J. exp. Biol. 150, 123–143 (1990)

Printed in Great Britain © The Company of Biologists Limited 1990

MODULATION OF CRAYFISH RETINAL SENSITIVITY BY 5-HYDROXYTRYPTAMINE

By HUGO ARÉCHIGA, ENRIQUE BAÑUELOS*, EUGENIO FRIXIONE, ARTURO PICONES AND LEONARDO RODRÍGUEZ-SOSA†

Departamento de Fisiología, Biofísica y Neurociencias, Centro de Investigación y de Estudios Avanzados del IPN, Apartado Postal 14-740, 07000 México, DF

Accepted 28 November 1989

Summary

The responsiveness of crayfish retinal photoreceptors to light was enhanced by exposure to 5-hydroxytryptamine (5-HT), either following injection into whole animals or following topical application to isolated eyestalks or retinas. The effect was measured as an increment in the amplitude of the receptor potential, and was dose-dependent in the range 10^{-6}–10^{-3} mol l^{-1} (injected as a 0.1 μl dose in intact animals). It was more pronounced at low levels of illumination and was reversibly blocked by methysergide.

The enhancement was a consequence of a dual effect: (a) retraction of the proximal pigment granules within the photoreceptors, with a corresponding increase in the light-admittance function of the retina; and (b) a direct effect, facilitating a membrane conductance increase which mediated the generation of the receptor potential. A set of axons in the lamina ganglionaris with a 5-HT-like immunoreactivity was found in the vicinity of the photoreceptor axons. 5-HT antagonists were capable of blocking the physiological retraction of pigment granules in photoreceptors at night, suggesting that 5-HT acts as a modulator during the nocturnal phase of the circadian cycle in the crayfish retina.

Introduction

The response of crustacean retinal photoreceptors to light varies through a 24-h cycle in a circadian manner, being higher at night than during the day (Aréchiga and Huberman, 1980; Bryceson, 1986). The physiological mechanisms underlying this rhythmicity are still largely unknown. The responsiveness of retinal photoreceptors to light can be regulated at two levels. (a) By changes in light admittance to the compound eye, which in turn depends on the position of two sets of accessory pigment granules which migrate longitudinally as a function of light

* Present address: ENEP Iztacala, UNAM, Los Reyes, Iztacala, México.
† Present address: Universidad Juárez Autónoma de Tabasco, Villahermosa, Tabasco, México.

Key words: retinal sensitivity, serotonin, crayfish.

intensity. The distal pigment granules are located inside long, slender cells whose long axis is parallel to that of the photoreceptors. The proximal pigment granules are within the photoreceptors themselves. In darkness, the distal pigment granules retract to the corneal end of the retina, and the proximal pigment granules retract to the axons of the retinal photoreceptors. Consequently, the photosensitive rhabdom region of the photoreceptors becomes more accessible to stray light. Under illumination, both sets of pigment granules are dispersed throughout the length of the retina, thereby reducing its photon-catching capacity. (b) By changing the gain of the phototransduction process itself. However, this is unknown in the crayfish (see Rao, 1985).

The physiological mechanisms regulating the position of the accessory pigments are only partly known. The distal pigment cells do not respond directly to light, but are the effectors of a neuroendocrine reflex initiated from extra-retinal photoreceptors (Aréchiga *et al.* 1985). This reflex involves the release of at least one peptide hormone, an octadecapeptide which triggers the migration towards the light-adapted position (Fernlund, 1976). What controls the dark-induced retraction of distal pigments is not clear. The proximal pigment granules migrate as a direct response of retinal photoreceptors to light (Olivo and Larsen, 1978; Frixione *et al.* 1979).

Both sets of pigments are synchronously influenced by light on either retina, and the illumination of one eye is capable of entraining the rhythmicity in the accessory pigments of the other (Barrera-Mera, 1978). Also, either under continuous illumination or in constant darkness, the position of both pigments undergoes circadian variations.

Although the modulation of light admittance to the eye could explain the diurnal variations in receptor responsiveness, a direct modulatory effect on photoreceptors has also been proposed, since circadian changes of response amplitude have been detected in animals devoid of both accessory pigments (Aréchiga and Huberman, 1980; Rodríguez-Sosa and Aréchiga, 1982).

In a first approach to this problem, the role of 5-hydroxytryptamine (5-HT) has been explored. This amine has been identified in various ganglia of the central nervous system and eyestalk of crustaceans (Beltz and Kravitz, 1983; Elofsson, 1983; Sandeman *et al.* 1988), both immunocytochemically and by high-pressure liquid chromatography (Elofsson, 1983; Laxmyr, 1984). It has also been detected in the haemolymph (Livingstone *et al.* 1980; Elofsson *et al.* 1982). Its content in the eyestalk has been shown to vary following a 24-h cycle (Fingerman and Fingerman, 1977). Physiologically, it is known to affect motoneurone activity in central ganglia (Kravitz, 1988) and peripheral neuromuscular transmission (Enyeart, 1981; Dixon and Atwood, 1985; Glusman and Kravitz, 1982). It has also been proposed as a neurohormone (see Kravitz, 1988) and as a neurotransmitter mediating the release of neurosecretory products (Fingerman, 1985). As to its role in visual processing in invertebrates, 5-HT has been shown to shift the phase of the circadian rhythm of activity in the optic nerve of isolated eyes of *Aplysia* (Eskin *et al.* 1982). It also enhances visual sensitivity in *Limulus* (Barlow *et al.* 1977).

The present experiments were aimed, consequently, at exploring a possible action of 5-HT on the crayfish retina. Some aspects of this work have been published in abstract form (Picones and Aréchiga, 1987).

Materials and methods

Electrophysiological recordings

The experiments were conducted upon adult crayfish *Procambarus clarkii*, of either sex and in intermoult. The animals were kept for at least 1 week before the experiments under 12 h: 12 h L: D cycles. The mass photoreceptor response to light (electroretinogram, ERG) in whole animals was recorded as described by Aréchiga *et al.* (1974) with a metal microelectrode 1–5 μm in tip diameter, inserted under the cornea and connected to an a.c. preamplifier to filter out slow movement artefacts. ERGs in isolated eyes were recorded as described by Rodríguez-Sosa and Aréchiga (1982) with a suction electrode and a d.c. preamplifier. The signals were displayed on a storage oscilloscope (Tektronix) and recorded on tape and on paper.

Single-cell recordings, either extracellular or intracellular, were made in isolated eyestalks or retinas following the techniques described in Picones and Aréchiga (1990). Solutions were injected into whole animals by means of chronically implanted cannulae, with the shaft glued to the carapace and the tip open to the body cavity. In isolated eyestalks or retinas, chemical substances were applied topically by adding them to the bathing fluid.

Determination of retinal pigment position

Experiments in whole animals

Once the animals had been left to adapt to either light or darkness, the eyestalks were excised and fixed by heat. They were kept in fixative (usually 5 % formaldehyde) overnight and the pigment position was micrometrically determined, as described by Aréchiga and Atkinson (1975).

Experiments in isolated eyes

Eyestalks excised from adult *P. clarkii* were further dissected and prepared for incubation as described in detail elsewhere (Frixione et *al.* 1979). Groups of four eyes were mounted in incubation chambers containing physiological solution (van Harreveld, 1936), with or without serotonin or its related drugs. The effects of these substances on the proximal pigment migration to either the dark-adapted or the light-adapted position were examined in eyes fixed by heat at different periods of incubation under each set of conditions. The proximal pigment position was quantitatively assessed by means of the normalized pigment index used in previous work (Frixione and Aréchiga, 1981). The results presented in the graphs were obtained from an average of 12 eyes for each point, representing three separate experiments.

Electron microscopy

Isolated eyestalks that had been incubated for 90 min (with or without 1 mmol l^{-1} serotonin) under normal laboratory illumination were fixed for 2 h with 4% glutaraldehyde made up in 0.1 mol l^{-1} sodium cacodylate-buffered van Harreveld's solution. The retinas were then severed from the eyestalks, divided into thin slices and postfixed for 1 h with cold 1% OsO_4 in cacodylate buffer. Ethanol and propylene oxide dehydration, Araldite embedding, thin sectioning, and double staining with uranyl and lead were carried out according to the usual methods of electron microscopy. The specimens were observed and micrographed with a Zeiss EM-9S2 electron microscope.

Chemicals

5-Hydroxytryptamine creatinine sulphate (serotonin) and methysergide bimaleate were obtained from Sigma Chemical Co. (St Louis, MO, USA). Cyproheptadine hydrochloride was supplied by Merck-Mexico, SA (México). Quipazine maleate, 5-methoxytryptamine and β-methylcarboxylate hydrochloride (TR 3369) were generously provided by the Section of Experimental Therapeutics, Department of Pharmacology and Toxicology, CINVESTAV (México).

Immunocytochemistry

For the immunocytochemical identification of 5-HT the eyestalks were dissected out at midnight from animals that had been dark-adapted for at least 2 h, and processed using the indirect immunofluorescence method (FITC) or the peroxidase–antiperoxidase (PAP) method (Sternberger, 1974). For the FITC method the tissue was fixed for 3 h at 4°C in 4% paraformaldehyde in 0.1 mol l^{-1} phosphate buffer and rinsed for 30 min in 30% sucrose dissolved in the same buffer. The tissue was frozen and sectioned (16–18 μm) in a cryostat. The sections were incubated with 5-HT antiserum (Immunonuclear, Stillwater) dissolved 1:400 or 1:200 in phosphate buffer with 0.30% Triton X-100, for 18 h at room temperature. Fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit IgG (generously provided by Dr J. Calderón, CINVESTAV, México) diluted to 1:20 in the same buffer was used as secondary antiserum. A Zeiss fluorescence microscope with epi-illumination and with filters for FITC was used for the examination of specimens. For the PAP method the eyestalks were fixed overnight in 4% paraformaldehyde in 0.1 mol l^{-1} phosphate buffer. After rinsing in buffer, the whole eyestalks were incubated in primary antiserum (18 h) and then treated according to the PAP method. The whole eyestalks were washed in 0.1 mol l^{-1} sodium phosphate and then postfixed in 25% glutaraldehyde for 1 h at room temperature. For electron microscope analysis the whole tissue was rinsed again and fixed in 1% OsO_4 in the same buffer for 1 h at room temperature. Dehydration was in ethanol, and propylene oxide and embedding was in Araldite. The thin sections were examined in a JEOL 2000 electron microscope. The specificity of the immunoreaction was tested using sections and whole eyestalks

incubated with antiserum inactivated by the addition of excess antigen (5-HT hydrochloride 500 $\mu g\,ml^{-1}$).

Results

Effect of 5-HT on retinal sensitivity in intact animals

Retinal sensitivity was explored in two ways in intact animals. One was by recording the mass receptor potential with gross metal electrodes (1–5 μm diameter at the tip), and exploring the time course of adaptation to darkness after a conditioning period of white light (5000 lx) exposure for 1 h. Brief (200 ms) light pulses were applied at increasing intensities (using a 565 nm wavelength light-emitting diode) until a 'threshold' response was attained. The amplitude usually chosen for this response was 50 μV, barely sufficient to be detected above the background noise. The series of stimuli were applied at 3 min intervals. It has been shown previously with this technique that the time course of dark adaptation in the crayfish retina can be described by two functions: an early phase, with a time constant of 12 min, essentially determined by the photoreceptor dark-adaptation and by the retraction of proximal pigment granules within the retinular cells; and a late phase, ascribed to the retraction of pigment granules in the distal cells of the compound eye (Rodríguez-Sosa and Aréchiga, 1982). Injection of 5-HT ($10^{-4}\,mol\,l^{-1}$ in 0.1 ml) produced a shift in the time course of the early phase of dark adaptation, which was essentially complete after 25 min (Fig. 1). No effects were seen during the late phase of dark adaptation (not shown in figure). In five preparations like the one shown in Fig. 1, there was an average enhancement of 0.13 ± 0.04 log units (mean±s.d.) in light sensitivity.

The effect of 5-HT on the mass retinal response was also explored in animals kept under regular stimulation with light pulses of 4 lx, applied at intervals of 5 min. During the intervals the animal remained in darkness. With this programme, the proximal pigment was kept in an intermediate position between the light- and dark-adapted positions (Olivo and Larsen, 1978; Frixione *et al.* 1979) and the distal pigment was maintained in the completely dark-adapted position. 10 min after injection, there was a clear enhancement of the response amplitude which lasted for over 60 min (see Fig. 2). The magnitude of this enhancement was dependent on the concentration of the injected dose, from a threshold of $10^{-6}\,mol\,l^{-1}$, to a maximum effect at $10^{-3}\,mol\,l^{-1}$.

The enhancing effect of 5-HT was found over a wide range of intensities (see Fig. 6).

Effect of 5-HT on retinal pigment migration in intact animals

To explore the possibility that retinal shielding pigment migration contributes to the enhancement of retinal responsiveness induced by 5-HT, the effect of this substance on both sets of pigments was measured directly. The proximal and distal pigment indices were determined as described in Materials and methods. (Note that the proximal pigment index ranges from a value of 0 in the dark-adapted

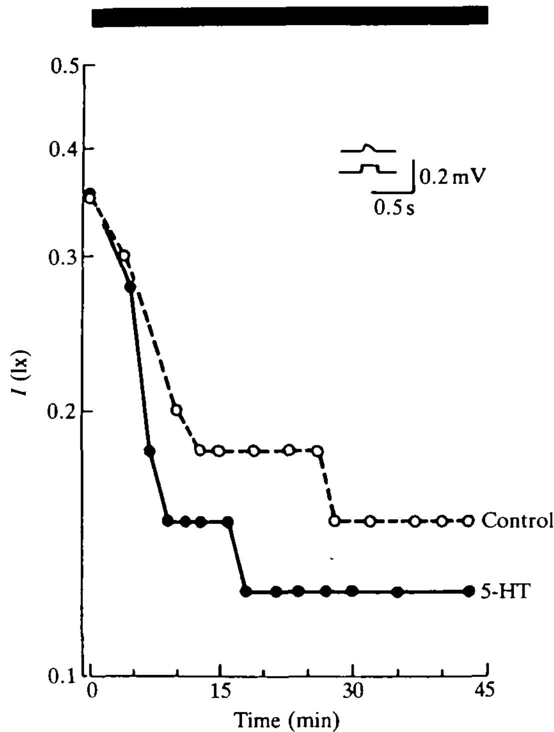

Fig. 1. The rate of dark adaptation is enhanced by 5-hydroxytryptamine (5-HT) injected into whole crayfish (100 μl, 10^{-4} mol l^{-1}). Ordinate: light intensity (lx) necessary to generate a criterion response of 50 μV amplitude. Abscissa: time (min) in darkness. (○) control; (●) after 5-HT. Inset, criterion response with photocell recording of light pulse.

photoreceptor to 1.0 in the fully light-adapted one.) Groups of four animals were kept in light-proof boxes and subjected to background illumination or darkness. One group was injected with 5-HT (total volume 0.1 ml), and the other group with saline solution. After injection, the animals were returned to the containers, and kept in darkness for a given time, then removed and killed and the position of the retinal pigments was determined. The migration of proximal pigment to the dark-adapted state was faster in the presence of 5-HT. The time course of pigment migration was similar to that seen for the electrical response (Fig. 2). Both effects were complete within 60 min. 5-HT did not affect the final position attained by either the proximal pigment (Fig. 2) or the distal pigment (data not shown). In some experiments, 5-HT was tested on animals maintained under continuous illumination at light intensities strong enough to keep the distal pigment in a light-adapted position. Under these conditions 5-HT was unable to induce any changes of distal pigment position.

To explore whether 5-HT could by itself trigger the migration of proximal retinal pigment, two groups of preparations were made. One group was kept

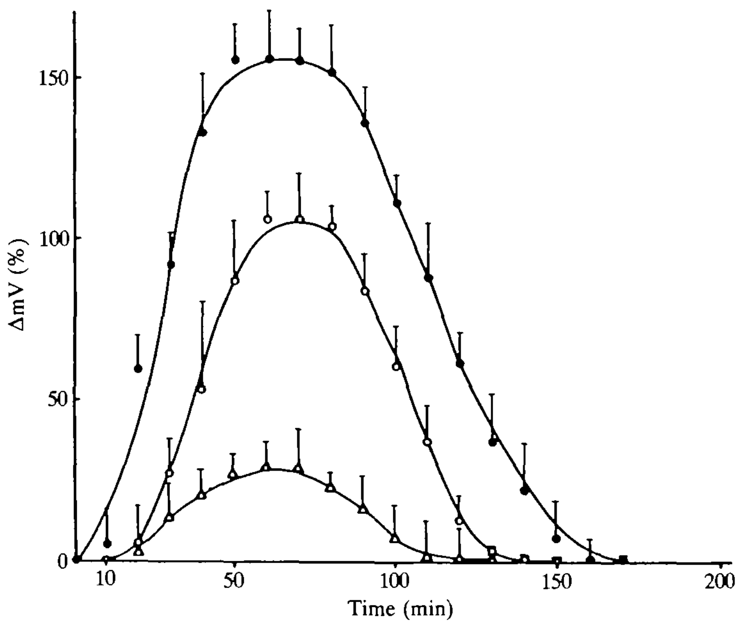

Fig. 2. The time course of enhancement of photoreceptor response amplitude after injection of three different concentrations of 5-HT (△, 10^{-6} mol l^{-1}; ○, 10^{-4} mol l^{-1}; ●, 10^{-3} mol l^{-1}). Ordinate, amplitude increase of the mass receptor potential as a percentage of the control value (ΔmV). Abscissa, time (min) after the injection. Each point is the average+S.E. of four determinations.

continuously at an adapting light intensity of 400 lx to produce a complete dispersion (light adaptation) of the proximal pigment. The other group was kept in continuous darkness. In a series of experiments with this protocol, animals were injected with different doses of 5-HT and killed 45 min after the injection, at which time the effect was fully established (Fig. 3). In the animals kept at 400 lx, and injected with 10^{-6}–10^{-9} mol l^{-1} 5-HT in 0.1 ml, a dose-dependent shift towards the dark-adapted position was promoted (Fig. 4). Even at high doses, there was no effect on the preparations kept in darkness. Again no effects were found on the position of the distal retinal pigments. The experiments were performed at different times of day, since the basal level of pigment position and the intensity of the light-induced response vary during the 24 h cycle. The results shown in Fig. 4 were from preparations tested near dusk.

Effects of 5-HT on isolated eyestalks

From the results obtained in whole animals, it seems clear that 5-HT enhances light responsiveness and promotes pigment retraction in retinal photoreceptors. To seek the site of action, experiments were conducted to test the effect of 5-HT applied directly to the retina. Groups of eyestalks were left to adapt fully to the dark before an adapting light was applied with an intensity sufficient to induce a complete dispersion of the proximal pigment. Test light pulses were then

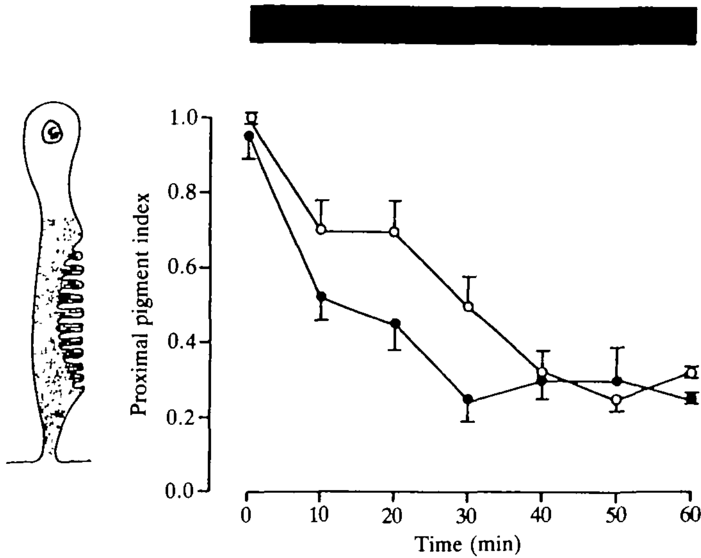

Fig. 3. The effect of 5-HT (10^{-5} mol l^{-1}) on the rate of dark adaptation of the proximal pigment granules inside photoreceptors in the whole animal. Abscissa, time in darkness; (○) control; (●) after 5-HT. Each point is the average and S.E. of four determinations (half the S.E. value is indicated). On the left is a schematic representation of a photoreceptor with pigments in partial dispersion.

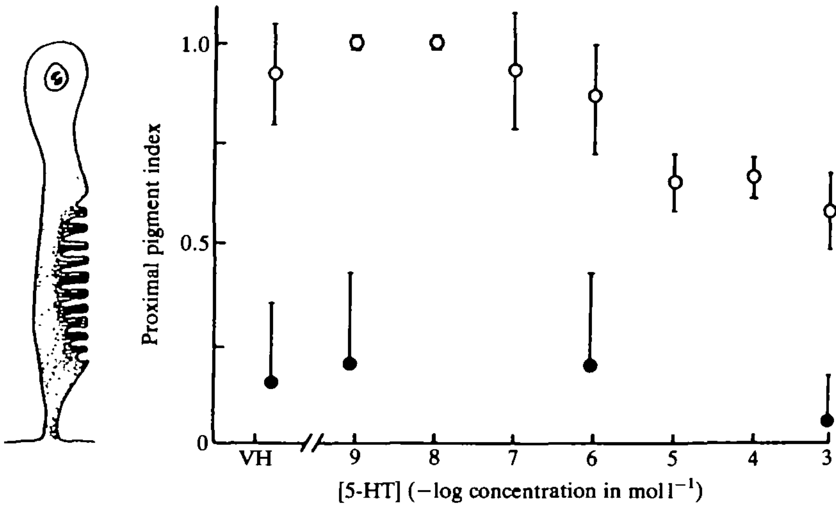

Fig. 4. The dose-dependence of the extent of proximal pigment migration in the presence of 5-HT, injected into whole crayfish, 45 min before the samples were taken. Animals were kept under 400 lx of adapting light (○) or in complete darkness (●). VH indicates animals receiving an injection of van Harreveld's solution. Each value is the average±S.E. of four determinations.

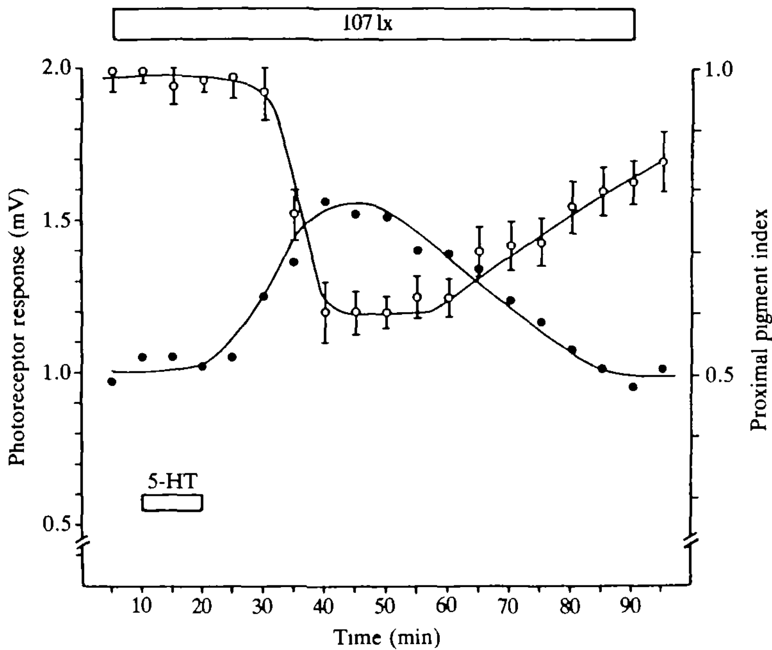

Fig. 5. The time correlation between the potentiation of the electrical response and proximal pigment migration, after topical application of 5-HT to isolated eyestalks. Left ordinate, amplitude of mass electrical response of photoreceptors (in mV, ●). Right ordinate, proximal pigment index (PPI). Preparations were kept under adapting light of 107 lx. Abscissa, time (min) under light adaptation. Light bar indicates time during which 5-HT was left to act. Each point for the PPI is the average±S.E. of six determinations.

superimposed on the background adapting light, and 5-HT was topically applied, left to act for 10 min, and then washed away. While the electrical responses from one eye were recorded, samples of eyestalks kept in the same dish were removed at 5 min intervals (six eyestalks per sample) and processed for proximal pigment determination. 10–15 min after 5-HT addition (at $1\times10^{-4}\,mol\,l^{-1}$ 5-HT in 0.1 ml) there was a potentiation of ERG amplitude concurrent with a partial retraction of proximal pigment (Fig. 5). Both were reversible after 1 h under these experimental conditions. Longer exposures to 5-HT resulted in more prolonged potentiation in this preparation. The potentiations and pigment migrations induced in isolated retinas in the presence of 5-HT were dose-dependent in the same range of concentrations tested in whole animals (10^{-6}–$10^{-3}\,mol\,l^{-1}$ in 0.1 ml).

In retinas isolated from previously dark-adapted animals (3 h), the activity from photoreceptors was recorded by means of extracellular suction electrodes. Once the electrode had been positioned, a series of control light pulses of varying intensities was delivered. A solution of 5-HT was then applied to the bathing fluid and, after another 30 min of dark adaptation, a new series of test light pulses was delivered within the same range of intensities as in controls. In some experiments,

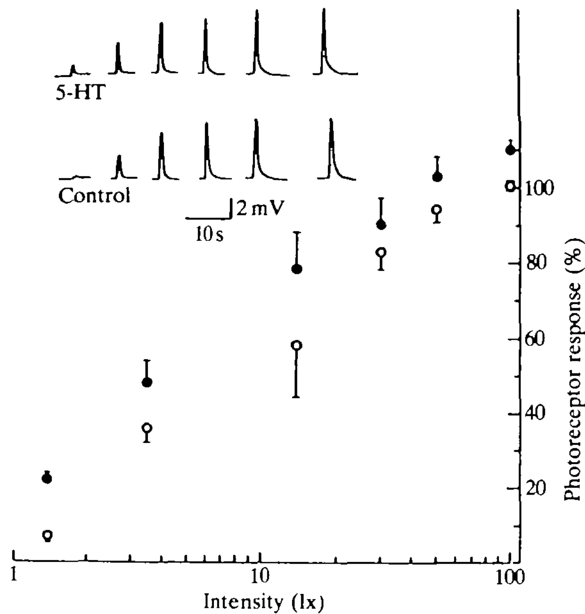

Fig. 6. The shift in *V*–log*I* curve of the photoreceptor response in the presence of 5-HT applited topically to an isolated retina (10^{-4} mol l^{-1}). Abscissa, light intensity of test light pulses; ○, control; ●, after 5-HT. Records in the upper part correspond to receptor potentials at different intensities of light. Top series, after 5-HT; bottom series, control.

the light pulses were applied in steps of increasing intensity; in others, a random sequence was programmed. No differences were found in the effect of 5-HT depending on the order of pulse application: there was an enhancement of the response, which was proportionally greater at low intensities of stimulating light (Fig. 6). For test pulses of 1.5 lx a more than twofold potentiation was observed. The range of concentration having an effect on the isolated eyestalks or retinas was the same as that for whole animals (10^{-6}–10^{-3} mol l^{-1}).

The response of the proximal pigment to 5-HT was similar in isolated eyestalks or retinas to that seen in the whole animal; that is, a retraction of the pigment to the dark-adapted position. In groups of retinas kept under continuous illumination of 200 lx (which is capable of promoting a complete dispersion of the proximal pigment), 5-HT induced a dose-dependent pigment retraction to a partial light adaptation. The time course of this effect was also explored. A series of experiments was conducted with a similar protocol to that followed for Fig. 3. The rate of dark adaptation was also enhanced by 5-HT in isolated eyestalks. Both the time course and the dose range for this effect (data not shown) were the same as in the whole animal.

The subcellular events underlying the retraction of proximal pigment granules induced by darkness have already been studied (Tsutsumi *et al.* 1981). For

comparison with the physiological response, the ultrastructural features of the 5-HT-induced pigment retraction were analysed. In the presence of 5-HT there was a conspicuous absence of pigment granules in the nuclear (distal) region of the photoreceptor (Fig. 7B), in contrast with the abundance of granules seen in the control cell (Fig. 7A). The volume occupied by the granules in the control cell was taken up by large vacuoles, similar to those seen in photoreceptors under dim light. No differences were observed in other subcellular features.

Effect of 5-HT-blocking agents

From the experiments described above it was clear that the effect of 5-HT on the retina was a direct one, not mediated by possible hormonal influences. However, the specificity of the effect was still uncertain. To explore this, several blocking agents were tested. The most potent effects were found with methysergide: topical application of $1\times10^{-3}\,mol\,l^{-1}$ methysergide abolished both the effect of 5-HT on the electrical response of retinal photoreceptors and the 5-HT-induced retraction of the proximal pigment (Fig. 8). In the electrophysiological experiments (Fig. 8A) a protocol similar to that used for Fig. 2 was used. Brief light pulses were applied at regular intervals and the electrical responses were recorded. Methysergide was first added to the bath, and 30 min later 5-HT was applied. No effect was observed. For pigment migration experiments (Fig. 8B) the isolated retinas were kept under continuous dim illumination, sufficient to induce a partial migration of the proximal pigment towards the light-adapted position. Whereas 5-HT induced the usual retraction, the effect was completely blocked when 5-HT was applied with methysergide. Methysergide by itself did not promote migration to the light-adapted position. The other 5-HT blocking agents tested were cyproheptadine hydrochloride, quipazine maleate, 5-methoxytryptamine and β-methylcarboxylate hydrochloride (TR 3369). They had less potent effects than those induced by methysergide. A more detailed account of the comparative actions of several 5-HT antagonists on proximal pigment migration is given by Frixione and Hernández (1989).

The demonstration of the blocking effect of methysergide suggested the possibility of exploring the physiological role of 5-HT in the control of retinal photoreceptors. As mentioned previously, the light responsiveness of the retina is enhanced at dusk when the retinal pigments migrate towards the dark-adapted positions, even in animals kept under continuous illumination. It was therefore decided to test whether, in whole animals, methysergide was capable of preventing this circadian trend. In an animal kept under 85 lx of continuous background illumination, at night (20:00 h), the proximal pigment underwent a partial retraction which was prevented by methysergide (Fig. 9). Again, in animals under continuous darkness, at night, methysergide did not promote any pigment migration by itself.

Localization of 5-HT in the retina

The possible physiological role of 5-HT in the retina was explored further by

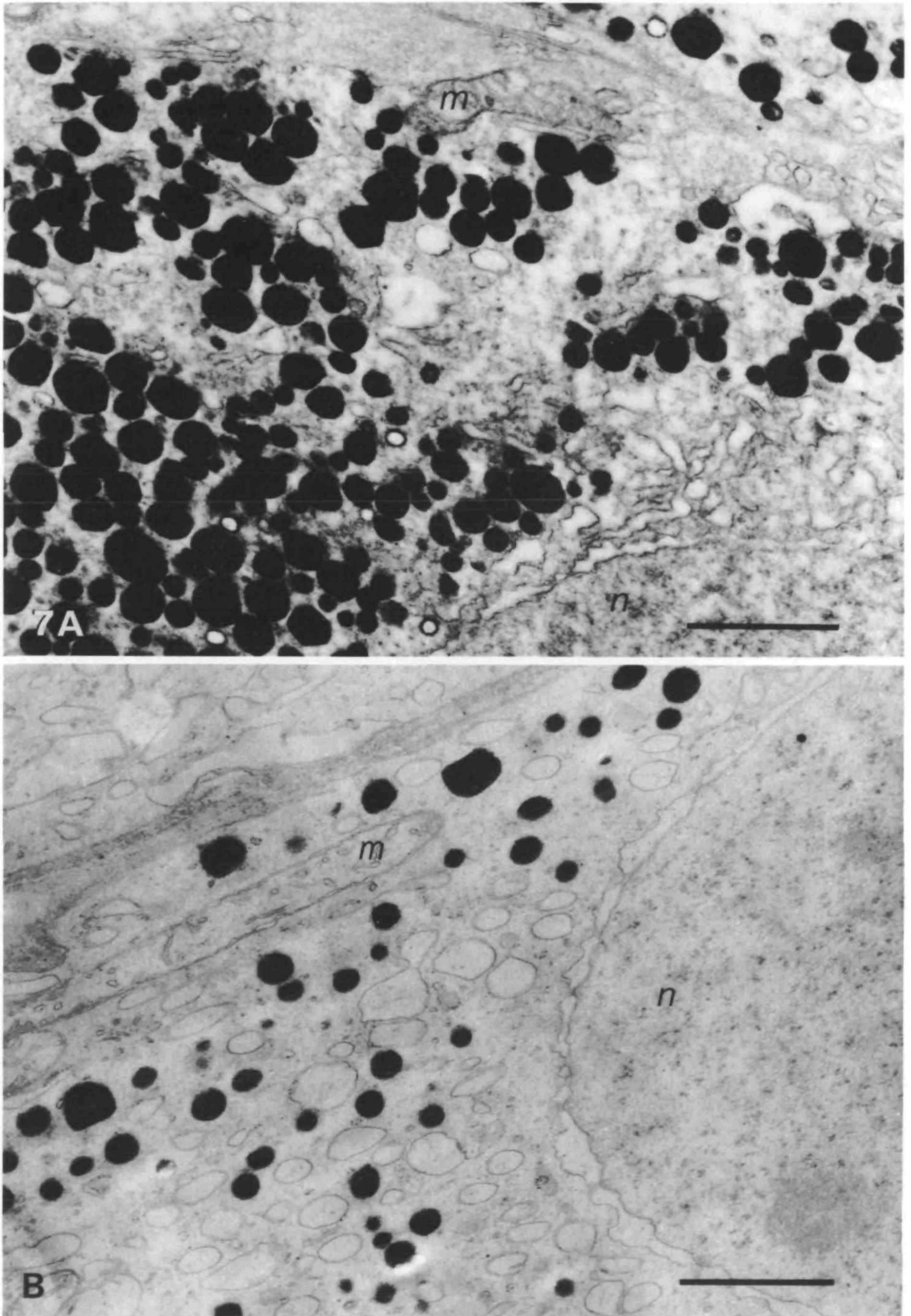

Fig. 7. Longitudinal sections of the perinuclear region of crayfish retinula cells. (A) Control preparation kept for 30 min under 100 lx background light. (B) Preparation under the same background illumination but incubated for 90 min in the presence of 10^{-3} mol l^{-1} 5-HT. *n*, nucleus; *m*, mitochondrion. Scale bars, 0.5 nm.

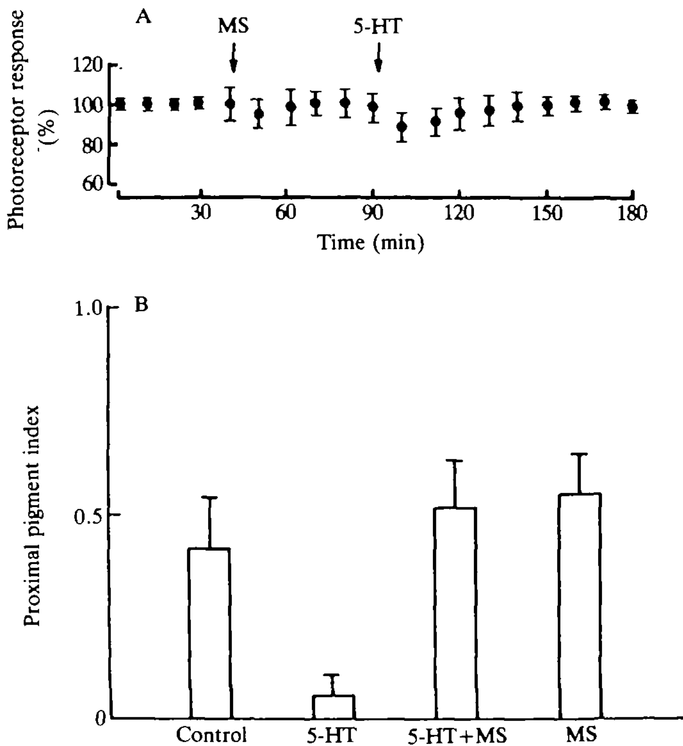

Fig. 8. Blockage by methysergide (MS) of 5-HT effects on the retina. (A) Lack of effect of 5-HT (10^{-4} mol l^{-1}) on mass receptor electrical response after previous incubation of the isolated retina in methysergide (5×10^{-3} mol l^{-1}). Each point is the average±S.E. of four determinations. (B) The retraction of proximal pigment is also blocked by methysergide. Bars indicate successively: control; the effect of incubation in 1×10^{-4} mol l^{-1} 5-HT solution (5-HT); 5-HT at 10^{-4} mol l^{-1} plus the addition of 1×10^{-3} mol l^{-1} methysergide (5-HT+MS); and the lack of a direct effect of methysergide. Each bar represents the average±S.E. of six determinations.

immunocytochemical localization in retinal sections. A positive reaction with an antibody against 5-HT was found, with an intensity that was strongly dependent on the time of day at which the retinas were removed: whereas at night all the preparations tested produced strong reactions, during the day the reaction was much weaker and often negative. A set of immunoreactive fibres was found, widely distributed over the lamina ganglionaris. Electron micrographs taken from sections at the level of the lamina revealed that the antibody-related label was present in axons ending in close apposition to the axons of the retinal photoreceptors, and did not penetrate further into the retina (Fig. 10). The photoreceptor axons could be clearly identified by the presence of large (up to 0.3 μm diameter) proximal pigment granules and mitochondria. No label was found inside these axons. We were unable to locate any synaptic structures between the immunoreactive fibres and the photoreceptor axons. Further evidence suggesting that

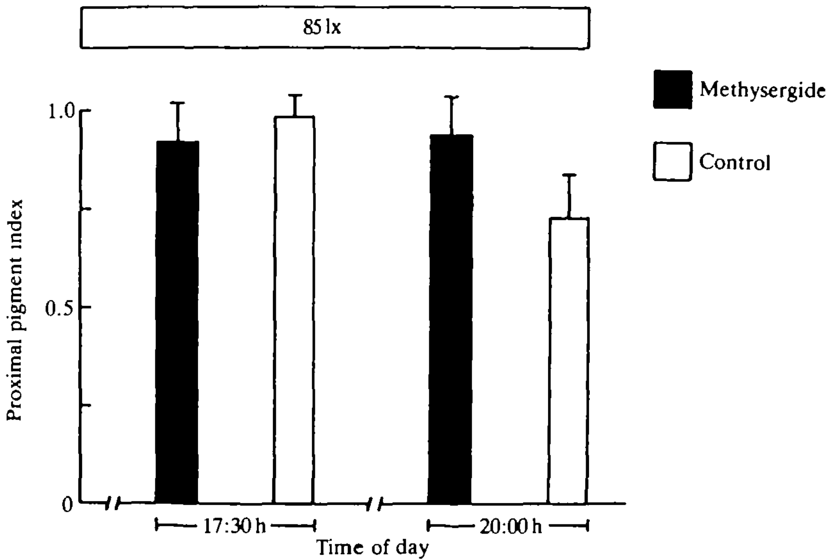

Fig. 9. Blockage of endogenous retraction of proximal pigment in the presence of methysergide. Whole animals, injected with methysergide, are fully light-adapted both during the day (17:30 h) and at night (20:00 h), whereas controls at night show a partial retraction of pigment, even when kept under moderate illumination. The experimental animals were injected with 100 μl of 1×10^{-3} mol l^{-1} methysergide and the controls with 100 μl of van Harreveld's solution. Each bar represents the average±S.E. of six determinations.

5-HT was endogenously contained in these fibres was the presence of dense-cored 5-HT-like immunoreactive vesicles, similar in size and shape to those associated with amine-containing terminals.

Conductance changes induced in photoreceptors by 5-HT

The enhancing effect of 5-HT on photoreceptor responses to light could be explained solely on the basis of the retraction it induces of the proximal pigment. However, as mentioned above, an alternative would be a dual effect, including a direct action on the electrical properties of the photoreceptors. This latter possibility was suggested by the enhancing effect of 5-HT on the electrical response of photoreceptors to light pulses of low intensity. From previous experiments it is known that the threshold illumination for triggering the dispersion of pigment granules in crayfish photoreceptors is about 10 lx (Frixione *et al.* 1979). However, 5-HT facilitated responses to test pulses below this intensity (Fig. 6). A possible direct effect on the membrane properties of photoreceptors was explored by intracellular recording of photoreceptor responses. In isolated retinas, we tested the effect of 5-HT on membrane potential and resistance, both in darkness and under test light pulses. Membrane resistance was monitored with 0.44 s hyperpolarizing pulses. The enhancing effect of 5-HT coincided with an increase in the light-induced conductance (Fig. 11). This facilitation existed

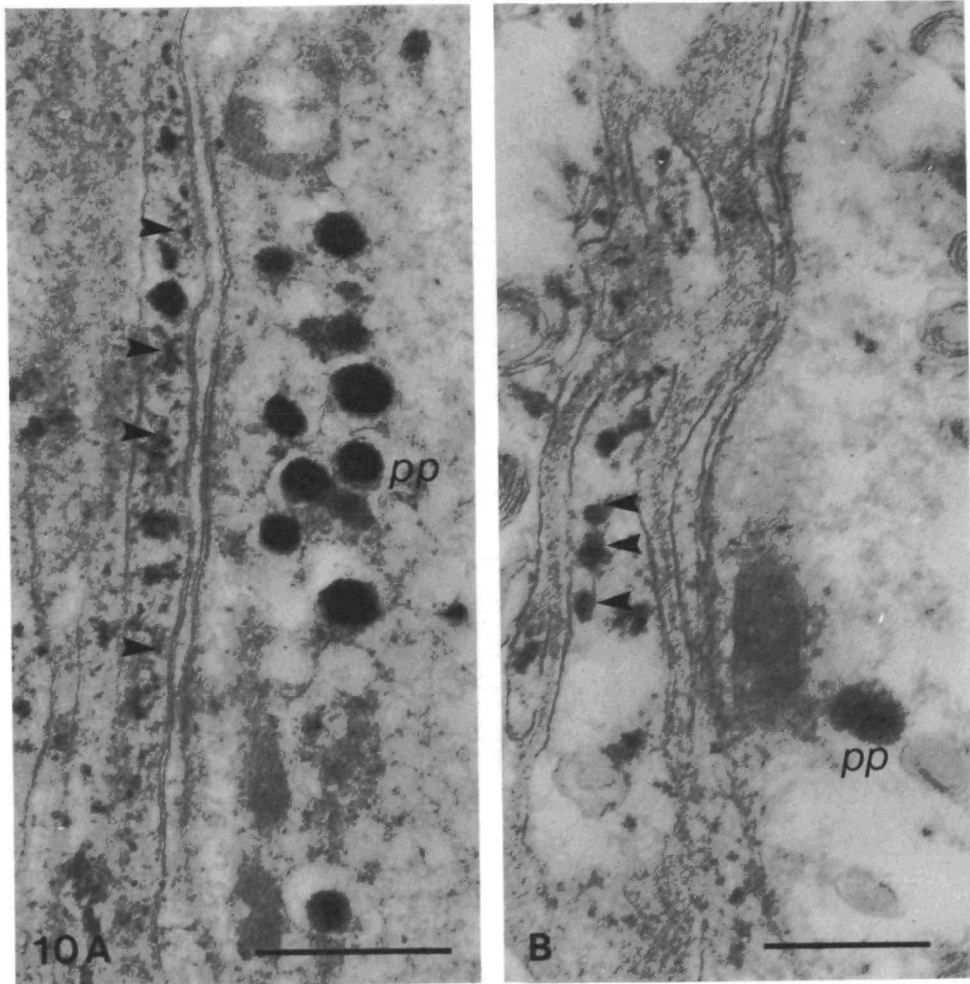

Fig. 10. Electron micrographs of the lamina ganglionaris; PAP method. 5-HT-like immunoreactivity (arrowheads in A) and 5-HT-like immunoreactive labelled vesicles (arrowheads in B) in axons closely apposed to photoreceptor axons. *pp*, proximal pigment granules. Scale bars, A 1 μm; B 0.5 μm.

throughout the photoreceptor response: that is, the initial transient response as well as the plateau phase and the depolarizing afterpotential. These effects were also blocked by methysergide.

Membrane potential and resistance were unaffected by 5-HT application in darkness. After 1×10^{-4} mol l^{-1} 5-HT had been topically applied, an enhancement ensued in the depolarization and conductance increase in response to test light pulses applied every 10 min (Fig. 12). No changes were detected in either variable during the periods of darkness. The effect on the light responsiveness lasted for more than 1 h after washing.

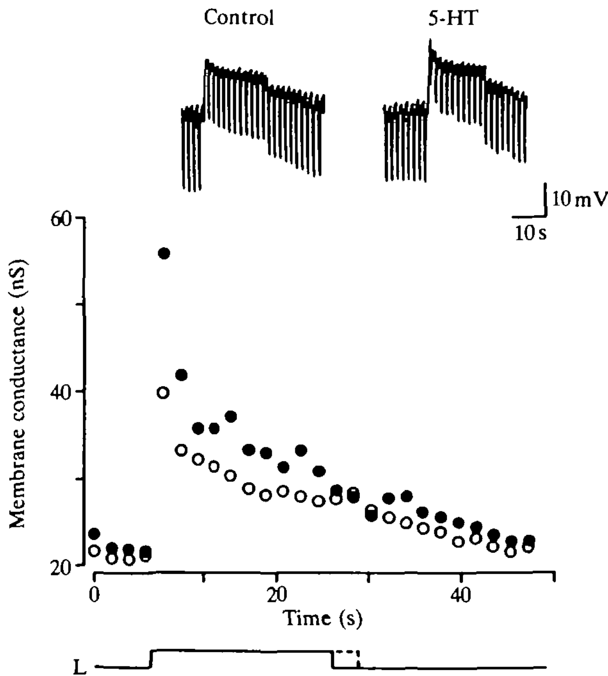

Fig. 11. The time course of the membrane conductance change during the receptor potential (control, ○) and after 30 min in the presence of 10^{-4} mol l^{-1} 5-HT (●). Recordings in the inset correspond to membrane potential deflections in response to 0.5 nA and 0.44 s hyperpolarizing current pulses (not shown). The receptor potentials are responses to the same light intensity (800 lx) but the pulse was 3.5 s longer in the control condition (bottom traces). Notice that the three components of the photoresponse (initial transient, plateau and depolarizing afterpotential) are all enhanced in the presence of 5-HT. The membrane conductance in the dark (pre-stimulation period) is almost unaffected.

Discussion

The effect of 5-HT on the crayfish retina appears to be a direct one, and its target is the photoreceptor cell. The possibility of an action through the distal retinal pigment can be ruled out, since the effect is well established within 30 min of 5-HT injection. The distal pigment migration takes at least that time to start (Aréchiga *et al.* 1974). Moreover, no changes in distal pigment position could be detected in our experiments, regardless of the basal state of light-adaptation or dark-adaptation. The effect on the photoreceptors could be conveyed through an axonal channel, given the presence of 5-HT-like immunoreactive fibres in the immediate vicinity of the photoreceptor axons. Since no synapses have been demonstrated at this level, one possibility is that 5-HT acts as a neuromodulator, released locally onto the photoreceptors. From our experiments, although a direct effect of 5-HT on the photoreceptors seems likely, one cannot rule out the possibility that 5-HT may be acting through other cells present in the retina. Our

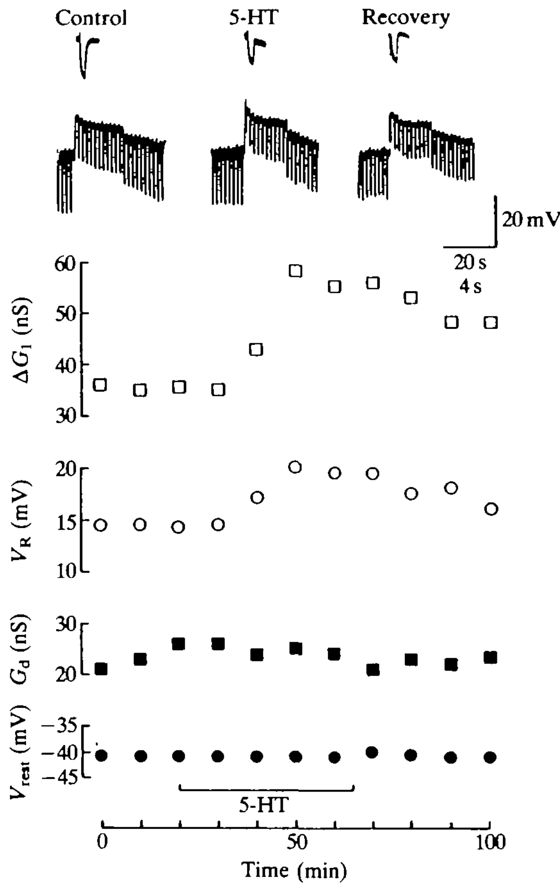

Fig. 12. The time course of 5-HT (10^{-4} mol l^{-1}) effects on the electrophysiological properties of photoreceptors. V_{rest}, resting membrane potential; G_d, membrane conductance in the dark (pre-stimulation period); V_R, peak of the receptor potential; G_l, maximal conductance change during the light response. The lower recordings correspond to the membrane potential during the intracellular injection of 0.5 nA and 0.44 s hyperpolarizing current pulses (not shown). The upper recordings are the first membrane voltage deflections during the light response. Notice that both V_R and G_l are enhanced in the presence of 5-HT, but V_{rest} and G_d remain practically unchanged.

preparations included part of the lamina ganglionaris, and only experiments on isolated receptors will provide a final answer to this point.

The site of origin of the 5-HT-like immunoreactive axons cannot be determined at the moment. Cell bodies have been identified in the vicinity of the lamina ganglionaris, but some are abundant at other levels of the eyestalk and some axons are even present in the optic nerve. It will be necessary to do further work on this point. Since 5-HT has also been demonstrated in the circulation (Livingstone *et al.* 1980), a possible concomitant action as a neurohormone cannot be ruled out. The doses of 5-HT that were found effective are certainly high. However, the figures

given correspond to the concentrations of the injected solutions. Since the volume used in our experiments was 0.1 ml, at least a 20-fold dilution in the haemolymph is to be expected in the intact animals, and there could be some breakdown before the substance reaches its target. In the isolated retinas, the diffusion barriers are another unknown variable, preventing a precise estimation of the real concentrations at the photoreceptors.

It seems likely that 5-HT mediates both the effect on proximal pigment migration and that on the electrical response to light acting through the same receptor, since methysergide blocks both actions (see Richardson and Engel, 1986; Peroutka, 1988), but more pharmacological evidence is required (see, however, Frixione and Hernández, 1989). The substrate(s) and mechanism(s) of action may, however, be different. It seems clear that the enhancement of receptor potentials does not require a previous retraction of proximal pigment, since the effect is still present in fully dark-adapted eyes. However, the magnitude of the effect is greater in light-adapted eyes, in which both mechanisms are operating. From previous evidence (Frixione and Aréchiga, 1981), it is known that the dispersion of the proximal pigment to the light-adapted position can be triggered by raising the intracellular levels of sodium and calcium. Since 5-HT increases the activity of Na^+,K^+-ATPase in a membrane fraction from retinal homogenates (Frixione and Hernández, 1989), it is possible that 5-HT might induce pigment retraction by stimulating a sodium pump. There is no indication, however, of a hyperpolarization of photoreceptors induced by 5-HT, but it is still possible that the effect is exerted at the level of photoreceptor axons, where the 5-HT-like immunoreactive fibres have been identified. A small change in membrane potential near the axon terminals might not be detected by an electrode at the opposite end of the receptor, as was often the case in our impalements.

In vertebrates, amines appear to influence the responsiveness of retinal elements by modulating the electrical coupling between neighbouring elements (see Neyton and Trautmann, 1986). No such connections have been demonstrated in the crayfish (Picones and Aréchiga, 1990) so we cannot postulate such a mechanism to explain our results. Since there are no changes in membrane conductances other than those evoked by light, it is difficult to envisage a direct effect on the opening or closing of ionic channels, as has been reported for this amine in other preparations (Crow and Bridge, 1985). The only demonstrated effect in our preparation is an enhancement of the light-induced conductance change, so it is tempting to suggest that the initial effect of 5-HT is to modulate light-sensitive cation channels responsible for the generation of the receptor potential. Since the effect is exerted both on the phasic and on the tonic phases of the receptor potential, and the conductance increase persists even during the late depolarizing potential, more than one channel type could be affected. Clearly, we need a further analysis of the ionic substrate of the 5-HT action.

From these data it can be speculated that 5-HT, by enhancing the magnitude of the receptor potential at low levels of illumination, could increase sodium and calcium entry, thus facilitating the dispersion of the proximal pigment (Frixione

and Aréchiga, 1981), which would, in turn, counteract the potentiating effect. It is interesting, therefore, that 5-HT also stimulates the sodium extrusion system, preventing pigment dispersion to the point of inducing a retraction of the pigment, which further enhances the responsiveness to light.

Since 5-HT levels in the eyestalks are higher at night (Fingerman and Fingerman, 1977), and given that 5-HT blocking agents abolish the pigment retraction which normally takes place at night, it seems reasonable to suggest that the release of 5-HT occurs preferentially at dusk and determines proximal pigment retraction as well as directly increasing photoreceptor sensitivity. The higher immunoreactivity observed in our preparations at night is in line with the role of 5-HT as an agent involved in the nocturnal phase of retinal sensitivity, but 5-HT could be one of several modulators. For instance, octopamine has been found to induce an effect opposite to that of 5-HT: it reduces the light-induced depolarization of crayfish retinal photoreceptors (L. Rodríguez-Sosa, J. L. Cortés and H. Aréchiga, unpublished results).

Another interesting aspect of the characterization of the modulatory influence of 5-HT on the crayfish photoreceptors will be the physiological conditions for its release in the retina.

The authors are greatly indebted to Ms Elizabeth Becerra and Mr Ricardo Mondragón for their technical assistance in the morphological aspects of this work and to Mr Víctor Anaya in electrophysiological experiments. The electron microscopy studies were made at the Unidad de Microscopía Electrónica (CINVESTAV). This work was supported by CONACyT fellowship to AP and LR-S and grant number P228-CCOX881151 to HA.

References

ARÉCHIGA, H. AND ATKINSON, R. J. A. (1975). The eye and some effects of light on locomotor activity in *Nephrops norvegicus*. *Mar. Biol.* **3**, 63–76.

ARÉCHIGA, H., CORTÉS, J. L., GARCÍA, U. AND RODRÍGUEZ-SOSA, L. (1985). Neuroendocrine correlates of circadian rhythmicity in crustaceans. *Am. Zool.* **25**, 265–274.

ARÉCHIGA, H., FUENTES-PARDO, B. AND BARRERA-MERA, B. (1974). Influence of retinal shielding pigments on light sensitivity in the crayfish. *Acta physiol. latinoam.* **34**, 601–611.

ARÉCHIGA, H. AND HUBERMAN, A. (1980). Hormonal modulation of circadian rhythmicity in crustaceans. In *Frontiers of Hormone Research*, vol. 6, *Comparative Aspects of Neuroendocrine Control of Behavior* (ed. C. Valverde and H. Aréchiga), pp. 16–34. S. Karger AG.

BARLOW, R. B., BOLANOWSKI, S. J. AND BRACHMAN, M. L. (1977). Efferent optic nerve fibers mediate circadian rhythms in *Limulus* eye. *Science* **197**, 86–89.

BARRERA-MERA, B. (1978). Neural coupling between left and right electroretinographic circadian oscillations in the crayfish *P. bouvieri*. *Comp. Biochem. Physiol.* **61**A, 427–432.

BELTZ, B. S. AND KRAVITZ, E. A. (1983). Mapping of serotonin-like immunoreactivity in the lobster nervous system. *J. Neurosci.* **3**, 585–602.

BRYCESON, K. P. (1986). Diurnal changes in photoreceptor sensitivity in a reflecting superposition eye. *J. comp. Physiol.* A **158**, 573–582.

CROW, T. AND BRIDGE, M. S. (1985). Serotonin modulates photoresponses in *Hermissenda* type-B photoreceptors. *Neurosci. Lett.* **60**, 83–88.

[illegible]ON, D. AND ATWOOD, H. L. (1985). Crayfish motor nerve terminal's response to serotonin examined by intracellular microelectrode. *J. Neurobiol.* **16**, 409–424.

ELOFSSON, R. (1983). 5-HT-like immunoreactivity in the central nervous system of the crayfish, *Pacifastacus leniusculus*. *Cell Tissue Res.* **232**, 221–236.

ELOFSSON, R., LAXMYR, L., ROSENGREN, E. AND HANSSON, C. (1982). Identification and quantitative measurements of biogenic amines and DOPA in the central nervous system and haemolymph of the crayfish *Pacifastacus leniusculus* (Crustacea). *Comp. Biochem. Physiol.* **71C**, 195–201.

ENYEART, J. (1981). Cyclic AMP, 5-HT, and the modulation of transmitter release at the crayfish neuromuscular junction. *J. Neurobiol.* **12**, 505–513.

ESKIN, A., LIN, C.-Y. AND MCADOO, J. (1982). Mechanisms of shifting the phase of a circadian rhythm by serotonin: involvement of cAMP. *Proc. natn. Acad. Sci. U.S.A.* **79**, 660–664.

FERNLUND, P. (1976). Structure of a light-adapting hormone from the shrimp *Pandalus borealis*. *Biochim. biophys. Acta* **439**, 17–25.

FINGERMAN, M. (1985). The physiology and pharmacology of crustacean chromatophores. *Am. Zool.* **25**, 233–252.

FINGERMAN, S. W. AND FINGERMAN, M. (1977). Circadian variation in the levels of red pigment-dispersing hormone and 5-hydroxytryptamine in the eyestalks of the fiddler crab, *Uca pugilator*. *Comp. Biochem. Physiol.* **56C**, 5–8.

FRIXIONE, E. AND ARÉCHIGA, H. (1981). Ionic dependence of screening pigment migrations in crayfish retinal photoreceptors. *J. comp. Physiol.* **144**, 35–43.

FRIXIONE, E., ARÉCHIGA, H. AND TSUTSUMI, V. (1979). Photomechanical migrations of pigment granules along the retinula cells of the crayfish. *J. Neurobiol.* **10**, 573–590.

FRIXIONE, E. AND HERNÁNDEZ, J. (1989). Modulation of screening pigment position in crayfish photoreceptors by serotonin: possible involvement of Na^+/K^+-ATPase. *J. exp. Biol.* **143**, 459–473.

GLUSMAN, S. AND KRAVITZ, E. A. (1982). The action on excitatory nerve terminals in lobster nerve–muscle preparations. *J. Physiol., Lond.* **325**, 223–241.

KRAVITZ, E. A. (1988). Hormonal control of behavior: Amines and the biasing of behavioral output in lobsters. *Science* **241**, 1775–1781.

LAXMYR, L. (1984). Biogenic amines and DOPA in the central nervous system of decapod crustaceans. *Comp. Biochem. Physiol.* **77C**, 139–143.

LIVINGSTONE, M. S., HARRIS-WARRICK, R. M. AND KRAVITZ, E. A. (1980). Serotonin and octopamine produce opposite postures in lobsters. *Science* **208**, 76–79.

NEYTON, J. AND TRAUTMANN, A. (1986). Physiological modulation of gap junction permeability. *J. exp. Biol.* **124**, 93–114.

OLIVO, R. AND LARSEN, M. E. (1978). Brief exposure to light initiates screening pigment migration in the retinula cells of the crayfish *Procambarus*. *J. comp. Physiol.* **125**, 91–96.

PEROUTKA, S. J. (1988). 5-Hydroxytryptamine receptor subtypes. *A. Rev. Neurosci.* **11**, 45–60.

PICONES, A. AND ARÉCHIGA, H. (1987). 5-Hydroxytryptamine modulates light response in crayfish retinal photoreceptors. *Soc. Neurosci. Abstr. 17th Annual Meeting*, **13**, 1341.

PICONES, A. AND ARÉCHIGA, H. (1990). Electrophysiological properties of crayfish retinal photoreceptors. *J. exp. Biol.* **150**, 111–122.

RAO, K. R. (1985). Pigmentary effectors. In *The Biology of Crustacea*, vol. 9, *Integument, Pigments, and Hormonal Processes* (ed. D. E. Bliss and L. H. Mantel), pp. 395–462. New York: Academic Press.

RICHARDSON, B. P. AND ENGEL, G. (1986). The pharmacology and function of 5-HT_3 receptors. *Trends Neurosci.* **9**, 424–428.

RODRÍGUEZ-SOSA, L. AND ARÉCHIGA, H. (1982). Range of modulation of light sensitivity by accessory pigments in the crayfish compound eye. *Vision Res.* **22**, 1515–1524.

SANDEMAN, D. C., SANDEMAN, R. E. AND AITKEN, A. R. (1988). Atlas of serotonin-containing neurons in the optic lobes and brain of the crayfish, *Cherax destructor*. *J. comp. Neurol.* **269**, 465–478.

STERNBERGER, L. A. (1974). *Immunocytochemistry*. Englewood Cliffs, New Jersey: Pren[illegible] Hall Inc.

TSUTSUMI, V., FRIXIONE, E. AND ARÉCHIGA, H. (1981). Transformations in the cytoplasmic structure of crayfish retinula cells during light- and dark-adaptation. *J. comp. Physiol.* **145**, 179–189.

VAN HARREVELD, A. (1936). A physiological solution for freshwater crustaceans. *Proc. Soc. exp. Biol. Med.* **34**, 428–432.