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Integration of hydrodynamic and odorant inputs by local interneurons of the crayfish deutocerebrum

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Summary

Intracellular electrodes were used to record from local interneurons in the olfactory lobes of the midbrain in the crayfish *Procambarus clarkii*. Cells that resembled previously studied central targets of olfactory receptor neurons on the lateral antennular flagellum were specifically examined for their responses to hydrodynamic stimuli. Initiation of water movement past the antennular flagellum, confined within an olfactometer, evoked a triphasic excitatory–inhibitory–excitatory postsynaptic potential lasting up to 2 s that generated spikes on depolarizing phases of the response sequence. Odorant pulses seamlessly imbedded in the water pulse past the antennule evoked purely excitatory, dose-dependent postsynaptic responses and associated spike trains. The latency of the initial phase of the response to water was approximately half as long as the latency of the response to odorant, suggesting that different afferent pathways are involved in responses to hydrodynamic and odorant stimuli, respectively. In some olfactory lobe interneurons that resembled previously described cells classified as Type I, conjoint stimulation of fluid onset and odorant evoked responses that were twice the amplitude of the summed response to either hydrodynamic or odorant stimulation alone, suggesting that the olfactory responses were potentiated by hydrodynamic input. Individuals of at least one other class of first-order interneuron that responded to both hydrodynamic and odorant stimulation were occasionally recorded from. These results indicate that multimodal integration of chemical and mechanical information occurs at the level of first-order sensory interneurons in the crayfish brain.

Key words: olfaction, glomerulus, olfactory lobe, antennule, crustacean, sensilla, *Procambarus clarkii*.

Introduction

Fluid environments disperse embedded odorants in chaotic spatial patterns characterized by turbulent eddy fronts of unpredictable amplitude, breadth and frequency (Dusenbury, 1992; Atema, 1996; Moore and Grills, 1999; Weissburg, 2000; Grasso and Basil, 2002). The distribution of odorants is inseparable from and temporally linked to advective movements and eddies within the fluid itself; therefore, animals that depend upon fluid-borne chemical signals for detecting food and other critical sources of chemical signals should possess nervous system elements tuned not only to rapid changes in odorant concentration but also to hydrodynamic shear within the fluid column.

Little is understood concerning the details of interactions between neural inputs from sensory receptors mediating coincident mechanical and chemical sources of peripheral excitation within the central nervous systems of aquatic animals. In crustacean chemoreception models, competing hypotheses have been advanced to account for the putative integration of chemical and hydrodynamic information in odorant-source tracking, variously implicating advection, eddies or edge effects in navigation of an animal toward odor sources (Moore et al., 1991; Basil and Atema, 1994; Weissburg and Zimmer-Faust, 1994; Atema, 1996; Guenther et al., 1996; Webster and Weissburg, 2001).

Crabs, lobsters and crayfishes all detect odorants using their antennules, which exhibit batteries of chemoreceptive sensilla. The lateral antennular flagella, in particular, possess aesthetascs and associated olfactory sensory neurons (ORNs) that are sensitive to amino acids and other dissolved organic molecules (Ache, 1972; Ache and Derby, 1985; Schmiedel-Jakob et al., 1989; Mellon and Alones, 1995; Mellon, 1996, 1997; Schmidt and Ache, 1996a,b), but they also possess mechanoreceptive setae (Schmidt et al., 1992; Schmidt and Ache, 1996a; Mellon, 1997; Cate and Derby, 2001, 2002). The extent to which hydrodynamic factors participate in or influence odorant detection, however, has remained poorly understood (reviewed in Atema, 1996; Grasso and Basil, 2002). Moreover, crustaceans routinely flick the lateral antennular flagella in the presence of odors and water movements (Reeder and Ache, 1980; Daniel and Derby, 1991; Mellon, 1997). Flicking transiently enhances the response of ORNs to odors, although the mechanism responsible remains

unclear (Schmitt and Ache, 1979; Koehl et al., 2002). There can be little doubt, however, that the act of flicking should itself generate hydrodynamic stimulation of the antennular sensilla.

Previous electrophysiological studies of olfactory interneurons in the crayfish brain from my laboratory did not consider effects of hydrodynamic stimulation of the antennules during responses to odorants, since a continuous, regulated flow of freshwater bathed the immobilized antennular flagella during the course of an experiment (Mellon and Alones, 1995; Mellon, 1996). Similar electrophysiological studies of neurons in the olfactory midbrain of the spiny lobster *Panulirus argus*, using a different stimulus regimen, did provide evidence for transient effects of rapidly introducing seawater past the antennule but they were not followed up in any detail (Schmidt and Ache, 1996b).

The present study was undertaken to re-evaluate the responses of local deutocerebral neurons to olfactory and hydrodynamic stimulation of the lateral antennular flagellum and to determine the interactive effects of these dual sources of sensory input. I used multiple stimulus routines in which different schedules of freshwater and odorant were flushed past the lateral antennular flagellum. The results indicate that at least two major classes of the local interneurons that have dendritic arborizations within the olfactory lobe (OL) receive input from mechanoreceptors as well as chemoreceptors on the lateral flagellum of the antennule and that interactions between chemosensory and mechanosensory inputs together determine the output dynamics of these neurons. Mechanosensory input potentiates the responses to odorants in some neurons and thus may serve to amplify weak chemical signals. Furthermore, the integration of olfactory and hydrodynamic information at this early stage in the central olfactory pathways supports theoretical views that cooperation between these two major sensory inputs is critical for accurate determination of odor sources by aquatic animals.

Materials and methods

Electrophysiological observations were made with sharp electrodes from interneuron somata in brain cell cluster 11 (Sandeman et al., 1992) and from processes within the OL in the crayfish *Procambarus clarkii* Girard. Animals were obtained from a supplier (Atchafalaya Biological Supply, Raceland, LA, USA) and were maintained in 400-liter tubs of filtered, circulating well water at 18°C on a light:dark cycle of 14 h:10 h. They were fed twice a week with crayfish chow (Carolina Biological Supply Co., Burlington, NC, USA).

For electrical recordings, crayfish were quickly decapitated using sharp scissors to cut through the cuticle just posterior to the cervical groove on the cephalothorax, transecting the stomach and circumesophageal neural connectives. The rostrum was removed, and the head capsule was rinsed in chilled crayfish saline (see below) and mounted dorsal side up in a recording chamber that separately accommodated the medial and lateral flagella of the right-hand antennule within an olfactometer, as described below. The median artery, which supplies the brain, and the right lateral cephalic artery, supplying both the brain and ipsilateral antennular sense organs, were then quickly cannulated with small glass pipettes and flushed continuously at 2 ml min^{-1} with chilled (15°C), oxygenated saline having the following composition (in mmol l^{-1}): NaCl, 205; KCl, 5.4; $CaCl_2.2H_2O$, 13.6; $MgCl_2.7H_2O$, 2.7; $NaHCO_3$, 2.4. The pH of the saline was adjusted to 7.4 with HCl.

The loose membrane lying over the dorsal aspect of the brain was removed with microscissors, and the perineural sheath applied to the accessory lobe and OL on the right side of the brain was torn using sharpened watchmaker's forceps. Loose glial cells and hemolymph were gently washed away using a saline-filled tuberculin syringe. This procedure exposed both the dorsal surface of the OL and cell cluster 11 to approach with microelectrodes. Sharp microelectrodes were pulled on a Brown-Flaming puller and were filled with 2 mol l^{-1} KCl or, when staining with neurobiotin, 1 mol l^{-1} KCl. The resistance of the electrodes measured in crayfish saline was consistently in the range of 50–200 MΩ.

The isolated crayfish head preparation and details of the fluid supply to the olfactometers are shown in Fig. 1. Fig. 1A shows a simplified diagram of the recording and stimulating situation using the isolated head preparation. Antennular flagella were inserted within the parallel tubes of an olfactometer, through which either water or odorant flowed in a controlled regimen. The olfactometers were two parallel polyethylene T-connectors, the cross-members of which penetrated one wall of the lucite recording chamber, with the stems of the Ts, through which both freshwater and odorant were introduced, pointing upwards. The antennular flagella were inserted into the cross-members of the Ts, with their distal ends extending well beyond the intersection with the influx stems. The bases of cross-members around the flagella were then sealed with Vaseline to prevent odorant and water from entering the recording chamber or saline from flowing past the flagella. The other end of the cross-member tubes constituted an exhaust for the water and odorant once they had flowed past the antennule. Fig. 1B shows the switching arrangement controlling the flow of water and odorants through the olfactometer. Water and odorant could be flushed through either of the olfactometers separately or in concert by means of the electrically controlled switches. The data in the present paper are confined to responses from OL interneurons following stimulation of the lateral flagellum alone. The standard stimulus paradigm was to switch on a 10-s water flow, which triggered the computer acquisition file, followed after a variable delay by interruption of the water by a 1–2 s odor pulse. Water and odorant flowed through the olfactometers under gravitational acceleration at 18 ml min^{-1}. The common feed from the switch to the antennule in the olfactometer constituted a 'dead space' volume of 0.016 ml, which was cleared in approximately 50 ms at the initiation of each stimulus. Normally, water onset constituted a much more vigorous hydrodynamic stimulus than the injection of odorant, which was nearly seamless when the respective flow rates were appropriately adjusted. A broad

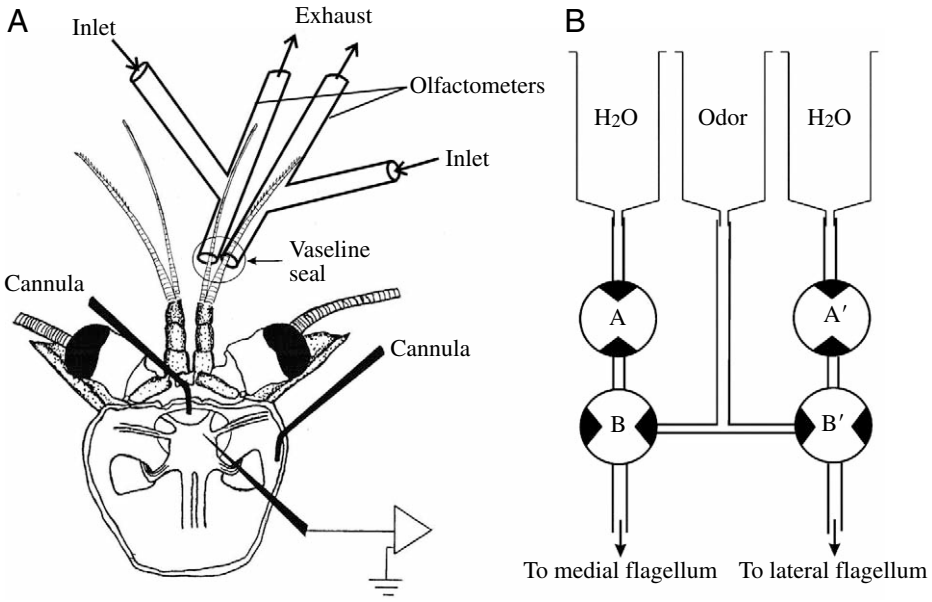

Fig. 1. (A) Diagram of the isolated, cannulated head preparation, including the olfactometer arrangement. (B) Organization of the solenoid switches controlling water and odorant flow to the antennular olfactometers. See text for details.

spectrum odorant was made up fresh for each experiment from 5 ml frozen aliquots of a 1% (w/v) tetramin solution, which were each dissolved in 95 ml of dechlorinated tap water. In some, but not most, preparations, morphological identification of interneuron types was pursued by injecting neurons with a 2% neurobiotin solution in 1 mol l^{-1} KCl. Crayfish heads were fixed overnight at 4°C in 4% paraformaldehyde made up in 0.15 mol l^{-1} Hepes buffered saline, pH adjusted to 8.2. Fixed, desheathed brains were then incubated at 4°C for 2 days with slow agitation in a solution of Hepes buffered saline, 0.5% Triton X-100 and 25 μg ml^{-1} Texas Red avidin D (Vector Laboratories, Burlingame, CA, USA). Brains were dehydrated, cleared in methyl salicylate and examined with an epiflourescence binocular dissecting microscope or, in some cases, with a laser scanning confocal microscope.

Results

Electrophysiological recordings from OL neurons

Sharp electrodes were used to record from interneurons having dendrites within the OL. Cells were penetrated either in cell cluster 11 or within the OL itself. Each approach had its positive and negative attributes. Recordings from dendritic processes of interneurons within the OL provided better electrical access to the synaptic events within the neuropil, but they were less stable with time. Penetrations of relevant neuronal somata and large neurites within cluster 11 were more difficult to obtain, due to the heterogeneous nature of the neurons present within the cluster, but they were generally more stable than neuropil recordings and thus permitted longer test series with odorants and hydrodynamic stimuli.

Dual response properties of Type I neurons

Cells resembling Type I cells (Mellon and Alones, 1995; Mellon, 1996) in their excitatory physiological responses to odorant stimulation and, in four cases, in their morphology, were most frequently encountered by recording electrodes, especially when penetrations were made at a depth of 300–400 μm within the central region of the OL. Tests were started following stabilization of the resting potential, which was usually between –55 and –65 mV. The current paper is based upon observations of 21 Type I-like neurons, obtained over a six month period. Although at least five additional types of neurons extend dendrites or axon terminals within the OL, Type I cells have the largest processes (up to 15 μm in diameter) and presumably are the largest targets within this neuropil. As shown in Figs 2–5, which are typical, these cells were excited by tetramin in a dose-dependent manner when it was injected seamlessly into the olfactometer during a long pulse of freshwater. Moreover, in both these and other Type I-like cells, the neurons responded not only to the odorant pulses but to the onset of water flow past the antennules as well. Fig. 2 shows responses of a Type I cell to both the leading edge of the water pulse and to different pulse durations of standard (0.05%) tetramin odorant embedded in a 10-s pulse of water. Very short odor pulses experienced more dilution by the ensuing tail of the interrupted water pulse and were thus less effective at stimulating the ORNs (Mellon and Alones, 1995). Fig. 3 shows responses of a different Type I neuron to 2-s pulses of standard tetramin and of three successive 10-fold dilutions of the standard. The responses of the cell, in terms of number of spikes, were a linear function of the $\log_{10}$ of different tetramin concentrations over the range tested. This cell was more typical than the one shown in Fig. 2 in that the response to the onset of water consisted of a sequence of depolarization–hyperpolarization–depolarization (E–I–E), with the initial depolarization usually generating one or two spikes. Fig. 4 records responses from another Type I neuron to illustrate the non-stimulatory nature of the injected fluid pulse from the odorant reservoir. Fig. 4A shows combined responses to water and standard odorant, whereas Fig. 4B shows the lack of response one minute later when the odorant reservoir was

Table 1. *Responses to a repeated hydrodynamic or odorant stimulus at 60-s and 120-s intervals*

	Trial			
	Hydro/60 s	Odor/60 s	Hydro/120 s	Odor/120 s
Mean	0	10.2	1.2	10.4
S.E.M.	N/A	±1.16	±0.36	±1.88

switched to freshwater. The responses to hydrodynamic stimuli in Type I-like neurons were more temporally labile than those to odorants in Type I neurons. A sequence of standard stimulus paradigms was presented to the cell shown in Fig. 3, alternately separated by 60 s and 120 s rest intervals. As shown in Table 1, the spike responses to water onset were absent on subsequent stimulus presentations that were separated by only 60 s, but they were present in most cases after a 120 s rest period. By contrast, the responses to a standard odorant stimulus were as robust following a 60 s interstimulus interval as they were after one lasting 120 s. A more dramatic illustration of this lability is shown in Fig. 5, in which records from a Type I neuron to successive standard stimulus paradigms are shown. A second stimulus set was initiated within 1.5 s following termination of a previous water pulse, at which point the input from hydrodynamic receptors was severely reduced while that to the odorant stimulus was undiminished.

The response latencies to water onset were also very different from the latencies to odorant presentation in Type I neurons (Fig. 6). The mean latencies of the excitatory postsynaptic potentials (EPSPs) generated by the neuron of Fig. 3 in response to water onset ranged from 408 to 448 ms in this cell; those in response to tetramin injection ranged from 854 ms for the most dilute stimulus to 598 ms for the highest concentration. Latency measurements for responses to water and standard (0.05%) odorant onset in seven other Type I neurons are summarized by the bar graphs in Fig. 7. The

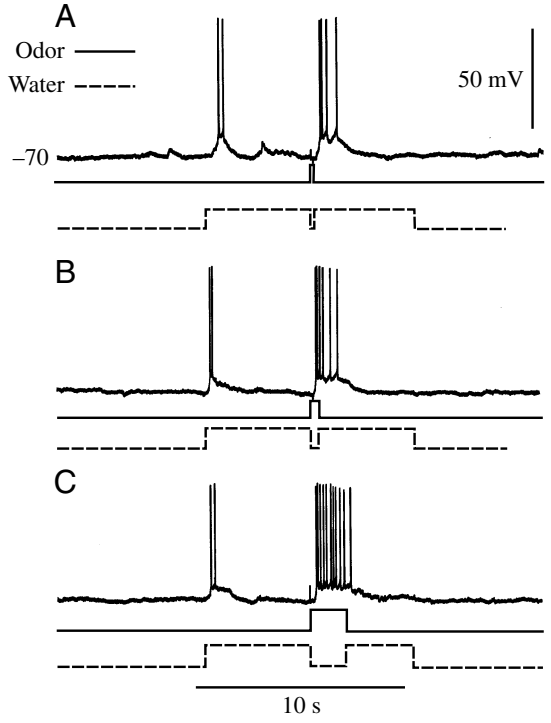

Fig. 2. Intracellular records from a Type I-like neuron recorded in the OL in response to (A) a 100 ms pulse of tetramin imbedded in a 10-s water pulse; (B) a 200 ms pulse of tetramin imbedded in a 10-s water pulse and (C) a 2 s pulse of tetramin imbedded in water. Freshwater flow past the lateral antennular flagellum was begun approximately 5 s prior to interruption by the odorant pulse in each case. In this and all other figures, water onset is indicated by upward deflection of the broken line, and odorant onset is indicated by upward deflection of the solid line.

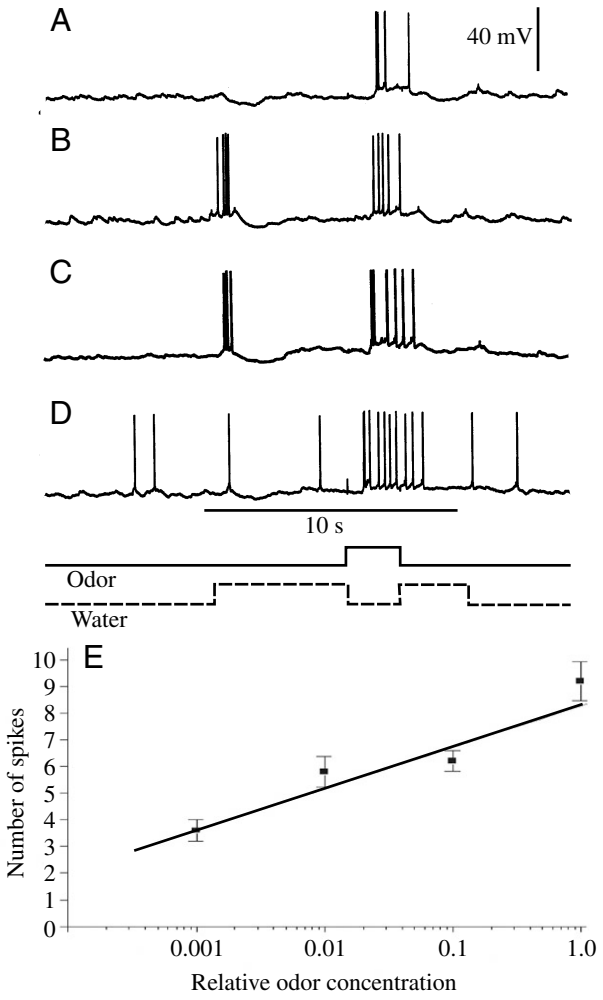

Fig. 3. Responses of a Type I-like neuron to water onset and to 2-s odorant pulses of standard tetramin and three serial 10-fold dilutions thereof, delivered as indicated by the routine shown below trace D. (A) Standard tetramin diluted 1000-fold; (B) standard tetramin diluted 100-fold; (C) standard tetramin diluted 10-fold; (D) standard tetramin. (E) Relationship between spike responses and the relative odor concentration. Each point is the mean ± 1 S.E.M. of five stimulus presentations. The straight line relationship is a graph of the equation $y=a(\log_{10}x)+b$, where a=1.55 and b=8.3. The correlation coefficient of the relationship is 0.95.

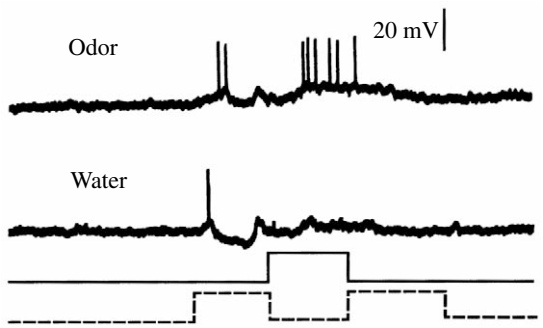

Fig. 4. Records from a Type I cell, illustrating its responses to water onset and a 2-s pulse from the odorant reservoir. When the reservoir contained 0.05% tetramin, the cell responded with a short burst of spikes (top trace); when the reservoir contained only water, there was no spike response. The initial onset of water produced the standard E–I–E response sequence.

majority of these data suggests that the input pathways for odors and hydrodynamic stimuli exhibit very different conduction times and must, therefore, be separate.

In two preparations, while recording from neurons exhibiting Type I response characteristics, the medial antennular flagellum as well as the lateral was stimulated with both water and odorant pulses to determine whether sensilla on this branch of the antennule are capable of driving OL interneurons. This was not the case in either instance, an example of which is shown in the records of Fig. 8; while not definitive because of the small number of cells examined, these tests suggest that chemosensory input arrives at Type I neurons only *via* lateral flagellar pathways. Other classes of OL interneurons were not tested this way, however, and possibly do respond to medial flagellar stimulation.

These findings support the conclusion that both odors and hydrodynamic stimuli are capable of exciting Type I-like neurons, presumably through different input pathways originating from sensory neurons associated with disparate sensilla on the lateral antennular flagellum. It was therefore important to determine whether there would be summation or other types of interaction between the responses to the two

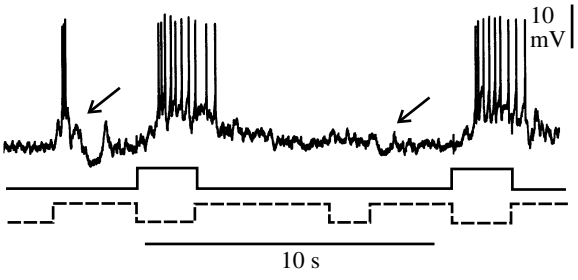

Fig. 5. Record from a Type I-like cell to illustrate the lability of the response to hydrodynamic stimulation. The initial stimulus sequence generated both a response to water onset (arrow) and to a 2-s odor pulse. When the sequence was repeated about 1 s following the end of the previous stimulus routine, the response to hydrodynamic input was greatly diminished (arrow).

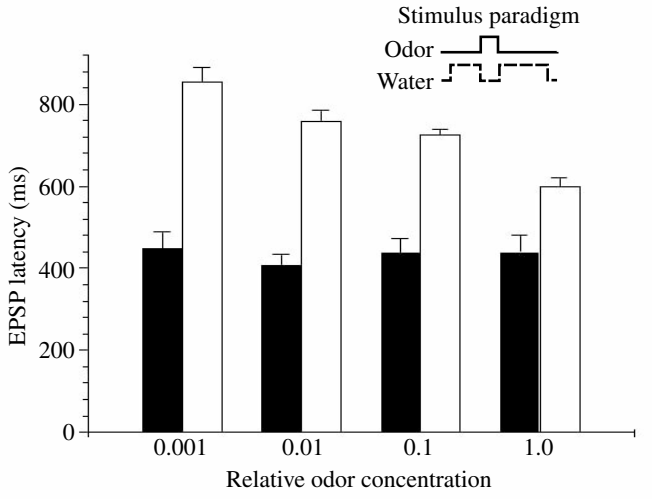

Fig. 6. Latency measurements from the Type I cell of Fig. 3. Filled bars are latency measurements to the EPSP generated by water onset, while the open bars are latency measurements from the immediately following response to a 2-s odor pulse. The odor response latency was, in every case, at least 25% longer than that for the hydrodynamic latency but was progressively shorter as the concentration of tetramin was increased. Each bar is the mean of at least five separate measurements ± 1 S.E.M.

modalities when presented together, as must usually occur in the natural environment. To test this, a different stimulus paradigm was used, in which a 2-s pulse of odorant was presented first, followed immediately by a 5-s water pulse. In this way, odorant and fluid onset were initially presented to the antennular flagellum together. The records of Fig. 9A show significant differences in the spike responses to this, as opposed to the standard, stimulus paradigm. Comparisons between the responses to the odorant-first routine, to the normal odorant-imbedded regimen and to the water onset step alone are shown graphically in Fig. 9B for the same neuron. In

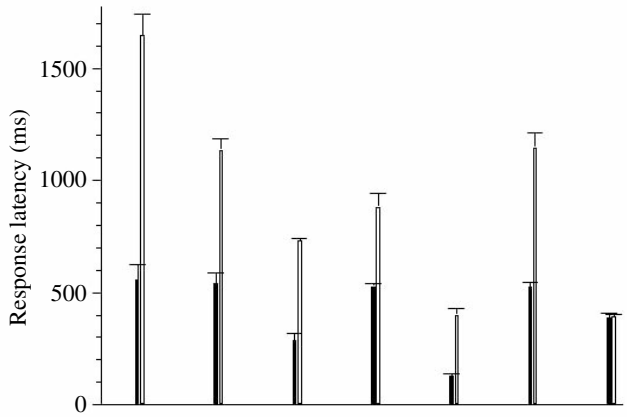

Fig. 7. Comparison of response latencies following water onset (filled bars) or odor (open bars) in seven additional Type I-like neurons. With the exception of one instance, the latencies to olfactory input were at least twice as long as those for hydrodynamic input. Error bars are ± 1 S.E.M.

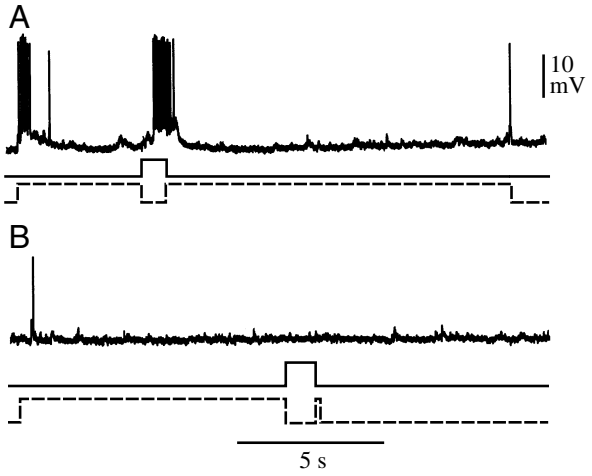

Fig. 8. Electrical records from a Type I-like neuron to water onset and to standard tetramin in response to stimulus regimes presented to the lateral flagellum (A) and to the medial flagellum (B) of the ipsilateral antennule. With the exception of a minimal response to water onset, the stimuli to the medial flagellum were ineffective.

20 separate trials, the mean number of spikes generated by the onset of water past the lateral antennular flagellum was 0.95, the mean response to seven trials of a 2-s pulse of 0.005% tetramin imbedded in the water pulse was 1.1 spikes, while the mean response to 23 trials of a 2-s pulse of 0.05% tetramin imbedded in the water pulse was 6.9 spikes. By contrast, the mean response to 10 presentations of the 2-s pulse of 0.005% tetramin prior to a 5-s water pulse was 3.2 spikes, or approximately twice the sum of the separate responses to water onset and to the imbedded odor pulse, and the mean response to 13 trials of a 2 s pulse of 0.05% tetramin prior to the water pulse was 14.6 spikes, again a doubling of the summed responses to a water pulse and to the imbedded odor pulse. The combination of onset of odor and fluid movement past the antennular flagellum thus caused an approximately 2-fold amplification of the interneuronal response compared with the sum of the responses to either stimulus alone. In this cell, the response latencies of the first spike to the stepped onset of fluid past the antennule were similar, whether caused by water alone or by the odorant, whereas the latency to the imbedded odorant, as in the cells of Figs 6 and 7, was between 50% and 60% longer.

Experimental data for combined hydrodynamic–odorant stimuli were then obtained from six additional Type I-like cells. Of these, two provided evidence of enhanced spike responses to a combination of odorant onset and fluid movement (Fig. 10) compared with responses to the odorant-imbedded paradigm, although the increase in the responses was not as robust as that for the cell in Fig. 9. These data support the hypothesis that, in a proportion of the Type I cells, a combination of hydrodynamic and odorant stimuli potentiates the response to either stimulus modality by itself. As noted previously, the initiation of fluid movement past the antennule normally generates a three-phase response, E–I–E, and, with

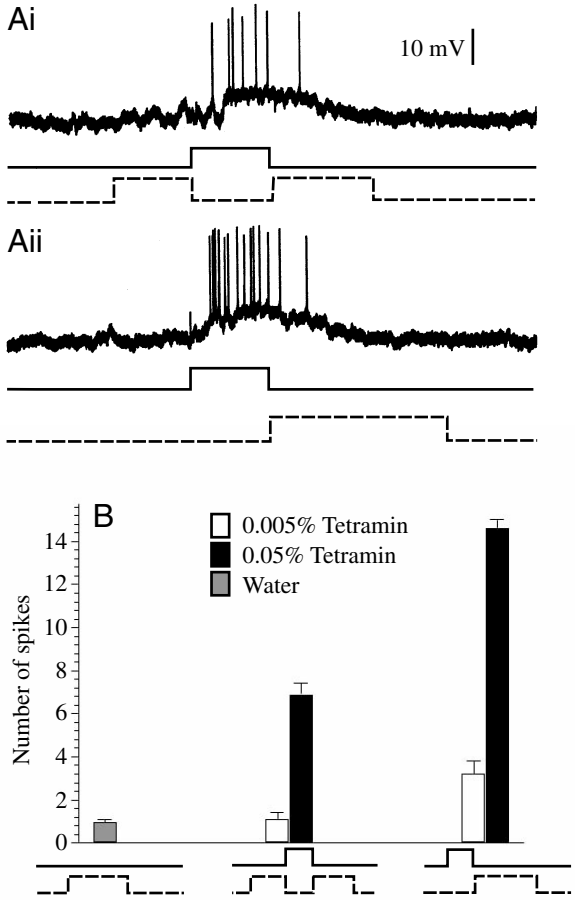

Fig. 9. (Ai,ii) Respective responses from a Type I-like interneuron to different stimulus routines, shown below each electrical trace. Responses to the odorant stimulus were doubled when it occurred prior to the water pulse. (B) Bar graphs documenting the potentiation of the neuronal response to two concentrations of odorant with different routines of hydrodynamic and/or odorant stimuli. Odorant pulses were 2 s in length. See text for further details.

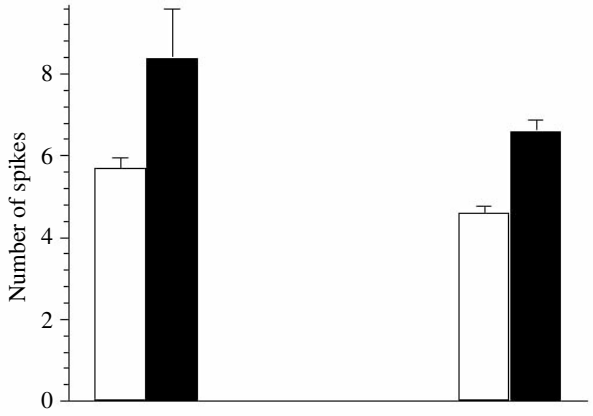

Fig. 10. Bar graphs of responses to water-first (open bars) or odor-first (filled bars) in two additional Type I-like neurons. Each bar is the mean of at least four observations ± 1 S.E.M. A *t*-test analysis between pairs of responses indicates that they are significantly different ($P<0.01$).

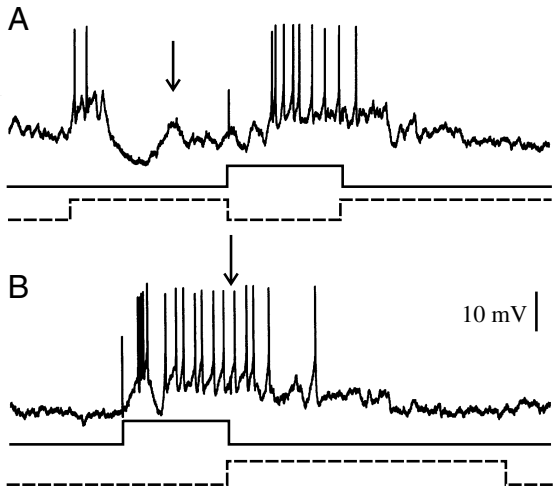

Fig. 11. Records from a Type I-like neuron in response to two different stimulus routines, as indicated beneath each electrical trace. In A, the arrow indicates the latency of the peak of the third (depolarizing) phase of the E–I–E response to the onset of fluid (water) movement past the antennule. In B, the arrow is at the identical latency to fluid (odor) onset and indicates that the third response phase would occur within the odor-response envelope, presumably allowing for summation. Odorant pulses lasted 2 s.

this paradigm, the third, excitatory phase will occur during the peak of the long-latency response to odors, as indicated from the data of Fig. 11. Summation of these response components would be expected to occur, possibly accounting for the potentiation when odorant and hydrodynamic inputs occur simultaneously. In addition, as shown in Fig. 12, the consistently more rapid rate of rise of the EPSP following an initial odor pulse, as opposed to an odor pulse imbedded within a long water pulse, may also influence the spike frequency response of these cells. The mechanism for this increased slope is not yet understood, but it may reflect differences in the rise times of EPSPs generated by hydrodynamic and odorant inputs, respectively.

In other Type I-like neurons, the sequence in which odor was presented to the antennular flagellum made little difference to the response magnitude. An example is shown in the records of Fig. 13. Here, it should be noted that the prominent depolarizing peak that normally follows the hyperpolarization evoked by fluid onset is missing from this cell's response profile; furthermore, the hyperpolarization is especially strong. Cell-by-cell variations in the strength of these segments of the response sequence would be expected to affect the magnitude of the delayed EPSP in response to odor.

Electrical recordings from Type II-like OL neurons

On three occasions, recordings from within the periphery of the OL yielded responses to hydrodynamic and odorant stimuli that may have originated in cells designated Type II in earlier studies (Mellon and Alones, 1995; Mellon, 1996). In that work, a continuous stream of water flowed through the olfactometer during the entire experiment, and, perhaps in response to this,

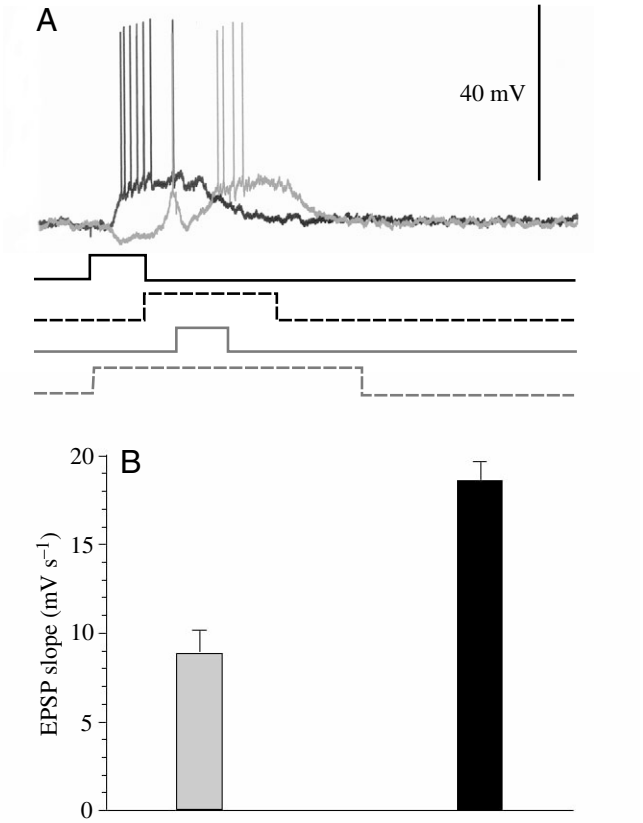

Fig. 12. (A) Records showing differences in the rate of rise of the response to odorant (gray trace and gray stimulus routine) and to a combination of odorant and hydrodynamic stimuli (black trace and routine). Odorant pulses lasted 2 s. (B) Bar graph showing differences in the slopes as mV s^{-1}. Each bar is the mean ± 1 S.E.M. of at least eight measurements. A *t*-test analysis showed the two means to be significantly different at the 0.005 level of confidence.

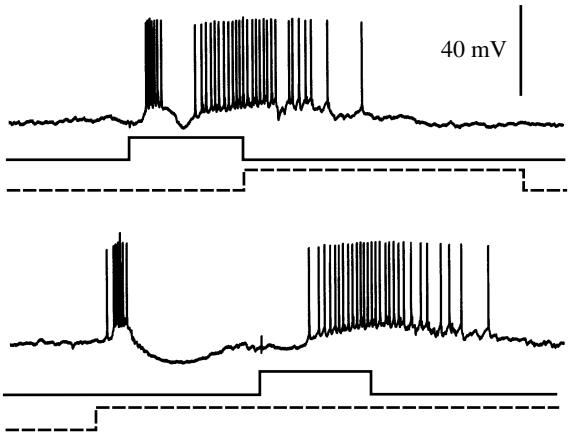

Fig. 13. Records from a Type I cell that showed no potentiation of the response to 2-s pulses of odorant in the different stimulus routines. In the lower trace, note the intensity of the second, hyperpolarizing phase of the response to hydrodynamic input and the absence of a third, depolarizing phase.

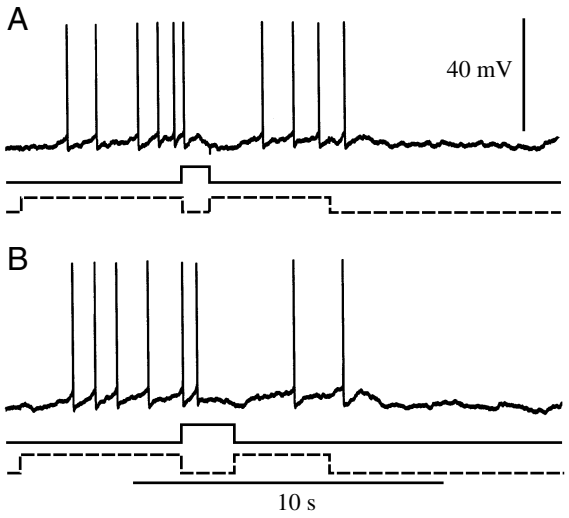

Fig. 14. Electrical responses from a Type II-like neuron recorded in the OL. Water onset was accompanied by spiking activity, which was inhibited in a dose-dependent manner by (A) 1-s and (B) 2-s pulses of 0.05% tetramin.

Type II neurons generated continuous ongoing spiking activity and responded to the introduction of odorants into the water stream by dose-dependent hyperpolarizing inhibition of spiking. In the present study, the lateral antennular flagellum was unstimulated except during specific routines when water and odorant pulses were admitted to the olfactometer. As shown in Fig. 14, the response to the onset of a long water pulse was initiation of a spike train that lasted throughout the period of the water pulse but was interrupted following the odorant pulse to an extent that depended upon odorant duration. While, with the clarity of prior knowledge, these cells might tentatively be identified as Type II, no morphological analysis was undertaken to confirm this.

Discussion

Anatomical evidence for dual sensory pathways

Data presented in this paper indicate that receptors for fluid flow are present on the lateral antennular flagellum and excite action potentials in Type I-like neurons of the crayfish midbrain. Although the receptors providing the input pathway for these responses have not been specifically identified, both pairs of antennae in aquatic crustaceans are in general well supplied with sensilla sensitive to both chemical and mechanical stimuli. In the Australian crayfish *Cherax destructor*, the ventral surface of the lateral antennular flagellum has, as in other macruran decapods, aesthetasc sensilla believed to mediate olfactory reception (Sandeman and Luff, 1974; Sandeman and Denburg, 1976; Ache and Derby, 1985; Ache et al., 1987). In addition, three other types of antennular setae have been described in this species: long (150 μm) guard hairs, short (75 μm) companion hairs and very long (200 μm) procumbent feathered hairs (Sandeman and Luff, 1974). All three non-aesthetasc sensilla types were also found on the internal flagellum of the antennule (Sandeman and Luff, 1974), as well as on the antenna (Sandeman, 1989). Although the procumbent feathered hairs in crayfish are non-innervated (Bender et al., 1984), innervation of at least one class of the other two non-aesthetasc sensilla has been assumed, since hydrodynamic stimulation of the lateral flagellum reliably evokes flicking reflexes, and chemical stimulation of the medial flagellum evokes reflex activity in the antennular slow depressor muscle (Mellon, 1997).

In the spiny lobster *Panulirus argus*, at least nine distinct types of setae in addition to aesthetasc sensilla are found on the antennular flagella, and all of them show evidence of being innervated by both chemosensory and mechanosensory neurons (Cate and Derby, 2001, 2002). Furthermore, previous studies have provided evidence for neurons in the spiny lobster deutocerebrum that are responsive to mechanical as well as odorant stimuli applied to both antennular flagella (Schmidt and Ache, 1996b). These cells have dendritic inputs within not only the olfactory lobes but also the lateral antennular neuropil (LAN), an integration center for mechanical and non-olfactory chemosensory inputs (Schmidt et al., 1992; Schmidt and Ache, 1992, 1996a,b). Studies of Type I and II broad-spectrum chemosensory interneurons within the OL of *Procambarus* have also provided anatomical evidence for dendritic arborizations within both the LAN and the OL (Mellon and Alones, 1995; Mellon, 1996). Since mechanoreceptive neurons on the antennules project axons to the LAN in lobsters (Schmidt et al., 1992), and because mechanical stimulation drives antennular flicking in *Procambarus*, it is assumed that mechanoreceptor axons terminate in the LAN; thus, both olfactory and mechanosensory afferents may provide inputs, *via* OL and LAN pathways, respectively, to Types I and II deutocerebral interneurons in crayfish.

Electrophysiological recordings from OL neurons in the present study document multimodal responses to both hydrodynamic and chemical stimulation of the lateral antennular flagellum in *Procambarus clarkii*. It is presumed, but not yet established experimentally, that the hydrodynamic stimuli are mediated by one or more of the setae types present on the lateral antennular flagellum. It is assumed that responses of OL neurons to chemical stimulation of the lateral antennular flagellum are primarily mediated by ORNs associated with aesthetascs on the ventral aspect of the distal half of the lateral flagellum, as these are the only classes of lateral flagellum receptors that innervate the OL directly. However, other classes of chemoreceptor axons may terminate within the LAN (Schmidt et al., 1992), and it is therefore possible that they could contribute to responses evoked in Type I and II deutocerebral interneurons.

Electrophysiological studies of Type I-like interneurons

Most of the electrophysiological responses from Type I-like neurons analyzed in the present paper were not verified by morphological examination of the cells involved, although they strongly resembled the responses to chemical stimulation from neurons that were analyzed morphologically in previous

studies (Mellon and Alones, 1995; Mellon, 1996). In our earlier studies, responses of Type I cells did not include evoked activity to hydrodynamic input, undoubtedly due to the different mode of stimulation used, where freshwater continuously flowed over the antennular flagella, probably causing adaptation of the mechanoreceptors comprising the afferent source of the responses. Furthermore, injection of odorants into the freshwater stream was effected seamlessly, further compromising any lingering hydrodynamic sensitivity.

Neurons judged to be Type I in our current study responded to the onset of water flow past the lateral antennular flagellum by a three-phase response profile that consisted of an initial depolarization, usually generating spikes, following the hyperpolarization lasting a second or more, and a second 'rebound' depolarization that sometimes generated a spike or two. Above a threshold water pulse duration, the length of the pulse had little influence on the form or the amplitude of the three-phase response, unlike the response to odorant input, which increased with longer stimulus pulses. The functional significance of the E–I–E sequence is not intuitively obvious, and, at this stage of investigation, one can only speculate about its role in the integration of mechanical and chemical inputs. In response to a purely hydrodynamic input, the initial, short-latency spike response is curtailed by an ensuing hyperpolarizing shift in the membrane voltage level, although this is usually terminated by a brief, third-phase depolarization. Consequently, the response to fluid shear alone will be phasic and brief. If the hydrodynamic input is accompanied by a simultaneous exposure to odorant, however, the third phase of the hydrodynamic response may summate with the long-latency response to chemical input and may potentiate it. Therefore, if response dynamics of local deutocerebral interneurons play a role in the interpretation of antennular inputs by the crayfish brain, the triphasic response generated by fluid movements could be important in behavioral choices. As suggested above, differences in the respective response-phase amplitudes, either because of adaptation to previous stimulation or due to intrinsic neuronal properties, could be expected to modulate the extent to which such interactions between stimulus categories can happen. Moreover, in those cells where potentiation of responses to odorants does occur in the presence of simultaneous hydrodynamic stimulation, there would be an obvious benefit to the organism in the form of increased sensitivity to dilute or weak odorants.

The cellular mechanisms involved in the enhanced rate of rise of the primary EPSP in some Type I-like neurons during simultaneous hydrodynamic/chemical stimulation (e.g. Fig. 12) are not immediately obvious; possibly, the steeper slope is simply a reflection of different synaptic dynamics of the short-latency, hydrodynamic-evoked response, but other explanations may be valid. For example, an enhanced membrane conductance during the inhibitory postsynaptic potential (IPSP) response phase, coupled with a raised membrane potential, could favor an increased excitatory synaptic current density due to the afference from the odor receptors, because of a reduced membrane time constant. Insights into the processes involved, however, must await computer modeling studies coupled with biophysical experimentation.

Observations on Type II-like interneurons

Type II-like neurons were recorded from on three occasions during the present study. Again, due to the revised stimulation paradigm, the responses of these cells were somewhat different from those originally described by Mellon and Alones (1995). As shown in Fig. 14, these neurons generated tonic impulse activity during water flow past the antennular flagellum, and this activity was inhibited in a dose-dependent manner by a seamless injection of odorant into the water pulse. Type II responses are essentially the inverse of those exhibited by Type I cells, and interactions between each of them and other, target neurons could also amplify weak odorant signals imbedded in eddies within the aquatic environment.

The crucial value of the present findings is the extent to which both hydrodynamic and odorant inputs interact to generate responses in large multiglomerular interneurons in the crayfish deutocerebrum. Even though the immediate role of any of these interneuron classes in detecting odor sources by the organism is not yet understood, the data presented suggest that such interactions may take advantage of the simultaneity of fluid movements and changes in odorant concentration, leading to the potentiation of chemical signals by large multiglomerular OL interneurons.

It has been suggested previously that flavored eddies, such as those in the wake of moving prey, may provide critical cues used in food-finding behavior for aquatic animals. Such eddy rheo-chemotaxis, as it has been called, would depend upon cooperativity between chemical and hydrodynamic inputs to locate the source of moving – or possibly, stationary – food odors (Basil and Atema, 1994; Atema, 1996; Guenther et al., 1996). Another hypothesis predicts that odor-gated rheotaxis, in which animals actively track upstream during smooth turbulent advection when prompted by the presence of odors, is critical to locating sources of chemical stimuli (Weissburg and Zimmer-Faust, 1994; Weissburg, 2000; Weissburg and Dusenbury, 2002). Both of these proposed behavioral mechanisms, as well as others, would depend upon higher-level central integration of conjoint activity of chemosensory and hydrodynamic inputs. Although converging mechanical and chemical inputs to the accessory lobes and lateral protocerebrum of the brain in crayfishes and lobsters have been reported previously (Sandeman et al., 1995; Wachowiak et al., 1996; Mellon, 2000), the present data illustrate that this integration can occur much earlier, in the central olfactory pathway at the level of presumed first-order interneurons within the deutocerebrum.

Finally, crayfish and other crustaceans flick their antennules during food-searching activities, a behavior that must certainly activate antennular hydrodynamic receptors. Thus, it is expected that mechanoreceptive inputs during antennular flicking would supplement activity from the aesthetascs, the spiking activity of which in response to odors, in spiny lobsters

at least, is enhanced by flicking (Schmitt and Ache, 1979). Observations in the current paper suggest that this enhanced input from the ORNs may be amplified at the level of some local interneurons by integration of concurrent input from hydrodynamic mechanoreceptors. It is quite possible, therefore, that interactions between olfactory and hydrodynamic inputs are most pronounced during flicking behavior, a time when the animal is actively searching for food-related odors and would be most attuned to positive interactions between these two critical sources of sensory input. Experiments are currently underway to specifically address this possibility.

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References

Ache, B. W. (1972). Amino acid receptors in the antennules of *Homarus americanus*. *Comp. Biochem. Physiol.* **42A**, 807-811.

Ache, B. W. and Derby, C. (1985). Functional organization of olfaction in crustaceans. *Trends Neurosci.* **8**, 356-360.

Atema, J. (1996). Eddy chemotaxis and odor landscapes: exploration of nature with animals sensors. *Biol. Bull.* **191**, 129-138.

Basil, J. A. and Atema, J. (1994). Lobster orientation in turbulent odor plumes: simultaneous measurement of tracking behavior and temporal odor patterns. *Biol. Bull.* **187**, 272-273.

Bender, M., Gnatzy, W. and Tautz, J. (1984). The antennal feathered hairs in the crayfish: a non-innervated stimulus transmitting system. *J. Comp. Physiol. A* **154**, 45-47.

Cate, H. S. and Derby, C. D. (2001). Morphology and distribution of setae on the antennules of the Caribbean spiny lobster *Panulirus argus* reveal new types of bimodal chemo-mechanosensilla. *Cell Tissue Res.* **304**, 439-454.

Cate, H. S. and Derby, C. D. (2002). Ultrastructure and physiology of the hooded sensillum, a bimodal chemo-mechanosensillum of lobsters. *J. Comp. Neurol.* **442**, 293-307.

Daniel, P. C. and Derby, C. D. (1991). Mixture suppression in behavior: The antennular flick response in the spiny lobster towards binary odorant mixtures. *Physiol. Behav.* **49**, 591-601.

Dusenbury, D. B. (1992). *Sensory Ecology.* New York: W. H. Freeman.

Grasso, F. W. and Basil, J. A. (2002). How lobsters, crayfishes, and crabs locate sources of odor: current perspectives and future directions. *Curr. Opin. Neurobiol.* **12**, 721-727.

Guenther, C. M., Miller, H. A., Basil, J. A. and Atema, J. (1996). Orientation behavior of the lobster: responses to directional chemical and hydrodynamic stimulation of the antennules. *Biol. Bull.* **191**, 310-311.

Koehl, M. A. R., Koseff, J. R., Crimaldi, J. P., McCay, M. G., Cooper, T., Wiley, M. B. and Moore, P. A. (2002). Lobster sniffing: antennule design and hydrodynamic filtering of information in the odor plume. *Science* **294**, 1948-1951.

Mellon, DeF. (1996). Dynamic response properties of broad spectrum olfactory interneurons in the crayfish midbrain. *Mar. Fresh. Behav. Physiol.* **27**, 111-126.

Mellon, DeF. (1997). Physiological characterization of antennular flicking reflexes in the crayfish. *J. Comp. Physiol. A* **180**, 553-565.

Mellon, DeF. (2000). Convergence of multimodal sensory input onto higher-level neurons of the crayfish olfactory pathway. *J. Neurophysiol.* **84**, 3043-3055.

Mellon, DeF. and Alones, V. E. (1995). Identification of three classes of multiglomerular, broad-spectrum neurons in the crayfish olfactory midbrain by correlated patterns of electrical activity and dendritic arborization. *J. Comp. Physiol. A* **177**, 55-71.

Moore, P. A. and Grills, J. L. (1999). Chemical orientation to food by the crayfish *Orconectes rusticus*: influence of hydrodynamics. *Anim. Behav.* **58**, 953-963.

Reeder, P. B. and Ache, B. W. (1980). Chemotaxis in the Florida spiny lobster, *Panulirus argus*. *Anim. Behav.* **28**, 831-839.

Sandeman, D. C. (1989). Physical properties, sensory receptors and tactile reflexes of the antenna of the Australian freshwater crayfish *Cherax destructor*. *J. Exp. Biol.* **141**, 197-217.

Sandeman, D. C. and Denburg, J. L. (1976). The central projections of chemoreceptor axons in the crayfish revealed by axoplasmic transport. *Brain Res.* **115**, 492-496.

Sandeman, D. C. and Luff, S. E. (1974). Regeneration of the antennules in the Australian freshwater crayfish, *Cherax destructor*. *J. Neurobiol.* **5**, 475-488.

Sandeman, D. C., Sandeman, R. E., Derby, C. and Schmidt, M. (1992). Morphology of the brain of crayfish, crabs, and spiny lobsters: a common nomenclature for homologous structures. *Biol. Bull.* **183**, 304-326.

Sandeman, D. C., Beltz, B. and Sandeman, R. (1995). Crayfish brain interneurons that converge with serotonin giant cells in accessory lobe glomeruli. *J. Comp. Neurol.* **352**, 263-279.

Schmidt, M. and Ache, B. W. (1992). Antennular projections to the midbrain of the spiny lobster. II. Sensory innervations of the olfactory lobe. *J. Comp. Physiol.* **318**, 291-303.

Schmidt, M. and Ache, B. W. (1996a). Processing of antennular inputs in the brain of the spiny lobster, *Panulirus argus*. I. Non-olfactory chemosensory and mechanosensory pathway of the lateral and median antennular neuropils. *J. Comp. Physiol. A* **178**, 579-604.

Schmidt, M. and Ache, B. W. (1996b). Processing of antennular input in the brain of the spiny lobster, *Panulirus argus*. II. The olfactory pathway. *J. Comp. Physiol. A* **178**, 605-628.

Schmidt, M. and Derby, C. D. (2005). Non-olfactory chemoreceptors in asymmetric setae activate antennular grooming behavior in the Caribbean spiny lobster *Panulirus argus*. *J. Exp. Biol.* **208**, 233-248.

Schmidt, M., Van Eckeris, L. and Ache, B. W. (1992). Antennular projections to the midbrain of the spiny lobster. I. Sensory innervation of the lateral and medial antennular neuropils. *J. Comp. Neurol.* **318**, 277-290.

Schmiedel-Jakob, I., Anderson, P. A. V. and Ache, B. W. (1989). Whole cell recordings from lobster olfactory receptor cells: Responses to current and odor stimulation. *J. Neurophysiol.* **61**, 994-1000.

Schmitt, B. and Ache, B. W. (1979). Olfaction: responses of a decapod crustacean are enhanced by flicking. *Science* **205**, 204-206.

Wachowiak, M., Diebel, C. E. and Ache, B. W. (1996). Functional organization of olfactory processing in the accessory lobe of the spiny lobster. *J. Comp. Physiol. A* **178**, 211-226.

Webster, D. R. and Weissburg, M. J. (2001). Chemosensory guidance cues in a turbulent chemical odor plume. *Limnol. Oceanogr.* **46**, 1034-1047.

Weissburg, M. J. (2000). The fluid dynamical context of chemosensory behavior. *Biol. Bull.* **198**, 188-202.

Weissburg, M. J. and Zimmer-Faust, R. K. (1994). Odor plumes and how blue crabs use them in finding prey. *J. Exp. Biol.* **197**, 349-375.