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9. Membrane preparations (130 μl) were first incubated with 5 μl of ethanol (total binding) or 5 μl of unlabeled excess drug suspended in ethanol (nonspecific binding) for 1 hour at 4°C, and then incubated with 5 μl of labeled drug for an additional 2 hours at 4°C. Nonspecific binding to the nanomolar receptor and micromolar receptor was studied by using unlabeled excess diazepam at final concentrations of 1.0 μ*M* and 2.5 m*M*, respectively. The wide range of [^{3}H]diazepam concentrations was obtained by either diluting the stock solution of [^{3}H]diazepam with ethanol or concentrating it with unlabeled diazepam.
10. For each binding condition in a single experiment, three identical assay mixtures were prepared. After the final incubation, three samples were taken from each assay mixture and placed on three separate Whatman GF/B glass fiber filters, thus providing triplicate values for each of the three identical mixtures. Filters were washed twice with 5 ml of buffer and each wash was conducted in less than 1.5 seconds. Each filter was placed in 10 ml of Aquasol and left at 4°C for at least 12 hours prior to liquid scintillation counting. The total quantity of [^{3}H]diazepam bound at each concentration was less than 10 percent of the initial amount of free [^{3}H]diazepam added to the assay mixture.
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Efferent Fibers to *Limulus* Eyes Synthesize and Release Octopamine

Abstract. *Octopamine synthesized in vitro from tyramine by* Limulus *lateral and ventral eyes was located by light microscopic and electron microscopic autoradiography in efferent fibers which innervate ventral photoreceptors and lateral eye ommatidia. Newly synthesized octopamine was released from efferent fibers in response to depolarization in high concentrations of potassium. We propose that octopamine is a neurotransmitter of efferent fibers that may modulate basic retinal processes such as photoreceptor sensitivity, photomechanical movements, and photoreceptive membrane turnover.*

Efferent innervation to retinas seems to be a feature common to the visual systems of many species (*1*) including that of the horseshoe crab *Limulus polyphemus* (*2*). In the *Limulus* visual system, a preparation that has been central to our understanding of basic mechanisms of vision (*3*), efferent fibers project from a circadian clock in the brain (*4*) and innervate all three types of eyes:

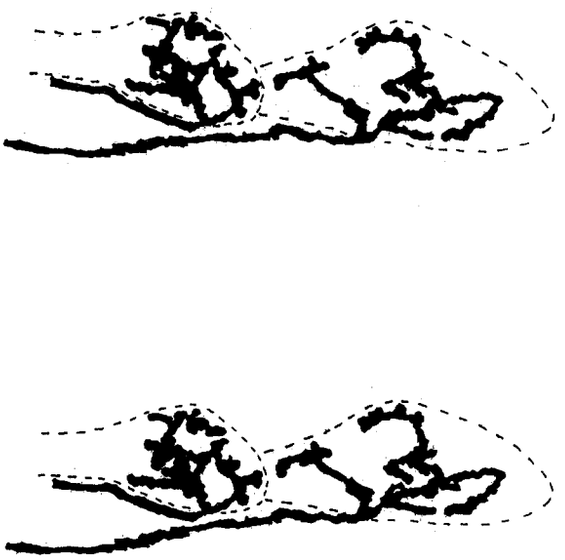

Fig. 1. Stereograph of a three-dimensional reconstruction of octopamine-synthesizing efferent fibers of the *Limulus* ventral eye. Regions of intense label seen in individual sections are part of a branched efferent innervation of photoreceptor cell bodies. Ventral eyes were incubated overnight in $6 \times 10^{-8}M$ [^{3}H]tyramine (New England Nuclear; 9.14 Ci/mmole), and then fixed (*10*), dehydrated, and embedded in Araldite 502. Thick sections (1 μm) mounted on glass slides were dipped in Kodak NTB-2 Nuclear Trak Emulsion, exposed for 7 to 14 days at 4°C, and developed with Dektol. Sections were stained with toluidine blue. Serial light autoradiographs were digitized and displayed by computer. Dotted lines show outlines of two photoreceptor cell bodies.

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lateral, median, and ventral (*2*). The functions of retinal efferents and the identity of their neurotransmitters are unknown in most species. In *Limulus*, however, complex effects of efferent input to lateral eyes have been characterized. These include the control or modulation of photoreceptor sensitivity and noise (*5*), photomechanical movements of photoreceptors and pigment cells (*6*), and photoreceptive membrane turnover (*7*). Previous studies from this laboratory (*8*) showed that significant levels of the biogenic amine octopamine are present in *Limulus* eyes where it is also synthesized from tyrosine and tyramine. Here we report results of light microscopic and electron microscopic autoradiography which show that octopamine is synthesized and stored within efferent fibers of both ventral and lateral eyes. We also demonstrate that newly synthesized octopamine is released from these fibers in response to depolarization in high concentrations of potassium. We suggest that octopamine is an efferent fiber neurotransmitter in the *Limulus* visual system where it may modulate many primary mechanisms in vision.

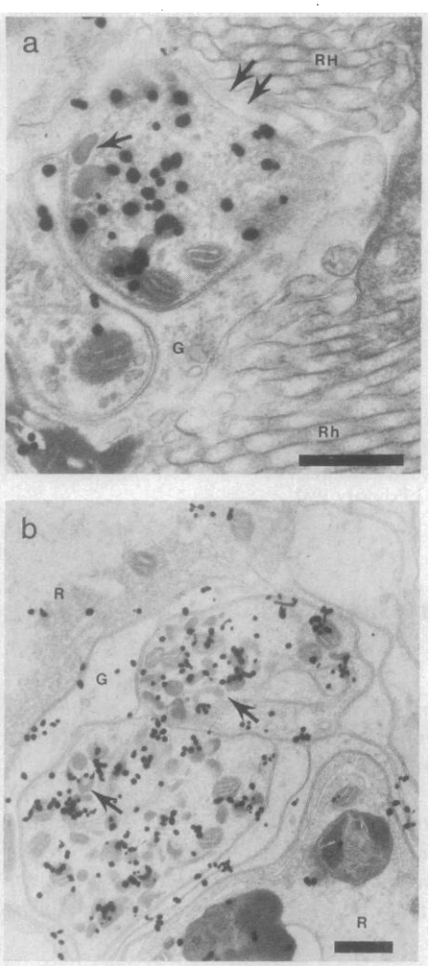

Fig. 2. (a) Electron microscopic autoradiography of a labeled efferent fiber containing dense granules (single arrow) inside a ventral photoreceptor cell body. The efferent is only partially surrounded by an unlabeled glial sheath (*G*) and there is direct contact (double arrow) between efferent and rhabdom (*RH*). Silver-gold thin sections were carbon-coated, dipped in Ilford L-4 fine grain emulsion, exposed for 4 weeks at 4°C, and developed in Agfa-Gevaert physical developer (*16*). Scale bar, 0.5 μm. (b) Electron microscopic autoradiography of two labeled efferent fibers in the lateral eye surrounded by an unlabeled glial sheath (*G*). Pleomorphic dense granules are seen in both fibers (arrows). *R*, retinular cell. Direct contact between efferents and rhabdoms have not yet been encountered; however, labeled efferent fibers were observed 1 to 3 μm from the ends of the rays of rhabdoms and in partitions between retinular cells. Lateral eyes were incubated with $1.3 \times 10^{-6} M$ [^{3}H]tyramine. Scale bar, 0.5 μm.

Octopamine was the major radioactive product found in *Limulus* ventral eyes that were incubated overnight with $6 \times 10^{-8} M$ [^{3}H]tyramine (*9*). Little or no [^{3}H]tyramine precursor remained in the preparations. Between 50 and 60 percent of the radioactivity in fractions rich in photoreceptor cell bodies was incorporated into octopamine and at least 20 percent incorporated into octopamine metabolites (*8*). Thus, approximately 70 to 80 percent of the total radioactivity closely associated with ventral photoreceptor cell bodies was in newly synthesized octopamine and octopamine metabolites. To locate newly synthesized octopamine, we incubated the tissues in [^{3}H]tyramine, fixed them in glutaraldehyde (*10*), and processed them for light micrographic and electron micrographic autoradiography.

In light micrographic autoradiographs of ventral eyes, punctate areas showing intense labeling were concentrated near the periphery of photoreceptor cell bodies and over their interior in the region of photosensitive rhabdoms. Occasional strings of heavy label were also scattered between large, diffusely labeled photoreceptor cell axons. A three-dimensional reconstruction of 16 serial (1-μm sections) autoradiographs (Fig. 1) suggested that regions of intense label were associated with fibers that penetrate photoreceptor cell bodies then branch extensively. Electron micrographic autoradiographic analysis of ventral eye preparations from four different animals confirmed that silver grains were clustered exclusively over fibers containing large, dense granules that are characteristic of efferent fibers (*2*) (Fig. 2a). Greater than 95 percent of the efferent profiles observed were intensely labeled, whereas the glia that surround efferents and photoreceptor cell cytoplasm were unlabeled.

A detailed electron micrographic examination of the distribution of octopamine-containing efferent fibers within ventral photoreceptor cell bodies revealed a striking association between efferents and rhabdoms. Of the 59 labeled efferent profiles observed, all were located near rhabdoms (*11*) and approximately one-third were in direct apposition to rhabdoms at interruptions in the glial sheath (Fig. 2a, double arrow). Efferent fibers do not make classical synapses (*2*). Rather, they appear to be neurosecretory with the photosensitive membrane as an apparent target.

The distribution of radioactivity in the *Limulus* lateral eye after incubation with [^{3}H]tyramine was also examined by electron microscopic autoradiography (Fig. 2b). Although the mixture of radioactive substances found in lateral eyes was more complex than in ventral eyes (30 to 40 percent of the total radioactivity was incorporated in octopamine, 50 to 60 percent in a mixture of tyramine and octopamine metabolites, and 5 to 10 per-

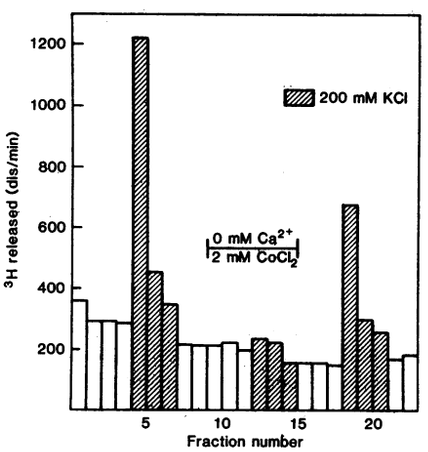

Fig. 3. Potassium-stimulated release of newly synthesized octopamine from fractions of ventral eyes rich in photorecepter cell bodies. The preparations were incubated overnight in the dark at 12°C in saline containing $6 \times 10^{-8} M$ [^{3}H]tyramine. All subsequent procedures were done in the light and at room temperature. The preparation was rinsed with large volumes of normal saline until the rate of efflux of radioactivity stabilized. Then the preparation was incubated with 200 μl of saline and the entire volume was collected and replaced at 5-minute intervals. The total radioactivity released during each 5-minute incubation period is plotted (*dis/min*). In some release experiments, 150-μl portions of fractions collected before, during, and after depolarization were desalted with 20 volumes 0.25 percent formic acid in acetone so that the radioactive substances released could be identified by high-voltage electrophoresis. (Open bars) Saline containing 16 m*M* KCl; (hatched bars) saline containing 200 m*M* KCl. Saline containing 0 m*M* Ca^{2+} plus 2 m*M* $CoCl_2$ was added as indicated by the bar. The NaCl and $MgCl_2$ concentrations were also changed to exactly compensate osmotically for the changes in KCl and $CaCl_2$.

cent remained in tyramine), areas showing intense labeling were similarly localized within dense granule-containing efferent fibers. Sections parallel to the cornea were surveyed for efferent profiles within ommatidia near rhabdoms. All efferent profiles in this region were labeled. We conclude that efferent fibers in both ventral and lateral eyes synthesize and store octopamine.

The ability of efferent fibers to release newly synthesized octopamine was tested in experiments such as that illustrated in Fig. 3. Ventral eyes were incubated overnight in [^{3}H]tyramine, rinsed with normal saline, and then depolarized with 200 m*M* KCl in the presence of saline containing either normal Ca^{2+} or zero Ca^{2+} plus 2 m*M* $CoCl_2$. Depolarization dramatically increased the rate of efflux of radioactivity from ventral eyes, and approximately 60 percent of this radioactivity was identified by high-voltage electrophoresis as octopamine. No octopamine was found in the rinses with normal saline. Octopamine release was reversibly blocked in saline containing zero Ca^{2+} plus $CoCl_2$; thus release is dependent on extracellular calcium. Potassium depolarization also stimulated octopamine release in the lateral eye.

Many observations now support the idea that octopamine, a known neurotransmitter of invertebrates (*12*), is a neurotransmitter in *Limulus* retinal efferents. We have shown that *Limulus* eyes contain and synthesize octopamine, that new synthesized octopamine is located exclusively in efferent fibers in both ventral and lateral eyes, and that octopamine is released from efferent fibers with depolarization. Octopamine-stimulated increases in adenosine 3′,5′-monophosphate have been measured in both ventral photoreceptor cells (*13*) and in lateral eyes (*14*), suggesting that octopamine receptors are present in both types of eyes. Low concentrations of octopamine injected into lateral eyes also mimic electrophysiological and anatomical effects of natural efferent activity (*15*).

Functions of efferent innervation to *Limulus* ventral eyes are not known. However, the frequent direct contacts between octopamine-containing efferent fibers and ventral photoreceptor rhabdoms suggest that octopamine may be involved in regulating sensitivity, rhabdom turnover, or other metabolic functions of the photoreceptor cell. The identification of octopamine as a likely neurotransmitter in efferents to lateral eyes opens up new possibilities for investigations of the mechanisms of known efferent effects. A thorough understanding of the effects of octopamine and efferent innervation in the *Limulus* visual system, a preparation that is already well characterized, may provide a basis for investigating efferent function in other species and give new information on basic sensory mechanisms.

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Naloxone Reverses Neonatal Depression Caused by Fetal Asphyxia

Abstract. *Pregnant near-term rabbits were given an intravenous dose of saline or the opiate antagonist naloxone and then asphyxiated. The fetuses were delivered by cesarean section and evaluated for respiration, color, muscle tone, response to stimulation, and general activity at 1, 3, 5, 10, 15, and 30 minutes of age. The naloxone-treated pups had significantly better scores during the first 15 minutes after birth than the saline-treated pups. Naloxone did not adversely affect the scores of nonasphyxiated pups. These data suggest that endogenous opiates worsen the neonatal depression caused by intrauterine asphyxia and that this effect can be reversed by naloxone.*

Endogenous opiates appear to play a role in the ventilatory response of neonates to asphyxia or hypoxia (*1–3*). In the neonatal rabbit the primary apnea induced by asphyxia is nearly abolished by naloxone, an opiate antagonist (*1*). The characteristic neonatal response to hypoxia—respiratory stimulation followed by respiratory depression—is also abolished by naloxone (*3*). However, naloxone does not affect the ventilatory response to hypercapnia or hypoxemia in the adult rabbit or human (*3, 4*). Thus, endogenous opiates appear to influence the ventilatory response to chemical stimuli only early in life.

Many of the characteristics of the neonate that has suffered intrauterine asphyxia mimic the effects of exogenously administered opiates: ventilatory depression, hypotonia or flaccidity, and depressed reflex responses and spontaneous activity (*5*). We therefore postulated that endogenous opiates might be implicated in the neonatal depression seen following intrauterine asphyxia.

On day 30 of pregnancy (full term is 31 days), rabbits were injected intravenously with naloxone (1 mg/kg) or saline (2.5 ml). Five minutes later, each doe was placed in a 2-liter chamber into which flowed nitrogen with 7 percent CO_2 at the rate of 20 liters per minute, rapidly replacing the air. Within 3 or 4 minutes the animal died. Eight minutes after being placed in the chamber, the doe was removed from the chamber and her uterus was exposed by laparotomy. At 10

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