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ORTHODROMICALLY AND ANTIDROMICALLY EVOKED LOCAL FIELD POTENTIALS IN THE CRAYFISH OLFACTORY LOBE

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Summary

A local field potential, consistent in form and duration, can be recorded from the olfactory lobe of crayfish following electrical stimulation of the outer flagellum of the antennule. The field potential is reversibly blocked by perfusion of the brain with low-[Ca^{2+}] saline or γ-aminobutyric acid and, to a lesser extent, histamine. Paired shocks to the antennule and antidromic electrical stimulation of olfactory lobe output neurones also partially block the field potential. Comparing the field potential with simultaneously recorded intracellular responses of olfactory interneurones reveals a coincidence between excitatory and inhibitory effects in the interneurones and the appearance of identifiable components of the field potential. We interpret the field potential to reflect the response of neural elements in the olfactory lobe to orthodromic activity in the axons of the olfactory receptor neurones on the antennule. We conclude from the blocking experiments that the greater part of the field potential stems from neurones in the olfactory lobe that are postsynaptic to olfactory receptor neurones. As such, it provides a robust indication of olfactory neurone activity.

Key words: crayfish, *Cherax destructor*, olfaction, field potential.

Introduction

Decapod crustaceans have chemoreceptors on their antennules, antennae, mouthparts, chelae and walking legs. The unique aesthetasc sensilla on the outer flagellum of the antennules, however, are the only chemoreceptors in decapods that have axons projecting exclusively to an area of glomerular neuropile, the olfactory lobes, in the brain. This set of receptors and the lobes to which they project are now generally identified as the ‘olfactory’ system of decapod crustaceans by analogy with the antennae and antennal lobes of insects and the olfactory epithelium and olfactory bulb of vertebrates. The output of the crustacean olfactory lobes is carried by projection neurones in the olfactory globular tract to the hemi-ellipsoid body (Mellon *et al.* 1992*b*; Mellon and Alones, 1997). This complex neuropile is located in the lateral protocerebrum near the optic neuropiles (Sandeman *et al.* 1992).

The many similarities between vertebrate and invertebrate olfactory systems, from receptor mechanisms through to the organization of the first synaptic neuropile into glomeruli, have been well documented (Ache, 1991; Hildebrand, 1995; Christensen *et al.* 1996; Hildebrand and Shepherd, 1997). Such parallels, in what appears to be an ancient and highly conserved sensory system, mean that comparative studies offer an opportunity to obtain general insights into the apparently tightly constrained neural mechanisms that enable animals to detect, sort and store information about many different odorants. A characteristic and universal feature of all olfactory systems is a multiplicity of input channels and a large number of small neurones in the first synaptic stage. Exploration of these first synaptic areas of vertebrates (olfactory bulb) has a long history, and early extracellular recordings were followed by intracellular studies resulting in accurate anatomical and physiological accounts of the neural elements and their connectivity. Despite this body of information, the olfactory system is not as well understood as other sensory systems and there is still uncertainty about some of its most fundamental functional properties (see review by Shipley and Ennis, 1996).

The exploration of invertebrate olfactory systems has followed a path similar to that taken for vertebrates and, while the diversity of invertebrates has meant that one cannot find the same close anatomical homologies evident among vertebrates (Andres, 1970), details of neural elements and their function in the olfactory systems of snails, insects and crustaceans are slowly emerging. One of the most surprising comparative aspects of these studies is that many electrical responses of higher-order olfactory cells to afferent stimulation, whether recorded as field potentials or intracellularly from single cells, are virtually identical in vertebrate and invertebrate systems (Christensen *et al.* 1996).

The olfactory system of crustaceans, in particular freshwater crayfish, marine lobsters and spiny lobsters, has quite recently received increased attention and has reached the point where the neural components in the first stages of the olfactory

pathway are known both anatomically and, in some cases, physiologically (Sandeman and Luff, 1973; Mellon *et al.* 1992*a*; Mellon and Alones, 1993; Sandeman and Sandeman, 1994; Wachowiak and Ache, 1994; Sandeman *et al.* 1995*b*; Gomez and Atema, 1996*a*,*b*; Wachowiak *et al.* 1996, 1997).

Like all other olfactory systems, the afferent and central pathways of the crustaceans involve large numbers of small neurones that present the investigator not only with certain technical difficulties in terms of obtaining electrical recordings from such small elements without destroying them, but also in drawing conclusions about how the system works from the responses of one element among many thousands. This difficulty can be overcome by recording from a large number of single neurones, but there are approximately 200 000 projection neurones in the olfactory lobe of a specimen of *Cherax destructor* with a carapace length of 6 cm, making the gathering of a representative sample a challenging task. A solution to this dilemma is to record the field potential generated by large numbers of neurones in the hope that, although such field potentials will not provide the precision of recording from the individual neurones, they can provide an overview of those neurones acting in concert that will not be seen at the level of the single cell. Studies of local field potentials in the olfactory systems of snails and insects have provided valuable insights and were instrumental in revealing that populations of olfactory neurones oscillate in synchrony following the application of odours to the chemoreceptors, a feature that may lie at the very core of the neural coding of odorants (Gelperin *et al.* 1996; Laurent, 1996).

We have found that orthodromic electrical stimulation of the outer flagellum of the crayfish antennule results in a large local field potential (LFP) in the olfactory lobe which is of relatively long duration and consistent in form. Field potentials in the olfactory lobe can also be evoked by antidromic stimulation of output projection neurones in the olfactory globular tract. Antidromic stimulation transiently inhibits orthodromic LFPs, suggesting the presence of a recurrent inhibitory pathway. In this report, we describe the nature of the field potentials and their correlation with simultaneously recorded intracellular responses from olfactory interneurones. We have also explored the effect on the field potentials of perfusing the brain with saline containing γ-aminobutyric acid (GABA), histamine, picrotoxin and cimetidine because particular areas of the crayfish olfactory lobe label strongly in response to antibodies to GABA and histamine. We conclude that the orthodromic field potential is a robust measure of olfactory activity and a useful tool in the study of olfactory processing in crayfish.

Materials and methods

An Australian freshwater crayfish, *Cherax destructor* (Clark), was used in this study. Animals were taken from ponds near Sydney, kept in aquaria in the laboratory and fed on carrot and ground beef. Experiments were performed on a semi-isolated, antennule–brain preparation, in which the cor frontale, situated immediately anterior to the brain, and the lateral blood vessel to one antennule were cannulated and perfused with cold (16–18 °C) saline (see below) from a common supply line (Fig. 1A). The brain cannula was secured in the lumen of the cor frontale with a ligature, whereas the lateral cannula lay free in the lumen of the dilated vessel to provide a pressure shunt. Saline flowed through the preparation at 60 ml h^{-1}. The preparation provided access to the dorsal surface of the brain while keeping the eyes, antennules and antennae intact. The oesophageal connectives were severed. Neuronal responses to illumination of the eyes, to electrical or chemical stimulation of the antennules and to touching the antennae were stable in such preparations for at least 8 h.

Physiology

Electrical stimuli were applied to the distal third of the external flagellum of the antennule (orthodromic) through two silver wires on which the flagellum rested (Fig. 1A) and through a glass suction electrode applied to the exposed olfactory globular tract (antidromic) in the protocerebral commissure on the contralateral side of the brain (Fig. 1B). Stimulation of the intact antennular flagellum, instead of exposing the olfactory nerve that lies within it, has the disadvantage of requiring much stronger currents to evoke potentials in the olfactory lobe. This was offset by the convenience of the method and by the lack of damage to the olfactory nerve. The need for high-intensity single-pulse stimulation was circumvented by applying a volley consisting of a pair of lower intensity 1 ms pulses, 10 ms apart. The olfactory globular tract stimulation was effective only when the cleaned tip of the suction electrode (diameter approximately 30 μm) rested firmly on the exposed surface of the tract. Small lateral displacements of the electrode tip or the presence of glial tissue between the electrode tip and the tract were enough to prevent the stimulus from reaching and activating the tract. Single pulses of 0.1 ms duration were applied to the olfactory globular tract.

LFP recordings were made with saline-filled, glass patch electrodes with an internal tip diameter of 3–5 μm and a resistance in saline (see below) of approximately 1 MΩ. The electrodes were coupled to a WPI DAM80 preamplifier with the high- and low-pass filter set to select a bandwidth from 0.1 Hz to 1 kHz.

LFPs recorded between a monopolar focal electrode in a localised region of neuropile and a reference (ground) electrode outside the neuropile reflect the sum of all currents that flow between the tissue in the vicinity of the electrode tip and the reference electrode. Currents flowing in opposite directions, for example, could partially or entirely cancel one another out and perhaps go undetected. Details of the potentials that are recorded depend on the nature of the neural tissue and the placement of the recording and reference electrodes (Rall and Shepherd, 1968; Hubbard *et al.* 1969; Galik and Conway, 1997), parameters that are often not simple to define or control in complex neuropile.

The heterogeneity of the crayfish olfactory lobes required us to take steps to standardise our recording procedure.

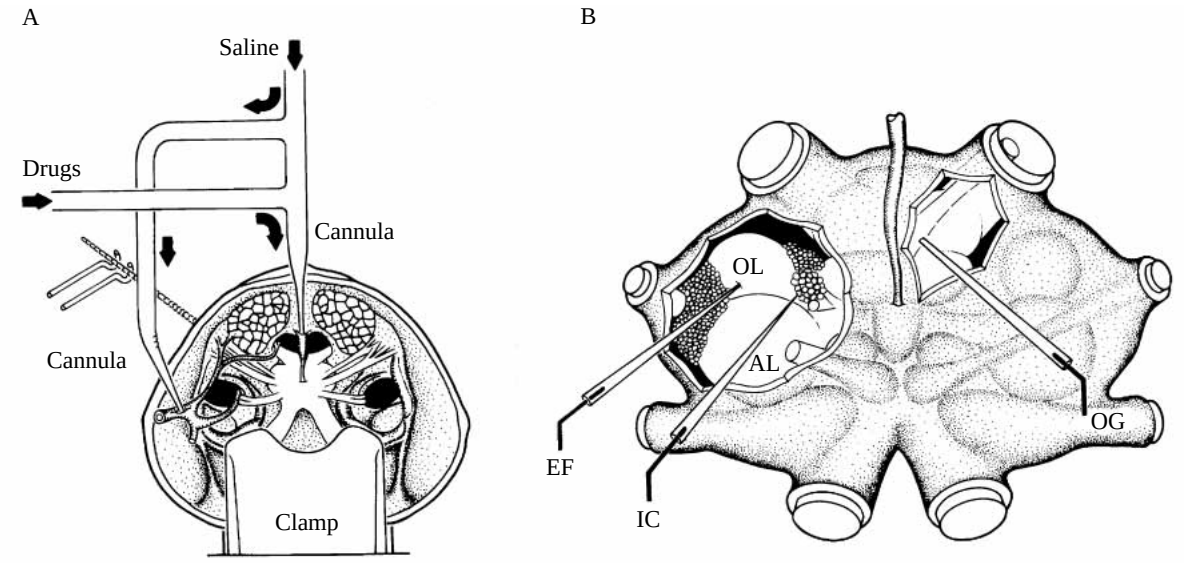

Fig. 1. (A) The isolated head preparation of *Cherax destructor* showing perfusion cannulae in the median artery of the brain and in the lateral anterior artery on the left side of the cephalothorax. The rostrum of the animal points away from the viewer (into the page), dorsal is to the top of the page and the ventral floor of the cephalothorax is fastened in the clamp. Neuroactive substances (drugs) were introduced along the sidearm through the T-junction, from where they were carried with the perfusing saline (arrows) into the median artery of the brain. Electrical stimuli were delivered to the outer flagellum of the left antennule through the two silver wires supporting it. (B) Dorsal view of the crayfish brain showing areas exposed in the isolated brain preparation. The sheath was removed over the olfactory (OL) and accessory (AL) lobes on the left side of the brain to allow local field potentials to be recorded with a glass patch electrode (EF) in the olfactory lobe and intracellular responses (IC) to be recorded with a glass microelectrode from the cell bodies of the interneurones in cell cluster 11. Removal of the sheath over the protocerebral tract on the right side of the brain provides access to the olfactory globular tract, which was stimulated with a glass suction electrode (OG).

Accordingly, our recording electrodes were all chosen to have very nearly the same tip diameters and were placed as nearly as possible at the same depth and in the same area of the olfactory lobe in all preparations. The reference electrode was always in the same position in the bathing solution near the surface of the olfactory lobe. Stimuli were applied at intervals of 20–30 s to exclude changes in the LFP amplitude due to fatigue or habituation. When such precautions were taken, the recorded LFPs repeatedly exhibited identifiable details.

Intracellular recordings from the cell somata of type III olfactory interneurones were made with KCl-filled (3 mol l^{-1}) microelectrodes with a resistance of approximately 80 MΩ. All recordings were digitised and stored on magnetic tape (DTR-1802, Biologic Instruments, France) and later analysed using a CED1401plus (Cambridge, UK) signal analyser.

Application of neuroactive agents

The effect of neuroactive substances on the LFP was explored by introducing these in saline from a microprocessor-controlled syringe pump (SP100i, WPI Instruments, Sarasota, USA) connected *via* a T-junction to the cannula entering the cerebral artery, where it mixed with normal saline before entering the brain (Fig. 1A). The cerebral artery enters the brain on its dorsal side. Its main trunk passes through the brain to the ventral side, where it divides into two large lateral branches extending directly to capillary networks permeating the olfactory and accessory lobes. Substances introduced into the cerebral artery are therefore brought rapidly into close association with all areas of the brain including the olfactory and accessory lobes.

A slight increase in flow rate in the perfusion cannulae was an inevitable consequence of injection of the neuroactive substances, but controls in which normal saline was used produced no observable effect. The lateral cannula also provided a release for pressure build-up in the medial cannula.

A disadvantage of this method is that it is not possible to assess accurately concentrations of neuroactive substance reaching the tissues, and we found that preparations varied in their individual sensitivity to applied agents. A source of this variation may lie in the architecture of the blood system: there are three large blood vessels that extend from the cor frontale, two of which go to the eyes. Tests with food dyes showed that, while a proportion of the perfusion fluid in the cor frontale always reaches the olfactory lobes, it also escapes to the eyes through small blood vessels, to the muscles of the gastric mill and from the median part of the brain when this was desheathed to gain electrode access to the olfactory lobe and olfactory globular tract. All of these factors could have contributed to the variability in sensitivity to applied agents that we found in different preparations.

Given the difficulty of controlling the above variables, we adopted a procedure in which the perfusion syringe always contained the neuroactive agent made up in saline to a concentration of 3 mmol l^{-1}. The quantity and rate at which the

agent was added to the median perfusion cannula were slowly increased until an effect was observed, and then further increased to test that the effect was dose-dependent. Wash-out and injection of saline controls from the syringe were always carried out after the administration of agents, and any preparation that exhibited irreversible change was immediately abandoned. Given the flow rates of the normal saline and added agents, and assuming complete mixing of agent and saline, estimates were made of the concentrations of agents that arrived at the entrance to the brain blood system. These never exceeded 150 μmol l^{-1} and were usually effective at concentrations from 40 to 100 μmol l^{-1}.

Salines and neuroactive substances

Normal saline contained 12 g of NaCl, 0.4 g of KCl, 1.5 g of $CaCl_2 \cdot 2H_2O$, 0.25 g of $MgCl_2 \cdot 6H_2O$ and 0.2 g of $NaHCO_3$ in 1 l of reverse-osmosis-treated water.

Ca^{2+}-free saline was prepared from normal saline from which the $CaCl_2$ was omitted. To preserve osmolarity, the quantity of NaCl was raised to 14.1 g l^{-1} (Weast and Astle, 1983).

All neuroactive substances, GABA (γ-amino-*n*-butyric acid, $C_4H_9NO_2$), histamine ($C_5H_9N_3 \cdot 2HCl$), picrotoxin ($C_{30}H_{34}O_{13}$) and cimetidine ($C_{10}H_{16}N_6S$), were obtained from Sigma Chemicals, St Louis, USA.

Results

Anatomical organisation of the olfactory lobe

The anatomy of the olfactory system of *Cherax destructor* has been described in previous publications (Sandeman and Luff, 1973; Mellon *et al.* 1992*b*; Sandeman and Sandeman, 1994; Sandeman *et al.* 1992, 1995*a*,*b*) and we limit ourselves here to those aspects that are relevant to the present study. Like other decapod crustaceans, olfactory receptor neurones in *C. destructor* are collected into special aesthetasc sensilla found only on the external ramus of the antennule. Axons from these receptors project exclusively to the spherical olfactory lobes in the brain. The olfactory lobes in *C. destructor* contain approximately 250 column-like glomeruli radiating out from the centre of the lobe, and the afferent axons enter these from the periphery and extend down their entire length (Sandeman and Sandeman, 1994) like those in other crayfish (Mellon and Alones, 1993).

The output of the olfactory lobes is *via* a fine-fibred tract, the olfactory globular tract. Axons in the olfactory globular tract from olfactory lobes on each side of the brain meet in a chiasm in the centre of the brain where some branch to both hemi-ellipsoid bodies in the protocerebrum. No axons have been seen to cross between the two olfactory lobes. A small neuropile, the olfactory globular tract neuropile, lies embedded in the axons of the olfactory globular tract near where it exits from the olfactory and accessory lobes. The large dorsal giant 5-hydroxytryptamine-immunoreactive (5HT-IR) neurone receives inputs from elements in this neuropile, which may link it to the olfactory system (Sandeman and Sandeman, 1994; Sandeman *et al.* 1995*b*).

Central neurones with their cell bodies in three separate clusters are associated with the olfactory lobe. These consist of two groups of local neurones with their somata located in two clusters (9 and 11), which lie medial to the olfactory lobe, and the projection, or output, neurones with somata in a large cluster lateral to the olfactory lobe (cluster 10) (Sandeman *et al.* 1992). There are approximately 70 000 local interneurones in cluster 9 and approximately 110 000 projection neurones in cluster 10 in *C. destructor* individuals that have a carapace length of 4 cm. Cell somata of projection neurones and local neurones in cluster 9 are characteristically small (10–15 μm in diameter), have nuclei that almost entirely fill the cell somata and have been referred to as 'globuli' cells (Hanström, 1925). Cell somata in cluster 11 range in diameter from 20 to 50 μm with the exception of the dorsal giant neurone, a unique 5HT-IR cell (Sandeman *et al.* 1992; Sandeman and Sandeman, 1994) that has a cell body 80–100 μm in diameter.

Orthodromic local field potentials

An extracellular recording electrode driven into the olfactory lobe in the absence of any stimulus to the antennule detects different kinds of activity at different levels. At the surface of the lobe, the electrode tip met mild resistance, presumably from a layer of glial cells and afferent axons. Advancing the electrode resulted in a large transient potential, probably associated with the electrode tip breaking through a glial cell layer and giving an immediate increase in the level of the recorded activity. Occasionally, single units were detected, but more usually the activity consisted of a compound mixture of slow and fast potentials. Advancing the tip further into the lobe was accompanied by a decrease in the level of baseline activity and sometimes by the presence of unitary action potentials. Depth measurements showed that electrode tips recording this level of activity lay 100–150 μm from the surface of the lobe, suggesting that they were at or close to the bases of the glomeruli.

In the initial stages of the investigation we sought some confirmation that the electrical activity in the lobe had some relevance to chemoreception and the antennule. We positioned an electrode so that the tip was 100–150 μm deep in the lobe, and irrigated the antennule with a Pasteur pipette containing first water and then a solution of fish food. Although qualitative and not pursued further, the test showed that an increase in electrical activity accompanied the presentation of fish food that did not occur when the antennule was flushed with water (Fig. 2). This result was reassuring, interesting and is worth further investigation, but the long duration and extreme complexity of the response made it unsuitable for the task in hand.

Electrically stimulating the outer flagellum of the antennule results in a complex local field potential (LFP) that can be detected with a recording electrode located approximately 50–150 μm from the surface of the lobe on the same side of the brain as the stimulated antennule. No LFP can be evoked by stimulating the internal branch of the antennule on the same side, nor by stimulating any of the other appendages of the head, ipsilateral or contralateral.

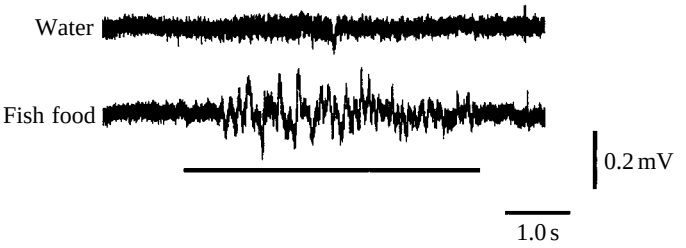

Fig. 2. Extracellular recordings from the olfactory lobe during irrigation of the external flagellum of the ipsilateral antennule first with water, which produces small spike-like potentials, and then with a solution of goldfish food, which produces larger compound potentials. The bar indicates the approximate duration of antennular irrigation.

The LFP increases in amplitude with an increase in stimulus intensity, eventually reaching a maximal amplitude beyond which an increase in stimulus intensity had no further effect (Fig. 3A). Application of such a supramaximal stimulus more frequently than once every 10–15 s led to a gradual decrease in the amplitude of the LFP, which then recovered its full amplitude after a few minutes' rest.

A fully developed LFP contains three broad 'components' (Fig. 3B,C) which change in polarity and amplitude depending on the electrode position in the lobe. In order to control for these variables, the electrodes were always positioned at a depth of 100–150 μm from the surface of the lobe, and stimulus strength was increased until supramaximal. Under these conditions, the LFP exhibited a form, although not necessarily an amplitude, that was consistent from one preparation to the next (N=53).

We have numbered the three components according to their appearance in an LFP produced by a supramaximal stimulus (Fig. 3C). Component 1 is positive-going and appears 50–70 ms after the stimulus to the antennules. It increases in size with an increase in stimulus amplitude and often exhibits inflections on the rising phase. Component 2 is large and negative-going. Its development with increasing stimulus intensities was more abrupt than that of component 1 (see fourth trace from the top in Fig. 3A). Component 3 is negative-going, slow and of long duration. It is often separated from component 2 by a small negative-going inflection, followed by a slow positive-going phase that may last up to 1.5 s after the stimulus. Component 3 sometimes terminates in an abrupt negative inflection (Figs 3A, 4A) and the resumption of the ongoing activity of the preparation, which was reduced during the entire evoked response. Component 3 has a lower threshold to stimulation than component 2.

A marked difference in LFP amplitude was often a feature of different preparations. We have no good explanation for this phenomenon. Adjustment of electrode depth, while producing the usual changes in form, neither increases the amplitude of 'poor' preparations nor decreases that of 'good' preparations. Overzealous aspiration of glial cells from the surface of the olfactory lobe during initial preparation sometimes resulted in a significant attenuation of the amplitude, but not shape, of the

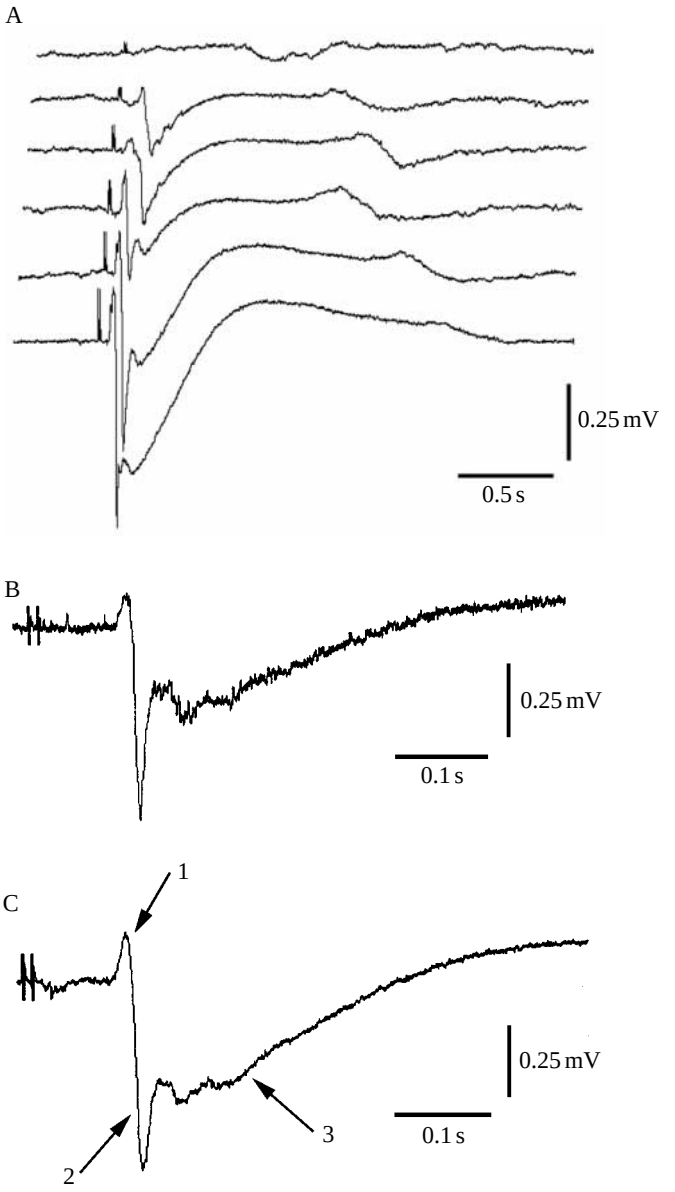

Fig. 3. The local field potential (LFP) in the olfactory lobe that results from electrical stimulation of the external flagellum of the ipsilateral antennule. (A) A gradual increase in the intensity of the stimulus to the antennule (from top trace down) leads to an increase in the amplitude of the LFP and the appearance of the different components. (B) The LFP from a different preparation displayed with a faster time base. (C) The average of 21 responses from the preparation in B. The three components of the LFP that can be consistently recognised are indicated by arrows 1–3. The duration of the LFP in response to a supramaximal stimulus is always between 1.5 and 2 s.

LFP, implying that one requirement for the appearance of a large LFP is a reasonably high resistance across the glomerular layer separating the electrode tip from the reference electrode in the fluid outside the lobe. Aspiration may damage the dense layer of afferent axons that spread over the surface of the lobe and lead to a reduction in the LFP. The size of the animals,

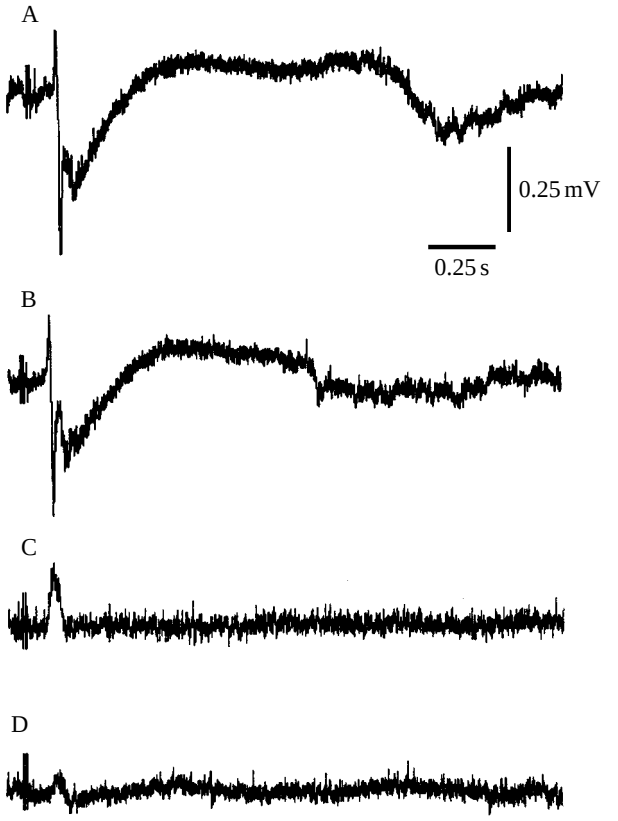

Fig. 4. Perfusion of low-[Ca^{2+}] saline into the brain first reduces the amplitude of component 2 and the duration of component 3 of the local field potential (A,B). Both components are then abolished, leaving a positive-going component 1 (C) which, although reduced in amplitude, persists with continued perfusion (D). Each trace is separated from the next by 20 s.

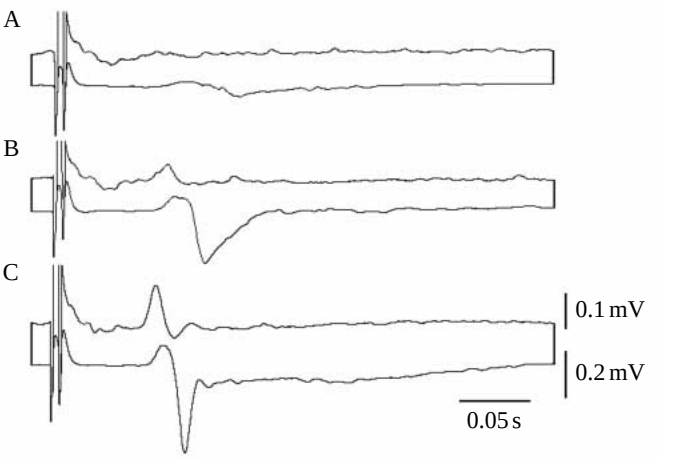

Fig. 5. Extracellular recordings from the antennular nerve bundle ventral to the olfactory lobe (upper trace in each pair) and the local field potential (LFP) (lower trace in each pair). (A) The stimulus to the antennule was just above threshold for the generation of a compound action potential, resulting in the appearance of component 3 of the LFP. (B) Components 1 and 3 of the LFP appear after a submaximal stimulus. (C) For supramaximal stimuli, the three components of the LFP are present. The compound potential precedes the peak of component 1. Inflections in the recording of the compound action potential are probably from the larger and more rapidly conducting axons of mechanoreceptors on the external flagellum.

and therefore the thickness of the antennular cuticle surrounding the olfactory nerve, also appeared to be a factor that influenced LFP amplitude. For this reason, we limited ourselves to animals with a carapace length of between 45 and 50 mm for all experiments.

Oscillatory potentials follow antennular stimulation in some preparations. Most often, oscillations occurred at submaximal stimulation of the antennule and were less pronounced with supramaximal stimulation. Oscillations were almost always present in preparations that had been treated with picrotoxin (see below).

Pre- and postsynaptic components of local field potentials

If the orthodromic LFP in the olfactory lobe is a reflection of all the neural elements that are activated by the olfactory receptor neurones, blocking synaptic transmission with low-[Ca^{2+}] saline should remove the contribution to the LFP of the postsynaptic elements. To do this experiment, Ca^{2+}-free saline was introduced with the normal saline into the brain to produce a final concentration of approximately half the normal value. The result was an immediate reduction in the amplitude of component 2 and in the duration of component 3 (Fig. 4A,B), followed by the abolition of both after about 40 s with continued perfusion (Fig. 4C,D). Component 1 was reduced in amplitude but persisted, suggesting that it represents, at least in part, activity in afferent terminals. The LFP was fully restored after re-perfusion with normal saline.

To test whether component 1 reflected activity in the afferent terminals in the lobe, we placed a second extracellular recording electrode ventral and medial to the olfactory lobe where the fine chemoreceptive axons branch away from the main nerve bundle of the antennule. A compound action potential recorded at this point slightly precedes component 1 of the LFP. Increasing the stimulus intensity from below threshold resulted in a simultaneous increase in the compound action potential and the LFP (Fig. 5A–C). Perfusion of the brain with low-[Ca^{2+}] saline resulted in the abolition of components 2 and 3 as before, but both a portion of component 1 and the compound action potential persisted (Fig. 6A–C). Washing out with normal saline restored the LFP to its former shape and size (Fig. 6D–F). The compound action potential changed very little during the experiment, but the collapse and recovery of the LFP show that the strongly negative-going part of component 2 is masking a positive component that rides on the falling phase of component 1 (Fig. 6B,E).

Low-[Ca^{2+}] saline also reduced ongoing activity in the lobe, which returned after replacement of the low-[Ca^{2+}] saline with normal saline. Low-[Ca^{2+}] experiments were carried out on eight animals. Simultaneous recordings were made from the afferents in four of these.

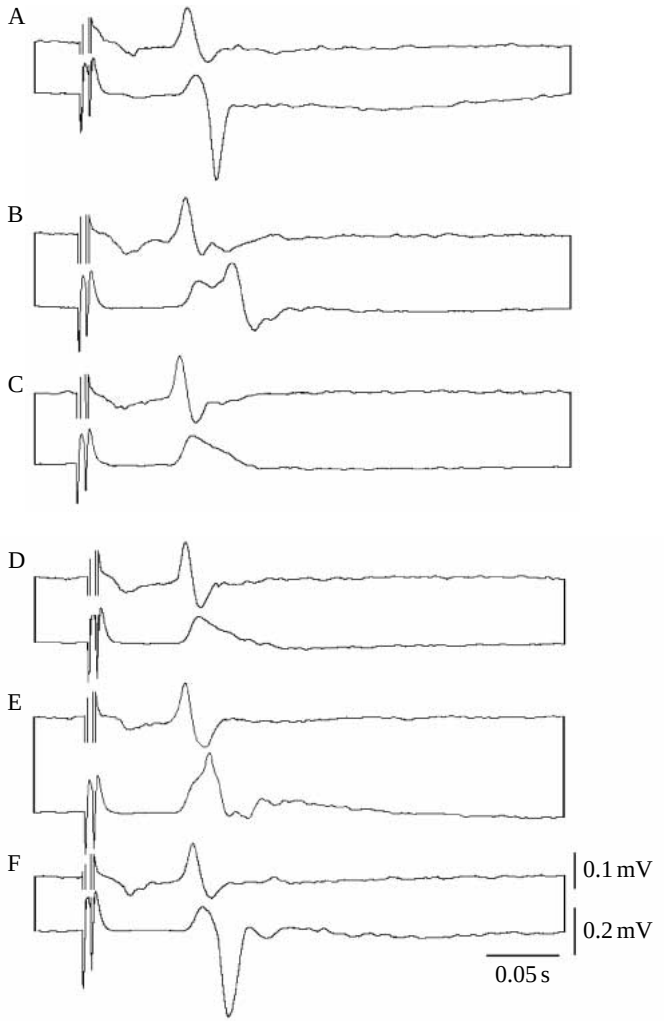

Fig. 6. Extracellular recordings from the antennular nerve bundle ventral to the olfactory lobe (upper trace in each pair) and the local field potential (LFP) (lower trace in each pair) during the perfusion (A–C) and wash-out (D–F) of low-[Ca^{2+}] saline. The compound action potential from the afferents does not change in either size or shape during the treatment with low [Ca^{2+}], but the LFP is reduced to a single positive-going potential (C,D). The strongly negative-going component 2 is the first to be affected by the low [Ca^{2+}], leaving a positive-going component that is usually masked in the supramaximal LFP (B). This is also removed by low [Ca^{2+}], but is the first to return on wash-out (E).

Antidromic local field potentials

LFPs produced by stimulation of projection neurone axons in the olfactory globular tract were recorded from the same depth in the olfactory lobe from which the orthodromic LFPs had been recorded. A single stimulus to the exposed surface of the olfactory globular tract in the contralateral protocerebral tract (Fig. 1B) results in a predominantly negative-going, biphasic LFP of short duration in the olfactory lobe. Stimulation of the axon bundles lying on either side of the olfactory globular tract did not produce an LFP in the olfactory lobe.

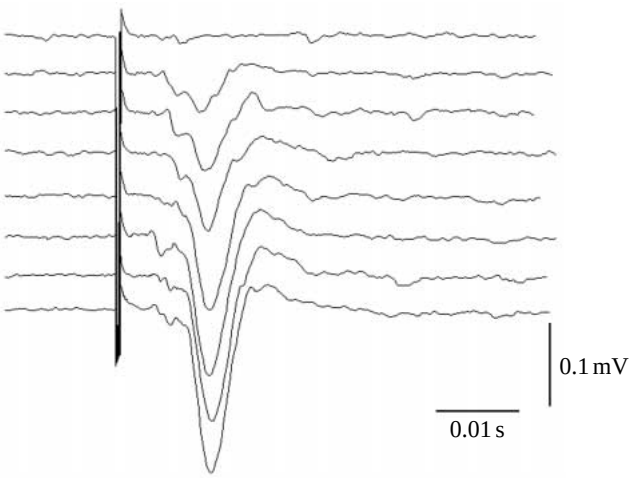

Fig. 7. Local field potentials (LFPs) recorded from the olfactory lobe following single electrical stimuli of increasing intensity applied to the exposed surface of the contralateral olfactory globular tract. The top trace shows the lowest intensity. The bottom trace shows supramaximal intensity, beyond which there was no further increase in LFP amplitude with an increase in stimulus intensity.

The amplitude of the LFP was graded, dependent on the intensity of the stimulus applied to the olfactory globular tract (Fig. 7) and could be produced by stimulation of either ipsilateral or contralateral branches of the olfactory globular tract.

In contrast to the complex orthodromic responses, antidromic LFPs appear to consist of one component, although a small inflection on the initial phase could indicate axons of different conduction velocities within the population that contribute to the compound potential. We estimated the conduction velocity of the axons mediating the antidromic LFP by measuring the distance between the stimulating and recording electrodes and the time delay between the stimulus and response. Conduction velocities ranged between 0.15 and 0.25 m s^{-1}, a result that is in accord with the small diameter of the fibres (less than 0.2 μm) found in the olfactory globular tract. Antidromic LFPs were recorded in 17 preparations.

Inhibitory effects of orthodromic and antidromic stimulation

Depression of ongoing olfactory lobe activity during orthodromic LFPs suggests that a relatively long-lasting inhibition is set in train by antennular stimulation. We tested the effect of this apparent inhibition on a second orthodromic LFP by applying two volleys to the antennule, separating them in time so that the second volley arrived during or after component 3 of the LFP evoked by the first stimulus.

The result of the double-stimulus experiment was that the second antennular stimulus evoked a purely positive-going LFP, implying that an inhibitory pathway had been activated but that this does not inhibit all the neural elements that make up the LFP (Fig. 8A,D). Increasing the interval between the stimulus volleys showed that this effect persisted for more than 5 s (Fig. 8B), and the LFP produced by the second volley

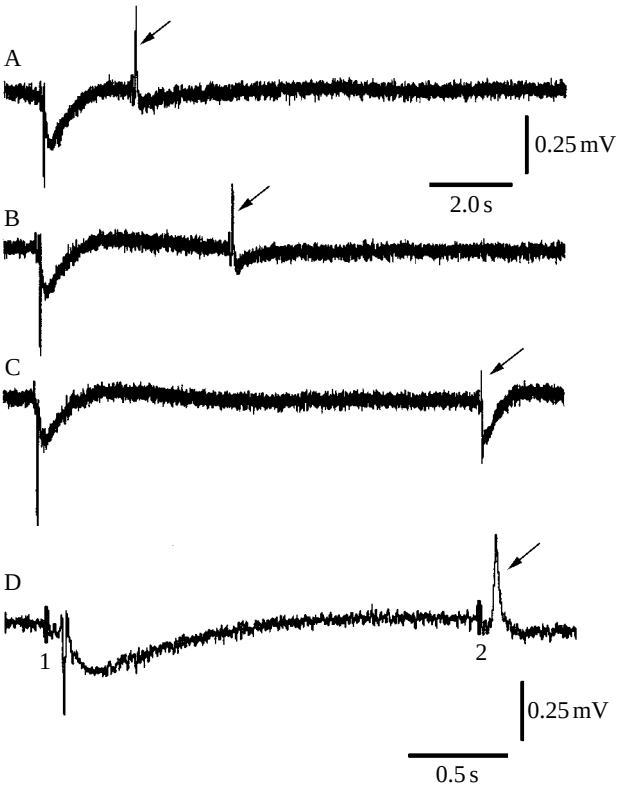

Fig. 8. Stimulus volleys (labelled 1 and 2 in D) delivered to the antennule and separated by a delay, result in a positive-going local field potential (LFP) (arrows) in response to the second stimulus volley (A,D). Increasing the interval between the stimuli allows the LFP in response to the second stimulus to recover its usual form (B,C), although full recovery does not occur until the interval is approximately 10–15 s.

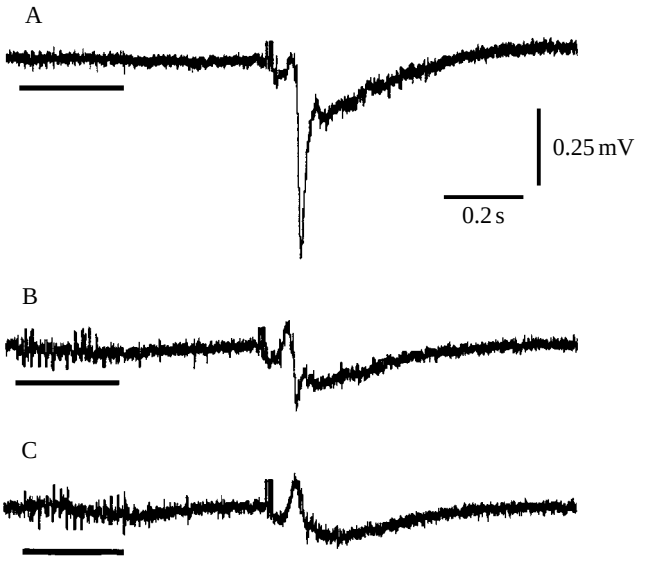

Fig. 9. A train of stimuli to the olfactory globular tract that precedes an orthodromic potential has little effect (A) unless the stimulus intensity is large enough to produce recordable antidromic local field potentials (LFPs). The orthodromic LFP is then reduced in amplitude (B). Increasing the antidromic stimulus intensity further reduces the amplitude of the orthodromic LFP (C). Antidromic stimulation of the olfactory globular tract is indicated by bars beneath the recordings.

recovered its normal shape only when the two volleys were separated by 10–15 s (Fig. 8C). This phenomenon was examined in six preparations.

Preceding the stimulus to the antennule with a train of antidromic stimuli to the olfactory globular tract reduces the size of the orthodromic LFP. This effect is graded and depends on the intensity of the antidromic stimulus train (Fig. 9). Low-intensity stimulation that produced small antidromic LFPs (Fig. 9A) did not affect the orthodromic LFP. Increasing the antidromic stimulus increased the amplitude of the antidromic LFPs and also the inhibitory effect on the subsequent orthodromic LFP (Fig. 9B,C). The effect of the antidromic stimulation on the orthodromic response was examined in 17 preparations.

Orthodromic LFPs and intracellular responses of cluster 11 local neurones

Some idea of LFP composition may be obtained by comparing it with simultaneously recorded intracellular responses of single olfactory interneurones. The local neurones in clusters 9 and 11 and the projection neurones in cluster 10 are all accessible to intracellular recording. To make recordings from a representative sample of the many thousands of neurones in clusters 9 and 10 was beyond the scope of this study, and we therefore focused our attention on the larger cells in cluster 11.

Cluster 11 contains the cell bodies of two separate groups of neurones, those with axons in the deutocerebral commissure and which constitute the input to the accessory lobes (Sandeman *et al.* 1995*a*) and those that have extensive branches in the olfactory lobes or olfactory lobes and accessory lobes and, to a lesser extent, in more medial areas of the deutocerebrum. Some of these, including the unique and very large dorsal giant neurone, label with antibodies to serotonin (Sandeman and Sandeman, 1994). A number of neurones with their cell bodies in cluster 11 in the crayfish *Procambarus clarkii* respond to electrical and chemical stimulation of the antennules, and three classes of multiglomerular, midbrain olfactory neurones have been characterised physiologically and anatomically (Mellon and Alones, 1995). Cluster 11 in *C. destructor* contains neurones that anatomically resemble the type III neurones of *P. clarkii* and so we use the same terminology for them. The cell body of a type III neurone in *C. destructor* lies close to the dorsal giant and could routinely be visually identified and impaled, providing a convenient single chemosensitive neurone to match against the LFP. Neurobiotin fills of the cell show it to have all its branches confined entirely to the olfactory lobe, where it extends to all glomeruli. The response of this cell in *C. destructor* to either electrical or chemical stimuli (fish food) applied to the antennule consisted of a short burst of action potentials

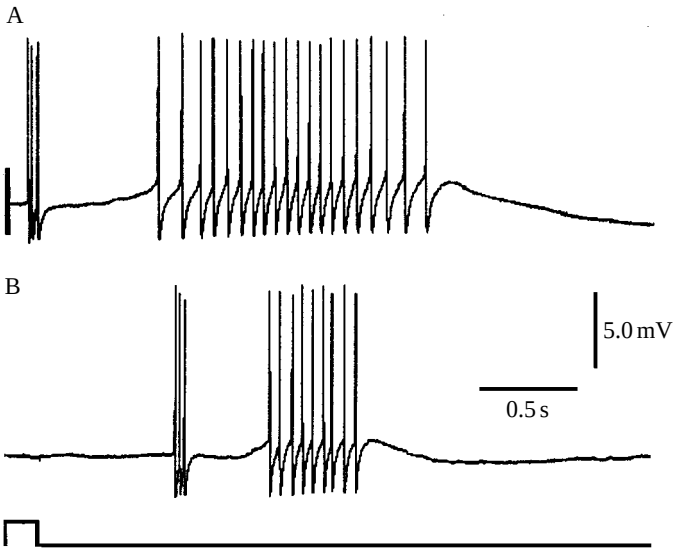

Fig. 10. The responses of a type III olfactory interneurone in cluster 11 to an electrical stimulus to the antennule (A) and to a pulse of fish food introduced into a continuous stream of water which was passing over the antennule (B). In both cases, the neurone responds with an initial burst of impulses followed by a pause and then a second burst. The stimulus marker in A is at the start of the trace. In B it indicates the introduction of the pulse of fish food into the olfactometer; there was a delay before the chemical reached the receptors on the antennule.

followed by a pause and then a second burst of action potentials (Fig. 10).

Simultaneous recordings of orthodromic LFPs and intracellular responses of type III olfactory lobe neurones showed that component 2 of the LFP coincided with the initial burst of action potentials in an olfactory lobe neurone and that component 3 of the orthodromic LFP coincided with the hyperpolarization and cessation of action potentials in the olfactory lobe neurone (Fig. 11A). The LFP is, however, not merely the extracellular expression of the type III olfactory neurone because action potentials in the neurone produced by depolarising it through the recording electrode were not accompanied by a field potential that we could record.

The type III olfactory neurones responded to repetitive stimulation of the olfactory globular tract with a train of action potentials, the frequency of which increased with the intensity of the stimulus. When these preceded an orthodromic stimulus, the orthodromic LFP was reduced, as expected, and was accompanied by a decrease in the number of action potentials in the first burst of the interneurone. Component 3 of the LFP and the hyperpolarization of the type III olfactory neurone were prolonged. (Fig. 11A–D). The LFP and intracellular responses of type III olfactory neurones were compared in six preparations.

Pharmacological modulation of the local field potentials

Immunocytochemical treatment of the brains of *C. destructor* with antibodies raised against GABA has shown

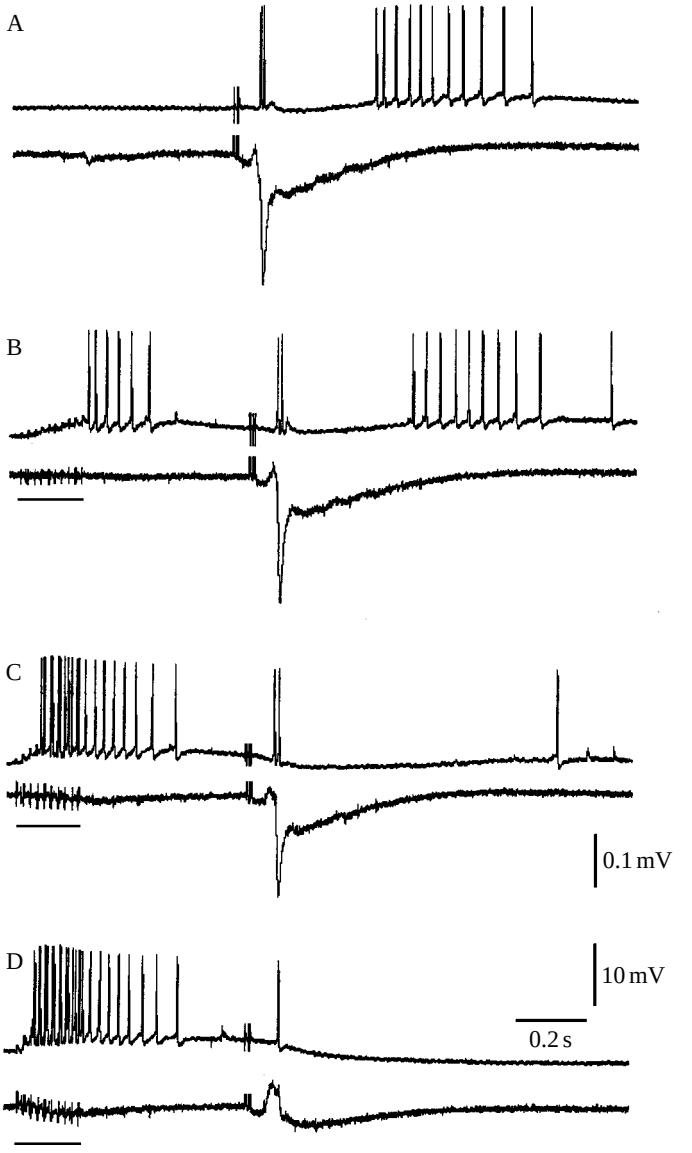

Fig. 11. Intracellular recordings from a type III olfactory interneurone in cluster 11 (upper trace in each pair) and the orthodromic local field potential (LFP) (lower trace in each pair). (A) An electrical stimulus to the antennule produces the characteristic burst, pause, burst of the olfactory lobe interneurone. The initial burst coincides with component 2 of the LFP, and the pause occurs during component 3 of the LFP. (B) Stimulation of the olfactory globular tract with a train of pulses also activates the olfactory lobe interneurone. (C,D) Increasing the stimulus intensity to the olfactory globular tract increases the firing frequency of the olfactory lobe interneurone, but enhances the inhibitory effect that follows the initiation of an LFP. The duration of the stimulus to the olfactory globular tract is indicated by bars beneath the recordings.

that, in common with spiny lobsters (*Panulirus argus*), the cell somata in cluster 9 and the peripheral ‘caps’ of the glomerular columns label strongly (Orona *et al.* 1990; Sandeman and Sandeman, 1995; Wachowiak and Ache, 1997). The olfactory lobes of crayfish and lobsters also label with antibodies raised against histamine suggesting that, among others, these two

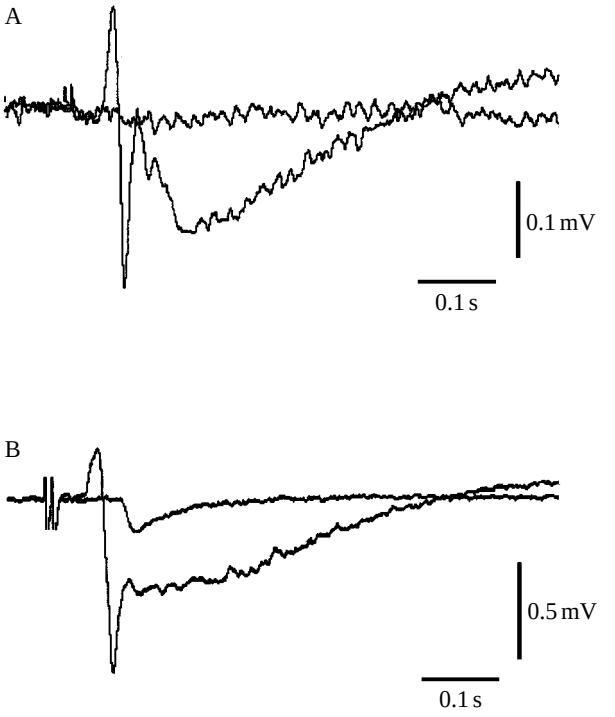

Fig. 12. (A) Two superimposed recordings from a preparation before and during perfusion of γ-aminobutyric acid (GABA) (approximately 50 μmol l^{-1}) into the brain show that GABA abolishes the entire orthodromic local field potential (LFP). (B) Two superimposed recordings from a preparation before and during perfusion of histamine (approximately 150 μmol l^{-1}) into the brain show that the effect of histamine is similar to that of GABA except that a long-latency negative-going component of the orthodromic LFP persists.

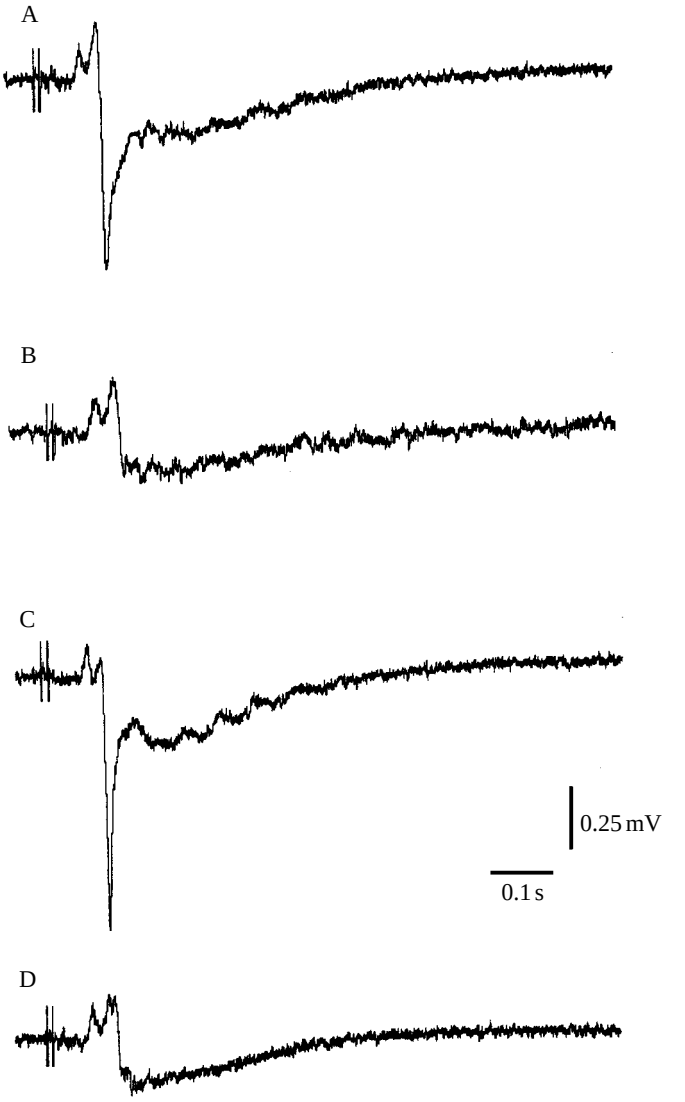

Fig. 13. A supramaximal stimulus to the antennule produces a normal local field potential (LFP) in the olfactory lobe (A) which is reduced to components 1 and 3 when the stimulus intensity is lowered to submaximal (B). During perfusion of the preparation with picrotoxin (approximately 100 μmol l^{-1}), the submaximal stimulus produces an LFP which is larger than that obtained with the initial supramaximal electrical stimulus (C). Washing out the picrotoxin restores the response to its previous submaximal level (D).

transmitters are involved in the neural processing that is carried out in the lobe (Orona and Ache, 1992; Wachowiak and Ache, 1997). We were interested to test the effect of these transmitters and their blockers on the orthodromic LFP because, for it to be useful as a tool for the exploration of the olfactory process, the LFP should at least show some modulation in response to the application of these substances and their blockers.

An electrical stimulus was applied to the antennule every 20–30 s before, during and after the addition of neuroactive agents to saline perfusing the brain. Action of the agent was judged by changes that occurred in the orthodromic LFP while the agent was included in the perfusate.

The effect of GABA on the orthodromic LFPs in *C. destructor* was unequivocal. At low doses (approximately 50 μmol l^{-1}), component 2 was first reduced in amplitude and then removed. Continued perfusion reduced and then abolished components 1 and 3 (Fig. 12A). Recovery from even high doses of GABA (approximately 150 μmol l^{-1}) was always rapid (often less than 1 min) and complete (GABA; N=9).

Perfusion of the *C. destructor* brain with histamine (approximately 150 μmol l^{-1}) has a complex effect on the orthodromic LFP. Component 2 is initially strongly potentiated in the early stages of the perfusion, but is then reduced and finally abolished along with component 1, leaving a longer-latency negative-going potential (Fig. 12B). The components reappear in the reverse order after wash-out, component 2 being the last to recover completely. Preparations took longer to recover from histamine, sometimes requiring up to 10 min before they exhibited an LFP with the original amplitude and form (histamine; N=6).

As a blocker of Cl^- channels, picrotoxin has often been used to prevent the action of GABA in invertebrate preparations (Watson and Burrows, 1987). It is therefore to be expected that the LFP would increase in size in the presence of picrotoxin if GABA were acting as an inhibitor. Perfusion of the brain with

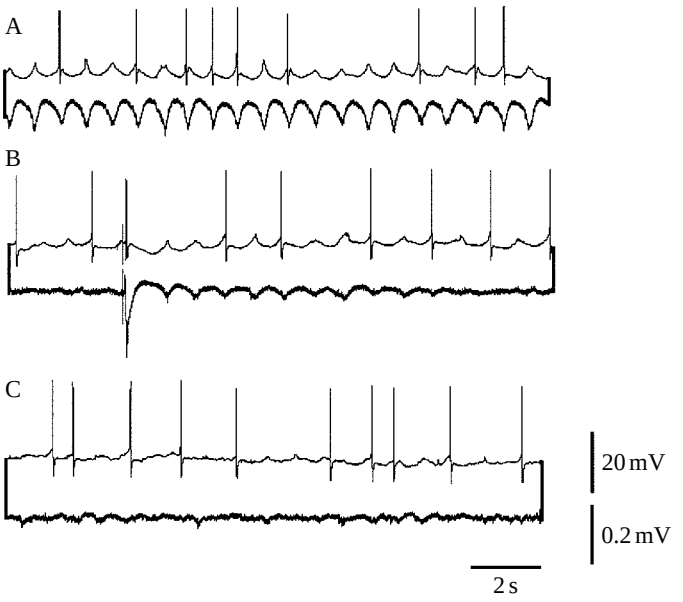

Fig. 14. Simultaneous recordings from a type III olfactory neurone (upper trace in each pair) and local field potentials (LFPs) from the olfactory lobe (lower trace in each pair) during the application of picrotoxin (approximately 100 μmol l^{-1}) to the brain. (A) The membrane potential oscillations and action potentials are synchronised with the continuous oscillatory field potentials. (B) During wash-out, the field potentials are damped out, but they appear again for several cycles after electrical stimulation of the antennule. Oscillation persists in the olfactory neurone. (C) After washing the picrotoxin out of the brain, the oscillations are no longer continuous, but small oscillations can still be detected over short periods in both the extracellular and intracellular recordings.

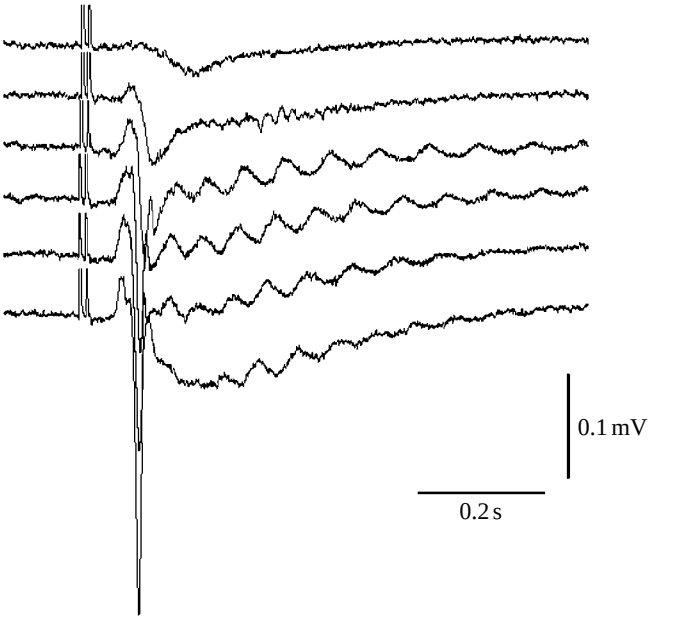

Fig. 15. A series of local field potentials (LFPs) evoked by stimulating the antennule at increasing intensity, from the top trace down, in a preparation that had been treated with picrotoxin (approximately 100 μmol l^{-1}) and then flushed with normal saline. Oscillations are most pronounced at submaximal stimulus intensities (third and fourth traces from the top).

picrotoxin (approximately 100 μmol l^{-1}) enhances the LFP so that a normally submaximal stimulus delivered to the antennule will produce an LFP that is sometimes even larger than can be evoked with supramaximal electrical stimulation (Fig. 13A–D).

Small, low-frequency oscillations of approximately 1–1.3 Hz were often seen in extracellular recordings from the olfactory lobe. These were seldom maintained for more than a few cycles and did not appear to be related to the antennular stimulus. Perfusion of the brain with picrotoxin resulted in these oscillations appearing in a continuous train in the extracellular recording and also as a synchronous membrane oscillation or action potential, in the type III olfactory neurones (Fig. 14A). The oscillations were not modulated by antennular stimulation except during wash-out with normal saline, when several oscillations followed a stimulus and then died away (Fig. 14B). After washing out the picrotoxin, the continuous oscillations ceased and the activity reverted to periods of relative quiet interspersed with a few small oscillations (Fig. 14C).

Faster oscillations of approximately 14 Hz were sometimes seen superimposed on component 3 of the LFP in preparations that had previously been treated with picrotoxin and to some extent in those that had not. These oscillations were more clearly expressed when the stimulus to the antennule was submaximal (Fig. 15) (picrotoxin; *N*=18).

Cimetidine blocks some histamine receptors and, if histamine is acting as an inhibitor in the crayfish olfactory lobe, then the application of cimetidine would be expected to enhance orthodromic LFPs by blocking the histamine inhibition. Perfusion of cimetidine (approximately 30 μmol l^{-1}) was followed by an increase in the amplitude of components 2 and 3 of orthodromic LFPs in very much the same way that was found for GABA, but cimetidine did not lead to increased activity or oscillations in the olfactory lobe (cimetidine; *N*=8).

Discussion

Orthodromic and antidromic local field potentials

The orthodromic LFP stems from neural elements in the olfactory lobe that are driven by the axons of the olfactory receptor neurones because it can be recorded only across the glomerular layer of the olfactory lobe and only after stimulating the external flagellum of the antennule ipsilateral to the olfactory lobe from which the recording is being made. Also, the latency of the response is such as to involve only axons that conduct very slowly (approximately 0.16 m s^{-1}), such as those known to project exclusively to the olfactory lobe from the olfactory receptor neurones (Sandeman and Denburg, 1976; Sandeman and Sandeman, 1994).

Orthodromic LFPs could include the activity of olfactory afferents which penetrate the entire length of the glomerular columns, local interneurones (cell bodies in cluster 9) that project across the glomerular caps, projection neurones (cell bodies in cluster 10) that project through the glomerular columns and large olfactory interneurones (cell bodies in cluster 11), some of which are serotonergic.

The gradual increase in amplitude of all components of the orthodromic LFP, when the stimulus to the antennule is graded in intensity, suggests that we are recording the summed activity of a large number of neurones. If any large elements (e.g. the dorsal giant or large olfactory neurones) are active, their individual signals must be submerged in the summed response because we seldom saw single action potentials of the large olfactory interneurones represented in the LFP recording, and on the few occasions that these did appear, they were only just visible above the general activity of the extracellular recordings.

Perfusion of the brain with low-[Ca^{2+}] saline decreases the ongoing spontaneous level of activity in the olfactory lobe and abolishes all but component 1 of orthodromic LFPs. Having the shortest latency of all components and being insensitive to low-[Ca^{2+}] saline is indicative of a part of the system unaffected by failure of synaptic transmission. Our suggestion is, therefore, that component 1, or at least that part of it remaining in the presence of low-[Ca^{2+}] saline, represents the activity of olfactory afferents.

Component 2 of orthodromic LFPs develops later than either components 1 or 3 and reaches its full size over a relatively small increment of stimulus intensity. It also has the largest amplitude of all components. Both these features suggest a large population of neurones that have very similar response latencies. Component 2 of the LFP coincides with the initial excitatory burst of the large type III olfactory interneurone. In lobsters (*P. argus*), three out of four types of local interneurones respond to electrical stimulation of the antennule with an initial burst followed by no, or a small, hyperpolarization. The fourth type responds with an initial burst and a much longer (1 s) hyperpolarization (Wachowiak *et al.* 1997). The projection neurones of spiny lobsters (*P. argus*), however, are fairly uniform in their production of an initial burst followed by a hyperpolarization lasting for 1–3 s (Wachowiak and Ache, 1994). Component 2 of the LFP in the crayfish *C. destructor* therefore probably reflects activity of both local and projection neurones, which could explain its fractionation into subcomponents during recovery from blocking with low-[Ca^{2+}] saline and during the application of histamine.

Component 3 of orthodromic LFPs has a low threshold and the longest duration. It is characterised by a general decrease in the extracellular activity recorded in the olfactory lobe and its termination is accompanied by a rebound of this activity. Component 3 coincides with the cessation of action potential activity in the type III interneurones and matches the hyperpolarising phases of the lobster local and projection neurones. Component 3 appears to reflect global and relatively long-lasting inhibition in the olfactory lobe.

In general terms, there seems to be a good overall correlation between the components of the crayfish LFP and the responses of local interneurones and projection neurones to electrical stimulation of the antennules in spiny lobsters, (Wachowiak and Ache, 1994; Wachowiak *et al.* 1997), insects (Christensen *et al.* 1993) and even salamanders (Christensen *et al.* 1996). It is becoming increasingly apparent that animal olfactory systems share many common design features (Hildebrand and Shepherd, 1997).

Antidromic LFPs are relatively small and uninflected, follow repetitive stimulation at high frequencies and are little affected by the application of low-[Ca^{2+}] saline. The time delay between the stimulus and the appearance of an LFP in the olfactory lobe suggests that it is being carried in axons with diameters of less than 1 μm with conduction velocities of 0.15–0.25 m s^{-1} and that the olfactory globular tract is the only tract between the protocerebral tract and the olfactory lobe that contains axons with such small diameters. A synaptic pathway could introduce conduction delays, but is excluded by the insensitivity of the greater part of antidromic LFPs to low-[Ca^{2+}] saline and repetitive stimulation. The greater part of the antidromic LFP, therefore, is most likely to be the summed action potentials in the axons of the projection neurones. The reduction of the antidromic LFP in the presence of GABA and histamine nevertheless signifies a synaptic involvement, perhaps associated with a recurrent inhibition operating *via* the projection neurones (see below).

Inhibition the olfactory lobe

Inhibition plays an important role in olfactory systems of both vertebrates and invertebrates (Shipley and Ennis, 1996; Waldrop *et al.* 1987; Christensen *et al.* 1993). A model of dual inhibition has been proposed for the olfactory lobe of spiny lobsters in which an early GABA inhibition is addressed, perhaps presynaptically, to the afferent input, followed by a later and long-lasting histamine inhibition which is addressed to the projection neurones (Wachowiak and Ache, 1997). The modulation of the LFP in the *C. destructor* olfactory lobe produced by perfusing the brain with GABA and histamine could be explained by such a model. GABA abolishes the entire LFP, including the initial positive-going component 1 that we assume comes from activity in the afferent terminals. Presynaptically applied inhibition at the top of the column, where the labelling to GABA is most intense, could remove this signal as well as any postsynaptic activity. Picrotoxin, in contrast, potentiates the LFP to such an extent that a submaximal stimulus to the antennule will result in a response that is larger than that produced by a supramaximal electrical stimulus, presumably by blocking the normally present depression of the afferent input by GABA. Histamine has a compound effect on the crayfish brain, and most of the LFP is removed after perfusion, suggesting that histaminergic neurones are also addressed to the afferents. A late negative-going component of the LFP persists, however, so that not all

afferent inputs to deeper layers of the lobe are blocked. Cimetidine enhances the LFP.

Our demonstration that antidromic stimulation of the projection neurones can block a significant proportion of the orthodromic LFP identifies the possibility of a new and unexpected inhibitory pathway operating *via* the output neurones of the olfactory lobe. The block affects component 2 of the LFP, which we identify above as the activity of the local and projection neurones. Simple collision between orthodromically and antidromically travelling action potentials in the axons of the projection neurones is not likely to be responsible because the delay between the last antidromic stimulus and the orthodromic stimulus can be as much as 250 ms.

We offer the alternative explanation that antidromically travelling action potentials in the projection neurones access a recurrent inhibitory pathway normally activated by the orthodromic passage of projection neurone action potentials leaving the olfactory lobe. The olfactory globular tract neuropile and the 5HT-IR dorsal giant neurone make interesting candidates for such a recurrent pathway. The olfactory globular tract neuropile is incorporated into the tract, close to the point where it leaves the olfactory lobe. Ultrastructural studies of this neuropile have shown elements that make synaptic contact with the 5HT-IR dorsal giant neurone, which branches widely within the olfactory lobe (Sandeman *et al.* 1995*b*), and serotonin can exert an inhibitory effect in crayfish central nervous systems (Glanzman and Krasne, 1983; Krasne *et al.* 1997; Yeh *et al.* 1997). The effect of serotonin on the LFP is still to be tested.

Oscillations

Oscillatory potentials have been reported in many olfactory systems (Gelperin *et al.* 1996) and have been modulated or induced by exposing olfactory receptors to odorants in many animals (vertebrates, Baumgarten *et al.* 1962; Beuerman, 1977; molluscs, Gelperin and Tank, 1990; Delaney *et al.* 1994; insects, Laurent and Davidowitz, 1994; Laurent and Naraghi, 1994). Such oscillations have attracted considerable attention because they could be part of the inner neuronal representation of odours detected by the receptor cells (Laurent, 1996; Wehr and Laurent, 1996).

The slow oscillatory potentials that accompany perfusion of the brain of *C. destructor* with picrotoxin do not yet qualify as a functional part of the olfactory process and may be a result of the removal of widespread inhibition. Systems containing strong negative feedback loops have the propensity to oscillate, particularly if their gains are altered (Laurent, 1996). Nevertheless, interneurones in the hemi-ellipsoid bodies of crayfish, on which the projection neurones terminate, generate regular bursts of action potentials with a repeat frequency of 0.5–1.0 Hz, and some of these neurones respond in a complex fashion to both electrical and odorant stimulation of the antennules (Mellon *et al.* 1992*a*,*b*; Mellon and Alones, 1997). The membrane potential of some local interneurones in the spiny lobster also fluctuates spontaneously at approximately 2 Hz (Wachowiak *et al.* 1997).

Oscillatory behaviour is, therefore, an inherent property of the crustacean olfactory system, and its appearance in the local field potentials described here is another confirmation that field potentials are a reliable manifestation of the activity of central olfactory neurones.

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