in juvenile p1 muscles even though electrical coupling was strongest in these preparations. This suggests that the actual variability in EJP amplitude at single synapses in the juvenile is even higher than what we have observed. Enhancement of transmitter release has been shown to be correlated with decreasing variability of postsynaptic responses (Glusman and Kravitz, 1982; Richards et al., 2003). Therefore, it is likely that an enhancement of transmitter release (as opposed to a change in electrical coupling strength) is responsible for the observed decrease in EJP amplitude variability during growth.

Growth-related changes in other crustacean mus-
cles have been the subject of previous studies. Both lobster walking leg muscles and crayfish abdominal superficial flexors grow continuously during postlar-
al life (DeRosa and Govind, 1978; Lnenicka and Mellon, 1983a,b). Because these older studies were done in muscles innervated by more than one motor neuron, the spacing between terminals of a single motor neuron are not known. At single synapses in both animals, presynaptic terminals grow and the number of synapses increases during growth (Atwood and Kwan, 1976; Pearce et al., 1985). This structural change is accompanied by an increase in transmitter release (DeRosa and Govind, 1978; Lnenicka and Mellon, 1983a,b). Interestingly, as is reported here, these studies also found little or no change in EJP
amplitude during growth (DeRosa and Govind, 1978; Lnenicka and Mellon, 1983a,b). In Drosophila, EJP amplitudes are maintained constant throughout larval life as well (Li et al., 2002), and a large number of genetic manipulations suggest that presynaptic output is homeostatically regulated by changes in muscle receptor function and muscle excitability to maintain motor synapse strength (Petersen et al., 1997; Davis and Goodman, 1998; DiAntonio et al., 1999; Paradis et al., 2001; Haghighi et al., 2003; Marek and Davis, 2003). Together, all of these studies argue that numerous regulatory processes must be called into play to maintain a fairly constant synaptic strength, despite enormous changes in muscle size.

**Does Regulation of Single EJP Amplitude Imply Constant Physiological Actions in Development?**

To date, most studies that have looked at the maintenance of motor synapse strength during development focused primarily on the regulation of the amplitude of single EJPs. The implicit assumption is that if the amplitude of a single EJP is maintained then the muscle response to patterned motor neuron input in vivo will also remain constant. In our study we have tested this assumption by measuring single evoked EJPs in p1, the p1 response to actual ongoing motor neuron input, and the response to stimulation that mimics in vivo motor activity. We found that although the amplitude of single EJPs in p1 are kept constant during growth, the response to both actual and mimicked bursting motor neuron activity is not kept constant. Juvenile p1 muscles undergo a larger envelope of depolarization than adult muscles in response to bursting input.

What differences at the juvenile and adult p1 neuromuscular junction account for a difference in response to bursting input? In our system, the duration of the juvenile EJP is significantly longer than that in adults. Due to temporal filtering, more distal inputs along a fiber are slowed down and contribute more to the late phase of the EJP recorded at a given site (Simon, unpublished modeling data). Because there are more synaptic contacts/length constant in the juveniles, distal synaptic input probably has a greater contribution to the postsynaptic response in juveniles than in adults. This could account for the longer EJP time course in juveniles. Alternatively, changes in membrane time constants and/or changes in the time courses of synaptic signaling could be responsible for the increased EJP duration. Regardless, a longer EJP leads to more summation in juvenile fibers, and this could account for part of the observed difference in response to bursting input.

In Drosophila, the time course of single EJPs also changes significantly during larval growth (Li et al., 2002) and after at least one genetic manipulation (Paradis et al., 2001). Because Drosophila abdominal wall muscles receive bursting motor neuron input (Budnik et al., 1990; Schuster et al., 1996b; Cattaert and Birman, 2001), the functional consequences of these changes in EJP time course could be substantial in the intact fly larvae.

The presence of wider EJPs in juvenile p1 muscles accounts at least in part for the larger envelope of depolarization in juvenile muscles in response to realistic rhythmic stimulation. In addition, changes in synaptic dynamics at the neuromuscular junction could also play a key role in determining the muscle response to bursting input. We did not attempt to measure facilitation and depression in high frequency bursts. However, at low stimulus frequencies where summation is not present, juvenile synapses show more initial facilitation and less depression with repeated stimulation than adult synapses. This change in synaptic dynamics probably contributes to the larger envelope of depolarization seen in juvenile muscles during high frequency bursts.

Overall, our findings highlight the importance of considering the in vivo activity of a neuromuscular system during studies of homeostasis at the neuromuscular junction.

We wish to thank Hugo Bellen for the gift of the synaptotagmin antibody, and Barbara Beltz for the use of the Wellesley College confocal microscope.

**REFERENCES**


Balice-Gordon RJ, Lichtman JW. 1990. In vivo visualiza-


Rall W. 1969a. Distributions of potential in cylindrical