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RESEARCH ARTICLE

Electrophysiology and transcriptomics reveal two photoreceptor classes and complex visual integration in *Hirudo verbana*

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ABSTRACT

Among animals with visual processing mechanisms, the leech *Hirudo verbana* is a rare example in which all neurons can be identified. However, little is known about its visual system, which is composed of several pigmented head eyes and photosensitive non-pigmented sensilla that are distributed across its entire body. Although several interneurons are known to respond to visual stimuli, their response properties are poorly understood. Among these, the S-cell system is especially intriguing: it is multimodal, spans the entire body of the leech and is thought to be involved in sensory integration. To improve our understanding of the role of this system, we tested its spectral sensitivity, spatial integration and adaptation properties. The response of the S-cell system to visual stimuli was found to be strongly dependent on the size of the area stimulated, and adaptation was local. Furthermore, an adaptation experiment demonstrated that at least two color channels contributed to the response, and that their contribution was dependent on the adaptation to the background. The existence of at least two color channels was further supported by transcriptomic evidence, which indicated the existence of at least two distinct groups of putative opsins for leeches. Taken together, our results show that the S-cell system has response properties that could be involved in the processing of spatial and color information of visual stimuli. We propose the leech as a novel system to understand visual processing mechanisms with many practical advantages.

KEY WORDS: Invertebrate vision, Opsins, Visual processing, S-cell interneuron, Medicinal leech

INTRODUCTION

Vision requires complex integration mechanisms. In most model species, investigating those at the level of individual neurons is complicated by the large number of neurons involved and the challenge of identifying specific neurons. Among animals with visual processing, the leech *Hirudo verbana* is a rare example in which all neurons can be readily identified. However, little is known about the neuronal mechanisms of visual processing in the leech. At the input level, the leech's visual system consists of several pigmented cylindrical eye cups within the head region, and a grid of non-pigmented photosensitive sensilla distributed across the entire body (Kretz et al., 1976). Several interneurons have been found to respond to visual stimuli (Kretz et al., 1976), but their response properties remain poorly understood. Among these, the S cell interneuron is especially intriguing. The S cell is an interneuron that is activated by salient stimuli of multiple modalities, including mechanical as well as visual stimuli (Magni and Pellegrino, 1978; Laverack, 1969; Bagnoli et al., 1973; Kretz et al., 1976), suggesting that it may be involved in multimodal sensory integration (Harley et al., 2011, 2013). A single (not bilateral) S cell is present in each of the 21 segments of the leech. Synaptic pathways between the S cell and both sensory and motor neurons have been reported within the segmental ganglia (Sahley et al., 1994). Importantly, S cells in adjacent ganglia are strongly coupled by electrical synapses (Frank et al., 1975). The electrical coupling between S cells is so strong that the whole S-cell system can be considered as a single syncytium that acts as a fast conducting pathway connecting the segmental ganglia (Peterson, 1984). Although direct proof is lacking (see Sahley et al., 1994), the general consensus in the field is that the S-cell system plays a key role in synchronizing general arousal throughout the nervous system of the leech.

Despite the S cell's purported central role in sensory processing, the neuronal pathways leading from photoreceptor cells to the S cell are not known. In addition, other basic questions regarding the S-cell system, including its role in light adaptation, its temporal and spatial integration properties, and its overall role in vision remain to be addressed.

It has long been known that *Hirudo* has the ability to visually detect the direction of water waves, and that – in combination with mechanical cues – it uses this information for prey localization (Dickinson and Lent, 1984; Carlton and McVean, 1993; Harley et al., 2011). This demonstrates that its visual system has the ability to process spatiotemporal patterned visual stimuli, despite the lack of image-forming eyes. S cells respond when the leech is presented with flashes of light as well as to stimuli associated with water waves (Lehmkuhl et al., 2018). Their multimodal response properties, along with the fact that the S-cell system spans the entire body, make them an intriguing candidate for further investigations.

Not much is known about the temporal properties of S-cell responses. However, an early study (Laverack, 1969) found that the S-cell response quite rapidly ceases when the leech is stimulated continuously either visually or mechanically, but that the S cell remains sensitive to tactile stimuli when visually desensitized. This finding is consistent with findings in other animals. For instance, the human central nervous system is well known to fairly quickly adapt to constant or repetitive stimuli, while remaining sensitive to different stimuli of the same or a different modality. This phenomenon is generally believed to enhance an animal's ability to detect ethologically relevant changes in its environment (Desimone and Duncan, 1995), though much about the underlying mechanisms remains to be fully understood.

Another early study of leech vision (Kretz et al., 1976) indicated that a single class of photoreceptors is involved in responding to light. Those photoreceptors, putatively found in both the head eyes

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Received 15 February 2019; Accepted 22 June 2019

Journal of Experimental Biology (2019) 222, jeb201764. doi:10.1242/jeb.201764

and the sensilla, respond most strongly to light in the green range of the visual spectrum. Unexpectedly, however, recent behavioral and electrophysiological experiments demonstrated that under certain specific circumstances, the S-cell system responded more strongly to UV than to green light. This phenomenon was observed especially when UV light was directed at the ventral side of the body wall, suggesting that the S-cell system may play a role in detecting and correcting the animal's orientation relative to the sun (Jellies, 2014a,b).

These results appear to require the presence of a second color channel, which has not been directly identified. There is, however, precedence for the existence of multiple photoreceptor classes in other leeches: molecular investigations in *Helobdella robusta* have found at least four distinct opsins (Döring et al., 2013). Unfortunately, the spectral properties of these opsins remain unknown because of a lack of physiological and molecular data.

In this paper, we present electrophysiological and transcriptomic evidence indicating the presence of at least two distinct color channels in *Hirudo*. Furthermore, we show that the S cells are involved in spatial integration and the implementation of differential adaptation to background light illumination, unveiling new roles for the S-cell system in vision and sensory integration.

MATERIALS AND METHODS

Animals and animal preparation

Adult leeches (*Hirudo verbana* Carena 1820) were obtained from Niagara Leeches (Niagara Falls, NY, USA) and maintained under standard conditions (Harley et al., 2011). At the time of the experiments, leeches had fasted for at least 2 months and weighed 1–1.5 g. Leeches were anesthetized with ice-cold leech saline (Tomina and Wagenaar, 2018) and immobilized ventral-side up on dark silicone (Sylgard 170, Dow Corning, Midland, MI, USA) using insect pins stuck in annuli without sensilla. The head of the leech was pinned against the dark silicone so that the eye cups faced the silicone and thus would not directly receive stimulation light. The body wall was opened from midbody segments M8 to M11 (or M7 to M10 in experiments on spectral sensitivity under full dark adaptation). The lateral roots of ganglia M9 and M10 (or M8 and M9) were transected, and the ganglia and connectives were gently separated from the body tissue without severing any other nerves. The wall of the ventral blood sinus ('stocking') was removed between the exposed ganglia. A thin strip of silicone (Sylgard 184) was slipped between the nerve cord and the body wall and pinned down on each side of the leech. Ganglia M9 and M10 (or M8 and M9) were pinned very close together onto the silicone strip and the connective between them was sucked into a suction electrode. The general setup is shown in Fig. 1A. The temperature of the leech was kept at 15–19°C throughout all experiments.

General electrophysiological setup

The electrophysiological setup consisted of a differential amplifier (Model 1700, A-M Systems, Sequim, WA, USA), an oscilloscope (TBAS 1046, Tektronix, Beaverton, OR, USA) and an A/D converter (Model 118, iWorks Systems, Dover, NH, USA). Recordings were performed inside a Faraday cage on a vibration isolation table (TMC 66-501, Technical Manufacturing Corporation, Peabody, MA, USA). Data were stored on a PC using LabScribe software (iWorks), and analyzed using custom-written code in Octave (https://www.gnu.org/software/octave/). To tightly control background illumination, the entire recording area was enclosed in black-out fabric (BK5, Thorlabs, Newton, NJ, USA). In addition, the room light was kept off during experiments, so that the only light sources in the room were indicator lights on electronic equipment and a computer screen. The light seal of the recording area was tested by means of a sudden substantial increase in ambient room light after the leech was fully dark adapted. The light seal was trusted only if this did not elicit a response.

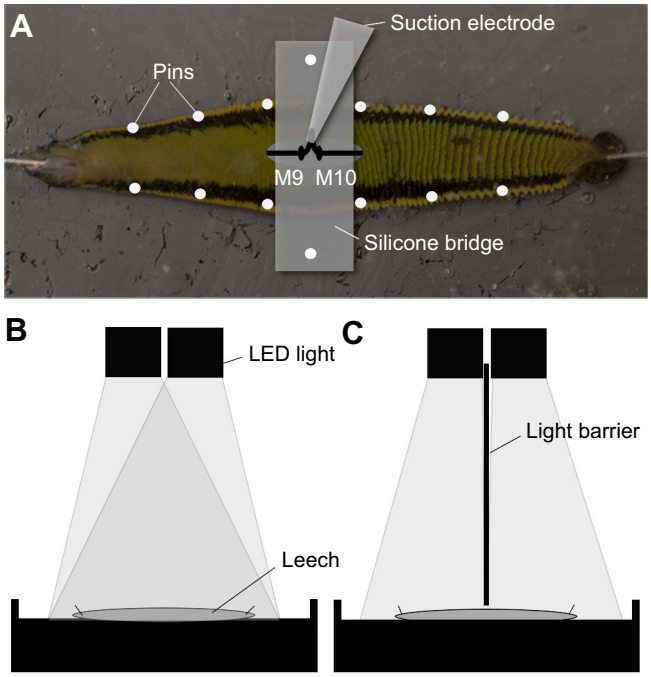

Fig. 1. Experimental setup. (A) Illustration of how leeches were pinned out, with a silicone bridge slipped underneath the nerve cord, the ventral body wall opening that was made between M8 and M11, and the suction electrode. (B) Orientation of the background and stimulus LED light sources relative to the leech for all experiments that tested the adaptation to background light illumination (Figs 3 and 4). (C) Orientation and placement of the LED light source and the barrier between the anterior and posterior side for the experiments on non-local adaptation (Fig. 6) and spatial integration (Fig. 7).

Measuring light intensity

Measurements were taken with a spectrometer (USB2000+ with a QP600-025-SR optical fiber and a CC3-UV-T cosine corrector; Ocean Optics, Dunedin, FL, USA) which was calibrated against a calibrated light source (DH-2000, Ocean Optics). All reported light intensities are absolute numbers from radiometric irradiance measurements, in units of photons cm^{-2} s^{-1}. To obtain controlled light intensities below the minimum intensity that the spectrometer could directly measure, we used calibrated neutral density filters placed in front of a brighter light source. Calibration of neutral density filters was performed independently for each relevant wavelength. All measurements were made with the cosine corrector of the spectrometer probe at the same distance and orientation relative to the light source as the leech would be in our actual experiments. Although we took great care to measure light intensities as accurately as possible, it should be noted that measuring absolute light intensities accurately is notoriously challenging: according to Johnsen (2012), measurement errors up to 10% (0.1 log units) are to be expected even in the best scenarios. We believe our measurements to be accurate to about that level. Furthermore, as all of our key results rely on relative light intensities, minor errors in absolute intensity values do not affect the interpretation of our results.

Spectral sensitivity measurements

Monochromatic light was generated by coupling a 150 W xenon arc lamp (Apex 70525 Monochromator Illuminator, Oriel Instruments, Stratford, CT, USA) to a monochromator (Cornerstone 130 1/8 m 74000, Oriel). In previous experiments using this system, we had observed a small secondary peak at approximately 300 nm below the primary peak wavelength. To eliminate this secondary peak, we used a long-pass filter (ET542LP, Chroma, Bellows Falls, VT, USA) for all primary wavelengths of 590 nm and above. The light intensity was controlled with a variable neutral density filter (50Q00AV.2, Newport Corporation, Irvine, CA, USA) mounted on a motorized rotator stage (NSR-12 controlled by a NewStep NSC200 controller, both Newport Corporation). Three additional neutral density filters (FRQ-ND1 and FRQ-ND2, Newport Corporation; NDUV30A, Thorlabs) that were mounted onto a manual filter wheel (FW1A, Thorlabs) were used to achieve light attenuation beyond the range of the motorized filter wheel. The duration of the stimulus was controlled with a shutter (VCM-D1, Uniblitz, Rochester, NY, USA). The light path also contained two lenses (LJ4395-UV and LA4306-UV, Thorlabs) that focused the light onto an optical fiber placed directly behind the shutter. (Lenses and fiber were chosen to transmit both UV and visible light.) At the end of the optical fiber was a lens that collimated the light so that a light spot with a diameter of 2.8–3.5 cm was projected onto the leech from a distance of 10–13 cm. The light source was positioned above the leech and illuminated the entire posterior ventral side ranging from the body wall opening at M10 to the rear sucker at an angle of no greater than 30 deg from normal.

Leeches were dark adapted for at least 30 min before starting a recording, and recordings were performed without background illumination. (We could not quantify stray background light, but estimate it to be below 10^8 photons cm^{-2} s^{-1}, or approximately 0.0002 lx, similar to the darkness under an overcast sky on a moonless night.) We recorded responses to 500-ms stimuli with the following peak wavelengths (in nm): 320, 350, 400, 455, 530, 590 and 655. The order of wavelengths that we tested was randomized. To generate response–log(intensity) curves, we used light intensities in a range of approximately 3 log units in steps of approximately 1/3 log units, always working in order of increasing light intensity, separately for each wavelength. Preliminary data (not shown) showed that it was critical to include prolonged recovery times between stimuli especially after a strong response to relatively high light intensity. To optimize the quality of obtained data, we allowed at least 1 min and up to 5 min between stimuli, depending on the stimulus light intensity and responses.

Adaptation to green and UV light

For these experiments, we used LEDs in combination with neutral density filters to achieve higher light intensities and a wider range of intensities than what was possible with the monochromator. The LEDs were controlled by a custom driver that provided a precisely regulated DC current to the LED; the neutral density filters served to extend the intensity range beyond the range of the driver. We specifically did not use pulse width modulation (i.e. control of the duty cycle of flicker) to avoid assumptions about the frequency response of the visual system. Schematic diagrams are available from the corresponding author on request.

For UV, we used LEDs with a dominant wavelength of 365 nm (LED Engin LZ1-10UV00, Mouser, Mansfield, TX, USA) and the same neutral density filters as above. For green light, we used 523 nm LEDs and OD-2 and OD-4 filters (NE20B-A and NE40B-A, Thorlabs). In this way, we achieved a green background light intensity range of 6 log units and a green and UV stimulus light intensity range of approximately 7.5 log units. The UV stimulus light (but not the UV background) was fitted with a filter (357/25x, Chroma AT) that eliminated a small secondary peak within the visual wavelength range. As UV illumination elicited a strong fluorescence of the exposed intestinal tissue at the body wall opening, we removed this tissue as much as possible, and closed up the body wall opening for the recording.

Each LED was mounted behind a condenser lens (ACL2520, f=20 mm, Thorlabs). The background and stimulus LED assemblies were mounted directly above the leech such that the angle between them was no more than 15 deg. The background illuminated the leech from a distance of 19 cm; the stimulus illuminated the leech from a distance of 11 cm. The illuminated area had a diameter of 9.5–10.5 cm. The leech was pinned out to a length of no more than 6 cm, so that the entire ventral side was illuminated by both the background and the stimulus (Fig. 1B). The green and UV background LEDs were mounted at fixed positions immediately adjacent to one another on a slider that allowed their positions to be switched. This ensured that the stimulus location and orientation were identical regardless of wavelength.

To quantify the adaptation to green background light, we tested six background intensities ranging from 3.4×10^{10} to 3.4×10^{15} photons cm^{-2} s^{-1} in steps of 1 log unit. Because the need to keep our experimental animals healthy throughout the experiment imposed time constraints on the duration of experiments, each leech (n=11) was tested with only three or four of the six background light intensities. (Specifically, we tested the lowest light level on 11 leeches, the second level on 6 leeches, the third on 4, the fourth on 3, the fifth on 5 and the highest on 10.)

As above, leeches were dark adapted for 30 min before recording, and additionally background adapted for 10 min every time we changed the background illumination or had to open the light seal to exchange neutral density filters. To generate response–log(intensity) curves for each background light intensity and stimulus wavelength (green and UV), we applied 2-s stimuli with intensities spanning 3 log units in steps of approximately 1/4 log units, in order of increasing light intensity. To prevent adaptation to the stimulus intensity, 3 min of only background illumination was provided between stimuli.

Local versus non-local adaptation

Two green through-hole LEDs (941-C505BGANCC0D0781, Mouser) provided differential background illumination to the anterior and posterior halves of the leech. A third such LED delivered flash stimuli to the posterior half of the leech. All LEDs were mounted at a distance of 9 cm from the leech; the stimulus LED was mounted immediately adjacent to the LED that provided background illumination to the posterior half of the animal. A light barrier consisting of blackout fabric was placed between the anterior and posterior halves of the leech to ensure controlled differential stimulation of the two halves (Fig. 1C). As above, we used neutral density filters to reduce light intensity beyond the range of the LEDs. These were mounted onto a slider so that they could be exchanged from the outside without opening the light seal of the recording area.

Two levels of background light intensity were used in these experiments: 3.9×10^{12} photons cm^{-2} s^{-1} (‘dark’) and 4.4×10^{13} photons cm^{-2} s^{-1} (‘light’). All combinations of light and dark background conditions were tested, always in the following order: (1) both halves dark; (2) both halves light; (3) posterior light, anterior dark; (4) posterior dark, anterior light; (5) both halves dark (as a control to test whether we could recover the initial response). For constructing response curves, the same range, step size, order of

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stimulation and stimulus duration were used as for the previous experiment.

Spatial integration

Background illumination intensity was 4.5×10^{11} photons cm^{-2} s^{-1}. The setup was otherwise the same as for the local versus non-local adaptation experiment, except that an additional stimulus LED was used to provide flashes to the anterior region. Order of stimulation was: (1) anterior only; (2) anterior and posterior together; (3) anterior only again to test whether we could recover the initial responses. After that, we cut the cord posterior to the recording site, which disconnected the posterior half of the body from our recording site, and tested for the influence of stray light by stimulating (4) posterior only (which potentially could affect the anterior side through stray light); and (5) anterior only, to test whether initial responses could be recovered. Stimulus duration, intensity range, step size, order of stimulation and time between stimuli were as before.

Data analysis

Action potentials from the S cell were identified as the largest spiking units in extracellular recordings from the nerve cord (Frank et al., 1975). Electrophysiological data were analyzed using custom-written programs in Octave (electrophysiology data and code used for analysis are available upon request from the corresponding author). As a measure of response strength, we counted S-cell spikes that occurred within a certain time window, starting when the stimulus was turned on. This time window was either as long as the stimulus duration (spatial integration and local versus non-local adaptation experiment), or slightly longer (spectral sensitivity experiment: 1.5 s; adaptation to background experiments: 2.5 s). Response–log(intensity) curves are standard logistics:

$$y = {}^1\!/_2 Y_0(1+\tanh[\alpha(x-x_{50})]), \quad (1)$$

where y is spike count, x is log intensity, Y_0 is the maximal spike count (plateau response), α is the slope of the curve and $x_{50}=\log(I_{50})$ is the light intensity (in log units) that elicits half-maximal response. For quantifying the light intensity for 50% response (I_{50}; Fig. 2), the plateau spike count (Y_0) was determined once per leech and then used for all wavelengths. Likewise, in Fig. 3, the plateau spike count was determined once per leech (for green stimuli) and used for all background levels and both UV and green stimuli. The same principle was used in subsequent figures, except that in Fig. 4 we used 35% of maximum as the critical value, because UV-on-UV stimuli often did not elicit 50% of maximum green-on-green response even at the highest intensities. To find the delay of the response (Fig. 3), we measured the time that elapsed from the beginning of the stimulus to the occurrence of the 3rd spike of the response.

Transcriptome analyses to identify opsins

Transcriptomic databases were generated from two separate tissue types: (1) a single head containing the eyes and (2) 100 isolated sensilla collected from the body. Tissues were dissected in ice-cold RNAse-free Gibco PBS (Thermo Fisher Scientific, Waltham, MA, USA). Tissues were briefly frozen in liquid nitrogen and ground using a mortar and pestle. RNA isolation was conducted using the RNeasy Lipid Tissue Kit (Qiagen, Valencia, CA, USA). To assess the quality of the RNA, extractions were subjected to spectrophotometric analysis utilizing a NanoDrop 1000 Spectrometer (Thermo Fisher Scientific) where the $A_{260/280}$ absorbance ratio yielded measurements around 2.0 for RNA extracts, indicating that all RNA measurements were relatively pure. RNA-seq utilized the Illumina HiSeq 2500 (75 bp) with Ribo-zero preparation at Cincinnati Children's Hospital Core Sequencing Facility (Cincinnati, OH, USA). The raw read FASTQ files were assembled using Trinity (Grabherr et al., 2011), CLC Genomics and Oases (Schulz et al., 2012) according to previously described methods (Rosendale et al., 2016). Expression was assessed by mapping reads based on parameters described in Rosendale et al. (2016). The quality of each transcriptome was assessed through evaluation of the Benchmarking Universal Single-Copy Orthologs (BUSCO) gene sets (Simão et al., 2015).

Opsin sequences were identified using the Blastx algorithm (Altschul et al., 1997) to identify orthologs to the previously

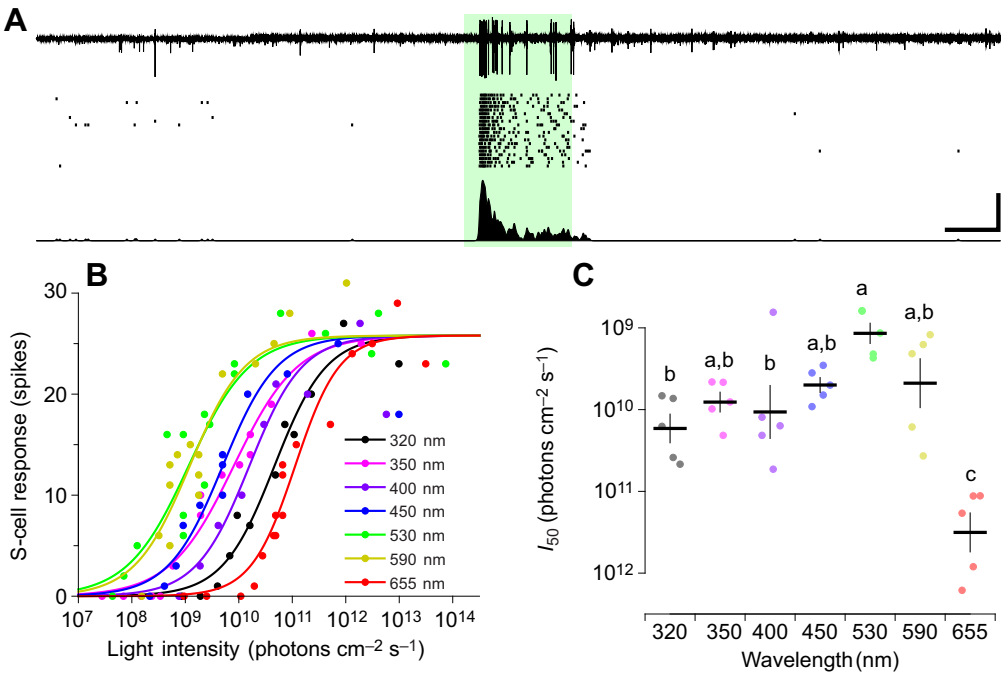

Fig. 2. S-cell responses to light stimulation and spectral sensitivity. (A) Responses to 2-s flashes of green light (530 nm, 1.5×10^{11} photons cm^{-2} s^{-1}; shaded area) presented to the posterior half of the ventral body wall. Top to bottom: representative raw extracellular trace; raster plots from 20 individual trials on a single leech; firing rate histogram of those trials. Scale bars: 1 s and 25 spikes s^{-1}. (B) Response curves to 500-ms flashes of light of various wavelengths (one representative leech). (C) Spectral sensitivity of S-cell responses (I_{50}, light intensity for 50% response). Dots represent individual animals; black lines mark means and s.e.m. Letters mark groupings from ANOVA/Tukey (at $P<0.05$; $n=5$).

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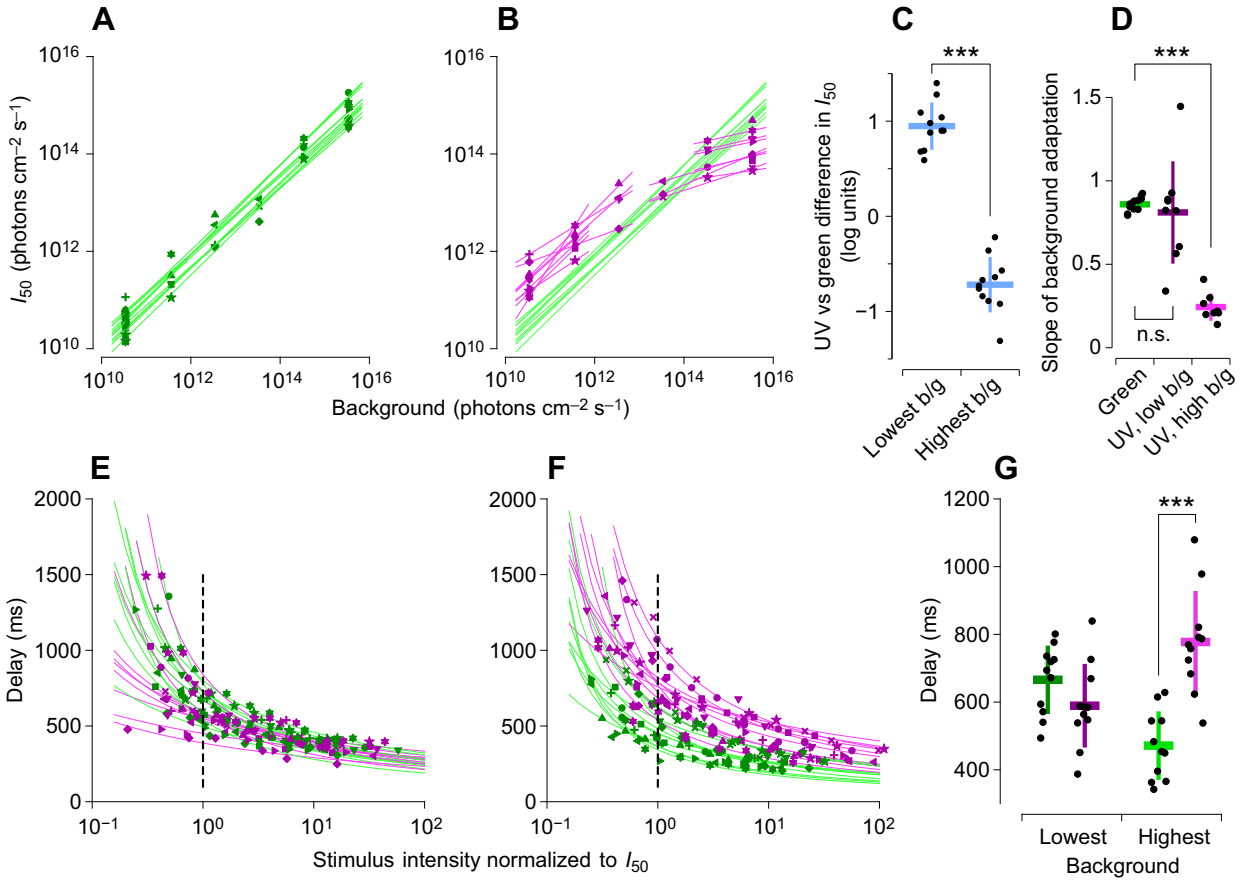

Fig. 3. Adaptation to intensity of background light. (A) Intensity of green stimulus light required to attain 50% of the plateau response (I_{50}, see Materials and Methods) as a function of green background intensity. Symbols indicate animals; lines are linear fits for each animal. (B) Intensity of UV stimulus light required to attain the same response as in A as a function of green background intensity. Lines are linear fits separately for the low-background (left) and high-background (right) regimes. (C) Difference in light intensity required to attain 50% of the plateau response when using UV light versus green light, at the lowest background (b/g) intensity (left) and at the highest background intensity (right). ***$P<10^{-7}$, *t*-test (n=11). (D) Summary of fit results from A and B. From left to right, slope of the response curve to green light (Green), to UV light in the low-background regime (UV, low b/g) and to UV light in the high-background regime (UV, high b/g). Black dots indicate the slopes of individual fits; bars indicate mean and s.d. across animals. ***$P<10^{-5}$, *t*-test (n=8). (E,F) Delay of the response to green and UV light stimulation at the lowest background light intensity (E) and highest background light intensity (F). The stimulus light intensity is plotted normalized to I_{50}. Symbols indicate individual leeches, lines are fits for each animal and the dashed line indicates the light intensity that elicited half-maximum response (I_{50}). (G) Summary of the data from E and F, showing the delay of the response at I_{50} for green and UV stimulation at the lowest (left) and highest (right) background light intensity. ***$P<10^{-5}$, *t*-test (n=11).

annotated opsin sequences of *H. robusta* (Döring et al., 2013) along with opsin sets obtained from arthropod and other invertebrate species from NCBI nr databases. These two different databases were used to identify potential functionality, as many annelid-specific opsins have not been fully characterized. A reciprocal BLAST against the invertebrate and arthropod databases was used to confirm whether predicted genes match opsins in other systems. Relationships between the opsin sequences and contigs were assessed through the use of MEGA5 (Tamura et al., 2011) to generate a neighbor joining tree after sequence alignment with CLUSTAL Omega (Sievers and Higgins, 2014). Illumina sequencing files have been deposited in NCBI SRA (Bioproject: PRJNA504032).

RESULTS

To examine the light-dependent responses of the S cells, we investigated their response strength as a function of the wavelength of the stimulus and adaptation to background illumination, tested whether the adaptation to background illumination is local or global, and quantified spatial integration. We focused specifically on the S cell's response to light stimulation of the ventral body wall.

General response properties

The S-cell system responded reliably and vigorously to stimulation of the ventral body wall of the leech with flashes of light. The typical response to a flash of green light presented against a dark background is illustrated in Fig. 2A. The response can be separated into two phases: (1) an initial transient phase characterized by high firing rates and (2) a sustained response that typically lasts beyond the duration of the stimulation with a substantially lower spike frequency.

Spectral sensitivity of dark-adapted leeches

To test the spectral sensitivity of the S-cell system, we applied 500-ms flashes of light of various wavelengths and intensities to the ventral body wall of dark-adapted leeches and recorded spike responses from the S cell using suction electrodes. For each wavelength, we constructed response–log(intensity) curves by fitting logistic functions (Fig. 2B). We then quantified the light intensity required to elicit half the maximum response (I_{50}; see Materials and Methods) for each leech to obtain absolute sensitivity profiles (Fig. 2C). In agreement with Kretz et al. (1976), we found the highest sensitivity in the green wavelength range. Certainly, these dark-adapted leeches failed to show the strong response to UV light reported by Jellies (2014a).

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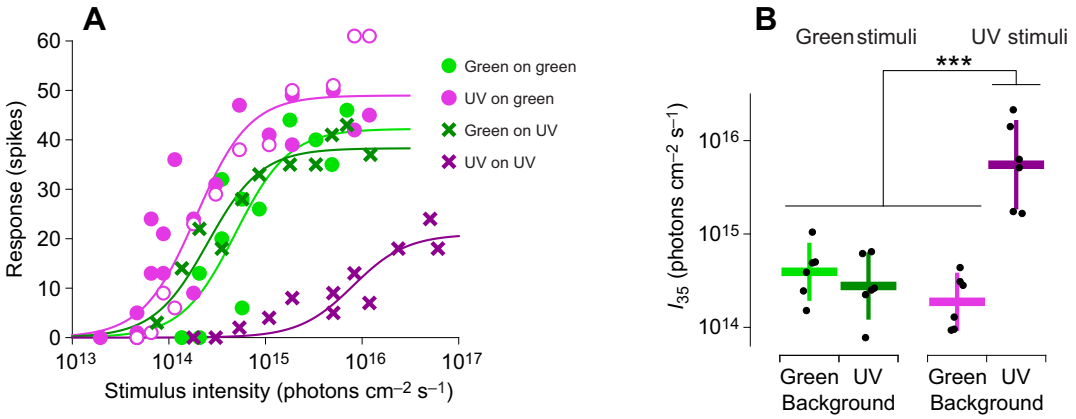

Fig. 4. Adaptation to the spectrum of background light. (A) Response to stimuli with green light (greens) and UV light (purples) on a background of either green light (circles) or UV light (crosses). Background intensity was 3.4×10^{15} photons cm^{-2} s^{-1} for the green background and 9.7×10^{15} photons cm^{-2} s^{-1} for the UV background (see Results, Physiological evidence for a second color channel, for rationale). Filled and open purple markers represent data collected under the same conditions at the beginning and end of the experiment, to confirm stability of responses. Data from one representative animal. (B) Stimulus light intensity required to elicit a response at least 35% as strong as the plateau response for UV stimuli on the green background (I_{35}). ***P<0.0001, Tukey test following ANOVA ($F_{3,15}$=32.5, P<10^{-6}, n=6 leeches). Colors as in A.

Physiological evidence for a second color channel

To investigate the possible existence of a masked secondary peak that would correspond to a second color channel, we performed a series of background adaptation experiments designed to unmask subtle secondary responses that otherwise remain hidden by the strong response to green light. We argued that increasingly intense green background light would increasingly adapt the green-sensitive pathway, so that increasingly strong flashes would be needed to activate it, regardless of the color of those flashes. In contrast, the effect of green background light on a possible second pathway that is only sensitive to UV light would be minimal.

Thus, we began by adapting leeches to a variety of background intensities of green light and measuring response curves to flashes of green light superimposed on those backgrounds. We found that over a range of nearly 6 log units, the response was approximately contrast invariant; that is, the intensity for half-maximum response (I_{50}) scaled almost in direct proportion to the background intensity (Fig. 3A): the slope of the best-fit lines was 0.86±0.04 (mean±s.d., n=11 animals; Fig. 3D).

We also presented these leeches with flashes of UV light against the same green background intensities, and found that at low background intensities (up to 10^{12} photons cm^{-2} s^{-1}), the intensity required to obtain half-maximum response again scaled nearly proportionally with the background intensity (Fig. 3B, left). The best-fit lines had a slope of 0.81±0.31 (mean±s.d.; n=9 animals), not significantly different from the 'green' slopes (t-test). This indicates that the responses to UV light were due to the same pathway that adapted to the green background light.

However, this trend did not continue at higher background intensities: At green background intensities above 10^{14} photons cm^{-2} s^{-1}, the intensity of UV light required to obtain half-maximum response no longer increased linearly with the background intensity (Fig. 3B, right). In this range, the best-fit lines had a slope of 0.24±0.08 (n=8), and at the very highest green background intensities, sensitivity to UV flashes was actually greater than to green flashes (Fig. 3C), suggesting that a second channel is activated by high-intensity UV light.

Further evidence for the involvement of two color channels in the S-cell response comes from analyzing response delays. At the lowest background light intensity, the delay of the response to UV stimulation was similar to that for green stimulation (on average 589±124 versus 666±101 ms, mean±s.d.; Fig. 3E,G), whereas at the highest green background light level, the delay of the response to UV was substantially longer than that to green stimulation (on average 777±151 versus 471±101 ms; t_{10}=8.8, P<10^{-5}; Fig. 3F,G). This difference could easily be explained if the two color channels have distinct temporal response properties or connect to the S cell via two pathways that introduce distinct delays. It would be harder to explain if there were only one color channel.

We next performed a direct test for the presence of two distinct color channels (UV and green) that contribute to the responses in high-intensity background light. We presented leeches with flashes of green or UV light on top of the highest intensity green background from the previous experiment, and also with those same flashes presented against a bright UV background. We purposefully chose the intensity of the UV background light such that green flashes against this background elicited similar responses to those against the green background (Fig. 4A, green curves). As expected, this required more photons of UV than green background light (9.7×10^{15} UV photons cm^{-2} s^{-1} versus 3.4×10^{15} green photons cm^{-2} s^{-1}); this merely confirmed that a substantial contribution to the response to green flashes came from a pathway that was more sensitive to green than to UV light, and that was hence also more susceptible to adaptation to green light than to UV light. Also in agreement with the previous experiment, UV flashes elicited slightly more spikes at slightly lower stimulus intensities against the bright green background than did green flashes (Fig. 4A, purple circles and curve). But crucially, UV flashes elicited far fewer spikes against the UV background (purple crosses and curve), even at very high intensities. This phenomenon was robust across animals: the photon flux required to elicited an equal response using UV flashes against a UV background was significantly larger than when using UV flashes against a green background or when using green flashes against either background color (Fig. 4B). The most parsimonious explanation is that the UV background specifically caused an adaptation of a mainly UV-sensitive pathway.

Transcriptomic confirmation of a second opsin

To obtain independent confirmation that the observed responses were indeed due to two color channels, we searched transcriptomes

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for putative opsin genes. We obtained these transcriptomes by performing RNA-seq on a tissue sample from the head (focusing on the head eyes) and on a tissue sample containing 100 sensilla isolated from the body wall. The quality of the resulting transcriptome was evaluated using three BUSCO gene sets (see Materials and Methods). BUSCO scores were over 80% for all assemblies and above 95% when the three sets were combined (Fig. 5A). This indicates that our *de novo* contig library has the completeness required for subsequent analyses.

Two putative opsins from *H. verbana* were identified through BLAST analyses against opsins from other invertebrates (Döring et al., 2013), and both had orthologs in another leech (Fig. 5B). Each of these had documented expression in both the head and the sensilla. Of the two putative opsins found in *Hirudo*, one (Contig139791; Table S1, Fig. S1) had BLAST hits with other invertebrate opsins other than those of leeches that are sensitive to blue and green wavelengths; the other (Contig156444; Table S1, Fig. S2) showed similarities to UV opsins from arthropods. We also performed a direct BLAST comparison against a previously described UV-sensitive opsin from another annelid, *Platynereis dumerilii* (Tsukamoto et al., 2017), and found a close match between it and our putative UV opsin (Table S1).

Orthologs of both our putative opsins in *H. robusta* showed similar results: three were likely blue and green sensitive and one had putative UV sensitivity. These transcriptomes suggest the presence of one blue- or green-sensitive opsin in *Hirudo* and one UV-sensitive opsin, supporting our physiological experiments.

Background adaptation affects only local sensory processing

Our experiments thus far showed that S-cell responses adapt to background light intensity. However, they did not show whether adaptation occurs in the sensory periphery, in the central nervous system or in both. In addition, if adaptation occurs in the nervous system, it could be a local phenomenon (limited to the segment or segments targeted by the light) or a global phenomenon (in which illumination of one or several segments would trigger adaptation throughout the animal).

To investigate these scenarios, we differentially adapted the anterior and posterior half of the ventral side of the body wall to two distinct green background light levels and tested the response to posterior green stimulation (n=5). As before, for each animal we established response curves as a function of log intensity (Fig. 6A) and calculated the light intensity that elicited 50% of the plateau response (I_{50}; Fig. 6B). As expected, the I_{50} for posterior stimulation strongly depended on the background light level on the posterior body wall (blue versus black symbols, or red versus green symbols; ANOVA, $F_{1,20}$=55, P<10^{-6}). In contrast, the background light level on the anterior body wall had no effect on the response to posterior stimulation (green versus black points, or red versus blue points). Thus, adaptation appears to be a local phenomenon.

S-cell responses integrate spatial information

The absence of non-local adaptation does not rule out the possibility that the S-cell system performs spatial integration. In fact, the intersegmental connections between S cells uniquely position the S-cell system to integrate information across the whole nervous system. To investigate that possibility, we stimulated either the whole leech or only the anterior half of the leech with green light while recording from an S cell located in the anterior half. We found that stimulating both halves together elicited a stronger response (Fig. 7). This indicates that the S-cell system integrates information pertaining to light stimuli from across the body.

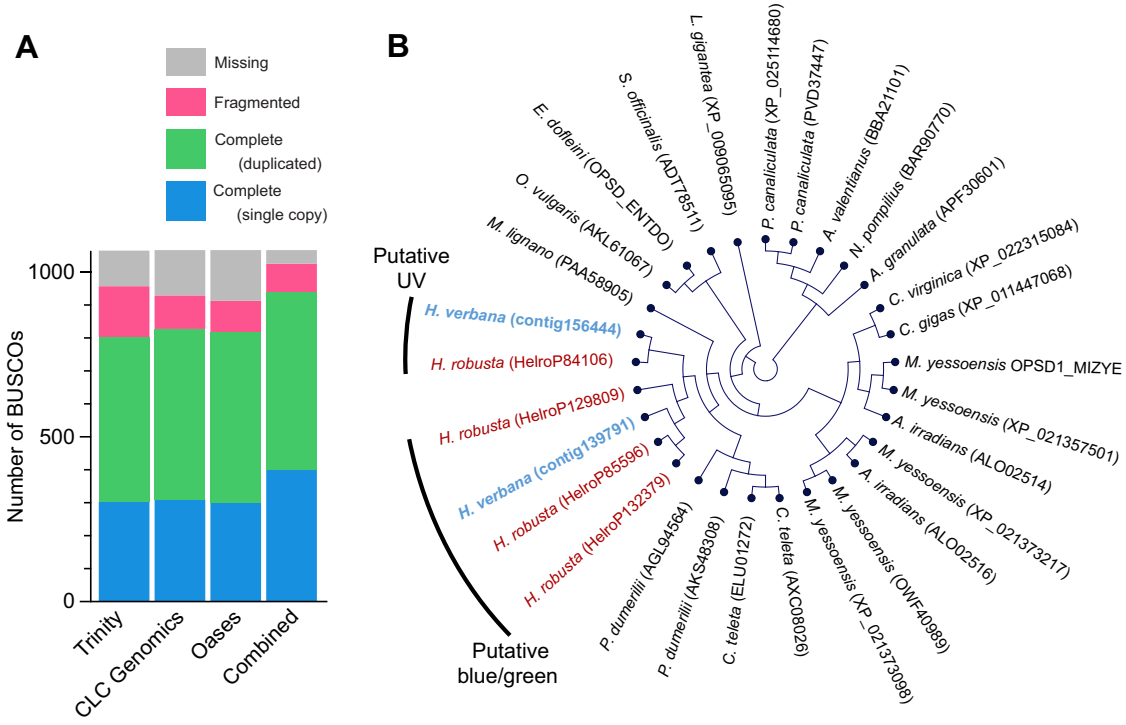

Fig. 5. Transcriptome analysis of putative opsins. (A) Benchmarking Universal Single-Copy Orthologs (BUSCO)-based quality assessment of contigs from *de novo* assembly. (B) Amino acid phylogeny based on alignment with CLUSTAL followed by sequence analysis and tree generation through the use of MEGA5 (Tamura et al., 2011). All nodes have at least 60% support. Red and blue indicate leech opsins.

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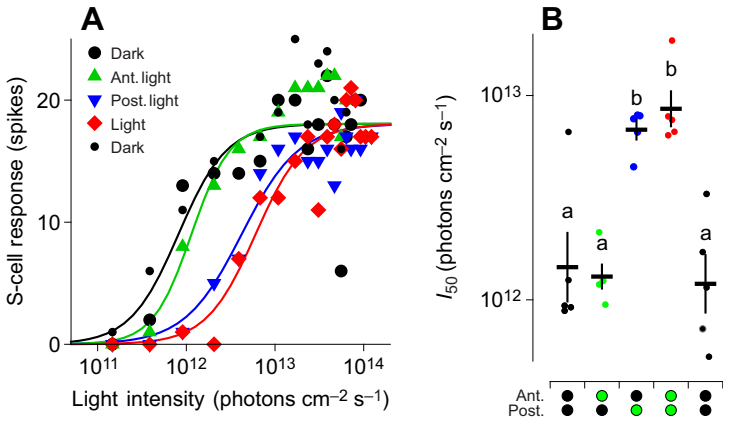

Fig. 6. Local and non-local adaptation in the S cell to green background light. (A) Responses to flashes of light presented to the posterior portion of the ventral body wall when the whole leech was dark adapted, when the anterior was light adapted, when the posterior was light adapted and when the whole leech was light adapted. A final repeat of the dark-adapted condition at the end of the experiment to confirm stability of responses is also shown. (B) Intensity of light required to obtain 50% of the maximum response to posterior stimulation (I_{50}), under the same series of conditions used in A. Symbols below the graph indicate light (green) and dark (black) adaptation for anterior (top symbol) and posterior (bottom symbol) body wall. Dots represent individual animals (n=5); black lines mark means and s.e.m.

To confirm that this integration occurs in the nervous system and that the responses are not merely due to stray light from the posterior illumination reaching sensilla in the anterior part of the animal, we performed control experiments in which we transected the nerve cord posterior to the recording site. Transecting the cord had no significant effect on responses to anterior stimulation, whereas posterior stimulation after transection was completely ineffective (except at extremely high light levels), confirming that the integration is indeed internal (Fig. 7A, open symbols).

To quantify these observations, we established response–log(intensity) curves as before. These curves indicated mainly a difference in the plateau (maximum) response (Fig. 7B): stimulating the whole leech versus only the anterior half resulted in a significantly stronger response (t_3=4.6, P<0.01, one-sided test, n=4). The light intensity needed to elicit 50% of the respective plateau responses was not significantly different (Fig. 7C; t_3=2.2, P=0.12, two-sided test, n=4). In one animal (data not shown) we additionally stimulated the posterior half by itself before transection, which elicited a strong response.

DISCUSSION

The leech *H. verbana* is an attractive system to investigate visual processing because of the animal's known behavioral responses to spatiotemporal visual stimuli such as water waves, despite the absence of image-forming eyes. However, even though several interneurons are known to respond to visual stimuli, their response properties are poorly understood. Among these, the S-cell system is especially interesting because of its putative involvement in multimodal sensory integration (Harley et al., 2011, 2013). To improve our understanding of the role of the S-cell system in visual processing, we here used a nearly intact leech preparation to quantify its spectral sensitivity under different background light conditions, to investigate spatial integration and to test whether light adaptation is local or global.

We began by quantifying the spectral response properties of the S-cell system, establishing for the first time absolute sensitivities for the leech visual system (Fig. 2). We confirmed earlier reports (Kretz et al., 1976) that the leech can adapt to a wide range of background light intensities. Under each of the tested background light intensities, the response range spanned approximately 2–3 log units of stimulus light intensities (Figs 2, 4, 6, 7), which is fairly typical for photoreceptors across the animal kingdom (Kawamura, 1993). When fully dark adapted, leeches responded to green flashes as dim as 10^8 photons cm^{-2} s^{-1} (Fig. 2), equivalent to the intensity of light on an overcast moonless night (Falchi et al., 2016).

Our physiological measurements support the existence of at least two distinct color channels (green and UV). Interestingly, the contribution of the two color channels to the response of the S-cell system is dependent on the background light level, which could explain the seemingly contradictory results of previously published studies. We found (Fig. 3) that only one color channel is active under dark background conditions and with green background illumination up to about 10^{13} photons cm^{-2} s^{-1}, or 22 lx (see Appendix). This is approximately equivalent to twilight conditions

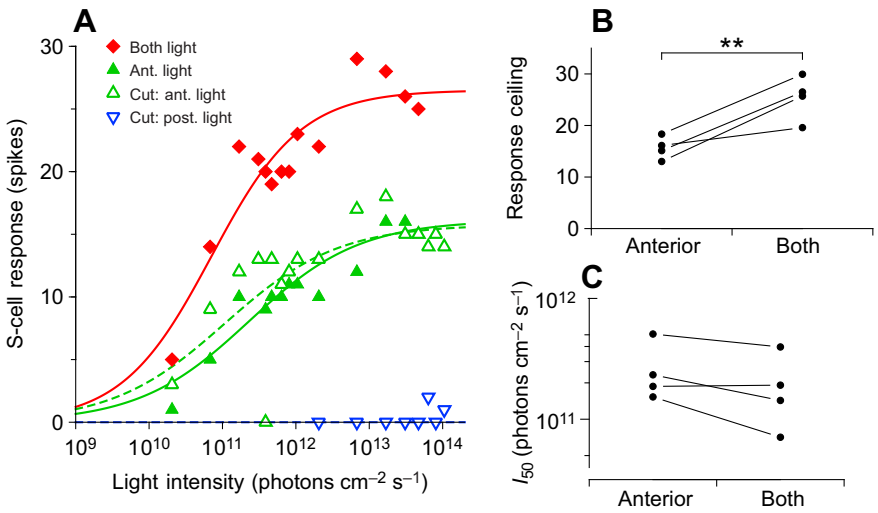

Fig. 7. Spatial integration in the S cell. (A) Response to light flashes presented to the anterior portion of the ventral body wall or to both anterior and posterior portions simultaneously. Open symbols indicate responses to stimulation of the posterior or anterior area after transecting the nerve cord immediately posterior to the recording site. Background light level was always 4.5×10^{11} photons cm^{-2} s^{-1}. (B) Maximum response (plateau of fitted curve) to light flashes presented to anterior and posterior portions simultaneously was higher than to flashes presented only to the anterior portion of the ventral body wall (t_3=4.6, **P<0.01, one-sided test; n=4). (C) Light intensity needed to elicit 50% of the respective maximum responses (I_{50}) for stimuli presented to the anterior portion of a leech or to both anterior and posterior portions simultaneously. The difference between these conditions was not significant (t_3=2.2, P=0.12, two-sided test; n=4).

(https://en.wikipedia.org/wiki/Lux, retrieved 3 January 2019). Under brighter background conditions, our results indicated that a second channel became active as well (Figs 3 and 4). This channel was predominantly UV sensitive. Both channels remained active even at the brightest green background illumination that we tested, 10^{16} photons cm^{-2} s^{-1} (equivalent to full daylight). However, under this background illumination – bright green background with no UV component – the sensitivity to UV was stronger than to green light (Fig. 4). We thus both confirmed the observations of Jellies (2014a, b) and explained the apparent conflict with the earlier results of Kretz et al. (1976).

The existence of two distinct color channels was further supported by our transcriptomic data, which indicated the expression of at least two distinct opsins within the body wall of the leech (Fig. 5). Similar opsins have previously been identified in another leech, *H. robusta*, and comparison with opsins from other invertebrate species is compatible with these opsins being responsible for the distinct green and UV sensitivity that underlie our physiological results. Future studies will be necessary to determine which specific cells express these opsins, confirm their specific sensitivity and determine the neuronal pathways that connect them to the S cell. Although the large differences in response delays (Fig. 3) suggest that the color channels comprise distinct neuronal pathways, it could be that the delays are explained by intrinsic differences of the opsins themselves, in which case it is even possible that the opsins are co-expressed in the same photoreceptor cell.

That said, many animals employ multiple photoreceptor classes that become active at different light levels; for instance, in humans, rods contribute to vision most strongly at low light levels, whereas cones only become active at higher light levels (Fain and Dowling, 1973; Ingram et al., 2016). Our results indicate that a similar differentiation between photoreceptors may exist in the leech.

As the S-cell system spans the entire length of the leech's body, it appears well positioned to integrate stimuli from different locations. We therefore investigated spatial aspects of the S cell's responses to light. In the first series of experiments (Fig. 6), we determined that adaptation to background illumination is local, suggesting that adaptation occurs in the sensory periphery or perhaps in the early stages of sensory processing. In the second series of experiments (Fig. 7), we determined that the S-cell system integrates stimuli from across the entire ventral body wall. We found that the maximum response of the S-cell increases with the size of the illuminated area, but there was no significant change in the light intensity required to elicit half of that maximum response. This suggests that the S-cell system pools (i.e. sums) responses. The existence of summation mechanisms is consistent with the organization of the S-cell system, as the individual S cells are strongly coupled to each other by electrical synapses across the entire length of the body of the leech (Frank et al., 1975), so that the whole S-cell system can be considered as a single syncytium that acts as a fast-conducting pathway connecting the segmental ganglia (Peterson, 1984). The combination of local adaptation with global integration means that the S-cell system can respond to small changes in illumination anywhere on the body, irrespective of whether that part of the body is exposed to bright background light or shade.

It has been suggested that the S-cell system plays a key role in synchronizing general arousal throughout the nervous system of the leech (see Sahley et al., 1994). Related functions could potentially include an involvement in the modification or activation of motor output, and facilitating or enhancing other effects of changes in sensory input.

Taken together, our results show that the response of the S-cell system to visual stimuli involves the integration of spatial and color information from visual stimuli, making the leech an ideal target for further investigations into the mechanisms and function of such integration.

Appendix

Converting units of light intensity

By definition, 1 W is 589 lm at 530 nm, which is the approximate wavelength of the green light used here. From basic physics, 1 photon has an energy of $E=hc/\lambda=3.74\times10^{-19}$ J. Thus, a photon flux of 10^8 photons cm^{-2} s^{-1} corresponds to an energy flux of 3.74×10^{-11} J cm^{-2} s^{-1}=3.74×10^{-11} W cm^{-2}=3.74×10^{-7} W m^{-2}. Given that 1 W equates to 589 lm, this is equivalent to $(3.74\times10^{-7})\times589$ lm m^{-2}= 2.2×10^{-4} lm m^{-2}=2.2×10^{-4} lx.

Acknowledgements

We wish to thank Elke Buschbeck and John Layne for the use of equipment and lab space.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A. Stowasser, D.A.W.; Methodology: A. Stowasser, J.B.B., D.A.W.; Software: D.A.W.; Formal analysis: A. Stowasser, A. Stahl, J.B.B., D.A.W.; Investigation: A. Stowasser, A. Stahl, J.B.B.; Resources: J.B.B.; Data curation: J.B.B., D.A.W.; Writing - original draft: A. Stowasser, J.B.B., D.A.W.; Writing - review & editing: A. Stowasser, J.B.B., D.A.W.; Visualization: A. Stowasser, J.B.B., D.A.W.; Supervision: D.A.W.; Project administration: D.A.W.; Funding acquisition: D.A.W.

Funding

Funding was provided by the National Institute of Neurological Disorders and Stroke (NS094403, to D.A.W.) and by the Burroughs Wellcome Fund in the form of a Career Award at the Scientific Interface (to D.A.W.). Deposited in PMC for release after 12 months.

Data availability

Illumina sequencing files have been deposited in the NCBI SRA: Bioproject PRJNA504032.

Supplementary information

Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.201764.supplemental

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