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ENDOGENOUS SEROTONIN AND SEROTONIN-LIKE IMMUNOREACTIVITY IN THE CENTRAL NERVOUS SYSTEM OF *LIMULUS POLYPHEMUS*

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ABSTRACT

Central nervous system (CNS) ganglia of*Limulus polyphemus* were studied for the presence of serotonin. When homogenates of the ganglia were extracted with trichloroacetic acid, dansylated and chromatographed, the results indicated the presence of endogens serotonin. To localize the endogenous serotonin, individual ganglia were studied using techniques of immunocytochemistry. Fixed sections revealed extensive anti-serotonin-like reactivity in the circumesophageal ganglia and in the chain of abdominal ganglia. In the circumesophageal ganglia a loose band of immunoreactive fibers was observed to have an orientation similar to the ganglia. Small clusters of cell bodies were observed also. Immunoreactive fibers were localized in the abdominal ganglia and their connectives. In each abdominal ganglion, an extensive plexus of immunoreactive fibers, as well as a small number of anti-serotonin staining cells, were readily observed. These results support the contention that serotonin is a putative CNS neurotransmitter in *Limulus*.

INTRODUCTION

It is becoming increasingly clear that the biogenic amines play significant roles in regulating neuronal activity in invertebrates. In the nervous tissues of the arthropod *Limulus polyphemus*, the catecholamines dopamine (DA) and norepinephrine (NE) have been found and they have been shown to have specific physiological effects (Augustine, et al., 1982; O'Connor, et al., 1982; Battelle and Evans, 1984). The indolamine serotonin (5-hydroxytryptamine) has been demonstrated in *Limulus* nerve cord and heart (Welsh and Moorhead, 1960). It has been shown to decrease *Limulus* heart rate and to closely mimic the action of the cardioinhibitory nerves (Pax and Sanborn, 1967). Additionally, it has been shown to be inhibitory to neurons in the abdominal ganglia (Walker and James, 1978; 1980). The abdominal ganglia have been shown to exert regulatory effects on cardiac function (Pax, 1968; Von Burg and Corning, 1971; Hoegler, 1980), and when serotonin is applied directly to the heart or when the abdominal ganglia are bathed with it, heart rate is decreased. Recently Chamberlain, et al. (1986) demonstrated serotonin-like immunoreactive neurons and processes in discrete neuronal groups in *Limulus* brain. Thus, a neurotransmitter role for serotonin in this animal is implicated. To further support the contention that serotonin serves a neurotransmitter role in *Limulus* we have conducted this study. Our objectives were to analyze central nervous system (CNS) tissues for the presence of endogenous serotonin and to survey these tissues for serotonin immunoreactive neurons and processes as revealed by immunocytochemistry.

EXPERIMENTAL PROCEDURES

Adult horseshoe crabs (*Limulus polyphemus*) were obtained from either the Marine Biological Laboratory, Woods Hole, MA or Gulf Specimen Co., Panacea, FL, and kept in the laboratory in containers of damp Spanish moss at an ambient temperature of approximately 5° -10° C.

Determination of Endogenous Serotonin

The brain, circumesophageal ganglia, and chain of abdominal ganglia were removed from cold anesthetized animals, weighed and homogenized in cold perchloric acid. Amines were extracted with alumina and reacted with a dansyl reagent. Then the dansyl derivatives were subjected to unidirectional chromatography in benzene:cyclohexane: methanol (44:5:1). Standards, which included serotonin, norepinephrine and octopamine, were extracted, dansylated, and chromatographed for comparison. All chromatographs were viewed under UV light. This procedure is essentially that described by Diliberto and DiStefano (1969).

Immunocytochemistry

Serotonin localization was studied using immuno–cytochemical techniques as described by Beltz and Kravitz

(1983). Briefly, CNS tissues were dissected out and fized overnight in cold 4% paraformaldehyde in 0.15M phosphate buffer (pH 7.4), washed in phosphate buffer containing 0.3% Triton X-100 (PBT), infiltrated with sucrose and sectioned 15-25μm in a cryostat. Sections were washed for an hour in PBT and incubated 2 hours at room temperature or overnight at 5°-10° C in anti-serotonin antiserum (from rabbits; Immuno Nuclear Corp.) diluted 1:500 or 1:1000 in PBT which contained 10% normal goat serum. Control sections were incubated in either PBT or antiserum preabsorbed with serotonin-BSA (bovine serum albumin) conjugate (Immuno Nuclear Corp.). After washing for an hour (six changes), all sections were again incubated in goat IgG produced against rabbit IgG under the same conditions as before (Boehringer Mannheim Company; diluted 1:25 or 1:50 in PBT containing 10% normal goat serum). Goat antibodies were conjugated with either rhodamine or horseradish peroxidase (HRP). After washing in buffer, rhodamine slides were coverslipped using 90% glycerol in 0.1M carbonate buffer (pH 7.4) and viewed using an Aus Jena epi-fluorescence microscope equipped with a 570-6/0 filter cube (green excitation; emission >570 nm). Slides treated with HRP were washed in phosphate buffer, developed in 3'3-diaminobenzidine tetrachloride (DAB), dehydrated in an ethanol series, cleared in xylene, and mounted with permount. Sections were viewed using bright field optics.

In a few experiments whole mount preparations of *Limulus* abdominal ganglia were studied. The chain of ganglia were dissected out as described above and pinned out in a sylgard dish. The enclosing sheath was removed and the chain of ganglia was fixed as above. After being washed in PBT for an hour, ganglionic chains were incubated in 2 ml microbeakers containing anti-serotonin antiserum (1:1000) overnight at 5°-10° C. Subsequently the ganglionic chains were washed for an hour in PBT and incubated at room temperature for 1.5 hours in goat anti-rabbit IgG labeled with rhodamine, washed for an hour, and then mounted with glycerol on depression slides. Preparations were viewed under the fluorescence microscope.

RESULTS

Determination of Endogenous Serotonin

Chromatography of dansylated compounds extracted from *Limulus* nervous tissue homogenate revealed at least two compounds. One exhibited an Rf value equal to that of standard serotonin (Figure 1).

The second compound appeared to be another amine derivative rather than a metabolite of serotonin. The finding of serotonin in *Limulus* nervous tissue agrees with published reports (Welsh and Moorhead, 1960). Immunocytochemistry In order to determine if endogenous serotonin is localized in neuronal elements in horseshoe crab nervous tissue, experiments were conducted using

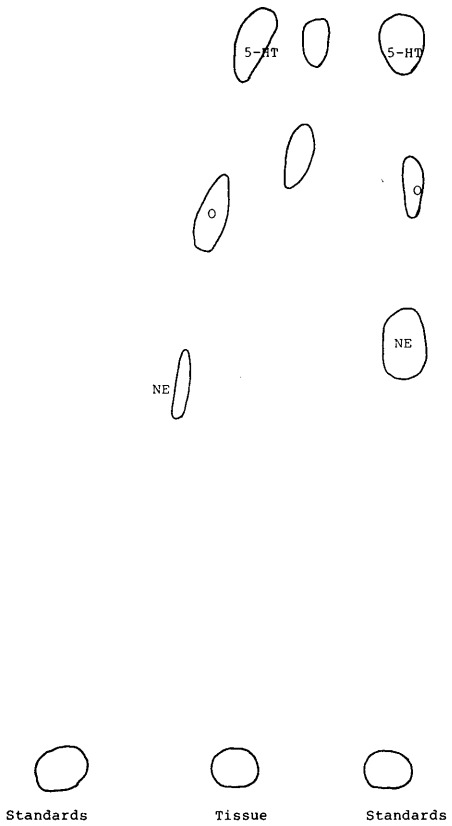


Figure 1. Chromatograph of tissue and standards extracts. The relative positions of the standards were determined in preliminary experiments.

Code: 5-HT Serotonin
O Octopamine
NE Norepinephrine

immunocytochemical techniques. Cryostat sections treated with anti-serotonin antisera revealed immuno–reactivity in the brain and circumesophageal ganglia. Small and large immunoreactive nerve fibers were observed in the ganglionic mass. These fibers formed a somewhat diffuse band which extended from the posterior part of the brain (ventral median ganglia), through the circumesophageal ganglia, and into the ventral nerve cord (Figure 2a).

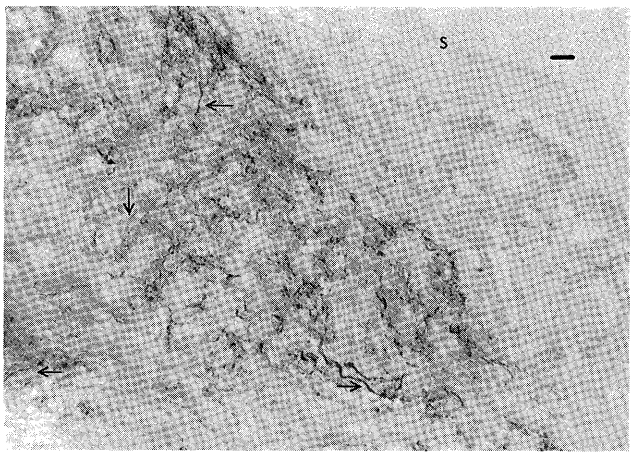
Of the immunoreactive fibers observed, few, if any, appeared to leave the CNS via the nerves to the legs given off by the circumesophageal ganglia. In observations made on cryostat sections, fibers were the dominant structures observed although cell bodies with their attached processes were seen (Figure 2b).

These cells occurred singly or in clusters and ranged in size from 15 to 30 μm. Areas of serotonin-like immuno–reactivity were noted in the chain of abdominal ganglia (Figure 3a).

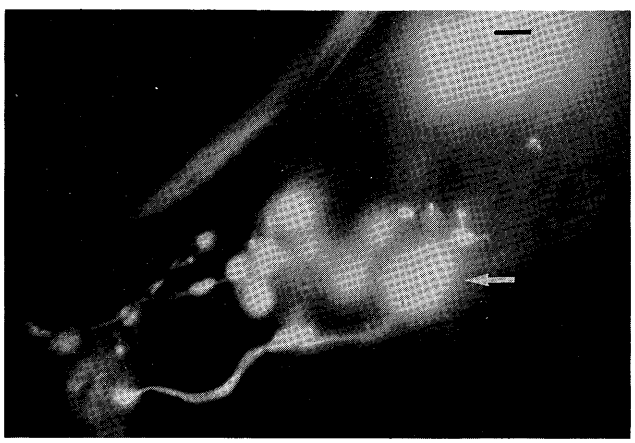
Immunoreactive fibers were seen in the ventral nerve cord (Figure 3b) and appeared to run its entire length.

Intense fluorescence was observed laterally in the

Figure 2. Photomicrographs of tissue sections from circumesophageal ganglia treated with anti-serotonin antibodies.



A. Immunoreactivity was localized in small and large fibers (arrows) and a plexus of fine fibers and varicosities which form a loose band near the medial enclosing sheath (S). Immunoreactivity was visualized using HRP (Bar=15μm).

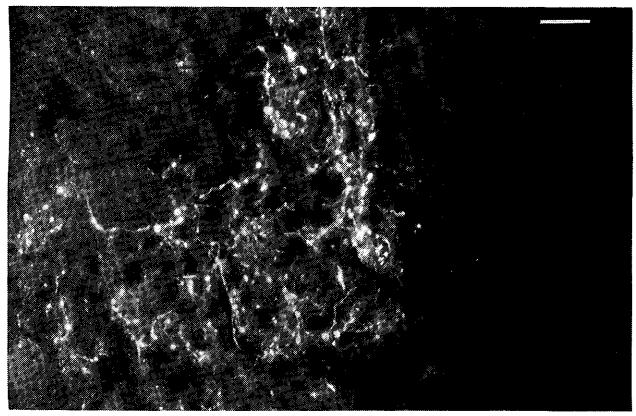


B. A cluster of immunoreactive cells visualized with rhoda mine. Note stained cell with process and varicosities (arrow). (Bar=10μm).

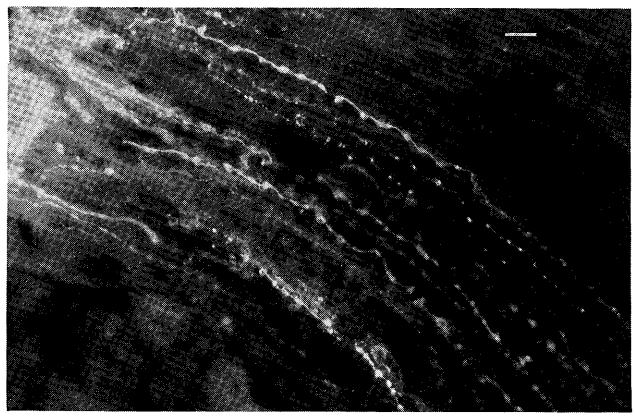
posterior quarters of each ganglion (bilateral observation; Figure 3a). Fibers entering the ganglion anteriorly tended to spread toward the areas of intense fluorescence. Upon close examination of these areas, large numbers of small immunoreactive fibers were observed. Additionally, these bilateral areas of intense fluorescence were connected posteriorly by a band of fibers which were oriented 90 degrees to the long axis of the ganglion. Cross sections of abdominal ganglia suggested the intense areas to be in the neuropil. In some of these cross sections, serotonin-like immunoreactive cells that were seen appeared singly (Figure 3c) and in some horizontal sections, cells appeared in small clusters (Figure 3d).

Most cells observed were in the size range 30-40μm. Although a large number of sections were studied, cells

Figure 3. Photomicrographs of tissue sections of abdominal ganglia treated with anti-serotonin antibodies.



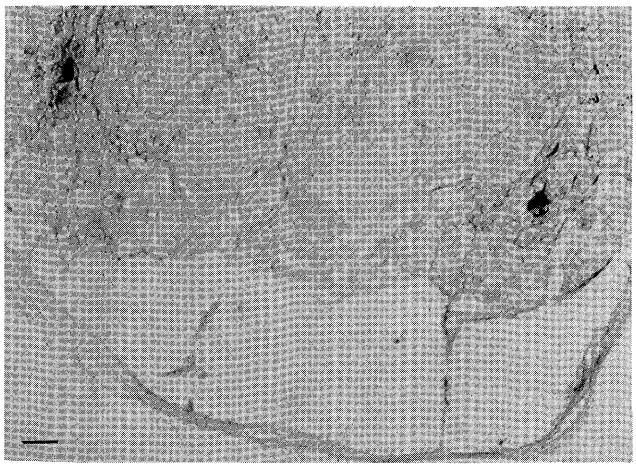
A. Immunoreactivity in the abdominal ganglia was visualized using rhodamine. The area shown is the posterior quarter of a representative ganglion (9th). Bar=30μm.



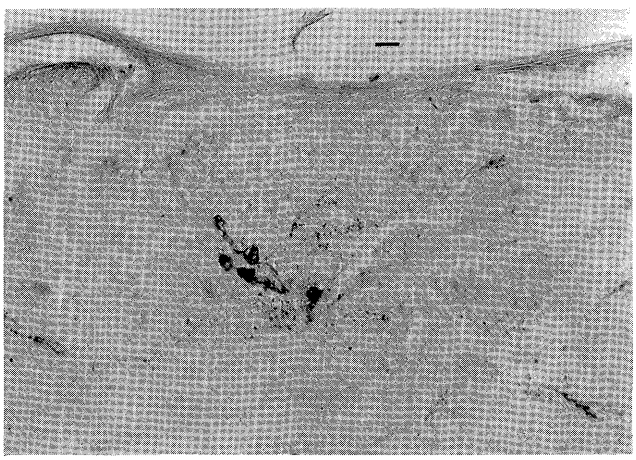
B. Immunoreactivity in the varicosities on the stained fibers. Bar=7μm.

were observed in only a few. While a serial reconstruction study was not conducted, it was clear that the number of immunreactive cells is small. Additional measures are needed to gain sufficient information on the immunoreactive cells in the abdominal ganglia. In some experiments, whole mount preparations were used. Observations from these preparations of the chain of abdominal ganglia revealed bilateral clusters of serotonin-like immunoreactive cells in each ganglion. Clusters consisted of six cells which were located anteriorly in the ganglia (figure 4).

The processes from these immunoreactive cells cound be followed for only short distances and appeared to join the band of fibers passing through the ganglion. A few immunoreactive fibers were observed in the ganglionic roots. However, the relationship of these ganglionic root fibers to the immunoreactive cells in the ganglion was not established. Controls showed low level background staining but no specific staining. Controls were incubated in PBT or in antiserum which had been preabsorbed with the



C. Cross section of ganglion showing a pair of immunoreactive cells located ventrally in the ganglion. Immunoreactivity was visualized using HRP. Bar=10μm.



D. Horizontal section of ganglion showing a cluster of cells (arrows). Bar=50μm.

serotonin-BSA conjugate. No difference was noted between PBT controls and preabsorption controls.

DISCUSSION

The occurrence of serotonin in *Limulus* nervous tissue has been established (Welsh and Moorhead, 1960) and we confirm that finding here. This biogenic amine has been proposed to be a neurotransmitter in *Limulus* (Pax and Sanborn, 1967) and our primary objective was to shed additional light on its localization in *Limulus* nervous tissue. We have extracted compounds from homogenates of *Limulus* central ganglia and chromatographic analysis indicated one of the compounds to be serotonin (Figure 1). This finding agrees with published reports and thus, was not surprising. In other experiments we confirmed this finding using techniques of high pressure liquid chromatography with electrochemical detection (HPLC-EC; Kerr, et al., 1986). These experiments suggested significant levels of endogenous serotonin in the central ganglia of *Limulus*.

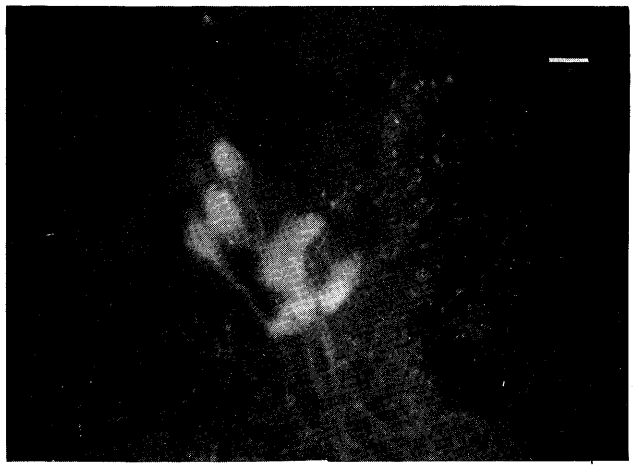


Figure 4. Whole mount preparation of abdominal ganglion. Note bilateral cluster of six stained cells. Bar=20μm.

Next, experiments were conducted using techniques of immunocytochemistry in an attempt to localize endogenous serotonin in discrete neural elements. Our study revealed significant serotonin-like immunoreactivity in *Limulus* central ganglia. The pattern of immunoreactivity observed in the brain is similar to that reported by Chamberlain, et al. (1986), who studied serotonin in *Limulus* brain, lateral eyes, and optic nerves, and found significant levels of endogenous serotonin only in the brain. Serotonin-like immunoreactivity was observed in sparsely scattered cells and fibers in discrete groups of neurons in the brain. No immunoreactive fibers were noted in the corpra pedunculata, lateral eyes, or optic nerves. Our findings agree quite well with this published report of serotonin-like immunoreactivity in Limulus brain. In addition, our observations revealed that a band of immunoreactive fibers extend from the posterior portion of the brain, through the circumesophageal ganglia and into the connectives of the ventral nerve cord. Prominent tracts of immunoreactive fibers were not observed to run into the large nerves to the legs. This observation does not preclude small fibers in those nerves. However, this observation may suggest that serotonergic neurons and fibers are more central than peripheral in function. Prominent tracts of fibers extend into and through the chain of abdominal ganglia (Figure 3b) and may be largely fibers of interneurons. However, immunoreactive fibers do appear to run the length of the ventral nerve cord and to traverse the ganglia by way of the ganglionic commissure connecting the bilateral areas of immunoreactivity. Large cells such as those seen in the nerve cord of the lobster (Beltz and Kravitz, 1983) or in Aplysia ganglia (Goldstein, et al., 1984; Ono and McCaman, 1984; Kistler, et al., 1985) were not observed. The absence of large immunoreactive cells (>100μm) is consistent with the observations of Walker and James (1978) who indicated a rather dispersed pattern of a few large cells in the abdominal ganglia. We noted, however, that a small number of immunoreactive cells (30-40μm) occurs in

Limulus abdominal ganglia and the cells appeared singly (Figure 3c) and in small clusters (Figure 3d) when viewed in cryostat sections. It was noted that immunoreactive cells were not seen in many sections, suggesting that the number of cells per ganglion is small and/or cell bodies are not as readily stained as their processes. Further observations with whole mount preparations revealed a pair of bilateral clusters of cells in each abdominal ganglion. Clusters contained six cells each, thus confirming the earlier observation.

The results reported here confirm the presence of endogenous serotonin in *Limulus* central ganglia and suggest its pattern of localization. A band of serotonin-like immunoreactive fibers extended the length of the ventral nerve cord and through the circular circumesophageal ganglia. Extensive plexuses of immunoreactive fibers extended from the band of fibers in the circumesophageal ganglia and in each abdominal ganglion. Immunoreactive cells associated with this system were observed also. These results lend strong support to the contention that serotonin serves as a central neurotransmitter in the *Limulus* nervous system. Additional studies are needed to demonstrate the relationship of presumptive serotonergic neural elements to peripheral structures. The function and organization of the clusters of serotonin containing neurons also need to be studied, as well as the connections between serotonergic neurons in the circumesophageal ganglia and those in the abdominal ganglia. Investigations in these areas are being pursued in our laboratory.

Acknowledgments

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