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Article

Behaviorally Related Neural Plasticity in the Arthropod Optic Lobes

Martín Berón de Astrada,[1,*] Mercedes Bengochea,[1] Julieta Sztarker,[1] Alejandro Delorenzi,[1] and Daniel Tomsic[1]
[1]Laboratorio de Neurobiología de la Memoria, Departamento Fisiología, Biología Molecular y Celular, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, IFIBYNE-CONICET, CP 1428 Buenos Aires, Argentina

Summary

Background: Due to the complexity and variability of natural environments, the ability to adaptively modify behavior is of fundamental biological importance. Motion vision provides essential cues for guiding critical behaviors such as prey, predator, or mate detection. However, when confronted with the repeated sight of a moving object that turns out to be irrelevant, most animals will learn to ignore it. The neural mechanisms by which moving objects can be ignored are unknown. Although many arthropods exhibit behavioral adaptation to repetitive moving objects, the underlying neural mechanisms have been difficult to study, due to the difficulty of recording activity from the small columnar neurons in peripheral motion detection circuits.

Results: We developed an experimental approach in an arthropod to record the calcium responses of visual neurons in vivo. We show that peripheral columnar neurons that convey visual information into the second optic neuropil persist in responding to the repeated presentation of an innocuous moving object. However, activity in the columnar neurons that convey the visual information from the second to the third optic neuropil is suppressed during high-frequency stimulus repetitions. In accordance with the animal's behavioral changes, the suppression of neural activity is fast but short lasting and restricted to the retina's trained area.

Conclusions: Columnar neurons from the second optic neuropil are likely the main plastic locus responsible for the modifications in animal behavior when confronted with rapidly repeated object motion. Our results demonstrate that visually guided behaviors can be determined by neural plasticity that occurs surprisingly early in the visual pathway.

Introduction

Motion vision provides essential cues for a wide variety of animal behaviors. It originated to fulfill two essentially distinct behavioral tasks. One task, which is based on the analysis of panoramic optic flow, is to inform the animal about its own movements. The other task, which is based on the processing of focal motion cues, is to allow the animal to know about the movement of prey, predators, and conspecifics. Because animal navigation imply sustained analysis of the optic flow, the visual processing involved in this task shows little change upon repeated or continuous stimulation. In contrast, behavioral and neuronal responses to repeated object motion often show fast and profound decline. Such decline, in the form of either habituation [1] or more-complex associative learning processes [2], represents constitutive mechanisms of an animal's adaptability [3].

The arthropod neural systems that have been studied most intensively and that are used to investigate object or target visual detection are: the system that contains figure detection (FD) cells in the blowfly [4]; the system that contains small target movement detector (STMD) neurons involved in the pursuit of prey, mates, or rivals in dragonflies and hoverflies [5, 6]; and the system that contains lobula giant movement detector (LGMD) neurons, which are involved in predator avoidance behavior in locusts (for a review, see [7]). In the last two models, the reduction of neuronal responses to repeated stimulus presentations has been observed (e.g. [1, 8]), but the site and mechanism of the neural plasticity have not been a subject of specific investigation (but see [9]). This issue, however, has been the main focus of interest in another arthropod model for visual research, the crab *Neohelice granulata*, which contains lobula giant (LG) neurons [10–12] that are involved in predator detection and escape [3, 13, 14]. The response of the LG neurons remarkably parallels the short- and long-term behavioral changes induced by repeated stimulus presentation [2, 15].

The visual nervous system of pterygote insects and decapod crustaceans is highly conserved [12, 16]. This system is composed of a compound eye retina and four optic neuropils: the lamina, the medulla, and the lobula complex, which comprises the lobula and the lobula plate neuropils. All the neuropils are organized into retinotopically ordered columnar units intersected by orthogonal tangential strata. Inside of each columnar unit are different cell types that are believed to code parallel channels of visual information (e.g., motion, color, e vector polarization). The small-field retinotopic columnar elements are supposed to feed different wide-field tangential neurons that, in turn, convey visual information to the midbrain to guide specific visually evoked behaviors.

The theoretical model for motion detection in arthropods is that motion information is first extracted in local columnar elementary motion detectors. Because of the small size of the elementary motion detectors, physiological information on motion processing in arthropods has been obtained mainly by recording from their wide postsynaptic tangential neurons (but see [17–19]). Electrophysiological studies on tangential neurons indicate that optic flow motion is processed in the lobula plate (for a review, see [20]), while focal target motion (object detection) would be mainly processed in the lobula ([5, 6, 11, 21], but see [22, 23]). The lobula neurons that are involved in object motion detection, such as the STMD neurons (dragonflies and hoverflies), LGMD neurons (locust), and LG neurons (crabs) mentioned above, have several commonalities. They are all wide-field tangential neurons that collect information from the retinotopic columnar mosaic and project to the midbrain. They respond more intensively to objects than to flow field motion, and they all show a marked response reduction to repeated stimulus presentations [1, 15, 24]. Our main objective here was to determine the location of the plasticity that accounts for the response decline of lobula tangential neurons using the crab as experimental model. Present results indicate that the locus of the decline

*Correspondence: martin@fbmc.fcen.uba.ar

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in the response to rapidly repeated object motion observed in arthropods is the lobula-afferent columnar neurons presynaptic to the lobula tangential neurons.

Results

Response Changes in Lobula Neurons

In the crab, the presentation of a visual danger stimulus (VDS) (see the Experimental Procedures) causes an intense escape response that declines very rapidly upon high frequency stimulus repetitions (intertrial interval [ITI] = 2 s) (Figures 1A and 1B_I). The effect of this massed training is short lasting because the response completely recovers after 15 min without stimulation (Figure 1B_{II}). Motion-sensitive LG neurons (Figure 1C) respond to the VDS with strong discharges of action potentials that are superimposed on noisy graded potentials (first traces of Figures 1D_I, 1F, and 1H). The morphological and physiological characteristics of these neurons have been described elsewhere [10, 11, 14]. Figure 1D_I shows an example of the rapid response decline of an LG neuron to massed VDS presentations, which fully recovers after 15 min without stimulation (Figure 1D_{II}). Figure 1E depicts the parallelism between the behavioral and the neuronal response changes to massed VDS presentations.

Some behavioral changes induced by training have been shown to be related, at least partly, to changes in the intrinsic properties of the neurons [25]. To study whether the reduction in the response of the LG neurons could be accounted for by a generalized change in the neuronal excitability, we injected a pulse of depolarizing current through the recording microelectrode before each training trial. Whereas the response to the visual motion stimulus declined across trials, the number of action potentials evoked by the pulse of current remained invariable (Figure 1F). Therefore, the drastic decline of the LGs response to rapidly repeated VDS presentations is not caused by a generalized change in the neurons excitability.

The recordings of Figures 1D_I and 1F suggest that the decrease in the number of spikes evoked by the VDS matches the decrease in the compound excitatory postsynaptic potential (EPSP). This is confirmed by quantitative analysis (Figure 1G). The reduction in the EPSP is observed to continue beyond the point at which spikes are no longer discharged (Figure 1H), which indicates that the decrement is not a consequence of the cell's spike output (e.g., by recurrent inhibition). In addition, the membrane potential of the LGs was never observed to hyperpolarize below the resting membrane potential (Figure 1H), and in those cells that presented spontaneous firing, the firing frequency was not altered during the last trials of the visual stimulation (data not shown). Altogether, the evidence indicates that the response reduction of the LG neurons to repeated VDS is not a consequence of increasing inhibition acting on these cells. To further investigate the locus of plasticity, we developed a preparation for assessing visual neural responses in vivo with calcium optical recordings. We began our investigation at the level of the LGs to assess whether the reduction of the neuronal output is already present at the LGs dendritic input or whether it stems from downstream signal processing within the neuron.

Optical Recordings of Lobula Tangential Neurons

Before performing calcium imaging recordings, we used fluorescent dextrans (dextran-Alexa Fluor 488 or dextran-tetramethylrhodamine) to investigate the projections of the neural elements that become stained with our procedure (see the Experimental Procedures). The same staining procedure was then used with dextran Calcium Green 1 for calcium imaging experiments. The neural elements stained with the three dyes appeared to be the same.

The LG neurons connect two layers of the lobula with the central brain. Their dendrites, which extend across large parts of the retinotopic map, converge toward the medial side of the lobula into a single main neurite [10, 11]. By applying fluorescent dextran into the medial side of the lobula and waiting for its neuronal uptake and retrograde transport, we stained those LG dendrites that extended toward the lateral side of the neuropil. Figures 2C and 2D show confocal images of one preparation in which we performed calcium optical recordings (the recordings shown in Figures 2E–2H). The white spot marked with an asterisk corresponds to the site of dye application in the lobula. We consistently found two horizontal strata stained in the lobula (corresponding to strata LMT2 and LMT3; from [12]), which are coincident with previous descriptions of the bistratified dendritic arborization of LG neurons made from intracellular stainings.

To perform the calcium optical recordings, we used visual stimuli that were delivered through a computer screen. LG neurons loaded with dextran Calcium Green 1 showed phasic on-off calcium responses to a light pulse (Figures 2E and 2F) that resemble the responses that are obtained with intracellular recordings (Figure 2F, inset) [10]. The signals occurred essentially in stratum LMT3 and were much weaker or undetectable in LMT2. The response to moving stimuli like the VDS also provoked calcium signals mainly in LMT3 (Figure 2G_I), with a temporal profile (Figure 2H_I) similar to that of the LG neurons electrophysiological response. Our next step was to look at the calcium response during repeated VDS presentation. To avoid illuminating the preparation with the microscope excitation light during the whole training duration, we restricted the acquisition of calcium signals to trials 1, 5, 10, and 15 of training (T1, T5, T10, and T15, respectively; Figures 2G_I–2G_{IV} and 2H_I–2H_{IV}). The repetitive presentation of the VDS produced a sharp and deep reduction in the calcium response (Figure 2I). The response, however, almost completely recovered at a test trial performed 15 min after training (Figures 2G_V, 2H_V, and 2I).

To be certain that the reduction in the calcium signal was caused by the repeated VDS presentation, we performed exactly the same recording protocol, but instead of presenting 15 training trials with the VDS, we delivered only two trials separated by the time corresponding to the separation between trial 1 and trial 15 (ITI ~118 s). The result in Figure 2J shows that the responses to the first and second VDS trial were very similar. Taken together, these results demonstrate that the decline in calcium responses observed in the dendrites of the LG neurons during VDS repetition is caused by the recurrence of visual motion stimulation. In the following section, we address whether such a decline results from a reduction in the response of the presynaptic columnar neurons.

Optical Recordings of Lobula-Afferent Columnar Neurons

The lobula of arthropods receives retinotopic visual information from the medulla via the second optic chiasma. To stain the columnar neurons that project from the medulla into the lobula, we locally applied fluorescent dextran in the medulla and then followed the stained neural projections into the lobula (Figure 3A). In all 16 preparations that we made, confocal reconstructions revealed stained retinotopic columnar

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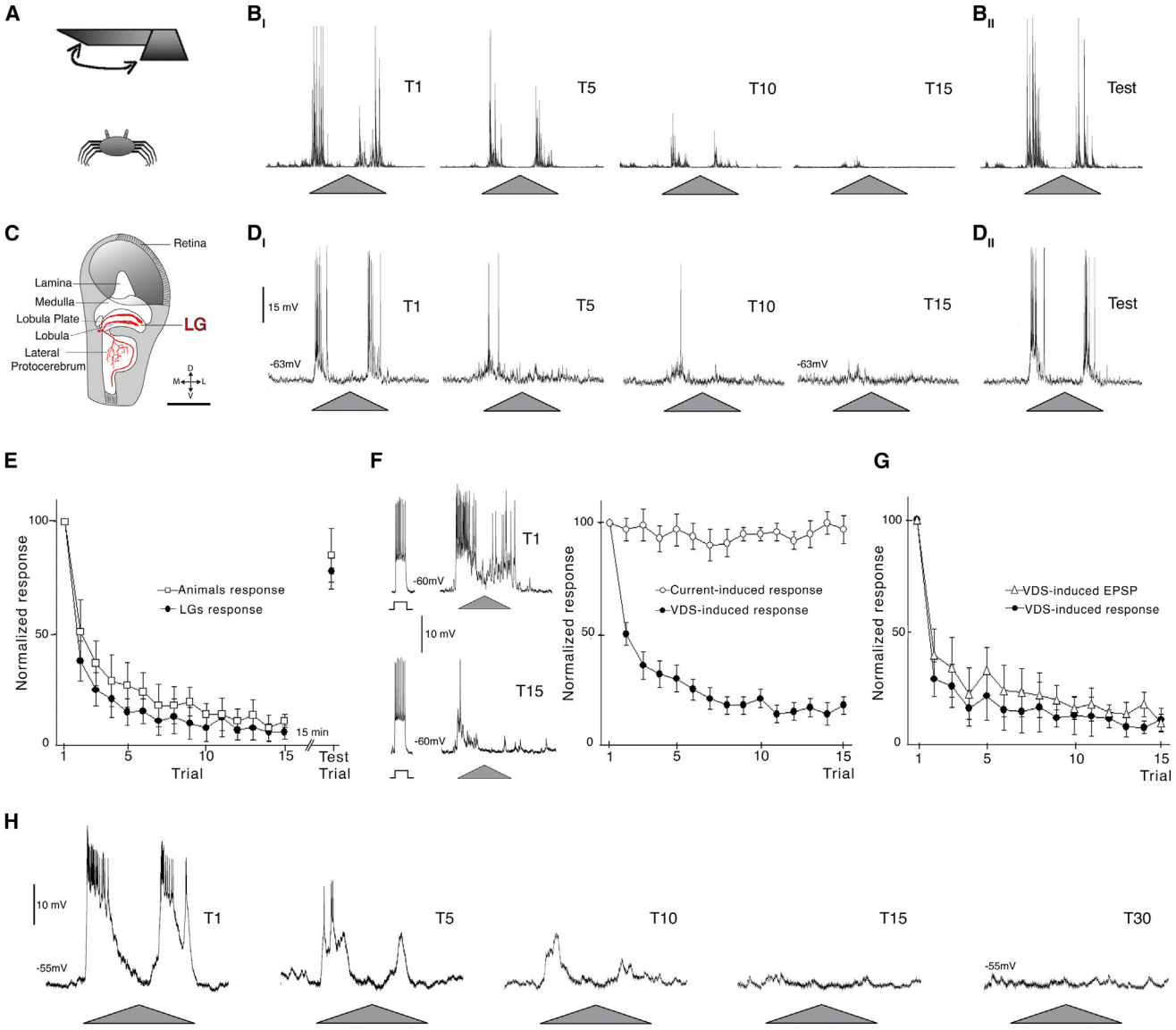

Figure 1. The Decrement in the Crab Escape Response Resulting from Repeated Visual Stimulation Is Reflected by the Performance of Lobula Giant Neurons

(A) The VDS used to elicit the escape response consisted of the displacement of a black rectangle. The motion cycle consisted of a 90° clockwise and counterclockwise excursion that lasted 2.2 s (the two top sides of the triangles below the traces represent the two directions of motion and the base 2.2 s of duration). A training trial includes two motion cycles separated by 2 s. For clarity, the figures show the responses to the first cycle.

(B) Intensity of the escape response of a single crab to a sequence of 15 training trials separated by 2 s. (B_I) shows the responses at trials 1, 5, 10, and 15, whereas (B_{II}) corresponds to a test trial performed 15 min after the last training trial.

(C) Scheme of the crab eyestalk containing the optic lobe with the four retinotopic neuropils (lamina, medulla, lobula plate, and lobula) and lateral protocerebrum. LG neurons (depicted in red) arise in the third neuropil and project to the midbrain.

(D) In vivo intracellular recording of an LG neuron in response to a sequence of 15 VDS trials separated by 2 s. (D_I) corresponds to responses at training trials 1, 5, 10, and 15, whereas (D_{II}) corresponds to a test trial performed 15 min after the last training trial.

(E) Averaged behavioral and neuronal responses from experiments, such as those illustrated in (B) and (D), respectively (behavior, n = 10 crabs; neurons, n = 8 from eight crabs).

(F) A 500 ms pulse of depolarizing current (initially adjusted to elicit 8–12 spikes) was delivered intracellularly to the LG neurons 1.5 s before each VDS trial. The electrophysiological recordings illustrate the response of an LG neuron to the pulse of current (left records) and to the VDS (right records) at trials 1 and 15. The number of action potentials elicited by the pulse of current and by the VDS was counted, normalized to trial 1, and averaged. The percentage of initial spike number at trial 15 evoked by VDS was 14% ± 5.2% and by current pulse was 97% ± 6.1% (t test $p < 0.001$ and $p = 0.67$, respectively; n = 8).

(G) In another set of experiments, we measured the compound EPSP, and the number of action potentials evoked by the VDS. The percentage of initial spike number and EPSP response at trial 15 was 7.2% ± 4.9% and 12.8% ± 5.7%, respectively (t tests, $p < 0.001$; n = 9).

(H) The responses of an LG neuron to 30 training trials with the VDS.

In (C), the scale bar represents 500 μm. M, medial; L, lateral; D, dorsal; V, ventral. The graphs show means ± SEMs.

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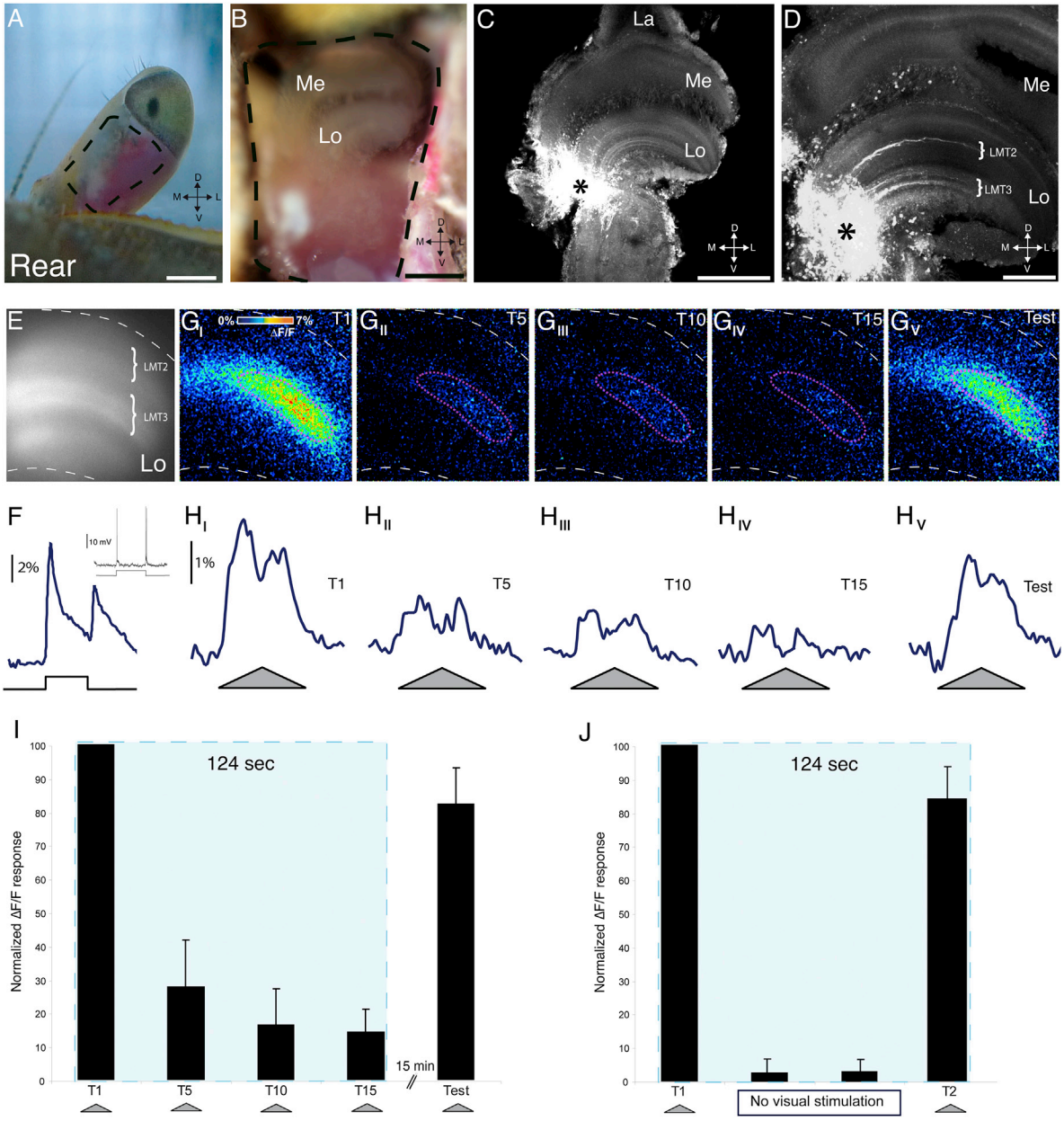

Figure 2. Calcium Responses at Dendrites of Lobula Tangential Cells Reflect the Neuronal Electrophysiological Output

(A and B) Posterior view of the right eye of the crab (A). A small window is opened in the cuticle to expose the optic ganglia (B). To stain the LG neurons, we applied fluorescent dextran on the medial side of the lobula.

(C) Confocal image obtained from such a staining (asterisk spots where dextran was deposited).

(D) Close up of the same preparation at a different lobula depth, in which both strata containing the dendrites of LG neurons are stained (LMT2 and LMT3 are lateromedial tangential strata 2 and 3, respectively; see the main text).

(E) Lateral side of the lobula stained with calcium-sensitive dye and imaged with a conventional epifluorescent microscope (CCD camera image) in a living animal. The eye looked frontally at a computer-controlled LCD monitor.

(F) Mean relative fluorescence change in one preparation of a lobula region comprising LMT3 stratum in response to a 1 s light pulse. The LG neurons' calcium response to a light pulse was on-off, similar to their electrophysiological response (inset).

(G and H) False-color-coded calcium response to visual stimulation with the VDS is shown in (G_I). Calcium responses to the VDS mainly occurred at LMT3. Time course of the mean relative fluorescence change in response to the VDS is shown in (H_I). Massed training with the VDS produced a steep reduction in the calcium response to the VDS (G_I–G_{IV} and H_I–H_{IV}). Calcium response of a test trial performed 15 min after training is shown in (G_V) and (H_V).

(I) Normalized average data. The percentage of the initial response at trial 15 was 14% ± 6.4% (t test, $p < 0.001$; $n = 6$). The percentage of the initial response at the test trial was 83.9% ± 8.5% ($n = 6$).

(J) Same recording protocol but applying only two VDS trials separated by the time that elapsed between trials 1 and 15 in the previous experiment. The percentage of initial response at trial 2 was 84.6% ± 7.8% ($n = 6$).

The dashed line in (G_I)–(G_V) represents the region of interest selected to analyze the calcium responses to visual stimuli. La, lamina; Me, medulla; Lo, lobula. Scale bars represent 500 μm in (A), 250 μm in (B) and (C), and 100 μm in (D). Graphs show means ± SEMs.

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projections into the lobula. Figures 3B–3E shows posterior views of such stainings. The white spot in Figure 3B corresponds to the site of dye application in the medulla (asterisk). As was described for the crayfish [26], in the crab the medulla columnar neurons arborize at three main lobula input levels: an outer (Li1), a medial (Li2), and an inner level (Li3) (brackets in Figures 3B–3E). Figure 3C shows a magnification of the lobula input strata. At the borders of these stainings, individual neurons can be readily recognized. Figure 3D shows a neuron that arborizes in the Li1 and Li3 strata; this neuron resembles a columnar neuron that we have been able to record electrophysiologically and stain intracellularly (Figures S1A and S1B available online). Figure 3E shows a neuron with short terminal arborizations that appear to be restricted to Li2.

To investigate the visually evoked calcium responses at the level of the lobula input strata, we loaded the neurons with dextran Calcium Green 1. Figure 3F shows the basal fluorescence of the lobula imaged by the conventional fluorescence microscope. Figure 3G shows the time course of the relative fluorescence change in response to a light pulse. Every preparation we have recorded from the three input strata showed phasic calcium responses to the onset and offset of a light pulse. These responses to the light pulse are consistent with the electrophysiological response that we obtained from a columnar neuron projecting from the medulla to the lobula (Figure S1C) and with responses obtained at the downstream LG neurons. The amplitude of the calcium responses in the three input strata showed no differences to the onset or offset of light (Figures 3G and 3J, left and middle columns, respectively). The visual stimulation with a vertical bar that moves horizontally across the frontal visual field provokes a calcium wave that sweeps across the lobula following the retinal positions of the motion stimulus (Movie S1). Figure 3H_I shows the calcium signal obtained in the frame of peak response during a first presentation of the VDS, and Figure 3I_I shows the time course of the relative fluorescence change within each input stratum. Responses to the VDS motion reached similar peak calcium signals at the three lobula input strata (Figure 3J, right columns). The repetitive presentation of the VDS provoked a rapid and deep reduction of the calcium responses (Figures 3H_I–3H_{IV}, 3I_I–3I_{IV}, and 3K). The same result was observed in the electrophysiologically recorded columnar neuron (Figure S1D). As expected from the animal's escape behavior and from the LG neuronal response, the response of the columnar elements recovered almost completely after 15 min without object motion stimulation (Figures 3H_V, 3I_V, and 3K).

Like in the previous section, we performed a control experiment in which we applied only two VDS trials separated by ~118 s. The calcium responses obtained to the first and second VDS trial were very similar (Figure 3L), confirming that the strong reduction in the calcium responses of the lobula columnar afferents is a consequence of the repeated VDS presentation.

Response Decrement to Rapidly Repeated Object Motion Is Specific to the Retina's Trained Area

The previous experiments showed that the response change in the LG neurons appears to be explained entirely by changes that occur in their columnar presynaptic neurons. As these columnar elements are arranged retinotopically, the LG's response reduction should be restricted to the retina's trained area. Figure 4A shows that the reduction of the elicited action potentials in the LGs that resulted from VDS massed training in one visual field (VF1) did not affect the response to a following VDS presented in a different visual field (VF2) (see the Discussion). Calcium responses of lobula-afferent neurons confirm that the response of the columnar elements mapping the untrained retina area remained unaffected by training in a neighboring area (Figure 4B). These results further indicate that the LG neuron's response declines after massed training is caused by a reduction only in the retinotopic input pathway that conveys the VDS information into the LG neurons.

Electrophysiological and Optical Recordings of Medulla-Afferent Columnar Neurons

The response decrement that we recorded in lobula-afferent neurons can be the consequence of plastic changes that take place in their presynaptic elements. Visual information flows retinotopically into the medulla directly from the retina through one photoreceptor (R8) and from the lamina through the first-order visual interneurons called lamina monopolar cells (LMCs). All LMCs thus far described in decapods have dendrites in the lamina and axons that end at various depths in the medulla [27]. To stain the crab's LMCs, we applied fluorescent dextran in the lamina (Figure 5A). In the medulla, we recognized stained R8 photoreceptor terminals, tangential cells, and other cell types, but the most prevalent elements were columnar projections that mainly arborize at different depths in the neuropil (Figures 5B and 5C). An outstanding feature of these terminal arborizations is their relatively large size (Figures 5D and 5E) [27]. Single cartridge monopolar cells (M1–M4) have been electrophysiologically characterized in the crayfish [29]. In brief, M1–M4 are nonspiking neurons that exhibit a hyperpolarizing light response and that frequently present a depolarizing off response. Likely because of the large size of their terminals, we were able to record intracellularly from several of these units. Figure 5G shows the electrophysiological response of a crab LMC to a light pulse, and Figure 5H shows the response of the same cell to a massed training with the VDS. In none of the LMCs we have recorded from did we find a significant reduction in the amplitude of their response to massed training with the VDS (averaged percentages of the initial response at trial 15 = 103% ± 6.1%, t test, $p = 0.62$, $n = 11$, data not shown).

The fact that we found no changes in the response of the recorded LMCs does not preclude the possibility that a specific subtype of these cells or any other type of cells that conveys visual information to the medulla could be affected by VDS repetition. Therefore, we performed a population analysis by conducting optical recordings in the medulla terminals of lamina-projecting neurons. For this purpose, we applied dextran Calcium Green 1 into the lamina, which stained the neurons that connect it to the medulla (Figures 5B–5F). Given the shape of the lamina, it was difficult to apply the dye within the area that maps the visual region stimulated with the VDS. However, in a few preparations, we obtained consistent responses to a light pulse ($n = 5$), and in one preparation the response to the VDS was strong enough to be confidently evaluated. In these experiments, we defined only one large region of interest, in which we analyzed the population's calcium response. The response of the neurons that projected into the medulla to a light pulse was a tonic response (Figure 5I). In the preparation in which we obtained a confident response to the VDS (the one shown in Figures 5B and 5C), the response consisted of a complex signal that showed no evident change in the amplitude or in the temporal profile along massed training with the VDS (Figure 5J). The evidence obtained from the electrophysiological and calcium imaging

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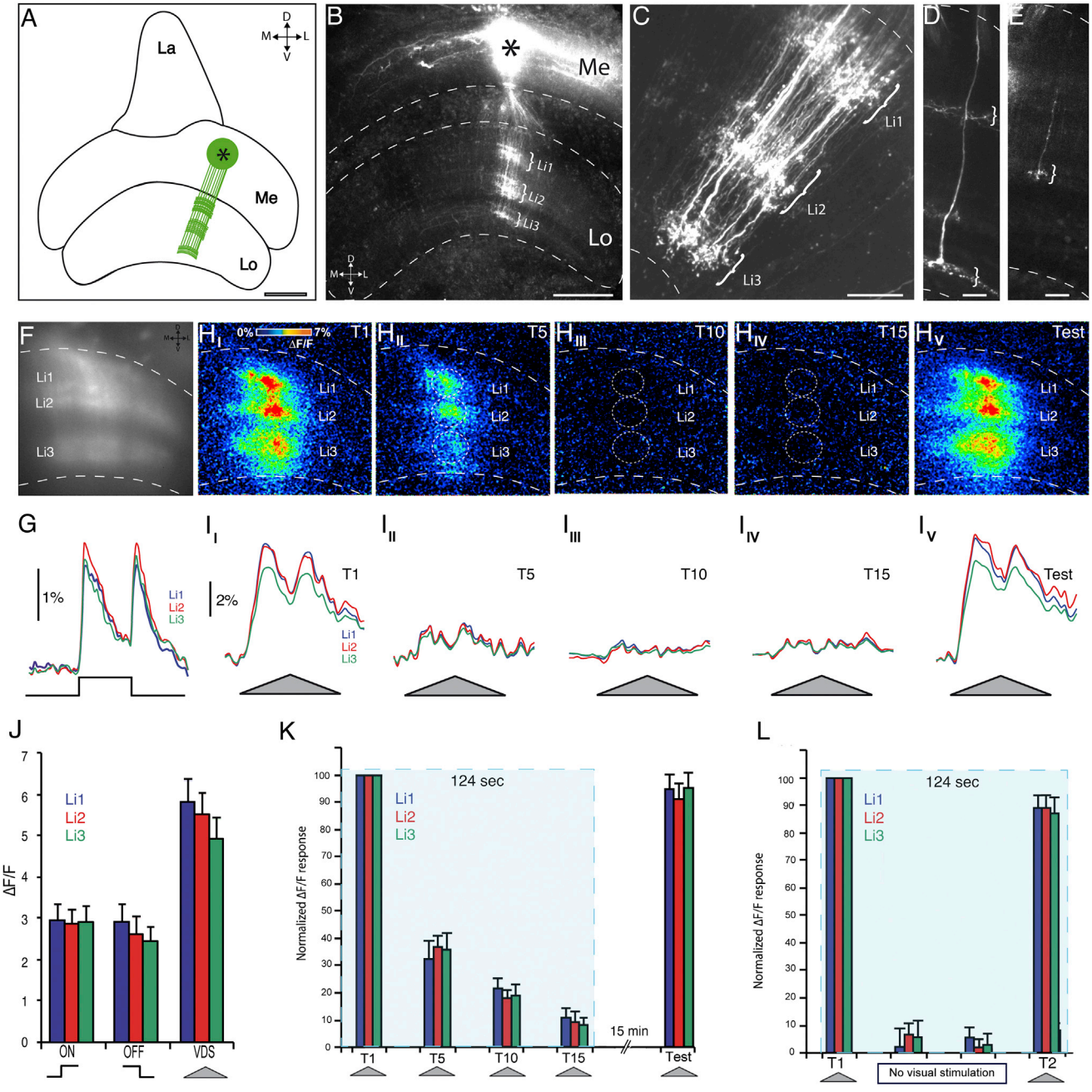

Figure 3. The Calcium Response of Lobula-Afferent Columnar Neurons Declines with Repeated VDS Presentations

(A) To stain the projections of medulla columnar neurons reaching the lobula, we applied fluorescent dextran in the medulla (asterisk).

(B) Confocal image obtained from such a staining. The projections of medulla columnar neurons arborize at three main lobula regions, called lobula input stratum 1, 2, and 3 (Li1, Li2, and Li3, respectively).

(C) Enlargement of the lobula input strata in another preparation.

(D and E) At the borders of the mass staining, individual neurons can be recognized. The neuron of (D) possesses its arborizations staggered at Li1 and Li3, while the neuron of (E) has arborizations only at Li2.

(F) Image from part of the lobula obtained with the conventional microscope, in which the projections of medulla columnar neurons were stained with calcium green.

(G) Mean relative fluorescence change in one preparation of lobula regions Li1, Li2, and Li3 in response to a light pulse.

(H and I) False-color-coded calcium response to visual stimulation with the VDS is shown in (H_I). Temporal course of fluorescence change in response to the VDS at Li1, Li2, and Li3 is shown in (I_I). Massed training with the VDS produced, at the three lobula input strata, a fast and profound reduction in the calcium response (H_I–H_{IV} and I_I–I_{IV}). Calcium response to a test trial performed 15 min after training is shown in (H_V) and (I_V).

(J) Averaged peak amplitude of the relative change in fluorescence in response to the onset and offset of the light pulse and to single VDS presentation (n = 6).

(K) Normalized average data from massed training with the VDS. The percentage of initial response at trial 15 was as follows: Li1, 10.1% ± 3.9%; Li2, 9.2% ± 3.7%; Li3, 8% ± 2.3% (t tests, $p < 0.001$; n = 6). The percentage of initial response at the test trial was as follows: Li1, 94.7% ± 5.7%; Li2, 91.9% ± 5.5%; Li3, 95.4% ± 5.6% (n = 6).

(legend continued on next page)

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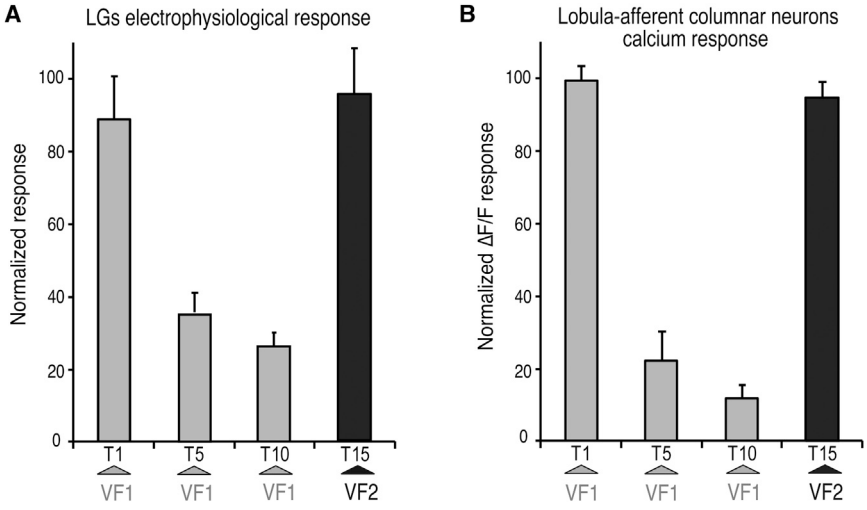

Figure 4. The Response Reduction Caused by VDS Repetition Is Retinotopically Specific

We presented 14 trials with a VDS located in one part of the crab visual field (VF1) and the following trial (trial 15) with an identical VDS located in a neighboring visual field (VF2). The angular distance between both of the stimulated visual fields was 10°. Because there could be variations in the responses evoked in different visual fields, a baseline trial with each one of the two VDSs was performed 10 min before the training and was used to normalize the responses in subsequent trials.

(A) Electrophysiological results from intracellular recordings of the LG neurons. The percentage of an initial baseline response at trial 15 was 96.8% ± 12.4% (t test, p = 0.81; n = 10).

(B) Calcium imaging results from lobula-afferent columnar neurons recorded as in Figure 3. Because the responses to massed training of the three input strata showed no differences (Figure 3K), here we averaged them. The percentage of an initial baseline response at trial 15 was 94.6% ± 4.5% (t test, p = 0.29, n = 6).

Graphs show means ± SEMs.

experiments of this section indicates that the visual information conveyed into the medulla by the neurons of the lamina is not affected by repetitive object motion.

Discussion

A fundamental function of the brain is to filter out the information that proves to be biologically irrelevant. Consequently, the reduction of behavioral responses to recurring visual object motion is a widespread phenomenon. In the crab, we have shown that behavioral changes consistently follow the reduction of LG neurons upon repetitive stimulation [2, 15]. By performing optical recordings, here we present conclusive evidence that the response decrement of the LGs to repetitive object motion arises by a drastic decrement in the response of retinotopic columnar elements that convey information from the medulla to the lobula. This decrement is restricted to the trained retinotopic elements, which enables the visual system to respond to novel motion stimuli appearing in new areas of the retina.

Implications of the Site of Response Decline

Each point on the compound eye retina of arthropods is represented at the first optic chiasma by more than 15 different units [27], whereas at the second optic chiasma, it is possibly represented by as many as 50 different units [30]. This surprisingly high number of elements has important implications with regard to the number of parallel visual channels that originate from only a few photoreceptors [31]. In dragonflies, the existence of descending interneurons in the ventral nerve cord that are sensitive to either self- or object movements indicates that the segregation of these two types of information is preserved downstream from the visual system [8]. Furthermore, whereas the response of the descending neurons that are sensitive to self-movements shows little or no habituation, the response to object movements shows a marked reduction in their activity when confronted with repeated stimulation [8]. Given the wide variety of tasks that visual information must serve, it is clear that adaptive response decrements to visual stimulation cannot occur at the very early stages of visual processing. Our results in Figure 5 confirm this notion because the response of the lamina columnar neurons that convey information into the medulla did not change with repeated visual motion.

The Site of Segregation of Optic Flow and Object Motion Information

In *Neohelice*, the optomotor reaction evoked by rotating a large visual field around the animal shows no apparent decrement after 45 min of continuous visual stimulation (unpublished data). This constancy of response contrasts with the rapid modification of the animal's escape response to repeated object motion (Figure 1E). The lobula giant neurons of the crab, similar to those studied in the lobula of insects, have proved to be unsuitable candidates to steer optomotor responses because they respond much less to panoramic flow than to focal motion and because they readily habituate to panoramic stimulation [11]. The candidate neurons for processing optic flow and guiding optomotor responses in the crab are those located in the lobula plate [12]. If lobula and lobula plate tangential neurons are both fed by columnar elements of the medulla, then why does the optomotor behavior not decline? There are at least two possible explanations. First, there could be two separate neural pathways [32], one that conveys optic flow information and another that conveys object motion information. Second, information regarding flow field and object motion would be involved with the same set of columnar elements, but these elements might react differently depending on the type of stimulation. For example, it could be that wide-field motion, working through a type of neural lateral

(L) Calcium responses in an independent group of animals recorded with the same protocol as in (K) but containing only two VDS trials separated by the time that elapsed between trials 1 and 15. The percentage of initial response at trial 2 was as follows: Li1, 89.1% ± 4.6%; Li2, 89.3% ± 4.9%; Li3, 86.9% ± 5.4% (n = 6).

The dashed line in (H_I)–(H_V) represents the regions of interest selected to analyze the calcium responses to visual stimuli. Scale bars represent 100 μm in (A) and (B), 25 μm in (C), and 15 μm in (D) and (E). Graphs show means ± SEMs.

See also Figure S1 and Movie S1.

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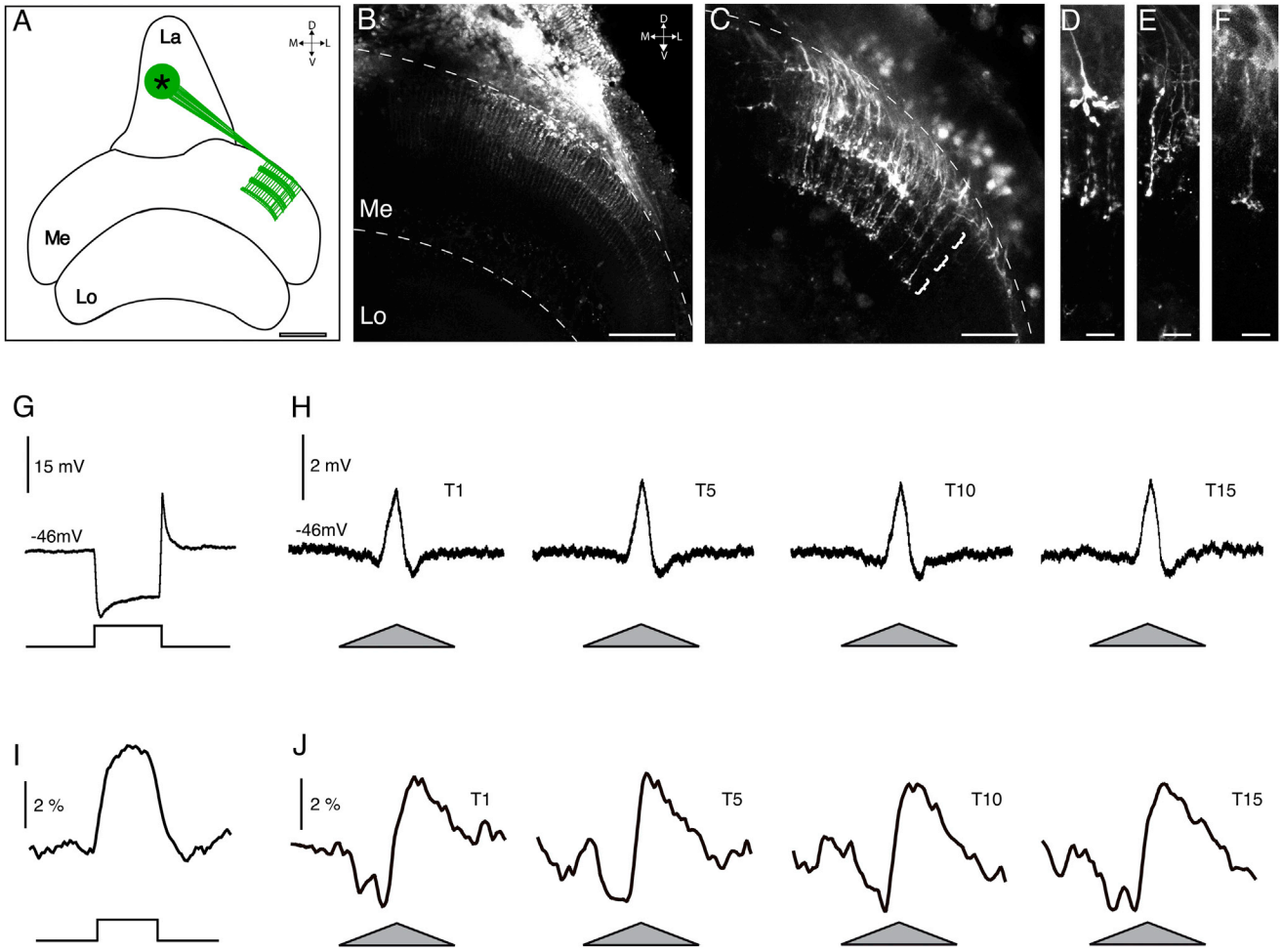

Figure 5. Information Conveyed from the Lamina to the Medulla Is Unaffected by Repeated VDS Presentations

(A) Neural elements that connect the lamina to the medulla were stained by applying fluorescent dextran into the lamina.

(B) Medulla confocal image of neural elements that link the lamina and the medulla.

(C) Close up at a different medulla depth of the projecting stained elements. The periodicity of the stained elements is the same as that of the visual columns [28]. This staining reveals at least one distal stratum with large arborizations and two proximal strata with smaller branches (brackets).

(D–F) Enlargement of a preparation in which individual stained elements can be recognized.

(G) Electrophysiological response of a LMC to a light pulse.

(H) Response of a LMC to 15 VDS training trials. Traces show responses at trials 1, 5, 10, and 15. The receptive field of monopolar cells is small, and the response profile that is recorded depends on when, during the motion, the stimulus shadows or unshadows the cell's receptive field. Quantification from several experiments is reported in the text.

(I) Population optical recording of the relative change in fluorescence to a light pulse of lamina-stained neurons obtained from their medulla neurites.

(J) Population calcium responses to repeated presentations of the VDS (same preparation of pictures in B and C).

Scale bars represent 100 μm in (A), 50 μm in (B), 25 μm in (C), and 5 μm in (D)–(F).

interaction, prevents the response decrement of columnar neurons that occurs with repeated object motion [33]. Further experiments aimed to explore the response of columnar elements to wide field motion will be required to disentangle how sustained versus decremental adaptive responses are addressed at the level of medulla columnar neurons.

Changes in Animal Responses Crucially Depend on the Frequency of Stimulus Presentation

In the field of learning and memory, it is well recognized that spaced training (low-frequency stimulation) provokes long-lasting behavioral changes, whereas massed training (high-frequency stimulation) renders shorter-term changes (e.g., [34, 35]). In *Neohelice*, spaced and massed training with the VDS have completely different behavioral effects. The reduction in the escape response that is induced by spaced training is slow and long lasting (days), generalized to nonstimulated retinotopic areas (i.e., bear translation invariance), and determined by an association with the training context (e.g., [2, 36]). On the other hand, the response changes that are induced by massed training are fast, short lasting, retinotopic specific, and independent of the training context. A large amount of evidence from pharmacological and molecular experiments has shown that the cellular and molecular mechanisms that underlie the behavioral modifications induced by spaced and massed training are completely different (for a review, see [37]). Previous studies [2, 15] indicate that the plasticity that underlies the behavioral changes induced by spaced training occurs in the LG neurons. Contrasting, the results of the present study clearly show that the neural plasticity that

underlies the behavioral changes induced by massed training occurs upstream from the LG neurons, in medulla columnar neurons. The emerging picture suggests that, in addition to the segregation of visual information into different parallel channels and neuropils, within the optic lobes of arthropods, there is also a segregation of the plastic loci and mechanisms that support different types of behavioral adaptive changes.

Experimental Procedures

Crabs used in the present study were *Neohelice granulata* (previously *Chasmagnathus granulatus*) collected in the field and kept in the laboratory as previously described [15]. The visual danger stimulus (VDS) used in behavioral and electrophysiological experiments consisted of the displacement of a black rectangle (subtended angles 30° × 10°). The motion cycle comprised a 90° clockwise and anticlockwise excursion (Figure 1A) that was completed in 2.2 s. For calcium imaging experiments, a computer-generated VDS was displayed on a monitor screen (Sony SDM-HS75P). Its angular size and motion velocity matched that of the VDS used for behavioral and electrophysiological studies. From the early studies in our laboratory, a stimulation trial has been defined as the presentation of two motion cycles separated by 2 s [15]. For clarity, here we show the responses to the first cycle. The training protocol corresponded to what is called massed training (high-frequency trial presentations) with an ITI of 2 s [15].

Our methods for behavioral and electrophysiological recordings have been previously described in detail [15]. The staining method for morphological and for calcium imaging experiments was identical. The optic ganglia were accessed by opening a small window in the cuticle of the eyestalk (Figures 2A and 2B), and a fine glass micropipette with fluorescent dextran conjugates deposited at its tip was gently inserted into the neuropil. The probe was removed after 5–10 s leaving behind a spot of dye. The animals remained undisturbed 2–3 hr to allow for dye uptake and transport within the neurons. Then, for morphological studies the optic ganglia were dissected and imaged with a confocal microscope. For in vivo calcium imaging, the animals were located under a conventional epifluorescence microscope. After the recordings, we dissected the optic ganglia and confirmed with the confocal microscope that the stained neural elements corresponded to the elements previously identified in the morphological studies. A one-sample t test was used to compare the neural responses at last training trial with their initial response level, i.e., a hypothetical value of 100%. For additional experimental details, see the Supplemental Experimental Procedures. Experimental procedures are in compliance with the Principles of Animal Care of Laboratory Animals published by the National Institutes of Health.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, one figure, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.05.061.

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