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RESEARCH ARTICLE

Light avoidance by a non-ocular photosensing system in the terrestrial slug *Limax valentianus*

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ABSTRACT

Although the eye is the best-studied photoreceptive organ in animals, the presence of non-ocular photosensing systems has been reported in numerous animal species. However, most of the roles that non-ocular photosensory systems play remain elusive. We found that the terrestrial slug *Limax valentianus* avoids light and escapes into dark areas even if it is blinded by the removal of the bilateral superior tentacle. The escape behaviour was more evident for short-wavelength light. Illumination to the head with blue but not red light elicited avoidance behaviour in the blinded slugs. Illumination to the tail was ineffective. The light-avoidance behaviour of the blinded slugs was not affected by the removal of the penis, which lies on the brain in the head, suggesting that the penis is dispensable for sensing light in the blinded slug. mRNA of Opn5A, xenopsin, retinochrome and, to a lesser extent, rhodopsin was expressed in the brain according to RT-PCR. Light-evoked neural responses were recorded from the left cerebro-pleural connective of the isolated suboesophageal ganglia of the brain, revealing that the brain is sensitive to short wavelengths of light (400–480 nm). This result is largely consistent with the wavelength dependency of the light-avoidance behaviour of the blinded slugs that we observed in the present study. Our results strongly support that the terrestrial slug *L. valentianus* detects and avoids light by using its brain as a light-sensing organ in the absence of eyes.

KEY WORDS: Extraocular photoreception, Opsin, Pulmonate, Spectral sensitivity, *Lehmannia valentiana*

INTRODUCTION

Most animals possess eyes as specialized sensory organs for photoreception. However, the presence of non-ocular photosensory systems, such as photosensitive brain neurons and dermal photosensing systems, has been reported in several invertebrate species (reviewed in Yoshida, 1979; Kartelija et al., 2003; Gotow and Nishi, 2009; Ramirez et al., 2011; García-Fernández et al., 2015; Kelley and Davies, 2016). Of these, photosensing by the brain is of special interest because of its possible direct involvement in light-evoked behavioural changes.

In gastropod molluscs, the existence of photoresponsive neurons in the brain has previously been reported (Hisano et al., 1972; Brown and Brown, 1973; Gotow, 1975; Pašić et al., 1977). Although the photosensitive brain neurons do not exhibit any specialized morphology, they exhibit excitatory or inhibitory responses upon light illumination. However, only a limited number of studies have investigated the relevance of such photosensitive neurons to any behavioural aspects of the animal. For example, photosensory neurons in the abdominal ganglion are thought to be responsible for the light entrainment of the circadian rhythm in *Aplysia* (Block and Lickey, 1973; Lickey and Zack, 1973), and for the pneumostome (PS) opening behaviour that is dependent on the tidal level in *Onchidium* (Shimotsu et al., 2010). However, these light-induced behavioural changes occur slowly over relatively long time scales.

The terrestrial gastropod slug *Limax valentianus* is nocturnal, and avoids light places in order not to lose water from its body. To do this, the slugs have a well-developed lens eye on the tip of their superior tentacles (STs), which can detect light with high sensitivity (Kataoka, 1975; Suzuki et al., 1979; Zieger et al., 2009; Land and Nilsson, 2012; Matsuo et al., 2017). Therefore, the slugs exhibit negative phototaxis behaviour, which is driven by comparing the intensities of incident light coming into the bilateral eyes (Crozier and Cole, 1929; Matsuo et al., 2014). However, we previously observed light-induced neuronal responses in extracellular recordings from the cut end of the cerebral commissure, and found that the responses still remained even if the eye was removed from the preparation (Matsuo et al., 2014). This suggests the presence of light-responsive neurons in the brain of the slug.

In the present study, we found that the slugs exhibited a light-avoidance behaviour even if their eyes had been removed bilaterally. This unexpected finding prompted us to examine whether the brain is the photosensor responsible for such eye-independent negative phototaxis behaviour and to analyse the spectral tuning properties of this behaviour. We also examined the expression of the opsin species in the brain that may be involved in the avoidance behaviour in the absence eyes. We discuss the roles of these opsins in the context of the expression and spectral tuning properties of the electrophysiological photoresponse exhibited by the isolated brain.

MATERIALS AND METHODS

Animals

The terrestrial slugs *Limax valentianus* Férussac 1822 were maintained in our laboratory for at least 25 generations as a closed colony. The slugs were fed a humidified powder mixture consisting of potato starch, rat chow and vitamins (for composition, see Fukunaga et al., 2006), and kept at 19°C in an incubator. The slugs were used 3–4 months after hatching. The behavioural experiments were performed at 19–24°C.

Surgery

To prepare blinded slugs for light–dark choice tests, the bilateral STs were amputated 24 h before the behavioural experiment (for details of the procedure, see Yamagishi et al., 2008). In the experiment where both the ST and the penis were removed, the penis was amputated from where it stuck out from the body when anaesthesia was injected into the body cavity. The surgery of the slugs used for the spot illumination test was performed 2 days before the experiment. In this

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case, the tip of the mantle (one-third or less) was also removed to enhance the penetration of light into the inside of the head. In all cases, approximately 500 µl of physiological saline (70.0 mmol l^{-1} NaCl, 2.0 mmol l^{-1} KCl, 4.7 mmol l^{-1} $MgCl_2$, 4.9 mmol l^{-1} $CaCl_2$, 5.0 mmol l^{-1} glucose and 5.0 mmol l^{-1} Hepes, pH 7.0) was injected into the body cavity after the surgery to facilitate recovery from the anaesthesia, and the slugs were kept in a plastic container lined on the base with moistened filter paper.

Light–dark choice test: group test

The experimental chamber was a transparent plastic container (200×130×30 mm), half of which was covered with black paper to screen light. White light was delivered from a desk lamp (ODS-27N, Panasonic, Osaka, Japan) from 300 mm above (see Fig. 1A). The integrated irradiance at the bottom of the chamber (measured using a photopower meter; Light Spex, Gretag Macbeth, Regensdorf, Switzerland) was 503.1±2.7 µW cm^{-2} with peak irradiance at 545 nm. Ten slugs were kept in the dark for approximately 15 min before the behavioural experiment. They were then placed on the bottom along the midline of the container (on the border between the light and dark areas) such that the head of the slugs was oriented in parallel with the midline. The orientation of the head was randomized. The number of the slugs in the light and dark compartments was counted after 1 and 2 h. The experiment was performed using 5–7 groups, each of which consisted of 10 slugs.

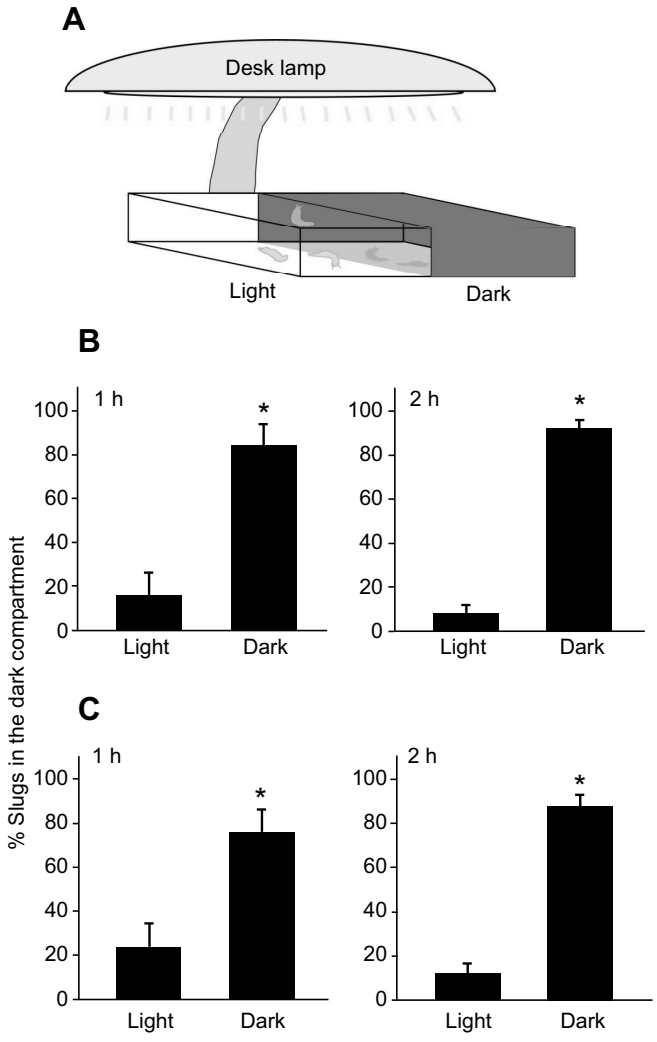

Fig. 1. Slugs avoid light in the light–dark choice group tests even if their eyes have been removed. (A) Experimental setup. (B) Percentage of the intact slugs that were in the dark compartment after 1 h (left) and 2 h (right). (C) Percentage of the blinded slugs that were in the dark compartment after 1 h (left) and 2 h (right). Means±s.e.m. (n=5 independent experiments). *P<0.01, Student's t-test.

Light–dark choice test: individual test

The experimental chamber was a small transparent plastic container (65×44×16 mm), half of which was covered with black paper to screen light. Monochromatic light was delivered by a xenon arc light system (500 W, model XW-500Q, Sanso, Tokyo, Japan), which consisted of a xenon arc lamp, neutral density filters and band-pass interference filters. The experimental chamber was illuminated from the lateral side. The intensity of monochromatic light was measured inside the plastic container using a photopower meter (model TQ8210, Advantest, Tokyo, Japan). The slug was individually placed on the bottom along the midline of the container, and its head was randomly oriented towards the light source or in the reverse direction. The position of the slug was recorded after 10 min. Each slug underwent tests with four different wavelengths of light (either 400, 480, 560 and 640 nm or 440, 520, 600 and 680 nm), with 50 min inter-test intervals. In the case of intact control slugs, 18 slugs were tested in each wavelength set, i.e. 36 slugs were used in total. For the blinded slug group, 42 (400, 480, 560 and 640 nm) and 41 (440, 520, 600 and 680 nm) slugs were used, i.e. 83 blinded slugs in total. The procedure for the behavioural experiment is provided in Table S1. The intensity of the light was 10 µW cm^{-2} (intact slugs) or 100 µW cm^{-2} (blinded slugs).

Spot illumination test

On the day of the behavioural experiment (2 days after surgery), the slugs were gently transferred to a glass Petri dish (100 mm diameter) with forceps to acclimate to the texture of glass, and were kept in the dark for 1 h. The behaviour of the slug was recorded using the nightshot device of a handycam HDR-PJ790V digital video camera recorder (Sony, Tokyo, Japan) from which near-infrared light was irradiated. Although Kataoka (1975) referred to the possible capacity of the slug's eye to sense infrared light, we did not find any sign of such an ability in our previous behavioural experiments (Matsuo et al., 2014) or in the electroretinogram of the eye (Matsuo et al., 2017). Monochromatic (440 or 700 nm) spot light was delivered by a xenon arc light system for 1 min while the slugs were crawling slowly. The intensity of the monochromatic light was adjusted beforehand with a photopower meter (model TQ8210). The time (s) during which the head (or tail) was within a lighted spot (approximately 30 mm diameter) was measured using Keynote software (Apple Inc., Cupertino, CA, USA). The photon flux density was 7.0×10^{14} photons cm^{-2} s^{-1} for both wavelengths of light.

RT-PCR

The slugs were deeply anaesthetized by injection of ice-cold Mg^{2+} buffer (57.6 mmol l^{-1} $MgCl_2$, 5.0 mmol l^{-1} glucose and 5.0 mmol l^{-1} Hepes, pH 7.0) into the body cavity, and the pieces of the body wall around the PS and at the dorsal side of the posterior part of the body (i.e. the body wall) were dissected out. Then, the tip of the ST, the brain (without the buccal ganglia) and the penis were isolated in a dish filled with cold physiological saline containing a 6-fold concentration of $MgCl_2$ (35.0 mmol l^{-1} NaCl, 2.0 mmol l^{-1} KCl, 28.0 mmol l^{-1} $MgCl_2$, 4.9 mmol l^{-1} $CaCl_2$, 5.0 mmol l^{-1} glucose and 5.0 mmol l^{-1} Hepes, pH 7.0). Total RNA was extracted from the isolated tissues by an acid guanidinium thiocyanate–

phenol–chloroform method (Chomczynski and Sacchi, 1987), and treated with DNase I as described previously (Matsuo et al., 2000). cDNA was obtained by reverse transcription (RT) using oligo-dT as a primer. The nucleotide sequences of the PCR primers were 5′-GGATGTCGGCTGCGCAATCAACG-3′ and 5′-GGCACATAGGAGAAACAAATGCTGAG-3′ for Opn5A (DDBJ/EMBL/GenBank accession no. LC440462), 5′-CGCCCAAACGACAGACCAGCAC-3′ and 5′-GCGTACAATTCCGCCAGGGACAAG-3′ for retinochrome (LC440464), 5′-CTGAGGACGTCGGTCTTGATAGTC-3′ and 5′-CACTGCGCACGGCAATGCTGAAAG-3′ for xenopsin (LC440461), 5′-CTGTGGGATGACAGCCAAGGAG-3′ and 5′-CGAAGTAGCGGACTGCGTGGAG-3′ for rhodopsin (also called G_q-coupled rhodopsin, LC223120), 5′-CTGGGTCTATGGAGATATCGGC-3′ and 5′-GATCTCGCGGTTACAAAAGTCTGG-3′ for Opn5B (LC440463), and 5′-GCTTACCAAGCTCCGACCCTCGTGG-3′ and 5′-CGTCACTACCTCCCCGTGCCGGGG-3′ for 18S rRNA (AB698077). The number of amplification cycles was 27, 28, 30, 25, 30 and 11 for Opn5A, retinochrome, xenopsin, rhodopsin, Opn5B and 18S rRNA, respectively. The PCR products were electrophoresed on 1% agarose gel and visualized with ethidium bromide under a UV illuminator.

Quantitative real-time PCR (qPCR)

cDNA was prepared as above from five sets of brain (without the buccal ganglia), STs, the body wall around the PS, and the body wall of the lower part of the body. PCR amplification and real-time monitoring were performed using a LightCycler 480 system (Roche Diagnostics, Rotkreuz, Switzerland). The nucleotide sequences of the PCR primers were 5′-GTGGCTCTTGTCGGCGTTG-3′ and 5′-TTCCAAATGGCTCAGGTGCG-3′ for Opn5A, 5′-TGCAAACTAATGGTGTCCATG-3′ and 5′-AGTCTAGTCCTGCTTCTTGTC-3′ for retinochrome, 5′-AGTGCCAAGGTCTCATGGAC-3′ and 5′-GAGGACCCTACAAGGCAAC-3′ for xenopsin, 5′-GCTCTGCGATTATCAAGGAC-3′ and 5′-TCACTTGAGGTGGGATGTAG-3′ for rhodopsin, 5′-CCTGGGTCTATGGAGATATC-3′ and 5′-CCAGGGTTATCATGCTGTTC-3′ for Opn5B, and 5′-CTAAAGCAATCGCCTCCTTG-3′ and 5′-ATAGACGAGGACTTGACG-TG-3′ for 18S rRNA. To delineate standard curves for absolute quantification, plasmid DNAs with inserts encompassing the PCR amplicons were prepared as templates. These inserts corresponded to bases 616–1131 of Opn5A, 780–1307 of retinochrome, 971–1568 of xenopsin, 1219–1670 of rhodopsin, 892–1194 of Opn5B and 1–1621 of 18S rRNA. The difference in the amount of cDNA between the tissue samples was normalized based on the copy number of 18S rRNA.

Electrophysiology

Photoresponses of the brain were recorded from the suboesophageal ganglia (SEG) at the cut end of the left cerebro-pleural connective. The brain was isolated from an anaesthetized slug as described above, and transferred to a dish filled with physiological saline. All neural connectives between the cerebral ganglia and the SEG were cut, and the cut end of the left cerebro-pleural connective of the SEG was suctioned by a recording electrode (approximately 100 µm i.d.). The tip of the reference electrode was placed in the physiological saline of the same dish. The preparation was kept in the dark for 1 h before starting the recording. The signal was amplified by a differential amplifier (model 3000, A-M Systems, Sequim, WA, USA) and recorded on a computer via an A/D converter (PowerLab2/26, ADInstruments, Dunedin, New Zealand). The signal was band-pass filtered between 5 and 50 Hz. Baseline spike activity in the dark was recorded for 5 min, and monochromatic light was subsequently delivered for 1 min. The intensity of the monochromatic light was adjusted beforehand using a photopower meter (model TQ8210). To delineate a response curve under illumination of equal irradiance (20 nW cm^{-2}), monochromatic light was delivered in a series either from short to long wavelengths or from long to short wavelengths in a counterbalanced manner among the brain samples (n=5+5). In the experiments measuring spectral sensitivity, each brain underwent a single series of monochromatic light illumination, and the light was delivered in the order from weak to strong irradiance. The inter-illumination interval (up to 60 min) ensured that the spike frequency returned to the baseline level. The number of spikes during the 1 min illumination was counted after the recording. Linear regression lines (log scale of wavelengths versus linear scale of spike frequencies) were drawn by the least squares method using the Excel 2016 software (Microsoft, Redmond, WA, USA). The recordings were performed at 19–22°C.

Statistical analysis

The data were statistically analysed using the two-tailed Student's t-test or χ^2 test. The data are expressed as means±s.e.m.

RESULTS

Light avoidance in the absence of eyes

We first confirmed the negative phototactic nature of the slugs (Crozier and Cole, 1929; Zieger et al., 2009; Matsuo et al., 2014; Fujisaki and Matsuo, 2017) in the light–dark choice group test (Fig. 1A). The intact control slugs successfully entered into the dark compartment after 1 and 2 h (P=1.3×10^{-5} and 2.5×10^{-7}; Fig. 1B). However, the slugs whose bilateral eyes had been removed by ST amputation 24 h beforehand similarly entered the dark compartment after 1 and 2 h (P=7.3×10^{-3} and 4.2×10^{-6}; Fig. 1C). The blinded slugs entered the dark compartment in a different manner from the control slugs: the former wandered about the experimental chamber until their successful escape, whereas the latter moved into the dark compartment more directionally and efficiently (Movie 1).

Wavelength dependency of avoidance behaviour

We next evaluated the wavelength dependency of the avoidance behaviour individually using a small experimental chamber in the light–dark choice individual test. Of the control slugs, most escaped into the dark compartment irrespective of the wavelength of the delivered light if the irradiance was high (100 µW cm^{-2}; Fig. 2A), but they escaped only from short wavelength light if the irradiance was low (10 µW cm^{-2}; Fig. 2B), consistent with the higher sensitivity of the slugs' eyes to short-wavelength light (Suzuki et al., 1979; Matsuo et al., 2017, 2019). We judged the avoidance behaviour based on whether more than half of the slug's body was within the dark compartment (criterion number 1). For the control slugs, the whole body was in the dark compartment in all the cases where slugs avoided light.

In contrast, the blinded slugs did not exhibit clear avoidance of 100 µW cm^{-2} monochromatic light when the same criterion was adopted to judge avoidance (Fig. 2C). However, the avoidance rates were higher in the short wavelength ranges if the avoidance was judged based on whether the head of the slug was within the dark compartment (criterion number 2; Fig. 2D).

Illumination of the head elicits avoidance behaviour

As the avoidance of light was more evident when it was judged based on the head position for the blinded slugs (Fig. 2C,D), we investigated whether illumination of the head was sufficient to elicit

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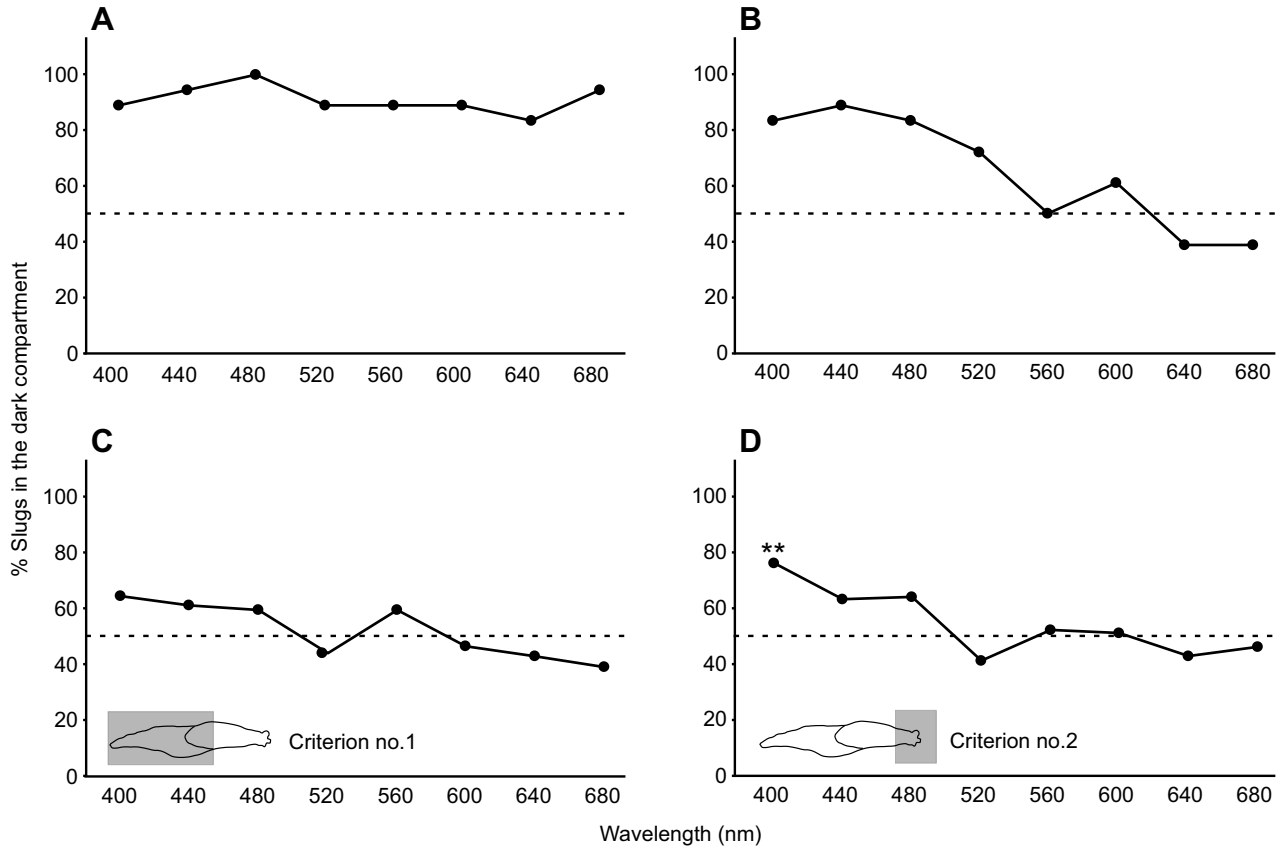

Fig. 2. Wavelength dependency of light-avoidance behaviour. (A,B) The avoidance rate of intact slugs to monochromatic light of equivalent irradiance: (A) 100 μW cm^{-2} (*n*=18), (B) 10 μW cm^{-2} (*n*=18). (C) The avoidance rate of the blinded slugs to monochromatic light of equivalent irradiance: 100 μW cm^{-2} (*n*=41 or 42). Avoidance in A–C was judged based on whether more than half of the body was within the dark compartment (criterion number 1). There was no significant difference among wavelengths (χ^2=11.54, d.f.=7). (D) Data from the same experiment as in C, but avoidance was judged based on whether the head of the slug was within the dark compartment (criterion number 2). ***P*<0.01, *post hoc* residual test after χ^2 test (χ^2=17.37, d.f.=7, *P*<0.05). Dashed lines indicate the 50% avoidance rate corresponding to the chance level.

avoidance behaviour. To enhance the penetration of light into the body, the tip of the mantle was removed during surgical removal of the ST, and a 2 day recovery period was given to ensure sufficient recovery from the surgical damage. The time during which the head of the slugs stayed within the light spot was significantly shorter when 440 nm monochromatic light (50 μW cm^{-2}) was delivered than when 700 nm monochromatic light with an equivalent photon flux density (31.4 μW cm^{-2}, i.e. 7.0×10^{14} photons cm^{-2} s^{-1}) was delivered (17.2±3.6 versus 32.1±3.6 s, *P*=0.0057; Fig. 3A). This difference in time reflects the aversion behaviour that was often observed during illumination with 440 nm monochromatic light (Movie 2). There was no such difference in the time during which the tail end of the slug stayed within the light spot if the light was delivered to the tail of the slug (48.6±4.2 versus 46.9±4.6 s, *P*=0.790; Fig. 3B).

Expression of opsin mRNA

We next analysed the expression of opsins, the well-studied photopigment genes in the animal kingdom, in different parts of the body by RT-PCR. As we recently found that five mRNA species belonging to the opsin family genes exist in *L. valentianus* (Matsuo et al., 2019), the expression of these mRNAs in the brain, the ST (including the eyes), the PS and the dorsal surface of the body (body wall, BW) was examined. We chose the PS because its light detection capability has been suggested in the pond snail *Lymnaea stagnalis* (Sunada et al., 2010). We detected all five mRNA species in the ST and three mRNA species in the brain (Opn5A, retinochrome and xenopsin; Fig. 4A), replicating previous findings (Matsuo et al., 2019). The expression of xenopsin mRNA was also detected in the BW and the PS, whereas retinochrome was barely detected in the BW and was not found in the PS (Fig. 4A). qPCR analysis further revealed that Opn5A mRNA was expressed in both the brain and the ST, and xenopsin mRNA was expressed at comparable levels among the four tissues examined (Fig. 4B). In the PS and BW, Opn5A, rhodopsin and Opn5B mRNA were all expressed at a low level (Fig. 4B), which is consistent with the agarose gel image from the RT-PCR (Fig. 4A).

Its location in the head and expression of as many as three to four opsin family genes may qualify the brain as a light sensor in the absence of the eye. However, the possibility that the penis, an organ overlying the brain in the head of the slug, functions as a non-ocular light detector could not be excluded. Indeed, we found that xenopsin mRNA was expressed in the penis (Fig. 5A). We therefore removed the penis in addition to the bilateral eyes from the slugs, and analysed their light avoidance behaviour in the light–dark choice test. The slugs also successfully avoided the light in this case after 1 h (*P*=0.0024) and 2 h (*P*=0.0083) (Fig. 5B). Therefore, the penis was dispensable for the non-ocular light-avoidance behaviour.

Spectral sensitivity of the photoresponse by the brain

Lastly, we examined whether the brain exhibits photoresponses *in vitro*. Indeed, light-evoked spike responses were recorded

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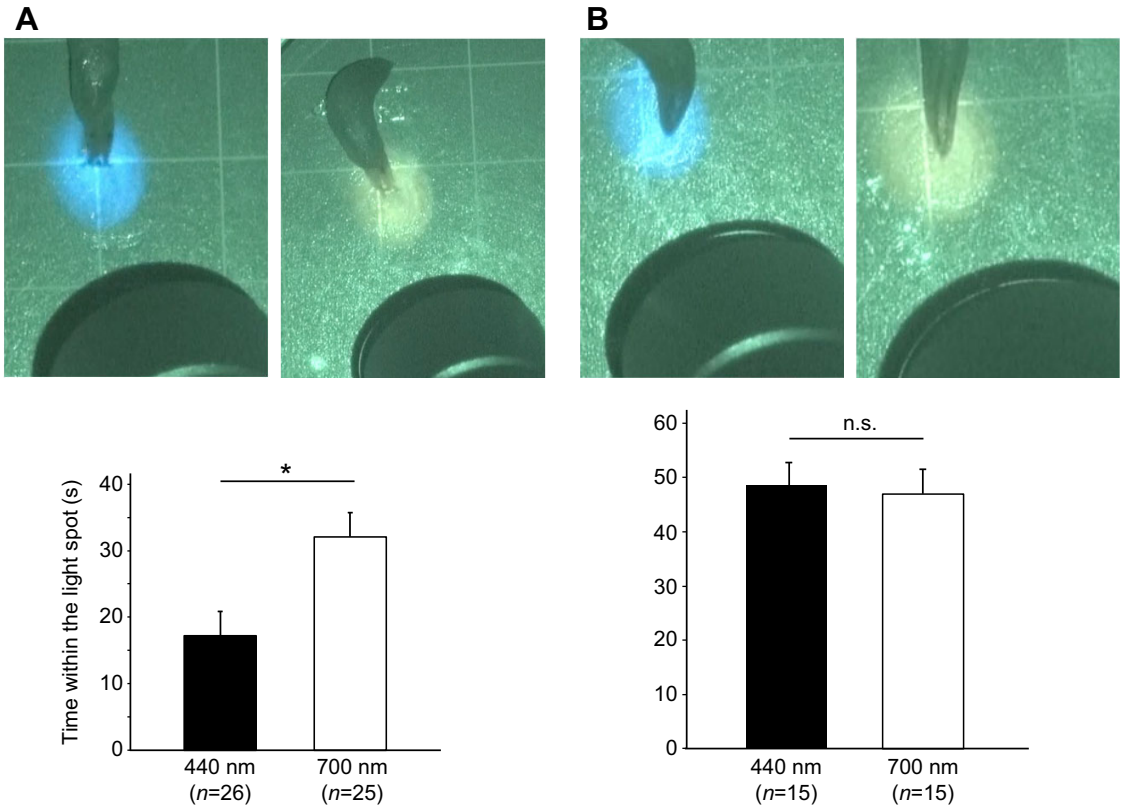

Fig. 3. Illumination with blue light to the head elicits avoidance behaviour in blinded slugs. (A) The duration that the head of the blinded slugs stayed within the light spot was shorter when the head was illuminated with blue rather than red light of an equivalent photon flux density. (B) There was no such difference when the light was directed to the tail of the slug. *P<0.01, Student's t-test. n.s., no significant difference.

extracellularly at the cut end of the cerebro-pleural connective of the left pleural ganglion (Fig. 6A). We then analysed the spectral properties of the photoresponse. Monochromatic light delivery of equivalent irradiance (20 nW cm^{-2}) gave rise to spike activity with a frequency that varied depending on the wavelength (Fig. 6B). The action spectrum was delineated based on the group data (n=10; Fig. 6C). The response peak was at 440 nm, and there was a small bump at 520 nm in this curve (red arrow in Fig. 6C), suggesting a response with a disproportionately large number of spikes to this wavelength of light. We further examined the response properties by changing the irradiance at six different wavelengths (400, 440, 480, 520, 560 and 600 nm; Fig. 6D; Table S2). Responses expressed as a function of photon flux density are shown in Fig. 6E. Of note, there was a small bump again when the spectral distribution of the expected spike frequencies was calculated as the intersection with 50 nW cm^{-2} (red arrow in Fig. 6F). A similar bump also appeared when the spectral distribution of the inverse of the expected photon flux density, which can be considered the spectral sensitivity, was calculated as the intersection point with 10 spikes min^{-1} (a red arrow in Fig. 6G).

DISCUSSION

We recently found that several opsin species are expressed not only in the photoreceptors of the eye but also in the brain of *Limax* (Matsuo et al., 2019; see Fig. 4). The presence of photopigment molecules implies the involvement of the brain in some behavioural aspects of *Limax*, and our present results support the idea that the brain serves as a photosensor in the light-avoidance behaviour of the blinded slugs. The present study is the first report on the role of photosensitivity of the brain in connection with negative phototaxis of molluscs. Of course, our present results do not exclude the possibility that the photosensing by the brain is also involved in the regulation of a photoperiodic system, as in some lower vertebrates (Menaker et al., 1970; Okano and Fukada, 2001; Wyse and Hazlerigg, 2009).

A possible involvement of dermal photosensing in the light-avoidance behaviour of blinded slugs should not be totally ignored, especially taking into account the reported ability of the dermal tissues such as the BW and the PS to convey light information to the brain in the pond snail *Lymnaea* (Stoll, 1972; Chono et al., 2002; Sunada et al., 2010). However, the PS and BW expressed only low levels of xenopsin, and our preliminary observations (H.N. and R.M., unpublished observations) and the results of Figs 4 and 5A in the present study indicate that xenopsin is expressed ubiquitously in the organs of the body that we investigated. In contrast, the brain expresses a higher level of several kinds of opsin genes (Fig. 4B). Although less likely, the involvement of non-opsin photoresponsive molecules cannot be completely ruled out because a certain type of transient receptor potential (TRP) channel functions as a photoresponsive molecule in the body wall of *Drosophila* larva (Xiang et al., 2010). However, an extraordinarily strong intensity of light is necessary to activate the photoresponsive neurons expressing this channel. Collectively, these findings make it more likely that the brain functions as a light sensor committed to the negative phototaxis behaviour of the blinded slugs.

The brain/CNS-dependent phototaxis/photomotor responses may be an evolutionarily conserved ability that is important when the eye is unavailable. Considering a wider range of animal taxa, there are reports of the brain's involvement in non-ocular photoreception in vertebrates such as zebrafish (Fernandes et al., 2012; Kokel et al.,

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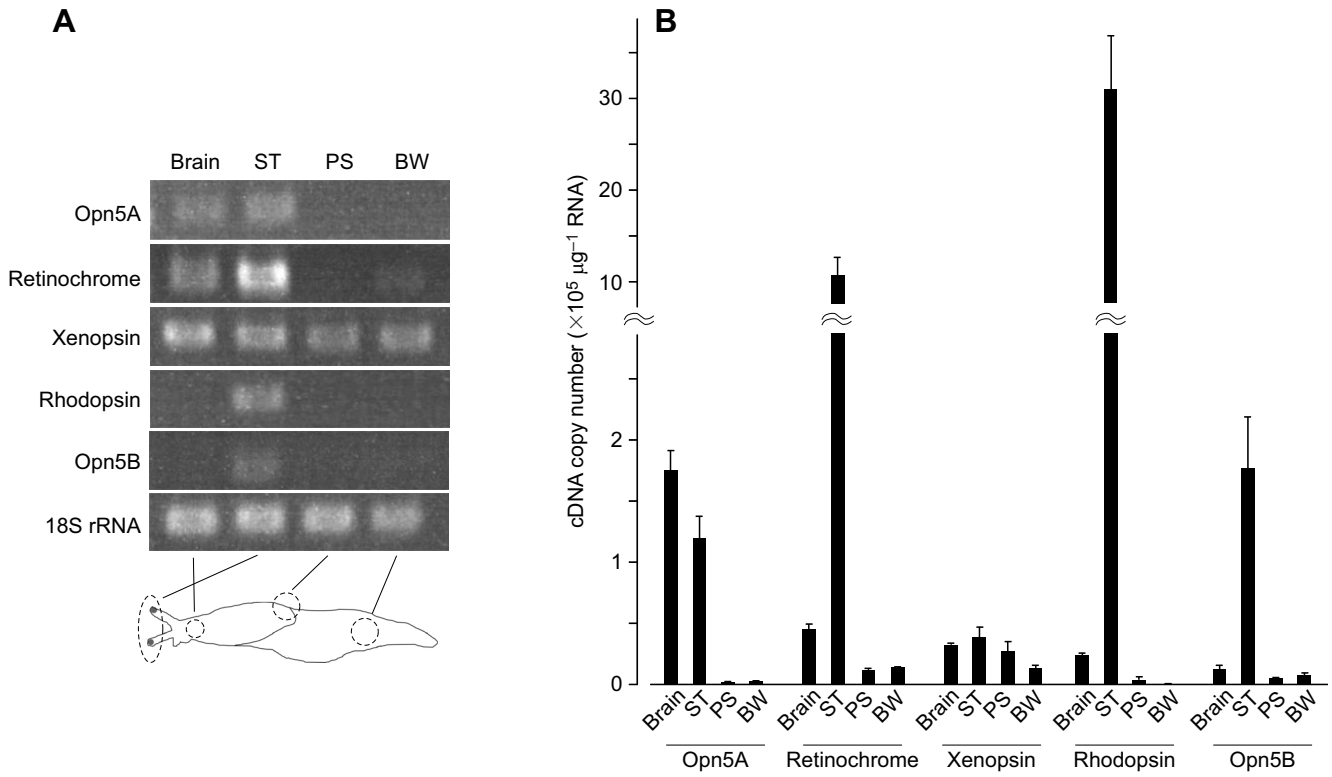

Fig. 4. Expression of five opsin genes in the tissues of the slug. (A) RT-PCR of opsin genes in the brain, the superior tentacle (ST), the pneumostome (PS) and the body wall (BW). 18S rRNA served as an internal control to demonstrate the equivalence of the amount of cDNA template. (B) qPCR analysis of opsin gene expression. Means±s.e. (*n*=5 biological replicates).

2013) and *Xenopus* tadpoles (Currie et al., 2016), and invertebrates such as insects (Hariyama, 2000), and crustaceans decapods (Simon and Edwards, 1990; Bobkova et al., 2003; Kingston and Cronin, 2015) and the amphipod *Talitrus* (Frelon-Raimond et al., 2005).

The present study also demonstrated that short-wavelength light is preferentially used by the non-ocular photosensory system (Figs 2, 3 and 6). We delivered light of equivalent irradiance in the behavioural experiment in Fig. 2. However, a larger percentage of animals was expected to avoid short-wavelength light if monochromatic light with an equivalent photon flux density was used instead. We and other groups also demonstrated that the eye of *Limax* is more responsive to short-wavelength blue light, especially when the eye is light adapted (Suzuki et al., 1979; Matsuo et al., 2017, 2019). The preferential use of short-wavelength light is thought to be a widely conserved phenomenon in the animal kingdom (Gehring and Rosbash, 2003; Erren et al., 2008), and the slugs also appear to have inherited this trait.

Previously, we demonstrated that negative phototaxis is induced by comparing the light intensity between the bilateral eyes (i.e. photo-tropotaxis; Matsuo et al., 2014). An observation supporting the notion that pulmonates can detect the edge between the light and dark area in their environment projected on the retina was also reported (Andrew and Savage, 2000; Fujisaki and Matsuo, 2017). However, spatial comparison of light intensity is expected to be difficult in the case of brain photoreception because of its small size and the blurring of images during light penetration through the body wall. Indeed, Zieger et al. (2009) failed to detect negative phototaxis behaviour when blinded slugs (*Arion rufus* or *Deroceras agreste*) were made to choose the direction of movement to either side wall (covered with black or white paper) in the arena. This result supports the idea that the brain is not optimized for spatial analysis of the light intensity in the environment.

Therefore, the temporal component seems to be more important for blinded slugs than the spatial component. Indeed, the blinded slugs wandered about until they successfully entered the dark

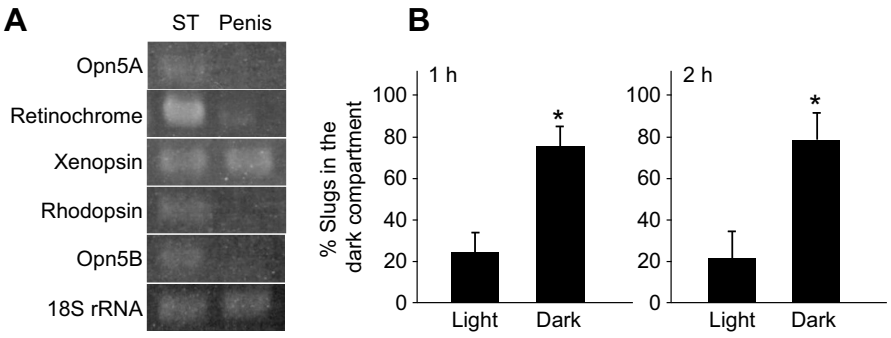

Fig. 5. The penis is dispensable for non-ocular light-avoidance behaviour. (A) RT-PCR of opsin genes in the cDNA derived from the ST and penis. 18S rRNA served as an internal control to demonstrate the equivalence of the amount of cDNA template. (B) Percentage of the eye- and penis-removed slugs that were in the dark compartment after 1 h (left) and 2 h (right) in the light–dark choice group tests (*n*=7). **P*<0.01, Student's *t*-test.

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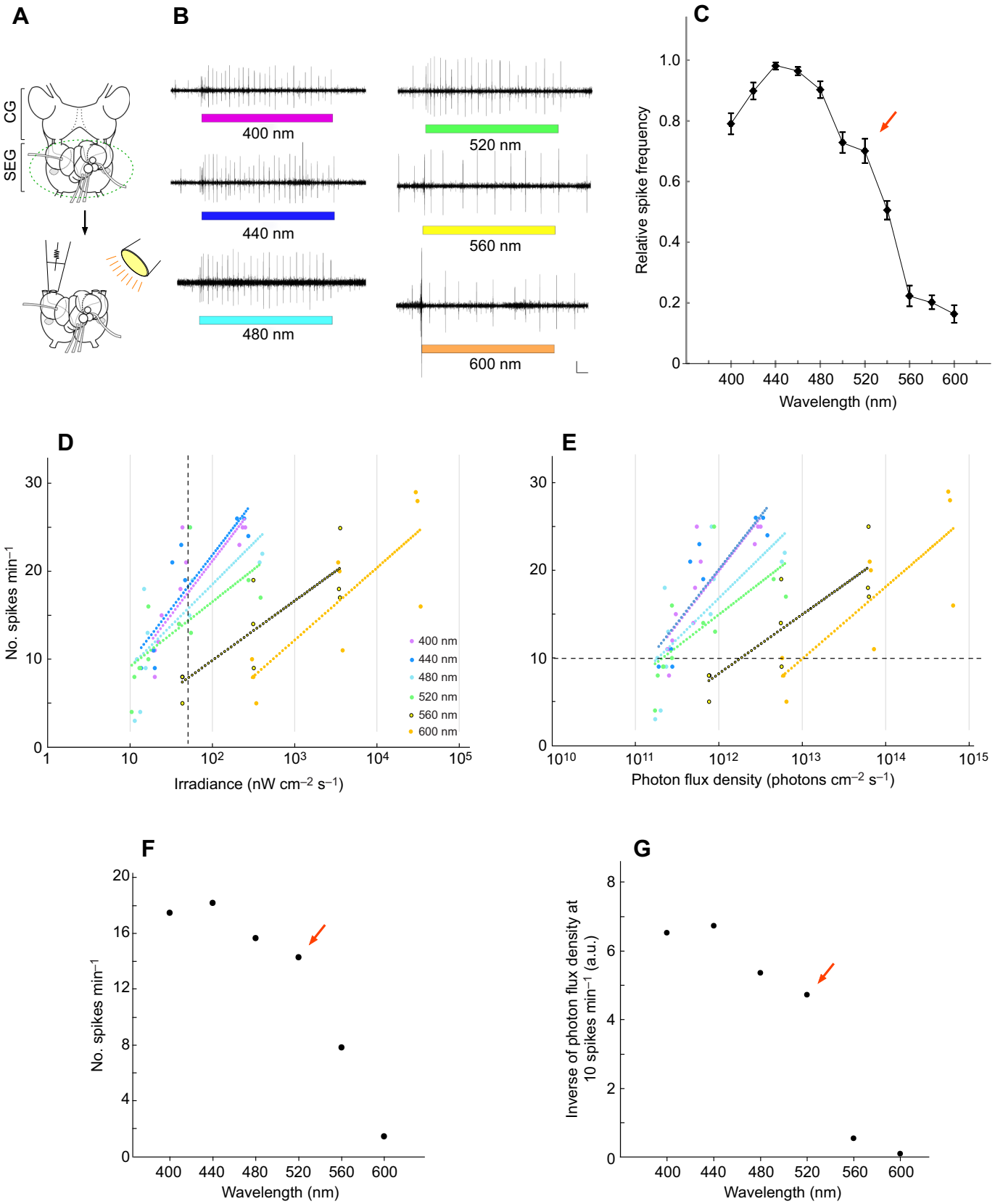

Fig. 6. See next page for legend.

compartment, whereas the intact slugs went more directly into the dark compartment (Movie 1). The intact slugs compare the intensity of light coming into the bilateral eyes or may find darker/brighter areas within the visual field (Zieger et al., 2009; Matsuo et al., 2014; Fujisaki and Matsuo, 2017). In contrast, the blinded slugs seem to compare the light intensity between the present and previous

Fig. 6. Spectral properties of light-evoked responses of the brain. (A) Schematic drawing of the preparation for the recording of brain photoresponses. CG, cerebral ganglia; SEG, suboesophageal ganglia. (B) Examples of spike responses to monochromatic light (20 nW cm^{-2}). Scale bars: 10 μV and 5 s. (C) Relative spike frequency evoked by monochromatic light. Means±s.e. (*n*=10). (D) Number of spikes expressed as a function of irradiance (shown on a log scale). The vertical dashed line indicates 50 nW cm^{-2}. (E) Number of spikes expressed as a function of photon flux density (shown on a log scale). The horizontal dashed line indicates 10 spikes min^{-1}. (F) Expected number of spikes evoked by 50 nW cm^{-2} monochromatic light, calculated as intersecting points of irradiance–spike linear functions (dashed line in D). (G) Relative value of the inverse of the photon flux density that evoked 10 spikes min^{-1} (dashed line in E). a.u., arbitrary units. Red arrows in C, F and G indicate a bump at 520 nm.

moments, and ceased to move when their surroundings became sufficiently dark (i.e. photo-klinotaxis). Slugs are thus capable of performing light-avoidance behaviours in two different ways, photo-tropotaxis and photo-klinotaxis. It also should be noted that van Duivenboden (1982) succeeded in detecting positive phototaxis behaviour in the blinded snail *L. stagnalis*. In her behavioural experiment, the snails were illuminated from a point light source on the side wall, which creates a spatial gradation of light intensity within the experimental chamber. It can be expected that a temporal component was introduced if the snails wandered about on the floor of such an experimental chamber.

Based on our behavioural experiments, the brain is less sensitive to ambient light than the eyes (Fig. 2). This is partly because of the absence of any lens-like structure in the brain that functions in collecting light to provide the photoreceptors with intensified light. Another reason may be the decrease in light intensity during penetration of the outer structures such as the mantle, the BW and the penis. For the mantle and the BW of *Limax flavus*, the spectral transmittance has been experimentally determined, and was found to be more than 0.8 and 0.85, respectively, even for short-wavelength (400 nm) light (Beiswanger et al., 1981). If the transmittance of the overlying structures was this large, their screening effect would explain only a small part of the poor light sensitivity of the blinded slugs, although there are currently no data available with respect to the transmittance of the penis and the oesophagus that usually overlie the brain and the SEG, respectively.

Another possible reason for the lower sensitivity of the brain to light is the difference in the sensitivity of the photoreceptor cells in the retina and the photosensory neurons in the brain. It is plausible that the highly developed microvilli and the dense distribution of the photopigment opsins in the eye photoreceptor cells enable photon capturing with a higher efficiency (Kataoka, 1975; Zieger et al., 2009). The photosensitive neurons of the brain are thought to lack any structures specialized for photoreception in *Onchidium* (Gotow and Nishi, 2009). However, taking into account that the isolated brain exhibited high sensitivity with respect to spike generation (Fig. 6), the relationship between the light-evoked change in spike frequency and the behaviour may not be simple.

It is currently not possible to ascribe the photoresponse of the brain to any one opsin species expressed there. Because retinochrome is an enzyme catalysing photoisomerization of chromophores (Ozaki et al., 1983), Opn5A and xenopsin are candidates based on the expression level in the brain (Fig. 4B; Matsuo et al., 2019). We observed in our preliminary experiment that the peak of the spectral sensitivity of Opn5A is at a longer wavelength than that of xenopsin and rhodopsin (R.M., unpublished observation). The involvement of Opn5A may thus explain the 'bump' in the spectral response/sensitivity curves at 520 nm (Fig. 6C,F,G). Further studies are required to identify the opsin molecules and the brain neurons that express them that are responsible for non-ocular photosensing.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: R.M.; Methodology: H.N., A.N., Y.M., R.M.; Investigation: H.N., A.N., Y.M., R.M.; Data curation: R.M.; Writing - original draft: R.M.; Writing - review & editing: H.N., A.N., Y.M., R.M.; Supervision: R.M.; Project administration: R.M.; Funding acquisition: R.M.

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Data availability
The nucleotide sequences of opsin genes have been deposited in DDBJ/EMBL/GenBank under accession numbers LC440461 (xenopsin), LC440462 (Opn5A), LC440463 (Opn5B), LC440464 (retinochrome).

Supplementary information
Supplementary information available online at
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