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BEHAVIORAL RESPONSES TO CHEMICAL STIMULATION OF THE OLFACTORY ORGAN IN THE SQUID *LOLIGO OPALESCENS*

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Summary

Behavioral experiments were carried out on restrained, but otherwise fully active, squid to test the chemoreceptive capabilities of the olfactory organ. Specific chemical substances stimulated high-pressure jet escape responses when ejected from a small pipette into the area immediately around the olfactory organ. These included squid ink and L-Dopa (3,4 dihydroxyphenylalanine) as well as agents that block voltage-dependent potassium channels, such as quaternary ammonium ions and 4-aminopyridine. Experiments designed to map chemosensitivity spatially identified the olfactory organ as the receptive site. Unilateral application of a topical local anesthetic to an olfactory organ selectively and reversibly abolished responsiveness on the treated side only. The olfactory organ can thus mediate detection of water-borne chemicals. This detection, in turn, is linked to motor control pathways involved in initiating escape-jetting behavior.

Introduction

Squids, cuttlefishes and octopuses (the coleoid cephalopods) are intelligent, highly mobile predators. Although they are largely visually oriented (Packard, 1972), these animals are also well equipped with complex vestibular (Budelmann, 1990), auditory (Hanlon and Budelmann, 1987; Budelmann and Bleckmann, 1988) and tactile (Wells, 1964; Wells and Young, 1975) capabilities. Sensory pathways for these modalities converge in the central nervous system and regulate important behavioral outputs, such as escape jetting, chromatophore display, mating, homing and long-distance migration (Messenger, 1983; Boyle, 1986).

Chemoreception is another sense that might be expected to be important to these animals, many of which are largely nocturnal, demersal or benthic. Although a rich literature on the chemosensory competence of many marine invertebrates exists (Grant and Mackie, 1974; Atema *et al.* 1988), comparatively little is known about chemoreception or chemotaxis in cephalopods. In *Octopus vulgaris* (Lamarck), chemoreceptors have been described on the sucker cups of the arms (Graziadei, 1962) and are thought to constitute the basis for the chemotactile sense that is evident in behavioral and learning experiments (Wells,

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1963; Wells *et al.* 1985). Morphologically similar receptors occur in the buccal lobes surrounding the beak ('lips') of squids (Emery, 1975) and cuttlefishes (Graziadei, 1965) and it has been proposed that they are involved in the initiation of feeding behavior when food touches the lips (LaRoe, 1971; Emery, 1975).

Evidence for detection of water-borne molecules arising from a distant source *via* a 'distance'-chemoreceptive sense, as opposed to a 'contact'-based chemotactile one, is more scarce in these animals, apparently being limited to two reports on *Octopus* (Boyle, 1983; Chase and Wells, 1986). Essentially nothing is known about the chemoreceptive capabilities of other cephalopods (Boyle, 1986). Morphological studies have revealed putative chemosensory cells in an elaborate structure called the 'olfactory organ' (Messenger, 1967; Woodhams and Messenger, 1974) that is present in all coleoids, and most prominently in squids. This organ is the site of a sensory epithelium containing several types of primary sensory neurons with distinctive morphologies (Emery, 1975; Wildenburg and Fioroni, 1990). In no case, however, has a function for this organ been demonstrated through behavioral or physiological experiments (Messenger, 1967) or have functional properties of the receptor cells been determined.

In addition to these studies on the olfactory organ itself, anatomical work on squid has focused on tracing the central projections of sensory axons. Afferent fiber tracts lead to the 'olfactory lobes' as well as to motor centers involved in the control of swimming and jetting, and relatively direct connections to the giant fiber pathway may occur in the magnocellular lobe (Messenger, 1979; Young, 1939). These findings suggest that sensory inputs from the olfactory organ may be a component of the neural control of escape jetting (Young, 1938; Wilson, 1960; Otis and Gilly, 1990). The optic gland, a region of the brain thought to be involved in the control of sexual maturation and reproduction, also receives major inputs from the olfactory lobes and from olfactory nerve axons (Messenger, 1979).

This paper is the first of two in which we address the function of the squid olfactory organ. Here we provide behavioral evidence that squids can detect water-borne chemical stimuli by means of this organ. These experiments also demonstrate that olfactory organ projections in the central nervous system do influence the motor centers that control escape jetting. The second paper (Lucero *et al.* 1992) describes the electrical characteristics of receptor neurons in the sensory epithelium of the olfactory organ and the manner in which chemical stimuli that produce behavioral responses *in vivo* act to modulate receptor excitability.

Materials and methods

Behavioral experiments and chemical stimulation

All experiments were carried out on *Loligo opalescens* Berry collected locally in Monterey Bay. Techniques for restraining the living animals were similar to those described by Otis and Gilly (1990), but in the present study recordings of neural activity were not carried out. Briefly, the dorsal mantle surface was attached to a

plastic support platform with cyanoacrylate cement, and the restrained animal was suspended in oxygenated sea water at 13–15 °C. The experimental tank had a volume of approximately 30 l, and a slow flow (2–3 l min^{-1}) of fresh sea water was maintained during experiments. Experimenters were shielded from the animal's view, and the animal was observed by means of a video camera. Great care was taken to avoid any sudden noises that could startle the animal and lead to aberrant escape responses. If an animal was deemed to be hypersensitive to spurious stimulation, the experiment was terminated and the associated results were discarded.

Chemical stimuli (test substances added to sea water) were delivered by pressure ejection at low rates, usually one stimulus every 1–2 min. Faster stimulation rates, at least with some substances, can lead to a habituation-like phenomenon that is characterized by intermittent, or even complete, loss of responsiveness (Long *et al.* 1989). In experiments designed to test the efficacy of different substances, 75–300 ms duration pressure pulses delivered fluid at a rate of approximately 1 $\mu l\,ms^{-1}$ from a port 0.65 mm in diameter positioned a few millimeters anterior and lateral to the olfactory organ. The ejection pipette was aimed posteriorly and oriented at an angle of approximately 45–60° with respect to the body axis. Generally, each test stimulus was bracketed by seawater controls. Test and control fluids were extruded only during pressure pulses; at other times the lines were closed to prevent leakage out of or siphoning back into the supply line. For some experiments, the stimulating probe also carried a pair of platinum wires and a fiber optic guide to transmit electrical and photic stimuli. Experiments designed to map sensitivity around the olfactory organ employed ejection pipettes of small diameter (0.2 mm), fluid delivery rates of approximately 2 $\mu l\,ms^{-1}$ and pulse durations of 50–75 ms.

Escape responses were detected as intramantle pressure transients (Otis and Gilly, 1990). The pressure transducer was not sensitive enough to record pressures associated with respiration or weak swimming. Only escape responses that were accompanied by measurable pressure transients occurring with a delay of less than 20 s following a chemical stimulus were scored as positive responses in our analysis of behavioral data. Jetting (and other) behavior was also documented by videotaping. Individual video frames were captured from the recorded tapes with a Sony videoprinter.

All chemicals were obtained from Sigma (St Louis, MO), with the exception of blue food coloring and Brilliant Blue FCF (McCormick, Inc., Baltimore, MD). Squid ink was prepared by mincing 1–4 ink sacs in 1 ml of sea water, briefly vortexing and diluting the sample into 20 ml of sea water. Other natural extracts were prepared by crude homogenization and brief centrifugation.

Electron microscopy

For scanning electron microscopy, the olfactory organ and surrounding tissue were dissected and pinned out for fixation in 2 % glutaraldehyde in artificial sea water containing 0.1 mol l^{-1} Hepes (pH 7.8). Samples were post-fixed in 1 % OsO_4

plus 0.1 mol l^{-1} Hepes and dehydrated in graded ethanol followed by critical point drying. Gold-coated specimens were examined in a Hitachi S-450 microscope.

Olfactory organ/nerve preparations were fixed for transmission microscopy in 1.5 % glutaraldehyde plus 10 mmol l^{-1} $CoCl_2$, 10 mmol l^{-1} $MgCl_2$, 25 mmol l^{-1} sodium cacodylate and sucrose to attain an osmolality of 980 mosmol kg^{-1} H_2O. Specimens were post-fixed with 0.5 % OsO_4, 0.8 % $K_3Fe(CN)_6$, 0.1 mol l^{-1} sodium cacodylate and sucrose, followed by 0.15 % tannic acid in 25 mmol l^{-1} sodium cacodylate plus sucrose, and finally stained *en bloc* with 4 % uranyl acetate in deionized water. Thin sections were examined in a Phillips 201 microscope operating at 80 kV.

Results

General anatomy of the olfactory organ

The location and overall form of the olfactory organ in *Loligo opalescens* is similar to that in *Lolliguncula brevis* (Emery, 1975). The organ is situated ventrally on the anterior aspect of a ridge of tissue that runs dorso-ventrally just posterior to the orbit (Fig. 1A). The sensory organ proper is a small knob-like structure located in a funnel-shaped cavity composed of muscular tissue (Fig. 1B). This knob is covered by a sensory epithelium, composed of several types of receptor cells, all of which project their axons into a well-defined 'olfactory' nerve leading to the brain, and by support cells bearing motile cilia (Emery, 1975; Wildenburg and Fioroni, 1990). The motile cilia are longer and more numerous than the specialized apical processes of the receptor cells, and their high density accounts for the distinctive appearance of the olfactory knob (Fig. 1C,D).

Secretory cells are also present in and around the olfactory knob (Barber and Wright, 1969). Examination of living material (an excised olfactory organ and surrounding tissues) under water-immersion optics reveals a robust flow of mucous over the sensory epithelium. This flow moves upwards and out of the funnel-shaped cavity and probably serves to cleanse the sensory epithelium continuously and to prevent fouling with particulate matter.

The sensory nerve that emanates from directly beneath the olfactory knob contains numerous small axons, 0.1–1.5 μm in diameter, that are wrapped tightly in fascicles by Schwann cell elements and densely staining extracellular material (Fig. 2A). Shortly after exiting from the base of the organ, this nerve is joined by a smaller nerve branch that emerges from the surrounding muscular tissue. Axons in this latter process are larger, 1.5–25.0 μm in diameter, and most are wrapped individually by Schwann cells (see Fig. 2B). The two nerves then run in parallel, though distinctly separated by connective tissues, for a few millimeters ventrally and slightly anteriorly where they enter the cartilaginous 'skull' through a common foramen. The sensory portion of the nerve then passes dorsally along the floor of the orbit to the region of the olfactory lobes of the brain (Messenger, 1979).

Demonstration of chemoreception and links to motor control

Small volumes (typically 100–300 μl) of sea water containing certain test

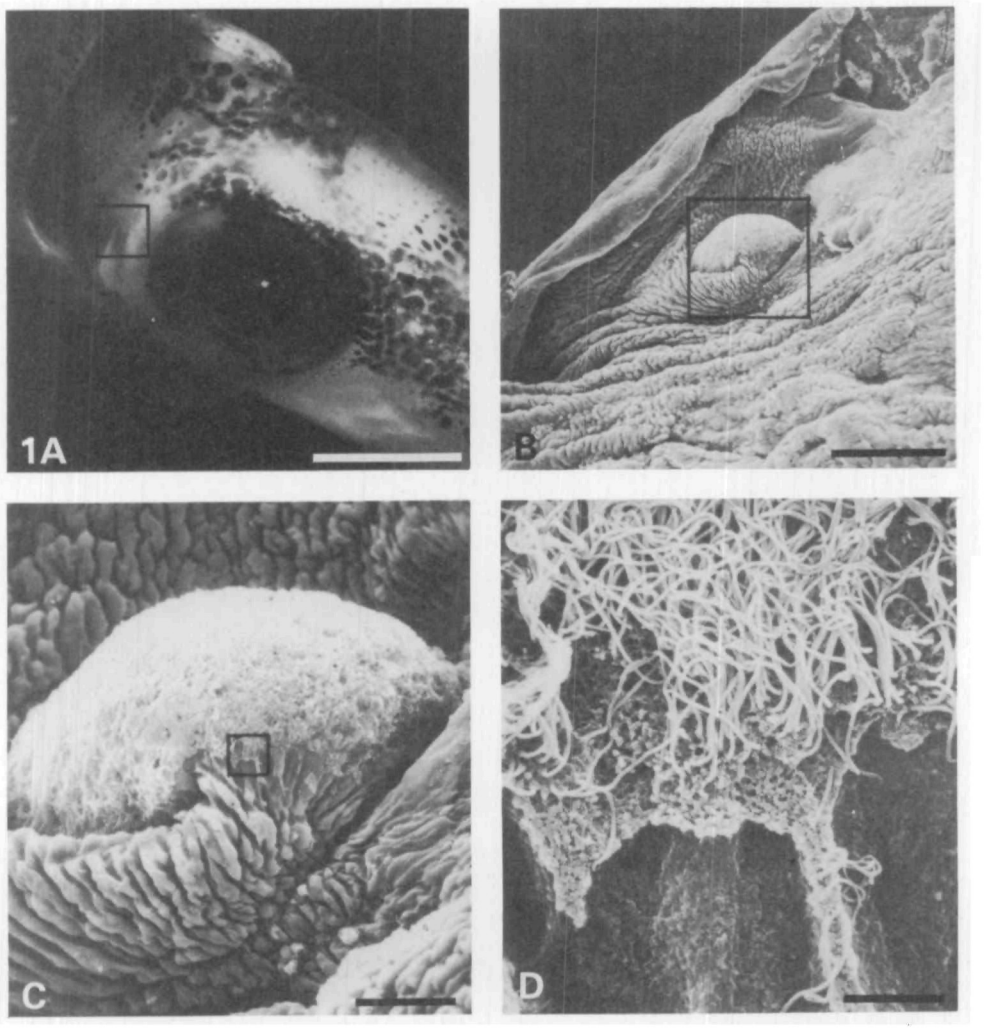
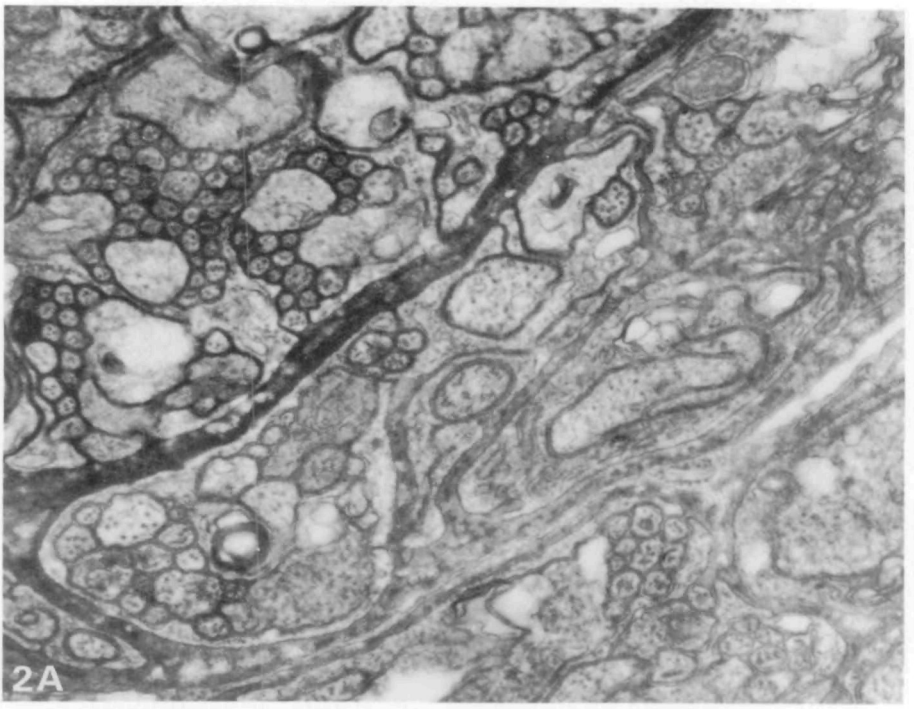


Fig. 1. Anatomy of the olfactory organ in *Loligo opalescens*. (A) The location of the olfactory organ in a living squid is indicated by the square posterior to the eye. (B) A scanning electron micrograph of fixed material shows the flap of muscular tissue at the base of which the olfactory knob lies. (C) An enlargement of the boxed area in B reveals the distinctive appearance of the sensory knob of the olfactory organ. (D) An enlargement of the boxed in area in C shows the dense mat of motile cilia that arise from supporting epithelial cells. Apical processes of receptor cells are buried beneath this mat and are not visible. Scale bars: A, 1 cm; B, 250 μm; C, 50 μm; D, 5 μm.

substances routinely elicit escape responses when ejected from a pipette into the region of the olfactory organ of a living, restrained squid. Control tests with sea water are ineffective in experiments of this type (see also below). An example of a typical series of high-pressure escape jets in response to a 300 ms pulse of diluted (1:500) blue food coloring is illustrated in Fig. 3. As demonstrated below (see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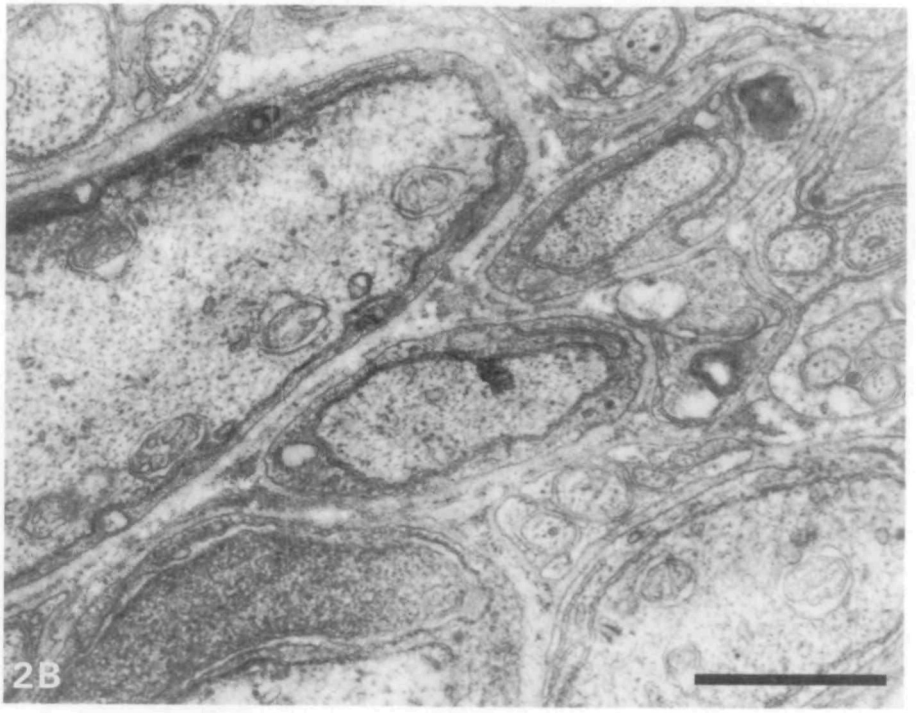
2B

Fig. 2. Transmission electron micrographs of transverse sections of the nerve leading from the olfactory organ to the brain. (A) The major portion of the olfactory nerve, presumably sensory in function, contains many fine axons grouped into fascicles by Schwann cells. Very few axons are individually wrapped by a Schwann cell. (B) A distinct portion of the nerve, separated from the sensory nerve by connective tissues, contains mostly large axons that are individually ensheathed by Schwann cells. This portion of the nerve probably contains motor as well as sensory fibers. Scale bar, 1 μm.

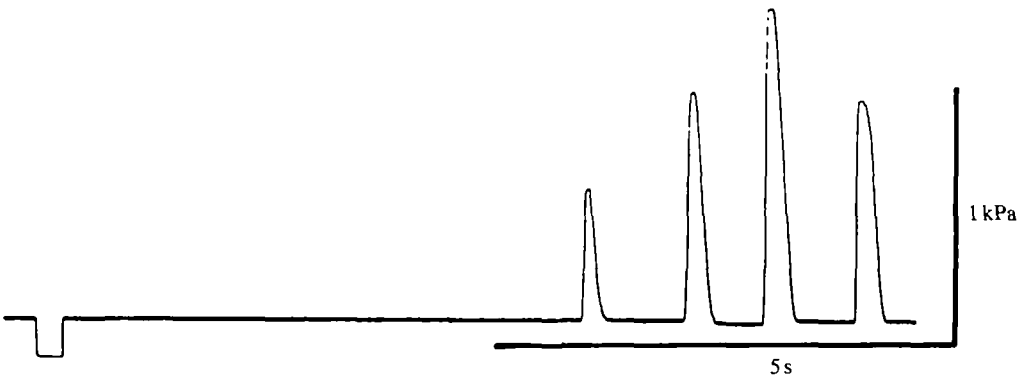

Fig. 3. Intramantle pressure transients associated with escape jetting behavior produced by chemical stimulation. Ordinary blue food coloring (1:500 dilution in sea water) was ejected from a pipette in the immediate vicinity of the olfactory organ, and four cycles of escape jetting ensued. See text for additional details.

Fig. 4), blue food coloring contains a colorless compound, propyl paraben (4-hydroxybenzoic acid propyl ester), that is the specific behaviorally active component.

Delays to the onset of escape jetting in response to chemicals are highly variable (2–20 s) and substantially longer than the delays in response to electrical shocks to the tentacles (0.2–2 s) or to visual (flash) stimulation (50–75 ms) (Otis and Gilly, 1990). Several complex behaviors transpire during the latent period for jetting following chemical stimulation. These include one or more cycles of mantle hyperinflation (Gosline *et al.* 1983), accompanied by a lifting and pointing of the arms anteriorly so that they roughly form a cone, and by aiming of the siphon. Chemical stimuli often also elicit strong, fin-driven swimming that commences 5–10 s before the first hyperinflation episode and jet (W. F. Gilly and M. T. Lucero, unpublished observations).

Although behavior like arm-lifting or fin-driven swimming might be useful to indicate whether animals respond to specific stimuli, these actions are obscured by normal respiratory–swimming behavior and are often relatively subtle. Because strong escape jetting is a more unambiguously defined behavioral output that is easily monitored, our behavioral analyses were restricted to this aspect.

Blue food coloring was originally intended as an inert tracking dye, and its

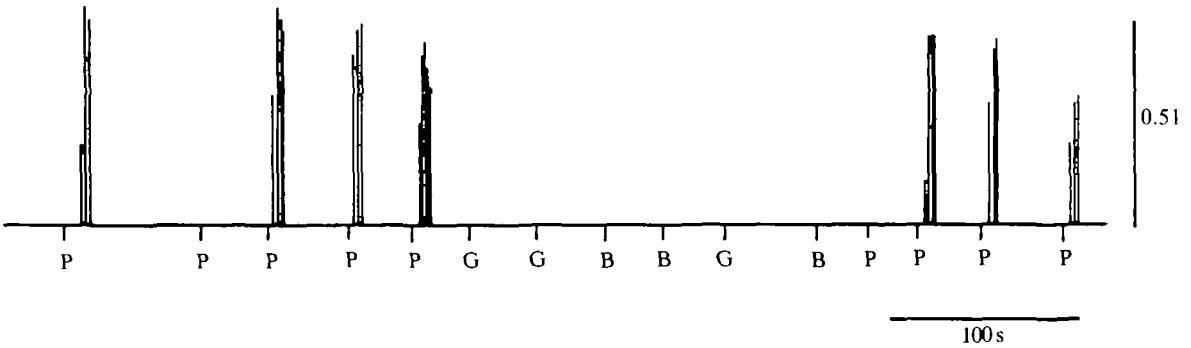

Fig. 4. Escape jets are produced by propyl paraben, the preservative in blue food coloring, but not by Brilliant Blue, the actual dye, or by Fast Green, a structurally related dye. 100 ms pulses of the following chemicals were delivered as indicated by the downwards marks: 50 μmol l^{-1} propyl paraben (P), 100 μmol l^{-1} Brilliant Blue (B) and 100 μmol l^{-1} Fast Green (G).

efficacy at stimulating escape responses was unexpected. This agent is a mixture of carrier (2 mol l^{-1} propylene glycol), dye (20 mmol l^{-1} Brilliant Blue) and preservative (10 mmol l^{-1} propyl paraben), and efforts were made to identify the active component. Fig. 4 shows the results of an experiment testing individual constituents and a structurally related dye, Fast Green. Only propyl paraben elicited escape responses. Tests with propylene glycol (20 mmol l^{-1} and 100 mmol l^{-1}) were also negative (not illustrated).

Experiments with blue food coloring were also carried out at different dilutions, and the results are summarized in Table 1. Although these dose–response data are limited, they clearly indicate that the probability of a stimulus evoking an escape jet increases as the stimulus becomes more concentrated. 'Threshold' for activity appears to occur with a dilution of approximately 1:1000, corresponding to a propyl paraben concentration of 10 μmol l^{-1}. Because of uncertainty concerning the actual stimulus concentration delivered to the olfactory organ (owing to mixing, etc.) and because of the restrictive method for scoring positive behavioral reactions (high-pressure jetting only), this value represents an upper limit for detectability. Similarly, dilution values cited in Table 1 must be taken as approximations.

Results similar to those described for blue food coloring and propyl paraben were obtained with a variety of other compounds, and these are discussed in the next section. We also tested many organic compounds that failed to produce escape jetting reliably. Marginal efficacy was observed with 5 mmol l^{-1} ammonium chloride, but results were inconsistent and may have been complicated by multiple effects of ammonium ions, which are impermeant, compared with ammonia molecules, which can penetrate cells. Substances that were non-effective included 5 mmol l^{-1} sodium nitrate, 20 mmol l^{-1} methionine, 1 mmol l^{-1} proline, 1 mmol l^{-1}

Table 1. *Efficacy of blue food coloring at different dilutions in eliciting escape jets in behavioral experiments when pressure-ejected onto the olfactory organ (150–300 ms pulses)*

Dilution	Concentration of propyl paraben (μmol l^{-1})	Positive test responses (%)	Trials (N)	Positive control responses (%)	Trials (N)	Number of squid
1:1000	10	14	7	0	8	1
1:500	20	68	22	0	20	3
1:200	50	47	19	0	17	1
1:40*	250	100	5	0	4	1

See text for additional experimental details. Responsiveness is given as the percentage of the trials (total number=N) that resulted in escape jets recordable as a pressure transient with a latency of <20 s.

Control stimuli consisted of sea water containing the following compounds that were not behaviorally active: 20–100 μmol l^{-1} Fast Green dye for the 1:000, 1:500 and 1:200 trials, 5 mmol l^{-1} betaine and 5 mmol l^{-1} isethionate for a 1:500 trial and 5 mmol l^{-1} menthol for the 1:40 trial.

* Green food coloring, rather than blue, was used for the 1:40 test.

taurine, 5 mmol l^{-1} menthol, 20 mmol l^{-1} trimethylamine *N*-oxide, and pH 5 sea water.

Application of a specific, colorless chemical (propyl paraben) to the general region of the olfactory organ can thus lead to escape responses driven by high-pressure jetting, and behavioral responsiveness is graded with stimulus intensity. A more rigorous identification of the olfactory organ as the actual receptive site is made below.

Stimulation of escape jetting by potassium (K^+) channel blockers

Electrophysiological studies on receptor cells of the olfactory organ indicated that propyl paraben is an effective blocker of voltage-controlled potassium channels (Lucero *et al.* 1992). With this in mind, a series of behavioral experiments was undertaken to assess the efficacy of other K^+ channel blockers in eliciting escape responses when applied to the olfactory organ. Although these substances might not be expected to be naturally occurring stimulants, they serve as useful probes for olfactory organ (and receptor cell) function.

Quaternary ammonium ions (e.g. tetraethylammonium, TEA^+) and aminopyridine compounds (e.g. 4-aminopyridine, 4-AP) are well-known K^+ channel blockers, and these substances are also potent activators of escape jetting *in vivo*. Results of an experiment with tetrabutylammonium (TBA^+) are shown in Fig. 5A. Following eight control stimulations, none of which produced an escape response, periodic application of a brief pulse of 20 mmol l^{-1} TBA^+ commenced for a period of 30 min. Every TBA^+ stimulus produced a strong escape jet with a

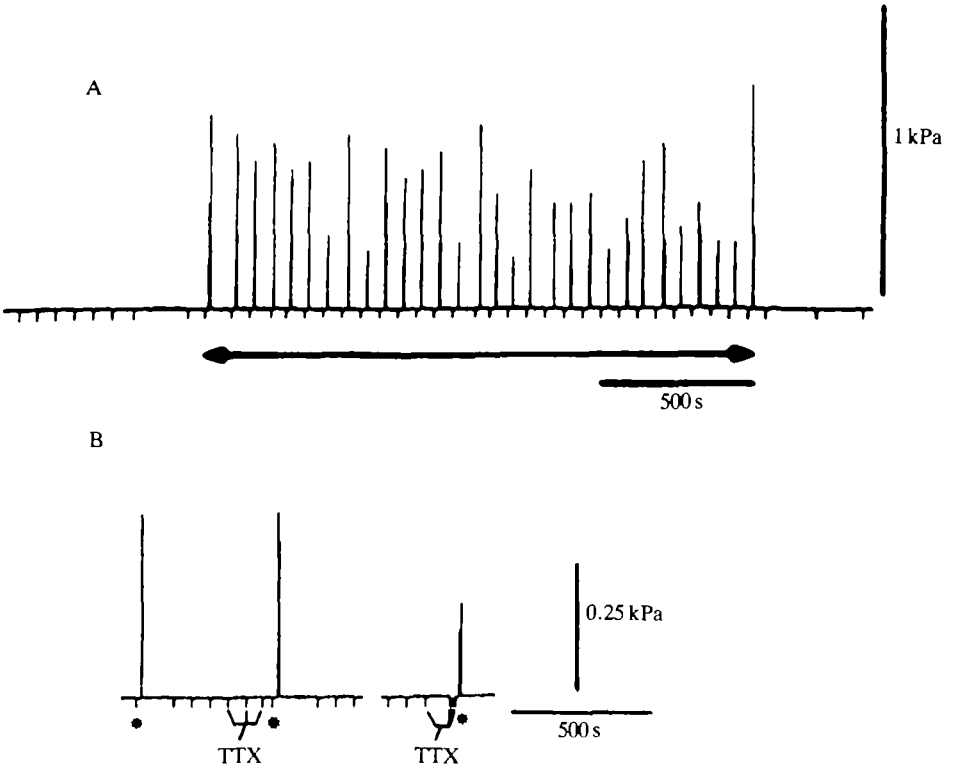

Fig. 5. Tetrabutylammonium (TBA^+, 20 mmol l^{-1}), a potassium channel blocker, reliably elicits escape jets, whereas tetrodotoxin (TTX), a sodium channel blocker, does not. (A) TBA^+ was applied during the time spanned by the arrows at each downward tick. Control stimuli (sea water) were delivered at the ticks before and after this period. (B) 20 mmol l^{-1} TBA^+ was used as a positive control (star), sea water as a negative control (unlabeled ticks) and 1 mmol l^{-1} TTX as a test substance. TTX neither produces escape jets nor interferes with the ability of TBA^+ to do so. See text for details.

mean latency of 15.1±3.8 s (1 S.D.). Three controls immediately following this series were again negative. Thus, TBA^+ is a potent stimulus that does not produce habituation at this frequency of stimulation (1 min^{-1}).

Table 2 summarizes results with TBA^+ and other quaternary ammonium salts at a single, high concentration. TBA^+ is more effective at triggering escape responses than is TEA^+, which in turn is much more effective than tetramethylammonium (TMA^+). This potency series mirrors the K^+ channel blocking action of these ions in receptor cells from the olfactory organ (Lucero *et al.* 1992).

4-AP is another specific K^+ channel blocker that is effective at producing escape responses at lower concentrations (see Table 2). Some form of habituation does develop with this substance, however, and animals tend to become unresponsive after several applications. For this reason experiments with 4-AP were limited.

Table 2. *Efficacy of K^+ channel blocker in eliciting escape jets in behavioral experiments when pressure-ejected onto the olfactory organ*

Chemical	Concentration ($mmol\,l^{-1}$)	Positive test responses (%)	Trials (N)	Positive control responses (%)	Trials (N)	Number of squid
Tetrabutylammonium (TBA^+)	20	92	38	7	93	7
Tetraethylammonium (TEA^+)	20	63	57	1	95	8
Tetramethylammonium (TMA^+)	20	21	19	0	47	4
4-Aminopyridine	10	100	5	3	35	2
	5	75	8	2	41	4
Methadone	1.0	83	6	0	16	1
	0.5	73	30	4	49	3

See text for experimental details.

Responsiveness is given as the percentage of the trials (total number=N) that resulted in escape jets recordable as a pressure transient with a latency of <20 s for both test substances and seawater controls.

Each substance was tested several times in a number of squid (indicated in right-hand column).

Methadone, a third blocker of K^+ channels (Horrigan, 1990) also reliably stimulates escape jetting at even lower concentrations. Methadone is intermediate between 4-AP and TBA^+ in producing habituation.

A specific sodium channel blocker does not cause escape jetting

Because all of the above compounds both block K^+ channels in isolated receptor cells (Lucero *et al.* 1992) and elicit vigorous escape jets in behavioral experiments, the possibility emerges that K^+ channel block is the receptor-level transduction mechanism for these substances. Not all of these compounds are selective K^+ channel blockers, however, and other alternative or additional pathways could be involved in their detection. Although the quaternary ammonium ions (TEA^+ and TBA^+) and 4-AP block only K^+ channels in the receptor cells, propyl paraben and methadone also reversibly block sodium (Na^+) channels (Lucero *et al.* 1992). Thus, changes in electrical activity in receptor cells due to Na^+ channel block might also lead to escape jetting behavior.

To test this idea, behavioral experiments were carried out with $1\,mmol\,l^{-1}$ tetrodotoxin (TTX), a highly specific Na^+ channel blocker. Fig. 5B illustrates results of such an experiment. The first stimulus in the record was $20\,mmol\,l^{-1}$ TBA^+, intended to serve as a positive control, and a strong escape jet was produced. Three seawater controls and three subsequent TTX applications all failed to produce a response, whereas a second TBA^+ stimulation did. In the right-hand portion of Fig. 5B, obtained later in the experiment, two seawater

controls were followed by a single TTX pulse and, 1 min later, by ten more pulses delivered in rapid succession. A single TBA^+ pulse at the end of the TTX train resulted in an escape jet. TTX evidently neither causes escape jetting nor prevents responsiveness to TBA^+.

Defining the location of chemosensitivity

Mapping

Two methods were used to map the spatial distribution of chemosensitivity in a living squid. In one case, a potent stimulus was applied at different locations, and at each site the duration of the pressure pulse was varied in order to identify a threshold value (the shortest pulse needed to elicit a high-pressure jet). Because pulse length is proportional to the amount of material ejected, the effective spatial spread of a stimulus will diminish as pulse duration decreases. We assume, therefore, that the stimulation site with the minimum threshold duration can be equated with the receptive site of highest chemosensitivity.

Fig. 6 shows results from an experiment in which the stimulant mixture contained 20 mmol l^{-1} TBA^+ and a 1:25 dilution of blue food coloring. The inset indicates the two axes along which the position of the stimulating pipette was varied (distances given in millimeters) with respect to the approximate position of the olfactory organ. Fig. 6A plots the duration of every pulse tested against the position along the horizontal axis, and the symbol type denotes whether a pulse produced an escape jet or failed to do so. Threshold duration (indicated by the dotted line) is approximately 75 ms around the site of the olfactory organ (upwards arrow on the abscissa) and increases dramatically in the anterior direction. Thus, pulses as long as 425 ms failed to produce any response when applied directly over the eye.

Fig. 6B shows analogous results for vertical displacements. Threshold duration, approximated by the dotted line, passes through a minimum near the immediate site of the olfactory organ and increases when the stimulating pipette is moved either ventrally or dorsally. The pulse at +16 mm hit the external surface of the mantle, missing the area of the olfactory organ altogether, and no responses could be obtained at this site. Similar mapping experiments carried out on five other animals were consistent with the idea that the olfactory organ is the site of highest chemosensitivity.

The second type of mapping experiment was designed to improve spatial resolution. A pipette containing a chemical stimulus (10 mmol l^{-1} TBA^+) and a tracking dye (5 mmol l^{-1} Brilliant Blue) served to deliver stimuli of 50 ms duration, and a video camera was used to track the stimulus plume. In this way, the actual site of impact for stimuli that produced escape jets, or failed to do so, could be directly defined in relation to the location of the olfactory knob.

Fig. 7A shows selected video frames (frame numbers indicated; 33 ms elapsed time per frame) from such an experiment taken sequentially after the onset of the stimulus ejection during frame zero. The stimulus stream initially bypassed the

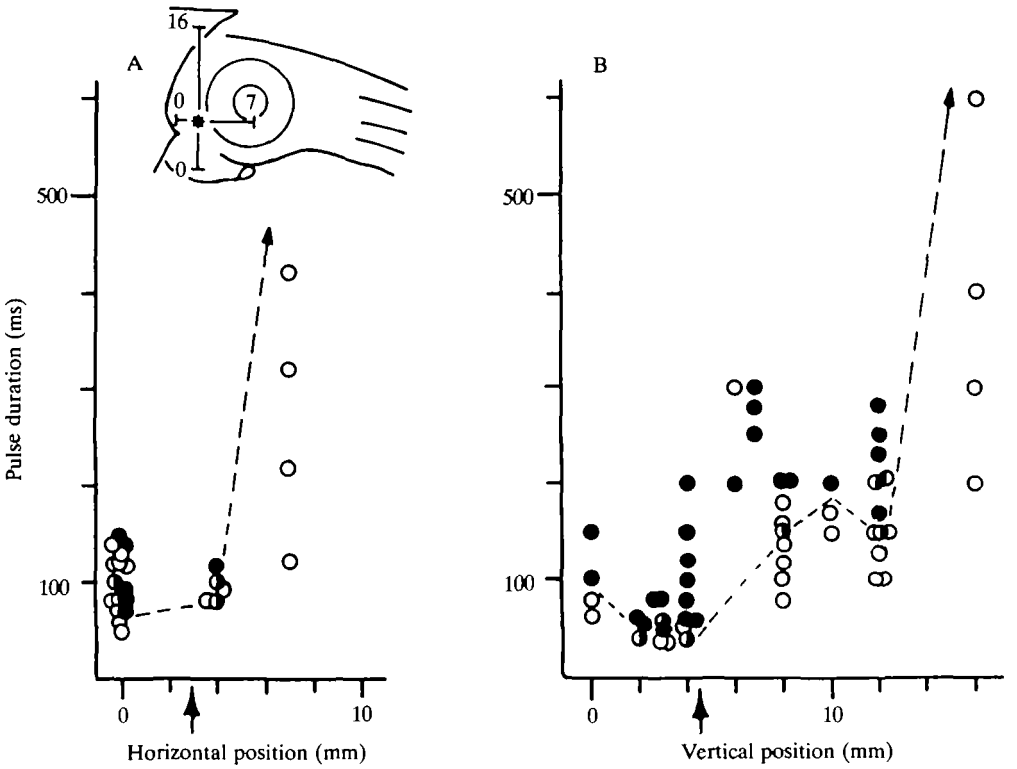

Fig. 6. Mapping the location of high chemosensitivity to the olfactory organ. A potent chemical stimulus (20 mmol l^{-1} TBA^+ and 1:25 blue food coloring in sea water) was delivered at different positions along two axes intersecting at the olfactory knob (star), as indicated on the inset (distances in mm). Pulse duration for ejecting the stimulant was varied in order to identify a threshold duration for each site. Filled symbols indicate a positive behavioral response in an individual trial; open symbols correspond to failure of a given pulse to produce an escape response. Threshold is thus defined by the transition zone (dotted line) between open and filled symbols at each location. (A) Varying the horizontal position of the stimulating pipette yielded low-threshold values adjacent to the olfactory organ (indicated by an arrow on the abscissa) and failed to produce responses over the eye (7 mm). (B) Changing the vertical position of the pipette reveals a minimum threshold near the site of the olfactory knob (arrow on the abscissa).

olfactory organ and entered directly into the mantle cavity with the inhalant respiratory current (Fig. 7Ai). During the exhalant phase of the respiratory cycle, the stimulus collected in a restricted area dorsal and posterior to the olfactory organ (Fig. 7Aii,iii) but did not actually reach it. The accompanying pressure record (Fig. 7C) indicates that no escape response occurred.

Stimuli that definitely reached the olfactory knob did elicit escape responses, as shown in Fig. 7B,D. In Fig. 7Bi the stimulus initially struck very close to the olfactory organ, and water currents then carried the plume directly over it

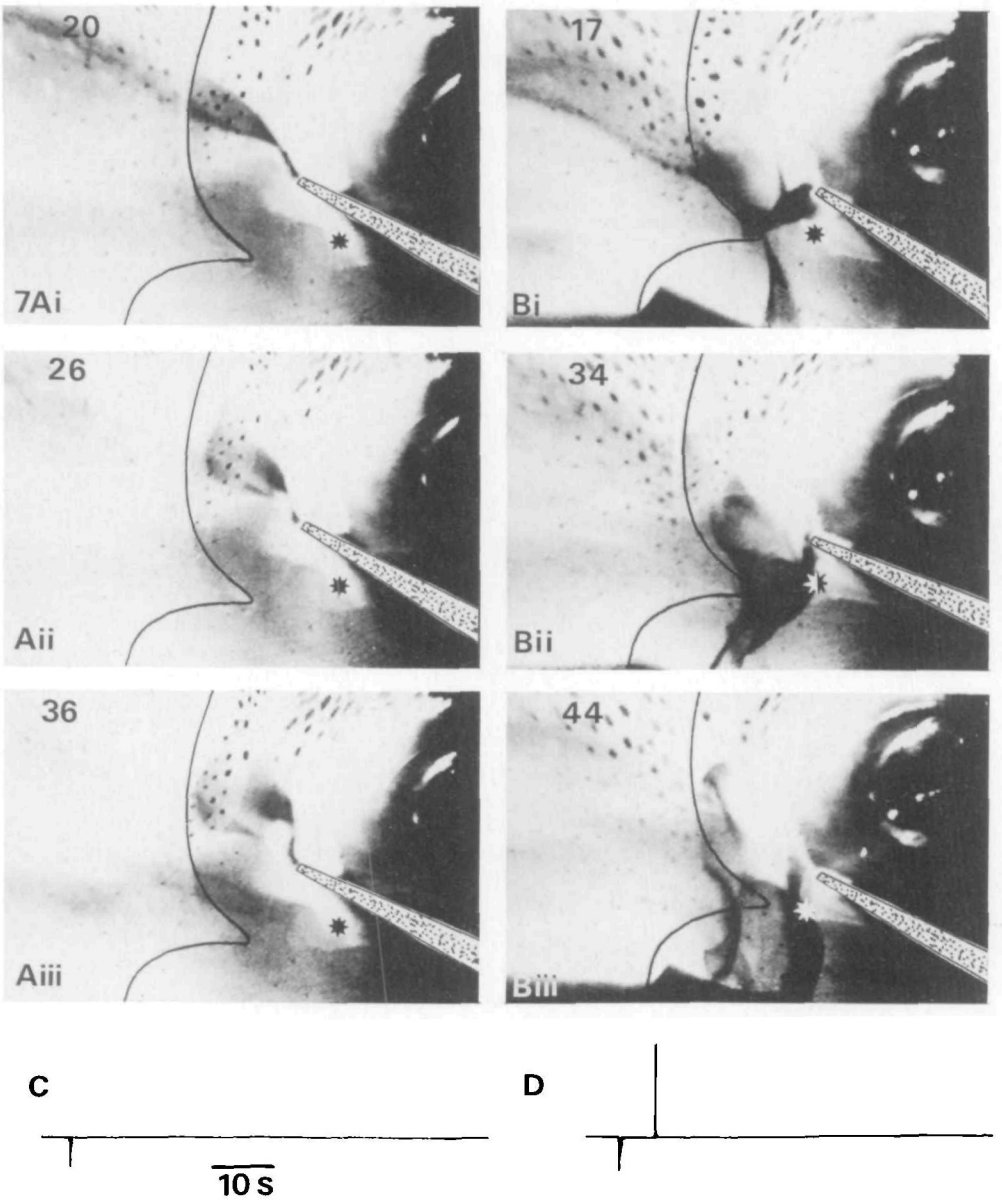

Fig. 7. Identification of the site of chemosensitivity with the olfactory organ. A chemical stimulus (10 mmol l^{-1} TBA^+) and a tracking dye (5 mmol l^{-1} Brilliant Blue) were delivered from a small pipette (stippled) during frame zero (not illustrated), and the stimulus plumes were tracked on video to determine whether they impinged on the olfactory knob (star). The anterior-most border of the mantle is indicated by the solid curve. Intramantle pressure was simultaneously recorded to indicate success or failure of the stimulus at producing an escape response. (A) Panels i–iii show numbered video frames taken after the pulse. The stimulus never reached the olfactory organ and failed to produce an escape jet as shown in the pressure recording in C. (B) Analogous results from a trial in which the stimulus touched the olfactory knob and produced an escape jet. (C,D) Pressure recordings corresponding to A and B, respectively. Vertical calibration was not recorded.

(Fig. 7Bii,iii). Fig. 7D demonstrates that an escape response occurred. This localization of responsiveness was seen in all three animals tested in such mapping experiments. Positive responses were not obtained when stimului unambiguously missed the olfactory knob, and definite hits reliably produced escape jets.

Chemical 'ablation'

Attempts were also made to localize chemosensitivity to the olfactory organ by specifically interfering with the functional integrity of the organ and then testing for loss of chemoreceptive ability in behavioral experiments. Surgical ablation of the olfactory organ in squid is not practicable because skin lesions do not heal and this approach was not attempted (see Messenger, 1967). Instead, temporary chemical ablation of the olfactory organ was performed by treating this structure with a potent local anesthetic to impair transduction in the receptor cells and/or afferent transmission in sensory axons of the olfactory nerve. Anesthetic treatment was performed on one side of the head only ('test' side); the contralateral, untreated organ served as the control.

Fig. 8 shows results of such an experiment. Before removal of the squid for anesthetic application at 9 min, high-pressure escape jets were produced by every stimulation (150 ms pulse of 100 μmol l^{-1} propyl paraben) of both control and test organs. After these trials, the squid was removed from the tank (still attached to the restraining platform), the region posterior to the right eye was blotted dry

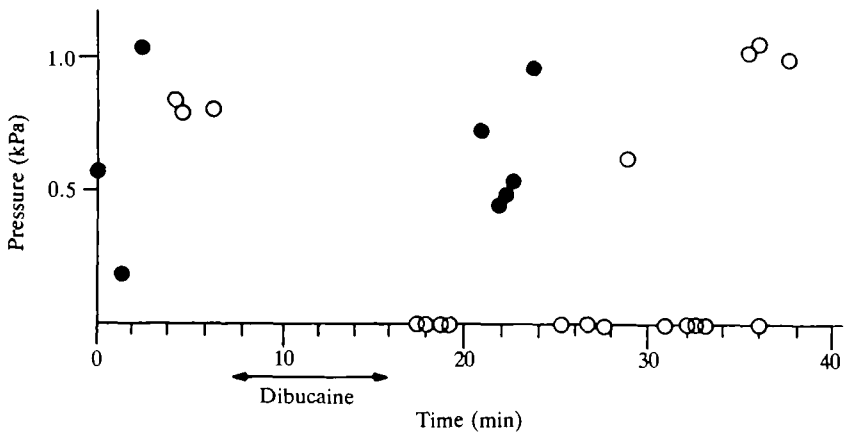

Fig. 8. Reversible impairment of chemoreceptive ability by treatment of the olfactory organ with a local anesthetic. Propyl paraben served as a chemical stimulant to produce escape responses in either the left (control) or right (test) olfactory organ, and the amplitudes of the resultant pressure transients (●, control; ○, test) are plotted as a function of time during the experiment. Before treatment of the left side with local anesthetic, both sides were responsive. Following application of anesthetic, the test side became unresponsive for approximately 20 min, after which time function returned. Stimuli delivered to the control side during the period of block remained effective at eliciting escape jets. See text for additional details.

(without touching the organ itself), and 10 μl of 0.5 mmol l^{-1} dibucaine (in sea water) was applied to the olfactory knob on this side. The animal was then remounted in the tank, and testing with propyl paraben resumed at 16 min. Stimulation of the anesthetized organ consistently failed to produce an escape response for the next 20 min (except for a single, possibly spurious, response at 29 min in Fig. 8). During this period of impaired function, stimulation of the untreated, control organ reliably produced escape jets. Complete recovery of the dibucaine-treated organ occurred by the end of the experiment.

Results consistent with those depicted in Fig. 8 were also obtained in a similar experiment on a second animal. These experiments, along with the mapping studies, support the idea that receptor cells of the olfactory organ mediate detection of water-borne chemical information.

Detection of and responses to naturally occurring substances

Experiments described thus far have employed fairly potent K^+ channel blockers as stimuli in behavioral studies. It is unclear, however, to what extent a squid in its natural environment would encounter such molecules. To explore the potential biological relevance of the olfactory organ, attention was turned to naturally occurring substances to which squid would be more likely to be exposed.

Crude extracts of tissues associated with reproduction were used as stimuli in behavioral experiments, but none reliably led to escape jetting behavior (or any other definite reaction). These extracts were prepared from spermatophores, egg jelly, nidamental and accessory nidamental glands, and gonads of both sexes.

Positive behavioral results were obtained, however, with a simple preparation of diluted squid ink (see Materials and methods) and with 1 mmol l^{-1} 3,4 dihydroxyphenylalanine (L-Dopa). L-Dopa is the precursor of melanin (Fox, 1976; Needham, 1974), the major pigment found in the ink sac of *Loligo opalescens* (Fox and Crane, 1942). Table 3 summarizes these results. Diluted ink was fairly variable in its effectiveness in different animals, and two of the six animals tested were completely unresponsive. L-Dopa was very effective in all three animals tested.

Squid thus appear to be capable of detecting the ink of members of their own species as well as L-Dopa, a compound that is probably present in the ink sac (Jimbow *et al.* 1984). Squid react to these naturally occurring substances with escape responses that would serve to remove them from the source of stimulation in their environment.

Discussion

It has been recognized for over a century that the cephalopod olfactory organ displays the anatomical attributes necessary for it to function in a chemosensory capacity (Zernoff, 1869; Messenger, 1967). It is covered by a sensory epithelium, projects a large afferent nerve into the brain, and is situated directly in the stream of inhalant respiratory current. The main point of this paper is to provide evidence that the olfactory organ in *Loligo opalescens* functions in a way that allows the

Table 3. *Efficacy of naturally occurring substances in eliciting escape jets in behavioral experiments when pressure-ejected onto the olfactory organ*

Substance	Squid	Positive test responses (%)	Trials (*N*)	Positive control responses (%)	Trials (*N*)
1:20 Ink	2OCT90-1	67	3	0	3
	2OCT90-2	0	6	0	3
	4OCT90-2	56	9	10	10
	23FEB91-1	11	9	0	12
	23FEB91-2	25	4	0	7
	23FEB91-3	0	4	0	5
	Total	26	35	2	40
1 mmol l^{-1} L-Dopa	23FEB91-1	83	6	0	6
	23FEB91-2	83	6	0	6
	23FEB91-3	67	3	0	3
	Total	80	12	0	12

See text for experimental details.

Responsiveness is given as the percentage of the trials (total number=*N*) that resulted in escape jets recordable as a pressure transient with a latency of <20 s.

Results are tabulated for individual animals and as pooled results.

animal to detect and analyze water-borne chemical information. Chemical stimulation can lead to the specific behavioral output of high-pressure escape jetting. To our knowledge the present study is the first to identify a function for this specialized organ in any coleoid cephalopod, and the first to describe a chemoreceptive sense in squid.

Chemoreception and the olfactory organ of squid

Although proof of the chemosensory function of the olfactory organ is considerably strengthened by our electrophysiological work (Lucero *et al.* 1992), results of the present study can largely stand on their own. First, mapping the spatial location of chemoreceptive ability by two different methods consistently led to identification of the olfactory organ as the chemosensitive site. Second, chemoreceptive ability could be temporarily impaired by topical application of a local anesthetic specifically to the olfactory organ. Third, only certain chemicals reliably acted to elicit escape jetting when applied to the olfactory organ, and their effectiveness was dose-dependent. This was clearly shown to be the case for the mixture of ingredients in blue food coloring.

A variety of compounds that block K^+ channels are all effective in producing behavioral responses, and these compounds lead to hyperexcitability and repetitive firing in receptor cells (Lucero *et al.* 1992). Similarly, squid ink and L-Dopa trigger escape reactions and also affect the firing pattern of the sensory neurons, but in the opposite way (i.e. inhibition). It thus seems that changes in the firing

rate or pattern of the receptor cells are integrated in the central nervous system, and that these highly processed outputs ultimately influence the motor centers that control jetting. The long behavioral delays to chemical stimulation (2–20 s) evident in our studies are probably set by this processing, and in nature might correspond to an important period of environmental assessment for the animal.

Tetrodotoxin, a specific Na^+ channel blocker, was found to be ineffective at producing escape responses in behavioral studies. This toxin blocks Na^+ channels in the receptor cells (Lucero *et al.* 1992), and it appears, therefore, that the density of Na^+ channels on the apical surface of receptor cells in the olfactory organ may be low. Each receptor cell is surrounded by a desmosome–tight junction complex (Wildenburg and Fioroni, 1990) that presumably acts as a diffusional barrier and interferes with solutes in sea water acting on the basolateral surface of the cell. The observation that quaternary ammonium ions produce escape responses when applied to the sea water bathing the apical surface of the receptor cells implies that K^+ channels susceptible to extracellular blockage by these ions are located on the apical surface.

One piece of evidence linking the olfactory organ and chemoreception that is missing from the present paper concerns recording the sensory discharge in the olfactory nerve in response to chemical stimulants that elicit escape jetting *in vivo*. Such recordings were attempted with a dissected olfactory organ/nerve preparation using conventional suction and hook electrodes but were unsuccessful. Although electrical stimulation of the sensory nerve or olfactory knob produced compound action potential waves that propagated slowly ($<0.5\,m\,s^{-1}$; data not illustrated), the small size of the sensory axons and their extensive wrapping by Schwann cell and connective tissue elements (Fig. 2A) make single-unit recordings very difficult. We were also unable to record a convincing summed sensory discharge in response to chemical stimulation, probably because of the same technical problems. In the accompanying paper (Lucero *et al.* 1992), however, we describe successful intracellular recordings from isolated receptor cells that show appropriate receptor potentials and changes in firing pattern upon application of those chemicals that produce escape jets in living squid.

Biological relevance of the olfactory organ

The results described in this paper indicate that the squid olfactory organ can mediate detection of water-borne chemicals, but they also reveal that many agents tested, both potentially attractive (e.g. amino acids, egg jelly, extracts of gonads) and potentially repulsive (e.g. ammonium, nitrate, low pH), failed to produce definite behavioral reactions. Many of these substances would be expected to stimulate other marine invertebrates, and their apparent failure to elicit responses when applied to the olfactory organ merits discussion.

Although negative results were obtained with many stimuli, our assay for detection was specifically limited to escape jet production occurring within tens of seconds following a stimulus. Attractive substances would not be expected to lead to escape jetting, and dramatic changes in ventilation rate which might indicate

'arousal' (Boyle, 1983) were not observed. Long-term effects on sexual maturation or encouragement of mating behavior would have been impossible to detect with our approach. Similarly, repulsive stimuli that failed to produce escape jetting did not induce obvious changes in the respiratory rhythm, nor did they lead to unusual chromatophore diplays that might have indicated alarm (Long *et al.* 1989).

At present, we have no evidence whether substances that do not lead to escape jetting can be detected by receptors in the olfactory organ or whether their detection leads to other behavioral responses like those suggested above. Additional behavioral assays for detection of such substances would clearly be valuable in further developing this work. Unfortunately, these assays might have to be limited to restrained animals in a way similar to the approach described here. Our results on the detection of K^+ channel blockers, squid ink and L-Dopa by the olfactory organ do not imply that receptors for these chemicals are restricted to this structure. Responsive cells could also exist at other sites known to contain chemosensory cells, e.g. around the lips or on the tentacles. Activation of these additional receptors could also lead to behaviors other than escape responses. Thus, application of stimuli to the sea water containing one or several free-swimming squid might be difficult to interpret.

Although the complete chemosensory competence of squid largely remains to be explored and defined, this paper provides an indication of at least one biologically important function mediated by the olfactory organ – the generation of escape responses. Our results lend credence to the idea, proposed originally by Watkinson (1909; cited by Messenger, 1967), that the olfactory organ serves to monitor water quality, and that the sensory information passed to the brain leads to avoidance or escape responses when the ambient water becomes tainted with noxious substances. Whether the olfactory organ serves only this purpose remains to be determined, but such a high degree of specialization would be surprising.

It is noteworthy that substances detected by the olfactory organ include biologically relevant compounds that may be released by other squid. Squid ink, for example, ought to be avoided in the environment, because its presence in the water would specifically indicate that another squid had been alarmed or attacked recently and nearby. Although the color of ink could serve as a visual alarm signal under conditions of sufficient illumination, chemical messengers such as L-Dopa, or an indole precursor to the melanin derived from it (Fox, 1976; Needham, 1974), would be more effective in the darkness of night or at great depths.

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