The Journal of Experimental Biology **199**, 1973–1982 (1996)
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JEB0306

EVIDENCE THAT HISTAMINE IS A NEUROTRANSMITTER IN AN INSECT EXTRAOCULAR PHOTORECEPTOR PATHWAY

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Accepted 2 May 1996

Summary

The pair of vasopressin-like immunoreactive (VPLI) neurones of the locust *Locusta migratoria* have cell bodies in the suboesophageal ganglion and extensive arborization throughout the central nervous sytem. The activity of the VPLI neurone is regulated by a spontaneously active excitatory descending interneurone (DI) that is, in turn, inhibited by an uncharacterised extraocular photoreceptor (EOP) system located in the brain. Light directed at the brain results in inhibition of DI activity, which thereby deprives the VPLI neurone of its major synaptic input. We present evidence that histamine plays an important role in the EOP–DI–VPLI pathway. Histamine mimics the EOP-mediated inhibition of the DI, and the H2-specific histamine antagonists cimetidine and ranitidine block its inhibitory action. Histamine application to various areas of the brain localises the area where histaminergic inhibition occurs; this region is confined to the medial protocerebrum. At least six bilaterally paired histamine-like immunoreactive neurones send axonal projections into this area. Depolarisation of the brain region containing the soma of these neurones with high-K^+ saline deactivates the VPLI neurone through the removal of the DI excitatory synaptic input.

Key words: cimetidine, circadian rhythm, histamine, insect, vasopressin, locust, *Locusta migratoria*, extraocular photoreceptor.

Introduction

Circadian rhythmicity is of fundamental importance to all animals. The central component is a clock which maintains the cycle at about 24 h; other cues, mostly light, act as the 'Zeitgeber' to synchronise the clock to external events. Insects, with their identified neurones and, in the case of *Drosophila melanogaster*, molecular genetics offer tractable material for the molecular and cellular analysis of these neuronal circuits mediating circadian rhythmicity.

Despite the experimental advantages offered by insects, the actual location of the clock and the light-sensitive 'Zeitgeber' neurones is somewhat controversial, although controversies may reflect species differences. In the cockroach *Leucophaea maderae*, for example, elegant lesion experiments by Stengl and Homberg (1994) have shown that the circadian clock is located in the optic lobes and that the successful regeneration of optic lobe neurones, immunoreactive to antisera against crustacean pigment-dispersing hormone (PDH), back into the midbrain correlates with the re-establishment of circadian locomotor activity. In contrast, Stengl (1995) has recently shown that crickets *Gryllus bimaculatus* and *Teleogryllus commodus* retain circadian motor activity after optic stalk transection and, therefore, that in these insects, a circadian pacemaker probably resides within the central brain. A similar outcome has been reported by Cymborowski *et al.* (1994) in the blowfly *Calliphora vicina*; removal of both optic lobes failed to abolish rhythmic locomotor activity, suggesting that the circadian oscillator resides in the brain.

Insect light-sensitive neurones sufficient and/or necessary for the entrainment of circadian rhythms have been found at a number of locations. For example, in one species of cricket, *Teleogryllus commodus*, Loher (1972) found that the compound eyes were necessary for the entrainment of stridulatory circadian activity to a light–dark cycle. However, work on other cricket species has demonstrated the sufficiency of an extraocular photoreceptor (EOP) for this function (Dumortier, 1972). A very clear example of an EOP entraining circadian activity is found in the moth *Manduca sexta*; extirpation of the compound eyes and ocelli has no effect on the synchrony of flight activity to the light–dark cycle (Truman, 1974). The extensive literature describing the behavioural and cellular investigation of insect circadian clocks and their 'Zeitgeber' has been reviewed (Page, 1985).

The molecular genetics of *Drosophila melanogaster* provides a different approach to the investigation of circadian circuits. Circadian locomotor activity in mutants lacking eyes and optic lobes is retained, again suggesting a brain location for the dipteran clock (Helfrich, 1987). Further evidence for the involvement of PDH-immunoreactive neurones in circadian rhythmicity is the recent demonstration that some of these in the *Drosophila* brain also express the *period* gene

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(Helfrich-Förster, 1995). The period protein plays a role in circadian rhythmicity (Siwicki *et al.* 1988).

In contrast to the work referred to above, which has inferred the presence and function of brain EOPs from the effects of light–dark regimes on the behaviour of operated and mutant animals, the present study characterises an EOP by examining its effects on an accessible identified postsynaptic neurone. The activity of the suboesophageal vasopressin-like immunoreactive (VPLI) neurones of the locust *Locusta migratoria* is influenced by the amount of light that illuminates the brain. In bright light, these neurones are inactive, but in darkness excitatory postsynaptic potentials (EPSPs) and an underlying depolarisation occur; these initiate action potentials (Baines and Bacon, 1994). Because this pattern of activity persists after the removal of the conventional photosensitive systems, the optic lobes and ocelli, it was concluded that light penetrating the head cuticle stimulates a brain EOP which in turn controls the activity of the VPLI neurones (Thompson and Bacon, 1991). Further characterisation of this system has determined that activation of the EOP inhibits a descending cholinergic interneurone (DI) that provides the major excitatory synaptic drive to the VPLI neurones (Baines and Bacon, 1994). Similar activity of the two VPLI neurones is ensured because they both receive synaptic input from each DI (Thompson and Bacon, 1991).

There is indirect evidence to suggest that the EOP–DI–VPLI circuit is involved in circadian rhythmicity. Analysis of isolated VPLI cell bodies using HPLC and radioimmunoassay shows that they contain three different arginine-vasopressin-related peptides: the monomer, F1 (Cys-Leu-Ile-Thr-Asn-Cys-Pro-Arg-Gly-amide), its antiparallel homodimer, F2, and the parallel homodimer, PDm (Baines *et al.* 1995). In the vertebrates, neurones of the suprachiasmatic nucleus (SCN) contain arginine-vasopressin, the release of which coordinates circadian rhythmicity (Reppert *et al.* 1987; Aronson *et al.* 1993). These SCN neurones indirectly receive photosensitive inputs arising from a subset of retinal photoreceptors (Foster *et al.* 1991) or, additionally in non-mammalian amniotes, from photoreceptive cells located in the pineal gland (Cassone, 1990). It is possible, therefore, that the EOP–VPLI system serves an analogous function in the locust. Although we have yet to identify postsynaptic targets, the function of the VPLI neurones is likely to be inhibitory. This is because their activity results in a reduction of the cyclic AMP content in the central nervous system (CNS), an effect mimicked by the peptide F1 (Baines *et al.* 1995).

This circuit therefore provides a unique opportunity to characterise an insect brain EOP. We present pharmacological evidence to indicate that the EOP inhibits the spontaneously active DI directly or indirectly (depriving VPLI of excitatory synaptic input) through histaminergic synapses. Application of histamine to various brain regions localises the area where the inhibition occurs to the medial protocerebrum. Immunohistochemistry with antisera to histamine identifies at least six immunoreactive neurones which have their arborizations in this area. Depolarisation of the brain regions containing the soma of these neurones with high-K^+ saline inhibits the activity of the DI.

Materials and methods

Animals

Adult *Locusta migratoria* L. were taken from a crowded colony in the School of Biological Sciences at the University of Sussex. The locusts were maintained on a 16 h:8 h light:dark cycle at 26–32 °C. Animals of both sexes were used in the first 2 weeks of adult life and were cold-anaesthetised at 4 °C in darkness for more than 1 h before use. The terms ventral and dorsal are defined on the basis of the neuraxis; within the head capsule, ventral is anterior and dorsal is posterior.

Chemicals

Unless otherwise specified, chemicals were obtained from the Sigma-Aldrich Chemical Co. (Poole, Dorset, UK). The histamine antiserum was a generous gift from Dr P. Panula, Helsinki, Finland. Insect saline consisted of (in mmol l^{-1}): 200 NaCl, 3 KCl, 9 $CaCl_2$, 5 Hepes, pH 7.2. In addition, high-K^+ saline contained 3 mol l^{-1} KCl.

Electrophysiology

The preparation used for recording from VPLI neurones in an isolated, tracheated brain and ventral nerve cord has been described previously (Thompson and Bacon, 1991). Briefly, the ventral surface of the suboesophageal ganglion was desheathed and the VPLI cell body initially identified by its 60 μm diameter, its consistent position and its greyish-white coloration. Neurones were impaled with thick-walled microelectrodes (20–30 MΩ) containing 2 mol l^{-1} potassium acetate. Drugs were applied with a dual-channel Picospritzer pressure-injection system (General Valve Corporation, Fairfield, NJ, USA) using nitrogen gas. For localisation of the EOP–DI synaptic contact area in the brain, a squared grid was mounted into the eyepiece of the dissecting microscope. This defined squares (200 μm×200 μm) on the brain surface, and drugs were applied in these different squares at depths of 0–750 μm, at 150 μm intervals, from the ventral surface. Depths were measured using the vertical calibration scale of the micromanipulator on which the pressure-injection pipette was mounted. In all experiments, one circumoesophageal connective was crushed so that only one of the two DIs synaptically excited the VPLI neurones. This procedure allowed the EOP–DI pathway to be characterised in one side of the brain without the input from the contralateral DI confusing the results.

Immunocytochemistry

Brains were fixed with 4% carbodiimide in 0.1 mol l^{-1} phosphate-buffered saline (PBS, pH 7.2) followed by fixation in 4% paraformaldehyde in PBS. Horizontal and transverse cryostat sections (25–30 μm) were cut at −18 °C, thaw-mounted on chrome-alum-gelatine-coated slides and kept at −20 °C until use. The histamine antiserum, diluted 1:10 000 in PBS containing 0.2% Triton X-100, was applied to sections for 14–16 h at 4 °C. Secondary antiserum (biotinylated goat-anti rabbit IgG; Vector Laboratories) was diluted 1:200 in PBS containing 0.2% Triton X-100, and slides were incubated, with

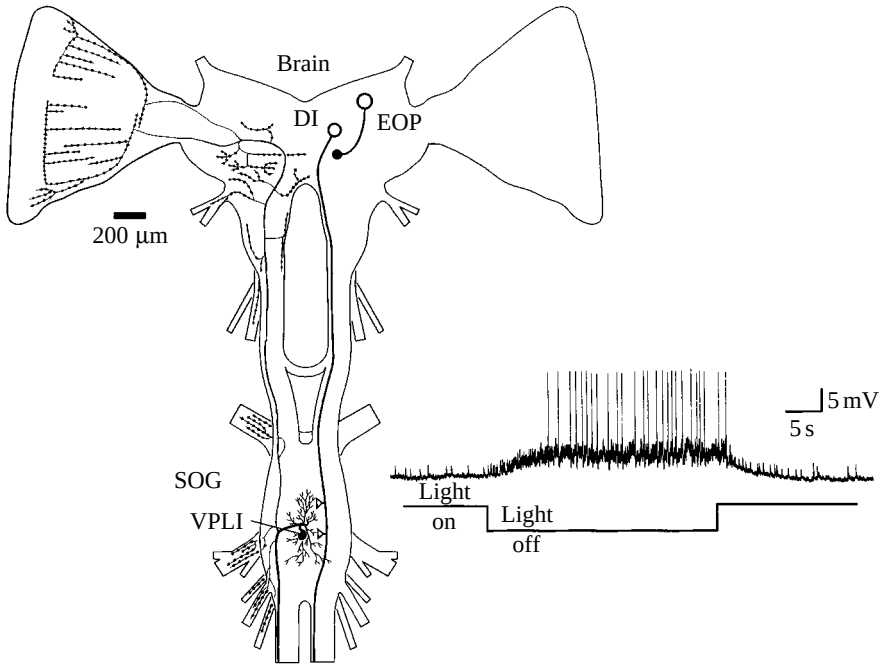

Fig. 1. Schematic drawing of the EOP–DI–VPLI system in *Locusta migratoria* (left) and an intracellular recording showing the modulation of VPLI neurone activity by light and dark (right). The VPLI neurone (only one of which is drawn) has its cell body in the ventral cortex of the suboesophageal ganglion (SOG) and extensive arborization throughout the brain and ventral nerve cord. It receives synaptic input from both the ipsi- (not shown) and contralateral descending interneurone (DI). The spontaneous activity of the DI is inhibited through the activation of an extraocular photoreceptor system (EOP) by light. The location and morphology of the DI and EOP shown in this figure are hypothetical. ●, inhibitory synapse, △, excitatory synapse.

gentle agitation, for 1 h at room temperature. The avidin–biotin–streptavidin complex (Vector Laboratories) was diluted 1:100 in PBS with 0.1 % Tween-20 and also incubated for 1 h at room temperature, with gentle agitation. Staining was developed using 0.03 % diaminobenzidine (DAB) and 0.01 % hydrogen peroxide in PBS for 5 min. Specificity controls were performed by omitting the primary antiserum and by liquid-phase preadsorption of the antiserum with histamine (5 mg ml^{-1} diluted antiserum for 12 h); both of these treatments abolished staining. The antiserum has been extensively characterised elsewhere (Panula *et al.* 1988).

Results

The activity of the cholinergic descending interneurone (DI), characterised physiologically but still morphologically unidentified (Baines and Bacon, 1994), was monitored by intracellularly recording the 4–5 mV EPSPs it evokes in the VPLI neurone. In bright light, the frequency of EPSPs is low (1.06±0.26 Hz, N=6, mean ± S.E.M.), whereas in darkness the frequency is significantly higher (4.62±0.52 Hz, N=6, mean ± S.E.M., $P \leqslant 0.001$, Student's paired t-test). Previous work has indicated that a light-activated EOP synaptically inhibits the spontaneously active DI (Baines and Bacon, 1994). The dark-evoked, DI-mediated increase in input to the VPLI neurones depolarises them sufficiently to fire action potentials (Fig. 1). The response to light/dark was determined for each preparation to ensure that the EOP–DI–VPLI circuit functioned normally.

The DI is inhibited by both GABA and histamine

Pressure-ejection of various transmitters into the intact brain was achieved by introducing the ejection microelectrode into the cut stump of the median ocellar nerve. Of the range of transmitters tested, only γ-aminobutyric acid (GABA) and histamine are capable of mimicking the activity of the EOP by inhibiting the DI (Fig. 2). In dim light (DI active), both GABA and histamine inhibit the appearance of EPSPs in the VPLI neurone (Fig. 2A,B). The inhibitory effect of both GABA (1 mmol l^{-1} for 50 ms) and histamine (100 μmol l^{-1} for 50 ms) is reversible and the EPSP rate always returns to control levels. Of the two transmitters, histamine is consistently the more potent, causing a clear inhibition that lasts approximately 30 s when applied at 100 μmol l^{-1}; GABA must be applied at a 10-fold higher concentration to cause similar inhibition (Fig. 2A,B). In darkness, where increased DI activity causes the VPLI neurone to fire action potentials, application of GABA (100 μmol l^{-1}, 100 ms) and histamine (10 μmol l^{-1}, 100 ms) both produce a clear inhibition (Fig. 2C). The other transmitters tested were 5-hydroxytryptamine (serotonin), dopamine, glutamate and octopamine; these are all ineffective inhibitors of the DI, even when added at 100 mmol l^{-1}. Because histamine is the most potent inhibitory transmitter tested, we repeated these experiments using a succession of increasing concentrations of histamine. The inhibitory effect on the VPLI neurone, through the removal of DI synaptic input, shows a clear dose–response relationship (Fig. 3).

The observation that both histamine and GABA inhibit the DI suggests that the underlying mechanism of inhibition may be the gating of a Cl^- channel; both transmitters gate Cl^- channels in other invertebrate systems (Hardie, 1989; McClintock and Ache, 1989). Under conditions of bright light, when the EOP is actively inhibiting the DI (i.e. VPLI is also inactive), injection of the Cl^- channel blocker picrotoxin (1 mmol l^{-1}, 1 s) into the brain evokes a sustained barrage of EPSPs and a large depolarisation of the VPLI neurone (Fig. 2D). Our interpretation of these data is that picrotoxin blockade of inhibitory Cl^- channels present on the DI allows the DI to become spontaneously active. These inhibitory Cl^-

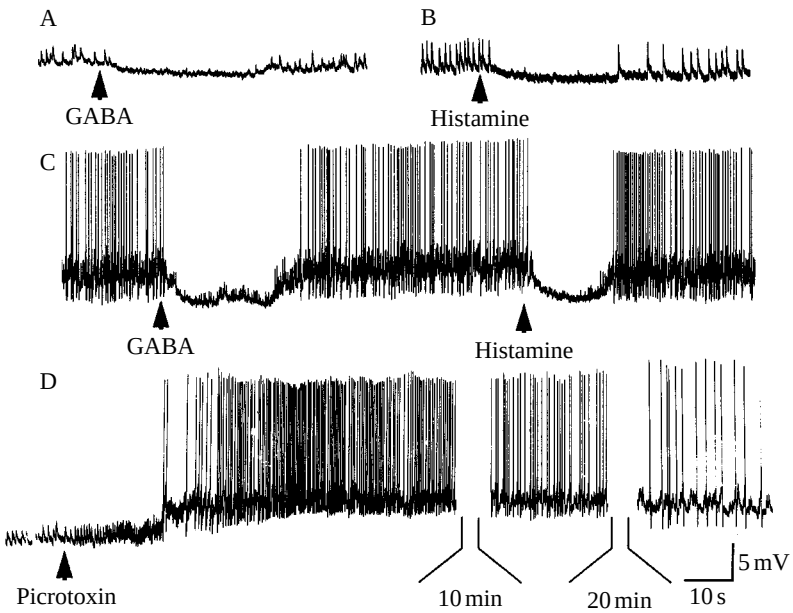

Fig. 2. GABA and histamine inhibit the activity of DI. (A) In dim light, pressure-ejection of GABA (1 mmol l^{-1}, 50 ms, applied at arrowhead) ventrally into the brain in the region of the median ocellar root inhibits the DI-mediated production of EPSPs in VPLI. EPSPs reappear approximately 35 s after GABA application. (B) Histamine application (100 µmol l^{-1}, 50 ms) has a similar inhibitory effect on the DI; EPSPs return after approximately 30 s. Experiment shown in A and B were performed in the light. (C) In darkness, where increased DI activity causes the VPLI neurone to fire action potentials, application of GABA (100 µmol l^{-1}, 100 ms) and histamine (10 µmol l^{-1}, 100 ms) both produce a clear, though reversible, inhibition of DI. (D) The Cl^- channel blocker picrotoxin, added in the light (1 mmol l^{-1}, 1 s) causes a rapid increase in EPSP frequency and depolarisation of the VPLI neurone. The effect of picrotoxin is long-lived; action potentials are still seen in the VPLI neurone 30 min after picrotoxin application. A–D are from different preparations. Experiments were repeated on four individual preparations for each transmitter and typical traces are shown.

channels would normally be activated by transmitter released from neurones within the EOP–DI pathway. Further support for this hypothesis comes from the observation that picrotoxin addition to a brain in darkness (EOP inactive, DI and VPLI active) does not further increase the activity of the VPLI neurone (data not shown).

Histamine is probably the EOP transmitter

To differentiate between histamine and GABA as the putative transmitter of the EOP, a number of selective antagonists for both transmitters were injected into the cut stump of the median ocellar nerve. Under conditions of bright light (EOP active, DI activity reduced, few EPSPs in the VPLI neurone), application of either of the two selective H2-receptor-specific histamine antagonists ranitidine (0.1 mol l^{-1}, 2 s) or cimetidine (10 mmol l^{-1}, 2 s) induces a rapid depolarisation of the VPLI neurone leading to action potentials, presumably by blocking the inhibitory action of the EOP (Fig. 4A,B). Of these two antagonists, cimetidine is the more potent. If the histaminergic antagonists ranitidine and cimetidine activate the DI (and consequently the VPLI) by blocking the activity of an EOP-released transmitter, then one would expect that in darkness, when the EOP is inactive, these antagonists would be ineffective. This experiment was performed and neither antagonist had any affect on the activity of the VPLI neurone (data not shown). The selective histamine H1-receptor antagonist chlorpheniramine maleate (0.1 mol l^{-1}, 2 s) has no effect (data not shown). Significantly, two selective GABA antagonists tested, 5-amino-*n*-valeric acid (0.1 mol l^{-1}, 2 s, Fig. 4C) and bicuculline (0.1 mol l^{-1}, 2 s, data not shown), are also ineffective in blocking the inhibitory action of the EOP.

The action of these antagonists suggests that histamine, and not GABA, is a neurotransmitter in the EOP–DI pathway. Thus, we tested the effectiveness of cimetidine in blocking the

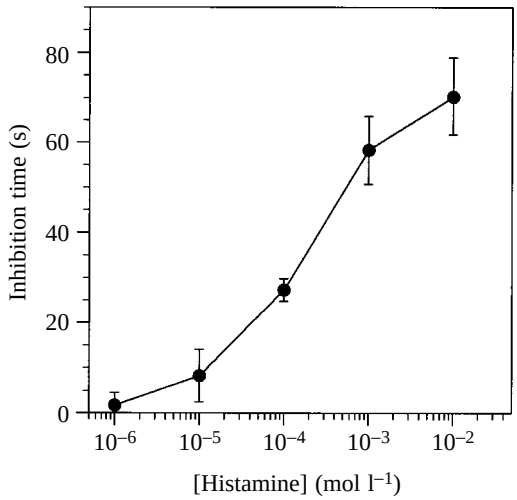

Fig. 3. Histamine inhibits the activity of the DI in a dose-dependent manner. Increasing concentrations of histamine (1 µmol l^{-1} to 10 mmol l^{-1}, 50 ms) were pressure-applied into the brain through the cut root of the median ocellar nerve in dim light. The effect this had on the activity of DI was measured as the time of inhibition (s) of EPSPs recorded in the VPLI neurone. The experiment was performed on three individual preparations where all concentrations were successively applied in an increasing series of concentrations, allowing a 10 min period between each application. Points represent mean values ± S.D. Concentration of histamine shown on the abscissa refers to pipette concentration and not the final concentration in the brain.

inhibitory action of histamine in our preparation. In cimetidine-pretreated brains (10 mmol l^{-1}, 2 s), the effect of histamine application into the brain (1–10 mmol l^{-1}, 200 ms) is totally blocked, but the inhibitory action of GABA (1 mmol l^{-1}, 200 ms) is unaffected (Fig. 5). The observation that cimetidine blocks the inhibitory effect of the EOP (Fig. 4) and the inhibitory effect of histamine, but does not block the effect of

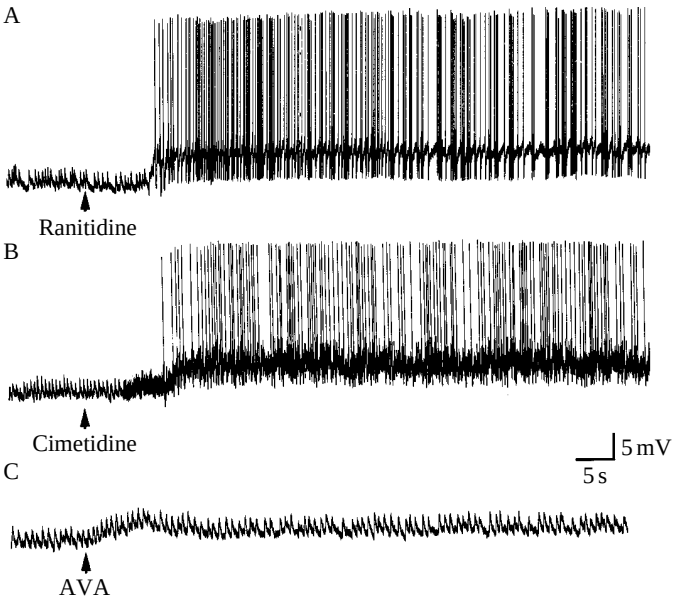

Fig. 4. Histamine antagonists block the inhibitory action of the EOP. (A) In bright light, the histamine antagonist ranitidine, pressure-ejected into the area of the median ocellar root in the brain (0.1 mol l^{-1}, 2 s), blocks the inhibitory effect of the EOP on DI. (B) The histamine antagonist cimetidine (10 mmol l^{-1}, 2 s) has a similar, but more potent, effect than ranitidine. (C) The GABA antagonist 5-amino-*n*-valeric acid (AVA; 0.1 mol l^{-1}, 2 s) fails to block the inhibitory effect of the EOP. The baseline shift following AVA application is an artefact of pressure-ejection. A–C are from different preparations. Experiments were repeated on three individual preparations for each antagonist and typical traces are shown.

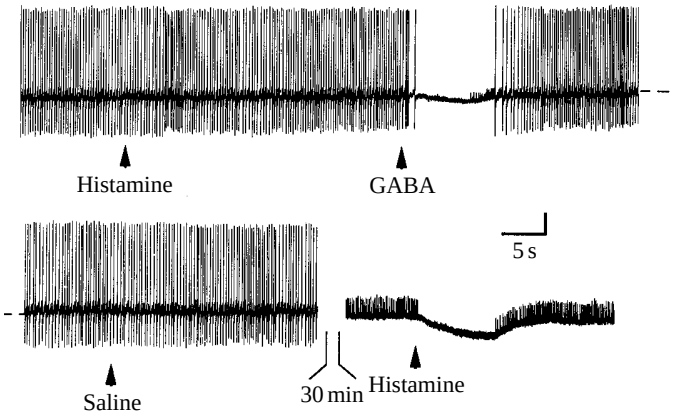

Fig. 5. Cimetidine blocks the action of histamine but not that of GABA. Application of histamine (1 mmol l^{-1}, 200 ms) to a brain pretreated with cimetidine (10 mmol l^{-1}, 2 s) fails to inhibit the DI. Application of GABA (1 mmol l^{-1}, 200 ms) under the same conditions produces a normal inhibitory response. Injection of saline alone (2 s) has no effect. 35 min after cimetidine application, histamine (1 mmol l^{-1}, 200 ms) is now able to inhibit the DI, thereby removing excitatory drive, to cause VPLI hyperpolarisation. However, after this prolonged recording period, the EPSPs produced in VPLI typically fail to fire action potentials (probably because of deterioration of the preparation). The lower trace is a continuation of the upper. The vertical bar represents 5 mV, except for the last part of the trace where it represents 2.5 mV. Experiments were repeated on three preparations and typical traces are shown.

GABA (Fig. 5), clearly indicates that histamine is part of the EOP–DI pathway and that GABA and histamine do not act at the same receptor.

The histaminergic synapse on the EOP–DI pathway is located in the medial protocerebrum

Our pharmacological evidence indicates that histamine plays an important inhibitory role within the EOP–DI–VPLI pathway. We therefore localised the site where inhibition by histamine occurs by pressure-ejecting this transmitter into various regions of the desheathed brain using a relatively blunt electrode. A standard histamine pulse (10 μmol l^{-1}, 10 ms) was applied just below the ventral surface on one side of the desheathed brain, in different zones delineated by a grid of 200 μm×200 μm squares (Fig. 6A). The response to histamine in each zone was quantified as the period of DI inhibition following ejection, indicated by monitoring the absence of EPSPs in the VPLI neurone in dim light. The area which exhibits the maximal response to histamine is in the medial protocerebrum (Fig. 6B). Adjacent squares show surprisingly little response to histamine, indicating that the inhibitory actions of histaminergic synapses are discrete. To localise further the synaptic site within the brain, histamine (10 μmol l^{-1}, 10 ms) was applied at varying depths within the zone that exhibited maximal inhibition; a sharper electrode (releasing less histamine) was used than in the previous experiment. The depth at which histamine optimally inhibits DI is 300–450 μm from the ventral surface (Fig. 6C); this is approximately in the middle of the brain.

A number of histamine-immunoreactive neurones could synapse with DI

Having localised the area in which the histaminergic synapses within the EOP–DI–VPLI pathway are likely to be found, we identified those histamine-like immunoreactive neurones that send projections into this area. Because the EOP is known to reside ipsilaterally within the brain (Thompson and Bacon, 1991), we did not examine the optic lobes for any histamine-like immunoreactive neurones. Of the 15 brain neurones that stain with histamine antibody on each side of the brain (Fig. 7A), at least six, all with ventral cell bodies, send projections into the brain region proposed to contain the EOP–DI synapse area (Fig. 7B,C). Specifically, these are two cell bodies (labelled a and b in Fig. 7A,C) in the posterior protocerebrum, one cell body in the anterior protocerebrum (labelled c) and one group of three cell bodies in the anterolateral protocerebrum (labelled d). The major axonal projections of these neurones are shown in Fig. 7C. Additionally, three weakly labelled neuronal cell bodies are located at a depth of approximately 50 μm. Their cell bodies are drawn in Fig. 7A (within the square with neurone c) but no axonal processes were immunostained and, thus, they could not be reconstructed. The other stained cell bodies, the lateral protocerebral cluster and the medial deutocerebral cell, do not send projections to the medial protocerebrum.

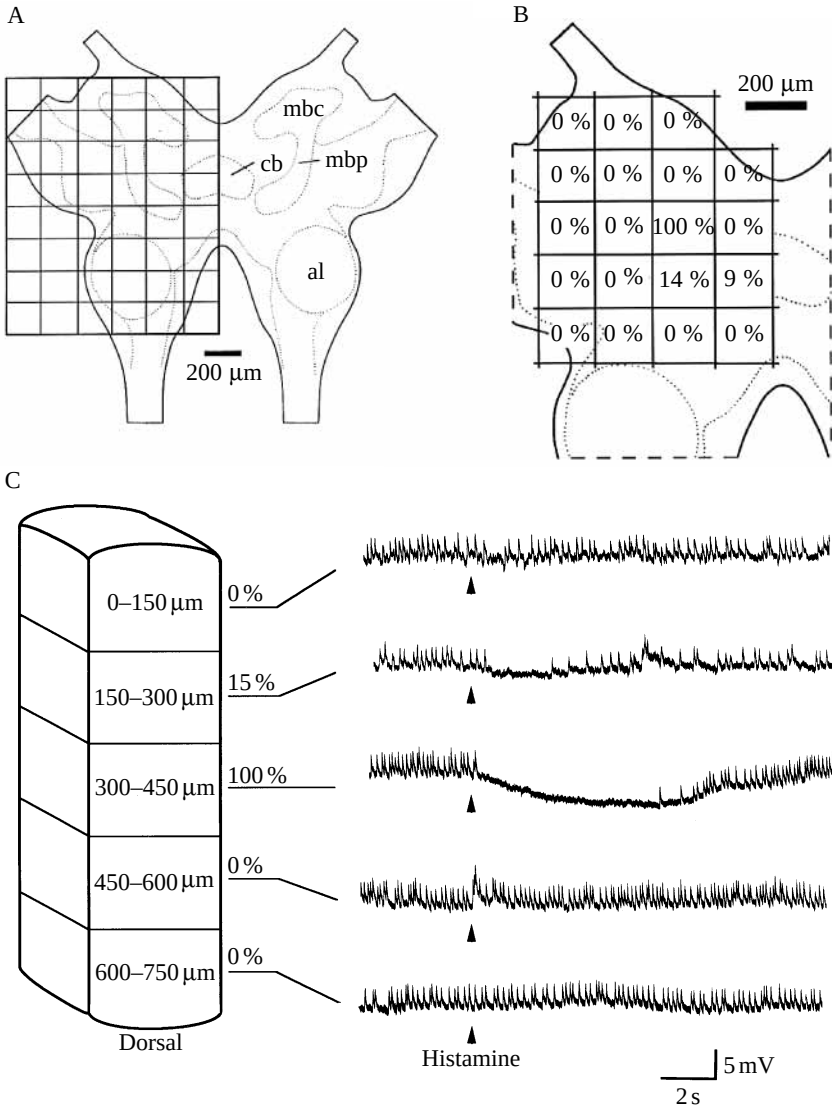

Fig. 6. Localisation of the EOP–DI synaptic region in the medial protocerebrum. (A) A 200μm×200μm grid is used to divide one side of the brain into defined areas. al, antennal lobe; cb, central body; mbc, mushroom body calyces; mbp, mushroom body peduncle. (B) The strength of synaptic contact between the EOP and the DI is assessed by applying histamine (10 μmol l^{-1}, 10 ms) just below the desheathed ventral surface of the brain in each square and measuring the inhibition of DI produced. For each area where inhibition is obtained, histamine application is repeated four times, with a 2 min interval between each ejection, and the mean value is calculated. The area that exhibits the longest inhibition time is set to 100 % and times from other regions are normalised to this. A region in the medial protocerebrum exhibits maximal inhibition; this is 22±4 s (three different applications) in this particular experiment. Two adjacent squares also show inhibition, but at a much reduced rate (2 and 3 s, 9 % and 14 % respectively). (C) The area showing maximal inhibition in B is probed at different depths; histamine application (10 μmol l^{-1}, 10 ms) is most effective at inhibiting DI activity at a depth of 300–450 μm from the ventral surface. Experiments were repeated on four preparations and typical traces are shown.

The DI is probably inhibited by more than one EOP

Mapping of histamine immunoreactivity identifies at least six possible candidate neurones that have appropriate projections to mediate inhibition of the DI. In an attempt to determine which, if any, of these neurones synapses onto the DI, groups of neurones were selectively depolarised by the localised application of high-K^+ saline to the ventral surface of the desheathed brain. Any resultant effect on DI activity was monitored by intracellular recording in VPLI neurone. Because of the close grouping of the neurones a/b and c/d, we could only test the relative effectiveness of these two areas, but not the individual neurones themselves. Application of high-K^+ saline (3 mol l^{-1} KCl, 20 ms) to each of these two areas inhibits activity of the DI. However, similar application of high-K^+ saline to other areas (not containing the neurones a–d) failed to affect the activity of the DI (Fig. 8). These data indicate that some of the neurones in both the a/b and c/d groups are activated, causing inhibition of the DI, and therefore may possibly be EOPs. Of course, we cannot rule out the possibility that we are also activating other non-EOP neurones that inhibit the DI, but since all the other inputs to the DI that have been described are excitatory (Thompson and Bacon, 1991), this seems unlikely.

Discussion

This study has characterised an insect EOP by an indirect method – intracellular recording in an accessible postsynaptic neurone. Our major finding is that the inhibitory pathway from the EOP to the DI involves histaminergic synapses. Using an antiserum to histamine, we have identified six putative candidate EOP neurones and present evidence to suggest that more than one of these inhibits the DI.

Is the EOP histaminergic?

There is substantial evidence to indicate that histamine is the neurotransmitter of insect primary photoreceptors. For example, neurones in the compound eyes of fly, moth and locust stain with histamine antibody (Maxwell *et al.* 1978; Nässel *et al.* 1988; Callaway *et al.* 1989; Schlemermeyer *et al.* 1989). Further, Elias and Evans (1983) demonstrated that the

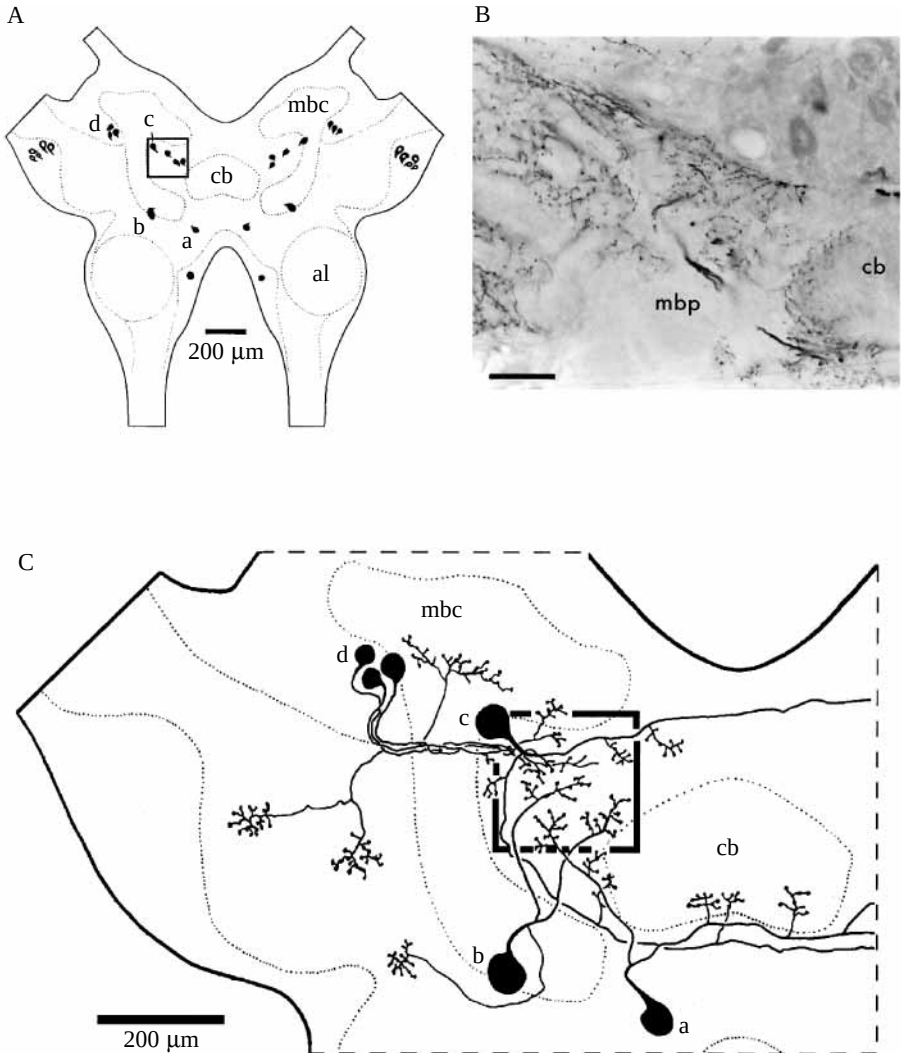

Fig. 7. Histamine-like immunoreactive neurones in the brain of *Locusta migratoria*. (A) Schematic representation of the positions of immunoreactive cell bodies. Black cell bodies are ventral, cell bodies with white nuclei are dorsal. Cell bodies a, b and c, and group d, send projections into the region presumed to containing the EOP–DI synapse. This region is delineated by the square determined in Fig. 6. (B) Horizontal cryostat section showing histamine-like immunoreactivity in the region of the square shown in A, 400 µm from the ventral surface; histamine ejection at this depth is most efficacious at inhibiting the DI. (C) The major anatomical features of cells a–d, reconstructed from serial horizontal sections. These neurones have arborizations in the area identified in A. al, antennal lobe; cb, central body; mbc, mushroom body calyces; mbp, mushroom body peduncle. The immunocytochemical staining was performed on 12 brains; no variation in the number of cell bodies (optic lobes excluded) was observed. Scale bar in B, 100 µm.

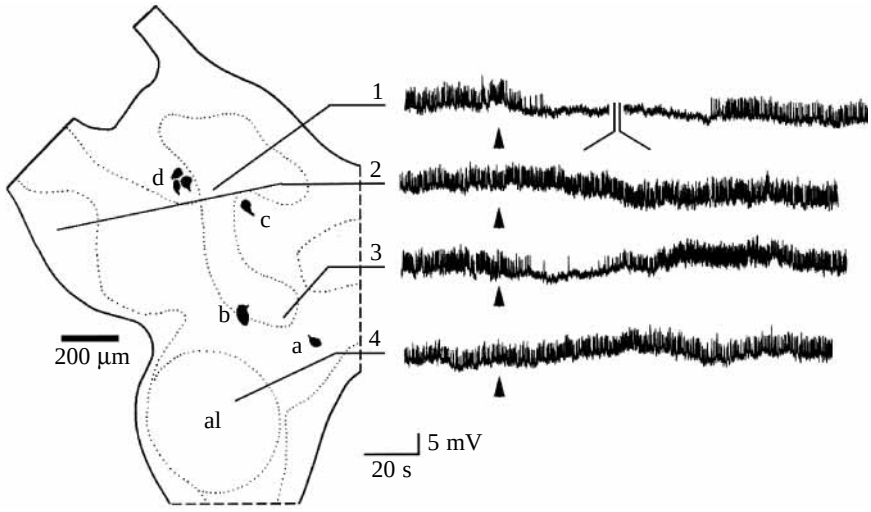

Fig. 8. More than one neurone probably inhibits the DI. Localised application of high-K^+ saline (3 mol l^{-1} KCl, 20 ms, arrowhead) onto regions of the ventral surface of the desheathed brain containing the cell bodies of the histamine-like immunoreactive neurones a/b and c/d inhibits the activity of the descending interneurone (traces 1 and 3). Application of high-K^+ saline to other regions of the ventral brain fails to inhibit the DI (traces 2 and 4). The break in trace 1 represents 60 s. Experiments were repeated on three preparations and typical traces are shown.

retinas of three insect species are able to synthesize histamine. Physiologically, application of the putative transmitter histamine mimics the action of the native neurotransmitter at the synapses between photoreceptors and large monopolar cells in the fly; no other transmitters tested have this effect (Hardie, 1987). Similarly, pharmacological investigation also suggests that locust ocellar photoreceptors release histamine as their major transmitter (Simmons and Hardie, 1988). Our

hypothesis that histaminergic synapses are involved in a locust brain EOP system is entirely consistent with these studies and adds to the body of evidence to indicate that the primary transmitter of insect photoreceptors (visual and extraocular) is histamine. However, more rigorous evidence that the locust EOP inhibits the DI directly through histaminergic synapses will require the identification of the DI. Voltage-clamp of the DI will allow us to compare the ionic basis of histamine action with that of the EOP transmitter. We are currently attempting to identify and map the morphology of the DI to allow these experiments.

Within the insects, histamine appears to be an inhibitory transmitter because it gates Cl^- channels (Hardie, 1989). Our data corroborate this, because we show that the action of histamine in our system is blocked by picrotoxin – a selective Cl^- channel blocker. GABA is also known to gate a Cl^- channel (Barnard *et al.* 1984), and we show here that it can also inhibit the DI. However, the observation that histamine antagonists block the action of histamine and the EOP, but fail to affect the inhibitory activity of GABA in this system, rules out the possibility that the EOP releases GABA. It does indicate, however, that the DI expresses distinct receptors for both histamine and GABA. This implies that the DI is under the control of other inhibitory inputs, in addition to possible histaminergic EOPs. Such alternative inhibitory inputs have not yet been described.

The receptor pharmacology for histamine in the insect CNS is not well understood. The most potent antagonists of histamine action in insect photoreceptors are H2-receptor-specific, notably cimetidine and ranitidine (Hardie, 1988). Our physiological studies confirm these findings; cimetidine and ranitidine block the effect of histamine and the EOP, while the H1-receptor-specific antagonist chlorpheniramine maleate is ineffective. There is, however, pharmacological evidence for H1-receptor-like binding sites in locust nervous tissue (Roeder, 1990).

Histamine-like immunoreactive neurones in the brain

The number (30) and distribution of histamine-like immunoreactive cell bodies in the brain of *Locusta migratoria* resemble those found in other insect species investigated: 20 in the cockroach *Periplaneta americana* (Pirvola *et al.* 1988); 20–40 in the moth *Manduca sexta* (Homberg and Hildebrand, 1991); 20 in the blowfly *Calliphora vomitoria* (Nässel and Elekes, 1992); 22–24 in *Drosophila melanogaster* (Pollack and Hofbauer, 1991; Nässel and Elekes, 1992). In all these species, histamine-like immunoreactive processes ramify extensively in brain neuropile although some distinct regions, such as the mushroom body peduncles, are not entered. Another point of particular similarity is the projection pattern of the lateral histaminergic cell cluster situated near the optic stalk. These project to both ipsi- and contralateral optic lobes. Some species differences, however, are found. For example, the mushroom body calyces are not innervated in *M. sexta* and *D. melanogaster*, but are innervated in both *Locusta migratoria* and *Leucophaea maderae*. The central body is innervated in *M. sexta*, *Leucophaea maderae* and *Locusta migratoria* but not in *D. melanogaster* (Pirvola *et al.* 1988; Homberg and Hildebrand, 1991; Pollack and Hofbauer, 1991; Nässel and Elekes, 1992; present study). Whether these differences are related to different functions of EOPs in these insects remains to be determined.

The DI is most likely to be inhibited by more than one EOP

The location of the histaminergic synapse in the EOP–DI pathway appears to be confined to a region of the ipsilateral medial protocerebrum approximately 300–450 μm from its ventral surface. The observation that the synapses lie deep in the brain corroborates an earlier proposal that the input sites of the DI lie deep in brain neuropile. This proposal was based on the observation that the DI was relatively insensitive to the spike-inactivating properties of bath-applied saline containing high levels of divalent cations, whereas the EOP was rapidly affected (Baines and Bacon, 1994). This indicates that the spike-initiation zone of the EOP is more superficial than that of the DI. Because of the requirement to concentrate photopigment, EOPs probably transduce light stimuli in their cell bodies (Sandeman *et al.* 1990; Wilkens and Larimer, 1972) and therefore the site of spike initiation is likely to be close by. It may be significant that the six histamine-like immunoreactive neurones identified as possible EOP candidates on the basis of their projections onto the medial protocerebrum all have their cell bodies just below the ventral surface of the brain. Indeed, it is the ventral, rather than the dorsal, surface of the brain that receives most of the light that penetrates the head cuticle. However, without other distinguishing features, it is difficult to identify which of these six neurones might possibly be the EOPs. The observation that crude extracellular stimulation of either of the two brain regions, which contain these six neurones, results in an inhibition of the DI suggests that more than one of them is able to inhibit the DI synaptically. There are alternative interpretations of these data: our extracellular ionic stimulation may have activated other (non-EOP) inhibitory inputs to the DI; alternatively, the high K^+ concentration issuing from the ejection electrode may stimulate the EOP from a number of positions. The latter explanation is particularly pertinent for neurone c because it sends a superficial branch near the cell bodies of neurones a and b, and could, in principle, be stimulated by application of high-K^+ saline at position 3 as well as position 1 (see Fig. 8). Despite these caveats, the most probable explanation for these data is that a number of histaminergic neurones provide inhibitory input to the DI.

Future directions

A recent study has shown that a number of neurones in the blowfly brain are immunoreactive to arrestin antibody (Cymborowski and Korf, 1995); arrestin participates in the phototransduction cascade in vertebrate rods (Pfister *et al.* 1985) and may be a good marker of invertebrate EOPs. Our next task will be to seek arrestin antigenicity in the locust neurones a, b and c and cluster d identified in the present study. We shall then attempt intracellular recording in some of these neurones; with cell bodies of up to 50 μm diameter, it should be possible to determine any synaptic effects on DI and to investigate the transduction mechanism of the EOP. In *Aplysia*

californica, voltage-clamping of the ventral photoresponsive neurone has shown that the hyperpolarising response to light is mediated largely by an outward K^+ current (Andreson and Brown, 1979) and, in the crayfish, injection of second messengers directly into the caudal photoreceptor has indicated that the transduction mechanism is mediated by inositol trisphosphate (Kruszewska and Larimer, 1993). These studies provide an intellectual framework for further investigation of the EOP in the locust brain.

We thank Dr Pertti Panula, University of Helsinki, Finland, for providing the histamine antiserum and Dr Kevin Thompson for practical help and comments on the manuscript. This work was supported by the BBSRC (UK) and a postdoctoral grant to C.T.L. from the Swedish Natural Science Research Council (NFR; Sweden).

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