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Application of a spike sorting procedure to analyze recordings in the crayfish ventral superficial flexor preparation: A high resolution approach to the study of neuromodulators on axons and synapses

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The crayfish ventral superficial flexor (VSF) is innervated by the ventral branch of the third nerve. This nerve and muscle system is tonic, and is responsible for posture control of the animal. The easy accessibility of the third nerve and its muscle fibers, along with its display of tonic activity in vitro, make this preparation ideal for observing axonal action potentials and synaptic responses. As a result, this preparation has often been adopted for undergraduate electrophysiology laboratories. This report describes application of a spike sorting procedure to simultaneously recorded traces from the third nerve and associated muscle fiber. This procedure allows for isolation of action potentials arising from each of the six axons in the third nerve. Separation of action potentials and their corresponding synaptic responses from large data sets, with sample sizes in the thousands, enables us to perform averaging and to analyze waveforms of action potentials and synaptic responses with a high resolution. With this high resolution approach, we document variations in the shape of action potentials in the third nerve and in synaptic potential in muscle fibers. The approach described here can be used for detailed study of the effects mediated by neuromodulators and drugs. An example of such an application is illustrated using 50 µM GABA. Several possible student projects using this approach are outlined and discussed.

Key words: crayfish; ventral superficial flexor; neuromuscular junction; action potential; synaptic transmission; spike sorting; GABA

The crayfish ventral superficial flexor (VSF) nerve muscle preparation was initially described by Kennedy and colleagues (Kennedy and Takeda, 1965) and later refined by others (Velez and Wyman, 1978a, b). It has been shown that the ventral superficial flexor muscle is innervated by six axons in the ventral branches of the third nerve (Kennedy and Takeda, 1965). The function of this nerve muscle system is assumed to be to maintain the posture of the animal because—unlike motor axons that innervate deep, phasic muscles controlling powerful tail-flip—axons innervating VSF are tonically active while the animal is at rest or performing terrestrial walking (Moore and Larimer, 1988). Due to the presence of tonic activity, the absence of fast muscle twitch, its relatively simple dissection and long survival period in vitro, this preparation has been widely used to study of axonal regeneration (Goransson et al., 1988; Worden et al., 1988) or adopted for undergraduate laboratory exercises. In a typical experiment for undergraduates, a suction electrode is used to record action potentials from the third nerve and an intracellular electrode to record synaptic responses from a muscle fiber in the VSF. A persistent problem has been the unambiguous identification of action potentials (AP) originating from the six axons that innervate the VSF. Another often raised question concerns the "right" shape of these action potentials and synaptic potentials. In order to seek clarity on these issues and to distinguish these axons on the basis of automated and objective measurements, we have applied a spike sorting technique to distinguish action potentials according to three parameters: the amplitudes of maximal and minimal peaks and of an after-potential corresponding to the after-hyperpolarization of an action potential recorded intracellularly. Action potentials thus distinguished can be averaged, compared and analyzed in detail.

The identification and separation of action potentials, together with simultaneous intracellular recording from muscle fibers, would allow for unambiguous correlation between action potential and synaptic response. This paired pre- and postsynaptic recording configuration would be a valuable tool for teaching synaptic transmission. In addition, spike sorting would make it possible to collect large data sets of paired pre- and postsynaptic activities. The large data sets would allow for averaging and improved signal to noise ratio, and enable detection of subtle changes mediated by neuromodulators. For example, neuromodulators released under physiological conditions may be present in relatively low concentrations such that their effects are difficult to quantify if only a small number of trials are inspected. Averaged responses from automatically sorted large data sets should provide far better resolution. Thus, the third nerve VSF preparation used in combination with spike sorting analysis could also be useful for drug testing, investigating dose response relationships and examining interactions among simultaneously applied neuromodulators.

In this report, we summarize the detailed analysis required to unambiguously distinguish action potentials originating from different axons. Variations in action potential shape and synaptic potential waveform are described. Finally, the effect of 50 µM GABA on this preparation is used as an example to illustrate the

improved resolution that can be achieved with this approach.

MATERIALS AND METHODS

Dissection and preparation. Crayfish, *Procambarus clarkii*, ~5cm length were used in all experiments. The animals were immobilized in ice and decapitated by cutting behind the eye stocks. The abdominal section, tail, of the animal was then separated from the thorax using scissors. The tail was pinned dorsal side up in a 100x25 mm petri dish after the dorsal carapace was removed. The petri dish had a layer of Sylgard, ~7-10 mm, and the remaining depth (~15-18 mm) was sufficient for the entire preparation to be submerged. (Larger animals, however, would need larger and deeper dishes.) The deep flexor muscle in the tail was removed in the dish under a dissection microscope.

We found it helpful to first separate the deep flexor (phasic) muscles along the midline such that the ventral nerve cord was visible during the dissection. Visualization of the ventral nerve cord facilitates the identification of dorsal branches of the third nerve that innervate the deep flexor. These branches should be cut before the phasic muscle fibers are removed, to ensure that the ventral nerve cord is not displaced or stretched in the process of clearing the deep muscles. Most deep flexor muscles are anchored to sternites, and the removal of these muscles involves cutting them close to the sternites. It is important not to damage the tonic VSF muscle fibers in the process of trimming away the phasic muscle because the former are also attached to the sternites. This dissection approach, pinning the tail dorsal side up and approaching from the top, has the advantage of enabling removal of most of the deep muscle such that the only tissue left is the ventral nerve cord and a thin layer of VSF. Since muscle fibers can release neuroactive compounds under stimulation or in response to neuromodulators (Pedersen and Febbraio, 2012), this dissection approach avoids complications related to the presence of a large mass of deep phasic muscle. However, removal of the phasic muscle is a lengthy dissection and may not be practical if insufficient laboratory sessions are allocated for this project. An alternative approach is to pin the animal ventral side up and approach the third nerve and VSF by removing the cuticle from between sternites (Baierlein et al., 2011). Although this is still a delicate dissection, the number of steps involved is smaller than those for the dorsal approach and is more practical for students to perform on their own.

Electrophysiology. A standard intracellular electrode filled with 3M KCl (~5MΩ in resistance) was used for recording from muscle fibers. Stable muscle recording was best obtained by aligning the electrode with the long axis of the muscle fibers, i.e., having the electrode approach muscle fibers from the anterior or posterior end of the animal.

Suction electrodes were used to record from the third nerve. These electrodes can be fabricated by melting and pulling plastic pipette tips and shaping the fine tip using heat from a soldering iron under a dissection microscope. These plastic electrodes can be reused if cleaned properly after use. However, making plastic pipette tips and shaping the tips take some skill, and may not be the best use of laboratory time for undergraduates. A better alternative is to make the suction electrodes by breaking off the tip of a glass electrode pulled for intracellular recording from muscle fibers. The tip of such a microelectrode should be large enough to accommodate a loop of the third nerve within the pipette. At the same time, the tip should be small enough that the nerve does not slip out when the negative pressure, i.e. suction, is released. Having the nerve fit snugly into the pipette tip also ensures that activity of the third nerve is recorded with optimal signal to noise ratio. Breaking the tip off an intracellular electrode should be done with a pair of #5 forceps and under a dissection microscope. It is helpful to tape an electrode with the appropriate tip size onto the stage of the dissection microscope to serve as a guide for students. The tip opening need not be flat; as long as the diameter is appropriate, the quality of recording will be assured.

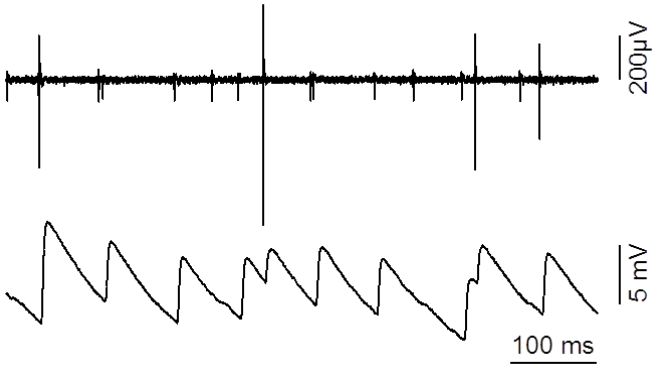

Figure 1. Simultaneous recording from the third nerve and a muscle fiber of the ventral superficial flexor. The upper trace shows a suction electrode recording from the third nerve. Spontaneous activity originating from neuronal networks in the ventral nerve cord initiated these action potentials. The lower trace shows intracellular recording from a muscle fiber of the ventral superficial flexor muscle. Every synaptic activity can be linked to an action potential in the upper trace. Not all action potentials trigger detectable synaptic responses.

Data analysis. Data acquisition can be performed with any of the standard software packages used in student laboratories. Since the suction electrode recorded AP is equivalent to the derivative of an action potential recorded intracellularly, a fast sampling rate (faster than 50 μs) is needed to fully capture the features of this AP. AC and DC amplifiers are used for the third nerve and muscle fiber recordings, respectively. Both channels should be acquired simultaneously. Data acquisition used in this report included a combination of National Instrument data acquisition board (NI PCI-6602) and the IGOR NIDAQ Tools package (Lake Oswego, Oregon).

The first step in the analysis is to set the AP detection threshold. With a low pass filter set to 3-5 KHz in an AC amplifier, the standard deviation of background noise in AP recording should be 3-5 μV. Setting the threshold to 3X the standard deviation, 10-15 μV, will typically give rise to one falsely identified AP with a peak-to-peak amplitude of

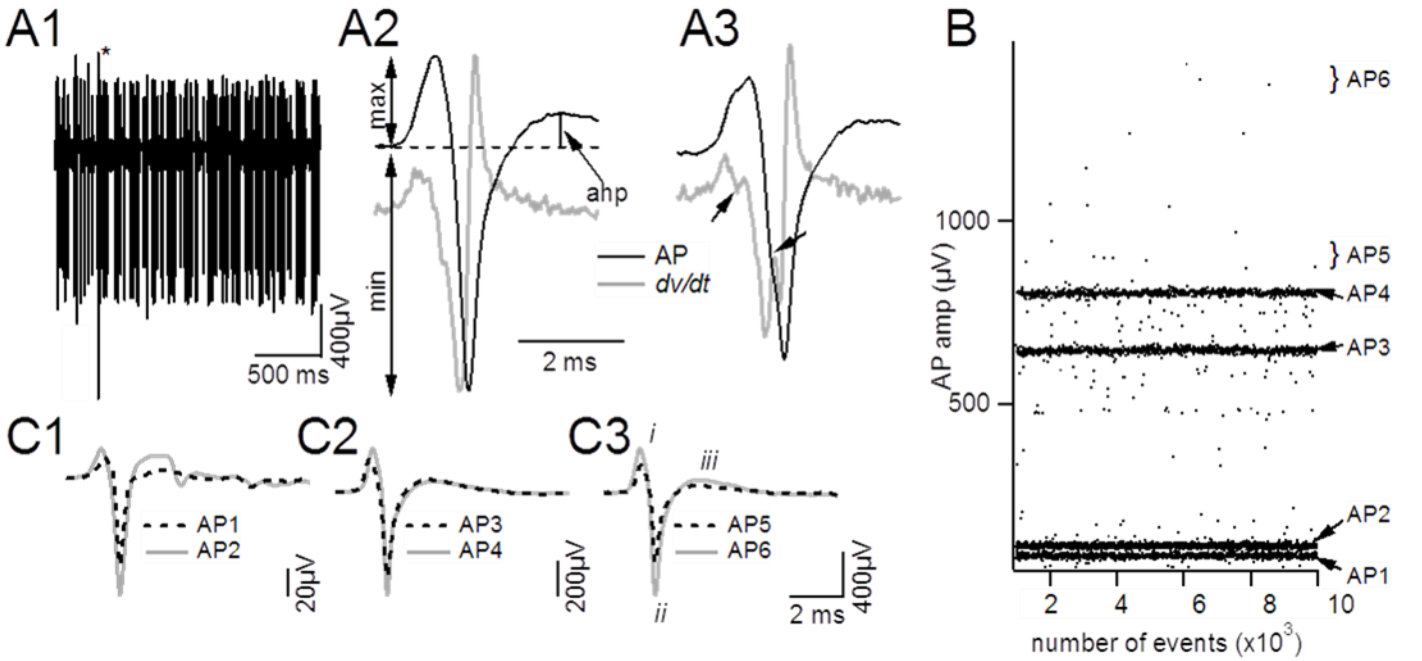

Figure 2. Data analysis based on spike sorting of third nerve action potentials. (*A1*) Superimposed traces (n=3) of third nerve recordings. Axons generating medium and small action potentials fire at high frequency while large axons fire less frequently (*). (*A2*) Expanded view of the large action potential (black trace) marked by the asterisk in A1. Overlaid on the action potential is the derivative of the action potential (grey trace). Also marked here—max, min and ahp—are the parameters measured by the spike sorting software. (*A3*) Another "large" action potential recorded from the same experiment. The derivative of this large action potential shows clear "glitches" (arrows) suggesting that the apparently large amplitude is due to the summation of two smaller action potentials fired almost simultaneously. (*B*) Peak-to-peak action potential amplitudes, i.e. maximum to minimum amplitude, plotted against numerical tags assigned to action potentials detected in a block of 80 traces. Each horizontal strip represents the activity of one particular axon. When the suction electrode recording is stable, i.e. the loop of the third nerve in the suction electrode does not slip out, the amplitude of each AP should show no sign of upward or downward drift. (*C1* to *C3*) Averaged action potentials recorded from the third nerve. Averages were obtained after aligning the minimal point of individual traces. The numbers of traces used for averaging were: 1308, 1977, 1267, 904, 10 and 4 for AP1 to AP6, respectively. "*i*", "*ii*" and "*iii*" in *C3* identify the three stereotypical components of an action potential. *A1* to *A3* share the same vertical scale. The vertical scales of derivative traces are not shown. *A2* and *A3* share the same time scale. *C1* to *C3* share the same time scale.

10-20 µV. Although this false AP can be easily avoided by setting the detection threshold to 5X standard deviation, ~20 µV, its presence can sometimes be educational when discussing signal detection. There are many public domain software packages that can perform sophisticated AP detection and sorting. Data presented in this report used a spike sorting procedure written in IGOR Macro language. A simple threshold detection method is used in this report, but some types of software use a more sophisticated filter matching approach to identify and classify action potentials. Due to the diverse file format used by different data acquisition setups, different degrees of flexibility in data format conversion, and different preferences among laboratory instructors, no "standard" software will be described here for detailed data analysis.

RESULTS

Figure 1 illustrates tonic activity recorded from the third nerve (upper trace) and from a muscle fiber (lower trace). Spontaneous activity continued unabated for 3-5 hours in unperfused preparations. Matching of action potentials in the third nerve with synaptic potentials served as a good talking point for the discussion of membrane excitability and synaptic transmission. Most students were impressed and excited by this continuous and vigorous activity, especially when they had performed the dissection themselves. The goal of this report is to advance the use of this preparation, from visual inspection and evaluation of a few pairs of traces to quantitative analyses of large data sets for more detailed and quantitative analysis. In addition, frequently encountered variations in action potential and synaptic potential shape are documented and clarified in the examples shown below.

Workflow of spike sorting analysis. Figure 2 illustrates the basic workflow for action potential analysis. Action potentials originating from the largest axon typically exhibited low firing frequency and could be missed if only a few seconds of data were inspected. Figure 2A1 contains 3 overlapping traces selected randomly from a set of 600, each trace having a duration of 2 seconds. The largest AP (*) in the figure appeared only once in three traces. (See Fig. 2A2 for the same AP displayed on an expanded time scale.) Detailed inspection of the action potential on an expanded time scale was needed to ensure that the large amplitude was not due to a summation of two smaller APs. Summation was detected in the form of abrupt changes in

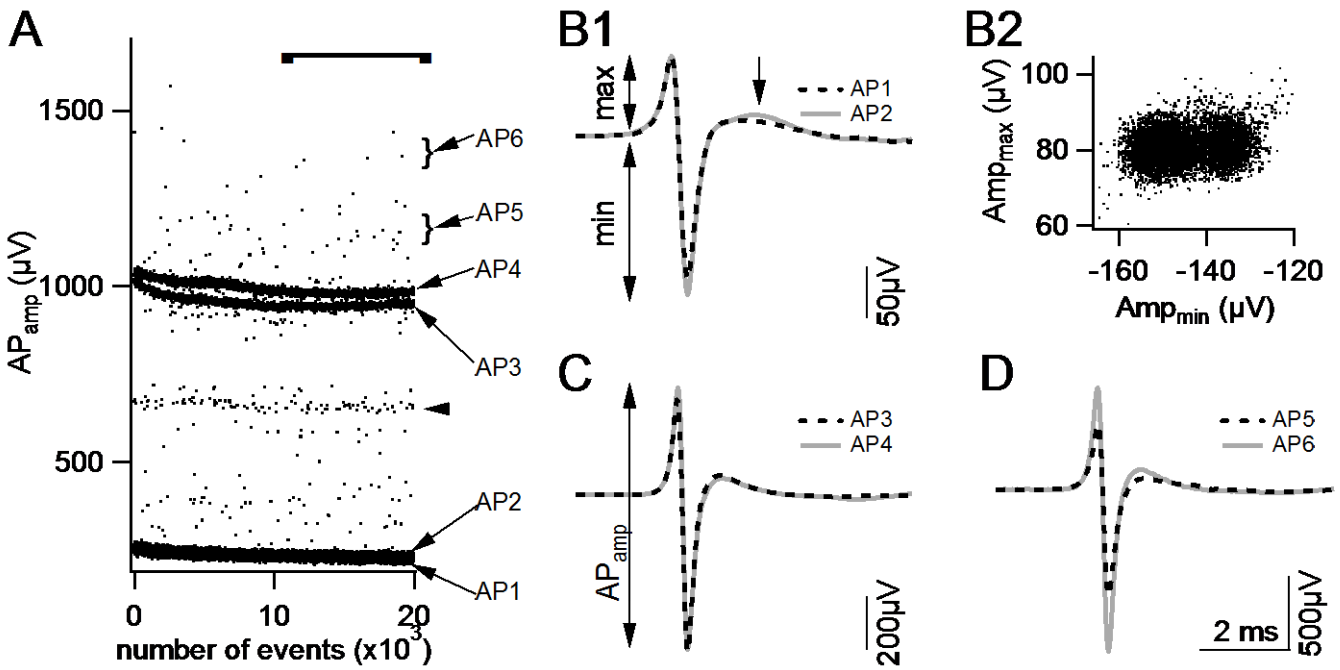

Figure 3. How to resolve action potentials originating from different axons but have similar amplitudes. (*A*) AP amplitude plotted again AP tag number. Note that the amplitude strip marked as AP1 and AP2 cannot be separated into two distinct strips visually. The bracket on top marks the section of data showing relatively stable amplitudes for all axons. Data under this bracket were selected for further analysis. (*B1*) Averaged action potentials differentiated on the basis of their minimal, instead of full, amplitudes. The arrow points to the difference in ahp between the two averaged APs. (*B2*) Maximal and minimal amplitudes of individual action potentials from the lowest strip in A plotted against each other. Side by side distribution of the two clusters suggests the presence of two action potentials with the same maximal amplitude but different minimal amplitudes. (*C*), (*D*) Averaged APs originating from other axons of the preparation shown in A. The numbers of trials used to obtain each average were: 1620, 2135, 683, 743, 31 and 5 for AP1 to AP6, respectively. Traces in *B1, C* and *D* share the same time scale.

the trajectory of action potential traces, better visualized as clear "glitches" in the differentiated AP traces. The grey traces in Fig. 2A2 and A3 are time derivatives of their corresponding AP traces (black). The large AP in A3 is not "real" because the glitches (arrows) suggest that it is the sum of two smaller APs that are slightly displaced in time. For comparison, differentiation of the AP in Fig. 2A2 did not reveal the glitches seen in A3. In some experiments, the presence of consistent synaptic responses correlating with the largest, but rare, action potentials lent further support to the likelihood of having identified the largest axon. Due to its low firing frequency, the largest axon is not ideal for analyses that require a large sample size. In most preparations, large data sets were obtained from the four smaller axons that fired at high frequency.

A simple threshold detection algorithm was used to detect action potentials that appeared frequently. The detection threshold was typically set to three times the standard deviation of baseline noise. Parameters measured from each detected action potential included the maximal and minimal peaks, and the amplitude of a late component corresponding to after-hyperpolarization of APs recorded intracellularly (ahp) (Fig. 2A2). An example of action potentials detected from 80 two-second long traces is shown in Figure 2B. Here the Y-axis displays AP amplitude, calculated from the sum of the maximal and minimal amplitudes, while the X-axis shows the numerical tag assigned to each detected action potential. A total of ~10,000 AP events were detected in this sorting session. Each action potential with a stable amplitude appears as a thick horizontal strip in this plot. (Scattered data points outside of the identified AP bands were mainly the result of random AP summation—see supplementary materials.) Identification and separation of action potentials was then achieved by selecting action potentials of specific amplitude. Traces in C1, C2 and C3 represent averaged action potentials identified according to their amplitude. All action potentials exhibited a characteristic triphasic waveform, with an initial, positive capacitive charging current (Fig. 2C3 *i*) followed by a negative, inward sodium current (*ii*) and finally finished with the positive, outward potassium current (*iii*). This characteristic AP waveform was consistent within the same recording session and among preparations, as long as the recording site was in the proximal or middle parts of the third nerve. Although AP shapes were consistent in most experiments, the amplitude of action potentials depended on the length of the third nerve sucked into the suction electrode, and the fit of the nerve within the electrode.

In some preparations, the identification of all six axons on the basis of amplitude alone was not immediately obvious. In this case, careful inspection of additional AP parameters could sometimes resolve the ambiguity. Figure 3A shows an example of action potential detected on the

basis of its full amplitude. (There is a slight downward trend initially, suggesting that the third nerve might have slipped out a little in the suction electrode. Data analyses shown in B to D in this figure were limited to the part under the bracket (Fig. 3A), where the amplitudes of all axons were stable.) AP3 and AP4 are clearly distinct. AP5 and 6 exhibited low firing frequency and the bands are not visually obvious. The bottom band, labeled AP1 and AP2, is unusually thick but there is no gap in this thick band to suggest the presence of two distinct action potentials. (The weak band identified by an arrowhead was due to the high incidence with which two APs fired almost simultaneously. This type of false band is typically thinner and can be identified by inspecting individual trials in expanded time scale or by using the criteria described Fig. 2A—see supplementary materials.) Averaged action potentials 3-6 were identified unambiguously and are shown in Figures 3C and D. AP 1 and 2 could not be separated on the basis of full amplitude alone. However, when the maximal and minimal amplitudes of individual APs from the bottom band in A were plotted against each other, the data clustered into two groups (Fig. 3B2). These clusters are distributed side by side, suggesting that they originate from two action potentials with similar maximal amplitude but with slightly different minimal amplitudes. Action potentials averaged from each cluster are shown in Fig. 3B1. As indicated in the scatter plot, the main difference between the two APs is in their minimal amplitudes. The hypothesis that the two clusters originate from different axons is further supported by the observation that the ahp amplitudes are distinctly different between the two averaged traces (Fig. 3B1 arrow) although the criteria for selecting individual traces for averaging did not involve ahp amplitude. Finally, synaptic potentials recorded simultaneously in this preparation showed that AP1 consistently evoked synaptic potential while AP2 did not. (Data not shown.)

Variations in action potential shape. Although action potential amplitude varied depending on the length of the nerve drawn into the suction electrode, the shape of action potentials was in most cases stereotypically triphasic. However, when the suction electrode was placed near the distal part of the third nerve, i.e. near the VSF, AP waveform became more variable. Figure 4 shows averaged APs recorded simultaneously from proximal and distal parts of the third nerve. The delay between the APs recorded from the two locations was long for small axons (AP1, 2) and short for large axons (AP5, 6), consistent with the known correlation between faster conduction velocity and larger axons. APs recorded by the proximal electrodes all displayed a similar triphasic waveform (black traces). However, those recorded simultaneously from the distal axon exhibited variations in the ahp component. Specifically, while AP1 and AP3 showed similar AP shapes for recordings obtained from proximal (black) and distal (grey) locations, AP2 at a distal location exhibited no apparent ahp (arrow). In other cases, the ahp exhibited spiky characteristics (AP4-6 circles) not observed in proximal recordings. The spiky component appeared

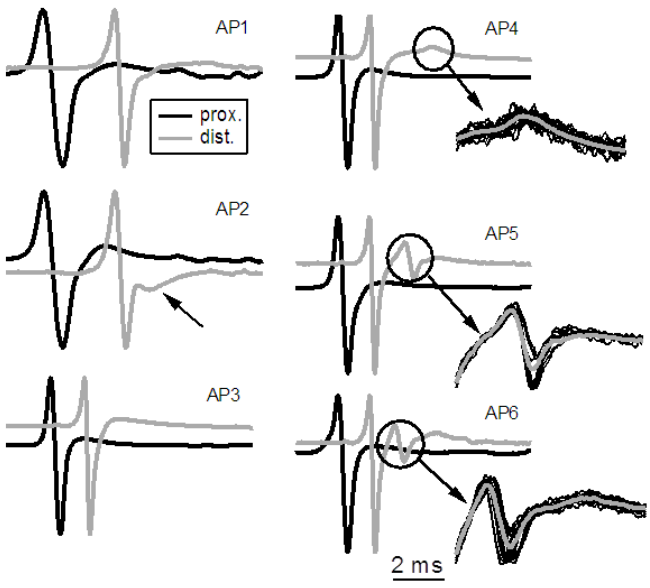

Figure 4. Comparison of action potentials recorded from proximal and distal parts of the third nerve. The black trace in each pair represents recordings from the proximal location while the grey trace represents those from the distal location. To facilitate comparison, amplitudes of each pair are normalized to the same height. The absolute amplitude of the proximal recording was always larger than that of the distal recording. These recordings were obtained simultaneously from the same preparation. The Arrow pointing to the grey trace of AP2 indicates the absence of ahp in this trace. The circles in AP4 to AP6 highlight the section of ahp that shows the "spiky" feature. Insets in AP4 to AP6 show superimposed single traces to illustrate the lack of (AP4) and the presence of (AP5 and AP6) trial to trial variation in the "spiky" part of ahp. All traces, except for those in the insets, share the same time scale.

consistently in individual traces in AP4 but showed some variability in delay for AP5 and AP6 (AP4-6 thin traces in insets). Greater variation in the ahp component of AP waveform was a consistent observation when the suction electrode was placed near the VSF. It is important to emphasize that the "spiky" ahp is not likely to have been due to firing of another axon, because no corresponding action potentials were detected in the proximal electrode. Instead, it is likely to be a reflection of rebound activity in the terminal region of these axons. Experimental tests of this hypothesis have not been published or performed.

It is apparent that AP duration in smaller axons (AP1, 2) is longer than that in larger axons (AP5, 6). Since there has been no published report on intracellular recording from axons of the third nerve, the duration of intracellular APs in each of the six axons is unknown. However, differences in duration of APs recorded with suction electrodes are likely to correlate with conduction velocity. Specifically, when action potentials travel through the loops packed into the suction electrode, those generated by smaller axons, with slower conduction velocity, should take longer to pass through the loop than those generated by larger, faster axons. This interpretation has implications for data analysis of suction electrode recordings. For example, a broader action potential after the application of a neuromodulator could be interpreted as a slowed

conduction velocity. This interpretation could be further tested by checking action potential conduction velocity, before and after the application of a neuromodulator, with dual recordings as illustrated in Figure 4. On the other hand, changes in action potential duration are difficult to verify unambiguously with an independent test, with the exception of intracellular recording from axons. Thus, it is easier to establish changes in AP conduction velocity than changes in AP duration when using suction electrode recordings.

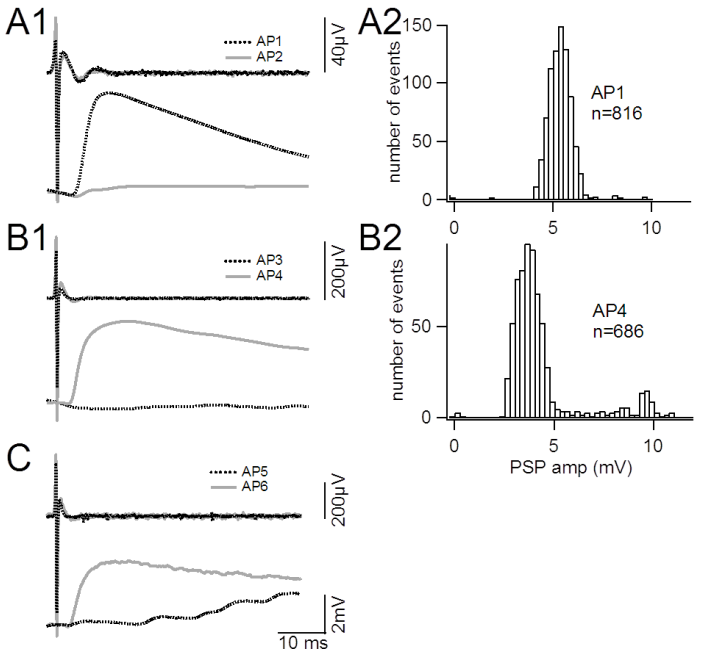

Figure 5. Simultaneously recorded action potentials and synaptic potentials. (*A1*) Averaged action potentials, upper traces, and synaptic potentials, lower trace, evoked by action potential 1 and 2 respectively. (*A2*) EPSP amplitude histogram from 816 trials evoked by AP1. EPSP amplitude centers around 5 mV. (*B1*) Averaged action potential and EPSP evoked by AP3 and AP4. (*B2*) EPSP amplitude histogram evoked by AP4. (*C*) Averaged action potential and EPSP evoked by AP5 and AP6. The sample size of AP/EPSP pairs for AP6 were too small to warrant a histogram. "n" in *A2* and *B2* represents the number of trials measured to compile the histogram. EPSPs in *A1, B1* and *C* share the same vertical scale. All traces in *A1, B1* and *C* share the same time scale.

Action potential sorting coupled with postsynaptic responses. Although synaptic responses evoked by individual axons can be readily identified by visually inspecting trace pairs recorded simultaneously from the third nerve and a muscle fiber, quantitative analysis of excitatory postsynaptic potential (EPSP) parameters such an amplitude, fluctuation of EPSP amplitude, synaptic delay, rise and decay times of EPSP are best achieved by measuring and averaging from a large number of trials. In order to couple synaptic responses with sorted action potentials, a short segment of the postsynaptic trace, starting 20 ms before an identified action potential and ending 70 ms after the action potential, was tagged with each detected action potential. Thus, when action potentials of a particular amplitude were selected and averaged, their matching postsynaptic recordings could also be identified, averaged and measured. Simultaneous recordings from the third nerve and VSF muscle fibers have shown that most muscle fibers are innervated by some but not all of the axons (Velez and Wyman, 1978a, b). Figure 5 shows an example of simultaneous records from a preparation where 3 of the 6 axons generated EPSP. (Although it has been established that the second largest axon (AP5) is most likely to be inhibitory (Velez and Wyman, 1978b), it has been difficult to unambiguously detect inhibitory postsynaptic potential. This is because crayfish muscle fibers have an efficient chloride transport system and it is difficult to shift the chloride equilibrium potential (Finger and Martin, 1986). Furthermore, AP5 often fires at a relatively low frequency and yields only a small sample size.)

In addition to trace averaging, synaptic responses evoked by individual action potentials were measured and compiled into amplitude histograms. The histogram constructed from EPSPs evoked by AP2 exhibited a single peak (Fig. 5A2). This single peaked histogram suggests a high degree of reliability in release, i.e. a high release probability. The histogram measured from AP4, evoked responses showed two distinct peaks with the large one (9.5mV) roughly two times of that of the smaller peak (4mV). Since the amplitude of quantal size has been shown to be about 100μV or less in muscle fibers with input resistance similar to that of VSF (Bittner and Harrison, 1970; Velez and Wyman, 1978a; Vyshedskiy and Lin, 1997), it is unlikely that the 5mV separation between the two peaks in Fig. 5B2 represents the amplitude of a single quantum. (See text describing Fig. 7A3 and B3 for further discussion of possible mechanisms for the multiple peaks in the EPSP amplitude histogram), Fluctuation analysis of quantal events in this preparation has only been successfully achieved with an en passant macropatch technique (Strawn et al., 2000). Nevertheless, the nearly complete absence of release failure, namely incidences of 0 mV responses, in either histogram suggests that the synapses established by both axons have a moderate to high release probability.

Variations in EPSP shape. The junctional potentials shown in Figure 5 are representative in shape, namely a single peak followed by a smooth decay. However, variations in the shape of synaptic responses do occur frequently. Figure 6 shows two APs and their corresponding synaptic responses recorded simultaneously from the same preparation. The synaptic potential evoked by AP4 shows monotonous decay (grey), whereas a second peak appears on the falling phase of the synaptic potential evoked by AP3 (dotted traces and arrow). The second peak associated with AP3 cannot be attributed to occasional axonal firing after AP3, smoothed out by averaging, due to the following analysis. We inspected individual traces of AP3 and selected trials that did not have AP events immediately before or after AP3 (Fig. 6B upper black thin traces). Muscle recordings corresponding to the selected AP3 traces (Fig. 6B lower black thin traces) show that EPSPs with the twin-peaked features appear in

nearly all trials. Since it is unlikely that there was any undetected action potential in this recording—all six axons were accounted for—the twin-peaked EPSP is likely to be due to two-phased release in the terminals. Thus far, we have not found conditions that correlate with, or predict the appearance of, twin-peaked EPSPs.

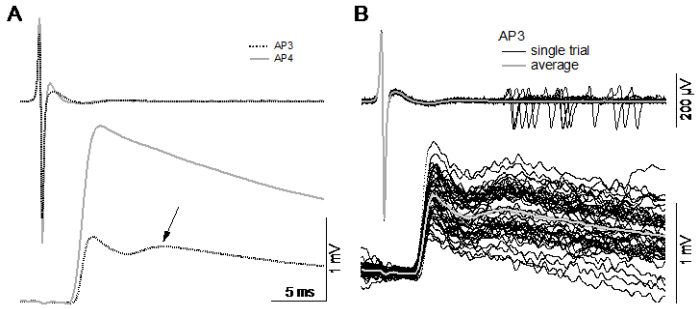

Figure 6. Variations in the time course of synaptic potential. (*A*) Paired action potential and EPSP recordings from AP3 and AP4 of a preparation. Although all EPSPs were recorded from the same muscle fiber, AP4 produced a smoothly decaying EPSP while EPSP evoked by AP3 shows a hump (arrow). (*B*) The hump identified in A is not due to another action potential timed to fire right after AP3. Single trials from AP3 were selected such that trials containing action potentials closely before or after the AP3 spike were excluded (upper black, thin traces). EPSPs evoked by these "clean" trials still show the hump (lower black, thin traces) and in the averaged trace (grey thick trace). All traces in *A* and *B* share the same time scale. AP traces in *A* and *B* share the same vertical scale.

Using the spike sorting approach to analyze the effects of GABA. To illustrate use of the spike sorting approach to evaluate the effects of a neuromodulator on axon and synapse, we used 50 µM GABA. GABA at this concentration has been shown to have a relatively small effect on $GABA_A$ receptors of crayfish muscle fibers (Takeuchi and Takeuchi, 1969; Adelsberger et al., 1996). It can therefore be assumed that inhibitory effects resulting from GABA at this concentration mainly involve presynaptic $GABA_B$ receptors. Figure 7 illustrates two action potentials, recorded simultaneously, and their corresponding synaptic potentials. In addition to the clear inhibitory effect on EPSP after GABA application, there was a distinct difference between the two axons. GABA mediated inhibition of EPSP was weaker for AP3 than for AP2. In addition, the onset of postsynaptic responses showed a clear delay after GABA for EPSP evoked by AP2 but not AP3. This increase in delay could be in part attributed to reduced AP conduction velocity. Based on the discussion in Figure 4, a decrease in conduction velocity might be expected to correlate with an increase in AP width. Such an increase in AP width in GABA was indeed observed for AP2, but not for AP3 (Fig. 7 A2 and B2) (Miwa et al., 1990).

Histograms compiled from EPSP amplitudes measured from single traces were also informative. In the case of EPSP evoked by AP3 (B3), GABA caused a left-shift in the main peak of the histogram and the appearance of a second, smaller peak (B3 black histogram). In the case of more severely inhibited EPSP mediated by AP2, the incidence of release failure accounted for 23 % of the trials in GABA (A3 black histogram). The mechanisms underlying appearance of the smaller peak (AP3 at 6 mV) and release failure (AP2 at 0 mV) are more likely to be due to AP conduction failure at branching points than to reflect quantal events associated with reduced release probability. Specifically, the 2mV (AP2) and 3mV (AP3) gaps between peaks of the EPSP histogram in GABA are too large for the quantal sizes expected in muscle fibers as large as those of VSF (see also comments on Fig. 5B2). One possible interpretation for the multiple peaks is that synapses originating from AP3 could be distributed in several terminal branches, in which case the high incidence of the 6mV EPSP in GABA may arise when synapses in one of the branches dropped out as a result of AP failing to propagate past a branching point and activate synapses in that branch (Spira et al., 1976; Smith, 1980). The appearance of the 0mV peak for AP2 would then be attributable to a complete failure of AP to reach any of the terminal branches of AP2. Since GABA has been shown to reduce AP amplitude in crayfish axons (Baxter and Bittner, 1981), presumably by way of both $GABA_A$ and $GABA_B$ receptors (Fischer and Parnas, 1996), an increase in incidence of branching point failure in GABA is a plausible interpretation. (The cause of the left shift in the major peaks—AP2 from 2.85 to 2 mV and AP3 from 11 to 9mV—could be due to a postsynaptic effect, namely a reduction in input resistance. Specifically, the left shift of the major peaks was equivalent to respective reductions of 70 and 81% in the amplitudes of the main peaks for EPSPs evoked by AP2 and AP3. Since EPSPs evoked by both axons were recorded from the same muscle fiber, a reduction in input resistance of the muscle fiber should result in a similar percentage reduction in amplitude for all excitatory synaptic inputs. This hypothesis could be tested by measuring the input resistance of muscle fibers before and after GABA application (Adelsberger et al., 1996), although the experiment, which requires two intracellular electrodes in the same muscle fiber, would be technically challenging for undergraduate students with limited bench hours.)

Inspection of action potentials also revealed variations in the effects of GABA. AP2 showed a clear reduction in its maximum and ahp amplitudes, while AP3 showed a slight increase in its minimum. Since the two APs were recorded from the same suction electrode, the reduction in only one of the two APs cannot be attributed to a gradual deterioration of recording conditions. In other words, it is reasonable to assume that factors affecting the quality of recording for AP2 must also affect AP3. The fact that AP2 was more severely affected by GABA than AP3 suggests that there were certain variations among axons in their sensitivity to GABA. The hypothesis of variation in GABA sensitivity is also supported by the observation that the increase in AP duration was clearly present in AP2 but not in AP3. It is important to emphasize that stability of suction electrode recording in this kind of pharmacological experiment is essential if the investigation involves analyses of AP waveform. At the same time, variation

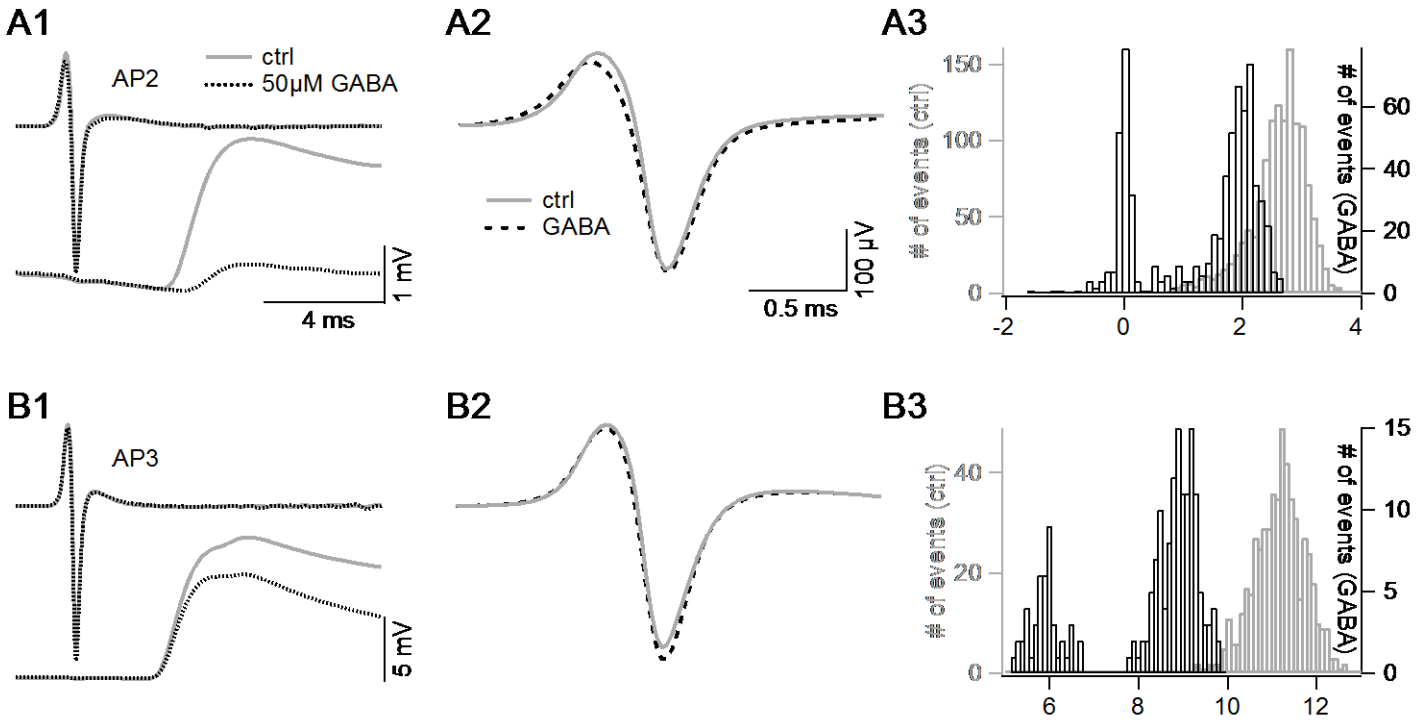

Figure 7. Impact of GABA at 50 μM on action potential and synaptic transmission. (*A1*) AP2 and its corresponding EPSP recorded before and after 50 μM GABA. The averaged EPSP was reduced to 15% of its control amplitude in 50 μM GABA. (*A2*) Changes in action potential waveform induced by GABA. Both the maximum and ahp show a slight reduction in amplitude in GABA. Furthermore, the overall time course of AP in GABA is slower, i.e. it has a slightly longer duration. (*A3*) EPSP amplitude compiled before (grey) and after (black) GABA. A peak around 0 mV in GABA indicates an increased incidences of release failure. (*B1*) AP3 and its corresponding EPSP recorded before and after GABA. The reduction of AP3 in the presence of GABA is smaller than that of AP2 in GABA. Note that EPSPs evoked by AP2 and AP3 were recorded simultaneously from the same muscle fiber. (*B2*) Comparison of AP3 recorded before and after GABA. There is no difference in the maximum or ahp, only a slight increase in the minimum. Unlike the comparison shown for AP2, no change in duration is detectable. (*B3*) EPSP amplitude histograms compiled before and after GABA for AP3 mediated EPSP. There is a clear leftward shift in the main peak, from 11 to 9 mV, and the appearance of a smaller peak, at 5.9 mV, after GABA application. *A1* and *B1* share the same time scale. *A2, B2* share the same time scale. AP traces in *A1, A2, B1* and *B2* share the same vertical scale.

among axons in their sensitivity to certain drugs should be viewed as an advantage, because axons with lower sensitivity to a drug can provide information on the stability of the suction electrode recording.

This variation in the GABA mediated response is not unique. In our laboratory, other agents such as h current blocker (ZD7288), 5-HT and high K^+ have also been shown to induce variable effects among axons. This variation has been a source of confusion for students but could also be used to encourage them to look at their data more closely and come up with a satisfactory conclusion. There are two important reminders to give students as they perform their data analysis. First, it is important that they check more than one axon in the same preparation, and more than one preparation, when they investigate the effect of certain drugs or neuromodulators. Second, axon-synapse pairs showing no response to a modulator should not be treated as failed experiments, especially when they coexist with pairs within the same preparation that are responsive. These nonresponsive pairs can be used as a reference for evaluating the stability of recording conditions or the viability of the preparation.

DISCUSSION

The goal of this report was to demonstrate the application of a spike sorting approach to analyze action potential shapes and postsynaptic responses recorded from the third nerve. The spike sorting routine enabled us to collect a large data set, allowed for signal averaging, and examined data with high resolution. Application of such analysis demonstrated the presence of diverse AP waveforms recorded with suction electrodes, and diverse synaptic potential shapes recorded from muscle fibers. Finally, 50 μM GABA was used to illustrate how the effects of drugs and neuromodulators can be evaluated by applying spike sorting to this preparation.

Other model systems could also take advantage of the spike sorting approach and develop into novel teaching or research tools. For example, the ventral nerve cord of *Drosophila* larvae is known to generate patterned motor commands (Cattaert and Birman, 2001), but since most muscle fibers in this preparation are known to be polyneuronally innervated, selective stimulation of a specific axon is difficult. Furthermore, different axons have been shown to generate distinct synaptic responses

(Kurdyak et al., 1994). Application of the spike sorting approach described here could help isolate individual axons along with their corresponding synaptic responses and offer an opportunity to quantitatively analyze changes in synaptic transmission in mutants.

Variation in the shape of action potentials recorded with a suction electrode. In nearly all cases, performing recordings by sucking a loop of the third nerve into a suction electrode gave rise to stereotypical triphasic action potentials. Data shown in Figure 4 demonstrate that action potential shape was not always stereotypical if the recording was obtained from the segment of the third nerve near VSF. Since the "spiky" ahp was only observed in distal recordings, it is reasonable to assume that the extra spiky component arose from activity in terminal branches. In studies of the lizard neuromuscular junction, the "extra peak" recorded at the heminode—a location analogous to the distal recording site of the third nerve used here—was made possible by K^+ channel blockers or by increasing extracellular Ca^{2+} (Lindgren and Moore, 1989). Therefore, the "spiky" ahp reported here could reflect local action potential rebound from deep after-hyperpolarization. The functional significance of this observation at the neuromuscular junction remains to be fully explored. A mechanistic explanation for the spiky ahp could be the topic for a student project. Specifically, the student could try to formulate a hypothesis to explain the "spiky" ahp, and design experiments to test their hypothesis, for example by using various channel blockers to enhance or block the spiky ahp.

Variation in the shape of postsynaptic synaptic potentials. The second unexplained observation reported here is the existence of synaptic potentials with a second peak (Figure 6). It is possible that the second peak in synaptic potential was due to the rebound spike that resulted in the spiky ahp, as discussed above. It is also possible that the second peak in synaptic potential was a consequence of the innervation pattern of the terminal branches. For example, an axon branch may make synaptic contact with one muscle fiber, but the same branch, or a different branch of the same axon, could also "loop back" and make additional synaptic contacts with the same muscle fiber. The second peak in synaptic potential could occur if the "loop" were sufficiently long that additional synaptic transmission occurred only after significant AP conduction delay. Thus far, there is no published explanation for the "twin-peaked" synaptic potential in this preparation. This observation could also be the basis for a student project, requiring incorporation of morphological analysis.

Most of the possible projects outlined above could be undertaken by performing experiments with the tools available in undergraduate laboratories, such as commonly available channel blockers and basic morphological analysis.

Potential laboratory projects for students using the spike sorting approach. A realistic estimate of the time required for student projects is an important consideration in planning a student laboratory schedule. In terms of data acquisition, a regular three to four hour laboratory session is adequate once students have learned all the necessary skills. In a typical scenario, it would takes one to two hours for students to start from dissection and reach a stage where they have both suction and muscle electrodes in place. It should take only ten to fifteen minutes to collect two hundred traces with each trace two seconds in length. Since axons in the third nerve fire at high frequency, a four hundred second period is likely to contain hundreds to thousands of events for each of the four smaller and higher frequency axons. Adding drugs or modulators and acquiring data again take another twenty to thirty minutes. Thus, by the end of a typical laboratory session, most groups in the laboratory should have two large data files, one control and one experimental.

Spike sorting and analyses are best done off line in the afterhours, on students laptops or desktops. The initial spike sorting step takes about four to six hours, best done by leaving the computer running overnight. Most students need input or reassurance when it comes to data inspection and evaluation. It is useful to allocate a specific session to discuss the results of the first pass analysis and the initial interpretations put forward by students. This session should be scheduled as early as possible such that students have a realistic idea about the time required for further analysis and the questions that might arise. Topics to be discussed could include: (*i*) potential issues with the software, (*ii*) the possibility of using a subsection of their data for further analysis, in the interest of time or due to considerations of data quality and (*iii*) whether additional experimental repeats are needed to clarify problems detected in the analyzed data. Since most biology majors are not used to this kind of lengthy data analysis, it is useful to allow another two weeks before their final presentations or reports are due. It is also useful to ask two students from the same group to independently analyze the same data set. This redundancy approach can foster discussion among students and can sometimes save time if one of the analyses was not performed correctly, or if analyzed results are lost due to computer accidents. During this period of off-line analysis, the instructor can check progress regularly, for example by setting aside 10 minutes at the beginning of lectures to check what stage students are at and address any questions they might have. In summary, the data analysis phase is slow in pace and requires regular exchanges between faculty, teaching assistant and students. It should also be pointed out that students often report that the lecture materials come together in their heads during this slow but deliberate data analysis phase.

In addition to laboratory projects designed to investigate the unresolved observations outlined in Figures 4 and 6, student projects using the spike sorting methods could take two additional approaches. In the first case, students could be given an unknown reagent. The goal would be for students to report the effects of this reagent on action potential and/or on synaptic transmission, and postulate possible mechanisms of action for this reagent. Each laboratory group could be given a different drug or

neuromodulator. This type of project could be completed over a period of 2 to 3 weeks, after students had learnt to dissect and record on their own.

Alternatively, it should be possible for students to initiate a project on their own. In this case, students would start by identifying candidate reagents to investigate. This first phase would involve justification of their choice of reagents, and assessment of the likelihood that the reagent will be active in crayfish. More importantly, students would need to justify why their choice of reagent is scientifically interesting and significant. The second phase would involve testing the reagent of choice. This type of student project would be more challenging, and the time line would need to be structured over the period of an entire semester. There would be two lines of laboratory work running in parallel. On the one hand, selection, assessment and justification of selected reagents would gradually take shape as the semester progressed. This aspect of the project could take the form of brief weekly presentations or discussions in the lab. Simultaneously, weekly laboratory sessions would be devoted to teaching students basic electrophysiological techniques, including intracellular, suction electrode recordings, data acquisition and analysis. After the choice of reagents had been debated and determined, students would have ~3 to 4 laboratory sessions to test their reagents. An hour-long power point presentation and written report would be generated at the end of the semester. A project on this scale could involve an entire class of about 10 students, such that all laboratory groups contributed and worked toward the same final goal. This type of project would serve as a good working example from which students could appreciate the entire scientific research process.

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