Photoresponse and Learning Behavior of Ascidian Larvae, a Primitive Chordate, to Repeated Stimuli of Step-Up and Step-Down of Light

I. KAWAKAMI, S. SHIRAISHI and M. TSUDA*

Department of Life Science, Himeji Institute of Technology, Harima Science Garden City, Akoh-gun, Hyogo 678-1297, Japan

(**Author for correspondence, e-mail: mtsuda@sci.himeji-tech.ac.jp*)

Abstract. Ascidians are lower chordates and their simple tadpole-like larvae share a basic body plan with vertebrates. Newly hatched larvae show no response to a stimulus of light. 4 h after hatching, the larvae were induced to swim upon a step-down of light and stop swimming upon a step-up of light. At weaker intensity of light, the larvae show the same response to a stimulus after presentation of repeated stimuli. When intensity of actinic light was increased, the larvae show sensitization and habituation of the swimming response to a stimulus after repeated stimuli of step-down and step-up of the light. Between 2 h 20 min and 3 h 40 min after hatching the larvae did not show any response to the first stimulus, but after several repeated stimuli they show swimming response to a step-down of light. A repeated series of stimulus cause sensitization. Between 4 h and 7 h after hatching, the larvae show photoresponse to the first stimulus, but after several repetition of the stimuli, the larvae could not stop swimming to a stimulus of a step-up of the actinic light. A repeated series of stimulus cause greater habituation. Both sensitization and habituation depend upon intensity of actinic light.

Key words: ascidian, habituation, larva, memory, photic behavior, sensitization, vision

1. Introduction

Ascidians are sessile marine invertebrates, but newly hatched tadpole larvae show the basic body organization of vertebrates [1]. The larva, composed of only 2600 cells, has a primitive nervous system [2, 3]. According to Katz [4] the central nervous system in the tadpole extends most of the length of the animal and is divided into two parts, an anterior prosencephalon and a caudal deuterocephalon. The prosencephalon contains a single large vesicle in which lie two sensory organs; the otolith and ocellus. These two sensory organs are thought to control the swimming behavior of the larvae.

The ascidian larva has a characteristic pattern of behavior consisting of an initial period when it swims upward (positive phototropism and negative geotropism) followed by a period when it swims or sinks downwards (negative phototropism) [5–7]. A generalized description of ontogenetic changes in larval behavior has been in the literature for nearly 80 years [5–7]. Many authors have noted that marked

variability in behavior occurs among the same age cohort within a given species [7]. This is partly because studies of the photic behavior of ascidian larvae are often casual observations made during studies of settlement and metamorphosis and field distributions [8].

In previous paper [9] we studied the photic behavior of the larvae of *Ciona intestinalis* during the course of development by a computerized cell-tracking system coupled to an electronic shutter controlling delivery of photo-stimuli with a time resolution of 0.1 s. Newly hatched larvae swim at an average speed of 1.4 mm/s, but show no response to light stimulus. 4 h after hatching, they were induced to swim by a sudden decrease in light intensity. During the course of development the maximal speed of swimming behavior increased with time until 8 h after hatching and then plateaued. Most of the larvae stop swimming due to settlement about 10 h after hatching. These results suggest that the larvae were induced to swim upon a step-down of the actinic light.

An action spectrum of motility of a hundred larvae were determined upon step-up and stepdown of photo-stimuli with different wavelengths and different intensities of light. On the basis of remarkable similarities of the action spectrum of photic behavior of larvae with the Dartnall's nomogram of rhodopsin [10], we propose a 'retinal pigment' as the functional photopigment in the ocellus of the ascidian larva. Existence of retinal protein in the ocellus of the ascidian larva was also confirmed by the time resolved difference fluorescence imaging method [11].

During the course of the measurements of action spectrum, we found the larvae show different photic behavior to repeated presentation of the stimulus, which depend strongly on the intensity of light and on the stage of development. In the present work, we study sensitization and habituation of swimming behavior of the ascidian larvae to a repeated stimulus of step-down and step-up of the light.

2. Materials and Methods

2.1. MATERIAL

Mature adults of *C. intestinalis* were collected from the dock near Murotsu, Hyogo, Japan. Eggs and sperm were obtained surgically from gonoducts and fertilized *in vitro*. Embryos were allowed to develop at 18 °C. Larvae which were collected with a pipette within 10 min after hatching were transferred to a transparent plastic container, 4 cm × 4 cm square and 1 cm deep.

2.2. BEHAVIORAL OBSERVATIONS

Swimming behavior of the larvae was monitored by nonactinic infrared illumination (wavelength 680–800 nm with the combination of cut-off filters, 0–68, and IR-cut filter, IRA-20A, Toshiba, Tokyo, Japan) at 18 °C in a constant-temperature incubator. The stimulation was a monochromatic light obtained by the combination of an interference filter (KL50; wavelength; 493 nm, full width at half maximum

Figure 1. Phototactic behaviors of the larvae were analyzed by the automated tracing system of Motion Analysis. (A) Typical computer generated paths for the larvae in the light (4 sec; dotted lines) and in the dark (4 sec; solid line). (B) Time course of the averaged swimming speed of the larvae was shown in the light (open bar) and in the dark (solid bar). The linear speeds was defined as the distance between consecutive larva-centers in a path divided by the time taken to travel this distance.

less than 18 nm, Toshiba, Tokyo, Japan) and a UV cutoff filter (L39, Toshiba, Tokyo, Japan). Intensity of actinic light was varied using neutral density filters (ND2, ND4, ND8, Kenko, Tokyo, Japan) in front of a 200 W slide projector. Intensity of actinic light was measured with a radiometer with a calibrated pyro-electrical crystal detector (Model 4090, SJI, Yellow Springs, OH). 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 a digitizing unit of the automated tracing system (Motion Analysis Corp., Santa Rosa, CA) 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). The video processor detected areas of high contrast in this case the edges of the bright larvae on a dark background.

3. Results

3.1. MOTION ANALYSIS OF SWIMMING BEHAVIOR OF LARVAE

The movements of ascidian larvae were recorded via video microscopy and videotape images were then digitized and analyzed by computer. The center of area of each larva outline (larvacenter) was calculated. Larva-centers in successive frames

Figure 2. Photoic behavior of ascidian larvae to a stimulus of step up and step down of light. (A) Time course of swimming speeds of the larvae upon a step-up (6 s; open bar) and a stepdown (7.5 s; solid bar) of the actinic light (wavelength; 493 nm, intensity of light was $1.3 \text{ J/m}^2 \cdot \text{s}$) from a slide projector. (B) The time course of swimming speeds upon three step stimuli, a step-up (6 s; open bar) and a step-down (1.5 s; solid bar) and then a step-up (6 s; open bar) of the actinic light.

were then connected into paths representing the two dimensional movement of individual larva in time. Figure 1A shows typical computer generated paths for the larvae in the light (4 sec) and in the dark (4 sec). The results suggest that the traces of most of the larvae in the dark are longer than those in the light.

The linear speeds, in millimeters per second, was defined as the distance between consecutive larva-centers in a path divided by the time taken to travel this distance [12, 13]. Figure 1B shows a response curve described the change in speed as a function of time upon addition of a stimulus. Under illumination, the swimming speed of the larvae is 0.37 mm/s. When the intensity of light was reduced (step-down of stimulus light), the larvae started swimming and the speed of the larvae reached a maximum (difference in speed; 2.0 mm/s) at 0.6 s and then decreased gradually in the dark.

3.2. PHOTIC BEHAVIOR OF LARVAE TO A SINGLE STIMULUS

Previously we reported the photic behavior of the larvae of *Ciona intestinalis* during the course of development [9, 14]. Newly hatched larvae swim spontaneously, but show no response to light stimuli. 4 h after hatching, they were induced to swim by a sudden decrease in light intensity. During the course of development the maximal speed of swimming behavior increased with time until 8 h after hatching and then plateaued. Most of the larvae stop to swim due to settlement about 10 h after hatching. Figure 2A shows time course of swimming speeds of the larvae upon a step-up (6 s) and a step-down (7.5 s) of the actinic light (wavelength; 493 nm, intensity of light was $1.3 \text{ J/m}^2 \cdot \text{s}$) from a slide projector. The swimming speed of the larvae reached a maximum (difference in speed; 3.0 mm/s) at 0.6 s after step-down of stimulus light and then decreased gradually in the dark. Figure 2B shows

the time course of swimming speeds upon three step stimuli, a step-up (6 s) and a step-down (1.5 s) and then a step-up (6 s) of the actinic light. The larvae started swimming to a step-down of the actinic light and reached to maximal speed at 0.6 s and then the swimming speed decreased gradually as Figure 2A. The swimming speed of the larvae slowed down abruptly to a third stimulus, a step-up of the light 1.5 sec after step-down of light. These results show that the larvae start swimming to a step-down of actinic light and stop swimming to a step up of the actinic light.

3.3. PHOTORESPONSE OF THE LARVAE TO REPEATED STIMULI

Figure 2B shows the photoreponse of the larvae to a single stimulus of step-down and step up of light during development. Until 4 h after hatching, no photoreponse of the larvae was observed to a stimulus of step-up and step-down of the actinic light. The swimming response of the larvae to a step-down and step up of stimulus light became obvious 4 h after hatching. Maximum speed of the larvae to a step-down of light stimulus did not change after repeated presentation of the stimulus. Moreover, swimming speed of the larvae reached original speed to a step-up of stimuli after more than 15 times.

When the intensity of actinic light was increased, swimming behavior of the larvae to repeated presentation of the stimulus show different pattern from those in weaker intensity of light. Figure 3 shows a temporal series of swimming speeds upon a repeated stimuli of step-up and a step-down of the actinic light (intensity of light was $3.2 \times 10^{-1} \text{ J/m}^2 \cdot \text{s}$) from 2 h to 4 h after hatching. 2 h after hatching, the larvae did not respond to a stimulus even repeated 15 times. 2 h 40 min after hatching larvae did not respond to first several stimuli but after repetition of stimulus of a step-up and step-down of the actinic light results in a gradual increase of swimming response. During the development, response to a stimulus after several repetition of a step-up and step-down of the light increased. These results suggest that ascidian larvae around 3 h after hatching have an ability of the simple form of learning, sensitization.

As shown in Figure 4, swimming response of the larvae to a first stimulus of a stepdown of light became obvious 5 h after hatching. When a stimulus of a step-up and step-down of the actinic light is repeated several times, the larvae continue to start swimming to a stepdown of the actinic light, but larva's response to a step-up of the actinic light declined and eventually can not stop swimming. Since the cessation of a response to a stimulus after repeated presentation of the stimulus, the larvae 5 h after hatching show habituation to a stepup of the actinic light.

Next, we examined the effect of intensity of actinic light on the sensitization of the larvae. Figure 5A shows that the photic behavior of the larvae to repeated stimuli at different intensity of light. At weaker intensity of light, the larvae did not respond to a stimulus even repeated 15 times. When intensity of actinic light reached ($3.2 \times 10^{-1} \text{ J/m}^2 \cdot \text{s}$), the larvae show sensitization.

Figure 3. Sensitization of swimming response of the larvae upon a repeated stimulus of stronger intensity of light. (wavelength; 493 nm, intensity of light was $3.2 \times 10^{-1} \text{ J/m}^2 \cdot \text{s}$) from 2 h to 3:40 h after hatching. a; 2 h, b; 2 h 20 min, c; 2 h 40 min; d; 3 h, e; 3 h 20 min, f; 3 h 40 min after hatching. The larvae did not respond to first 1 stimulus but after repetition of stimulus of a step-up (open bar) and step-down (solid bar) of the actinic light results in a gradual increase of swimming response.

Figure 5B shows that the photic behavior of the larvae to repeated stimuli at different intensity of light 6 h after hatching. At the weak light of intensity, the larvae start swimming to a step-down of stimulus light and then stop swimming to a step-up of light stimulus even repeated more than ten times. However, at the stronger intensity of light ($3.2 \times 10^{-1} \text{ J/m}^2 \cdot \text{s}$), the larvae could not stop swimming to a stimulus of a step-down of light, that is, the swimming speed of the larvae did not reach original speed but increased after repeated presentation of stimulus. These results suggest the larvae around 6 h after hatching show the habituation to step-up of light at stronger intensity ($3.2 \times 10^{-1} \text{ J/m}^2 \cdot \text{s}$).

Figure 4. Habituation of swimming response of the larvae upon a repeated stimulus of stronger intensity of light. (wavelength; 493 nm, intensity of light was $3.2 \times 10^{-1} \text{ J/m}^2 \cdot \text{s}$) from 4 h to 9 h at 1 h interval after hatching. Panels b-f, over 5 h after hatching the swimming response of the larvae to a single stimulus. When a stimulus of a step-up (open bar) and step-down (solid bar) of the actinic light is repeated, the larvae continue to start swimming to a step-down of the actinic light, but larva's response to a step-up of the actinic light declined.

4. Discussion

The present study is concerned with photic behavior of ascidian larvae and ontogenetic changes in learning behavior to repeated stimuli of light. In previous paper, we showed the first quantitative analysis of photic behavior of the ascidian larvae during the course of development. As shown in Figure 2, the larvae start swimming to a step-down of actinic light and stop swimming to a step up of the actinic light. It is still not known how photo-excitation of photoreceptor can control motor-neuron.

In vertebrate photoreceptors, ion channels on the plasma membrane are closed in the light, and neurotransmitters from the synaptic terminal are not released. In the dark, ion channels on the plasma membrane are open and induced release of

Figure 5. Dependency of light intensity on sensitization and habituation of photic response of the ascidian larvae. (A) Sensitization was observed at 3 h after hatching. (B) Habituation was observed 6 h after hatching. Intensity of light at 493 nm is (a) $1.3 \times 10^{-1} \text{ J/m}^2 \cdot \text{s}$, (b) $3.2 \times 10^{-1} \text{ J/m}^2 \cdot \text{s}$, (c) $1.6 \times 10^{-1} \text{ J/m}^2 \cdot \text{s}$, (d) $8.0 \times 10^{-2} \text{ J/m}^2 \cdot \text{s}$, (e); $4.0 \times 10^{-2} \text{ J/m}^2 \cdot \text{s}$.

neurotransmitter. As a result, vertebrate photoreceptors hyperpolarize upon illumination.

Photoreceptors of ascidian larvae hyperpolarize to light, as vertebrate does [15]. Thus, a step-down of actinic light to ascidian larvae induces to release of neurotransmitter from the photoreceptor and a step up of the actinic light stop to release of neurotransmitter. Assuming released neurotransmitter from photoreceptors activates neural networks involved in swimming behavior of ascidian larvae, present results in Figure 2 can be reasonably explained, that is, the larvae start swimming to a step-down of actinic light and stop swimming to a step up of the actinic light.

The photoreceptor responsible for phototaxis of the ascidian larva is still controversial. In previous paper [9, 14] we showed that the action spectrum for photobehavior of the fitted to Dartnall's nomogram [10] assuming the absorption maximum of the pigment is located at 503 nm. These results strongly suggested that a retinal protein serves as photoreceptor the visual pigment of ocellus in the ascidian larva. Histochemical method was applied to identify the localization of retinal protein in ascidian tadpole [11]. The retinal proteins were reduced by sodium borohydride to N-retinyl opsin and the localization of N-retinyl opsin in the larvae were visualized by the time-resolved difference fluorescence imaging method. The fluorescence of the N-retinyl protein is localized at posterior wall in the ventricle of the prosencephalon, that is the ocellus.

Quite recently we obtained cDNA clones showing significant homology to vertebrate opsins in a project analyzing expressed sequence tags of *Ciona intestinalis* larvae [16]. The corresponding mRNA, named *Ci-opsin1*, was solely expressed in photoreceptor cells of the ocellus by whole-mount *in situ* hybridization. Phylogenetic analysis revealed that Ciopsin1 is closely related to the vertebrate visual pigments and clustering of Ci-opsin1 with the vertebrate retinal and pineal opsins.

Robert et al. [17] showed that the pineal eye of *Xenopus laevis* embryos and larvae are essential for swimming responses to light dimming by behavioral and electrophysiological experiments. Present results of shadowing behavior of ascidian larvae and homology of ocellus photopigment to vertebrate pinopsin suggest the origin of vertebrate eye might be pineal like eye and locate inside the brain like ascidian ocellus.

In the present work we report that ascidian larvae showed simple learning, sensitization and habituation, to a stimulus after repeated stimuli of step-down and step-up of the light, at different developmental stage [14]. At weaker intensity of light, the ascidian larvae showed similar response either to a single stimulus or repeated stimuli of the actinic light. However, intensity of actinic light was increased, the larvae to a single stimulus and repeated stimuli showed quite different swimming behavior. Sensitization of swimming behavior of the larvae can be seen around 3 h after hatching. The larvae at this stage did not show photic response to the first stimulus in any intensity of light. However, repetition of stimulus of stronger intensity of light ($3.2 \times 10^{-1} \text{ J/m}^2\cdot\text{s}$) results in a gradual increase of

swimming response. The sensitization of swimming behavior to a step up of light happened only when intensity of actinic light is over ($3.2 \times 10^{-1} \text{ J/m}^2 \cdot \text{s}$).

The ascidian larvae show habituation of swimming behavior 5 h after hatching. After this stage, swimming response of the larvae to a single stimulus of a step-down of light became obvious. As shown in Figure 5B repetition of a stimulus of a step-up and step-down of the actinic light results in larva's response to a step-up of the actinic light declined and eventually can not stop swimming. This is the simple form of learning, habituation.

Ascidian larvae showed two different forms of learning behavior sensitization and habituation, at different developmental stage. Origin of sensitization and habituation is not known in the present work, but it may be assumed to be a consequence of the complete development of the central nervous system of the larva, which is composed of 40 neurons [2, 3].

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan to M.T. (Nos. 07408019, 11480194, and 13480223).

References

1. Satoh, N.: *Developmental Biology of Ascidians*, Cambridge Univ. Press., 1994.
2. Nicol, D. and Meinertzhagen, I.A.: Development of the Central Nervous System of the Larva of the Ascidian, *Ciona intestinalis* L. I. The Early Lineages of the Neural Plate, *Dev Biol.* **130** (1988), 721–736.
3. Nicol, D. and Meinertzhagen, I.A.: Development of the Central Nervous System of the Larva of the Ascidian, *Ciona intestinalis* L. II. Neural Plate Morphogenesis and Cell Lineages during Neurulation, *Dev Biol.* **130** (1988), 737–766.
4. Katz, M.J.: Comparative Anatomy of the Truncate Tadpole, *Ciona intestinalis*, *Biol. Bull.* **164** (1983), 1–27.
5. Grave, C.: *Amaroucium pellucidum* (Leidy) from *constellatum* (Verrill). I. The Activities and Reactions of the Tadpole Larva, *J. Exp. Zool.* **30** (1920), 238–257.
6. Mast, S.O.: Reactions to Light in the Larvae of the Ascidians, *Amaroucium constellatum* and *Amaroucium pellucidum* with Special Reference to Photic Orientation, *J. Exp. Zool.* **34** (1921), 148–187.
7. Svane, I. and Young, C.M.: The Ecology and Behavior of Ascidian Larvae, *Oceanogr. Mar. Biol. Rev.* **27** (1989), 45–90.
8. Dybern, B.I.: Biotope Choice in *Ciona intestinalis* L. Influence of Light, *Zool. Bidr. Uppsala.* **39** (1963), 589–601.
9. Nakagawa, M., Miyamoto, T., Ohkuma, M. and Tsuda, M.: Action Spectrum for the Photophobic Response of *Ciona intestinalis* (Ascidieacea, Urochordata) Larvae Implicates Retinal Protein, *Photochem Photobiol.* **70** (1999), 359–362.
10. Dartnall, H.J.A.: The Interpretation of Spectral Sensitivity Curves, *Brit. med. Bull.* **9** (1953), 24–30.
11. Ohkuma, M. and Tsuda, M.: Visualization of Retinal Proteins in the Cerebral Ganglion of Ascidian, *Halocynthia roretzi*, *Zool. Science.* **17** (2000), 161–170.

12. Sundberg, S.A., Alam, M. and Spudich, J.L.: Excitation Signal Processing Times in *Halobacterium halobium* Phototaxis, *Biophys J.* **50** (1986), 895–900.
13. Sager, B.M., Sekelsky, J.J., Matsumura, P. and Adler, J.: Use of a Computer to Assay Motility in Bacteria, *Anal Biochem.* **173** (1988), 271–277.
14. Tsuda, M., Kawakami, I., Miyamoto, T., Nakagawa, M., Shiraish, S. and Gouda, M.: Photore-sponse and Habituation of Swimming Behavior of Ascidian Larvae, *Ciona intestinalis*, In: H. Sawada, H. Yokosawa and C.C. Lambert (eds.), *The Biology of Ascidians*, Springer-Verlag, Tokyo, New York, 2001, pp. 153–157.
15. Gordman, A.L.F., McReynolds, J.S. and Barnes S.N.: Photoreceptors in Primitive Chord-ates: Fine Structure, Hyperpolarizing Receptor Potentials, and Evolution, *Science*. **172** (1971), 1052–1054.
16. Kusakabe, T., Kusakabe, R., Kawakami, I., Satou, Y., Satou, N. and Tsuda, M.: *Ci-opsin1*, a Vertebrate-Type Opsin Gene, Expressed in the Larval Ocellus of the Ascidian *Ciona intestinalis*, *FEBS Lett.*, in press.
17. Roberts, A.: Pineal Eye and Behavior in *Xenopus* Tadpoles, *Nature* **273** (1978), 774–775.