Neurotransmitter synthesis in *Limulus* ventral nerve photoreceptors[1]

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Summary. Radiochemical precursor compounds for neurotransmitters were incubated with *Limulus* ventral nerve photoreceptor preparations. Octopamine was preferentially synthesized by a photoreceptor rich fraction of the nerve, acetylcholine was made by a photoreceptor poor fraction, and γ-aminobutyric acid was made about equally well in both fractions. The possibility that the ventral nerve photoreceptor cells serve a neurosecretory function in the adult *Limulus* is discussed.

The horseshoe crab *Limulus polyphemus* is an ancient type of chelicerate arthropod with 3 pairs of photoreceptors. There are 2 large, prominent lateral eyes, 2 small median eyes and a pair of nerves containing photosensitive cells underneath the cuticle of the animal's ventral surface. These ventral photoreceptors are considered rudimentary larval eyes. The photo-sensitive cells are large (up to 200 µm × 60 µm) and scattered along a nerve that runs inside an arterial blood vessel. The large size and relatively simple organization[3] of the cells of *Limulus* ventral eyes have allowed them to be used as a model for arthropod photoreceptors and in studies of visual transduction[4-12]. However, relatively little information is available on the biological function of these photoreceptors in the adult animal.

In this communication we show that octopamine, a possible neurosecretory substance in invertebrates[13,14], is synthesized by the photoreceptor rich portion of the ventral nerve in adult *Limulus*. Acetylcholine (ACh) is preferentially synthesized by the part of the nerve trunk without receptors, and γ-aminobutyric acid (GABA) appears to be synthesized equally well in both portions of the ventral nerve.

Experimental procedures. The 2 ventral nerves were dissected from a series of animals. After removal of the surrounding blood vessels, nerves were incubated individually in saline (462 mmoles NaCl, 16 mmoles KCl, 26 mmoles $CaCl_2$, 8 mmoles $MgCl_2$, 11 mmoles glucose, 10 mmoles Tris, 10 mmoles maleic acid adjusted to pH 7.4 with NaOH) with 1 of the following ^3H labelled neurotransmitter precursor compounds: choline (69.5 Ci/mmole) for acetylcholine; glutamate (47 Ci/mmole) for GABA; tyrosine (51 Ci/mmole) for noradrenaline, dopamine and octopamine; tyramine (10.6 Ci/mmole) for octopamine; tryptophan (17.9 Ci/mmole) for serotonin. All radioisotopes were purchased from New England Nuclear, Boston, Mass., USA. The final concentration of radioactive precursor in the incubation medium was 10 µCi/ml. Nerves were incubated in the dark for 12–17 h at 15 °C. After incubation, each nerve trunk was divided in the light into 2 fractions: the 1st, the photoreceptor-rich fraction (P), contains most of the photoreceptor and at most 1/4 of the total amount of nerve tissue; the 2nd fraction, the photoreceptor-poor fraction (N), contains very few photoreceptor cells and about 3/4 of the mass of the tissue. Each sample was extracted with 15% 1 N formic acid in aceton and the soluble fraction was subjected to high voltage electrophoresis, as described by Hildebrand et al.[15], to separate the labelled precursor from its products.

In some experiments a 2nd dimension using paper chromatography was used to verify further the identity of the radioactive products. In these cases, the solvents used were secbutanol:pyridine:acetic acid:H_2O, 300:5:20:50; and methylethyl ketone:propionic acid:H_2O, 200:56:55.

The amount of radioactivity that co-migrated with a particular putative neurotransmitter was quantified, using liquid scintillation spectroscopy. Electropherograms were cut into 1-cm strips; each strip was put into a scintillation vial and incubated in 0.1 N HCl for 30–60 min to elute radioactivity from the paper. Liquid scintillation fluid (Aquasol from New England Nuclear, Boston, Mass.) was added to the vials and samples were counted in a Packard liquid scintillation counter. The efficiency of counting was in the range of 15–20%.

Results. Synthesis of possible neurotransmitters from radioactive precursors. The results of screening experiments designed to search for transmitter products formed from ^3H-labelled precursors compounds are summarized in the table. With choline as a precursor compound, there was no ^3H-ACh formed in the photoreceptor-rich P-fraction of nerve trunks, but ACh was made in the photoreceptor-poor N-fraction. With glutamate as a precursor compound, on the other hand, both the P-fraction and the N-fraction produced radioactive GABA. There were about twice as many counts of GABA formed in the N-fraction as in the P-fraction; this difference could easily be accounted for by the larger amount of tissue present in the N-fraction. When tryptophan was used as a precursor compound, we saw no evidence of serotonin production, even though we could

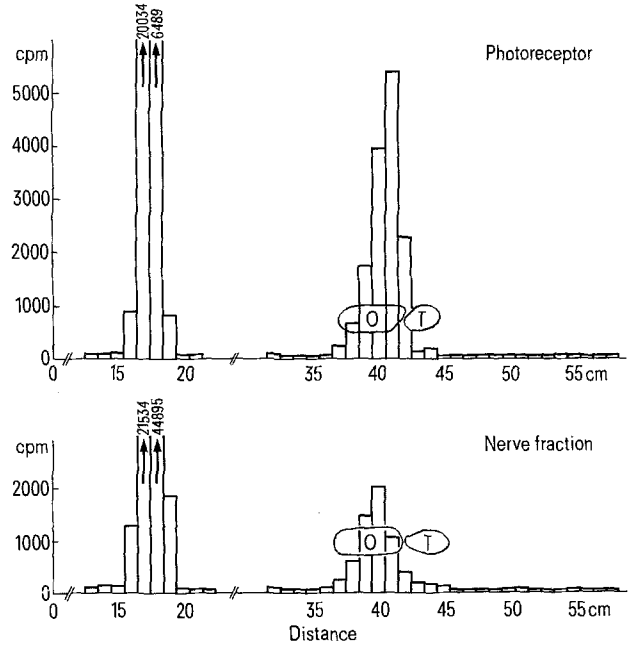

Fig. 1. Radiochromatogram of high voltage electropherogram showing peak of radioactivity that co-chromatographs with octopamine in *Limulus* ventral nerve. The positions of standard compounds: O, octopamine and T, tyramine are indicated. In the photoreceptor-rich P-fraction (upper curve) the radioactivity of the octopamine area is ca. 3-fold greater than in the photoreceptor-poor N-fraction. There is also a slow running peak of radioactivity in both electropherograms that is unidentified and likely to be a tyramine metabolite. Abscissa is distance from origin O; ordinate-radioactivity eluated from 1-cm wide strips of chromatogram.

demonstrate that both fractions of the nerve trunk took up large amounts of precursor. Finally, incubation with tyrosine did not generate any norepinephrine or dopamine in either the P- or the N-fractions, but a radioactive substance was found that ran slightly faster than dopamine, to the octopamine region of the electropherogram. This was studied in greater detail by using tyramine and tyrosine as precursor compounds, and further verifying the identity of the products by paper chromatography after electrophoresis.

A typical result with tyramine as precursor compound is shown in figure 1. The photoreceptor-rich P-fraction formed large amounts of a radioactive product migrating to the octopamine region of the electropherogram (figure 1, upper trace). Little or no radioactive tyramine remained in the nerve, but a very large peak of an unidentified metabolite was also found. In the photoreceptor-poor N-fraction, the results were similar but only $\frac{1}{3}$ as many counts were seen in the octopamine peak, despite the fact that this fraction of the nerve has the greater volume (figure 1, lower trace). This experiment was repeated several times with similar results. In 1 experiment with tyrosine and 1 with tyramine as precursor, the acid extracts of the tissue were divided into several fractions that were subjected to 2-dimensional separations. In all cases, all of the radioactivity migrating to the octopamine region of an electropherogram, ran to the same locations as an octopamine standard in the paper chromatographic separations.

Endogenous octopamine. Using a modification of the assay described by Molinoff et al.[16], ventral nerves were assayed immediately after dissection for endogenous octopamine without previous incubation with precursors. We could not detect octopamine in any of the preparations tested. In 1 experiment, the tissue we used contained about 70 photoreceptor cells and still we found less than 0.5 pmoles of octopamine.

Neutral red staining of cells. In the leech and the lobster, neutral red has been used as a vital stain for amine-containing cells[13,17,18]. We tested the same stain (0.5 mg/ml neutral red in saline) in the *Limulus* ventral nerve. Within 2 min after application of neutral red, photoreceptor cells were conspicuously stained with the dye (figure 2).

Discussion and conclusions. The results presented in this communication suggest that the *Limulus* ventral nerve photoreceptor can preferentially synthesize octopamine. Since octopamine seems to function as a neurosecretory substance in other invertebrates[13,14], the results raise the possibility that these photoreceptor cells might play a neurosecretory role in adult *Limulus* as well. The presence of large darkly stained granules of 0.05 µm–0.4 µm diameter within these neurons[19], and their staining with neutral red, support this suggestion. On the other hand, our failure to detect endogenous octopamine is of concern. It raises the possibility that octopamine is not the substance actually contained within and released by these cells (possibly the unidentified radioactive metabolite is, see figure 1) or that much of the amine is released by the cells during our dissection of the tissue in the light.

An additional concern is the reported close association between ventral eye photoreceptor cells and efferent fibres containing dense core granules[3,20].

In our crude dissections of photoreceptor cells from the ventral nerve, we may not have cleaned the cell bodies of all adhering efferent fibres. Therefore the possibility exists that some of the octopamine synthesis we observe is coming from the efferent fibres and not the photoreceptor cells themselves. Clearly further study is required to resolve these problems.

The very active synthesis and accumulations of an unidentified metabolite during incubations of ventral nerve photoreceptor cells with tyramine is clearly of interest. Studies designed to characterize the metabolite are underway. So far it is clear that the *Limulus* metabolite does not have the same mobility in pH 1.9 electrophores as either of the amine metabolites formed in lobster[21], nor does it co-migrate with synephrine, the n-methyl derivative of octopamine[22].

If the cells do function as neurosecretory neurons, their location on a nerve trunk within a blood vessel might be ideal. They might function by releasing octopamine or a related substance directly into the haemolymph in response to light. In this regard, the recent findings of Barlow and

Indication of transmitter synthesis from radiochemical precursors by the high voltage electrophoretic characterization of metabolic products

n	^{3}H-labelled precursor	Transmitter	Found in fraction P	N
2	Choline	Acetyl choline	(−)	+
2	Glutamate	GABA	+	++
1	Tryptophan	Serotonin	(−)	(−)
2	Tyrosine	Norepinephrine	(−)	(−)
		Dopamine	(−)	(−)
		Octopamine	+	+
3	Tyramine	Octopamine	++	+

(−), No significant peak of radioactivity found for the transmitter in question; +, significant peak of radioactivity; n, number of individual nerves incubated.

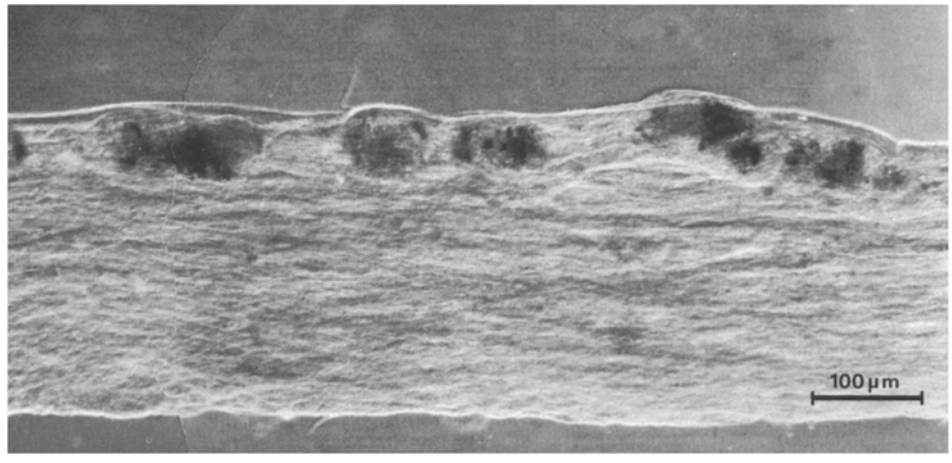

Fig. 2. Light-micrograph of *Limulus* ventral-nerve several photoreceptor cells stained with neutral red. Staining was performed in 0.1 mg/ml solutions of neutral red in saline for 5 min (Original W. Schröder, unpublished).

Chamberlain[23-25] are particularly relevant. These authors have reported that an endogenous clock mechanism seems to be involved in regulating both the sensitivity of the visual system and the extent of locomotor activity of *Limulus*. A photosensitive neurosecretory cell, releasing a neurohormone capable of modulating neuronal activity, might be the simplest way to generate such diurnal activities.

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Daily variation in the eye's 5-HT stores

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Summary. Eyes from mice have been assayed for 5-HT content at various times during the day. 5-HT levels are highest midway in the light period and lowest during the dark period. In general this daily variation conforms with other published reports for variation of 5-HT stores in brain and pineal.

Daily variation in 5-hydroxytryptamine (5-HT) stores in brain and pineal are well documented[1-3]. Another neuronal structure with well established 5-HT stores is the eye[4,5]. We have wondered if 5-HT varies upon a daily basis in the eye and if so whether that variation conforms in its pattern to brain and pineal.

Methods. Albino male mice of the CFW strain (Carworth Farms, New City, N.Y.) between 19 and 21 weeks of age were used. They were kept upon a lighting regimen of 10 h light and 14 h dark. The mice were killed by cervical dislocation at 4 different times, 2.5, 5.5 and 8.5 h into the light period and 4 h into the dark period. Eye pairs were removed, cleaned of extraneous tissues and weighed. Eye pairs were then homogenized by hand and assayed for 5-HT using the method of Quay[6]. An Aminco-Bowmen spectrophotofluorometer was used and blanks and standards were prepared and carried through the whole procedure and read interspersed with the tissue samples.

Results. As seen in the table, there are daily variations in 5-HT level in the eye. The lowest levels are during the dark period and the highest during the mid-light period, with the difference being on the order of 2.5 fold.

5-HT levels in the eye of the mouse expressed as ng 5-HT per mg wet weight eye tissue. Times of measurements are shown as h after the onset of light or darkness

	Time measured			
	Light +2.5 h	Light +5.5 h	Light +8.5 h	Dark +4 h
n	8	8	8	6
$\bar{X}$	0.48	0.99	0.65	0.38
SEM	0.048	0.084	0.044	0.026

n, number of measurements; $\bar{X}$, mean value; SEM, standard error of the mean.

Discussion. 5-HT has been known in the eye for some time, as have the enzymes associated with its metabolism[4,5,7]. 5-HT synthesis from exogenous precursor has been described and can be modified by alterations in the lighting regimen[8], however, there are differences in eye indoleamine biochemistry when compared to other structures[9,10]. Light related normal variations in the 5-HT stores of brain and pineal have been reported a number of times, and in general the levels are low during the dark period and high during the light period, although the patterns are not all duplicates of each other[1-3]. Since, as we have found here, the eye shows a similar kind of variation the same caution concerning time of day that is used in other indoleamine studies must also be exercised in investigations of the eye.

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