

Serotonin and Inhibition in *Limulus* Lateral Eye

A. R. ADOLPH and F. J. TUAN

From the Neurophysiology Laboratory, Retina Foundation, Boston, Massachusetts 02114

ABSTRACT The response to light of one ommatidium is reduced or suppressed by simultaneous illumination of neighboring ommatidia. The mechanism of this lateral inhibition may be chemical synaptic transmission, based on the physiological findings of a number of investigators and on the following evidence. The fine structure of the neuropil of the lateral plexus exhibits numerous clear vesicles (ca. 400 Å), dense-core vesicles (ca. 700–1400 Å), Golgi regions, and other morphological features of neurochemical synapses. The indolealkylamine, serotonin (5-HT), even in nanomolar concentrations, has a potent inhibitory action. An initial, potent inhibitory dose of 5-HT produces a long lasting desensitization to subsequent doses. The desensitization affects lateral inhibition evoked by light stimulation of neighboring receptors, i.e. crossed-desensitization. Eye tissue extracts contain 5-HT and melatonin (MLT) at a level greater than 1 µg/g wet tissue and perhaps as high as 20–30 µg/g, as determined by two-dimensional thin-layer chromatography (TLC) and *o*-phthalaldehyde fluorescence assay techniques. Subcellular fractionation on sucrose gradient indicates a peak in 5-HT and MLT content associated with an intermediate density fraction. 5-HT may be an inhibitory transmitter for lateral inhibition. One pathway for metabolism of 5-HT in the lateral eye may be via *N*-acetylserotonin and melatonin.

The lateral eye of *Limulus* has long served as the prototypal visual receptor (Hartline, 1968; Wolbarsht and Yeandle, 1967). Among the interesting features of this eye is the lateral inhibition between neighboring ommatidial receptors. The response to light of one ommatidium is reduced or suppressed by simultaneous illumination of neighboring ommatidia. The mechanism of lateral inhibition has been extensively studied from the physiological viewpoint (Hartline et al., 1961; Wolbarsht and Yeandle, 1967, Hartline, 1968) and these studies provide strong physiological evidence that the mechanism of lateral inhibition is some form of chemical synaptic transmission. Among this evidence is the following: lateral inhibition produces in the inhibited cell a membrane potential hyperpolarization accompanied by a conductance increase to an ionic species having a negative intracellular equilibrium po-

tential (Purple and Dodge, 1965). There is a significant delay between the occurrence of a burst of spikes in neighboring axons and the inhibition of spikes in a test receptor's axon (Ratliff et al., 1966). This delay is too long to be accounted for by conduction times in the various axons. Lateral inhibition exhibits facilitation phenomena (Ratliff et al., 1966) as well as fatigue effects (Lange, 1965).

The pharmacology and neurochemistry of the possible chemical synaptic basis of lateral inhibition has, nonetheless, received relatively little study (Adolph, 1966; Behrens and Wulff, 1970). There have only been a few studies of its histology (Hartline et al., 1961; Gur et al., 1972), as well. This paper reports the results of our recent work especially in these latter two areas, on the lateral inhibitory mechanism.

METHODS

1. *Animals* Adult *Limulus polyphemus*, 10–12 inches or larger across the carapace, were obtained from the Marine Biological Laboratory, Woods Hole, Mass. The animals were maintained in a 150 gallon aquarium (Instant Ocean, Aquarium Systems, Inc., Eastlake, Ohio) at 17°C. The aquarium light environment was not controlled; for part of the time of the experiments, fluorescent lighting during a “working day” on, nighttime off, cycle was used. Most of the experiments, however, were done during a period when the animals were under conditions of natural illumination through windows adjacent to the aquarium, and consequently subject to seasonal variations in light-dark ratios.

2. *Optical* A large (ca. 1.5 mm diameter) and a small (ca. 70 μ m diameter) light spot were transmitted directly to the corneal surface by two individual fiber optic bundles (American Optical Corp., Bedford, Mass.). The corneal ends of the two bundles were held in an adjustable two-electrode holder (Narashige, Labtron Scientific Corp., Farmingdale, N. Y.) which allowed their relative positions to be adjusted. The two-electrode holder was further manipulated by a microdrive with four degrees-of-freedom of movement (Narashige) so that the two fiber optic bundles could be ultimately positioned with eight degrees-of-freedom movement. The light source for the larger bundle was a 50 watt tungsten-halogen lamp (Klinger Scientific Apparatus Corp., Jamaica, N. Y.) whose filament image was focused on the fiber bundle end through a heat-absorbing filter, any desired neutral density filters, and an electromagnetic shutter (Uniblitz, Velmex, Inc., Holcomb, N. Y.). An 18 watt tungsten lamp, beamed through a similar filter and shutter chain, illuminated the aperture of a microscope substage condenser which focused the filament image on the smaller fiber bundle. Either bundle alone, or both combined, could be used as the stimulus source in experiments on single receptors, a group of receptors, or the interaction between a group and a single receptor. Both lamps were operated at nominal ratings and without modification of their visible spectra.

3. *Electrophysiological* A thin (ca. 1 or 2 mm) slice of lateral eye, including the bulk of optic nerve fibers in a bundle 1–2 cm long, was mounted with its corneal surface accessible to optical stimulation, in a 10 ml perfusion chamber. The chamber

could be drained and refilled in 20–30 sec. The vascular sheath was removed from the optic nerve and bundles with several nerve fibers were teased from the optic nerve. Extracellular responses were recorded from fibers within a bundle by means of a drawn-glass capillary suction electrode and AC preamplifier (Grass P-15, Grass Instrument Co., Quincy, Mass.). The output of the preamplifier was displayed along with stimulus indicator signals on a multitrace CRO (Tektronix 565, Tektronix, Inc., Beaverton, Oreg.) and recorded on an FM data recorder (H-P Sanborn 3917B, Hewlett-Packard Co., Waltham, Mass.). Spikes were counted either manually from filmed records made with a Grass C-4 camera or electronically by a gated event-counter (Heath EV-805, Heath Co., Benton Harbor, Mich.). Occasionally, thicker slices of eye were used (up to 5 mm) in the experiments on light-evoked lateral inhibition where some searching of the cornea for suitable fiber optic locations was necessary.

4. *Pharmacological* The bathing medium in the perfusion chamber was artificial sea water (ASW), pH ca. 8, prepared from a commercially available salt mix (Instant Ocean, Aquarium Systems, Inc.). The ASW was also used as the carrier medium for the 5-HT creatinine sulfate (Sigma Chemical Co., St. Louis, Mo.) and melatonin (Sigma) drug solutions. The final bath concentrations of perfused drug solutions were usually at a level between 1 and 100 μM . 25 experiments form the basis of the electrophysiological and pharmacological findings presented in subsequent sections.

5. *Neurochemical* Lateral eyes were rapidly excised using a cast-cutting saw and the eye tissue between cornea and internal chitin margin was frozen, in small glass vials, in liquid nitrogen. For each TLC procedure, the tissue from eight eyes was pooled. The pooled tissue was homogenized in 3 ml of 0.05 N NaOH (Miller and Maickel, 1970), centrifuged, floating and sedimented impurities removed, and reduced in volume to about 20 μl by drying in vacuo. The extract was applied to a TLC plate (Eastman Silica Gel 6060, Eastman Organic Chemicals, Rochester, N. Y.) and developed together with several standard concentrations of serotonin-creatinine sulfate and melatonin (Sigma Chemical Co.) as position markers in an *n*-butanol:glacial acetic acid:water (60:15:25) solvent system. The appropriate spots were scraped from that primary, one-dimensional TLC, eluted with 95% ethanol, and rechromatographed on secondary TLC. The secondary TLC was run in two dimensions with the solvent system for one of the dimensions the same as the primary TLC and the second dimension solvent system consisting of methyl acetate:isopropanol:25% ammonia (9:7:4). Spot locations were determined by fluorescence under UV light. The method used for quantitative assays of tissue indolealkylamines evolved through several stages. Initially the method of Maickel et al. (1968) was used as an indication of 5-HT content. When the presence of significant amounts of indolealkylamines other than 5-HT was suspected, we employed the procedure of Miller and Maickel (1970), incorporating more sophisticated differential solvent extraction techniques. Quay's (1963, 1969) fluorimetric procedure was used in attempting to compare the assay levels of 5-HT and its possible metabolites *N*-acetylserotonin (NAS) and 5-hydroxyindole-3-acetic acid (5-HIAA). The possibility of cross-contamination and quenching effects discussed by Fischer and Aprison (1972) were also considered, finally leading to our present technique, a modification of the

TLC identification method, which combines solvent extraction, preparative-layer chromatography, spot elution and *o*-phthaldialdehyde fluorimetry. In the last step of the combination method, the eluted spots are reacted with *o*-phthaldialdehyde in 10 N HCl at 110°C for 10 min, and fluorescence measured in a Turner 110 fluorometer (G. K. Turner Associates, Inc., Palo Alto, Calif.) at 360 nm excitation and 470 nm emission. For the sucrose gradient fractionation, the homogenate was centrifuged 8 min at 1000 rpm (Sorvall GLC-1, Ivan Sorvall, Inc., Norwalk, Conn.) and supernatant applied to a 1.0–1.7 M sucrose gradient in 10 mM tris(hydroxymethyl)amino-methane (Tris) buffer, pH 7.0–7.5. This was centrifuged 45 min at 30,000 rpm (Beckman L3-50, SW50L rotor Beckman Instruments, Inc., Fullerton, Calif.). Fractions were collected from punctured tubes and extracted with diethyl ether, spotted and developed on TLC plates. The relative amounts of identified components of each fraction was determined from their spot intensities. The neurochemical data presented in the paper is representative of 12 of the best TLC, sucrose gradient, and assay experiments from a total of 128 thin-layer, column, and paper chromatography, solvent extraction, isotope, and sucrose gradient experiments.

6. *Histological* Tissue was fixed in 4% glutaraldehyde (buffered by either phosphate or Earle's buffer), postfixated in 1% osmium tetroxide, embedded in Araldite-Epon (Ladd Industries, Burlington, Vt. and Fisher Scientific Co., Pittsburgh, Pa.) and sectioned 600–900 in an LKB Ultratome (LKB Instruments, Inc., Rockville, Md.). Sections were stained with uranyl acetate/lead citrate and viewed with a Philips 300 electron microscope (Philips Electronic Instruments, Mount Vernon, N. Y.).

RESULTS

The extracellular spike response of a single receptor, optically isolated through stimulation by a 70 μ fiber optic bundle is illustrated in Fig. 1. After an initial period of 5 sec during which the receptor alone is stimulated, a group of neighboring receptors is activated for 3 sec. It is a relatively large group of neighbors underlying a fiber optic bundle 1.5 mm in diameter. There is an initial silent period produced by the strong transient phase of the group's response, followed by a steady response at a frequency lower than the firing rate during the initial 5 sec test period. After the 3 sec inhibitory period, the illumination of the group of neighboring ommatidia is removed and the test receptor remains stimulated in isolation for 2 sec more. The sequence in Fig. 1 illustrates the effect produced by a constant intensity of inhibitory stimulation on test responses to a series of decreasing intensity stimuli. The inhibitory decrement is a constant percentage of test frequency, i.e., the absolute decrement in response to constant inhibitory stimulation is a linearly decreasing function of test response.

This electrophysiological evidence of lateral inhibition is hardly novel (Hartline, 1968) but is included here to illustrate the effect as an aspect of identification of a putative inhibitory neurotransmitter. In addition, the methodology is combined with pharmacological techniques in studying cross-desensitization effects and those results are presented later in this section.

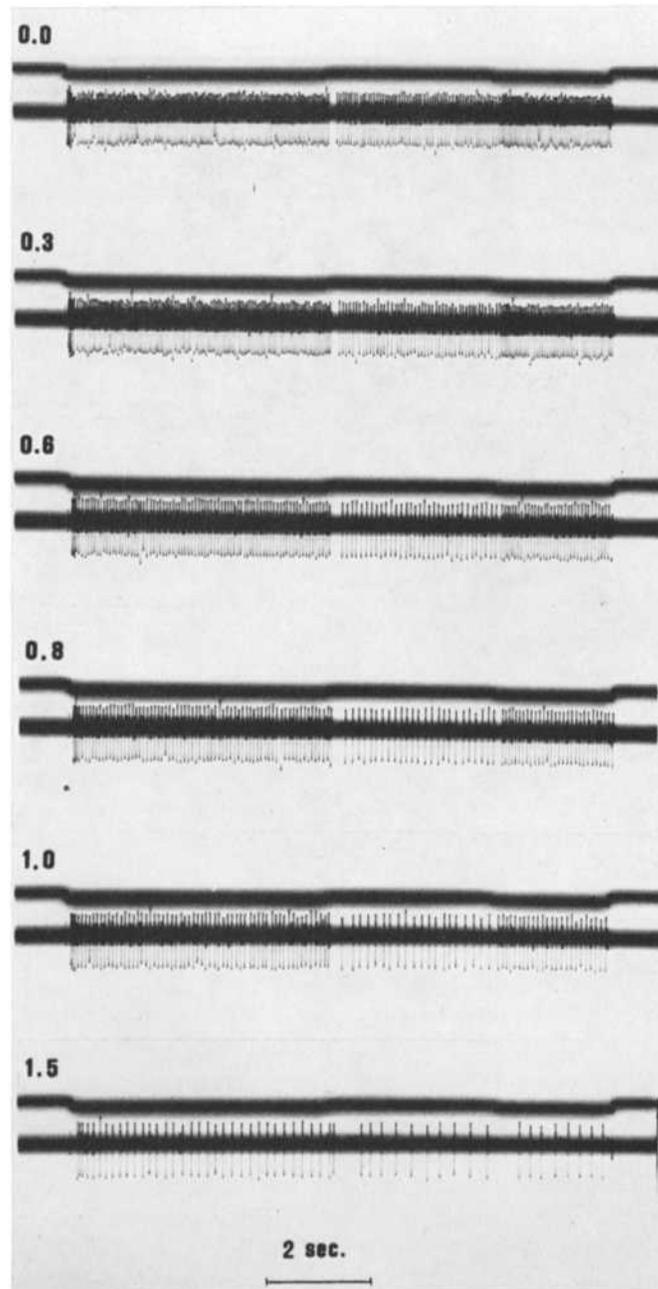


FIGURE 1. Lateral inhibition of the spike response of a single, optically isolated, ommatidium by a group of neighboring ommatidia. Test receptor stimulated for 10 sec. Inhibitory group stimulated for 3 sec beginning 5 sec after test stimulus onset. Numbers to left of records indicate log-attenuation of test stimulus intensity by neutral density filters. Inhibitory stimulus constant for each run. Upper trace, shutter signals (down: test, up: inhibitors); lower trace, optic nerve response of test receptor.

The morphological locus of the mechanism of lateral inhibition is believed to be the neuropil of the lateral plexus formed by branches of the eccentric cell axons (Hartline et al., 1961; Gur et al., 1972). The fine structure of this region exhibits a number of features associated with chemical synapses seen in a wide variety of neural tissue (Eränkő, 1971). Fig. 2 shows a cross-section through a neuropilar region with numerous collaterals containing prominent mitochondria and vesicles. These vesicles are primarily clear type of 400–500 Å diameter. Other structures, relatively devoid of vesicles but containing occasional mitochondria, endoplasmic reticulum, and vacuoles, are probably glial cell processes. The neuropilar structures are shown at a somewhat greater magnification in Fig. 3. Basement membrane surrounding the group of neural elements is quite obvious. Large pigment granules, within what are probably proximal pigment cells, can be clearly seen. A Golgi region, from which newly formed vesicles are thought to arise, indicates vesicular synthetic capability within the neuropil region.

A new feature is apparent in Fig. 3: dense-core vesicles. A longitudinal section of neuropil in Fig. 4 shows numerous dense-core vesicles and neurofibrillar structures within the neural process. Basement membrane and glial cell components are also apparent. There is a broad range of dense-core vesicle sizes from less than 700 Å to more than 1400 Å in diameter, although they appear to be separated into two distinct size groups: one group with a mean diameter of 825 ± 90 Å and the other of 1300 ± 100 Å. The smaller vesicles constituted about one-third, and the larger group, two-thirds of the rather limited sample of dense-core vesicles. It is conceivable that the different sizes are due to tangential sections of large vesicles, although such an artifact would more likely result in a continuous distribution of vesicle diameters rather than the essentially nonoverlapping subgroups seen thus far.

The relative number of small (400 Å), clear vesicles greatly exceeds the number of dense-core vesicles in a region such as shown in Fig. 2. There are many membrane-enclosed structures of neural, glial, and supporting-cell type, tightly packed. In contrast, regions illustrated by Figs. 3 and 4, show many fewer membrane-enclosed structures, they are less tightly packed and frequently separated by distinct basement membrane borders. There are fewer vesicles of either type in these regions although the dense-core type predominate. One might speculate that Fig. 2 illustrates one of the highly branched regions of the neuropil while Fig. 3 and 4 show relatively unbranched neuropil (Gur et al., 1972).

Pharmacological studies show that serotonin (5-HT), among a number of putative neurotransmitters which includes γ -aminobutyric acid (GABA), β -alanine, and glycine, has the most potent inhibitory action (Adolph, 1966; Behrens and Wulff, 1970). Fig. 5 shows the effect of perfused 5-HT on light-evoked spike responses, recorded from single ommatidia in thin slices of

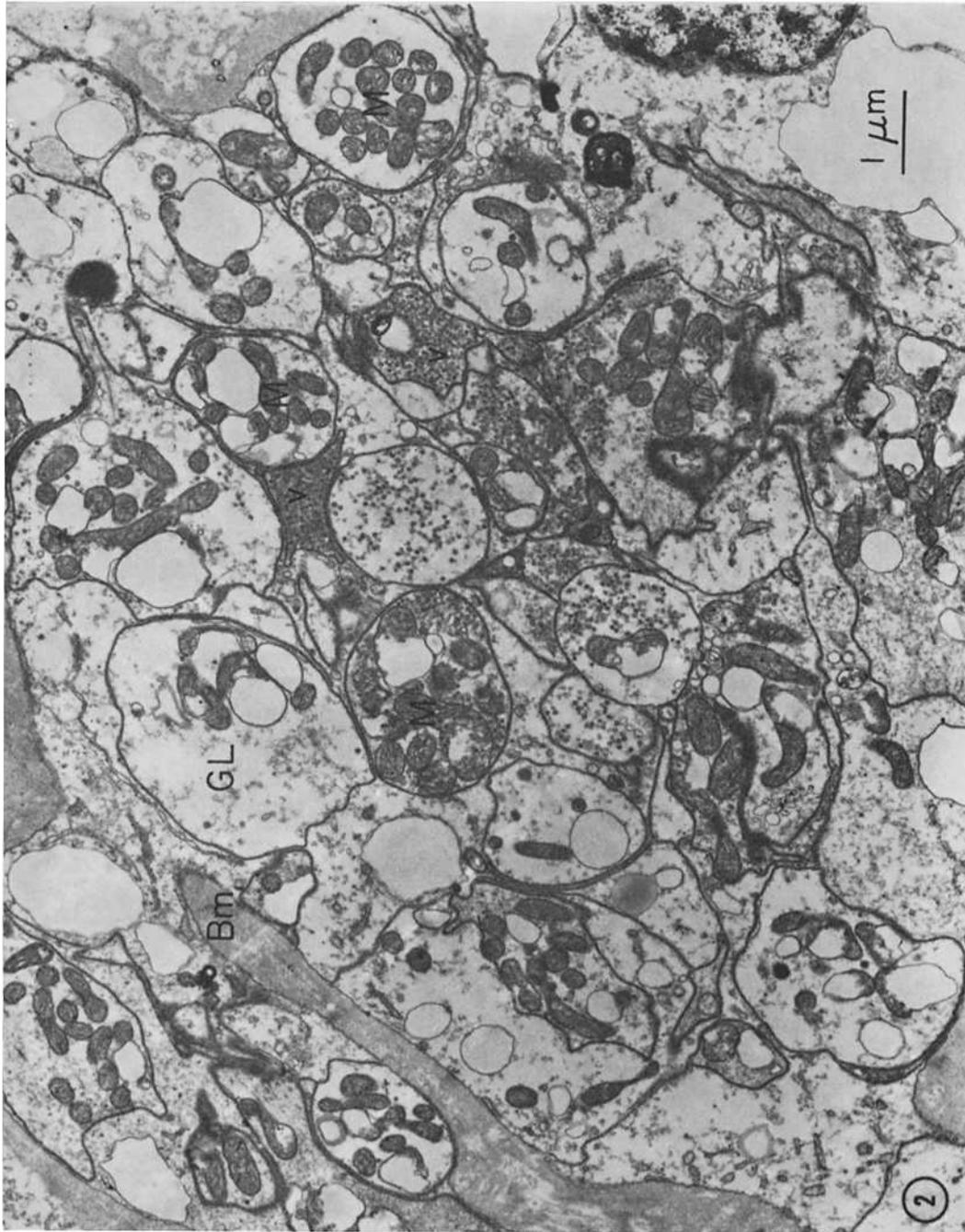


FIGURE 2. Electron micrograph of neuropil region in lateral eye. Primarily cross-sections of neuropilar elements. V, clear vesicles, ca. 400 Å diameter; M, mitochondria; GL, glial element; Bm, basement membrane.

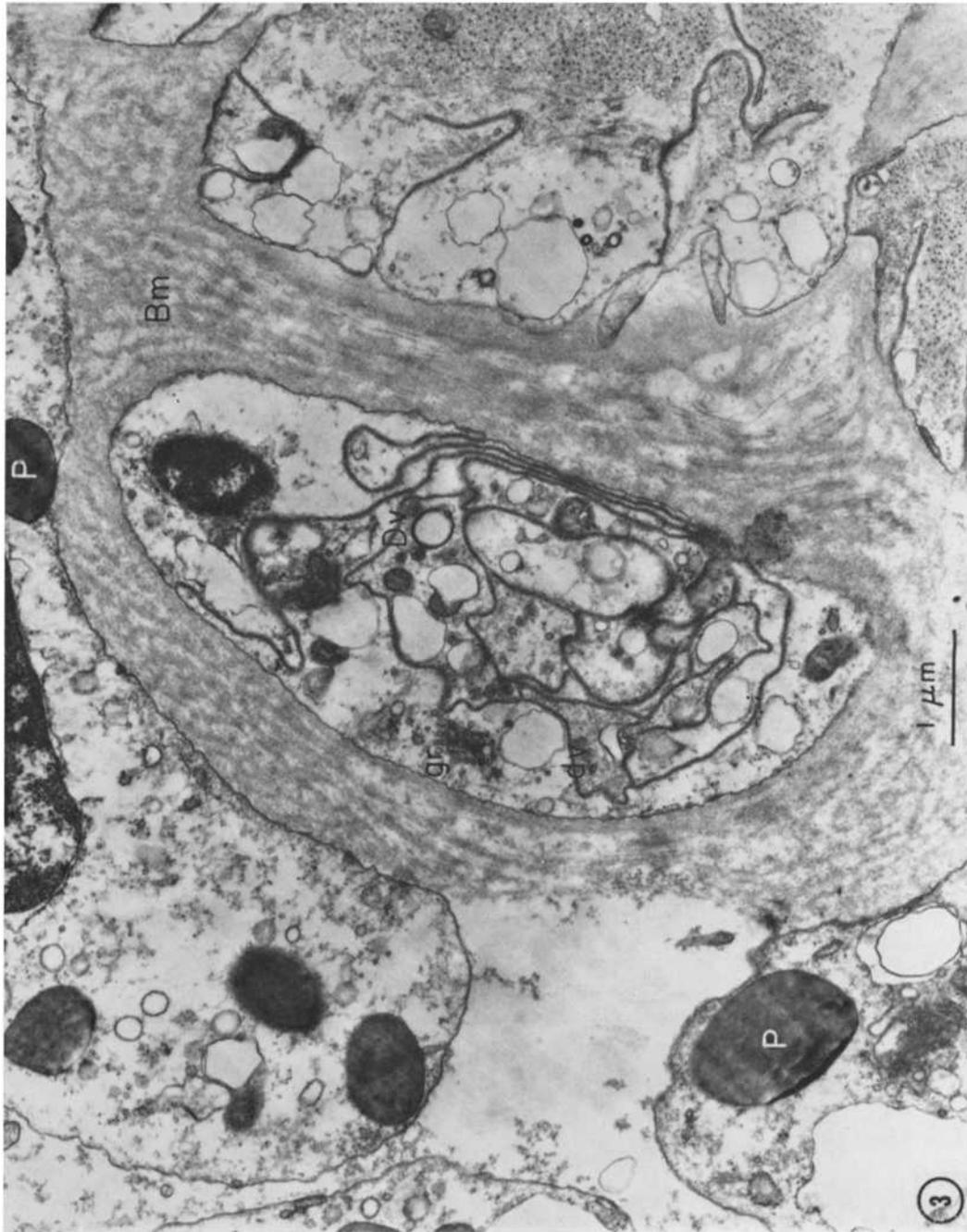


FIGURE 3. Electron micrograph of neuropil region showing Golgi region (gr), large dense-core vesicles (Dv), small dense-core vesicles (sv), and pigment granules (P).



FIGURE 4. Electron micrograph of longitudinal section of neuropil region which shows neurofibrils (Nf), small- and large-dense-core vesicles (dv, Dv) as well as glial elements (GL).

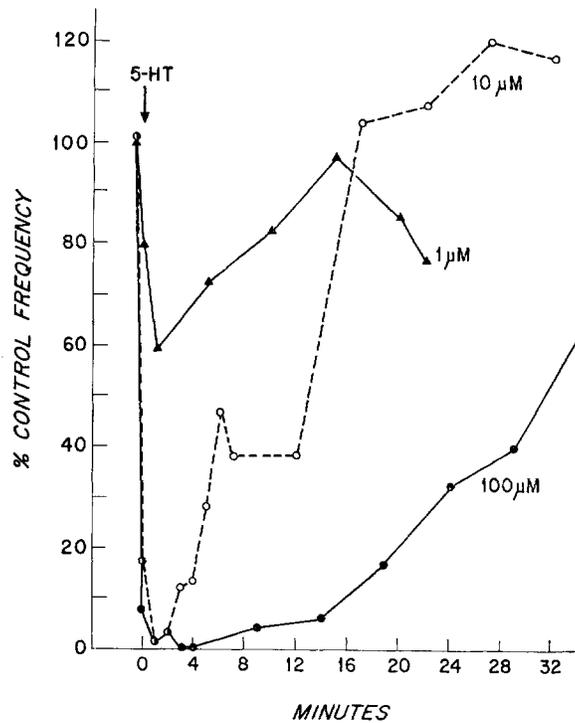


FIGURE 5. Inhibitory effect of serotonin (5-HT) at different molar concentrations on light-evoked spikes from single ommatidium in lateral eye of *Limulus*.

lateral eyes by extracellular suction electrodes. 1 ml portions of 5-HT, at the concentrations indicated on Fig. 5, were perfused into the 10 ml of ASW bathing the slice; the actual 5-HT concentrations finally acting on the receptive regions of the ommatidia were most likely further diluted. The inference is that 5-HT, even in nanomolar concentrations, is a strongly effective inhibitor of spike activity. The rate of initial action is approximately the same for the different 5-HT concentrations; the primary differences in effectiveness appear to be in absolute level of inhibition and recovery time. If one defines the inhibitory effect as the area under the inhibition-time curve, then inhibitory effect is an approximately linear function of log 5-HT concentration.

An initial, potent, inhibitory dose of 5-HT produces a long lasting desensitization to subsequent and equal 5-HT doses, as shown in Fig. 6. Directly perfused 5-HT also desensitizes the natural, light-evoked, inhibitory mechanism mediating lateral interaction between neighboring receptors. This cross-desensitization effect is illustrated in Fig. 7. Conditions similar to those used in the experiment shown earlier in Fig. 1, were used in crossed-desensitization experiments. The additional factor involved was the perfusion of the eye slice with 5-HT. The inhibitory decrement does not recover in parallel with the

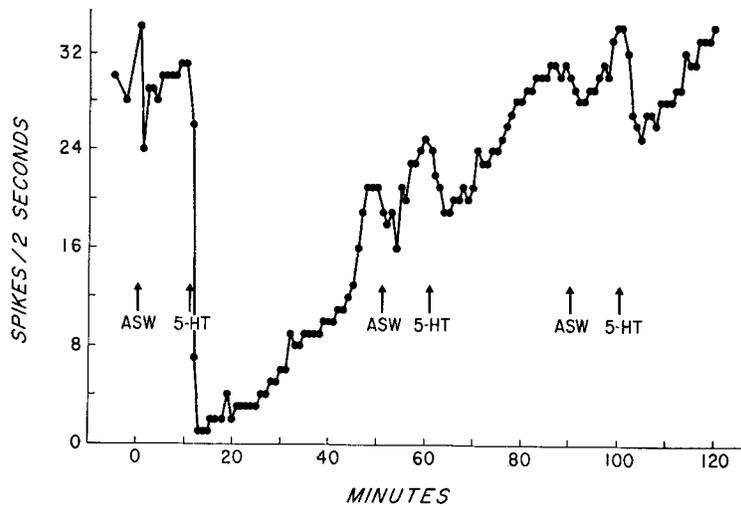


FIGURE 6. Desensitization effect of initial dose of 5-HT on subsequent 5-HT doses. ASW, artificial seawater; 5-HT, 100 μ M serotonin-creatinine sulfate in ASW.

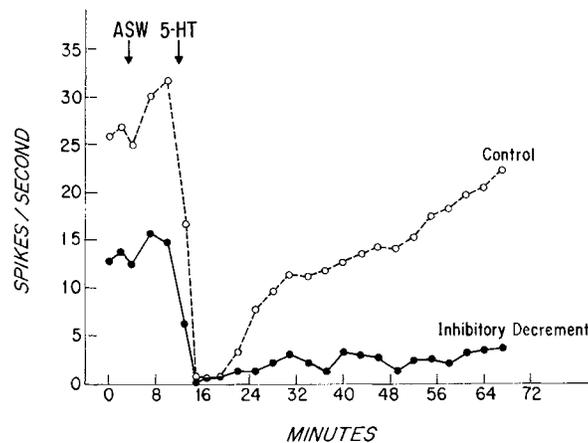


FIGURE 7. Crossed-desensitization of light-evoked lateral inhibition by perfused 5-HT. Control-test receptor response without lateral inhibition. Inhibitory decrement-difference between control test response and test response during lateral inhibition, i.e. reduction in firing rate produced by inhibition.

recovery of the control response which suggests that the perfused 5-HT specifically desensitized the inhibitory synaptic mechanism (cf. Fig. 6), in addition to directly decreasing the light-evoked response of both test and inhibitory receptors, as previously illustrated by Fig. 5. If perfused 5-HT has only a direct effect of inhibiting spike firing in ommatidia, perhaps via a non-specific action on the cell membrane, then there is no reason a priori to expect differential rates of recovery from 5-HT inhibition of inhibiting ommatidia or

inhibited ommatidium. These desensitization and crossed-desensitization effects may be related to the so-called "inhibitory fatigue" described by Lange (1965) and others, in which continued and repeated natural or antidromic inhibition becomes less effective or "desensitized" with time.

Another aspect to identifying a putative neurotransmitter in addition to the physiologically proper pharmacological action, is its presence in the eye tissue and the presence of a metabolic mechanism capable of its synthesis and inactivation. Identification of the indoleamine was accomplished by two-dimensional thin-layer chromatography (TLC). One identified component was 5-HT, as illustrated in the TLC reproduction in Fig. 8. Authentic 5-HT, which was spotted over the element to be identified, moved to the exact same location on the TLC as did the eluent spot, with the result that identification was unambiguous. A second component of the tissue extract, and an initially unsuspected one, was identified by similar technique as melatonin (MLT). A representative TLC identification of melatonin is depicted in Fig. 9. In this case, the overspotting of authentic MLT was done for only one dimension of separation. The serotonin and melatonin appear to be morphologically localized in eye tissue structures possessing densities intermediate between 1.0 and 1.7 M sucrose, after fractionation and centrifugation in a continuous

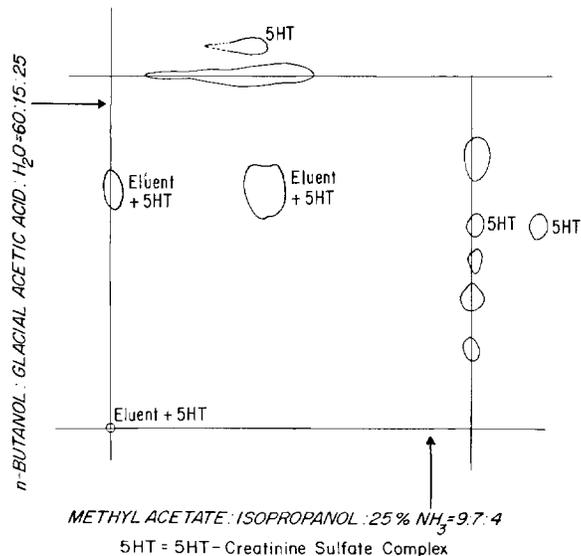


FIGURE 8. Identification of 5-HT in eye tissue extract by two-dimensional thin-layer chromatography. Rechromatograph of material from putative 5-HT location on primary one-dimension TLC separation, with overspotting of authentic 5-HT. See text for details of technique. Two right-hand and two upper tracks, mixture of seven authentic indolealkylamines (5-HT, 5-HIAA, 5-MIAA, 5-HTP, 5-MT, NAS, MLT), and authentic 5-HT only.

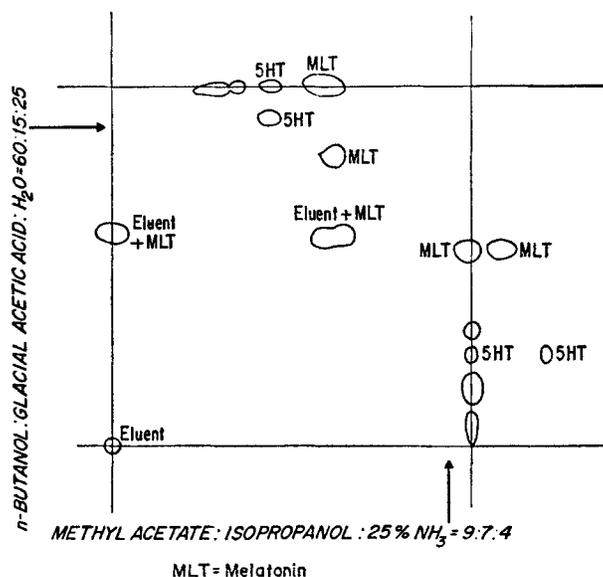


FIGURE 9. Identification of melatonin (MLT) in eye tissue extract by TLC. Rechromatograph of material from putative MLT location on primary one-dimension TLC separation, with overspotting of authentic MLT. See text for details of technique.

sucrose gradient (Fig. 10). The fine structure of the fragments in the appropriate region of the gradient has not yet been characterized.

In contrast to the consistent and reproducible qualitative results obtained by the chromatographic techniques and described above, the results of quantitative assays were variable and dependent on the specific fluorimetric procedure employed. According to the Maickel et al. (1968) procedure, nine measurements indicated an average 5-HT content of 3.5 $\mu\text{g/g}$ wet tissue, with a range of 1.1–7.7 μg . In eight determinations of 5-HT level using the Miller and Maickel (1970) method, an average of 60 $\mu\text{g/g}$ was found, with a range of 16–132 μg . This method did not detect melatonin. The Quay (1963, 1969) procedures did extract melatonin, but did not detect 5-HT. The combination technique described in the Methods section, which utilized solvent extraction, chromatographic separation/identification, and OPT-fluorimetry, gave 23 $\mu\text{g/g}$ 5-HT and 31 $\mu\text{g/g}$ MLT.

It should be noted that most of the techniques in the literature dealing with the measurement of indolealkylamines have been developed and applied to mammalian central nervous tissue rather than nerve tissue in marine invertebrates. This may have caused some of the indicated difficulties in quantitative assay. There is also the possibility that this primitive eye contains an, as yet, unidentified indoleamine which cross-reacts in one of the assay methods but not the others. In addition, these determinations were made on extracts from animals coming from a loosely controlled environmental milieu: a

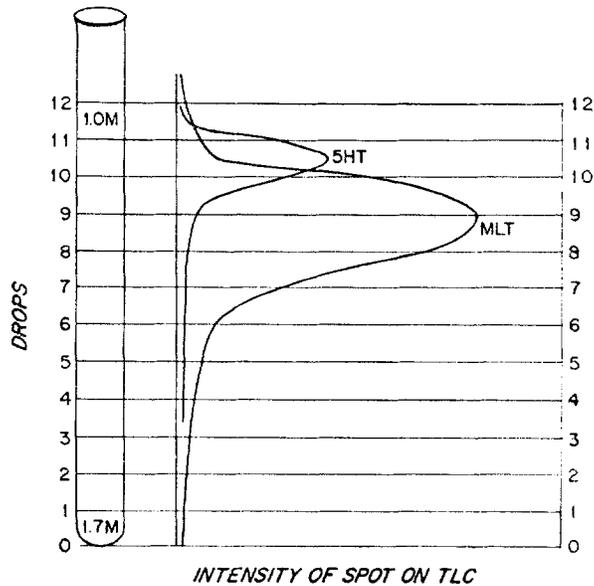


FIGURE 10. Sucrose gradient fractionation of eye tissue homogenates. Each of the 12 fractions was developed by TLC and the relative concentrations of the identified 5-HT and MLT components gauged by spot intensity. See text for details of technique.

natural light/dark cycle via building windows, and at different times of the year and thus different light/dark cycles. The tentative nature of the *quantitative* assays must therefore be emphasized.

The pharmacological effect of MLT on light-evoked spike activity is characteristically different from the effect of 5-HT and is illustrated in Fig. 11. Perfusion with $100 \mu\text{M}$ MLT is essentially ineffective on spike frequency; an unphysiologically concentrated 100 mM dose of MLT produces a moderate inhibitory effect which is quickly reversed by a seawater flush. Subsequent perfusion of the eye by 5-HT produces the typical, potent inhibition of spike activity. It is apparent that MLT is effective in inhibiting spike activity only in high concentration and that it does not desensitize the receptor to subsequent 5-HT action.

Two alternative metabolic pathways for 5-HT are shown in Fig. 12. The common pathway in mammals is oxidation, catalyzed by a monoamine oxidase (MAO) to 5-hydroxyindole acetic acid (Erspamer, 1966). In such a system, MAO inhibitors such as iproniazid and nialamid, for example, suppress the metabolism of 5-HT and increase its concentration in the tissue. We have found that a wide variety of MAO inhibitors, indicated in Table I, are essentially ineffective in either prolonging or increasing natural lateral inhibition or the effect of perfused 5-HT, or in raising the assay level of en-

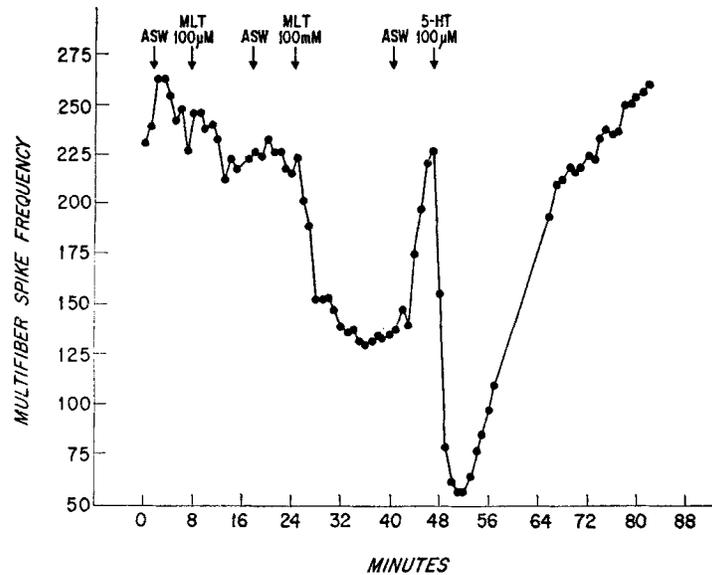


FIGURE 11. Comparative inhibitory effect of melatonin and serotonin on light-evoked spike activity. Response of several receptors simultaneously recorded by extracellular suction electrode pick-up from their optic nerve fibers. Multifiber spike frequency of the composite spike record.

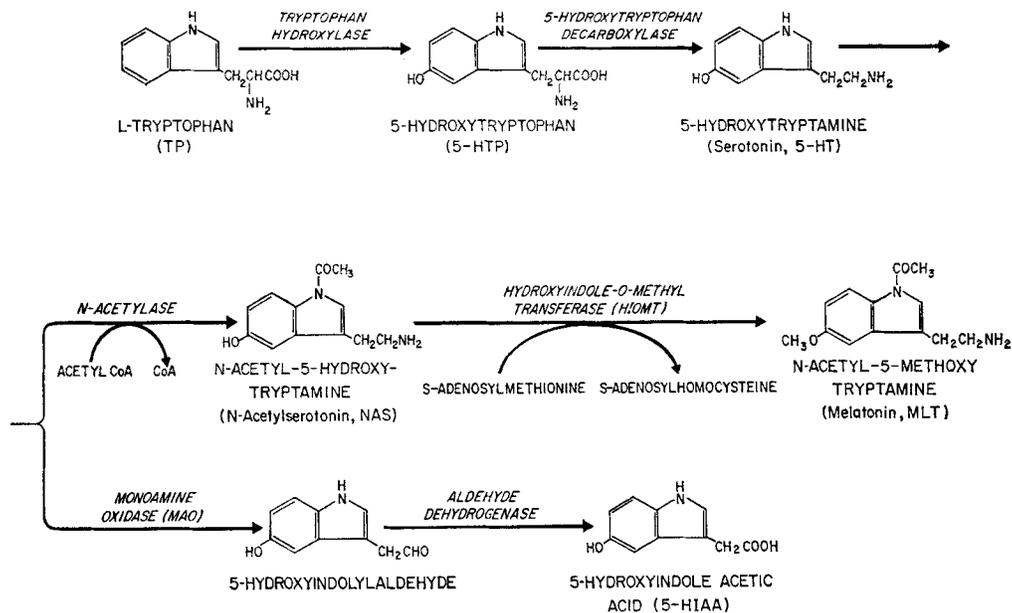


FIGURE 12. Schematic representation of possible inhibitory neurotransmitter metabolism in *Limulus* lateral eye.

TABLE I
MAOI EXPERIMENTAL CONDITIONS

Type experiment	MAOI	Concentration	Method	Period
Pharmacological	Iproniazid	0.2-2 mg/g eye tissue	In bath	Up to 2 hr during experimental run
	Pargyline	2 mg/g eye tissue (Average weight of eye slice about 50 mg)	In bath	
Neurochemical	Pargyline	2 mg/g eye tissue	Homogenate	Varied with different experiments and MAOI from 2 to 24 hr
	Pargyline	100 mg/kg whole animal	Intracardiac	
	Iproniazid	100 mg/kg whole animal	Intracardiac	
	Nialamid	10 mg/kg whole animal	Intracardiac	
	Tranlycypromine	2 mg/kg whole animal	Intracardiac	
	Dibenamine (antisymphathetic)	100 mg/kg whole animal (Average weight of whole animal about 1 kg)	Intracardiac	

dogenous 5-HT, in lateral eye tissue. Furthermore, we have not found the 5-hydroxyindole acetic acid (5-HIAA) metabolite in our TLC identification procedures, nor have we found isotope-labeled 5-HIAA in response to eye tissue incubation with 5-HT-¹⁴C.

Another pathway for 5-HT metabolism is the one associated with pineal melatonin synthesis and also shown in Fig. 12. It has *N*-acetylserotonin (NAS) as a metabolic intermediate (Erspamer, 1966). One possible interpretation of the electrophysiological action of high concentrations of MLT is that MLT is exerting its effect here not directly on the synaptic receptor, but indirectly by reversing or inhibiting a rate-limiting step in the catabolism of 5-HT by a feedback action on hydroxyindole-*o*-methyl transferase (HIOMT) or *N*-acetylase. Another possibility is that MLT stimulates 5-HT release. Administration of MLT in rats markedly increases midbrain 5-HT concentration although the mechanism of this action is not definitely known (Antón-Tay, 1971). Speculation concerning a metabolic relationship between the identified 5-HT and MLT in lateral eye tissue, cannot be resolved without further pharmacological and neurochemical studies.

DISCUSSION

A number of criteria, applicable at the cellular and subcellular levels, and useful for distinguishing synaptic transmitters in invertebrates have been proposed by Gerschenfeld (1966). Included among these criteria are the requirements that (a) the putative transmitter substance be present and stored in bound form in the neural tissue, (b) synthesizing enzymes be present, (c) inactivating or metabolizing enzymes be present, (d) the substance

can be collected after nerve stimulation, e.g. by superfusion, (*e*) receptors to the substance exist in the postsynaptic cell, and (*f*) the substance and the transmitter induce identical physiological actions. The results presented in our paper either directly satisfy a number of the criteria or are strong indications that the results of further experimentation will meet the requirements.

The ultrastructural identification of numerous vesicles, of clear and dense-core type, suggests the presence and storage sites of a neurotransmitter in the neuropil of the lateral eye. This is all that can be said based on the data presented here. There has been some work on vesicles in mammalian nervous tissue which may eventually prove relevant to *Limulus* eye. Hökfelt (1971) presents good evidence that intraneuronal monoamines in both the peripheral and central nervous system, including the pineal, of mammals are localized mainly to granular (dense-core) vesicles of small and large type. Synaptic boutons containing small (ca. 400 Å), clear vesicles show isotope labeling after administration of tritiated 5-hydroxytryptophan, a 5-HT precursor, in rat medulla (Hashimoto, 1971). Perhaps one, or both, of these types of vesicles is the storage site for a putative 5-HT transmitter in *Limulus* eye tissue.

Although MLT in the eye tissue does not in itself prove that the 5-HT is inactivated by metabolism to MLT (requirement *c*), it is a strong indication of such a mechanism. Wurtman et al. (1968) indicate that the enzymatic mechanism for producing melatonin from 5-HT, i.e. HIOMT, is found in the retina in lower vertebrate forms such as fish and amphibia. Further study of the possible intermediate step involving *N*-acetylserotonin in *Limulus* eye, including the quantitative aspects of metabolic turnover, is necessary. The crossed-desensitization between 5-HT and light-evoked lateral inhibition, and the inhibitory action of perfused 5-HT, directly satisfy the requirement (*e*) that postsynaptic receptors exist. Finally, some aspects of the identity of physiological action induced by 5-HT and the actual transmitter (*f*), such as the inhibition of spike activity, action in highly diluted concentrations, desensitization vis-à-vis inhibitory fatigue, are further indirect evidence that 5-HT, or a related indolealkylamine, may be the inhibitory transmitter in *Limulus* lateral eye.

Serotonin metabolism via a pathway similar to the one found in vertebrate pineals is suggestive of several possible hypotheses. First, the *Limulus* lateral eye has evolved into an invertebrate analog of the vertebrate pineal organ. The photoreceptor structural and electrophysiological properties of vertebrate pineals, especially those of amphibia and reptilia, may be evidence for such an analogy (Wurtman et al., 1968). The relationship between light sensitivity, pineal activity, and neurohumoral control of reproductive behavior in vertebrates is vaguely understood at present. In *Limulus*, the role of vision in affecting behavior has been difficult to discern. Much of the

animal's ordinary activity appears to be in response to chemical sensory inputs, i.e. gustatory and olfactory. However, its annual reproductive activity seems highly correlated to patterns of daylight and darkness in its environment and may reflect neurohumoral control mediated through visual input.

A second hypothetical aspect of a serotonin-melatonin system in *Limulus* involves the mechanism of light and dark adaptation, at least in this invertebrate. Melatonin is a substance known to exert humoral control of pigment migration in animals, especially fish and amphibia (Wurtman et al., 1968). One phase of dark/light adaptation in invertebrates is controlled by the dynamics of shielding pigment migration within rhabdomeric regions of reticular cells (Höglund, 1966). It is tempting to postulate a scheme in which incident light level, by controlling the level of inhibitory activity in the eye and the metabolism of the inhibitory transmitter, i.e. 5-HT, regulates the level of melatonin and ultimately controls shielding pigment mobilization and adaptation.

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REFERENCES

- ADOLPH, A. R. 1966. Excitation and inhibition of electrical activity in the *Limulus* eye by neuropharmacological agents. In *Functional Organization of the Compound Eye*. C. G. Bernhard, editor. Pergamon Press, Inc., Elmsford, N. Y. 465.
- ANTÓN-TAY, F. 1971. Pineal-brain relationships. In *The Pineal Gland*. G. E. W. Wolstenholme and J. Knight, editors. Churchill Livingstone, London. 213.
- BEHRENS, M. E., and V. J. WULFF. 1970. Neuropharmacological modification of response characteristics of sense cells in the *Limulus* lateral eye. *Vision Res.* 10:679.
- ERÄNKÖ, O., editor. 1971. Histochemistry of Nervous Transmission. *Progr. Brain Res.* 34:147.
- ERSPAMER, V., editor. 1966. 5-Hydroxytryptamine and Related Indolealkylamines. Handbook of Experimental Pharmacology, Vol. 19. Springer-Verlag New York, Inc., New York.
- FISCHER, C. A., and M. H. APRISON. 1972. Determination of nanomole levels of 5-hydroxytryptophan, 5-hydroxytryptamine, and 5-hydroxyindoleacetic acid in the same sample. *Anal. Biochem.* 46:67.
- GERSCHENFELD, H. M. 1966. Chemical transmitters in invertebrate nervous systems. In *Nervous and Hormonal Mechanisms of Integration*. S. E. B. Symposium XX. Academic Press Inc., New York. 299.
- GUR, M., R. L. PURPLE, and R. WHITEHEAD. 1972. Ultrastructure within the lateral plexus of the *Limulus* eye. *J. Gen. Physiol.* 59:285.
- HARTLINE, H. K. 1968. Visual receptors and retinal interaction. In *Les Prix Nobel en 1967*. 242. Elsevier, Amsterdam.
- HARTLINE, H. K., F. RATLIFF, and W. H. MILLER. 1961. Inhibitory interaction in the retina and its significance in vision. In *Nervous Inhibition*. E. Florey, editor. Pergamon Press, Inc., Elmsford, N. Y. 241.
- HASHIMOTO, P. H. 1971. Morphological studies on the mechanism of adrenergic transmission in the central nervous system. II. Minute vesicles. *Progr. Brain Res.* 34:207.

- HÖGLUND, G. 1966. Pigment migration and reticular sensitivity. *In* Functional Organization of the Compound Eye. C. G. Bernhard, editor. Pergamon Press, Inc., Elmsford, N. Y. 77.
- HÖKFELT, T. 1971. Ultrastructural localization of intraneuronal monoamines—some aspects of methodology. *Prog. Brain Res.* 34:213.
- LANGE, G. D. 1965. Dynamics of inhibitory interactions in the eye of *Limulus*: experimental and theoretical studies. Ph.D. thesis, The Rockefeller University, New York.
- MAICKEL, R. P., R. H. COX, J. SAILLANT, and F. P. MILLER. 1968. A method for the determination of serotonin and epinephrine in discrete areas of rat brain, *Int. J. Neuropharmacol.* 7:275.
- MILLER, F. P., and R. P. MAICKEL. 1970. Fluorometric determination of indole derivatives, *Life Sci. Part I Physiol. Pharmacol.* 9:747.
- PURPLE, R. L., and F. A. DODGE. 1965. Interaction of excitation and inhibition in the eccentric cell in the eye of *Limulus*. *Cold Spring Harbor Symp. Quant. Biol.* 30:529.
- QUAY, W. B. 1963. Differential extractions for the spectrofluorometric measurement of diverse 5-hydroxy- and 5-methoxyindoles, *Anal. Biochem.* 5:51.
- QUAY, W. B. 1969. Fluorometry of 5-hydroxytryptamine (serotonin). *Fluorescence News.* 4(5):5.
- RATLIFF, F., H. K. HARTLINE, and D. LANGE. 1966. The dynamics of lateral inhibition in the compound eye of *Limulus*. I. *In* Functional Organization of the Compound Eye. C. G. Bernhard, editor. Pergamon Press, Inc., Elmsford, N. Y. 399.
- WOLBARSH, M. L., and S. S. YEANDLE. 1967. Visual processes in the *Limulus* eye. *Annu. Rev. Physiol.* 29:513.
- WURTMAN, R. J., J. AXELROD, and D. E. KELLY. 1968. The Pineal. Academic Press Inc. New York.