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Direct evidence for the role of pigment cells in the brain of ascidian larvae by laser ablation

Motoyuki Tsuda*, Daisuke Sakurai and Muneki Goda

Department of Life Science, Graduate School of Science, Himeji Institute of Technology, Harima Science Garden City, Kouto 3-2-1, Akoh-gun, Hyogo 678-1297, Japan

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

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Summary

The anterior sensory vesicle of ascidian larvae contains a single large vesicle in which lie two distinct types of pigment cells, anterior and posterior. The ultrastructure of these pigment cells suggests that the anterior pigment cell is an otolith, presumably used for gravity detection, and the posterior pigment cell is an ocellus, used for photoreception. However, there is no direct experimental evidence for this assignment of function. Upward swimming behaviour occurring during the initial period of larval life was examined before and after laser ablation of the anterior pigment and posterior pigment cells. Posterior pigment cell-ablated larvae retained the upward swimming behaviour, but anterior pigment cell-ablated larvae lost it. These results suggest that the anterior pigment acts as a gravity sensor. The negatively phototactic swimming during the latter part of larval life was also examined before and after laser ablation of the anterior pigment or posterior pigment cells. Anterior pigment cell-ablated larvae retained the phototactic response, but posterior pigment cell-ablated larvae lost it. These results suggest that the posterior pigment of the sensory vesicle is involved in the negatively phototactic, downward swimming behavior. The effect of pressure on swimming behaviour was studied, and a putative pressure-detection organ was found not to be involved in the larval swimming behaviour. These are the first published experimental results that permit a functional role in ascidian larval behavior to be assigned to the sensory organs.

Key words: otolith, ocellus, eye, ascidian, larva, swimming behavior.

Introduction

Ascidians are considered chordates, because their tadpole-like larvae share a basic body plan with those of vertebrates (Sorrentino et al., 2000; Corbo et al., 2001). The classical close relationship between protochordates and the vertebrates, based on similarities in embryological development and the ascidian larval structural plan, has received recent support from molecular studies. On the basis of gene expression, a correspondence between the classic partitioning of vertebrate brain and the structure of the ascidian larval nervous system has been proposed (Wada and Satoh, 2001).

The ascidian larva, composed of only 2600 cells, has a primitive nervous system that is said to comprise only about 100 neurons (Nicol and Meinertzhagen, 1988a,b; Meinertzhagen and Okamura, 2001). Not only do ascidian larvae have among the smallest numbers of neurons in any central nervous system (Meinertzhagen and Okamura, 2001), but the central nervous system (CNS) in the tadpole also 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 anterior sensory vesicle is a single large vesicle in which lie two types of pigment cells, the anterior and posterior pigment cells, called the otolith and the ocellus, respectively. The ultrastructure of these pigment cells has been described by Dilly (1969, 1962), Eakin and Kuda (1971), Torrence (1986) and Ohtsuki (1991). The otolith is a spherical mass of pigment granules connected to the floor of the sensory vesicle by a narrow stalk. The ocellus is cup-shaped and contains many small pigment granules. In addition to the ocellus and the otolith, a third type of presumptive sensory organ has been described in some species, consisting of globular bodies with membranous tubules and a ciliary organ. The function of this sensory receptor is unknown. Eakin and Kuda (1971) proposed that it may function as a hydrostatic pressure detector, but others (Dilly, 1969; Reverberi, 1979) have characterised it as a photoreceptor.

The ascidian larva has a characteristic pattern of swimming, consisting of an initial period when it swims upward followed by a period when it swims or sinks downwards to settle and metamorphose. In nature, the initial phase serves to distribute the larvae, and in the second phase the larvae seek out the undersides of eelgrass blades or other suitable sites for attachment and metamorphosis. The putative sensory organs such as the ocellus, otolith and globular bodies are thought to guide the swimming behaviour of the larvae.

The larvae of *Ciona intestinalis*, one of the species studied in most detail, have been reported to pass through photopositive and photonegative phases (Dybern, 1963). Svane and Young (1989), however, suggest that *Ciona intestinalis* larvae remain photonegative during their entire free-swimming period. Many observations have shown that larvae at the beginning of the free-swimming period swim upward both in the dark and in light. Our observations showed that within 3 h of hatching, the larvae do not show any photoresponse (Nakagawa et al., 1999; Tsuda et al., 2001). These results indicate that a negative response to gravity or hydrostatic pressure effectively leads the larvae to the water surface. Our motion analysis results, which are consistent with many other observations, showed that larvae start swimming when the intensity of light is decreased (Tsuda et al., 2003), suggesting that larvae swim downward due to a negative photoresponse. We also showed that when stimulated by repeated onset and cessation of light, the larvae exhibited sensitization and habituation of the swimming response (Tsuda et al., 2003).

The functions of three putative sensory organs of ascidian larvae – the ocellus, otolith and pressure organs – have been suggested from morphological information alone. To date there has been no direct evidence for their function from physiological and behavioural studies. In the present work, the functions of these putative sensory organs were studied using the behaviour of free-swimming larvae. Geotropism (movement in response to gravity) was studied by observing the upward swimming stage (within 3 h of hatching) with or without laser ablation of the anterior and posterior pigment cells. Phototropism (movement toward or away from light) was studied by observing the photic behaviour of the larvae with or without laser ablation of the anterior and posterior pigment cells. Prestropism (response to pressure) was studied by recording the effect of hydrostatic pressure on the swimming behaviour of the larvae.

Materials and methods

Ciona intestinalis L. were collected from docks near Murotsu and Aioi, Hyogo, Japan. Eggs and sperm were obtained surgically from gonoducts and mixed *in vitro* (Nakagawa et al., 1999). Cross-fertilised 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. The larvae found near the surface of the water due to negative geotaxis were collected with a pipette within 20 min after hatching and used for observations.

Laser microsurgery

Larvae that demonstrated negative geotactic ability were anesthetized with 0.02% MS-222 (Ethyl m-aminobenzoate methanesulfonate, Nakarai, Kyoto, Japan). Larvae in the anesthetized condition were transferred to a chamber in a glass slide with a plastic spacer (6 mm in diameter, 150 μm in depth) and covered with coverslip. They were examined under Normarski DIC optics using an Axioplan 2 microscope (Carl Zeiss, Jena, Germany) attached, *via* the epifluorescence port, to a small nitrogen laser (Micropoint Ablation Laser System; MP VSL337, Carl Zeiss, Jena, Germany) coupled with a dye laser [Coumarin dye (440 nm)]. To ablate the anterior pigment cell in the sensory vesicle (i.e. the otolith), the laser microbeam was focused onto the short stalk that interconnects the vesicle wall and its distal foot in the lumen of the vesicle. The posterior pigment cell (i.e. the ocellus) was ablated by focusing the laser onto the centre of the pigment. Intact control and ablated larvae were transferred to a thin quartz optical cell (10 mm×40 mm×3 mm) at 18°C in a constant-temperature incubator (AG-HC090X, Nihon-ika, Osaka, Japan).

Behavioural observation

Upward swimming behaviour of the larvae

Both intact and ablated larvae were transferred to a thin quartz optical cell (width×depth×length=10 mm×40 mm×3 mm) in order to observe the two-dimensional (2-D) upward swimming behaviour. The larvae in the thin optical cell were illuminated by nonactinic far-red illumination (wavelength 640 nm; 20 far-red photodiodes were placed by the side of the cell) and visualised as bright larvae on a dark background. In a side-on view, bright larvae on the dark background were monitored with a CCD camera (30 frame s^{-1}; Photron FASTCAM-Net, Osaka, Japan) which was rigidly mounted on the side of a constant-temperature incubator (AG-HC090X, Nihon-ika, Osaka). The 2-D movement of a larva in time was analysed using modular software, 'Image Tracker PTV' (Digimo Corp., Osaka, Japan).

Photic behaviour of the larvae

The photic behaviour of 100 larvae was analysed by the ExpertVision system as shown in previous papers (Nakagawa et al., 1999). Intact control and ablated larvae were transferred to a transparent plastic container (width×length×depth, 50 mm×60 mm×15 mm). In order to prevent geotaxis of the larvae, the depth of the seawater was 5 mm. The swimming behaviour of the larvae was monitored by non-stimulus 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 the constant-temperature incubator. 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), a UV cut-off filter (L39: Toshiba, Tokyo, Japan) and neutral density filters (Kenko, Tokyo, Japan) in front of a 300 W slide projector. 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 digitising 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. Analysis was done using modular software 'ExpertVision' (Motion Analysis Corp., Santa Rosa, CA, USA). The video processor detected areas of high contrast which, in this case, were bright larvae on a dark background. The centre of each larva in successive frames was connected into paths representing the 2-D movement of each individual larva in time. The linear speed, in mm s^{-1}, 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).

Swimming behaviour under hydrostatic pressure

The effect of hydrostatic pressure on the swimming behaviour of the larvae was studied. Larvae collected within 3 h after hatching were transferred to the optical quartz cell (width×depth×length=10 mm×40 mm× 10 mm) connected to a gas pressure apparatus (High Select GH-Type; Chiyoda Seiki, Tokyo, Japan).

The larvae in the optical quartz cell were illuminated by nonactinic far-red illumination (wavelength 640 nm; 20 far-red photodiodes were placed by side of the cell) and visualized as bright larvae on a dark background. In side-on view, bright larvae against a dark background were monitored with a CCD camera (30 frames s^{-1}; Photron FASTCAM-Net, Osaka, Japan), which was rigidly mounted on the side of a constant-temperature incubator (AG-HC090X, Nihon-ika, Osaka, Japan). The 2-D movement of a larva in time was analysed using modular software 'Image Tracker PTV' (Digimo Corp., Osaka, Japan).

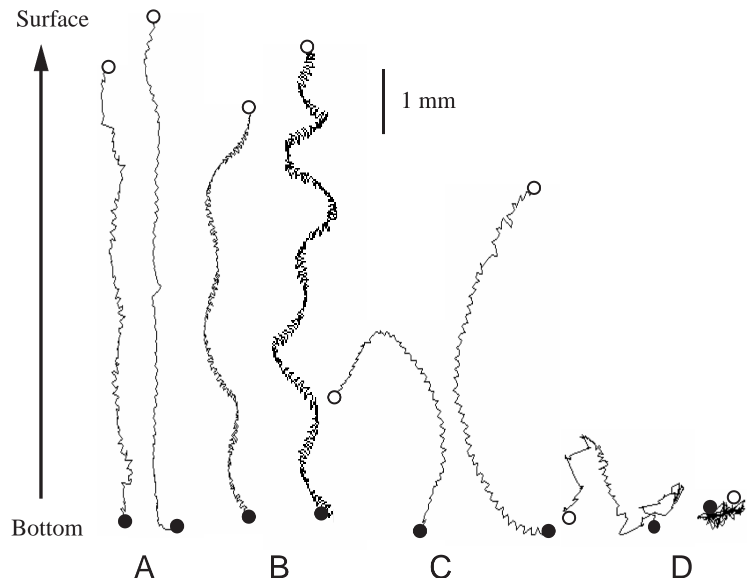

Fig. 1. Swimming paths of intact larvae collected within 3 h of hatching as measured by a CCD camera. The initial position of the larva is marked by a closed circle and the final position by an open circle. Most of the intact larvae swam upward, but followed different paths that were characterized as straight (A), spiral (B), curved (C) and random (D).

Results

Upward swimming behaviour

The ascidian larva has a characteristic pattern of behaviour consisting of an initial period when it swims upward followed by a period when it swims or sinks downward. Newly hatched larvae of *Ciona* in a small container usually swim upwards until they reach the surface film. Once attached they become quiescent. In addition to symmetrical, bilateral tail movements during swimming, larvae exhibit a single or several tail flicks to one side, resulting in several different movement traces.

To determine whether the anterior pigment and/or posterior pigment cells play a role in this tendency to swim upwards, the swimming behaviours of four groups of larvae (intact, pre-anesthetized, anterior pigment and posterior pigment cell-ablated) were tested. 1–2 animals from each group were placed on the bottom of an optical quartz cell, and were recorded with a CCD camera from a side window (10 mm×40 mm) as they swam horizontally. They were illuminated by 20 far-red photodiodes placed on each side of the optical cell.

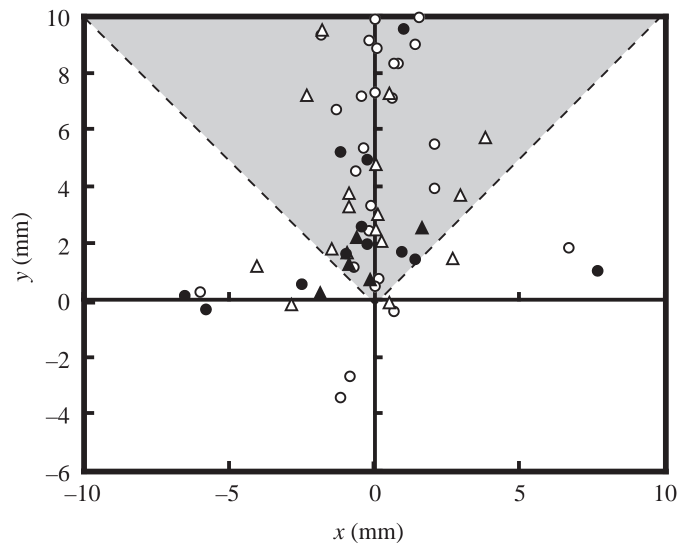

Fig. 2. Swimming direction and pattern of 60 intact larvae in the x–y plane. Each point in the x–y plane shows the final position (x_f, y_f) of the intact larvae after swimming from the initial position at the origin (0, 0). Different symbols denote the different patterns of swimming exhibited by the larvae, i.e. straight (open circle), spiral (open triangle), curved (solid circle) and random (solid triangle). Most of the intact larvae collected within 3h of hatching swam upward. The shaded area represents 45° from the y axis (see Fig. 5).

Fig. 1 shows several traces from CCD recordings of the paths taken by the intact larvae within 3 h after hatching. The x, y coordinates of the swimming paths were measured every 20 ms. This allowed us to plot the 2-D swimming paths and the x, y coordinates of the initial (x_i, y_i) and final (x_f, y_f) positions of the larvae. Most of the intact larvae showed upward swimming behaviour, but produced different patterns of traces such as straight (Fig. 1A), spiral (Fig. 1B), curved

(Fig. 1C) and random (Fig. 1D). Some of the larvae swam diagonally or erratically (Fig. 1D). Swimming directions and patterns of the intact larvae in the *x–y* plane are shown in Fig. 2. Each point in Fig. 2 shows the final position (x_f, y_f) of the larvae after swimming, where the coordinates of the initial position (x_i, y_i) of each larva were adjusted to the origin of the coordinate axes (0, 0). The different symbols used for the points in Fig. 2 represent the different patterns of swimming behaviour of the larvae, i.e. straight (open circle), spiral (open triangle), curved (solid circle) and random (solid triangle). These results suggest that most of the larvae initially (within 3 h of hatching) swam upward, though some of them swam diagonally or erratically.

Fig. 3 shows the sensory vesicle in the prosencephalon of a larva, before and after laser ablation. The laser was focused on either the anterior pigment or posterior pigment cell (Fig. 3A). Ultrastructural studies of the anterior pigment cell suggest that a single pigment-containing cell is connected to the floor of the sensory vesicle by a narrow stalk (Torrence, 1986; Otsuki, 1991). When the laser is focused onto the stalk, the pigment granule detaches from the wall of the vesicle in the lumen, and is thus defocused, as shown in Fig. 3C. The posterior pigment cell is in the pigment cup of the ocellus, and the photoreceptor is located inside the pigment cup. To ablate photoreceptors in the ocellus, the laser was focused onto the center of the posterior pigment cell, as shown in Fig. 3D. Fig. 4 shows swimming directions and patterns of the anterior pigment (Fig. 4A) and posterior pigment (Fig. 4B) cell-ablated larvae in the *x–y* plane.

In order to evaluate any statistical difference between the patterns before and after lesion, the results in Figs 2 and 4 were re-examined. The larvae whose final position was within (shaded area) or beyond 45° of the *y*-axes in Figs 2 and 4 were counted for each swimming pattern and the probability of each swimming pattern was plotted (Fig. 5). The final position of 77% of the 60 control larvae (A in Fig. 5) was located within 45° of the *y*-axes. 72% of the 69 posterior pigment cell-ablated larvae also finally located within 45°, as for the intact controls, but only 27% of the 56 anterior pigment cell-ablated larvae located finally within 45° of the *y*-axes. The majority of the control and the posterior pigment cell-ablated larvae showed a straight or spiral swimming pattern, but those of anterior pigment cell-ablated

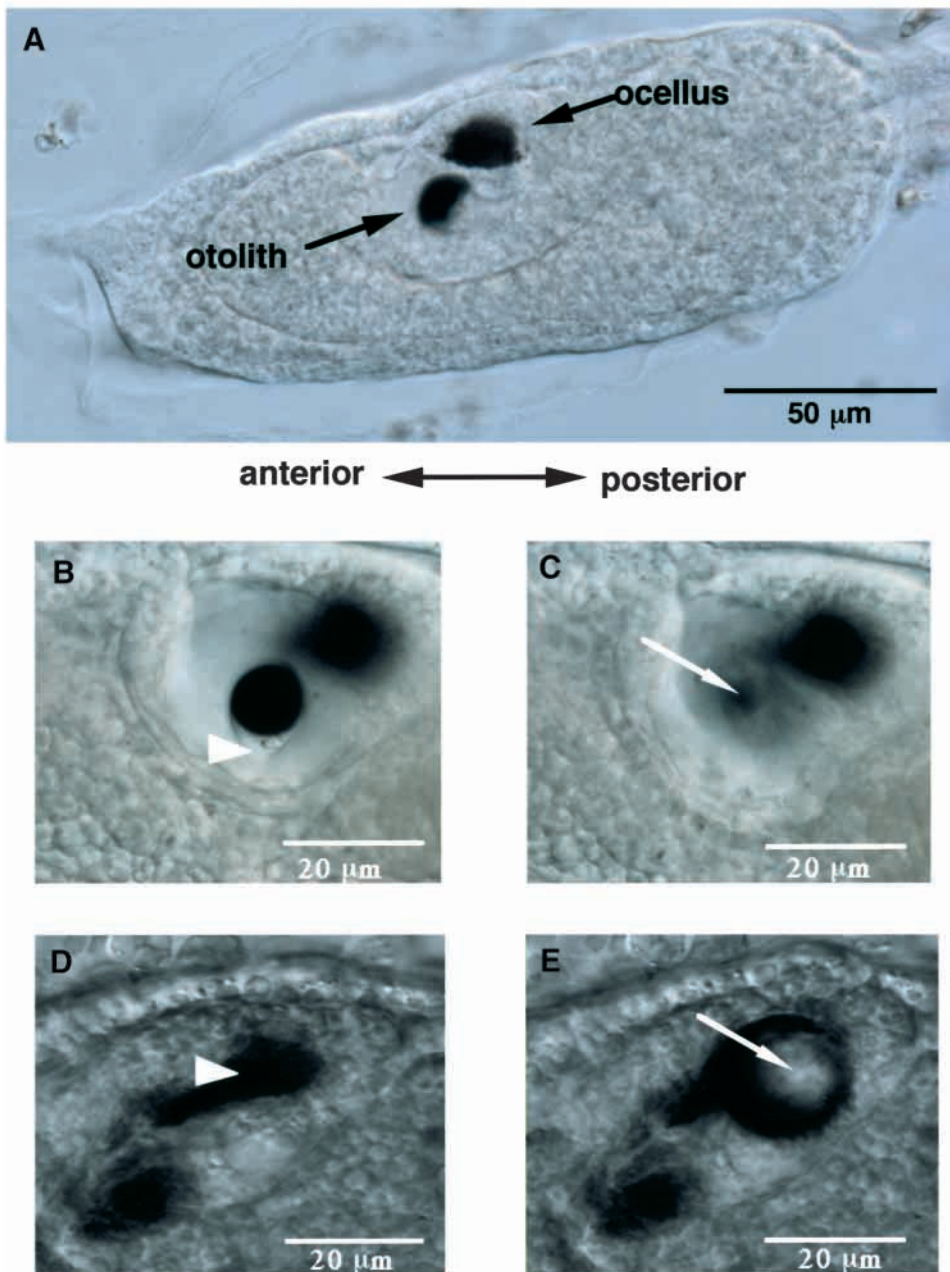

Fig. 3. Laser ablation to the anterior and posterior pigment cells in the sensory vesicle of the larvae. (A) Trunk of the larva, showing the anterior (otolith) and posterior (ocellus) pigment cells. (B) When the stalk of the otolith (arrowhead) is ablated by laser, the anterior pigment cell (C; arrow) is detached from the wall in the lumen of the sensory vesicle and is thus defocused. (D) The laser is focused onto the center of the posterior pigment cell (arrowhead), making a hole was made in the center of the posterior pigment cell (arrowhead) (E). Bars, 50 μm (A); 20 μm (B–D).

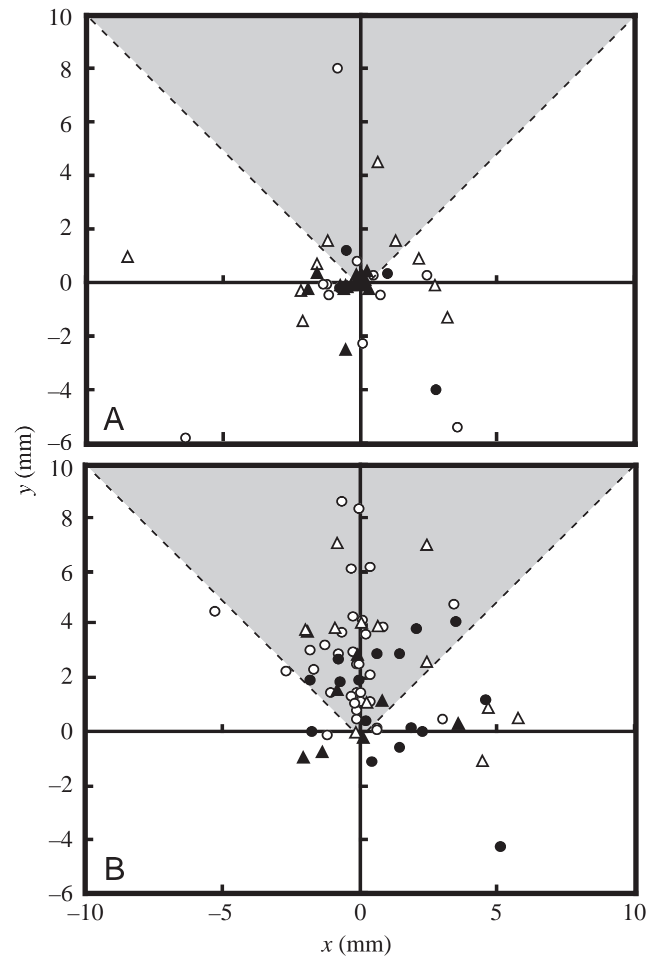

Fig. 4. Swimming directions and patterns of larvae with anterior (A) and posterior (B) pigment cells ablated in the *x–y* plane. (A) The ablation of the anterior pigment cell greatly reduced upward swimming (*N*=69). (B) Upward swimming of the larvae with posterior pigment cells ablated was not affected (*N*=56). Both pigment cell-ablated larvae followed similar swimming paths; straight (open circle), spiral (open triangle), curved (solid circle) and random (solid triangle). The shaded area represents 45° from the *y* axis (see Fig. 5).

larvae showed a random swimming pattern. Thus, ablation of the anterior pigment cell greatly reduced upward swimming and increased the extent of random swimming behaviour, but ablation of the posterior pigment cell affected neither swimming direction nor pattern.

These results suggest that the posterior pigment cell is not linked to the upward swimming behaviour.

Photobehaviour

In previous papers (Nakagawa et al., 1999; Tsuda et al., 2001), we showed that larvae of *Ciona intestinalis* changed their photic behaviour during the course of development. Newly hatched larvae showed no response to a light stimulus at any intensity. 4 h after hatching, larvae were induced to start swimming upon cessation of illumination, and to stop swimming upon onset of illumination (Tsuda et al., 2003). The maximum speed of swimming increased with time up to 8 h after hatching and then plateaued.

Fig. 6A shows swimming speeds of intact larvae with time in response to repeated stimuli consisting of the onset (for 6 s periods) and cessation (for 1.5 s periods) of light (494 nm; 5.0×10^{-3} J m^{-2} s^{-1}) from the slide projector. Intact larvae started swimming when the light was switched off, reaching a maximum speed at 0.6 s, after which the swimming speed decreased gradually. The swimming speed of the larvae slowed abruptly (after 1.5 s) when the light was switched on. These results show that larvae start swimming in response to the cessation of light and stop swimming with its onset. Fig. 6B shows the photoresponse of the pre-anesthetized larvae using the same photo-stimuli as in Fig. 6A, showing that anesthesia had no effect on the photoresponse of the larvae.

After being anesthetized, either the anterior pigment or posterior pigment cell in the sensory vesicle was ablated by laser as shown in Fig. 3, and Fig. 6 shows the swimming photoresponse of larvae, anterior pigment (Fig. 6C) and posterior pigment (Fig. 6D) cell-ablated 5 h after hatching. Anterior pigment cell-ablated larvae start swimming in response to the cessation of light and stop swimming with its onset (Fig. 6C), like the intact or anesthetized controls (Fig. 6A,B, respectively). By contrast, the posterior pigment cell-ablated larvae did not show any response to light cessation or onset (Fig. 6D). These results clearly show that the posterior pigment cell, but not the anterior pigment cell, is responsible for the photoresponsive component of swimming behaviour.

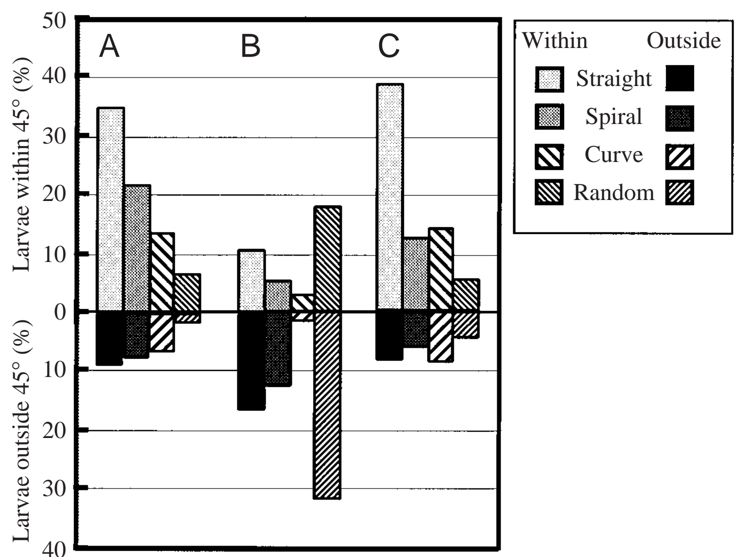

Fig. 5. Statistical difference in swimming behaviour of larvae before and after lesion. The number of the larvae whose final position was located within 45° of the *y*-axis in Figs 2 and 4 (shaded areas) was counted for each swimming pattern and the percentage of each swimming pattern was plotted. (A) The 60 control larvae. (B) The 56 larvae with anterior pigment cells ablated. (C) The 69 larvae with posterior pigment cells ablated.

Effect of pressure on swimming behaviour

Besides the otolith and the ocellus, the larvae of *Ciona intestinalis* possess a third type of presumptive sensory organ. It is situated in the dorsal posterior wall of the neural vesicle and projects into its lumen, and consists of numerous globular or ovoid bodies. Its function is still disputed; according to Dilly (1962, 1969) it is a second type of photoreceptor; Eakin and Kuda (1971), on the other hand, consider it to be a pressure-detection organ. In contrast to the otolith and ocellus, these globular bodies do not contain pigment cells and the diameter of the bodies are as small as 2 μm. The larvae of *Ciona* possess more than ten such bodies and thus it is very difficult to ablate all of them by laser. Instead, we investigated the effect of pressure on the swimming behaviour of the larvae to ascertain whether the pressure organs are involved in controlling the upward and downward swimming behaviours. *Ciona* larvae swim actively in more-or-less regular short bursts, which may last for several minutes, but usually last 1–5 s or less, with interburst intervals of 5–20 s. Pressure was applied to the optical cell containing the larvae 5 s after beginning to record the behaviour by CCD camera. Fig. 7A shows the time profiles of the swimming speeds of the bursts of 12 individual larvae 2 h after hatching. The larvae swam actively in more-or-less regular bursts, which lasted for 1–5s. Application of a hydrostatic pressure of 2 atm (1 atm=1.013×10^5 Pa) was applied to the quartz optical cell (Fig. 7B) neither caused swimming larvae to stop, nor induced stationary larvae to start swimming. The starting times of the individual bursts after the observation in Fig. 7B were measured at five different pressures, from 1.1 to 2.0 atm, corresponding to a sea depth of approximately 1–10 m (Fig. 7C), but no larvae were induced to stop or to start swimming. Thus swimming frequency or swimming period are not affected by pressure, which suggests that the presumed pressure-detection organ is not involved in the control of larval swimming behaviour.

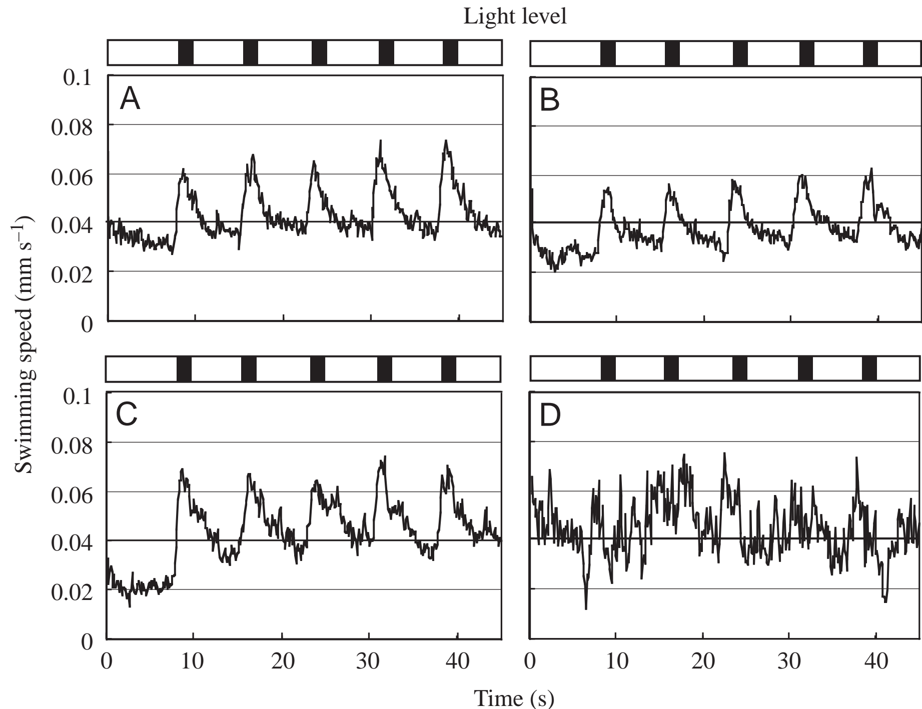

Fig. 6. Swimming speeds of the intact larvae (A), larvae that had recovered from anesthetia (B), anterior- (C) and posterior- (D) pigment cell-ablated larvae in response to repeated stimuli consisting of the onset (6 s light period) and cessation (1.5 s dark period) of light (494 nm; 5.0×10^{-3} J m^{-2} s^{-1}). Intact larvae (A) started swimming when the light was switched off, and slowed swimming speed when the light was switched on. Pre-anesthetized (B) and the anterior pigment cell-ablated larvae (C) showed the same photoresponse as intact larvae. Posterior pigment cell-ablated larvae (D) showed no photoresponse whether the light was switched on or off.

Discussion

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. The putative sensory organs (ocellus, otolith and globular bodies) are thought to control the swimming behaviour of the larvae (Svane and Young, 1989). The anterior sensory vesicle is a single large vesicle in which lies two types of pigment-containing cells, the anterior and posterior pigment cells, respectively called the otolith and the ocellus. The ultrastructure of these pigment cells has been described by Dilly (1962, 1969), Eakin and Kuda (1971), Torrence (1986) and Otsuki (1991). The anterior pigment cell, the otolith, is composed of three parts: a body, which contains the nucleus, the statolith, a spherical mass of black pigment contained within a vacuole, and a narrow neck projecting from the body into the ventral wall of the sensory vesicle and then widening to form a large, anchoring foot. Movements of the pigment-containing statocyte body in response to gravity or inertia are presumably sensed by the receptor (Torrence, 1986). However, the mechanism by which gravity and direction are sensed by the statocyte is controversial. Dilly (1962, 1969) and Eakin and Kuda (1971) proposed that deformation by movements of the statocyte body produces generator potentials, either directly in the adjacent neuron or in the neck of the statocyte, which are then transmitted to the neuron. However, this hypothesis does not suggest great directional sensitivity. Torrence (1986) and Otsuki (1991) found that two dendrites protrude from the wall near the junction of the statocyte and the sensory vesicle, which are responsible for sensing movements of the statocyte. As the

granule moves within the statocyte body, it may induce the deformation of the dendrites, providing the animal with gravity detection.

If this is the case, when the statocyte is removed from the sensory vesicle, the larvae should be unable to recognize the direction of gravity or the direction of the horizontal plane. When the otolith was removed from the sensory vesicle by laser ablation, the larvae lost upward swimming behaviour, as shown in Fig. 4A. One explanation for these results is that without the statocyte body, the otolith lacks a way to deform the dendrites and thus the animal can no longer sense the direction of gravity. Since ascidian larvae do not exhibit a photoresponse at this initial swimming stage, the larvae swim upward by using gravity orientation.

As shown in Fig. 5, the swimming behaviour of the larvae with or without the otolith differs not only in the upward component but also in the pattern of swimming. Because of their placement beside the neck of the statocyte in the intact larvae (Torrence, 1986; Otsuki, 1991), the two dendrites (anterior and posterior), would be differentially deformed and comparison of the activities of the two sensory neurons by ganglion cells could provide the animal with directional information.

The initial period of larval life is characterized by upward swimming. Larvae do not show a photoresponse for the first 4 h after hatching (Kajiwara and Yoshida, 1985; Nakagawa et al., 1999). As shown in Fig. 7, pressure changes between 1.1 and 2 atm did not induce larvae either to start or to stop swimming. Thus it is concluded that the otolith is solely responsible for the upward migration of ascidian larvae after hatching.

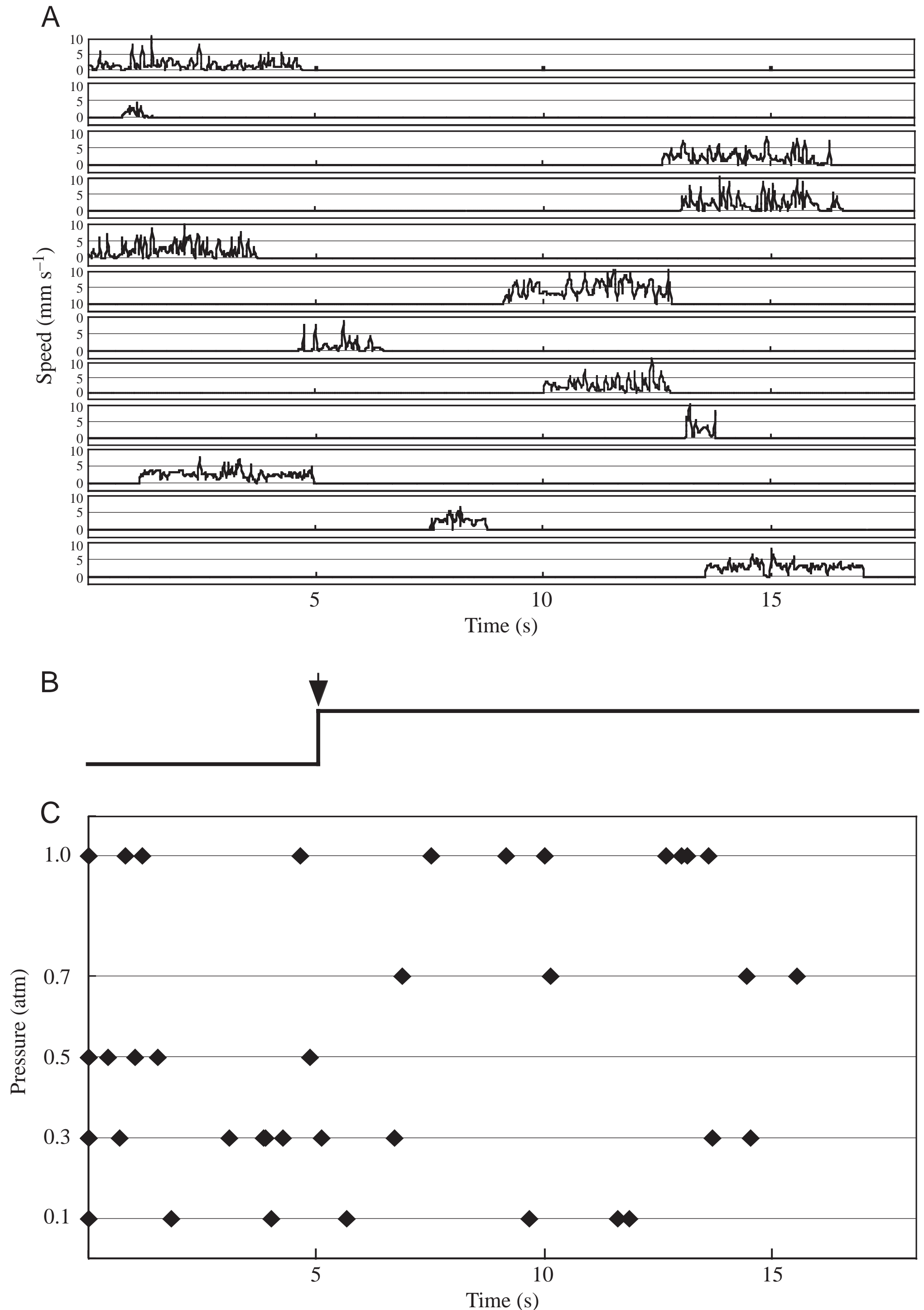

Fig. 7. Effect of hydrostatic pressure on swimming behaviour. (A) Swimming speed of individual larvae within 3 h of hatching at 2 atm pressure. (B) Time course of the hydrostatic pressure, applied 5 s after beginning behavioural measurements (arrow). (C) The time at which larvae began to swim at different pressure levels (1.1 to 2 atm). Swimming was not correlated with application of hydrostatic pressure.

In the latter half of their life, larvae sink downward, which in nature presumably aids them in selecting a suitable site for attachment and metamorphosis. In a previous paper, we showed that larvae of *Ciona* show a response to shading 4 h

after hatching (Nakagawa et al., 1999). They were induced to start swimming upon the cessation of illumination, and to stop swimming upon the onset of illumination (Tsuda et al., 2003). The photopigment responsible for phototaxis in the ascidian larva is still controversial. It was shown that the action spectrum of larval photic behaviour was similar to the absorption spectrum of human rhodopsin (Nakagawa et al., 1999; Tsuda et al., 2001). The presence of rhodopsin in the ocellus was shown by a retinal protein imaging method (Ohkuma and Tsuda, 2000). Three opsins of *Ciona intestinalis* have been cloned and expression patterns in larvae detected by whole-mount *in situ* hybridization. *Ci-opsin1* mRNA was found only in photoreceptor cells of the ocellus (Kusakabe et al., 2001). *Ci-opsin2* is homologous to *Ci-opsin1* and is expressed on the dorsal side of the sensory vesicle, but not in the ocellus (Kusakabe et al., 2002). Since Ci-opsin3 is a retinal G-protein-coupled-receptor (RGR) homologue and functions as a retinal photoisomerase (Nakashima et al., in press), it is not responsible for phototaxis. Thus, the two opsins Ci-opsin1 and Ci-opsin2 are candidates for the visual pigment responsible for phototaxis in the ascidian larvae.

Electron microscopic examination of the *Ciona* larvae shows that the ocellus consists of three parts: the pigment cup, the photoreceptor cells and the lens cells (Dilly, 1964). A single cup-shaped cell filled with membrane-bound pigment granules lies between the lens and the retinal cells. The whole structure of the retinal cells was visualized by antibody against Ci-arrestin (Nakagawa et al., 2002; Horie et al., 2002). The pigment granules were arranged to prevent stray light from falling on the photoreceptor endings. The photoreceptor membranes of the retinal cell were situated inside the concavity of the pigment cup cells, which were stained by antibody against Ci-opsin1 (Kusakabe et al., 2002).

As shown in Fig. 3D, the laser was focused onto the center of the posterior pigment cell to ablate the photoreceptors in the ocellus. The posterior pigment cell-ablated larvae lost all response to a light stimulus, i.e. both the cessation of light and its onset (Fig. 6D). Since the posterior pigment cell contained only Ci-opsin1, Ci-opsin1 is responsible for the photoresponse during larval swimming.

In addition to the ocellus and the otolith, a third type of presumptive sensory organ has been described in some species, consisting of globular bodies with membranous tubules and a ciliary organ. The function of this organ is unknown. Eakin and Kuda (1971) proposed that it may function as a hydrostatic pressure detector; however, the present work shows that the globular bodies are not involved in the swimming behaviour of the larvae (Fig. 7).

In conclusion, the functions of two distinct pigment cells in the brain vesicle were determined by laser ablation. The larvae with anterior pigment cells ablated lost the upward swimming behaviour. Thus, the anterior pigment acts as the statocyte body in response to gravity or inertia and the deformation of the dendrites of mechanoreceptors may provide the animal with a means of gravity detection. The larvae with posterior pigment cells ablated lost all response to a light stimulus. Ci-opsin1, the photopigment of the ocellus, is responsible for the photoresponse during larval swimming. Since swimming behaviour is not affected by pressure, the reason for the presence of any pressure detector remains uncertain.

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