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Sensitization and Habituation of the Swimming Behavior in Ascidian Larvae to Light

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ABSTRACT—Ascidian larvae of *Ciona intestinalis* change their photic behavior during the course of development. Newly hatched larvae show no response to a light stimulus at any intensity. At 4 hr after hatching, larvae were induced to start to swimming upon the cessation of illumination, and to stop swimming upon the onset of illumination. At a weaker light intensity (5.0×10^{-3} J/m^2·s), the larvae showed similar responses to either a single stimulus or repeated stimuli of onset and cessation of light until 10 hr after hatching. At a stronger light intensity (3.2×10^{-1} J/m^2·s), when the stimulus was repeated, they showed sensitization and habituation of the swimming response.

At 3 hr after hatching the larvae failed to show any response to an initial stimulus at any intensity of light, but after several repeated stimuli (sensitization) they showed a swimming response at light intensities above 4.0×10^{-2} J/m^2·s. At 5 hr and with intensity above 1.0×10^{-2} J/m^2·s, the larvae showed photoresponses to the first stimulus, but after several repetitions the larvae failed to stop swimming upon the onset of light (habituation). A repeated series of stimuli at stronger intensities of light caused greater habituation; this habituation was retained for about 1 min. Since the larval central nervous system in *Ciona* is comprised of only about 100 neurons, learning behavior in ascidian larvae should provide insights for a minimal mechanism of memory in vertebrates.

Key words: ascidian larva, habituation, sensitization, swimming behavior, photoresponse

INTRODUCTION

Ascidians are generally thought to most closely resemble the ancestors of all chordates and their simple tadpole-like larvae share a basic body plan with those of vertebrates (Corbo *et al*., 2001). The larva, composed of only about 2600 cells, has a correspondingly simple nervous system (Nicol and Meinertzhagen 1988a, 1998b; Meinertzhagen and Okamura, 2001). The central nervous system in the ascidian tadpole extends most of the length of the animal and is divided into three parts, an anterior sensory vesicle, a visceral ganglion and a caudal nerve cord (Katz, 1983; Nicol and Meinertzhagen, 1991). The sensory vesicle contains two sensory organs, a gravity sense organ (otolith) and an eyespot (ocellus) (Eakin and Kuda, 1971), which are thought to control the swimming behavior of the larva.

The ascidian larva has a characteristic pattern of behavior consisting of an initial period when it swims upward followed by a period when it swims or sinks downwards (Svane and Young, 1989). Generalized descriptions of ontogenetic changes in larval behavior have been in the literature for nearly 80 years (Grave, 1920; Mast, 1921, Svane and Young, 1989; Tsuda *et al*., 2001). Many authors have noted the marked variability in behavior occurs among the same age cohort within a given species. This is partly because studies of the photic behavior of ascidian larvae are often casual observations made during studies of settlement (sinking downward), metamorphosis or field distributions (Dybern, 1963).

In a previous paper we studied the photic behavior of the larvae of *Ciona intestinalis* by a computerized cell-tracking system (Nakagawa *et al*., 2000; Tsuda *et al*., 2001). Newly hatched larvae swam at an average speed of 1.4 mm/s, but showed no response to light stimuli. By 4 hr after hatching, the average speed of the larvae slowed down to 0.6 mm/s, but they were now induced to swim at the turning off of light. During the course of development the maximal speed of swimming increased with time until 8 hr after hatching and then plateaued. Most of the larvae stopped swimming and started to settle at about 10hr after hatching.

An action spectrum of the motility of the larvae was determined from the responses to the onset and cessation of photo-stimuli of different intensities (Nakagawa *et al*.,

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2000; Tsuda *et al.*, 2001). On the basis of the similarities between the action spectrum of photic larval behavior and Dartnall's nomogram for rhodopsin (Dartnall, 1953), we proposed a "retinal pigment" as the functional photopigment in the ocellus of the ascidian larva. Further evidence for a retinal photoreceptor pigment in the ocellus of the ascidian larva has subsequently been obtained by a retinal protein fluorescence imaging method (Ohkuma and Tsuda, 2000) and by cDNA cloning (Kusakabe *et al.*, 2001).

During the course of the measurements of the action spectrum, we found that larvae show different photic behavior to a stimulus after repeated presentations of the stimulus, behavior which depended strongly on the intensity of light and on the stage of development. In the present work, we investigated the origin of such differences and find they are based on the sensitization and habituation of swimming behavior of the larvae.

MATERIALS AND METHODS

Material

Ciona intestinalis were collected from docks near Murotsu and Aioi, Hyogo, Japan. Individuals were maintained in running seawater at 18°C and continuously illuminated for a few days with a 15 W fluorescent lamp 30 cm from the animals, to prevent uncontrolled spawning. Eggs and sperm were obtained surgically from gonoducts and mixed *in vitro*. Cross-fertilized eggs from each individual were separately maintained at 18°C after several washes with a large volume of artificial seawater (Marine Art BR, Senju, Osaka, Japan). Just before hatching, late tailbud embryos were transferred to an Erlenmeyer flask. About 100 larvae near the surface of the flask were collected with a pipette within 20 min after hatching and transferred to a transparent plastic container, 4 cm × 4 cm square and 1 cm deep. In order to avoid geotaxis of the larvae, the depth of the seawater was 5 mm.

Behavioral observations

The swimming behavior of the larvae was monitored by nonstimulus far-red illumination (wavelength 680-800 nm with the combination of cut-off filter, O-68, and IR-cut filter, IRA-20A: Toshiba, Tokyo, Japan) at 18°C in a constant-temperature incubator (AG-HC090X, Nihon-ika, Osaka, Japan). The stimulus was monochromatic light obtained by the combination of an interference filter (494 nm with a full width of <18 nm at half maximum, KL-series: Toshiba, Tokyo, Japan) and an UV cut-off filter (L39: Toshiba, Tokyo, Japan). The intensity of stimulating light was varied using neutral density filters (ND2, ND4, ND8: Kenko, Tokyo, Japan) in front of a 300 W slide projector. The intensity of stimulus light was measured using a radiometer with a calibrated pyroelectrical crystal detector (Model 4090: YSI, Yellow Springs, OH, and USA). Delivery of the light stimulus was controlled by means of an electromagnetic shutter (C-79-1: Chuo Precision Industrial Co. Ltd., Tokyo, Japan). The shutter was coupled to the digitizing unit of an automated tracing system (Motion Analysis Corp., Santa Rosa, CA, USA) that controlled the delay between an event marker used to initiate data acquisition and the delivery of the stimulus.

The motion of the free-swimming larvae was recorded by an infrared sensitive CCD camera (XC-77: Sony, Tokyo, Japan) that was connected to a video processor. Video data were collected at a frame rate of 10 Hz for a period of 15 s in each experiment. Analysis was done using modular software, "ExpertVision" (Motion Analysis Corp., Santa Rosa, CA, and USA). The video processor detected areas of high contrast, which in this case were the outlines of the bright larvae on a dark background. The center of the profile of each larva was determined. The centers in successive frames were then connected into paths representing the two-dimensional movement of each individual larva in time. The linear speed, in millimeters per second, was defined as the distance between consecutive centers in a path divided by the time taken to travel this distance (Sundberg *et al.*, 1986; Sager *et al.*, 1988)

RESULTS

Photic behavior of larvae in response to a single stimulus

Fig. 1A shows the time course of the swimming speeds of larvae at 6 hr after hatching to the onset (6 s) and the cessation (7.5 s) of a light stimulus (494 nm, intensity 3.2×10^{-2} J/m^2·s) from the slide projector. The swimming speeds of the larvae reached a maximum (difference in speed; 3.00 ±0.15 mm/s) 0.6 s after the cessation of the stimulus and then decreased gradually to the original speed in the dark. Fig. 1B shows the time course of swimming speeds after a three step light stimuli, on (6 s), off (1.5 s) and then on (6

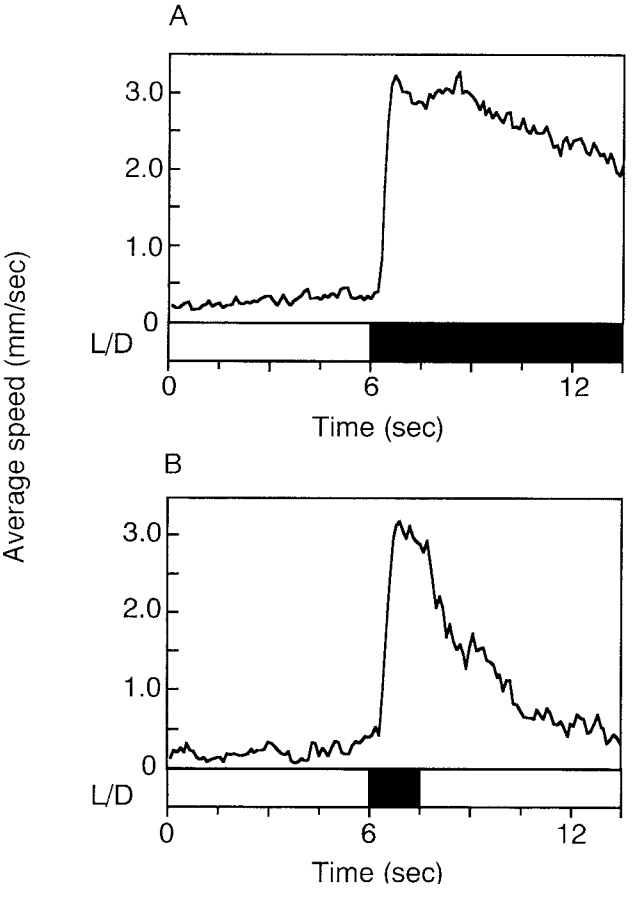

Fig. 1. Photoic behavior of ascidian larvae to an on/off light stimulus. (A) Time course of swimming speeds of the larvae upon onset (6 s, open bar) of the actinic light (494 nm; intensity, 1.3 J/m^{2}s) and then its cessation (7.5 s, solid bar). (B) Time course of swimming speeds upon three step-stimuli, onset (6 s, open bar), darkness (1.5 s, solid bar), and then onset (6 s, open bar) of the actinic light.

s). The larvae started swimming at the cessation of the light stimulus, reached a maximum speed at 0.6 s, and then the swimming speed decreased gradually, as in Fig. 1A. The swimming speed of the larvae slowed abruptly in response to the third stimulus, the onset of light, 1.5 sec after darkness occurred. These results show that larvae start swimming in response to the cessation of light and stop swimming with its onset.

Photoresponse of larvae to repeated light stimuli of low intensity

The photoresponse of larvae to a single light stimulus

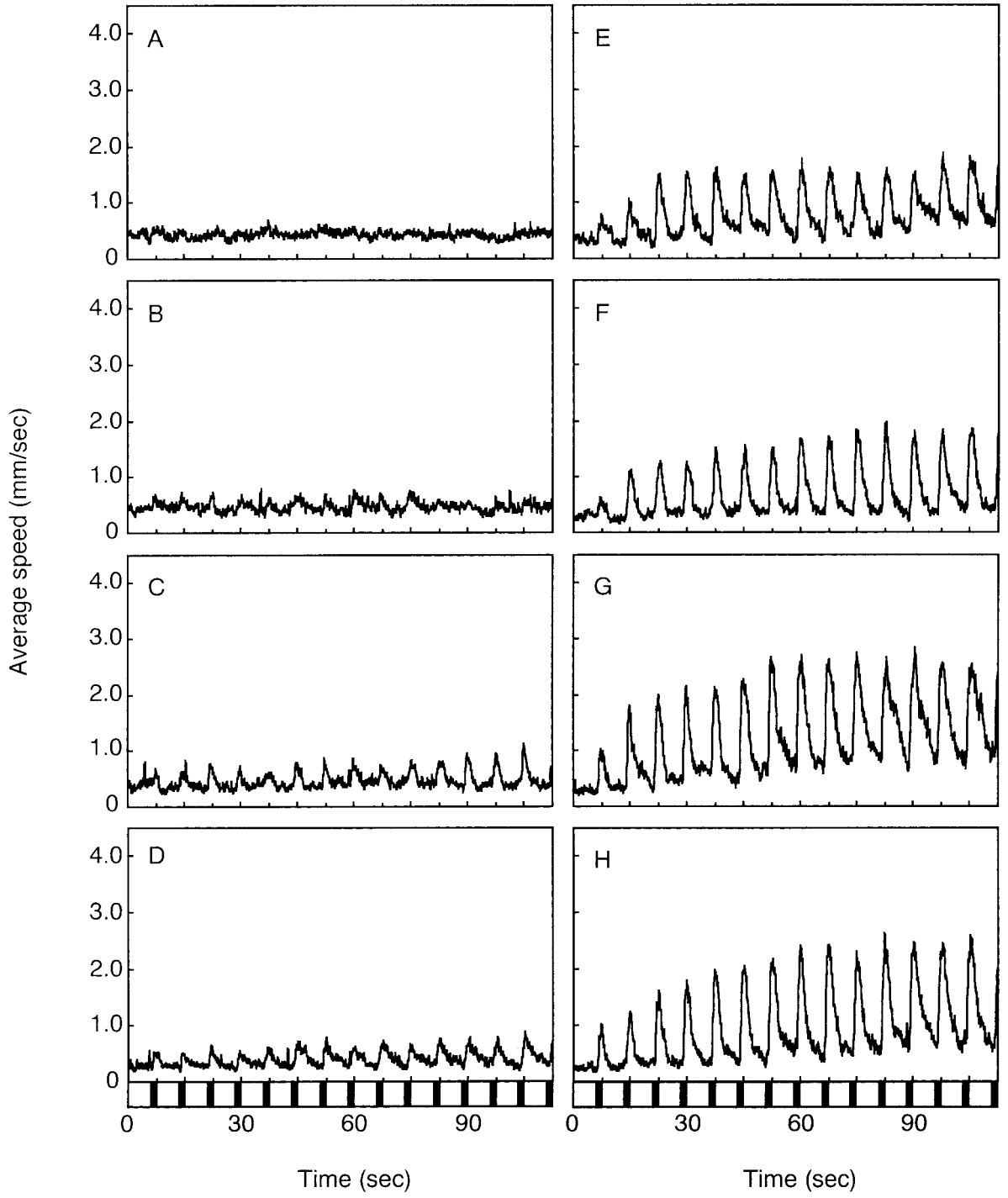

Fig. 2. Time series showing the swimming speeds of larvae after repeated onsets (6 s; open bar) and darkness (1.5 s, solid bar) with a weaker intensity light stimulus (494 nm; intensity, 5.0×10^{-3} J/m^{2}s). Panels A to H correspond to the photobehavior of larvae from 2 to 9 hr after hatching. Each point shows the average speed of about 100 larvae.

changes during development (Nakagawa *et al.*, 2000). Fig. 2 shows a temporal series of swimming speeds in response to repeated stimuli consisting of the onset (6 s) and cessation (1.5 s) of light (494 nm; 5.0×10^{-3} J/m^2·s). Until the 4 hr after hatching, no larval photoresponse was observed to repeated stimuli of a light being turned on and off. The swimming response of larvae to the cessation of the light became observable at 4 hr after hatching. The swimming speeds of the larvae reached a maximum (difference in speed: 0.86±0.24 mm/s) 0.6 s after the cessation of light and then decreased to the original speed. The maximum speed of the larvae to the cessation of light did not change after repeated presentations of the stimulus. Moreover, the swimming speed of the larvae returned to the original speed

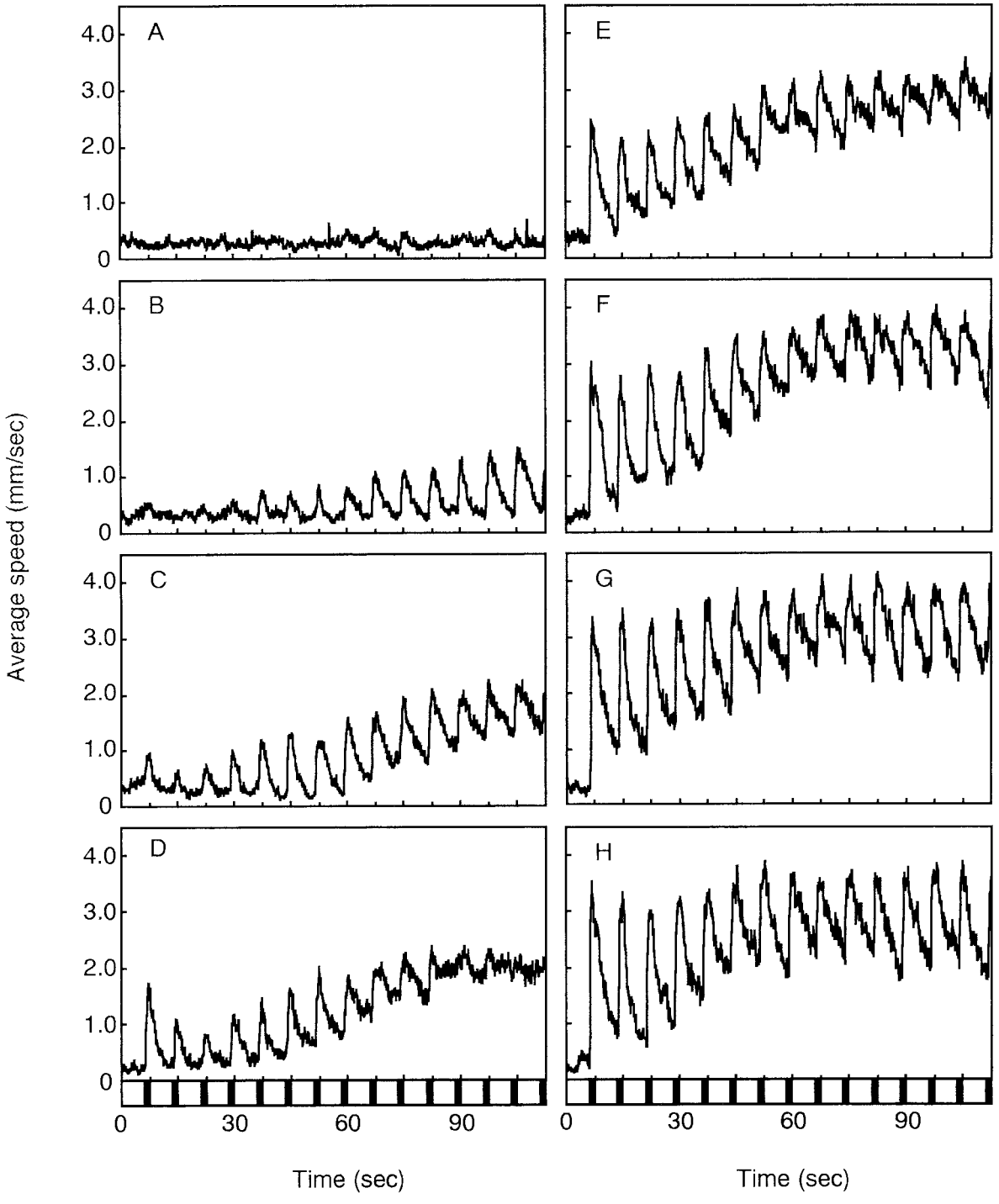

Fig. 3. A time series showing the swimming speeds of larvae after repeated light onset (6 s, open bar) and darkness (1.5 s, solid bar) with a stronger intensity of light (494 nm; 3.2×10^{-1} J/m^{2}s). Panels A to H correspond to the photobehavior of larvae from 2 to 9 hr after hatching. Each point shows the average speed of about 100 larvae.

in response to the onset of the light even after more than 15 repetitions.

High light intensities induce sensitization and habituation of the photic response

When the intensity of the light was increased, the swimming response of the larvae to the onset and cessation of light changed with time. Fig. 3 shows a temporal series of swimming speeds in response to repeated light (500 nm; 3.2×10^{-1} J/m^2·s) onset (6 s) and cessation (1.5 s). Although the larvae did not respond to the first light stimuli 3 hr after hatching, repetition of the same stimulus resulted in a gradual increase in the swimming response. This is a simple form of learning, called sensitization.

By 5 hr after hatching a swimming response of larvae to a single light off stimulus became obvious. At weaker intensities of light, by contrast, the larvae start swimming to the cessation of light and then stop swimming to an onset of light, even when the stimulus is repeated more than 15 times, as shown in Fig. 2. However, if the stimulus is repeated with a stronger light intensity (3.2×10^{-1} J/m^2·s), the larvae continue to start swimming to the cessation of the light, but the response to the light onset gradually declines so that eventually the larvae fail to stop swimming. This cessation of a response to a stimulus after repeated presentation of the stimuli is called habituation. Larvae in 5 hr or more after hatching show habituation to the onset of the stimulus light.

Stimulus intensity dependence of the larva's sensitization and habituation

The swimming response of the larva to repeated on and off light stimuli depends strongly upon the intensity of the light (Figs. 2, 3). We studied light intensity dependence of sensitization and habituation in larvae. The light intensity was varied between 1.3 J/m^2·s and 5.0×10^{-3} J/m^2·s. The temporal series of swimming speeds to repeated stimuli at a given light intensity were measured in two to four independent experiments.

Sensitization of the larva's photic behavior was clearly observed 3 hr after hatching (Fig. 3). At this stage the larvae did not show any photic response to the first few stimuli, but repeated stimuli of stronger light resulted in a gradually increasing swimming response. Since the cessation of light enhances the larva's swimming speed, an enhancement index (E_n) of the swimming response after n repetitions was defined as the area of the average speed of the larva versus time for the time interval between 0 and 1.5 sec in the dark, after the light was turned off (Fig. 4). An index of sensitization (S_n) after n repetitions was then calculated as the difference between E_n and E_1 ($S_n=E_n-E_1$) for a given light intensity. Even though more than 100 larvae were used to obtain the average linear speed of free-swimming larvae for each trial, the values for S_n at a given intensity of light were con-

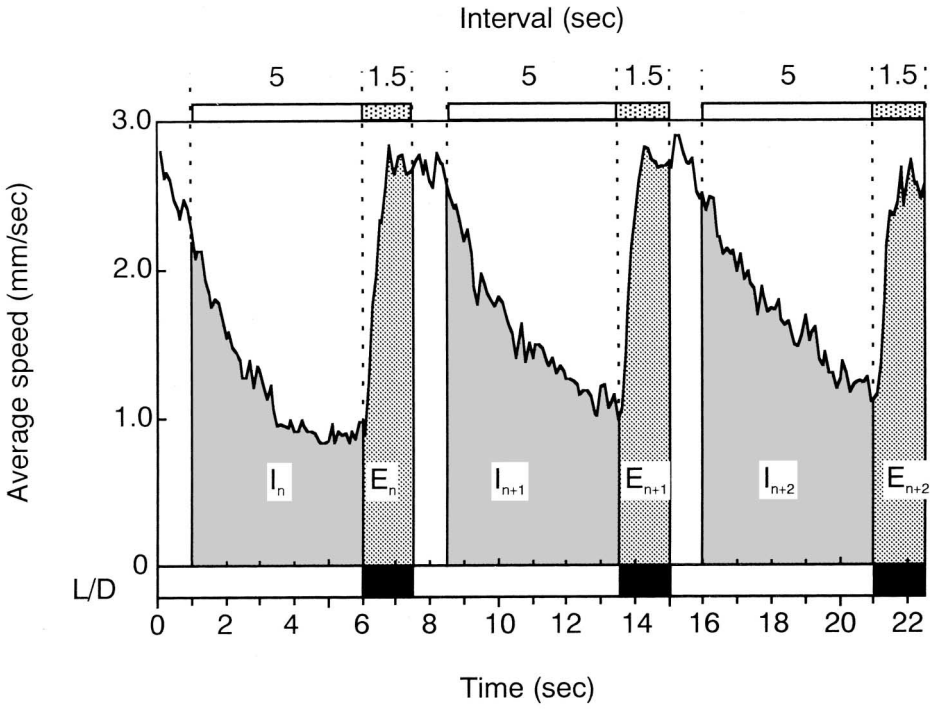

Fig. 4. The index of enhancement (E_n) of the swimming speeds of larvae at n repetitions is defined as the area of the average speed of the larvae between 0 and 1.5 sec in the dark after the light offset. The index of sensitization (S_n) after n repetitions is the difference between E_n and E_1 ($S_n=E_n-E_1$) at each light intensity. An index of inhibition (I_n) of swimming speed at n repetitions is defined as the area of the average speed of the larvae between 1.0 and 6.0 sec after the onset of light. An index of habituation (H_n) after n repetitions is the difference between I_n and I_2 ($H_n=I_n-I_2$) at each light intensity.

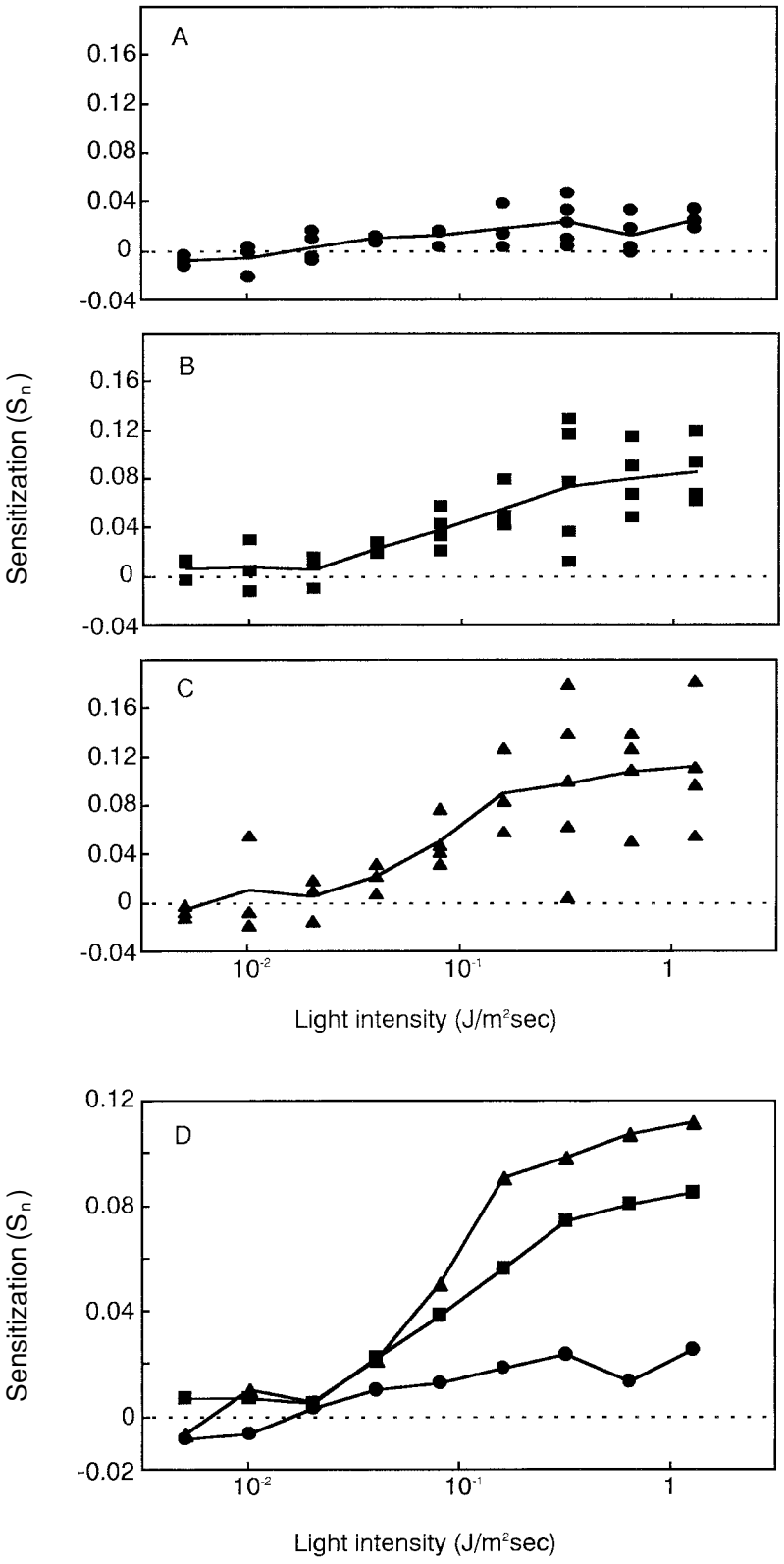

Fig. 5. Values for the index of sensitization (S_5, S_{10}, S_{15}) at the 5th, 10th, 15th repetition of the stimulus as a function of light intensity. A, S_5 (●); B, S_{10} (■); C, S_{15} (▲). For each, a solid line at a given number of repetitions is plotted against the average linear speed of the larvae. These lines are replotted at D.

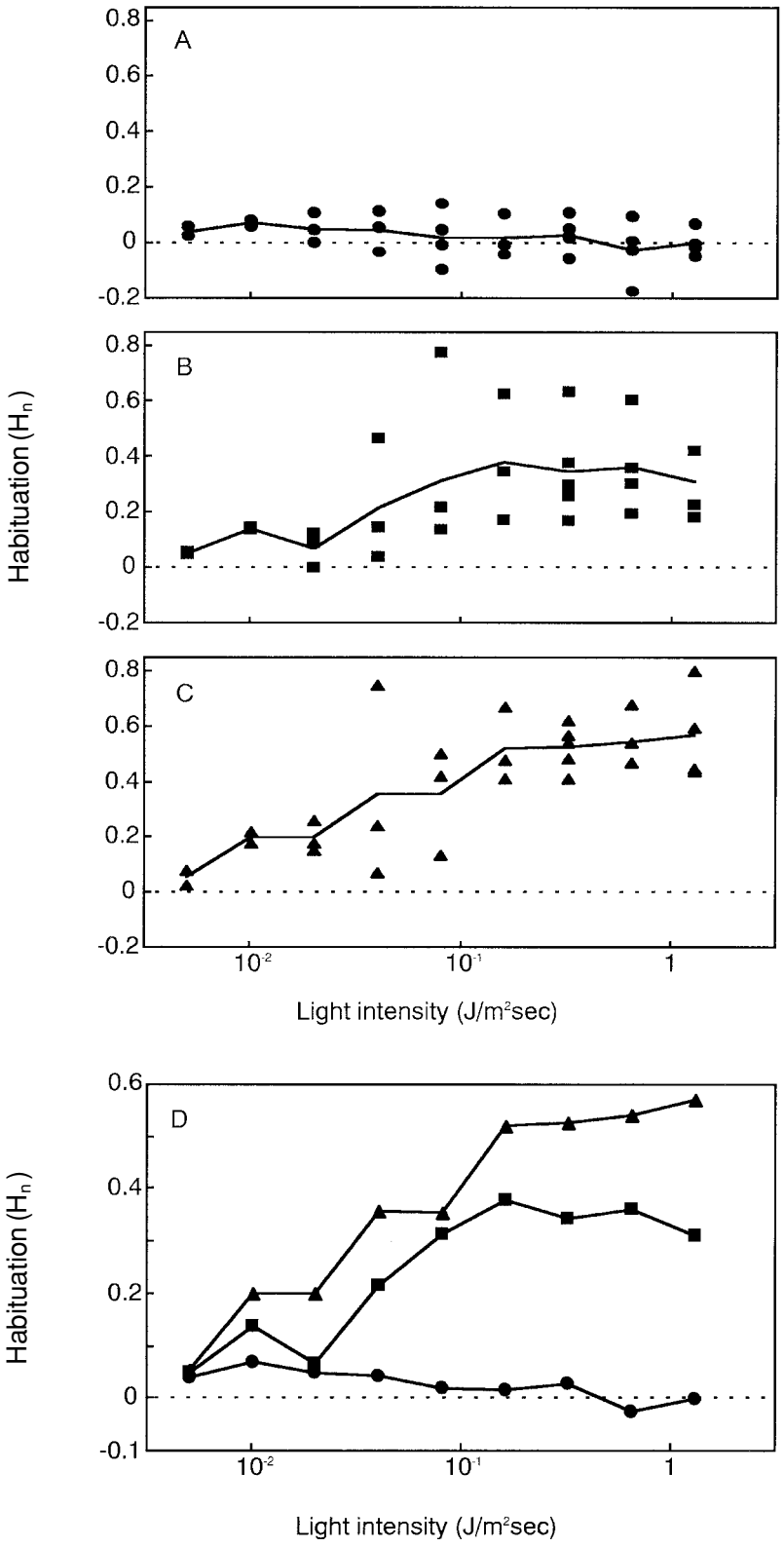

Fig. 6. Values for the index of habituation (H_5, H_{10}, H_{15}) at the 5th, 10th, 15th repetition of the stimulus as a function of light intensity. A, H_5 (●); B, H_{10} (■); C, H_{15} (▲). For each, a solid line at a given number of repetitions is plotted against the average linear speed of the larvae. These lines are replotted at D.

siderably scattered. At least three independent experiments were therefore performed. The values for the sensitization index at the 5th, 10th, and 15th repetition of stimuli (S_5, S_{10}, S_{15}) were plotted against the intensity of light (Fig. 5A, B, C, respectively). The average values for the sensitization index (Fig. 5D) revealed that the larvae show a level of sensitization to repeated stimuli which increased with the number of repetitions at a light intensity at or exceeding 5.0×10^{-2} J/m^2·s.

Habituation of the photic behavior of the larva was observed 5 hr after hatching (Fig. 3). The swimming response of larvae to a single stimulus of light-off became obvious but after repeated presentations of the stimulus the larvae failed to stop swimming to a light-on stimulus. As an index of inhibition (I_n) of the swimming response to a light-on stimulus after n repetitions, we defined the area of the average speed of the larvae between 1 and 6 sec after the onset of light (Fig. 4). An index of habituation (H_n), was calculated as the difference between the values of I_n and I_2 ($H_n = I_n - I_2$).

The values for the index of habituation at the 5th, 10th, 15th repetition of the stimulus (H_5, H_{10}, H_{15}) were next plotted for different intensities of light (Fig. 6A, B, C, respectively). The experiments at a given light intensity were performed at least three times, as before. Since the values of H_n at a given light intensity were scattered, the average values from these independent trials were replotted (Fig. 6D).

These results showed that the larvae show habituation to stimuli repeated more then 10 times over a wide range of light intensities from 1.0×10^{-2} to 3.2×10^{-1} J/m^2·s.

Storage of memory

Next we asked how long ascidian larvae could store the memory of a change in light intensity. Repetition of relatively strong light stimuli results in a gradual decrease in the swimming speeds in response to the light onset among larvae older than 5 hr after hatching (Fig. 3), thus showing habituation. Larvae 5 hr after hatching were exposed to 15 presentations of light-on, light-off stimuli. To examine the duration over which memory is stored, habituated larvae were next kept in the dark for a given period of time and then they were stimulated again by repeated on/off light stimuli. The observed photic behavior of larvae who sat for 30 or 45 sec in the dark, after they had first received a stimulus presentation of 15 times, was unaltered. By contrast, the photic behavior of larvae at 120 sec and 150 sec in the dark, after similar prior presentations of a set of 15 stimuli, had recovered to the level of that to the first stimulus before habituation. The values for the index of habituation (H_{15+t}) were plotted as a function of the period of time (t) for which they were kept in the dark after habituation (Fig. 7). The results thus suggest that habituated larvae can store a memory for about 1 min.

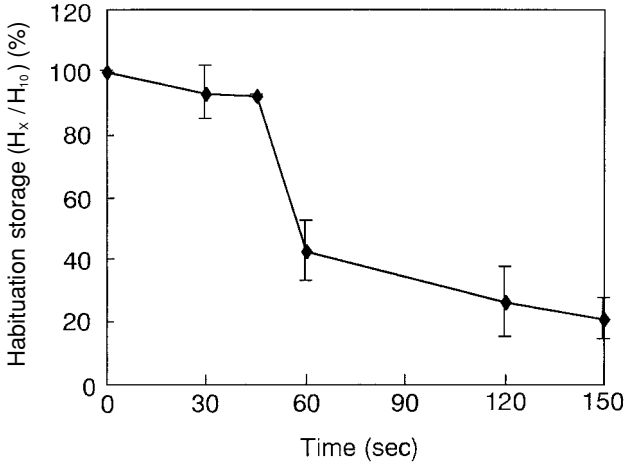

Fig. 7. Larvae 5 hr after hatching were exposed to 15 repeated stimuli of the onset and cessation of light and the habituated larvae kept in the dark for given period of time (t), before being stimulated again by the onset or offset of light. Values for the index of habituation (H_{15+t}) were plotted against the time period (t) in the darkness.

DISCUSSION

Photic behavior of ascidian larvae

Even though the behavior of ascidian larvae is highly variable, both within and among species, the shadow response, in which an abrupt decrease in light intensity elicits active swimming, occurs almost universally (Svane and Young, 1989). We confirm this behavior in quantitative terms for the larvae of *Ciona intestinalis*, which 4 hr after hatching start swimming in response to a decrease of light intensity and stop in response to the onset of light (Figs. 1, 2).

Shadow responses are seen in the larvae and young of both teleosts and amphibians (Blaxter, 1968,1969; Chamalbert *et al.*, 1991; Forward, 1996). Roberts (1978) has, for example, shown that young *Xenopus laevis* tadpoles start swimming when the illumination is dimmed. Pinealectomy of the tadpoles blocks the responses to dimming, but this behavior is not affected when both lateral eyes are removed (Jamieson and Roberts, 2000). This evidence suggests that the pineal eye can initiate swimming when the illumination is dimming (Roberts, 1978; Foster and Roberts, 1982).

It is still not known by what neural pathway light-evoked excitation of photoreceptors can initiate swimming. In vertebrate photoreceptors, ion channels on the plasma membrane are open in the dark, but in the light they close, so that vertebrate photoreceptors hyperpolarize in response to illumination (Tomita, 1965; Ebrey and Koutalos, 2000). Associated with this hyperpolarization neurotransmitter release is interrupted. The pineal complex of lower vertebrates contains photoreceptors similar to those of the retina (Meissl, 1997). These photoreceptors are in synaptic contact with ganglion cells, discharges in which are decreased by light and enhanced by dimming in most animal groups examined (agnathans, elasmobranchs, teleosts and amphibians) (Dodt 1973). An off-response, generated by dimming, in pineal sensory ganglion cells can initiate swimming by neural pathways that must presumably connect ultimately to the motor neurons.

Photoreceptors of ascidian larvae likewise hyperpolar-

ize to light, just as do those of the vertebrate retina (Gorman *et al*. 1971). Our behavioral observations suggest ascidian larvae behave just like *Xenopus laevis* tadpoles do, by starting to swim in response to the turning off of a background light and stopping swimming at the onset of the light. These results suggest that the structure and function of the ocellus of the ascidian larva is essentially same as the pineal eyes of lower vertebrate. An alternative explanation from comparative embryogenesis suggests that the ascidian ocellus is the surviving right partner of the paired lateral eyes of a common ancestor of both chordate and urochordate groups (Sorrentino *et al*., 2000).

The photopigment responsible for phototaxis of the ascidian larva is still controversial. In earlier papers (Nakagawa *et al*., 2000; Tsuda *et al*., 2001) we showed that the action spectrum for photic behavior fitted Dartnall's nomogram (Dartnall, 1953) assuming that the absorption maximum of the pigment is 503 nm. These results suggested that a retinal protein serves as the visual pigment in photoreceptors of the ascidian ocellus. Histochemical methods have been applied to the localization of retinal protein in the ascidian tadpole. The retinal protein is reduced to N-retinyl opsin and the localization of N-retinyl opsin in the larva visualized by the time-resolved difference fluorescence imaging method (Ohkuma and Tsuda, 2000). Fluorescence due to the N-retinyl protein is localized at the posterior wall of the sensory vesicle, that is, to the ocellus. Recently cDNA clones have been obtained showing significant homology to vertebrate opsins (Kusakabe *et al*., 2001; Kusakabe *et al*., 2002). The corresponding mRNA, named *Ci-opsin1,* is expressed solely in photoreceptor cells of the ocellus as assayed by whole-mount *in situ* hybridization. Phylogenetic analysis revealed that Ci-opsin1 is closely related to vertebrate opsins and that there is a clustering of Ci-opsin1 with both the vertebrate retinal and pineal opsins. These combined data, together with behavioral and electrophysiological results on ascidian larvae and lower vertebrates, suggest that the ocellus of the ascidian larva and the pineal eye and lateral eyes of vertebrates have a single origin.

Sensitization and habituation

The simple nervous systems of invertebrates have been widely used in reductionist analyses of habituation, sensitization and classical conditioning. To provide greater understanding of the cellular and biochemical requirements for behavioral plasticity in the short and long term (Kandel, 2001). As distant relatives of ancestral chordate groups, ascidians have been promoted for the study of the features of the vertebrate nervous systems. Not only do ascidian larvae contain some of the smallest numbers of neurons in any central nervous system (Meinertzhagen and Okamura, 2001) but these are housed in a simple tadpole-like larva which shares a basic body plan with vertebrates (Corbo *et al*., 2001). Since the swimming behavior of the ascidian larva is controlled by light, it is presumed to originate with signals arising in the ocular photoreceptors (17 cells: Nicol and Meinertzhagen, 1991) to be processed by cells in the posterior sensory vesicle and to converge on motor neurons (10 neurons: Meinertzhagen and Okamura, 2001) in the visceral ganglion that innervate the tail.

In the present work we report that at different developmental stages, ascidian larvae show simple learning behavior, sensitization and habituation, to repeated on/off light stimuli. At weak light intensities, the larvae show similar response either to stimulus or to repeated stimuli. However, as the intensity of stimulus light increases, the larvae begin to show a quite different swimming behavior to repeated stimuli as compared to a single stimulus. Sensitization of swimming behavior in the larvae can be seen at around 3 hr after hatching. The larvae at this stage do not show photic response to the first stimulus at any intensity of light. However, repetition of the stimulus at stronger intensities of light (3.2×10^{-1} J/m^2·s) results in a gradual increase in the swimming response. These results show that sensitization of swimming behavior of the larvae to repeat stimuli depend upon the intensity.

Sensitization does not appear at low light intensities (less than 2.0×10^{-2} J/m^2·s) even when the stimulus is repeated 15 times. However, sensitization become obvious when the intensity of actinic light exceeds 4.0×10^{-2} J/m^2·s, even when there are only five repetitions. These results suggest there is threshold of light intensity required to induce sensitization of photobehavior in ascidian larvae.

In contrast to sensitization, larvae show habituation to the onset of light at a wide range of light intensities, from 1.0×10^{-2} to 3.2×10^{-1} J/m^2·s, when the stimulus is repeated more than 10 times. Habituation did not appear at the strongest light intensity investigated, 3.2×10^{-1} J/m^2·s, when the stimulus is repeated fewer than five times.

Overall, our results show that the dependencies of sensitization and habituation on light intensity are quite different. Apart from these operational characteristics, the origin of neither sensitization nor habituation in the larval brain is clear. One of the explanations is synaptic, stronger light possibly releasing larger amounts of transmitters from photoreceptor cells or their target neurons, so as to change the strength of the synaptic connections between precisely interconnected cells (Castellucci, *et al*., 1970). In our preliminary observations, nitor-L-arginine methyl-ester (L-NAME) which is an inhibitor of nitric oxide synthase (NOS) completely diminished the habituation of the larvae. The existence of NOS in the posterior sensory vesicles of the ascidian larva has also been demonstrated by the NADPH diaphorase reaction (Kawakami and Tsuda, unpublished). These results suggest that a NMDA receptor-NOS-cGMP signal cascade may be involved in the learning behavior of ascidian larvae.

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