ARTICLE
A Classic Improved: Minor Tweaks Yield Major Benefits in Crayfish Slow-Flexor Preparations

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Action potentials and the postsynaptic potentials they evoke fill the pages of neuroscience textbooks, but students have relatively few opportunities to record these phenomena on their own. However, the act of making such recordings can be key events in a student's scientific education. The crayfish abdominal slow flexor muscle system is a well-established platform for recording spikes and PSPs. It enables students to see nerves and the muscles they innervate, record spontaneous spikes from several motor axons in these nerves as well as PSPs in their postsynaptic muscle fibers, and interpret these recordings quantitatively. Here we describe an improved method for preparing the slow-flexor system for recording that employs transmitted illumination through the stereo microscope's conventional substage lighting. Oblique transmitted lighting allows students to see the nerve and muscles fibers in each segment clearly and position recording electrodes accurately under visual control. Because students can see the nerves, muscles, and recording electrodes, broken electrode tips are relatively uncommon and the first successful recordings come more quickly.

Many kinds of neurons in the CNS have the same pattern of multineuronal, multiterminal innervation that occurs on these muscle fibers. To visualize these innervation patterns on these fibers, we describe an immunohistochemical protocol that labels the GABAergic inhibitory motor axon and all the synaptic vesicles in the synaptic terminals on these muscle fibers. Dual-color images reveal extensive branching of the axons and fields of presynaptic terminals, only some of which are double-labeled for GABA.

Key words: crayfish, extracellular recording, PSPs, immunohistochemistry, GABA

A major challenge in many undergraduate neurophysiology courses is choosing a preparation that will demonstrate critical theoretical concepts, yet also be one that students can use successfully. The crayfish slow-flexor system is used in many teaching laboratories to introduce students to electrophysiological recording, principally because when the ventral nerve cord is cut anterior to the abdomen, some of the motor neurons that innervate the slow flexor muscles fire tonically. Thus, students can record action potentials from these axons without needing to stimulate anything (Johnson et al., 2014).

Postural adjustments of the abdomen in large-tailed decapod crustaceans are made by two sets of slow muscles in each segment, the superficial flexor and superficial extensor muscles (Kennedy and Takeda, 1965). These antagonist muscles are innervated by separate sets of motor neurons in each segment (Wine et al., 1974; Leise et al., 1986, 1987). Kennedy and Takeda (1965) demonstrated that the superficial flexor muscles on each side of each abdominal segment in crayfish are innervated by a set of six motor neurons, a pattern that is the same in every animal. The preparation is also excellent for introducing students to intracellular recording with sharp microelectrodes because the postsynaptic muscle fibers are big and slow. This "slow" property is useful because the muscle fibers do not twitch and displace the intracellular electrode (Atwood, 1976, 2014). Several carefully-crafted descriptions of the slow flexor preparation appropriate for student labs have been published, e.g., Wyttenbach et al. (2014); Paul et al. (1997). We have developed certain improvements in the dissection, extracellular electrodes, and immunohistochemistry procedures that allow students to see more through the stereo microscope, to get better recordings, and to understand more about the anatomical organization of these multineuronal, multiterminal neuromuscular synapses. Here we describe these improvements and illustrate the difference they make.

MATERIALS AND METHODS

Crayfish, *Pacifastacus leniusculus*, of both sexes were anesthetized by chilling them on ice for 10 minutes. The walking legs and the swimmerets on each abdominal segment were removed. A single cut was made through the ventral carapace and ventral nerve cord between the 3rd and 4th walking legs. The muscles beneath the dorsal carapace at the juncture of the thorax and abdomen were severed and the dorsal section of the thorax removed, leaving the 4th and 5th thoracic ganglia connected to the abdominal nerve cord (Fig. 1*A*). Using fine tungsten-carbide bladed scissors, two cuts were made along the length of the ventral abdominal carapace, lateral to the swimmerets but medial to the lateral projections of each pleural plate (Fig. 1*AB*). The abdomen was pinned out ventral side up in a dish lined with Sylgard 184 (Dow Corning, Midland MI), and submerged in normal saline.

The fast flexor muscles were carefully detached from the ventral carapace by gripping and gently lifting the ventral carapace at its anterior end with toothed forceps while cutting each segment's fast flexor muscles at their

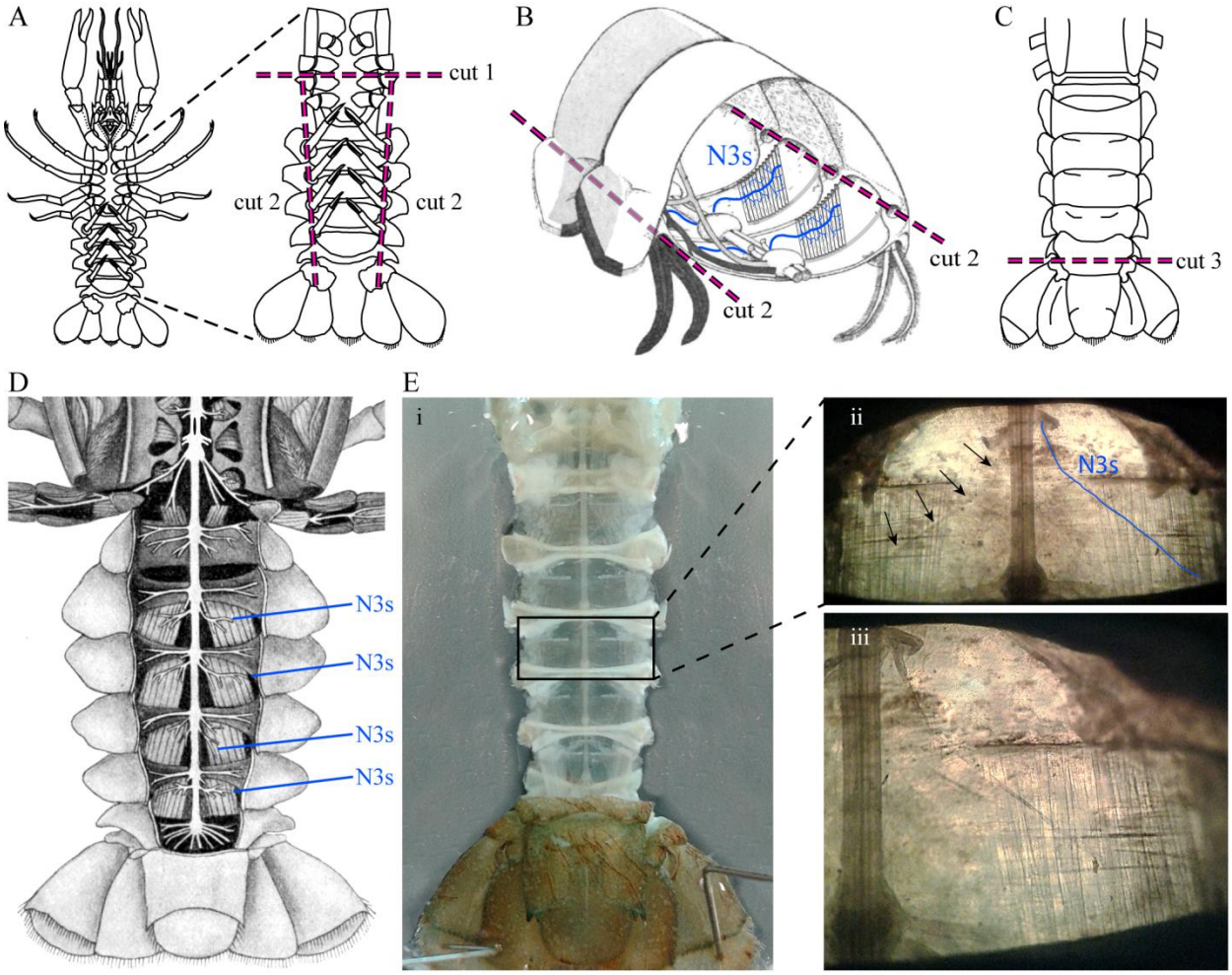

Figure 1. Preparation of the slow-flexor systems for electrophysiological recording. ***A*** The muscles beneath the dorsal carapace at the juncture of the thorax and abdomen are severed, and a single transverse cut (*cut 1*) is made in the ventral carapace between the 3rd and 4th walking legs to enable removal of the dorsal and more anterior parts of the thorax. ***B*** Two incisions (*cuts 2*) are made down the length of the abdomen lateral to the swimmerets but medial to the pleural plates. The abdomen is then pinned out ventral side up in a dish and covered with normal crayfish saline. The fast flexor muscles are detached from the ventral carapace by cutting them near their insertions on each sternum. ***C*** The dorsal carapace is removed with a single cut (*cut 3*) through the dorsal carapace posterior to the 5th abdominal ganglion. The tail fan is left attached, but the swimmerets are removed to improve the light path. ***D*** A cartoon of the preparation showing the nerves (*N3s*) that innervate the slow flexor muscles in the right side of segments A2, A3, A4, and A5. ***E*** Photographs of a finished preparation illuminated with transmitted light. The box in ***Ei*** indicates the areas magnified in *Eii* and *Eiii*. ***Eii*** shows the bilateral set of slow flexor muscles in segment A3, with the *N3s* on the right side traced in blue. Arrows mark the location of the left *N3s* as it projects from the ventral nerve cord to the muscles. ***Eiii*** shows the slow flexor muscles on the right side of *Eii* at higher magnification. ***B*** is modified from Hughes and Wiersma (1960)***,*** and ***D*** is modified from Keim (1915).

attachments to the sternum with small spring scissors. With this approach, the deep branch of the third segmental nerve (*N3*) that innervates the fast flexor muscles in each segment is clearly visible. These deep branches must be cut to prevent stretching or tearing the nerve's delicate superficial branch (*N3s*) that innervates the slow flexors. To avoid ambiguity in this paper, we have italicized *N3s* to mark it as a name, not a plural of N3.

When all the fast flexor muscles were severed, the ventral carapace with the ventral nerve cord and slow flexor muscles intact and attached was freed with a single cut through the dorsal carapace posterior to the 5th abdominal ganglion, leaving the tail fan attached to the preparation (Fig. 1*C,E*). The preparation was turned over and pinned out dorsal side up in a dish lined with transparent Sylgard. Any remaining fast flexor muscle tissue was cleared away to expose all of the slow flexor muscles and the *N3s* fully (Fig. 1*E*). The dish was removed to the stage of the stereo microscope at the recording work station, where transmitted light was used to illuminate the preparation (Figs. 1*Eii*, 1*Eiii*).

Different species of crayfish differ in the extent to which their hypodermis is pigmented. We have noticed numerous pigment spots in the ventral exoskeleton of

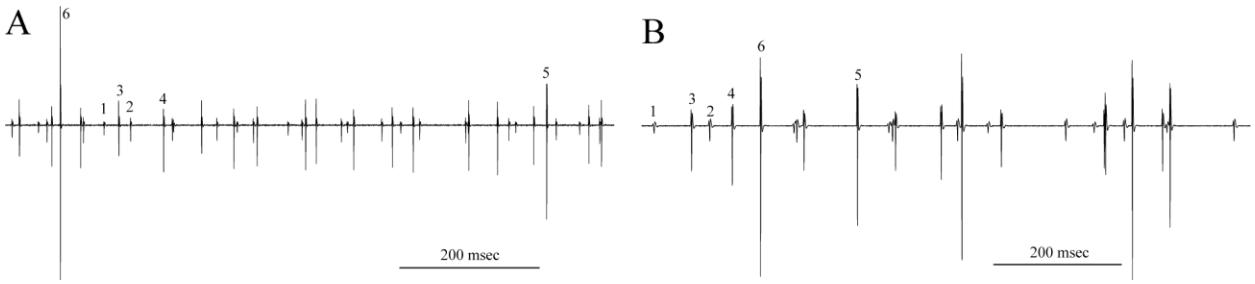

Figure 2. Examples of recordings from the *N3s* innervating muscles in segment A2 in which all six slow flexor motor units (labeled 1 through 6) are spontaneously active. These recordings are from different preparations.

individual *Procambarus clarkii* that interfere with transmitted illumination. We recommend *Pacifastacus*.

Recording electrodes.

Sharp microelectrodes were prepared by pulling 1.0 mm borosilicate glass capillary tubing with internal filament (Sutter Instruments, Novato CA), and filling with 2.5M KCl. They had tip resistances between 15 – 30 megaohms. Glass suction electrodes were prepared by pulling standard microelectrodes from 1.5 mm OD thick-walled borosilicate glass capillary tubing (Sutter Instruments). Tubing with and without internal filaments worked equally well for suction electrodes. Under a stereo microscope, the tapered shank of the microelectrode was scored at the desired diameter with a diamond-tipped pencil or a piece of ceramic tile (Model CTS, Sutter Instruments) and broken off. The jagged edges of the electrode tip were carefully smoothed by brushing the tip across the ceramic tile. These electrodes were inserted into a suction electrode holder (Model MEW-F15A, Warner Instruments, Hamden CT) fitted with two silver wires. One wire is integral to the holder and lies in the lumen of the inserted electrode. It was connected to one conductor of the two-conductor shielded cable attached to a high-gain differential preamplifier. The other silver wire was soldered to the cable's second conductor, and serves as the reference electrode. A length of PE tubing connects the holder to a plastic syringe used to apply suction. Johnson et al. (2007) describe construction of a similar slightly less expensive electrode holder.

Recording methods.

Extracellular recordings using these suction electrodes were amplified and filtered with an A-M Systems Model 1700 amplifier (A-M Systems, Carlsborg WA). Microelectrode recordings were made using an npi SEC 05 amplifier (npi electronik, Tamm Germany). Signals from both extracellular and intracellular recordings were digitized with a Digidata 1322A digitizer and pClamp software (Molecular Devices, Sunnyvale CA) and recorded as computer files for further analysis.

Normal saline and low-Ca^{++} high Mg^{++} saline solutions.

The composition of the normal and experimental salines are given in Table 1. The osmolarity of each solution was measured with a vapor pressure osmometer, Vapro 5520 (Wescor; Logan UT).

In experiments where solutions were changed during microelectrode recording, the preparation was superfused continuously using a gravity-fed inlet and a vacuum outlet to maintain a stable fluid level.

Immunohistochemistry.

Crayfish, *Pacifastacus leniusculus*, of both sexes were anesthetized by chilling them on ice for 10 min, and then exsanguinated by transfusion with 60 ml cold saline. The ventral carapace was removed as described above, fixed on ice for 1 hr in 0.2% glutaraldehyde and 2% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS; Sigma D5773), followed by a post-fix for 20 min in 4% PFA in PBS. The tissue was then washed 3x10 min in PBS. Selected abdominal segments with their slow flexor muscles were removed whole, taking care that the *N3s* remained fully attached to the muscles. To stabilize the muscles, the anterior and posterior sterna and the ventral carapace of the segment were left attached. The segments were pinned out in a Sylgard-lined dish, washed 3x10 min in 0.1M glycine in PBS to block any residual aldehyde fixative, and sent through an ascending and descending ethanol series (25, 50, 70, 80, 95, 100% and back again to PBS) to permeabilize the tissues. Non-specific labeling was blocked by a 1 hr incubation in a blocking solution composed of 10% normal goat serum (NGS), 3% bovine serum albumen (BSA) and 0.3% Triton-X 100 in PBS. The tissue was incubated for 3 days in the primary antibodies [mouse anti-synapsin 3C11 at 1:30 (DSHB, Iowa City IA); rabbit anti-GABA AB141 at 1:750 (EMD Millipore, Billerica MA)] in blocking solution at 2-4°C, and then washed 3x20 min in PBS. At this point, the slow flexor muscles were completely removed from the ventral carapace and incubated free-floating in secondary antibodies (goat anti-rabbit 488 and goat anti-mouse 568, both at 1:500; both Life Technologies, Grand Island NY) in PBS and 0.3% Triton for 1 hr in the dark at room temperature. The muscles were washed 3x20 min in PBS, and dehydrated through the ascending ethanol series. Upon reaching 100% ethanol, a glass cover slip was placed on the muscles to prevent folding or curling, and methyl salicylate was slowly infused into the dish. The cleared muscles were mounted dorsal side down in methyl salicylate in a Permanox dish (Fisher Scientific, Pittsburg PA) with a cover slip base. A small, thick piece of glass was placed on top of the muscles to prevent movement during imaging.

Labeled muscles were imaged with an inverted confocal

microscope (Olympus FLUOVIEW; Olympus America Inc, Center Valley, PA) equipped with krypton (488 nm) and argon (568 nm) lasers, using an Olympus 10x 0.3 NA UPlanFl, a 20x 0.7 NA UPlanApo, or a 60x Water 1.2 NA UPlanApo lens. All images were scanned at 1024 x 1024 pixels, and step sizes ranged from 0.75 to 1.5 µm. Images were converted to 24-bit TIF images using Fluoview software, where the gamma and intensity were adjusted to enhance the background intensity. The resulting images were then transferred to Adobe Photoshop for further adjustment of brightness, contrast, and sharpness.

RESULTS

Transillumination of the slow flexor muscles. Because the ventral carapace of this species of crayfish is largely translucent, the alternative dissection of the abdomen described in the Methods produces a preparation that can be viewed through a stereo microscope using transillumination. A frosted substage mirror used to shine light obliquely from a conventional substage lamp nicely illuminates the muscles and the ventral nerve cord. This allows the observer to resolve individual muscle fibers in each segment and to see the *N3s* running from the nerve cord to innervate these muscles (Fig. 1*E*). Given that the Sylgard lining the dish is transparent, adjustment of the lighting by rotating the substage mirror is simple. If the microscope is not equipped with a frosted mirror, covering a clear, flat mirror with a stretched piece of Parafilm (parafilm.com) works equally well.

Because the observer can soon learn to see the unstained sheet of muscle fibers and the *N3s*'s that innervate them (Fig. 1*E*), s/he can then maneuver the suction electrode into contact with a selected *N3s* and observe the nerve as s/he draws it into the electrode. If an audio monitor is attached to the recording preamplifier, the observer can hear the increasing signal from spontaneous spikes as the nerve is drawn into the electrode.

Glass electrode tips versus gel-loading tips. In teaching labs, there is a strong incentive to use inexpensive, tough electrodes that will survive the inevitable errors beginners make. One widely-used design employs commercial gel-loading tips to contact the *N3s* (Wyttenbach et al., 1999). We have been unable to find any such tips that are small enough to hold the *N3s* snugly so that recordings are stable. On the other hand, glass tips made under a stereo microscope by breaking off glass microelectrodes to an optimal tip diameter yield excellent stable recordings. Glass electrodes are more fragile than plastic tips and the initial cost of the holders is greater, but the huge advantage of these glass tips is that recordings made with them are stable for long periods; the signal-to-noise ratios don't change. If one goal of the lab is to compare spike heights and infer how many motor units are active, this stability is crucial. If the glass tips are used with well-made electrode holders, replacing a broken tip during a class from a stock of prepared tips is quick and straightforward. In our experience, when the observer can see the electrode tip, the muscle fibers, and the nerve, broken tips are uncommon.

Segmental differences in spontaneous activity in N3s units. The slow flexor muscles in each abdominal segment are innervated by separate sets of motor neurons (Wine et al., 1974; Leise et al., 1986). In different segments of the abdomen, the orientation and insertions of slow flexor muscles differ. Observers naturally attend to the middle of a preparation, to segments 3 and 4, because the slow flexor muscles of these segments are often most cleanly exposed. Recordings from the *N3s* in these middle segments usually reveal four spontaneously active units (Kennedy and Takeda's units 1 through 4), but unit 5 – the peripheral inhibitor – and unit 6 are normally silent. We have observed that the levels of spontaneous activity in motor neurons innervating the muscles in abdominal segments 1 and 2 are often higher than those of more posterior segments, and that in some preparations all six units are spontaneously active in these anterior segments (Fig. 2). This is particularly advantageous when students are recording simultaneously from *N3s* and a postsynaptic muscle fiber because they can then observe EPSPs and IPSPs from each motor neuron without needing to stimulate units 5 or 6 (Fig. 3).

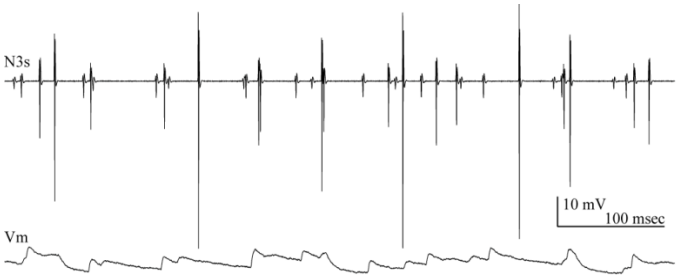

Figure 3. Paired recordings from the *N3s* and a postsynaptic slow flexor muscle fiber (Vm) in segment A2. The intracellular recording from the muscle fiber (Vm) shows both EPSPs and IPSPs that follow spikes in certain different *N3s* motor axons. The maximum hyperpolarization here during an IPSP was -51 mV. In this example, all six motor axons fire but not all six synapse with this muscle fiber.

Paired recordings from presynaptic axons and postsynaptic muscle fibers. Given the preparation illustrated in Figure 1, students can position a suction electrode on a selected *N3s*, aim accurately for a selected muscle fiber innervated by that *N3s*, and penetrate it under visual control. In transmitted light, they can also see the tip of the microelectrode and so are less likely to break it. The resulting recordings of presynaptic spikes and postsynaptic potentials (Fig. 3) allow students to address questions of multineuronal innervation, patterns of innervation, and relationships of presynaptic spike size and PSP amplitude.

In these slow muscles, tension is strongly correlated with the fiber's membrane potential, and these fluctuations in potential are tracked by fluctuations in mechanical force (Kennedy and Takeda, 1965). Intracellular recordings from neurons in active nervous systems show similarly complex fluctuations of their membrane potentials that are also due to integration of PSPs from multiple presynaptic units., e.g., (Smarandache et al., 2009).

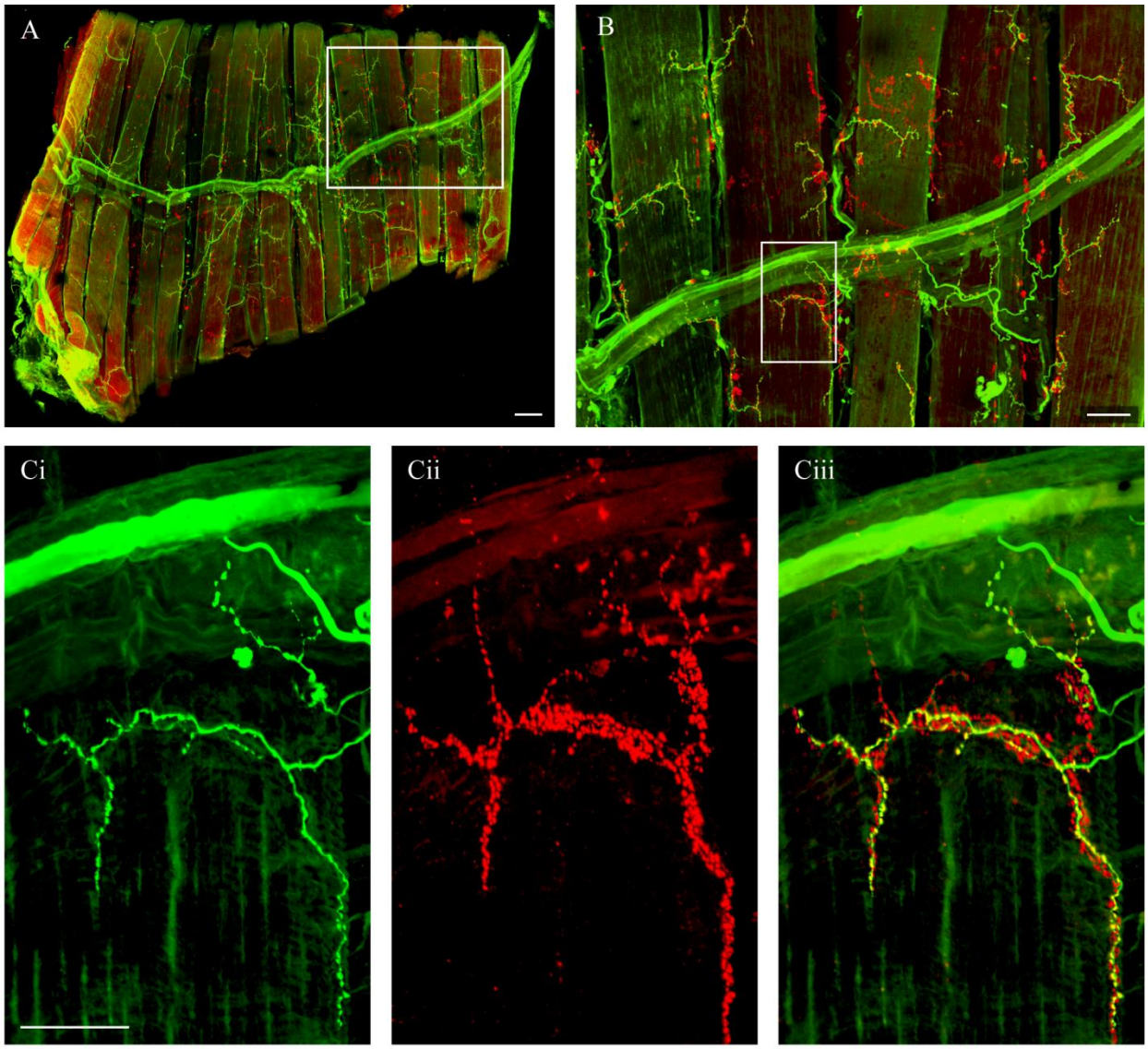

Figure 4. Double labeling of synaptic vesicles using anti-Synapsin antibody (red) and GABAergic motor axon using anti-GABA antibody (green). ***A*** Full merged montage of a slow flexor muscle on the left side of segment A2 imaged at 10x. The *N3s* that innervated the muscle fibers is visible as it crosses the muscle from right to left. The single GABAergic axon is visible in the nerve. Up is anterior, left is lateral, and scale bar is 200 µm. The box marks the region magnified in ***B***. ***B*** A montage of a region of the same muscle at 20x magnification. The box outlines the area of 60x magnification displayed next. Scale bar is 100 µm. ***C*** High-resolution images (60x) of fine branches of the nerve showing separately the GABAergic axon (***Ci*** green), the distribution of synapsin in clusters of synaptic vesicles (***Cii*** red), and the areas of colocalization between anti-GABA and anti-Synapsin (***Ciii*** yellow). Scale bar is 50 µm.

Anatomy of multineuronal synaptic contacts. One feature of this preparation that is particularly salient for teaching is that each of these muscle fibers is innervated by several axons, one of which is inhibitory (Kennedy and Takeda, 1965; Velez and Wyman, 1978; Mulloney and Hall, 1990; Lnenicka, 2014). Multineuronal innervation is widespread in the CNS. To visualize these synaptic connections, we used two antibodies to label the protein synapsin on all synaptic vesicles and the neurotransmitter GABA in unit 5, the GABAergic inhibitor (Mulloney and Hall, 1990). This anti-GABA antibody labels the entire cytoplasm of neurons that have elevated concentrations of GABA. The resulting two-color whole mounts reveal the green branches of the GABAergic axon (Fig. 4), the numerous clusters of red synaptic vesicles that mark each synaptic contact (Fig. 4*B*, 4*C*), and the yellow subsets of vesicles that contain GABA (Fig. 4*C*). By inspection of these whole mounts, students can observe that each muscle fiber is the target of many synaptic contacts, but only some synapses have GABA immunoreactivity. The other contacts must be from other presynaptic axons that use a different neurotransmitter.

Experimental tests of Ca^{++} dependence of synaptic transmission. The slow flexor muscles in each segment are not heavily ensheathed by connective tissue, so access to the motor axons and synapses is relatively unrestricted. This makes it easy to change the solutions bathing the preparation. We took advantage of this feature

to compare three different low-Ca^{++}/ high-Mg^{++} saline solutions (Table 1) that have been used at different times in our lab to block chemical synaptic transmission (del Castillo and Engbaek, 1954; Katz, 1962). These solutions differed in their Mg^{++} and Ca^{++} concentrations. Although the calculated osmolarity of all these solutions are the same, their measured osmolarities differed (Table 1).

The effectiveness of each solution in blocking transmission was measured as a reduction in the size of a muscle fiber's PSPs, which were monitored continuously. Bath application of each low-Ca^{++} solution was preceded and followed by application of normal saline (Table 1). Shortly after applying the experimental solution, PSP amplitudes began to decrease, and within five minutes they were reduced to less than 35% of control sizes (Fig. 5). One solution, #3 in Table 1, which had 20 times more Mg++ and about 5 times less Ca^{++} than normal saline, was clearly more effective than the others. It achieved an almost complete block within five minutes, which was fully reversed when the low-Ca^{++}/high Mg^{++} solution was replaced again by normal saline (Fig. 5).

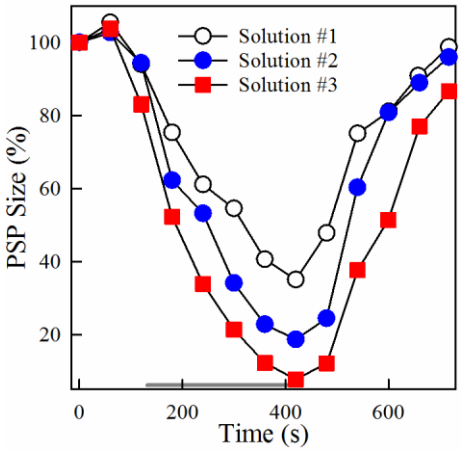

Figure 5. Plots of PSP sizes during and after perfusion with different experimental solutions show the sensitivity of synaptic transmission to the ratio of extracellular Ca^{++} and Mg^{++}. All three curves are from the same preparation. The most successful block of transmission is caused by the 3rd solution, which achieved 98% reduction in PSP amplitude within 5 minutes, and a nearly complete recovery within 6 minutes. The grey bar marks the interval when the low Ca^{++} solutions were applied.

DISCUSSION

These modifications of the classic student preparation are each minor, but when used together they lead to significant improvements in student outcomes: the pace at which naive students get their first successes with extracellular recordings and their first paired recordings of presynaptic spikes and PSPs is accelerated and the quality of their recordings is markedly improved.

The lab we teach devotes one three-hour period to this preparation, and it is important that students get something out of it within that time. The lab is organized into nine groups of three students each, so teaching the dissection is out of the question. When students walk in, there is a completed preparation (Fig. 1*E*) on the stage of each stereo microscope, with a suction electrode connected to the recording apparatus.

Students begin by looking carefully at the preparation to find nerves and muscles, and only then swing the electrode into place and start to make recordings. To make nine fresh preparations in the hour before the lab opens takes three moderately experienced people working side-by-side steadily, but the heuristic result is worth that effort. In the first lab where we used this dissection and this organization, all nine groups made good recordings and were able to compare recordings from left and right sides of the same segment and from different segments. There were two broken glass tips, and these took little time to replace. Were we able to teach a second three-hour lab that added microelectrode recording to this foundation, we are sure that most groups would succeed in making recordings like Figure 3.

The superiority of glass-tipped suction electrodes over plastic electrodes for student labs has been advocated before in these pages (Johnson et al., 2007; Inam et al., 2014), and our results confirm those authors' recommendations. We also find that the meager amount saved making electrode holders does not compensate for the faculty time spent in construction or, particularly, the teaching time lost repairing equipment during a lab.

The recordings students make can be analyzed quantitatively in more than one way. When the recordings are stable and have high signal-to-noise ratios, e.g., Figure 2, using a ruler to count size classes and differences in

	Normal Saline†	Solution #1	Solution #2	Solution #3
NaCl	195 mM *11.4 gm/L*	175 mM *10.2 gm/L*	138.6 mM *8.1 gm/L*	138.6 mM *8.1 gm/L*
$CaCl_2$•$2H_2O$	13.5 mM *1.98 gm/L*	2.7 mM *0.4 gm/L*	1.4 mM *0.2 gm/L*	2.5 mM *0.38 gm/L*
$MgCl_2$•$6H_2O$	2.6 mM *0.53 gm/L*	26 mM *5.28 gm/L*	52 mM *10.6 gm/L*	52 mM *10.6 gm/L*
Measured Osmolarity (Calculated Osmolarity)	422 mOsm (463.8)	412 mOsm (461.3)	402 mOsm (463)	422 mOsm (466.3)

Table 1. Composition of Normal Crayfish Saline and Experimental low-Ca^{++} high-Mg^{++} salines †.

† Each solution also contained 5.4 mM KCl (*0.4gm/L*), 4.7 mM Maleic Acid (*0.55gm/L)*, and 10 mM Trizma Base (*1.21gm/L*) at pH 7.4. Osmotic values for each solution are calculated from molecular weights, assuming complete dissolution of the salts.

spike frequency is effective, and gets them looking at their data. These recordings also lend themselves to digital spike-sorting procedures (Inam et al., 2014), and cases where the students' own analysis differs from the digital analysis could be particularly valuable points in their education.

Because the slow-flexor muscles are not surrounded by a sheath of connective tissue, the preparation is also useful for pharmacological experiments. The results of our low-Ca^{++} tests (Fig. 5), for example, show that bath-application of test solutions is an effective approach here.

The protocol for double-labeling synapsin and GABA is workable for well-equipped lab courses with time for more than one lab-session devoted to this preparation, but even courses without these resources can use the double-label figures to teach the anatomy of this system and to introduce the concepts of immunohistochemistry. Seeing the patterns of synaptic connections on these muscle fibers illustrated by these double-labeled images (Fig. 4), will also increase students' understanding of what they are attempting to record and how synaptic integration works.

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The colored images in this paper are available as PowerPoint slides at http://npb.ucdavis.edu/npbdirectory/mulloney_abdominal_ganglia/index.html.

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