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Teaching Basic Neurophysiology Using Intact Earthworms

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Introductory neurobiology courses face the problem that practical exercises often require expensive equipment, dissections, and a favorable student-instructor ratio. Furthermore, the duration of an experiment might exceed available time or the level of required expertise is too high to successfully complete the experiment. As a result, neurobiological experiments are commonly replaced by models and simulations, or provide only very basic experiments, such as the frog sciatic nerve preparation, which are often time consuming and tedious.

Action potential recordings in giant fibers of intact earthworms (*Lumbricus terrestris*) circumvent many of these problems and result in a nearly 100% success rate. Originally, these experiments were introduced as classroom exercises by Charles Drewes in 1978 using awake, moving earthworms. In 1990, Hans-Georg Heinzel described further experiments using anesthetized earthworms.

In this article, we focus on the application of these experiments as teaching tools for basic neurobiology courses. We describe and extend selected experiments, focusing on specific neurobiological principles with experimental protocols optimized for classroom application. Furthermore, we discuss our experience using these experiments in animal physiology and various neurobiology courses at the University of Bonn.

Key words: action potentials; giant fibers; earthworms; extracellular recordings; threshold; refractory period; conduction velocity; facilitation of conduction velocity; spatial size of action potentials; flight reflexes; synaptic depression and facilitation; giant motorneuron

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Basic neuroscience textbooks usually introduce the nervous membrane with its passive and active properties as a foundation for understanding principles of neural computation (e.g., Kandel et al., 2000; Bear et al., 2007). Exercises that accompany basic neuroscience lectures are often challenging: they require expensive equipment, complicated dissections, and a high level of instructor expertise or take too much time (usually 2.5-3 hours available per unit). As a result, animal experiments are replaced by models and simulations, limited to a handful of standard experiments, like the frog sciatic nerve preparation, or classroom demonstrations by instructors. Although necessary equipment has become cheaper and more widely available, dissections remain a major obstacle because they are time-consuming, raise ethical concerns about animal experiments, have lower success rates, or fail to meet the curriculum requirements.

In our opinion, experiments that record from giant fibers in intact earthworms (*Lumbricus terrestris*) circumvent these problems and generally cover many aspects that can be found in corresponding chapters of introductory neuroscience textbooks. In this article, we describe our experience using such experiments in introductory animal physiology and various neurobiology courses at the University of Bonn. We present a set of nine experiments, selected to cover important neurobiological topics and focus on their classroom usage and specific teaching goals.

Originally, experiments that measure giant nerve fiber activity in awake, moving earthworms were introduced by Charles Drewes and colleagues (Drewes et al., 1978). Extending these experiments, C. Drewes later presented a thorough description of classroom experiments (Drewes, 1999). All these experiments use awake, moving earthworms and apply mechanical stimuli to elicit giant fiber activity. Inspired by the first descriptions of C. Drewes, a German publication by Hans-Georg Heinzel introduced similar experiments that used anesthetized earthworms and electrical stimulation (Heinzel, 1990). These experiments covered further action potential (AP) properties like refractoriness and even synaptic depression, habituation, and facilitation (Heinzel, 1990).

Background
Earthworms possess three giant fibers within their ventral nerve cord, one median and two lateral fibers. These fibers are responsible for flight reflexes, twitches of the worm (Fig. 1b; Bullock, 1945; Bullock and Horridge, 1965). Skin sensory cells of the worm front end are connected to the median giant fiber (MGF) that has a diameter of up to 0.07 mm (Bullock, 1945; Guenther, 1973). The two lateral giant fibers (LGF) have a diameter of up to 0.05 mm and receive their input mainly from skin sensory cells of the hind end. These two fibers have segmental cross-connections and can, therefore, be regarded as a single functional unit (Bullock, 1945; Guenther, 1973). In general, earthworms and similar Oligochaetes have the advantage that recordings from these fibers can be performed without any dissection. Because the skin and body muscle wall are thin, it is sufficient to place worms upon arrays of electrodes that make contact with the outer ventral part of the body (Fig. 1a). This has obvious advantages, including the use of either awake or anesthetized worms and the animals survive the experiment intact.
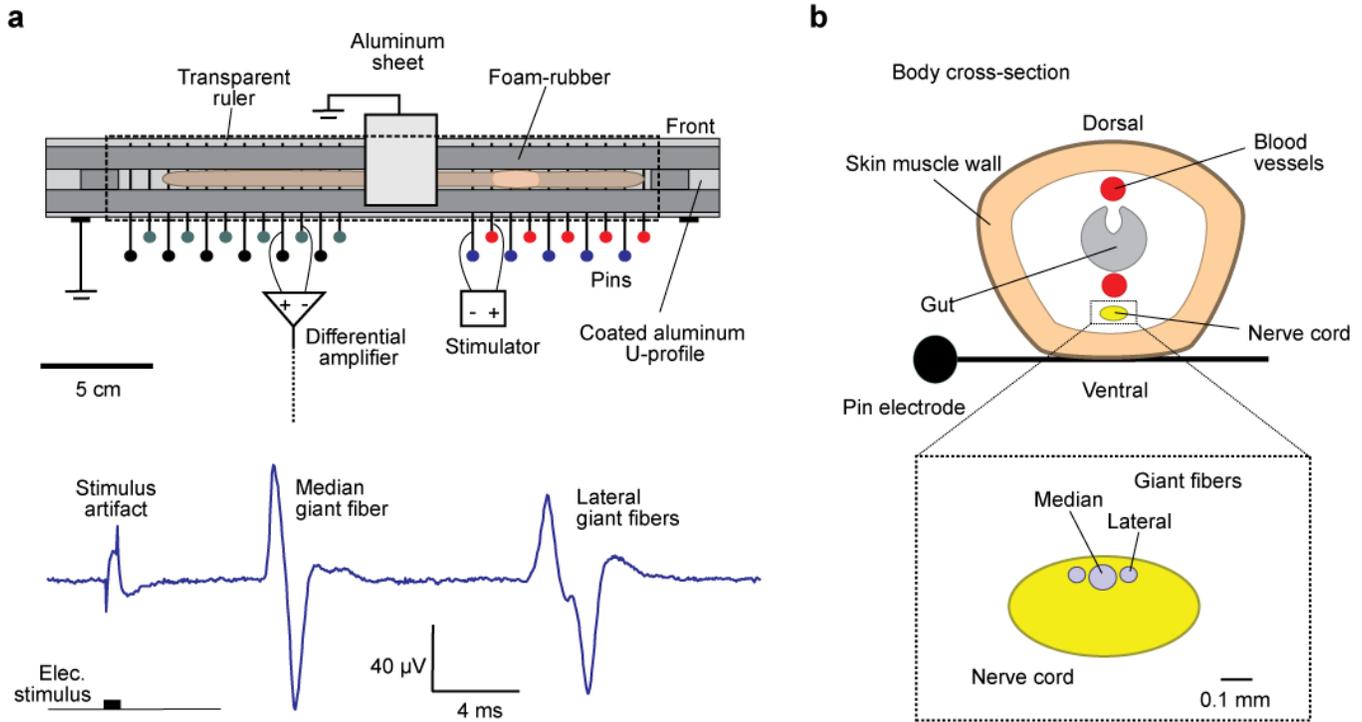
Figure 1. Experimental setup. *(a)* The setup for recording giant fiber activity in intact earthworms is illustrated. Basically, the intact earthworm is placed in a cage that maximally restricts locomotion. An array of electrodes comes into contact with the outer ventral side of the worm. These electrodes are plain stainless steel household pins and serve as recording, as well as stimulation electrodes. A transparent ruler, clamped above, completes the cage. This ruler can be shifted to allow mechanical stimulation of the worm. The trace shows an extracellular recording of the median and lateral giant fiber responses following an electrical stimulus (classroom data). *(b)* A body cross-section through the earthworm is shown. The nerve is located ventrally, close to the pin electrodes beneath the worm. The inset illustrates the dorsal location of the three giant fibers within the ventral nerve cord.

In awake worms, the specific giant fiber pathway can be stimulated by touching appropriate skin sensory cells (front or hind end), giant fiber activity can be recorded, and reflexes can be observed. Because awake worms can move and might escape, performing these experiments requires training and generally increases the experimental duration. Therefore, anesthetized worms should be used when possible. With rather weak anesthetics, it is easy to achieve a level of anesthetization such that the muscles and skin sensory cells are inactive, but the giant fibers and giant motorneurons are still responding. In this situation, action potentials are elicited by electrical stimulation, using the same array of electrodes that is used for recording (Fig. 1a). The APs of both pathways can then be separated by adjusting the stimulus amplitude and looking at the timing of the responses (Fig. 1a, trace).

For the MGF pathway, the components of the flight reflex are known (Fig. 2). A mechanical stimulus to the front end leads to activation of skin sensory cells, which activate a sensory interneuron that connects to the MGF. If the stimulus is strong enough, a positive feedback loop of the MGF can lead to a second MGF response. The MGF is connected to the longitudinal muscles of the body wall via six segmental giant motorneurons. Activation of these muscles finally leads to twitches of the worm.
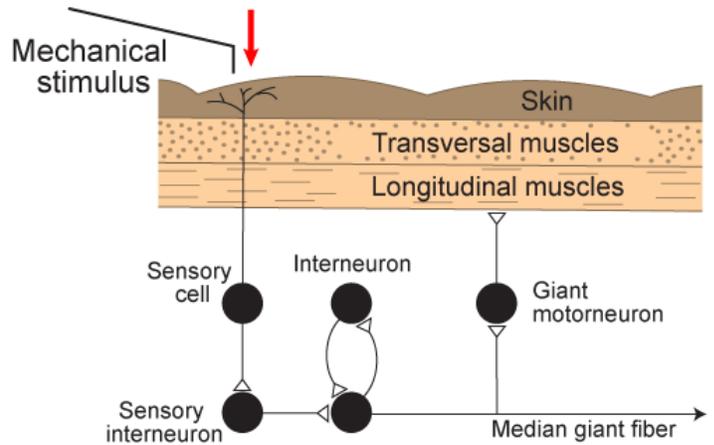
Figure 2. Components of the flight reflex, mediated by the MGF pathway. Mechanical stimulation of the worm front end leads to activity in skin sensory cells. This activates sensory interneurons that are connected to the median giant fiber. The MGF is connected to segmental giant motorneurons that elicit contraction of longitudinal muscles in the body wall. A positive feedback loop, via a single interneuron, can enhance the flight reflex by eliciting a second, or even several more, action potentials of the MGF.

Target students

The presented experiments are used in undergraduate animal physiology (first year) and neurobiology courses for advanced undergraduates and introductory graduate levels.   These courses are conducted as part of the bachelor and master level biology programs, as well as a life science informatics masters program at the University of Bonn.   Furthermore, the experiments are regularly conducted as live demonstrations for the public, in lectures, practical exercises for visiting upper level high school classes (ages 16-19), and in training courses for high school teachers.

Selected experiments and covered theory

Our main focus was the selection of a set of experiments that can accompany an introduction to neuroscience. As we participate in various courses where students had to meet different prerequisites prior to attending our classes, we decided to design these experiments in a modular fashion.   Each experimental module can be performed independently and requires approximately 15-30 minutes in the classroom.   This allows the selection of subsets to comply with specific course requirements (allotted time, learning objectives, etc.).   Overall, we present nine experiments that each focus on specific theoretical topics:

1. The action potential – shape, duration and threshold
2. Absolute and relative refractory periods of action potentials
3. Spatial dimensions of action potentials
4. The conduction velocity
5. Influence of stimulus duration on action potential threshold
6. Synaptic depression
7. Reflex facilitation and habituation
8. Reflexes and facilitation of conduction
9. Temperature dependency of conduction velocity

MATERIAL AND METHODS

Animals

In the described experiments, earthworms (*Lumbricus terrestris*) are used.   These are easy and inexpensive to obtain from fish bait or gardening suppliers and can be kept in the lab refrigerator for about 4-6 weeks. Furthermore, it is often possible to obtain large specimens which are easy to handle. In principle, other Oligochaetes may be used as well (Drewes, 1999; Krasne, 1965).

Experimental setup

The basic setup is illustrated in Figure 1a. An earthworm is placed in a cage that (I) allows external recording from the giant fibers, (II) prevents awake worms from crawling away, and (III) serves as a simple Faraday's cage.   In principle, a simple aluminum U-profile is covered with foam rubber which is also used to construct a worm sized cage. Household pins can be pushed through the rubber and these are used to record from and stimulate the giant fibers. Finally, a transparent ruler covers the worm and

completes the cage (Fig. 1a). For further illustration on the cage, several photos of our setup are shown in the supplementary material.

In addition to the worm cage, only standard lab equipment is necessary. An ordinary electrophysiological workstation is sufficient for these experiments, similar to setups required for other classroom exercises, such as the frog sciatic nerve preparation. We usually let 2-3 students work at each workstation. This workstation should consist of two amplifiers for differential recordings, a unit for electrical stimulation, and any kind of visualization equipment (e.g., oscilloscope or computer).   The extracellular amplifiers may be cheap, with 1k amplification and a 0.3-3 kHz band-pass filter being sufficient.   The isolated unit for electrical stimulation should be capable of producing single, double, or frequent pulses up to 10 V amplitude (or current pulses up to 10mA), as well as stimulus durations between 0.05 and 50 ms.   The equipment for visualization of the recordings may consist of oscilloscopes or analog/digital converters, plus appropriate PC/software interfaces. In the past, we have applied both, custom made equipment as well as packaged workstations for education purposes (see Discussion). With rather low-end, custom made amplifiers and the described worm setup, we regularly achieve signal-to-noise ratios of 20:1 (100 μV signal: 5 μV peak-to-peak noise).

In the following sections, we describe the two basic setups that are required to conduct the experiments, with detailed descriptions of stimulus parameters and experimental conditions for each experiment being presented in the Results section.   In principle, the electrophysiological equipment should be connected to the worm cage as shown in Figure 1. The recording (and stimulus) electrodes are connected to the pin electrodes that touch the ventral skin surface of the worm.   The distance of each pair of electrodes should be about 1 cm. In our experience, pins that make contact with the last 4 cm of the worm should be used. The best recording and stimulation is achieved where the worm is thinner.   The clitellum should be avoided for recording, as this part of the worm is especially thick.   A list of suggestions for successful experiments is provided in the supplementary material. We use this list as a quick troubleshooting guide for students. Depending on the specific experiment, we either apply mechanical or electrical stimuli, and this requires slight changes to the setup.

Mechanical stimulation (Basic setup 1)

If reflexes are observed, earthworms have to be awake and the stimulation has to be pathway specific. Therefore, we recommend mechanical stimulation, either using the stimulator described in the supplementary material or a simple bristle to touch the worm (Drewes, 1999). We recommend using the stimulator, as this allows measurements of latency.   The qualitative stimulus strength can be assessed by looking at the amount of skin indentation during a stimulus.   We usually use the categories weak, medium and strong.

Electrical stimulation (Basic setup 2)

In experiments where the behavioral responses are not observed, we use anesthetized earthworms. As mechanical stimulation would not work in anesthetized worms, electrical stimuli are used to generate action potentials in the giant fibers. These stimuli are applied through pin electrodes that are not used for recording. In this setup, it is important to shield the worm itself with a broad piece of aluminum foil that touches the skin surface of the worm in between the sites of stimulation and recording. This minimizes stimulus artifacts caused by current conducted over the moist skin surface.

Anesthetization

The earthworms are anesthetized in a 0.2% aqueous solution of Chlorobutanol (1,1,1-Trichloro-2-Methyl-2-Methyl-2-Propanol), in tap water. It is important not to use distilled water as this might kill the worm. The worms remain in the anesthetic solution for about 10-15 minutes, until the skin muscles are flaccid. Then, the worms are washed and used, with their skin slightly moist. This keeps the worms alive and improves recording and stimulation.

RESULTS

Experiment 1 (Basic setup 2)
The action potential – shape, duration and threshold

In this experiment, students measure action potentials (APs) of the median (MGF) and lateral (LGF) giant fibers. They analyze the main features of an action potential and the influence of the fiber diameter on the threshold for action potential generation (Fig. 1a).

Electrical stimuli with 0.5 ms duration and increasing amplitude are delivered. Beginning at an amplitude of 1 V and increasing in 1 V steps, the MGF usually starts responding at some value below 10 V. As soon as the amplitude is reached where the MGF starts responding, students can determine the threshold more exactly by varying the voltage around this stimulus amplitude in 0.1 V steps. Using this, now known, MGF threshold, the amplitude is increased again, until the LGF starts responding. Then, the LGF threshold is determined.

While the actually measured thresholds vary, the MGF threshold should always be lower than the LGF threshold (Table 1). The MGF threshold is typically around 1 - 4 V. This observation illustrates the relationship between fiber diameter and threshold – if the diameter of a nervous fiber is increased, the threshold for eliciting action potentials is decreased. Furthermore, the MGF response precedes the LGF response (Fig. 1a, trace). This decrease in latency illustrates that an increase in fiber diameter increases the conduction velocity of action potentials along the fiber. Sometimes, the lower conduction velocity of the LGF leads to plateaus between the peaks of the biphasic action potential recording (Fig. 1a).

This experiment can also be used to illustrate the type of recording. If the polarity of the recording electrodes is inverted, the recorded biphasic AP also becomes inverted.

We usually conduct this experiment first in our courses, as the thresholds are required for several experiments and have to be repeated every time the position of the stimulus

or recording electrodes is changed, the worm moved, or another worm is used.

#	MGF thresholds [V]	LGF thresholds [V]
1	1.2	4.2
2	1.8	3.6
3	1.5	4.3
4	2.9	4.7
5	2.8	4.3
6	1.6	3.4
7	1.3	3.2
8	2.7	5.8
9	2.5	4.6

Table 1. Action potential thresholds of the median and lateral giant fibers. This table shows a representative sample of student measurements; for each measurement, the MGF and LGF thresholds are corresponding pairs.

Experiment 2 (Basic setup 2)
The absolute and relative refractory period of action potentials

The refractory period is a direct consequence of the kinetics of ion channels that cause action potentials. It can be divided into the absolute period, where no further APs can be elicited, and the relative period, where more current is necessary (Kandel et al., 2000). In this experiment, students look at these changes in membrane excitability shortly following an action potential.

There are two methods to measure the refractory period. First, it can be determined by measuring the amplitude of the second AP, where double pulses of 0.5 ms duration and varying intervals are applied (Fig. 3a, green dots, method 1). The second method determines the refractory period through the thresholds that are required to generate a second AP at different stimulus intervals (Fig. 3a, red dots, method 2) - this is the classical textbook approach.

In the first case (method 1), starting at intervals of 35 ms and decreasing the intervals continuously until the second AP vanishes, the response of the MGF is measured. To prevent superposition of the MGF and LGF responses, the stimulus amplitude is set to a value between their previously determined thresholds. Although this approach does not allow a precise measurement of the period of absolute refractoriness, it does provide evidence for the changes in excitatory processes, even at larger double pulse intervals. This approach is significantly faster, allowing students to perform the experiment in 15-30 minutes.

Figure 3 illustrates the main results of this experiment. At large stimulus intervals, students observe the usual prediction: each stimulus leads to a response of the MGF. However, when students are asked to lower the stimulus interval, the second MGF action potential is abolished at an interval of about 1 – 2 ms. Now, we have reached the absolute refractory period of the first elicited action potential (Fig. 3a; Table 2). Using this approach, the relative refractory period starts at about 14 ms, the interval at which the second AP has only 90% of its maximal amplitude.
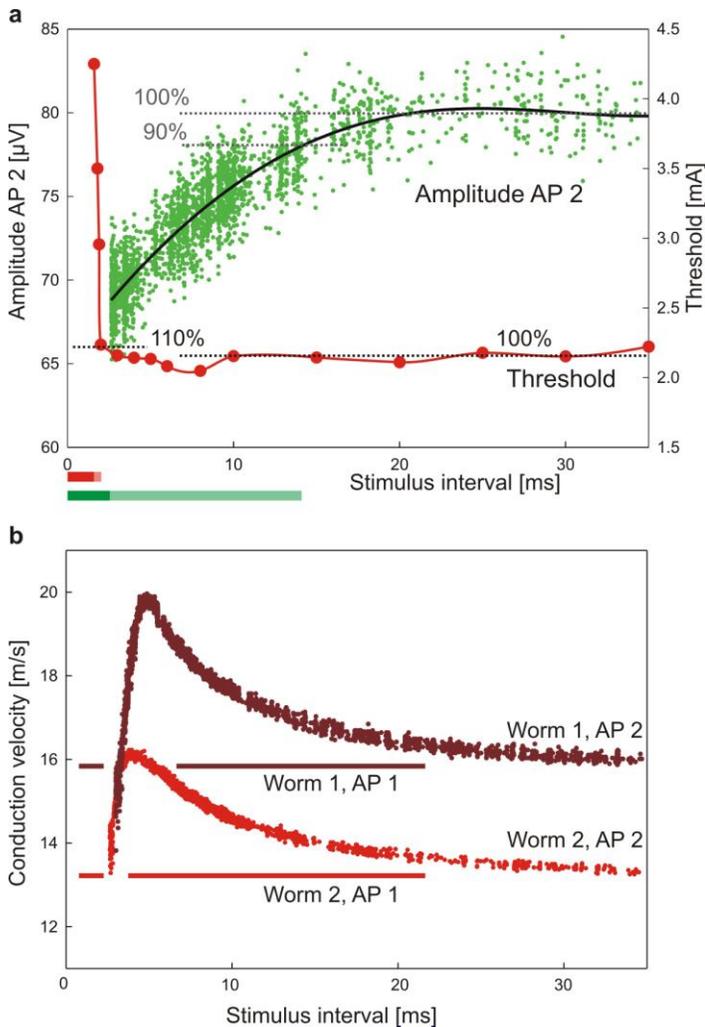
Figure 3. Changes in membrane excitability following an action potential. *(a)* Example of action potentials elicited by double pulses. The measurement was performed using method 1, varying intervals but constant width and strength (0.5 ms, 2.8 mA). At intervals larger than 20ms, the amplitude of the second AP (green dots) is the same than the first AP. At some point, the second AP becomes smaller (relative refractory period, light green bar), until it completely vanishes (absolute refractory period, dark green bar). Using method 2 (measuring the threshold for eliciting a second AP), we get somewhat different values for the refractory periods (dark red and light red bar). *(b)* Comparison of the conduction velocities of the first and second AP of two different earthworms. If the second AP is elicited 5-8 ms after the first AP, it has a higher conduction velocity. However, close to the absolute refractory period, it has a lower velocity.

The absolute refractory period is precisely calculated by measuring the threshold for AP generation (method 2). This is the point where more current cannot elicit a second AP (Fig. 3a, 1.2 ms). The relative refractory period, starting at a time where 110% of stimulus strength is necessary, is more difficult to define with this approach, because obtaining data points for the threshold curve is much more time consuming than to generate thousands of

amplitude values with method 1.

If more time is available in a course, we also conduct double recordings and let students measure the conduction velocity, as well as obtain more data points between 0.5 and 3 ms. Plotting and comparing the conduction velocities of both elicited APs (Fig. 3b) students can observe that at intervals of about 3 - 20 ms, the conduction velocity of the second AP is increased. This is a phenomenon which has been coined 'facilitation of conduction velocity' (Bullock, 1951). The ion channels engaged in such phenomena and their possible role in shaping the patterned motor output of neural networks are currently under investigation in the crustacean stomatogastric nervous system (Ballo and Bucher, 2009). At very short intervals (1-5 ms), the second AP not only gets smaller, but slows down in conduction velocity. This is another direct consequence of the imminent changes of excitability. In addition to illustrating mechanisms behind the refractory period, this experiment provides some data to compare the refractory periods of nerves with muscle cells of the human heart.

#	Estimated absolute refractory period [ms]	Estimated relative refractory period [ms]
1	0.9	9
2	0.7	4
3	1.2	14
4	1.3	2
5	1.0	14
6	2.8	12
7	2.6	5
8	1.1	14
9	1.7	10

Table 2. Estimates of the absolute and relative refractory period durations obtained by method 1 (observing the amplitude of the second AP). Paired, representative classroom data.

Experiment 3 (Basic setup 2)
The spatial dimensions of action potentials
In the described experiments, differential, extracellular recordings are performed, i.e., voltage differences are measured over distant recording sites. Here, the shape of the recorded potential depends on the negative potential wave. The potential wave itself depends on ion channel properties of the specific nervous tissue. This experiment illustrates these principles by looking at changes in the shape of the recording when the distance between the recording electrodes is changed from 10 mm to 20 mm (Fig. 4c I and II). The stimulus amplitude is set above the LGF threshold to analyze both giant fibers.

This experiment allows students to observe the effects of changing electrode distances between two measurements. Alternatively, they can perform simultaneous recordings with four amplifiers (Fig. 5). Here, all minus inputs were connected to the same pin and the plus inputs were connected to pins at 2, 5, 10 and 15 mm distance.
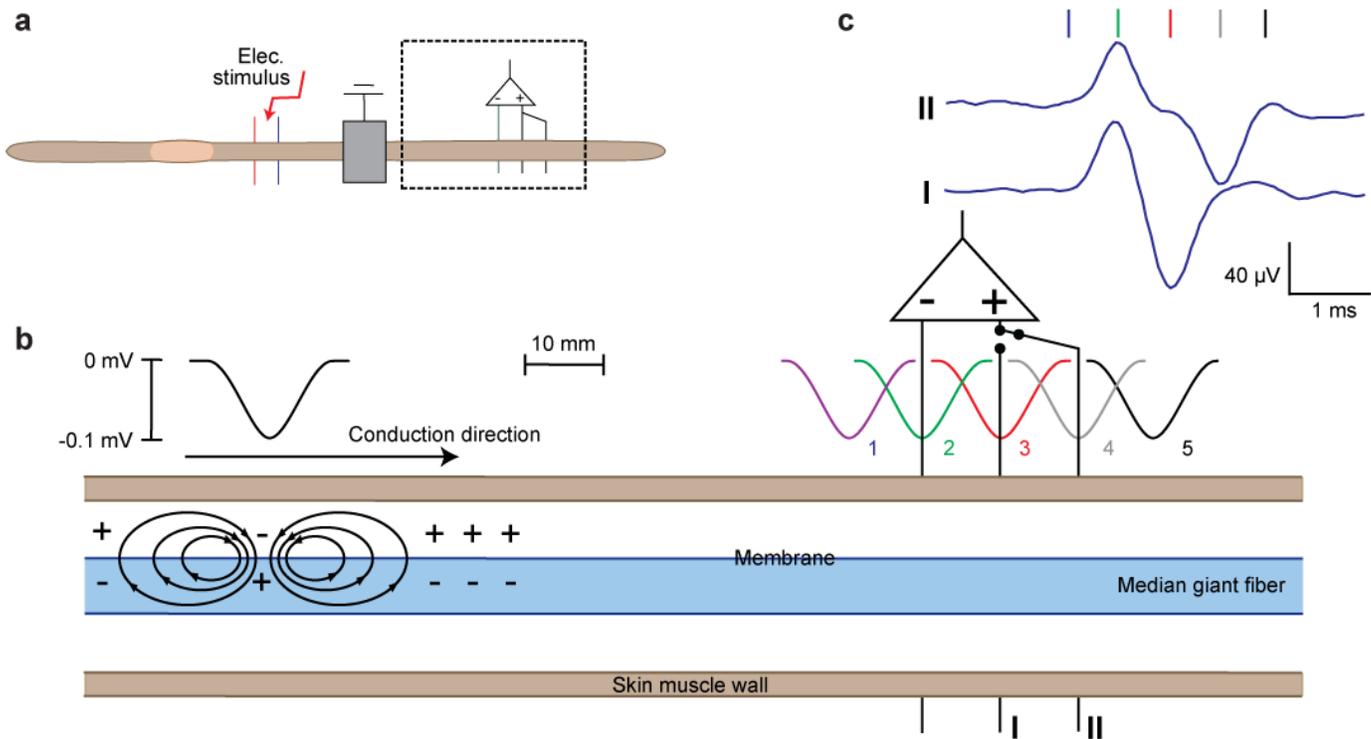
Figure 4. Spatial dimensions of action potentials. (a) Experimental setup. (b) The conduction of action potentials. An electrical stimulus leads to the generation of an action potential by depolarizing the membrane; in this case the median giant fiber. This depolarization leads to a negative potential wave caused by current loops. As the negative potential wave is conducted along the fiber, the amplitude of the recording depends on the difference between the two electrodes (+/-). This difference is dependent on electrode distance, i.e., if the negative potential wave fits in between the electrodes (setting II), there is no potential difference measured for a short period of time and this results in a plateau showing up in the recordings. (c) Examples of classroom traces for electrodes placed at distances of 1 and 2 cm (I/II).

At a distance of 10 mm, the LGF, and at a distance of 15 mm, the MGF responses usually start showing plateaus between the positive and negative peaks. The plateau is the visible result of the negative potential wave fitting in between the two electrodes. Therefore, for a short period of time, no voltage difference is measured. In contrast, a decreased distance of 5 mm or 2 mm usually leads to decreased AP amplitudes (often better observed in the MGF responses) - the distance of the electrodes is smaller than the spatial dimensions of the negative potential wave. This experiment is a powerful and simple demonstration that experimental design has to be thoughtful and that the results have to be questioned thoroughly. Even the worm action potentials are big in terms of spatial wave lengths (1-1.5 cm). This always comes as a big surprise for students. In humans, these can even be bigger, e.g., 10 cm for fibers with 100 m/s conduction velocity and APs of 1 ms duration.

Experiment 4 (Basic setup 2)
Conduction velocity
The conduction velocity of a nervous fiber is one of the main features that are used to classify different nerves and to illustrate the influence of fiber diameters. One typical textbook example is the classification of human sensory and motor nerves. In this experiment, students compare two different ways to measure/estimate the conduction
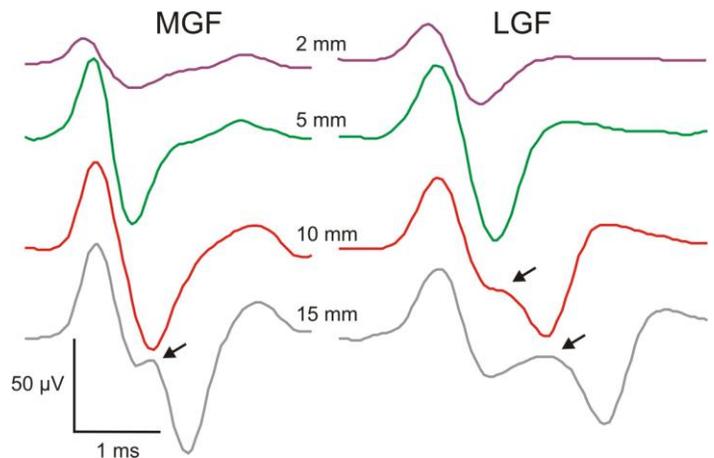
Figure 5. Spatial dimensions of the MGF and LGF responses. An example of four recordings of the MGF and LGF with different electrode distances (2, 5, 10 and 15 mm) is shown. The plateau of the MGF is given at a distance of 15 mm and for the LGF there already is a plateau at a distance of 10 mm (black arrows).

velocities of the MGF and LGF. To obtain the responses of both pathways, the electrical stimulus is set to 0.5 ms duration and an amplitude 0.5 V above the previously determined threshold of the LGF.

In this experiment, students learn how to calculate the

conduction velocity using two different approaches (Fig. 6). The first method estimates the velocity using the distance between the sites of stimulation and recording (Rec. 1 in Fig. 6), as well as the recorded interval between the stimulus and the positive peak of the MGF potential. The LGF velocity is obtained accordingly. The second method is a classical way to measure the velocity, using a double recording setup. Here, the distance of the two recording sites and the time interval between their recorded potentials are used. While the first method assumes that action potential generation at the site of electrical stimulation does not require any extra time, the second method provides an accurate measurement of the mean conduction velocity of the piece of axon between the two sites of recording. For both methods, it is important that electrode distances are measured, otherwise velocities cannot be calculated.

Electrode (-/-) distance (mm): 50		
#	ΔT between maxima of MGF recordings (ms)	ΔT between maxima of LGF recordings (ms)
1	3.0	7.3
2	3.2	7.1
3	3.1	7.2
4	3.0	7.2
5	3.3	7.3
Mean value:	3.1	7.2
Velocity (m/s):	16.1	6.9

Table 3. Typical conduction velocities of the MGF and LGF. In this case, the velocities were calculated using method (b), a double recording. The table shows exemplary classroom data.
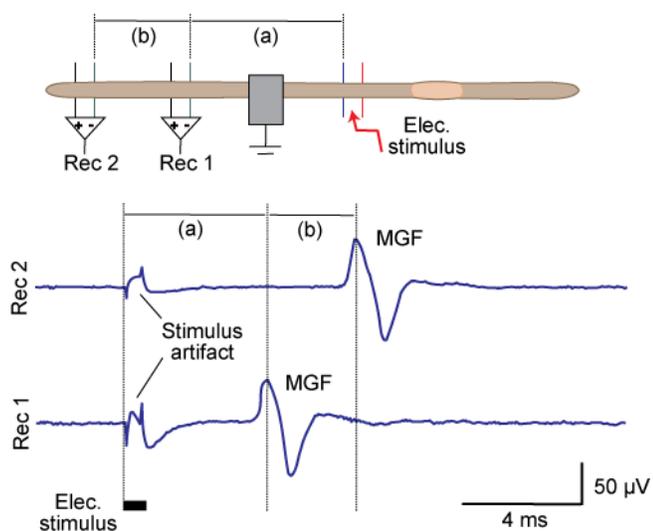
Figure 6. Methods to calculate the conduction velocity. If possible, a double recording is used to calculate the conduction velocity (b). Here, the time between the two recording electrodes (-/-) and the distance between these is used for the calculations. If a double recording is not an option, the distance between the stimulus and the recording electrodes as well as the latency can be used to estimate the conduction velocity (a).

A typical classroom example of measurements using a double recording is shown in Table 3. This shows that the conduction velocity of the LGF is always lower than that of the MGF. Using Figure 4 as an example, even a third method can be discussed. If a plateau between the peaks of an AP is observed, the distance between the electrodes and the peak-to-peak time interval can be used to calculate the conduction velocity as the potential wave is smaller than the electrode distance.

Experiment 5 (Basic setup 2)
Influence of stimulus duration on action potential threshold
The necessary stimulus duration and amplitude that elicit an AP depend on the required current to charge the membrane capacitance. In this experiment, students vary the stimulus duration (e.g., 20, 2, 0.5, 0.1, 0.05 ms), and

for each stimulus duration, determine the threshold of the MGF as described in experiment 1. Alternatively, we use either stimulus generators which deliver pulses with adjustable voltages or generators which deliver current pulses. Both give similar curves, but the latter method is preferable because the amount of current determines the charging of the membrane. This stimulation is also independent of the skin resistance of worms, which are sometimes more or less wet. Using these thresholds, students then determine the amplitude-duration dependency for the MGF and LGF. This dependency is a common measure of the excitability of a nerve or muscle. Two values are defined for comparison: chronaxy and rheobase. The rheobase is the minimal electric current of a stimulus with infinite duration that results in an AP, while the chronaxy is the shortest duration of an electrical stimulus where the threshold amplitude is twice the rheobase (Woodbury, 1965). Using their data, students should be able to plot a strength-duration curve and obtain values for the chronaxy and rheobase of the MGF and LGF (Fig. 7). These can be used to discuss the influence of underlying principles (voltage, current, ion channel function for AP generation).

Experiment 6 (Basic setup 2)
Synaptic depression
In each segment of the earthworm, the MGF connects to six giant motorneurons which further connect to the longitudinal muscles (Fig. 2). In this experiment, students analyze the reversible (synaptic) depression of these giant motorneurons using frequent stimulation (Guenther, 1972). At first, the electrical stimulation should be set to a frequency of 0.5 Hz or slowly repeated manual stimulation. With this slow frequency, the stimulus amplitude is adjusted between the MGF and LGF thresholds and the worm is tested whether responses of the giant motorneurons can be seen. The motorneuron responses are easy to distinguish: they occur about 1-2 ms after the MGF response, and their responses result in a broader and irregular potential (Fig. 8b). This is the summed response of the giant motorneurons in the according segment. As soon as the response of the giant motorneurons is recognized, a 30 Hz stimulus should be applied for 20-30 seconds, all the while recording the responses. This

should eliminate the response of the giant motorneurons (Fig. 8b). After a break of 1-2 minutes, slow stimulation (0.5 Hz) usually allows the recovery of all or at least some of the six motoneurons.

Figure 8. Synaptic depression of giant motorneurons. *(a)* Experimental setup. *(b)* In weakly anesthetized worms, the MGF response is followed by the summation of the action potentials of six segmental giant motorneurons. By overdrawing all responses to the 30 Hz stimuli, it becomes clear that the motorneuron response does not diminish continuously but in steps, according to failure of one synapse after the other.

This demonstration of the plasticity of a specific, identified circuit that is responsible for flight reflexes is a powerful example to discuss reflex circuitry and the possible cellular processes that may cause the synaptic depression. Students should discuss the biological relevance of this depression, e.g., the worm crawling through rougher substrate and adapting its reflex strength. Students sometimes confuse why the measured giant motorneuron responses look different than the giant fiber potentials (often they think that we are actually measuring synaptic APs). Therefore, we stress the point that the giant motorneuron response is a summed potential, i.e., the summation of up to 6 different APs and that sudden changes in its shape or amplitude can be explained by different thresholds of individual motorneurons. If students are lucky, they might even observe that the response of the motorneurons declines in a step-wise fashion (Fig.8). This is direct evidence that we are looking at a summed potential. However, most times only two or three steps are seen and not six.

Figure 7. Strength-duration curves. *(a)* Representative student measurements of the MGF strength-duration curve. In this example, we used 24 different stimulus durations and amplitudes up to 2.8 mA. The minimal electric current (rheobase) of the MGF is at 0.19 mA and the chronaxy is at 129 µs. *(b)* The strength-duration curve of the LGF shows a rheobase of 0.3 mA and a chronaxy of 177 µs.

This is one of the most complicated experiments, as it requires a defined level of anesthetization. While the worm should not be moving anymore, the giant motorneurons still have to be responding. Usually, students have to test several worms with varying exposure to the anesthetics (5-10 min) before they get a suitable worm. As soon as the responses of the giant motorneurons can be seen in a worm, the experiment should be conducted quickly to minimize the chance of the worm waking up. This experiment should only be conducted if at least 30 minutes are available; experience has shown that most students get results within 30-45 minutes.

Experiment 7 (Basic setup 1)
Reflex facilitation and habituation

In awake earthworms, withdrawal reflexes in response to mechanical stimulation can be observed. In this experiment, stimuli are applied to the very front end of the earthworm to analyze the MGF pathway (Fig. 9a). Students apply several stimuli and note the qualitative strength of each, as determined by visual control of skin indentation (weak, medium, strong). Because the worm is still wiggling around in its cage, 10 successive stimuli are applied, 10-30 seconds apart. In the second part of the experiment, 10 successive stimuli with minimal intervals are applied. It is important to keep an eye on the animal as it might move and to keep note of the strength of the stimuli.
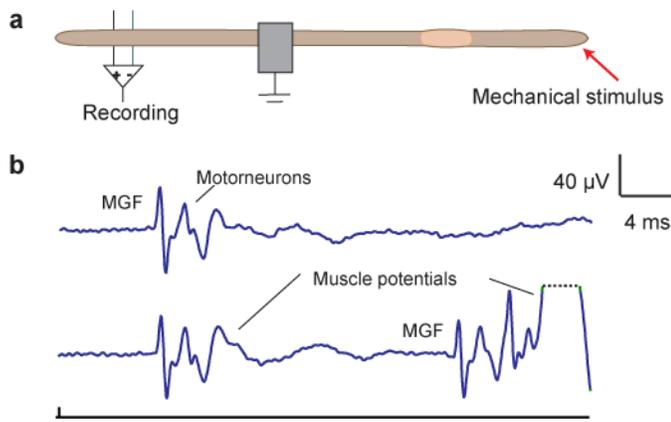
Figure 9. Mechanical stimulation of the worm front end, activating the MGF pathway. *(a)* Experimental setup. *(b)* Two traces of recordings following a mechanical stimulus are shown. In the upper trace, the stimulus leads to a response of the MGF, followed by motorneuron responses. The lower trace shows the action of the positive feedback loop. The first MGF response is followed by the motorneurons and a few muscle potentials. In addition, a second MGF response can be observed after a delay (positive feedback loop), again followed by motorneuron responses and now much stronger muscle potentials (facilitation).

Usually, students can identify the different components of the flight reflex pathway in their recordings (Fig. 2). After weak stimuli, the already known MGF action potential can be seen, followed by the summed response of the giant motorneurons. In addition, muscle potentials can be seen because the worms are awake. These can be distinguished from nerve potentials by their duration and, sometimes, their gigantic size, often even exceeding amplifier range (Fig. 9b). Stronger stimuli often activate the already described positive feedback loop (see Introduction, Fig. 2). This leads to a second MGF response, which usually contains increased muscle potentials, as more fibers get recruited (Fig. 9b, compare traces). A comparison between observed behavioral responses qualitatively corresponds to these findings (i.e., the earthworm should have twitched stronger). This experiment provides direct evidence of the positive feedback loop. Furthermore, the larger muscle potentials that follow the second MGF response are the result of facilitation. After frequent stimulation of the worm in short intervals, the strength of the twitches decreases. This is caused by a decrease in motorneuron activity and concordant with the results of experiment 6, demonstrating habituation within the reflex circuit.

Another goal of this experiment is that students understand the difference between electrical and mechanical stimulation. After electrical stimulation, we get responses of MGF and LGF pathways. However, even strong mechanical stimuli to the front end fail to elicit a response of the LGF. The explanation is simple: skin sensory cells of the front end are not connected to the LGF pathway.

Experiment 8 (Basic setup 1)
Reflexes and facilitation of conduction
In contrast to experiment 7, mechanical stimulation of the

worm hind end leads to activation of the LGF pathway (via connected skin sensory cells). In this experiment, students observe and describe the behavior of the worm (e.g., are there twitches and, if so, how do they look?) and classify their mechanical stimuli into the classes weak, medium and strong. The stimuli should be 10-30 seconds apart. To study facilitation of conduction, a double recording has to be performed.

Students should note that no MGF responses can be observed in this case and, again, explain how this differs from electrical stimulation.
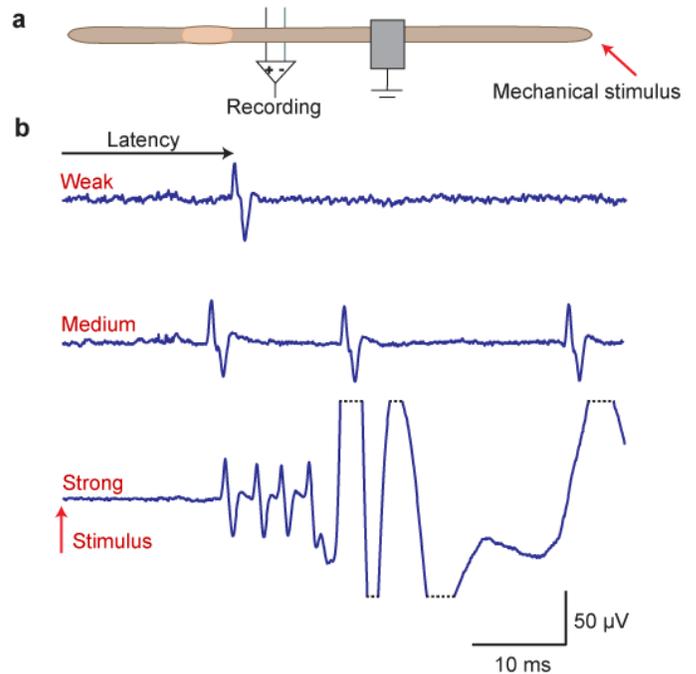
Figure 10. Mechanical stimulation of the worm hind end, activating the LGF pathway. *(a)* Experimental setup. *(b)* Classroom measurements following three stimuli with different strengths are shown. After weak stimuli, usually one AP with a high latency is recorded. The number of APs increases with stimulus strength, while their latency decreases. After strong stimuli, muscle potentials are typically seen, along with strong twitches of the worm.

Even with just a few stimuli, they should be able to see that less APs occur after weak stimuli and if the time of the stimulus is available, also that their latency is higher (Fig. 10). After stronger stimuli, more than three LGF responses can occur, often followed by muscle potentials. These gigantic potentials can be distinguished from APs by their size and duration (Fig. 10).

In courses with more experimental time, we ask students to perform double recordings and calculate the conduction velocities of the first and second elicited LGF action potentials (Fig. 11). Usually, a 10% increase in conduction velocity can be expected if the first two APs are compared (Table 4). Using their results, students should discuss possible physiological processes that could explain the facilitation of conduction, compare these results with corresponding data from the double pulse experiment (Fig. 3b), and discuss the biological relevance.
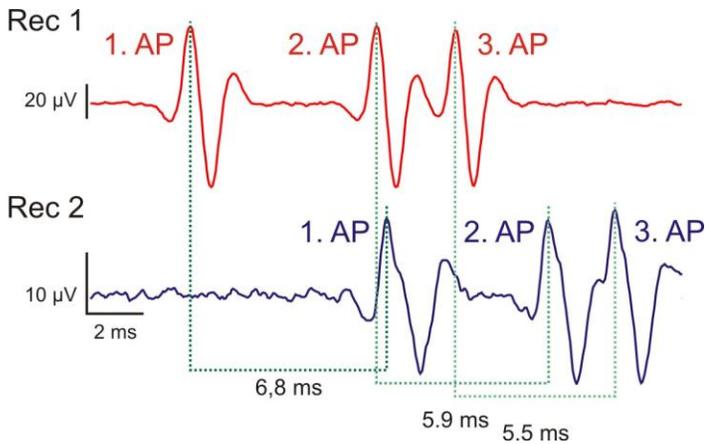
Figure 11. Conduction velocities of LGF responses. With a double recording, students can measure the decrease in conduction delay in subsequent APs. In this example, students measured the conduction velocities of the first three LGF responses. In this measurement, the intervals between the two recordings were 6.8 ms (first AP), 5.9 ms (second AP) and 5.5 ms (third AP) and the distance of the two recording sites was 70 mm. The calculated conduction velocities increase from 10.3 m/s for the first AP, 11.9 m/s for the second AP to 12.7 m/s for the third AP.

#	Velocity 1st AP (m/s)	Velocity 2nd AP (m/s)	%-Change
1	9.3	10.5	13
2	8.5	9.5	12
3	8.0	8.8	10
4	7.8	8.5	9
5	9.1	10.0	10

Table 4. Facilitation of conduction. Classroom calculations of conduction velocities are shown. Usually, a >10% increase in conduction velocity can be observed if the first two APs following medium or strong stimuli are compared.

Experiment 9 (Basic setup 2)
Temperature dependency of conduction velocity

As all biochemical processes, the generation of action potentials is dependent on the temperature. The van't Hoffs rule is a simple description of this dependency - a 10 degree rise in temperature leads at least to a two-fold velocity increase of the biochemical reaction. In this experiment, students cool down the worm and calculate the conduction velocity of the responses as a measure of these changes in biochemical processing speeds (as the conduction velocity of action potentials depends on the kinetics of ion channels).

In order to calculate the conduction velocities, a double recording should be performed. The electrical stimulus should be set to 0.5 ms duration and its amplitude should exceed the LGF threshold. During the experiment, the worm is constantly cooled down with icepacks around the cage, while students monitor the temperature with thermometers. As the worm cools down over time, repeated measurements of the conduction velocity should be performed, until the worm surface has approximately the same temperature as the icepacks.

These measurements are then used to plot the temperature-velocity characteristics. In our experience, this experiment takes some time, as the worms only cool down slowly. In advanced courses, we place the last 30 mm of the worm (recording site) on cheap peltier elements and apply 10 Hz stimuli during the cooling of the worm. With more sophisticated data analysis software, we then let students process all these measurements to generate a temperature-velocity curve as shown in Figure 12. However, only a few data points are necessary to show the temperature dependency of the conduction velocity.
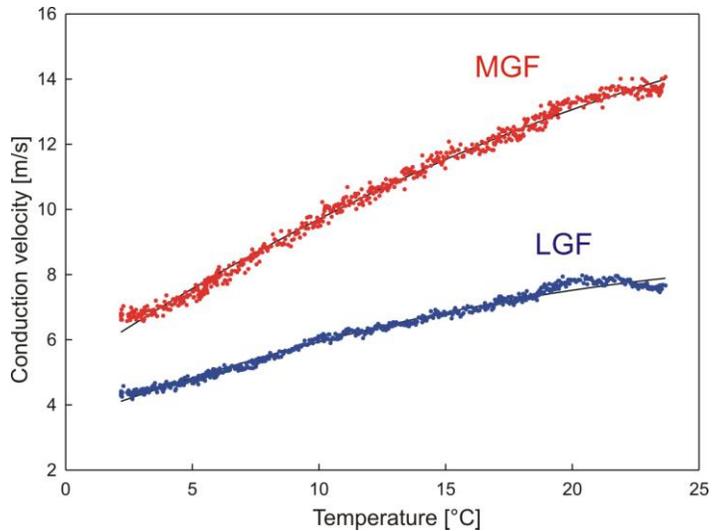
Figure 12. Temperature dependency of the conduction velocity. A classroom calculation of the conduction velocity of the MGF (red line) and LGF (blue line) is shown. In this case the experiment starts at room temperature and over time, the worm is cooled down to 2 °C.

DISCUSSION

In general, recordings from giant fibers are a good choice for neurobiological experiments. They are often easy to access, usually do not show spontaneous activity, and their size makes them easy to handle and position electrodes. This is also true for recordings of giant fibers in earthworms, with experiments using dissected worms already being a standard classroom exercise.

In our opinion, the described set of experiments is very suitable to accompany introductory courses in neuroscience, touching many fundamental principles, and even making it possible to link cellular phenomena to behavior. These experiments are so successful that we apply at least a subset in virtually every neuroscience course we teach. In addition to the obvious advantages, only basic electrophysiological equipment is necessary and experiment-specific materials can be constructed from widely available and cheap items found at any hardware and household store.

Course implementations

Usually, students work in groups of 2-3 per setup. With 20 minutes allocated per experiment, we conduct experiments

1, 2 and 4 in the neurobiology part of a basic animal physiology course with 2.5 hours of experimental time. In this course, students generally do not have any experimental experience. Therefore, we have to give an introduction to the equipment and techniques in the first hour. In this course, all groups are able to finish the first two experiments in the allotted time, with a few groups not having finished the double recordings of experiment 4. These students are provided with data from other groups. In other courses, where we have two full days available (2 x 6 h), we perform all experiments.

In all our courses, we use analog-digital converters and software interfaces. In basic courses, we use one of the commercially available neuroscience teaching stations. In advanced courses, we use more sophisticated equipment that is also used in our research.

Student experience

Given the feedback that we got over the last years, these experiments provide a rather gentle introduction to neurobiological experiments and animal experiments in general. The animals remain intact, the anesthetization is clearly reversible with worms waking up regularly during the courses and the electrical stimulation is weak (<10 V). In our experience, most students find the experiments very easy to conduct, which allows them to focus on the covered theory.

Several years ago, we performed these experiments using oscilloscopes instead of the computer interfaces we use today. It seems that students are less afraid to make mistakes using the software than they were changing settings on the oscilloscopes. This encourages more students to try and perform the experiments independently.

Using the student lab reports and individual student feedback to assess the quality of these experiments in terms of student experience, it seems that the simplicity of performing the experiments and their extremely high success rate help students really focus on the theory and give them a pleasant introduction into neurobiological experiments.

Teaching experience

We think that instructors with a general background in neurobiology will find these experiments easy to learn. Especially from instructors who already perform neurobiological classroom experiments and started to use experiments as described in Heinzel (1990), we did get very positive feedback about the simplicity, success rate, and covered theory.

In the more than 20 years of experience, an experiment only failed three times: once the lab technician used distilled water for the anesthetics, the second time, the anesthetics was confused with a formaldehyde solution and the third time, an experiment failed during a live demonstration caused by a defective power supply ground (this took long to identify). In all other cases where problems occurred, these were minor and could be fixed during the course.

The short introduction time for course assistants, the general simplicity of the experiments, and the fact that in worst case, the instructor is able to conduct all experiments within 45 minutes, gives much confidence that all students will experience a successful exercise. We also think that it is important to emphasize that these experiments have the potential to be applied in neurophysiology courses of all levels, from advanced high school to graduate levels.

Further possibilities

We have presented a set of experiments specifically tailored to the teaching goals of our introductory neurobiological courses. Furthermore, as presented in other publications, these experiments can be easily extended, such as looking at sensory maps (Drewes, 1999).

All the experiments and resulting insights can be obtained by just having a close look at a few raw measurements. However, most of the experiments are easily extended into advanced levels of classroom exercises. By using real research equipment, students can generate much data within a short time and perform quantitative, advanced analysis. Aspects of active research are touched, such as facilitation of conduction. This provides a bridge between low-level, 'old-fashioned' earthworm experiments and new publications (e.g., Ballo and Bucher, 2009).

The continued positive feedback we get from students and colleagues as well as the recent redesign of the experiments finally led to writing this article. This has, again, confirmed our opinion that these experiments have the potential to be commonly applied in neurophysiology courses of all levels.

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SUPPLEMENTARY MATERIAL

Photos of the earthworm cage

Supplementary figure 1: Assembled earthworm cage. The aluminum U-profile is covered with foam rubber through which pin electrodes are pushed.
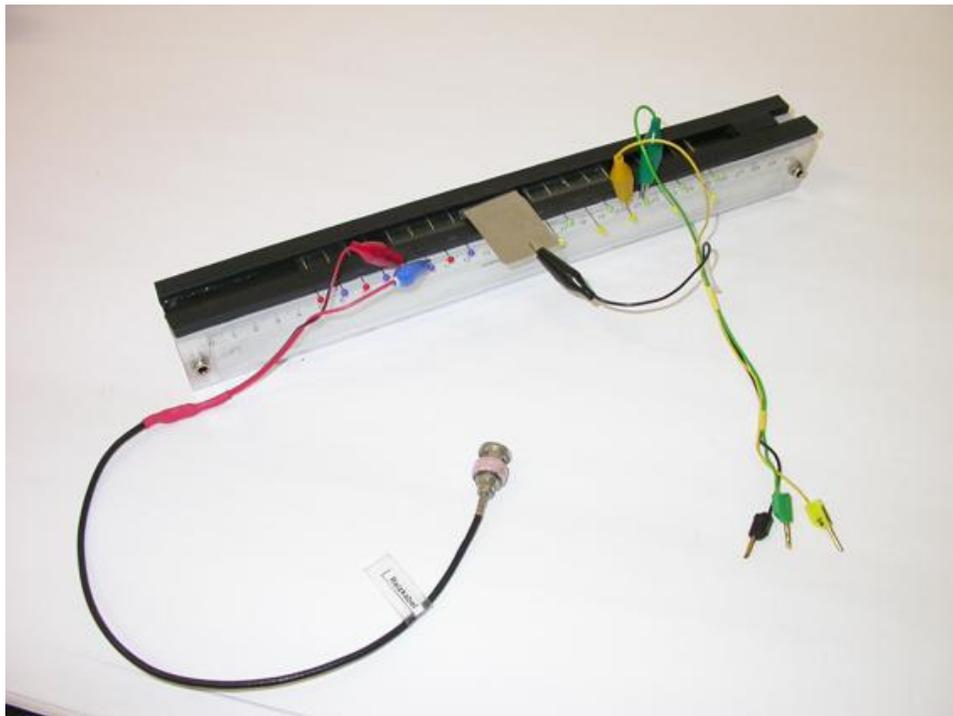
Supplementary figure 2: Connecting equipment to the earthworm cage. In this case, equipment for electrical stimulation is connected to the cage.
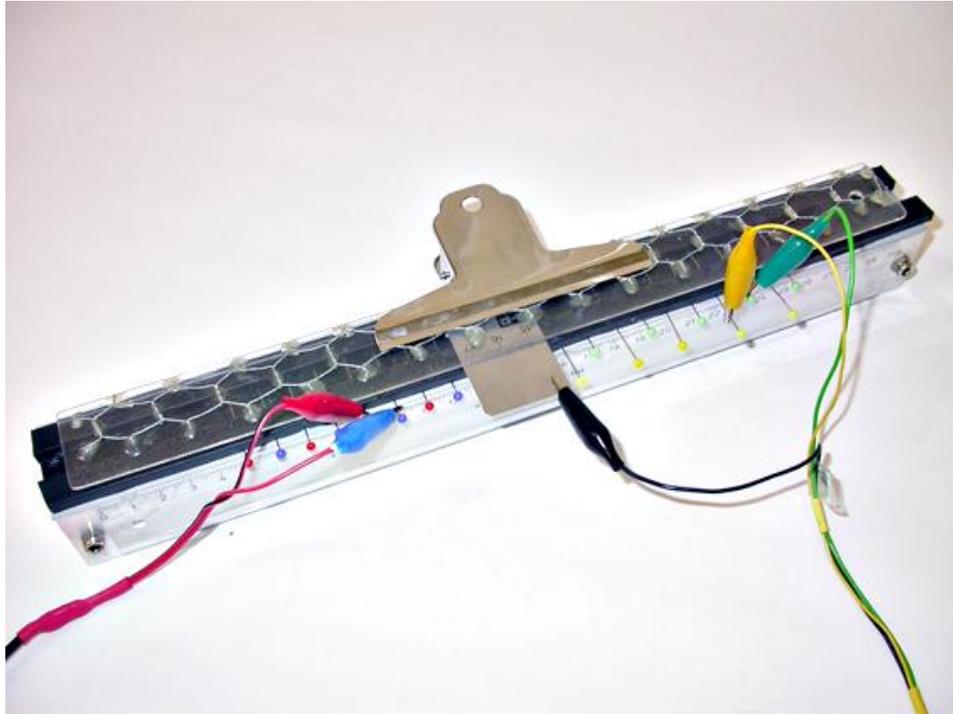
Supplementary figure 3: Completely assembled earthworm cage.

A cheap mechanical stimulator that allows the measurement of the time of stimulus
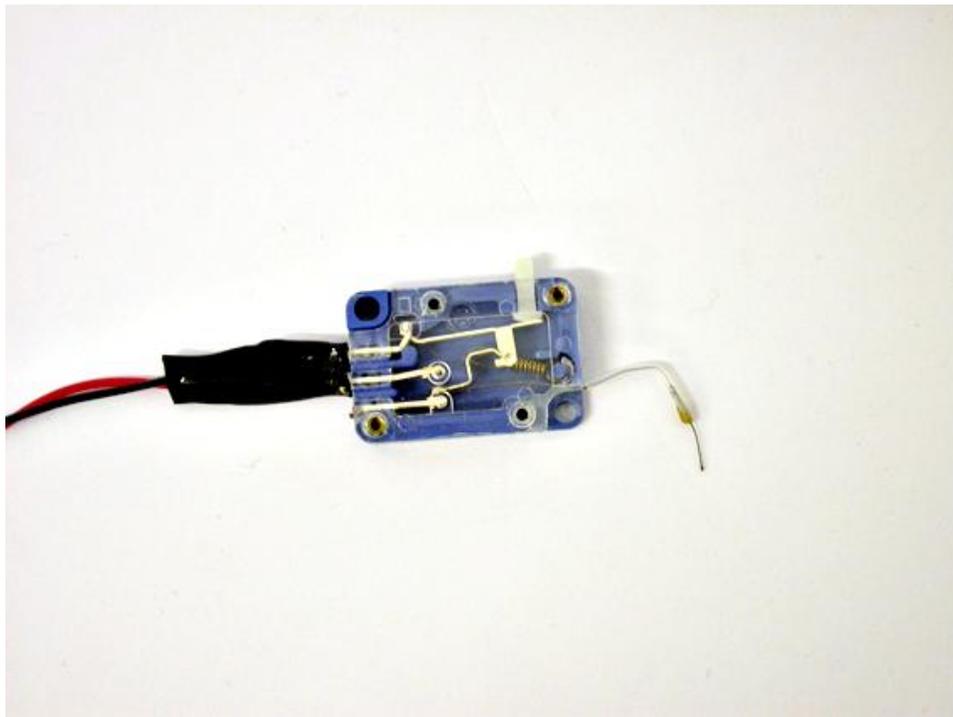
Supplementary figure 4: Design of the mechanical stimulator. A standard electrical switch can be modified by gluing a bristle to touch the worm onto the inner mechanics. The closing of the switch can be monitored with a simple electrical circuit, e.g., looking at the short circuit.
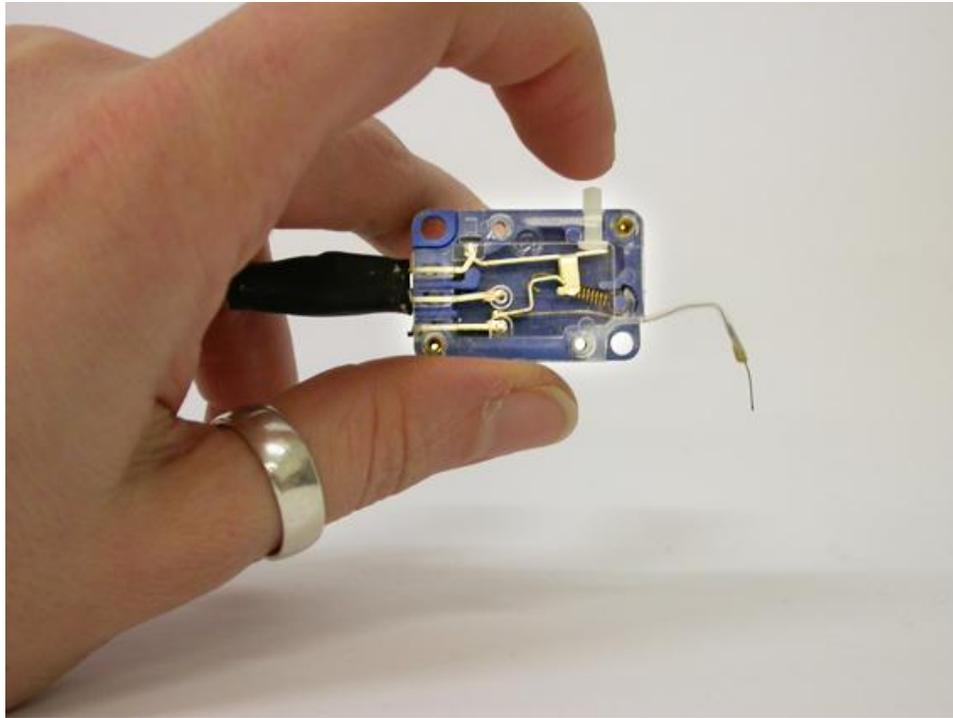
Supplementary figure 5: Using the mechanical stimulator.
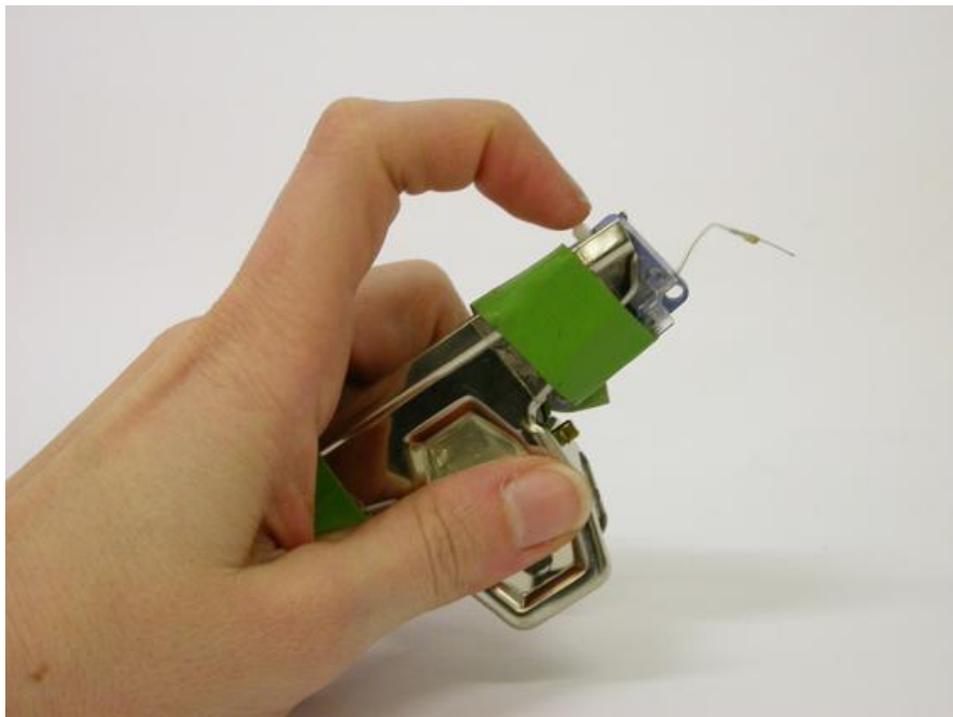
Supplementary figure 6: Assembled mechanical stimulator. For easier handling, we attach the switch to a paper clip.

List of necessary equipment per setup
- Earthworm cage
- Two standard extracellular amplifiers (1k amplification, 0.3-3 kHz band-pass filter)
- Isolated stimulator for electrical stimuli with adjustable stimulus amplitude (0-10 V) and optimal a stimulus duration between 50 µs and 50 ms, hardware & software trigger, functionality to deliver double-pulses with pulse intervals between 1 and 50 ms.
- Oscilloscope or analog-digital converter and PC/software interfaces with a minimum of two channels.
- Anesthetic: 1,1,1-Trichloro-2-Methyl-2-Methyl-2-Propanol (e.g., sciencelab.com)
- Appropriate cables and connectors, aluminum foil or Faraday's cage
- Manual mechanical stimulator: either a simple bristle or the described stimulator

General advice for successful experiments
- Use a moist tissue as a dummy-worm to test the setup
- Make sure that the ventral side of the worm has contact with the pin electrodes
- Make sure that all connections are correct
- Do not forget to use the small piece of aluminum foil between the stimulus and recording electrodes when applying electrical stimuli. You will not be able to see the giant fiber responses as the stimulus artifact gets really big. Sometimes it helps to move the grounding foil around a little bit to improve contact with the skin of the worm.
- Look at the timing between stimulus and giant fiber response: if the interval is less than 1 ms, you are looking at the stimulus artifact. This can also be identified as it should change in size when the amplitude of the electrical stimulus is changed.
- If the worm still moves in experiments using anesthetized worms: do not try too long, take another worm.
- The recordings are very tiny: try to change the position of the recording electrodes, preferably in the last 50 mm of the worm. If this does not help, change the position of the stimulation electrodes (if applicable).
- If you are giving electrical stimuli with amplitudes close to 10 V and you still do not measure anything, make sure that the stimulus electrodes are placed behind the clitellum. If this does not help, try changing the worm and also make sure that the negative electrode is closer to the hind end than the positive electrode to prevent an anodic block (very rare).