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SHORT COMMUNICATION

Physiology and morphology of sustaining and dimming neurons of the crab *Chasmagnathus granulatus* (*Brachyura*: *Grapsidae*)

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Abstract In crustaceans, sustaining (SN) and dimming (DN) neurons are readily identified by their distinct responses to a light pulse. However, morphological identification and electrophysiological characterization of these neurons has been achieved only in the crayfish. This study provides a description of SNs and DNs in a second crustacean species, the crab *Chasmagnathus*. SNs and DNs of the crab arborize extensively in the medulla and the axons project to the midbrain. Upon a light pulse, SNs depolarize and increase the firing rate while DNs hyperpolarize and reduce firing. These responses are highly consistent and their magnitudes depend on the intensity of the light pulse. When stimulated with a wide-field motion grating, SNs respond with a modulation of the membrane potential and spike frequency. We also characterized the responses of these neurons to a rotating e-vector of polarized light. SNs show the maximum depolarization when the e-vector approaches vertical. In contrast, DNs show maximal depolarization to near horizontal e-vector orientations. The semi-terrestrial crab and the crayfish inhabit unique light environments and exhibit disparate visual behaviors. Yet, we found that the location, morphology and physiology of SNs and DNs of the crab are nearly identical to those described in the crayfish.

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Introduction

In most crustaceans (malacostracans), the visual system is composed of the retina and four retinotopically arranged optic neuropils that are named, from periphery to center: lamina, medulla, lobula and lobula plate (Strausfeld 1998; Harzsch 2002; Sztarker et al. 2005). The lamina has been studied in great detail in several species and its organization appears to be highly conserved even among species with different lifestyles and ecologies (Sztarker et al. 2009). However, it is not known whether such similarities also exist for downstream neuropils that may be responsible for more specific and behaviorally relevant visual processing.

It has been over 50 years since Wiersma and coworkers first recorded the two types of light intensity sensitive elements in the crustacean visual system that they called sustaining (SN) and dimming (DN) neurons (Waterman and Wiersma 1963; Wiersma and Yamaguchi 1966, 1967). They were also able to functionally identify SNs, DNs and several motion sensitive neurons in different crustacean species (e.g. Wiersma and Oberjat 1968; York and Wiersma 1975, reviewed in Wiersma et al. 1982). However, because the recordings were obtained extracellularly from the fibers in the protocerebral tract, the location and morphology, as well as many physiological aspects of these cells, remained unknown. Subsequently, using intracellular recording and dye injections, SNs and DNs were morphologically identified and further characterized in crayfish while motion sensitive neurons have been investigated in a crab (see below).

Crayfish SNs are wide field visual interneurons that respond to a pulse of light with a strong depolarization and

a tonic discharge of action potentials that persists for the duration of the stimulus. Both the frequency of the discharge and the amplitude of the excitatory postsynaptic potential depend on the intensity of the light pulse. DNs appear to be the exact opposite of the SNs; they discharge continuously in the dark and are inhibited by the onset of illumination (reviewed in Wiersma et al. 1982; Glantz and Schroeter 2002). In crayfish, SNs and DNs are considered to be the principal output neurons of the medulla (Glantz and McIsaac 1998). Because of their maintained response to motion grating stimulation and their connection to optomotor neurons, SNs have been implicated in compensatory optomotor responses (Glantz and Nudelman 1988). Additionally, SNs and DNs were shown to be sensitive to polarized light; the former responding optimally to e-vectors close to the vertical, while the later showing maximal sensitivity for e-vectors close to horizontal (Glantz and McIsaac 1998; Glantz 2001).

In this paper, we characterize the properties of SNs and DNs in the semi-terrestrial crab, *Chasmagnathus granulatus*. Over the last two decades, *Chasmagnathus* has been established as a model system for the neurobiology of memory using a visual learning paradigm (Romano et al. 2006; Tomsic et al. 2009). In order to identify the neural circuits underlying the memory of the crab, we performed anatomical and electrophysiological studies in the optic lobe of the crab, which resulted in the first identification of individual neurons subserving long-term visual memory in an arthropod (Tomsic et al. 2003). In addition, these studies contributed to our understanding of the neural organizations and physiological properties of the crustacean optic neuropils (Berón de Astrada and Tomsic 2002; Sztarker and Tomsic 2004, 2008; Sztarker et al. 2005, 2009; Medan et al. 2007; Oliva et al. 2007). Up to this point, our work has mainly focused on motion sensitive neurons from the lobula of the crab.

In order to compare the visual nervous systems of two disparate decapod species, we decided to investigate in the crab two classes of medullary neurons that had been well characterized in the crayfish, the sustaining and dimming neurons. Our results show that the location, morphology, and sensitivity to visual stimulation of SN and DN in the crab are nearly identical to those found in the crayfish.

Materials and methods

Animals

Animals were adult male *Chasmagnathus granulatus* (Dana 1851) crabs, 2.7–3.0 cm across the carapace, weighing approximately 17 g. We collected crabs in the rías (narrow coastal inlets) of San Clemente del Tuyú, Argentina. In the laboratory, the crabs were kept in plastic tanks (35 cm × 48 cm × 27 cm) filled to 2 cm depth with diluted seawater at a density of 20 animals per tank. Water used in tanks and other containers during the experiments was prepared using hw-Marinex (Winex, Hamburg, Germany), salinity 10–14‰, pH 7.4–7.6, and maintained within a temperature range of 22–24°C. The holding and experimental rooms were maintained on a 12 h:12 h light:dark cycle (lights on 07:00–19:00 h) and the experiments were run between 08:00 and 19:00 h.

Crabs were fed rabbit pellets every 3 days (Nutrients, Buenos Aires, Argentina), water was changed 6 h after feeding. Experiments were performed within the first 2 weeks of the crab's arrival in the laboratory.

Physiological preparation and recordings

We performed intracellular recordings from the optic lobe as described by Berón de Astrada et al. (2001). Briefly, the crab was held in an adjustable clamp that allowed free movements of the walking legs but reduced movements of the chelae. The eyestalks were cemented to the carapace at an angle of approximately 70° from horizontal. A tangential cut performed with a scalpel was made to remove a small piece of cuticle (about 500 μm in diameter) from the tip of the eyestalk without causing damage to the ommatidial area. Once the dissection was complete, the clamp with the crab was mounted inside the recording setup with a magnetic holding device. The glass microelectrode was then positioned and advanced through the opening in the cuticle. Microelectrodes (borosilicate glass; 1.2 mm outer diameter, 0.68 mm inner diameter), were pulled on a Brown-Flaming micropipette puller (P-97, Sutter Instrument, Novato, CA, USA) yielding tip resistances of 40–60 MΩ when filled with 3 M KCl. A bridge balance amplifier was used for intracellular recordings (Axoclamp 2B, Axon Instruments, Foster City, CA, USA). The output of the amplifier was monitored on an analog oscilloscope, digitized at 3.5 kHz (Digidata 1200; Axon Instruments) and recorded in a computer using Clampex 6 (Axon Instruments).

The general experimental protocol was as follows. After a neuron was impaled, we tested its response to a brief light pulse. When responses resembled those published for SNs or DNs of the crayfish, a curtain in the front of the cage was lowered, and the neuronal responses to the different visual stimuli (see below) were recorded. All experiments were performed at the cell membrane resting potential.

Dye injection and cell morphology

Following characterization of the response to visual stimuli, the cell was iontophoretically injected with Neurobiotin. Electrode tips were backfilled with 5%

Neurobiotin, 50 mM Tris buffer in 500 mM KCl solution, backed up with 3 M KCl. Cells were injected for 10–20 min using 1- to 4-nA positive current. We injected only one neuron per animal. The trace was allowed to diffuse for 1–3 h in the living animal. We then anesthetized the crab on ice and dissected the optic lobes and the supraesophageal ganglion. Following dissection, we immersed the tissues in 4% paraformaldehyde in phosphate buffer (pH 7.2) to be fixed overnight. After five 20-min washes with PTA (PBS 0.1 M, Triton X 2% vol/vol, and sodium azide 0.1% wt/vol; pH 7.4), ganglia were incubated overnight with avidinrodamin (1/3,000 v/v in buffer PTA) at 4°C with constant shaking, after which they were again washed five times with PTA. The ganglia were then dehydrated in ethanol and cleared in methyl salicylate. Cleared ganglia were imaged as whole mounts and scanned at 2- to 5-μm intervals with a confocal microscope equipped with a Helium/Neon laser (Olympus FV 300). Images, saved as three-dimensional stacks, were adjusted for brightness and contrast, and illustrations were obtained by merging individual serial sections with ImageJ 1.33U (National Institutes of Health, Bethesda, MD). The morphologies of filled neurons were reconstructed by tracing and hand drawing the neuronal structure from the series of individual optical sections.

Visual stimuli

The electrophysiological setup was enclosed in a Faraday cage completely sealed to prevent external visual stimuli from reaching the animal. The roof and walls of the cage were painted white and the surface of the vibration-dampened table was black. Visual stimuli consisted of light pulses from a Xenon-filament projector lamp located outside the cage. The light was guided by an 8 mm diameter fiber optic. A shutter, which was controlled by the acquisition software, controlled the duration of the pulse. To ensure a maximum response in the neurons, the light pulse intensity at the eye was high (130 Wm^{-2}), and illuminated the entire eye. Lower intensities were attained using neutral density filters. The grating stimulus was identical to that described by Medan et al. (2007). Briefly, it consisted a pattern of vertical bars moving across an arrangement of three computer screens that surrounded the crab. The grating spatial frequency was 0.03 cycles/deg and the temporal frequency was 6 Hz.

Linearly polarized light was delivered by a system similar to that used in experiments with the crayfish (Glantz and McIsaac 1998). Light from an optic fiber passed through a wax-paper diffuser/depolarizer followed by a linear polarizer (HN38S, Polaroid Co., Waltham, MA, USA) mounted on a motorized rotation device. The e-vector of the stimulus was monitored with a photodiode covered by a vertically oriented scrap of polarizer film. The degree of polarization exceeded 99% and stray polarization was <1.0%. The polarized stimulus illuminated the entire surface of the eye with a total maximum power of ~18 Wm^{-2}, and the filter was always rotated clockwise.

Results

Identification of crab sustaining neurons

Our method for in vivo intracellular recording in the crab involves descending the glass microelectrode vertically through a small hole in the tip of the eyestalk. While descending through the vertically arranged neuropils, we usually penetrate neurons with distinct and recognizable responses to a pulse of light (Berón de Astrada et al. 2001). Among these, there are neurons with response profiles that clearly resemble those observed in the SNs and the DNs of the crayfish. Recording from these neurons in the crab proved to be more difficult than recording from the giant motion sensitive neurons in the lobula of this animal using the same methodology. While impalements of the lobula neurons usually remain stable for about an hour (Medan et al. 2007), recordings from SNs or DNs rarely exceeded 10 min. The lower stability is likely due to the shallower penetration of the electrode required to record from these elements (the medulla instead of the lobula). Presumably, the electrode is more exposed to mechanical disturbances such as those caused by hemolymph movements produced by heartbeats or by the movements of the animal legs. Consequently, the whole set of visual stimuli could be hardly ever tested in the same neuron.

We recorded and stained intracellularly six sustaining neurons in *Chasmagnathus*; Fig. 1a shows representative recordings from two of them (see also Fig. 3). In dim light (20 mWm^{-2}), the cells presented different levels of spontaneous activity, ranging from 0 to near 20 Hz. This is likely due to differences in the amount of pre-exposure to light during the procedure of neuronal searching, and to the short time for dark adaptation imposed by the stability of the recording. The response to a step increase in illumination of a crab SN consists of an excitatory postsynaptic potential (EPSP) with an initial, highly depolarized transient phase, followed by a less depolarized tonic phase. The EPSP elicits a discharge of action potentials that persists for the duration of the stimulus. The termination of the light pulse elicits a hyperpolarizing response (off response) that decays over several hundred milliseconds. Recordings from different SNs may show differences in the sizes of the EPSP and spike amplitudes, which are likely due to variability in the site of impalement (Berón de Astrada et al. 2001).

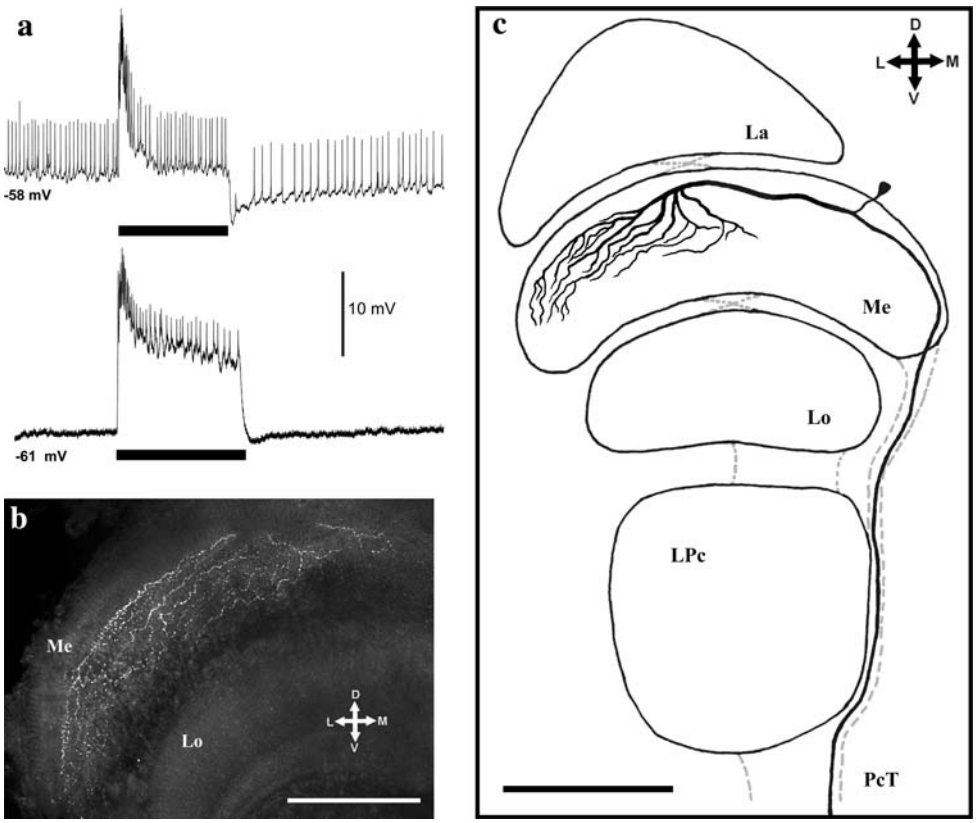

Fig. 1 Sustaining neurons (SNs) of the crab. **a** The characteristic response of SNs to a 1 s pulse of light (*horizontal bar*) is illustrated by recordings from two different animals. **b** Image stack of 10 confocal sections (4 µm each) corresponding to the SN with the light response shown in **a** (lower trace). **c** Reconstruction of the morphology of this neuron from the whole series of optical sections. *La* lamina, *Me* medulla, *Lo* lobula, *LPc* lateral protocerebrum, *PcT* protocerebral tract, *D* dorsal, *V* ventral, *L* lateral, *M* medial. *Scale horizontal bar* in **b** and **c**: 100 and 200 µm, respectively

Three out of the six dyes injected SNs were sufficiently stained to permit morphological characterization. Figure 1b shows part of the dendritic arbor in the medulla, corresponding to a 40 µm stack obtained by merging 10 individual 4 µm optic serial sections. The cell morphology shown in Fig. 1c was reconstructed from the whole series of individual optical sections. The other two stained SNs had similar morphologies. The dendritic arbor is located in the medulla and a main neurite traverses the neuropil lateromedially. Near the middle part of the neuropil, this neurite branches into a tree that expands horizontally toward the lateral side of the medulla within a single stratum. Based on previous studies on the anatomical organization of the optic neuropils of *Chasmagnathus* (Sztarker et al. 2005), the stratum containing the dendritic arbor of SNs is the lateromedial tangential 1 (LMT1). The main neurite of the SN gives rise to an axon fiber that leaves the medulla through the medial pole of the neuropil. The axon descends by the medial side of the lateral protocerebrum and continues toward the midbrain through the protocerebral tract. The cell body is located just above the medulla and connects to the axonal fiber through a short neurite.

Identification of crab dimming neurons

Figure 2a shows recordings obtained from two DNs of *Chasmagnathus*. The response profile to a light pulse recorded intracellularly from a crab DN consists of a compound IPSP that reduces the spontaneous tonic discharge of action potentials. The termination of the light pulse gives rise to a strong depolarization and increased discharge that lasts several hundred milliseconds. Similar response profiles were obtained in four neurons from different animals (see Fig. 3). As is the case for SNs, the amplitude of the postsynaptic potential and spikes recorded in DNs depend on the site of cell impalement.

Three neurons were sufficiently stained to allow the morphological characterization of this cell type. Figure 2b corresponds to a 40 µm stack of 10 individual 4 µm optic serial sections that shows part of the dendritic arbor of a DN in the medulla. Figure 2c illustrates the general morphology obtained by reconstruction from the whole series of optical sections. A main neurite that traverses the medulla lateromedially gives rise to a tree of dendritic arborizations that run horizontally in a single stratum, which corresponds to layer LMT1, according to Sztarker et al. (2005). In comparison to SNs, the dendritic branches of DNs taper into more narrow processes. The main trunk leaves the medulla by its medial side as an axon fiber that descends via the protocerebral tract toward the midbrain. The cell body is located above the medulla and is connected to the axonal fiber by a short neurite.

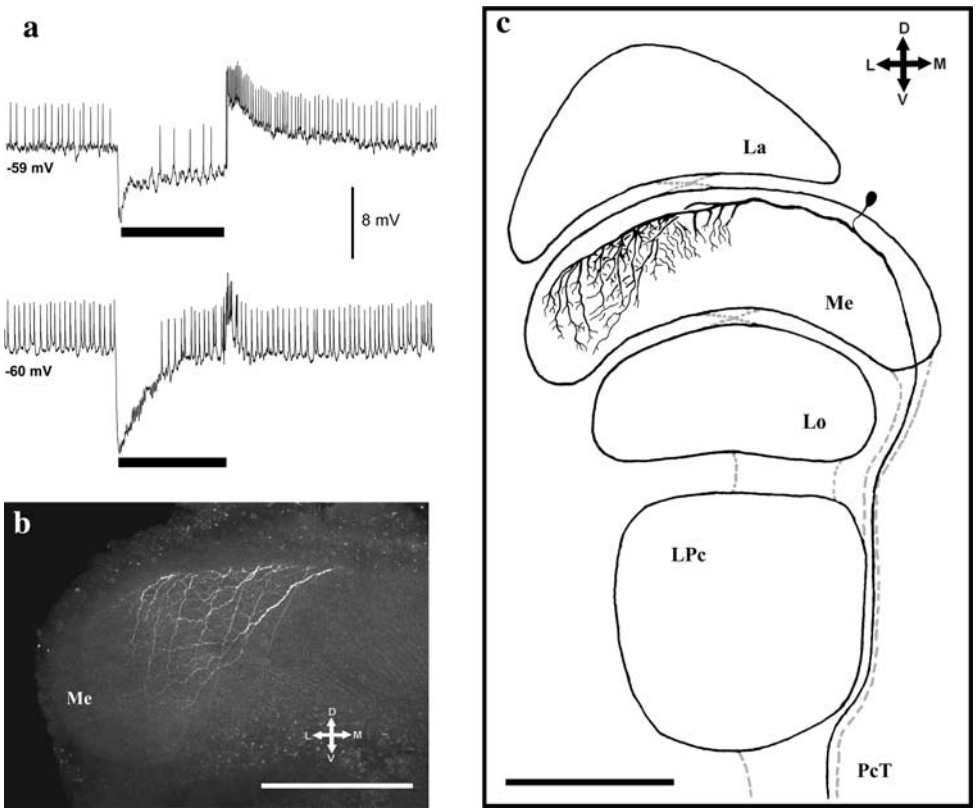

Fig. 2 Dimming neurons (DNs) of the crab. **a** Two recordings from different crabs showing the typical response of DNs to a light pulse. **b** Confocal image stack of 10 individual 4 µm optic serial sections that shows part of the dendritic arbor of the DN in **a** (*upper trace*). **c** Morphological reconstruction of this neuron from the whole series of optical sections. References as in Fig. 2

Responses to light intensity, motion grating and polarized light

The responses of SNs and DNs to repeated light pulses of equal intensity are highly consistent. On the other hand, when stimulated with pulses of different intensity, these neurons respond with changes in voltage and spike frequency that reflects the intensity of the light stimulus (Fig. 3a). Upper trace in Fig. 3b shows the response of a SN to a pattern of vertical bars moving across an array of three computer monitors that surrounded the crab. When stimulated with the moving grating, SNs showed a transient response to stimulus onset, followed by a non-adapting response that included modulations of the membrane potential and spike frequency. The consistency of this response contrasts with the rapid adaptation to the same stimulus observed in motion sensitive neurons from the lobula of the crab (Fig. 3b lower trace; Medan et al. 2007).

Crab, crayfish and stomatopod photoreceptors are sensitive to the e-vector of light (Shaw 1966; Kleinlogel and Marshall 2006; Glantz 2007). Furthermore, there is evidence for polarization sensitivity in visual interneurons of the lamina and medulla of crustaceans (Leggett 1976; Glantz 1996a, b). These neurons show a much higher sensitivity to a time-varying e-vector than to flashes at stationary e-vector angles (Glantz 2001). A distinctive feature of the SN polarization sensitivity as studied in the crayfish with a rotating polarizer is that they responded optimally to e-vectors near the vertical. Conversely, the DNs examined were inhibited by the vertical e-vector and their dynamic responses were typically maximal for e-vectors close to horizontal (Glantz and McIsaac 1998; Glantz 2001). Remarkably, the four SNs and two DNs examined for polarization sensitivity in the crab showed a maximum response for near vertical and horizontal e-vectors, respectively (Fig. 3c). Both the membrane potential and the spike rate exhibit strong modulations. The firing of SNs ceases when the e-vector approaches the horizontal, whereas the firing of DNs ceases when it is near the vertical. The similarities in e-vector orientation preference of SNs and DNs of the crab and crayfish can be observed by comparing recordings of Fig. 3c with those of Fig. 4 in Glantz (2001).

Discussion

Sustaining and dimming neurons were first identified by their responses to a light pulse following extracellular recordings in the protocerebral tract of several decapod species (reviewed in Wiersma et al. 1982). Although the SNs and DNs are thought to be the principal output neurons of the crustacean medulla (Glantz 2001), intracellular and morphological studies of these elements have been

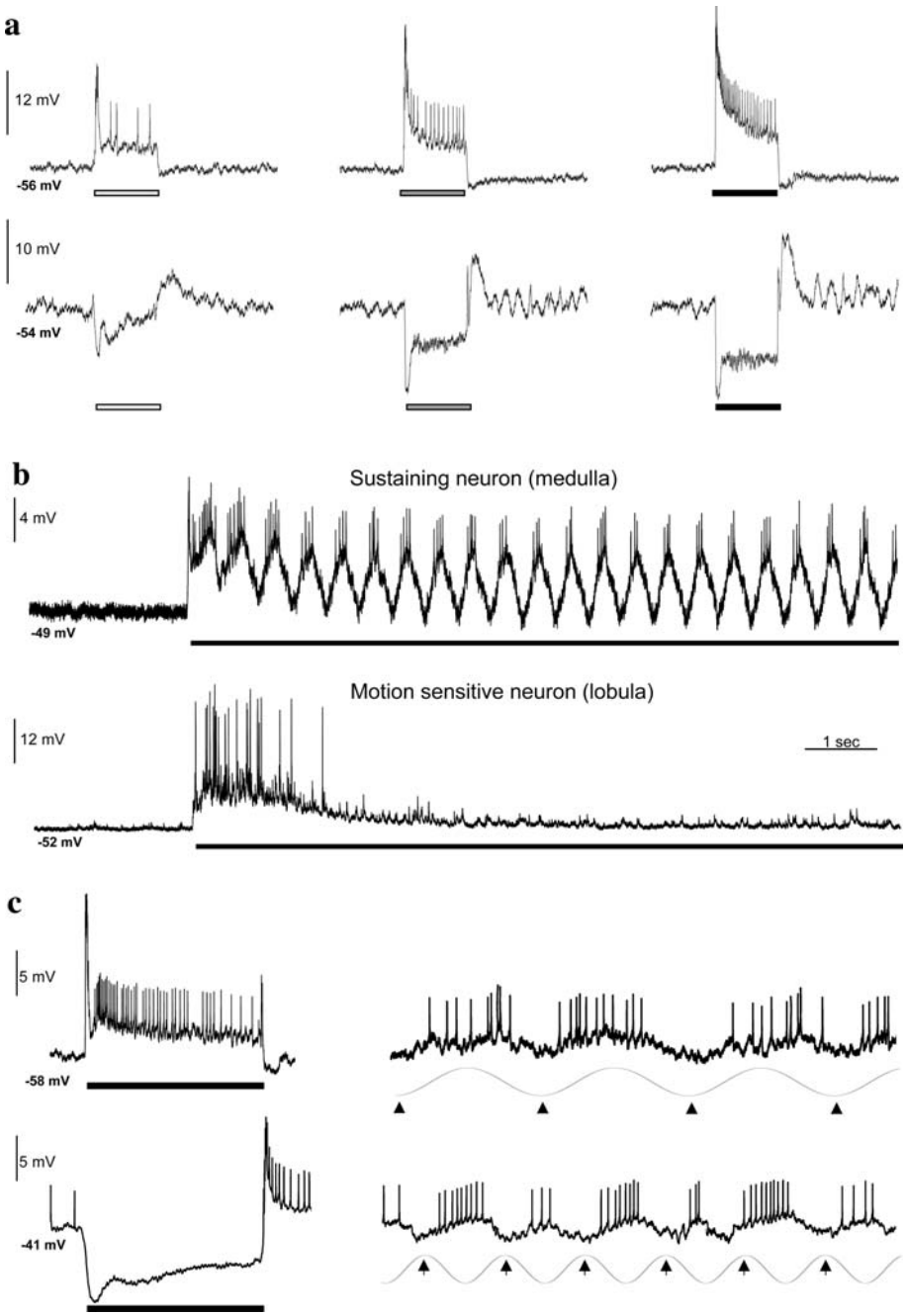

Fig. 3 SN and DN responses to different stimuli. **a** The traces show the responses of a SN (*upper row*) and a DN (*lower row*) to 1 s light pulses of increasing intensity. In the DN shown here the spikes are attenuated, suggesting that the recording was taken from a distal dendrite. *Light gray*, *gray* and *black bars* correspond to stimulus intensities of 10, 50 and 130 Wm^{-2}, respectively. **b** When stimulated with a square wave grating moved around the crab, the SN (*upper trace*) responds with modulations of the post-synaptic potential and the spike rate. Following the transient response to the onset of grating movement, the response reaches a steady state that is maintained throughout the stimulus presentation (*horizontal black line*). Conversely, the response of movement detector neurons from the lobula (*lower trace*) to this stimulus rapidly vanishes (see Medan et al. 2007). The grating spatial frequency was 0.03 cycles/deg and the temporal frequency was 6 Hz. **c** SN and DN response to polarized light. The traces show the response of a SN (*above*) and a DN (*below*) to a pulse of unpolarized light (*left*) and to the light transmitted by a rotating polarizer (*right*). Sinusoidal lines below the traces represent the e-vector monitored by the photodiode (see "Materials and methods"). Note that the membrane potential of the SN approaches the maximum hyperpolarization when the e-vector approaches horizontal (*arrowhead*). In contrast, the DN shows maximal hyperpolarization to near vertical e-vector orientations (*arrows*). Polarizer rotation moved at 60°/s for the SN and 120°/s for DN

achieved only in the crayfish. The present study provides the first morphological description and initial physiological characterization of SNs and DNs from a crustacean distinct from the crayfish, the crab *Chasmagnathus*.

The SN of the crab responds to a light pulse with a sustained depolarization and increased rate of spike discharge, both of which reflect the intensity and duration of the light stimulus. DNs respond to the light pulse with a hyperpolarization and reduction in spike frequency. Both types of responses contain a peak and a plateau component (Figs. 1, 2). The responses obtained in the crab are nearly indistinguishable from those obtained in the SN and the DN of the crayfish (for comparisons with the response of crayfish SN, see Kirk et al. (1983a) Fig. 5a, or Waldrop and Glantz (1985) Fig. 2; for comparison with a crayfish DN see Pfeiffer-Linn and Glantz (1991) Fig. 2). When stimulated with a moving grating, SNs of the crab and crayfish are also very similar (compare Fig. 3b, upper trace, with traces of Fig. 2a in Glantz et al. (1995)).

The morphology of the crab SNs closely resembles that of the crayfish (compare Fig. 1 in this paper to Fig. 1 in Kirk et al. 1983b). In both species, the neuron has a wide arborization of branches that extends in a single stratum across a wide portion of the retinotopic mosaic of the medulla. These branches, which intersect the columnar projections of transmedullary neurons (Kirk et al. 1982; Sztarker et al. 2005), eventually converge into a single fiber that exits the medulla by its medial side. From there, the axon descends along the protocerebral tract toward the midbrain. The cell body is connected by a thin neurite to the main medullary fiber. In crayfish, the description and illustrations of the morphology of SNs are limited to the optic lobe. Therefore, the anatomical projection of the axon fiber beyond the protocerebral tract is not documented. Unfortunately, we were also unable to track the SN axon in the midbrain of the crab, probably due to the distance between the optic lobe and the midbrain (>20 mm).

The DNs of the crab consist of a wide dendritic arborization that extends lateromedially along a single stratum of the medulla. The axon fiber exits the medulla by the medial side and descends along the protocerebral tract. As for SNs, the projections of DNs in the midbrain remain unknown. Unfortunately, there are only a few published illustrations of the morphology of DNs of the crayfish. However, the available drawings and descriptions resemble the DNs described here in the crab (see Fig. 2 in the present paper and Fig. 8 in Pfeiffer and Glantz (1989)).

The responses of crab SNs and DNs to a rotating e-vector resemble those obtained to similar stimuli in crayfish. In crayfish, SNs are known to constitute an ensemble of 14 cells with different receptive fields (Kirk et al. 1982). All of the crayfish SNs responded optimally to e-vectors near the vertical. Conversely, the fewer dimming fibers examined to date were all inhibited by the vertical e-vector (Glantz 2001). Although we have few recordings of polarization sensitivity in SNs and DNs of the crab, all cells exhibit the same e-vector preferences as those found in crayfish.

The organization of the lamina (the first optic neuropil) of different crustacean species with distinct lifestyles and ecologies is highly conserved. Here, we qualitatively compare the principal output neurons of the medulla (the second optic neuropil) of a crab with previous studies of these neurons in the crayfish. Our results show that the SNs and DNs of these two species are morphologically indistinguishable, and the neurons behave similarly for all the stimuli we tested. Such similarities are remarkable when considering that, crayfish and semi-terrestrial crabs are dissimilar in ecology and behavior, and that the Astacidea (crayfish) and Brachyura (crabs) diverged over 325 million years ago (Porter et al. 2005). Differences in visually guided behaviors that distinguish these species likely arise at deeper neuropils within the decapod nervous system, perhaps at the level of the motion detectors neurons in the lobula.

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