Developmentally regulated multisensory integration for prey localization in the medicinal leech

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SUMMARY
Medicinal leeches, like many aquatic animals, use water disturbances to localize their prey, so they need to be able to determine if a wave disturbance is created by prey or by another source. Many aquatic predators perform this separation by responding only to those wave frequencies representing their prey. As leeches' prey preference changes over the course of their development, we examined their responses at three different life stages. We found that juveniles more readily localize wave sources of lower frequencies (2 Hz) than their adult counterparts (8–12 Hz), and that adolescents exhibited elements of both juvenile and adult behavior, readily localizing sources of both frequencies. Leeches are known to be able to localize the source of waves through the use of either mechanical or visual information. We separately characterized their ability to localize various frequencies of stimuli using unimodal cues. Within a single modality, the frequency–response curves of adults and juveniles were virtually indistinguishable. However, the differences between the responses for each modality (visual and mechanosensory) were striking. The optimal visual stimulus had a much lower frequency (2 Hz) than the optimal mechanical stimulus (12 Hz). These frequencies matched, respectively, the juvenile and the adult preferred frequency for multimodally sensed waves. This suggests that, in the multimodal condition, adult behavior is driven more by mechanosensory information and juvenile behavior more by visual. Indeed, when stimuli of the two modalities were placed in conflict with one another, adult leeches, unlike juveniles, were attracted to the mechanical stimulus much more strongly than to the visual stimulus.

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Key words: development, leech, mechanosensory, multisensory, vision.

INTRODUCTION
An ability to localize prey using water waves is found in a wide range of aquatic predators including seals, arthropods, frogs, fish, arrow worms and leeches (Bleckmann and Barth, 1984; Dehnhardt et al., 1998; Elepfandt, 1984; Horridge and Boulton, 1967; Kanter and Coombs, 2003; Lang, 1980; Mann, 1962; Russell and Roberts, 1974; Young et al., 1981; Zimmer, 2001). For this information to guide effective prey localization behavior, prey-induced water disturbances must be separated from those arising from other sources (such as wind, rain or predators). Some animals make this separation by exclusively responding to a narrow range of wave frequencies that represent their prey (Bleckmann and Rovner, 1984; Lang, 1980).

It is known that medicinal leeches will localize the source of water waves (Carlton and McVean, 1993; Young et al., 1981) but the frequency dependence of this response has not yet been studied. Thus, it is unknown if they only respond to specific frequencies of water disturbance. Furthermore, it is unknown whether the frequency response changes during development. This becomes an interesting issue as leeches exhibit changes in prey preference with their development (Elliott, 2008; Keim, 1993; Sawyer, 1986; Wilkin and Scofield, 1990; Wilkin and Scofield, 1991), which, if they are responding to prey-like water disturbances, should be accompanied by changes in their frequency preferences such that they localize prey appropriate to their life stage. Here, we test this idea by measuring the responses of medicinal leeches at three life stages to a range of wave frequencies.

Leeches have two sensory systems capable of sensing water movement: mechanosensory hairs along their body; and simple eyes that sense changes in light level that occur as surface waves cast shadows on their body (Carlton and McVean, 1993; Derosa and Friesen, 1981; Dickinson and Lent, 1984; Friesen, 1981; Kretz et al., 1976; Phillips and Friesen, 1982; Young et al., 1981). Both of these sensor types have an extensive distribution over the body of the leech (Blinn et al., 1986; Friesen, 1981). Similar arrays of sensors allow for accurate localization of prey in other animals (Elepfandt, 1982; Elepfandt, 1984; Franosch et al., 2003; Maclver et al., 2001; Müller and Schwartz, 1982; Nelson and Maciver, 1999). In the leech, each of the 21 mid-body segments has seven pairs of mechanosensory sensilla that are sensitive to even minute water movements (Blinn et al., 1986; Friesen, 1981; Young et al., 1981). Co-localized with these is a visual sensillum: a simple pit eye. In addition, the leech has five pairs of simple eyes located on its head (Kretz et al., 1976; Peterson, 1983; Peterson, 1984). While neither type of eye is capable of image formation, the head eyes are capable of sensing the movement direction of a beam of light (Kretz et al., 1976; Peterson, 1983). Furthermore, although individual visual sensilla on the body are non-image forming, the whole grid receives enough information to, in principle, determine the location of a water disturbance (Carlton and McVean, 1993; Peterson, 1984; Schwab, 2006). While both the visual and the mechanosensory systems can be used to orient toward waves, it remains unknown whether they are both actually capable of localizing the source of waves. Here, we assess
the capability of these individual sensory systems in wave source localization behavior, measure their tuning curves and examine their relative influence on the behavioral response of the animal.

MATERIALS AND METHODS

Animals

For this study we examined the responses of three different sizes of leech. As leeches exhibit salatory growth whereby after each feeding they have a period of rapid growth, these sizes relate to the number of feedings that the leech has had, and thus to its age (Elliott and Tulliet, 1986; Sawyer, 1986; Wilkin and Scofield, 1991). Adult (3.6±0.3 g) and ‘adolescent’ (1.2±0.16 g) leeches of the species Hirudo verbana (Carena, 1820) were obtained from Niagara Medical Leeches, Inc. (Westbury, NY, USA). Juveniles (<0.01 g) of the same species were generously provided by the Kristan lab at University of California, San Diego. Adults and adolescents were fasted for 6–10 months so that they were inclined to exhibit prey localization behaviors. Leech mass was measured just prior to testing (i.e. post-fasting). Juveniles were tested within 3 months of hatching and were seeking their first meal.

Leeches were maintained in tanks of 20–30 individuals in a temperature-controlled room held at 16.0°C with a 12h:12h light:dark cycle. Sample sizes appear in Table 1.

Adults and adolescents were tested individually whereas juveniles were tested in groups of approximately 10 individuals. To accommodate this, for juveniles we used N to indicate the number of groups, and n to indicate the total number of animals tested.

Behavioral arena

Behavioral testing was performed in a saucer-shaped arena (diameter: 83 cm) filled with water (18±0.5°C) to a depth of 20 mm. This resulted in the diameter of the water reaching 75 cm. Approximately 0.2 kg of white aquarium gravel was placed on the floor of the arena as this was found to aid in quiescence (C.M.H., unpublished observation). The arena was placed on an air table to isolate it from external vibrations.

Stimulus

A function generator (Pasco Scientific, Roseville, CA, USA) was used to drive a speaker (Pasco Scientific) that was connected to a thin aluminium rod with a clear plastic circular foot (diameter: 4.7 cm) at its tip. The foot was placed such that it lay flat on the surface of the water. This system was used to induce sinusoidal vertical movements in the foot at various frequencies between 0.5 Hz and 12 Hz. The driving voltage for the speaker was adjusted to maintain a constant wave amplitude. These voltages were determined for each frequency condition, except that only IR lighting was present. Thus, no visual cues could reach the leech in this condition but visual cues could not. To further ensure the absence of any visual cues, only dim red light [which leeches are insensitive to (Kretz et al., 1976)] was used in the experimental room during these experiments.

<table>
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For juveniles, N is used to indicate the number of groups, and n is used to indicate the number of animals tested.

Stimulus conditions

Multimodal

The arena was illuminated using a 750 W halogen flood lamp (McMaster-Carr, Santa Fe, CA, USA). The wave generator was placed in the behavioral arena, 7±1 cm from the edge of the water.

Mechanical

Like in the multimodal condition, the wave generator made waves directly in the behavioral arena, but the only light present was from four infrared (IR) light-emitting diode (LED) lamps (PN850, Pinecomputer, Covina, CA, USA) placed around the arena. Additionally, we placed blackout curtains around the arena to block stray light. As leeches are blind to IR light (Kretz et al., 1976), mechanical cues could reach the leech in this condition but visual cues could not. To further ensure the absence of any visual cues, only dim red light [which leeches are insensitive to (Kretz et al., 1976)] was used in the experimental room during these experiments.

Visual

Waves were created in an arena with a clear plastic bottom that was independently suspended above the behavioral arena which contained the leeches. Thus, the two arenas were mechanically isolated from each other. By shining the 750 W halogen lamp through the clear-bottomed arena onto the behavioral arena, we were able to transmit the visual cues from the waves without making the mechanical cues available to the leech. To ensure that visual cues were arising from the waves and not from the visual aspect of the stimulator foot movement, we placed the stimulator foot 2.5 cm above the water. We found that its movement alone was not enough to evoke localization behavior [1/11 individuals found the stimulus; a number not significantly different from when no stimulus was present (2/13)].

As a control (‘dark control’) the arena was set up as in the visual condition, except that only IR lighting was present. Thus, no visual cues could have influenced the leeches.
cues would have been available to the animals. The water in the top arena was stimulated at 2 Hz. This allowed us to assess whether or not any non-visual information was available to leeches in the visual condition.

Conflict
The mechanical stimulator was placed 7±1 cm in from the edge of the water as before. A digitally rendered visual stimulus was presented to the leeches using an LCD projector (EX50, Epson, Long Beach, CA, USA) mounted 1.5 m above the arena. This stimulus consisted of green bars spreading circularly outward on a black field moving at 2 Hz and emanating from a disc the size of the physical stimulus. Responses to this type of stimulus were no different from responses to waves in the visual condition. It was positioned such that the origin of the visual stimulus was 120 deg away from the mechanical stimulator.

This set-up allowed us to disconnect the stimulus that produced visual cues from the stimulus that produced mechanical cues. The projector was necessary as a means of creating non-mechanical waves in this complex set-up. Complex visual waves arising from the combination of our visual stimulus and the physical water waves were not detected with the naked eye. However, we are unable to rule out the possibility that they were detected by leeches.

We then compared the results from the conflict with the same individuals responding to projected visual stimuli, purely mechanical stimuli and no stimulus at all. During the experiments, which provided either no stimulus or a purely mechanical stimulus, we projected a background, i.e. a random pattern of 0.64 cm black and green squares. This pattern was equal in brightness to the projected visual stimulus. This equivalent brightness is important as background brightness can impact the baseline activity level in juveniles (supplementary material Fig. S1).

Stimulus protocol
The stimulus type (multimodal, visual, mechanical, conflict, projected or dark control) for each day was chosen at random. In addition, a random number generator was used to determine the frequencies at which each animal or group was tested. Individuals or groups thereof did not repeat frequencies within the same stimulus protocol.

Adults
A shadow was projected into the arena using a piece of cardboard measuring 7 cm × 7 cm. This shadow encouraged the leeches to reach a quiescent state (C.M.H., unpublished observations) (Gee, 1913; Herter, 1936; Mann, 1962). Prior to starting the trial, the leech was required to remain within the shaded region for 1 min. Once this time had passed, the shadow was removed and the stimulator was activated. Stimulation lasted for 5 min. During trials with only IR lighting, a shadow could not be projected and instead the leech had to remain still for a minimum of 30 s. If a trial could not be completed due to a leech’s failure to become quiescent within 20 min from the time an animal was first placed in the arena, or if it left the arena three times, that trial was abandoned and the animal was not retested that day.

Juveniles
Juveniles were separated into groups of 10±1 individuals. A group was placed within the testing arena and was given 15 min to disperse throughout the arena and reach relative quiescence. Small variations in the number of individuals occurred because of their failure to leave the transfer pipette or because they escaped from the arena during the 15 min dispersal period. We did not require complete quiescence of juveniles. Their activity was then recorded for 5 min during stimulation.

Data acquisition
When the halogen lamp was used, videos were acquired using a Logitech pro 9000 webcam (Fremont, CA, USA) suspended above the arena. For trials with IR light or projected visual stimuli, a Logitech c600 webcam was used that had the IR filter removed. With either camera, video was acquired at 25 frames s⁻¹.

Behavior Finds
‘Finds’ were defined as an individual remaining within a radius of 7 cm from the center of the stimulus for a minimum of 30 s. This timing was chosen to separate pauses in forward movement occurring because the individual was actually investigating the stimulus from those occurring between crawling steps as a normal part of the behavior or during transitions to swimming. We examined 50 pauses from seven individuals as they explored the arena without stimulation. These pauses ranged from 2 s to 193 s with a median of 6 s; 98% of these lasted 16 s or less. As exiting the ‘find’ radius may require up to two crawling steps that take on average 4–6 s each (Baader and Kristan, 1995; Stern-Tomlinson et al., 1986), we posited that if a leech spent 30 or more seconds near the stimulus,
it was not simply crawling past. Such events were thus counted as a ‘find’.

For adults and adolescents, a maximum of one find was counted per trial. In the conflict condition, finds for the two stimuli were counted independently. The ‘find rate’ was defined as the number of trials in which the stimulus was found divided by the total number of trials. Error bars in figures representing adults and adolescents are binomial standard deviations for this data.

Juveniles were tested in groups, and we did not keep track of individual identities. We counted the total number of finds per 5 minute trial, and it was possible for one individual to contribute more than one find to the total; however, it was not common for individuals to leave and immediately re-enter the find radius. For a given trial, the ‘find rate’ was defined as the total number of finds divided by the number of individuals. The find rates plotted in the figures are the means of the values for each trial with the same stimulus condition. The error bars for juveniles are the standard deviations between trials.

RESULTS

Responses to multimodal stimuli

We tested the leech’s ability to localize the source of water waves of six frequencies between 2 Hz and 24 Hz (Fig. 1). These responses were compared with the probability of the leech localizing the stationary stimulus. Adult leeches found the stimulus significantly more often when it produced waves at mid-range frequencies (8 Hz and 12 Hz) than when it was stationary ($P<0.01$ and $P<0.05$, respectively, $\chi^2$ test) or when it produced low-frequency (2 Hz) or high-frequency (20 Hz) waves ($P<0.05$, $\chi^2$; Fig. 1A).

In juveniles, all frequencies of stimuli evoked a greater find rate than when no stimulus was present. However, they responded most robustly to low frequencies. The find rate at 2 Hz for juveniles was significantly higher than that at any of the other frequencies tested ($P<0.01$, ANOVA; Fig. 1B) or when the stimulus was stationary ($P<0.0001$, ANOVA).

Responses to unimodal stimuli

To individually examine the contributions of visual and mechanical information to localization behavior, we performed experiments under two conditions: one that provided only mechanical cues from the stimulus; and another where only visual cues were available. For the former, the mechanical stimulus was presented while the arena was illuminated using only IR light, which is not visible to leeches. For the latter, the stimulus was created in a clear-bottomed arena mounted above the behavioral arena (Carlton and McVean, 1993) while the whole set-up was brightly illuminated with visible light. This resulted in the creation of visual waves within the lower arena without any physical movement of the water in that arena. We noted that in the visual condition juveniles had a much lower find rate than adults. This is likely a result of their reduced baseline activity under conditions when bright light was present (supplementary material Fig. S1).

While nearly all frequencies of mechanical stimuli evoked a response greater than when there were no waves present, the peak find rate for this modality in both adults and juveniles occurred at 12 Hz (Fig. 2A,C). In both age groups this find rate was significantly greater than the find rate at 2 Hz ($P<0.05$, $\chi^2$ for adults and $P<0.01$, ANOVA for juveniles).

In contrast, for the purely visual condition, the peak find rate in both adults and juveniles was at 2 Hz (Fig. 2B,D). This find rate was significantly higher than when no stimulus was present ($P<0.05$, $\chi^2$ for adults and $P<0.005$, ANOVA for juveniles) and when the same experiment was performed in the dark ($P<0.05$, $\chi^2$ for adults). In addition, the find rate at 2 Hz was significantly higher than at 12 Hz ($P<0.05$, $\chi^2$, for adults and $P<0.05$, ANOVA for juveniles).

Thus, despite the fact that the peak find rates of adult and juvenile leeches were different for multimodal stimuli (Fig. 1), their respective response profiles to unimodal stimuli closely resembled one another (Fig. 2). For both juveniles and adults the peak find rate for mechanical stimuli was at 12 Hz whereas it was 2 Hz for visual stimuli. Strikingly, the adult multimodal response showed a strong

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Fig. 3. Responses to conflicting stimuli. Two stimuli were placed in different locations within the arena. One gave only mechanical cues while the other gave only visual cues. Adult (A) and juvenile (B) leeches were tested on their ability to find these locations (x-axis and y-axis) when either only the mechanical stimulus was active (blue), only the visual stimulus was active (red), both were active ('conflict' condition, green) or neither was active ('control', black). The error bars represent standard deviations (adults, n = 16 for the other three conditions) or inter-group standard deviations (juveniles, N = 9 groups for each condition; total number of animals: 90 for control, 91 for mechanical only, 88 for visual only, 92 for visual stimuli (open symbols) did not differ significantly from their responses for adults were more driven by mechanical cues and juveniles more by visual cues.

Responses to conflicting stimuli
To test this hypothesis, we examined the relative dominance of each sensory modality by simultaneously presenting a purely visual stimulus in one region of the arena with a purely mechanical stimulus in another. Specifically, a 2Hz projected visual stimulus (see Fig.3C) and a 12Hz mechanical stimulus were simultaneously presented in locations 120° apart in the arena. When conflicting visual and mechanosensory stimuli were present, adult leeches preferentially localized the mechanical stimulus (Fig.3A). In fact, for adults, the find rate for the mechanical element of conflicting stimuli was not significantly different from that of individuals exposed to purely mechanical stimuli. Both of these find rates were significantly greater than when the mechanical stimulator was present but not active (P<0.001, χ²). Furthermore, adults exhibited no orientation toward the visual element of the stimulus: the find rate for the visual element of conflicting stimuli was not significantly greater than when this element was absent.

In contrast, the juvenile find rate for both the mechanical and visual elements of the conflicting stimuli were both significantly higher than when no stimulus was present (P<0.005, P<0.001, χ²; Fig.3B). In fact, juveniles presented with simultaneous visual and mechanical stimuli at different locations localized each stimulus as readily as they would a single stimulus in unimodal conditions.

Responses of adolescent individuals to multimodal stimuli
As the frequency tuning curves of juvenile leeches were very different from those of adults (Fig.1A,B), we hypothesized that there should be an intermediate period when the animal transitions from a juvenile response to an adult response. To test this hypothesis, we exposed leeches weighing approximately 1.2g to multimodal wave stimuli. We found that these adolescents responded strongly, and equally, to both the frequencies preferred by juveniles and those preferred by adults (Fig.4; cf. Fig.1). This demonstrates that there is a gradual transition between the juvenile response profile and that of adults.

DISCUSSION
Our results clearly show that leeches are able to localize the source of water waves. In addition, sources of certain wave frequencies are preferentially localized over others. But what could these wave frequencies correspond to? While involvement in social behavior is possible, it is unlikely as the behavior of a small group of leeches is not influenced by others within the group (Bisson and Torre, 2011). It is also possible that this behavior is related to prey localization; a notion that is further supported by the reduction of this behavior in leeches which have been recently fed (Young et al., 1981). Serological studies of Hirudo spp. have shown that mammalian blood is only found in the stomachs of adult leeches weighing at least 3.5g (Elliott, 2008; Keim, 1993; Wilkin and Scofield, 1990). Adult leeches are thought to prefer mammalian blood because it allows them to grow faster and have more young per cocoon (Davies and McLoughlin, 1996; Elliott, 2008; Sawyer, 1986; Sineva, 1944; Sineva, 1949). In contrast, stomach contents of wild-caught medicinal leeches weighing less than 3g have only contained blood of non-mammals such as frogs, fish and birds (Elliott, 2008; Keim, 1993; Wilkin and Scofield, 1990). It is thought that young leeches rely more on alternative prey because their jaws are too small to pierce the skin of mammals (Bennike, 1943; Blair, 1927; Elliott and Kutschera, 2011; Hoffman, 1960; Mann, 1962; Sawyer, 1986; Sineva, 1944; Sineva, 1949; Sineva, 1950). In fact, this prey switching phenomenon has been documented in many species of jawed leech [e.g. Nephelopsis obscura (Davies et al., 1978); Erpobdella punctata (Davies et al., 1981); Erpobdella testacea (Dall, 1983)] but has yet to be found in species lacking jaws. Thus, jawed leeches must exhibit behavioral changes that enable them to find the appropriate prey for their life stage.

Behavioral studies on leeches of the distantly related genus Erpobdella showed that some species exhibited a robust localization response to prey animals and only a limited response to non-prey (Blinn and Davies, 1989). Likewise, if Hirudo only responds to those select frequencies representing their prey, then the frequency tuning of that response should change with different life stages. Indeed, we found that sources of certain wave frequencies were localized more readily than others and that this response profile was life-stage-dependent (Fig.1). Specifically, adult leeches more readily localized the source of a 12Hz water surface wave than those of 2Hz or 20Hz waves (Fig.1A). In

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contrast, juveniles more readily localized the source of 2Hz waves than those of 12Hz waves or higher (Fig. 1B). The response profile of adolescent leeches was different from both juveniles and adults (Fig. 4), and indeed, leeches’ food preference at each of these three life stages is distinct (Elliott, 2008; Keim, 1993; Sineva, 1944; Sineva, 1950; Wilkin and Scofield, 1990).

How does this change in frequency tuning come about? Do the sensors change their sensitivity or is another mechanism at work? To answer this question, we investigated prey localization behavior when cues from only a single sensory modality were available to the animal. We found that the tuning curve of the mechanosensory system does not change as the leech matures (Fig. 2A,C), and neither does the tuning curve of the visual system (Fig. 2B,D). Instead, the age-related changes in the multimodal tuning curve appear to be due to changes in the weights accorded to these individual cues. This notion is further supported by the responses of juveniles and adults to conflicting stimuli, i.e. when visual and mechanical cues indicated two different prey locations, adults followed the mechanical cues as if the visual cues were absent. In contrast, juveniles would follow either cue to its source (Fig. 3). These data suggest the exciting possibility that the gain of individual sensory systems is changed with life stage to match changes in prey preference.

One may suspect that the mechanism responsible for age-related changes in frequency tuning may arise from changes in the body size and, thus, distribution of the sensors. Such a change in the sensory array would carry with it changes in the relevant stimuli. However, a condensed array, such as that of the juveniles, would respond better to higher frequencies than would an extended array, as that seen in the adults. Furthermore, rather than noting a change in the frequency response solely in the multimodal case, under this scenario we would notice it for each individual modality because the visual and mechanosensors exhibit the same spacing changes as the animal grows. Several alternative mechanisms could underlie this change. One possible mechanism is that between the juvenile and adult life stages leeches gain 250% more sensory afferents and have extraordinary changes in their mechanosensory system: the diameter of their sensilla increases and they gain cilia on each sensillum (Gascoigne and McVein, 1993; Peinado et al., 1990). Such a change could result in an increase in the influence of mechanosensors because they are both increasing in sensitivity and size during this time period while the visual system is not changing. Alternatively, the change could occur as the result of a change in synaptic strength of either excitatory or inhibitory connections (Baltzley et al., 2010; Chiang et al., 2006; Lockery and Kristan, 1989). Lastly, a neuromodulatory mechanism could be responsible. Such mechanisms have been noted in central pattern generators (CPGs) in many animal species and have been hypothesized to allow for CPG-controlled behavior to adapt to conditions surrounding the animal at that moment, developmental stage or evolutionary stage (Baltzley et al., 2010; Briggman and Kristan, 2008; Crisp and Mesce, 2003; Fenelon et al., 1999; Fenelon et al., 2004; Gaudry and Kristan, 2009; Katz, 1998; Lockery and Kristan, 1991; Newcomb and Katz, 2007). Interestingly, in most of these examples the excitability of the system is modulated by serotonin, which in Hirudo is known to result in the decreased sensitivity of mechanosensors during feeding (Gaudry and Kristan, 2009; Gaudry and Kristian, 2010). It is worth noting that leeches can also change the sensitivity of their skin by merely adjusting its topology (Rodriguez et al., 2004). However, this mechanism may not operate on a developmentally relevant timescale.

Similar adaptive changes in sensory tuning have been noted in several other species. For example, the salamander Hynobius leechii undergoes morphological changes in its mechanosensors during maturation, resulting in a more robust response to the 1Hz waves created by their mating display (Park et al., 2008). And the mottled sculpin (Cottus Bairdi) modulates its dependence on different sensory modalities in response to environmental factors that change the reliability of a sensory signal; lake populations of this fish rely chiefly on mechanosensors for prey localization, while river populations utilize other senses, presumably because river currents make the mechanosensory signature of prey harder to detect (Coombs, 1999; Coombs and Grossman, 2006). In both examples, the ability to change the gain of individual sensors provides the animal with the necessary plasticity to adapt to changing conditions. The leech, with its simple, easily accessible nervous system provides us with an exciting new model for studying neural mechanisms behind these adaptations.

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