Serotonin Depletion by 5,7-Dihydroxytryptamine Alters Deutocerebral Development in the Lobster, *Homarus americanus*

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ABSTRACT: The olfactory and accessory lobes constitute prominent histological structures within the larval and mature lobster deutocerebrum, and both are associated with a dense innervation from paired serotonergic nerve cells, the dorsal giant neurons (DGNs). During development, the cell bodies of the DGNs are the first central somata to express serotonin (5-HT), and the onset of their 5-HT immunoreactivity coincides with the beginning of accessory lobe formation. In contrast, the olfactory lobe anlagen emerge much earlier and grow in the apparent absence of serotonin. The role of serotonergic input for the development of these brain structures was investigated in lobster embryos after serotonin had been depleted pharmacologically with the neurotoxin 5,7-dihydroxytryptamine. A ~90% reduction of serotonin was confirmed in eggs using high-performance liquid chromatography with electrochemical detection. Morphometric analyses suggested that serotonin depletion dramatically slowed the growth of olfactory and accessory lobes, although glomeruli differentiated at the normal time in both areas. The toxin exhibited a high degree of specificity for serotonergic neurons and associated target regions, and serotonin depletion persisted for at least 2 months following treatment. The goal of future experiments is to determine which of the cell types that innervate the olfactory and accessory lobes are affected by toxin treatment, thereby resulting in the retarded growth of these areas. © 1997 John Wiley & Sons, Inc. *J Neurobiol* 33: 357–373, 1997

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INTRODUCTION

Serotonergic neurons are among the earliest neurons to develop in the central nervous system of animals including mammals (Lauder and Bloom, 1974; Fujimiya et al., 1986), crustaceans (Beltz et al., 1990; Sandeman and Sandeman, 1990), insects (Taghert and Goodman, 1984; Kent et al., 1987), annelids (Glover et al., 1987), and mollusks (Goldberg and Kater, 1989). Their precocious development led to the idea that serotonin (5-HT) plays specific roles in the developing nervous system (Lauder, 1991). Indeed, experimental studies repeatedly link serotonin with crucial developmental changes in a variety of organisms. For example, serotonin has trophic effects on neuronal proliferation (Lauder et al., 1981) and synaptogenesis (Lipton and Kater, 1989). *In vitro* experiments have shown that serotonin can inhibit (Haydon et al.,

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1984) or facilitate (Chubakov et al., 1986) synapse formation, thereby influencing neuronal connectivity. *In vivo*, perturbation of serotonin levels in invertebrate organisms during embryogenesis has profound effects on the development of specific identified neurons (Goldberg and Kater, 1989). Serotonin can stimulate (Mercer et al., 1996) or inhibit (Haydon et al., 1984) growth of specific target neurons during defined developmental periods. It also appears to be important in the maintenance of synapses in mature vertebrate organisms (Okado et al., 1993; Chen et al., 1994). In combination, these experiments suggest that serotonin plays a prominent role in the development and maintenance of neuronal architecture and connectivity.

These roles, combined with diverse modulatory actions in the mature nervous systems of vertebrate and invertebrate organisms, strongly implicate a dual function for serotonin: as a neurotransmitter or modulator on the one hand, and as a developmental architect on the other. In both of these roles, serotonin's actions may be mediated by its ability to alter intracellular calcium or activate second-messenger systems (Lipton and Kater, 1989). There also is evidence that serotonin may antagonize activation of the *N*-methyl-D-aspartate (NMDA) receptor (Holohean et al., 1992), an element which appears to be involved in the activity-dependent refinement of axonal projections (Constantine-Paton et al., 1990; Roskams et al., 1994).

In lobsters, as in vertebrates, the complete serotonergic system is composed of relatively few neurons that ramify extensively throughout the nervous system (Beltz and Kravitz, 1983). In the crustacean midbrain, or deutocerebrum, the serotonergic dorsal giant neurons (DGNs) branch ipsilaterally throughout olfactory and accessory lobes and provide the predominant serotonergic input to these regions (Fig. 1) (Sandeman and Sandeman, 1987, 1994; Beltz et al., 1990; Sandeman et al., 1995). Functionally, the olfactory lobes receive first-order sensory input from chemosensory receptor cells located on the antennulae (or first pair of antennae). The accessory lobes, which neither receive primary sensory input nor send projections to the periphery, are thought to be higher-order processing centers (Sandeman et al., 1995).

The serotonergic system of neurons forms relatively early during lobster embryonic development (Beltz et al., 1990). Serotonin is first detectable immunocytochemically by 10% embryonic development (E10%) in fibers in the protocerebrum, and fiber labeling follows a roughly anterior–posterior pattern of emergence in the brain and ventral nerve cord. Neuronal somata contain detectable immunoreactivity by E40–45%, and the full complement of ~100 serotonergic neurons found in the adult (Beltz and Kravitz, 1983) are immunocytochemically labeled by E50%. Therefore, by mid-embryonic life, the basic pattern of serotonergic cells and fibers has been laid down. The early establishment of serotonergic neurons is in contrast to the appearance of octopamine, proctolin, and dopamine in the lobster nervous system (Beltz et al., 1990, 1992; Cournil et al., 1995; Schneider et al., 1996). Each of these substances appears in neurons relatively late during embryonic development, and the acquisition of the complete set of adult-staining neurons is a protracted event, continuing throughout larval and into juvenile life.

The DGNs are among the first somata to label immunocytochemically for serotonin (by ~E40%) (Beltz et al., 1992). These cells and their projections are stable throughout larval, juvenile, and adult life. Coincident with the appearance of serotonin in these cell bodies and their fibers, the accessory lobes emerge as small concentrations of neuropil just medial to the olfactory lobes. The coordinated appearance of serotonin in the DGNs and the first growth of the accessory lobes has led to the hypothesis that serotonin might be involved in the assembly and/or growth of these neuropil regions. To test whether serotonin promotes growth and differentiation in its target regions, the morphological development of deutocerebral structures was investigated in lobster embryos in which serotonin was depleted pharmacologically by treatment with 5,7-dihydroxytryptamine (5,7-DHT). 5,7-DHT, a neurotoxic analogue of serotonin, has been used extensively in both vertebrates and invertebrates to reduce serotonin levels and thereby study the physiology, morphology, and function of serotonergic neurons (for review, see Cook and Orchard, 1993).

The stages chosen for 5,7-DHT injection were selected based on previous studies that examined the development of the brain in embryonic and larval lobsters (Beltz et al., 1990; Helluy et al., 1995, 1996). In those studies, the timing of serotonin appearance in various brain regions was established (Beltz et al., 1990), the first appearance and enlargement of the olfactory and accessory lobes were described (Helluy et al., 1995), and developmental increases in the numbers and sizes of glomeruli in these regions were documented (Helluy et al., 1996). From these data, significant periods and events in the development of the brain were identified. For instance, the first appearance of olfactory lobe glomeruli and the emergence of the accessory lobes occur by E40–45%, just after the first immunocytochemical detection of serotonin in the deuto-

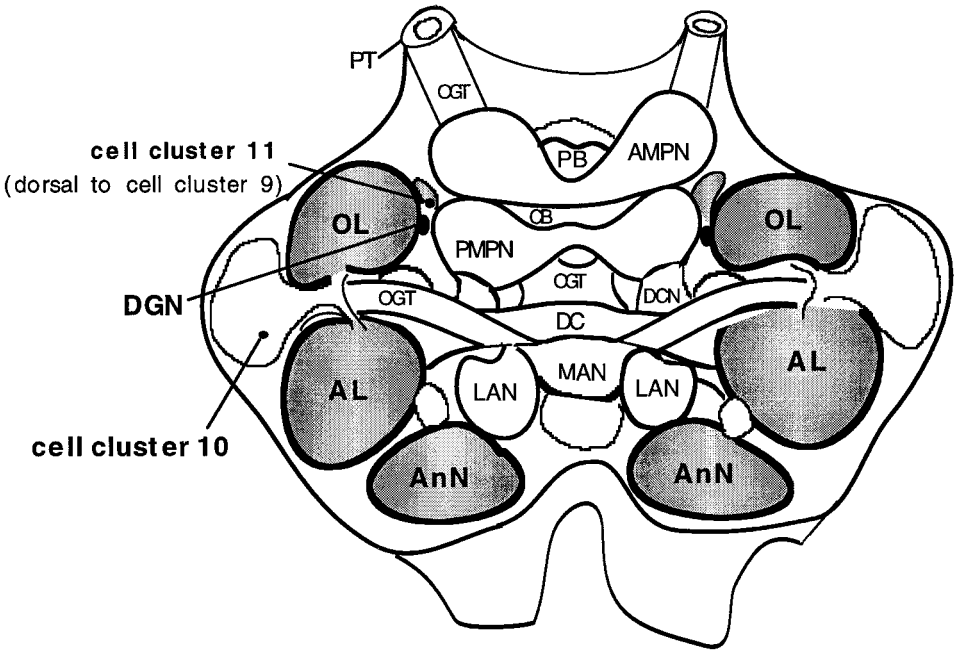


Figure 1 Schematic diagram of a juvenile American lobster brain (collapsed in the dorsal–ventral plane). Protocerebral structures: AMPN = anterior medial protocerebral neuropil; CB = central body; OGT = olfactory globular tract; PB = protocerebral bridge; PMPN = posterior medial protocerebral neuropil; PT = protocerebral tract. Deutocerebral structures: AL = accessory lobe; DC = deutocerebral commissure; DCN = deutocerebral commissure neuropil; LAN = lateral antenna I neuropil; MAN = medial antenna I neuropil; OL = olfactory lobe. The deutocerebral giant neurons (DGNs) are located in cell cluster 11. Tritocerebral structures: AnN = antenna II neuropil.

cerebrum [Fig. 2(A)] (Helluy et al., 1996). Just before hatching, many developmental processes accelerate (Helluy and Beltz, 1991; Helluy et al., 1993), and the influence of steroids during this time (Snyder and Chang, 1991) make the hatching period particularly interesting. Accessory lobe glomeruli begin to form during the transition from the first to second larval stage, and the numbers of both olfactory and accessory lobe glomeruli stabilize by the fourth postembryonic stage when the juvenile lobsters become benthic (Helluy et al., 1996). Therefore, in addition to assessing the long-term effects of serotonin depletion on the growth of deutocerebral structures, we also focused our attention on specific periods when important histogenic events normally take place.

The results we present here suggest that serotonin may be involved in the embryonic growth of the olfactory and accessory lobes. However, neither the emergence of the accessory lobes, nor the formation of glomeruli in the olfactory or accessory lobes, is disrupted by toxin treatment. Finally, effects of 5,7-DHT in the developing lobster brain are long-lasting, and appear to be limited to serotonergic target regions.

MATERIALS AND METHODS

Embryonic and Larval Maintenance and Developmental Staging

Embryonic lobsters develop over a period of 9–10 months in eggs attached to the ventral surface of the mother's abdomen. During this period embryos undergo a complex series of changes, including at least two molt cycles within the egg [Fig. 2(A)] (Helluy and Beltz, 1991). The hatching process is also closely tied to an impending molt from prelarva to larval stage 1, which is completed just after hatching. Following three free-swimming, pelagic larval stages, the transition to the fourth stage brings about an anatomical, physiological, and behavioral metamorphosis in which the lobster settles to the substrate and from then on leads a benthic life (Herrick, 1895; Charmantier, 1987).

Determining the developmental ''age'' of lobster embryos is complicated by the fact that they undergo a period of developmental arrest during the later stages of development, even at constant temperature. Thus, ''real time'' does not provide a good basis for assessing an animal's developmental stage; a quantitative scheme, the ''eye index'' (Helluy and Beltz, 1991), has been used instead. This measure of development is based on a combination of the average extent of eye pigmentation (the

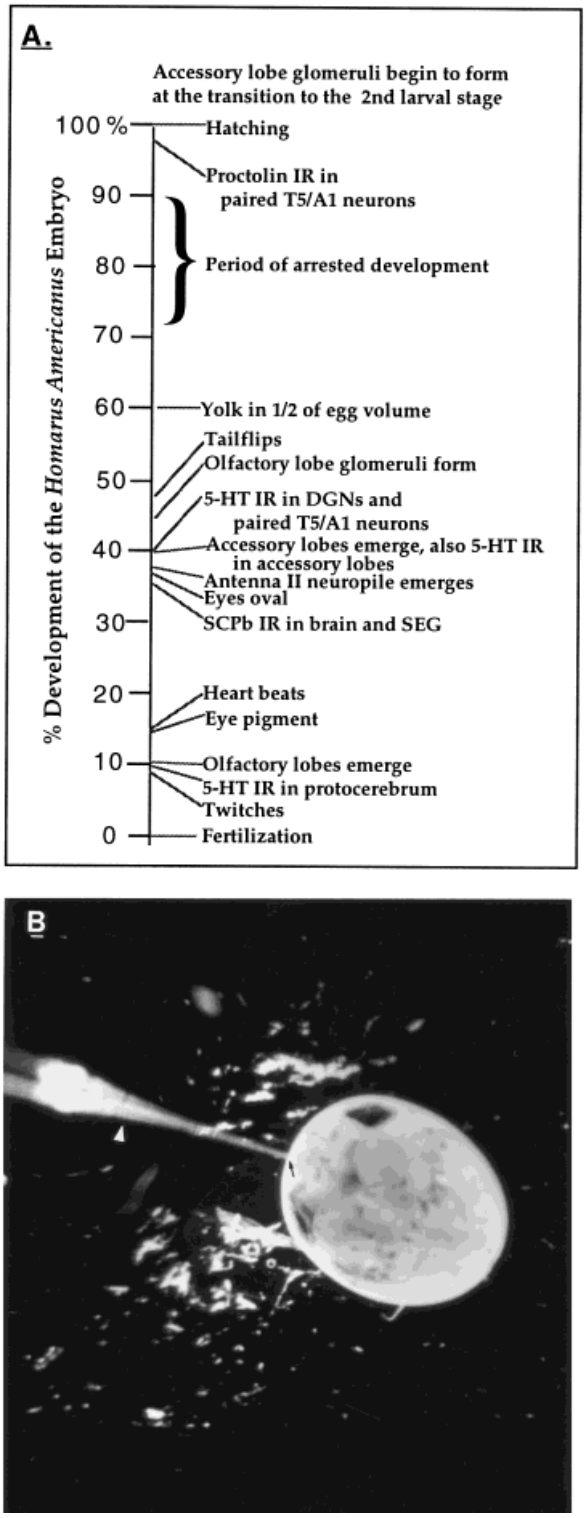


Figure 2 (A) Sequence of occurrence of selected developmental events relative to the percentage of embryonic development, based upon the eye-index staging system (Helluy and Beltz, 1991). Note that the time span of embryogenesis at 18°C constant temperature is ~5 months; at seasonal temperatures in the wild, embryogenesis spans 9–10 months. (B) The neurotoxin (5,7-DHT) was injected via a micropipette (white arrow) into the dorsal hemolymph sinus (black arrow) just anterior to the brain of this embryo.

average of the length and width of eye pigmentation) with additional developmental landmarks. The index ranges on a percentage scale from fertilization or E0% to E100% at hatching. Larval staging was based upon the detailed anatomical features described by Herrick (1895), which are summarized in Beltz et al. (1990). Egg-bearing female lobsters were obtained locally or from D. E. Aiken's laboratory in New Brunswick, Canada, and maintained in circulating, filtered sea water tanks at the New England Aquarium (Boston, MA). As needed, embryos were placed in netting baskets in a circulating artificial sea water tank (16–18°C) on a 12:12 h light/dark cycle at Wellesley College. At the time of hatching, first-stage larvae were removed daily from the egg baskets and placed in kreisels, where their development proceeded through the fourth stage.

Pharmacological Depletion of Serotonin

Embryos were removed from an egg-bearing female and staged according to methods described above. After penetrating the egg membranes and the body wall with a fine glass micropipette [Fig. 2(B)] connected to a microinjection apparatus (Sutter Instruments), test solutions were injected into the dorsal hemolymph sinus. ''5,7-DHT'' embryos received 0.13–0.26 μL of 10^{-2} *M* 5,7-DHT (Sigma Chemical Co., St. Louis, MO) in a vehicle solution (physiological saline, 1% ascorbate, and 0.05% boiled food coloring as a visual marker). Initially, a concentration of $\sim 10^{-3}$ *M* 5,7-DHT is present just after injection, when the toxin is diluted by the hemolymph. This concentration slowly decreases as 5,7-DHT is metabolized and oxidized within the egg. ''Control'' eggs were injected with the vehicle solution alone. ''Normal'' eggs were removed from the same mother as experimental and control eggs. They were handled and observed under the microscope, but were not injected.

Injections were administered once each week for a variable number of weeks depending upon the goals of the experiment. Three to four days after the last (in most cases, the fourth) injection, embryos from the three experimental groups (normal, control, and 5,7-DHT) were sacrificed and the nerve cords were dissected and prepared for one of four assays [Fig. 3]: (I) Immunocytochemical methods were used to test for the presence of serotonin; (II) serotonin content in whole embryos was quantified using reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection; (III) morphometric data were compiled using histological sections and three-dimensional reconstruction analysis (Eutectics Electronics, Inc.) of the olfactory lobes, accessory lobes, and antenna II neuropils; and (IV) immunocy-

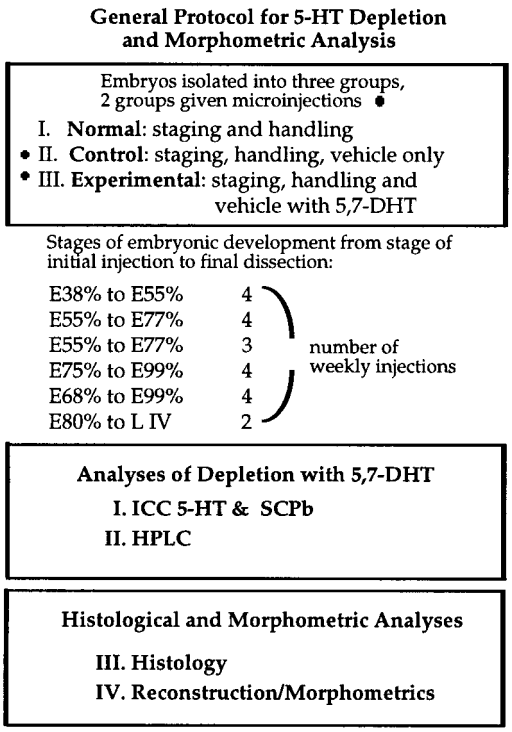


Figure 3 Flow chart of the 5,7-DHT experimental protocol, including the stages of embryos and the technical analyses used.

tochemical techniques were used to assess the pattern of localization of small cardioactive peptide b (SCP_b). SCP_b is present in the same brain regions as serotonin but does not, to our knowledge, co-localize in the same cells (Langworthy et al., 1997). This substance, which should not be disrupted by the 5-HT depletion, therefore serves as one type of control. In some experiments, embryos were allowed to mature for extended periods of up to 2 months after the final 5,7-DHT injection, and the permanency of the depletion was assessed using immunocytochemical analyses.

Immunocytochemistry

Methods for processing whole mounts of ganglia for serotonin immunocytochemistry followed the techniques of Beltz and Burd (1989) and Beltz et al. (1990). Embryonic brains with the thoracic nerve cord attached were dissected, fixed in 4% paraformaldehyde, rinsed, and incubated in 0.1 *M* phosphate buffer (PB) with 0.4% Triton X-100 (PBTx) and 8% normal goal serum (NGS). Tissues were incubated in primary antibody (rabbit anti-serotonin antiserum; ETI, Ridgefield Park, NJ) diluted 1:2500 in PBTx for 72 h at 4°C with mild agitation, followed by secondary antiserum (biotin anti-rabbit; Vector Labs, Burlingame, CA; dilution 1:200) and avidin–biotin–horseradish peroxidase complex (ABC; Vector Labs). Following rinses, ganglia were reacted with 3′,3-diaminobenzidine (DAB) (Sigma; 1 mg/2 mL PB). Finally, preparations were dehydrated, cleared in methyl salicylate, and mounted in DPX (Fluka).

For immunocytochemistry for SCP_b, a monoclonal primary antiserum generated against molluskan SCP_b was obtained from the Monoclonal Laboratories at the University of Washington, and applied at a dilution 1:15 in 0.1 *M* PBTx. Visualization of the antibody using avidin–biotin methods was identical to that for serotonin described above. It is likely that this antibody binds to an endogenous FLRFamide compound found in lobster tissues instead of to authentic SCP_b (Trimmer et al., 1987; Arbiser and Beltz, 1991). This antibody was used despite this specificity issue, because it provides robust and consistent labeling with avidin–biotin methods in areas where serotonin also is found (Langworthy et al., 1997).

Whole mounts of brains and thoracic nerve cords, immunocytochemically processed for 5-HT and SCP_b, were rated for labeling intensity. Using blind scoring methods (random presentation, no knowledge of experimental group identity), 6 samples/group were rated by at least three observers. The intensity and appearance of labeling in specific areas of brain and nerve cord were scored according to a scale ranging from no staining (0) to intense staining (4). One-way analysis of variance (ANOVA) was used to look for significant differences in these ratings of 5-HT–immunoreactive labeling.

HPLC Methods

In several experiments, single embryos from the three experimental groups (normal, control, and 5,7-DHT) were removed at various stages of development, but always at the same time of day (9–11 A.M.), and assayed for levels of serotonin by reverse-phase HPLC with multielectrode electrochemical detection (HPLC-EC). After removal of the yolk, embryos were transferred to microcentrifuge tubes containing 50 μL of 0.1*N* perchloric acid (PCA) and mechanically homogenized using a Teflon pestle. Insoluble residue, consisting of denatured proteins and cell debris was pelletted for 15 min at 15,000 rpm on a tabletop centrifuge (Beckman Microfuge). An aliquot of the clear supernatant was diluted fivefold with mobile phase, and a 20-μL sample was applied onto a reverse-phase C_{18} column (Spherisorb; 3 μm, 100 × 4.6 mm) for isocratic separation. The mobile phase contained 20 m*M* sodium phosphate (2.4 g/L monobasic anhydrous; FW: 120.0; Sigma S-0751), 1.85 m*M* heptanesulfonic acid (375 mg/L sodium salt; FW: 202.2; Sigma H-2766), 0.27 m*M* ethylenediaminetetraacetic acid (EDTA) (80 mg/L anhydrous; FW 292.2; Sigma EDS), and vol/vol 16% MeOH and 4% acetonitrile as organic modifiers. The final solvent buffer was adjusted to pH 3.25 with concentrated phosphoric acid (ACS reagent; FW: 98.0; Sigma P-6560), filtered through a 0.22-μm filter, and operated at ambient temperature with a flow rate of 1.7 mL/min. Eluted compounds were detected electrochemically on an ESA model 5100A detection system with four potentiostats and a four-channel coulometric detector cell. A/D converters (MacLab) and a strip-chart

program (MacLab Chart v. 3.3.3) recorded the chromatograms, and chromatography software (MacLab™ Peaks v. 1.3.1) was used to integrate areas below the peaks. Detector potentials were set at 250 mV (channel 1), 350 mV (channel 2), 450 mV (channel 3), and 550 mV (channel 4) with a guard cell screening at 150 mV. Under this set of conditions serotonin was oxidized at detectors 1 and 2 [Fig. 4(A,B)]. The detection limit was in the range of 1–2 pg 5-HT injected on column. Recovery rates were close to 100% and no further corrections were applied.

Histological and Morphometric Analyses

Intact embryos separated from the egg envelopes and the yolk, and the brains of larvae were fixed in Bouin's solution, rinsed in PB, and infiltrated, embedded in JB-4 plastic medium, and polymerized. Sections (4 μm) were cut serially with a JB-4 microtome, stained with 0.1% Toluidine blue in 0.2 *M* carbonate buffer, and viewed through a Nikon Optiphot microscope with a camera lucida attachment. The outlines of the left and right olfactory lobes, accessory lobes, and antenna II neuropils in each section of the brain were traced on a magnetic data pad linked to the computer system, digitized and integrated into 3D displays; surface areas and volumes also were calculated using this computer reconstruction system (Eutectics Electronics, Inc.). The volumes for the left and right antenna II neuropils and the olfactory and accessory lobes were treated separately, and ANOVA statistical analyses were performed to test for significant differences between the volumetric values.

RESULTS

The consequences of serotonin depletion by 5,7-DHT were evaluated from two perspectives: (a) The effectiveness of toxin injections in reducing serotonin levels was assessed qualitatively and quantitatively with immunocytochemical and HPLC methods. (b) The long-term effects of serotonin depletion on the growth and differentiation of the olfactory, accessory, and antenna II neuropils were examined using histological and morphometric methods.

Effects of 5,7-DHT on Serotonin Levels

Immunocytochemistry for Serotonin and SCP_b. The patterns of labeling for 5-HT and SCP_b are distinctive, even at early embryonic stages [Fig. 5(A and D respectively)]. Serotonin labeling in the ventral nerve cord and brain was comparable in normal and control embryos, while 5,7-DHT-injected embryos showed a dramatic loss of serotonin immunoreactivity [Fig. 5(A–C)]. The effects of 5,7-DHT treatment were so pronounced that cell bodies and neurites were either not stained or barely distinguishable from the background of surrounding tissues. Using blind ratings to evaluate immunocy-

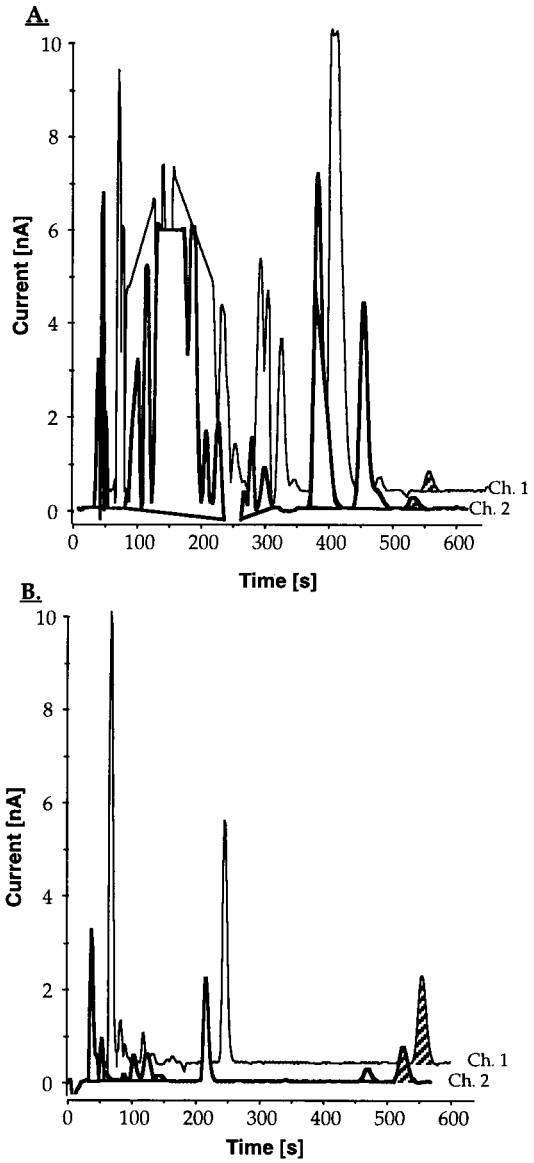


Figure 4 Chromatograms for detectors 1 (250 mV) and 2 (350 mV). (A) Six stage-matched normal embryos (E80% and E94%) with the yolk removed were pooled for HPLC analysis of amines to quantify serotonin levels. The small shaded peaks at 500–550 s represent the endogenous levels in the pooled samples. (B) Identification of serotonin was based on comparison with external standards run the same day with respect to (a) retention time, (b) maximum oxidation potential, and (c) peak area ratio on different detectors. Moreover, the addition of exogenous serotonin to tissue samples increased the amplitude of this peak without affecting its retention time. Known standards were dissolved in 0.1*N* perchloric acid (PCA) at a concentration of 1 mg/mL with final concentrations (5 pg amine/μL) made from stock each day.

tochemical preparations, 5,7-DHT nerve cords and brains showed a significant loss of labeling compared with the brains of normal and control animals [Fig. 6(A)]. In contrast, the distribution and intensity of labeling for SCP_b immunoreactivity in the brain were unaffected by 5,7-DHT treatment [Fig. 5(D)].

A longitudinal experiment, in which animals were treated with toxin and groups were sacrificed at successive developmental stages, was designed to test the permanency of depletion after 5,7-DHT treatment: first injection at E68% → sacrifice at E80% → sacrifice at E99%. Blind ratings of the immunocytochemical preparations at each stage [Fig. 6(B)] demonstrated that very little staining was evident, regardless of the length of time that followed injection. Sensitivity to the toxin, or to the reduced serotonin levels, became a concern when these embryos had difficulty hatching into larvae. In an attempt to improve the survival rate, embryos were injected later in development with only two injections, 1 week apart. These 5,7-DHT–treated embryos also showed a significant reduction in serotonin immunolabeling which lasted throughout embryonic stages and larval life, nearly 2 months after toxin treatment (first injection at E80% → sacrifice at E82% → sacrifice at E92% → sacrifice at postembryonic stage IV) [Fig. 6(C)].

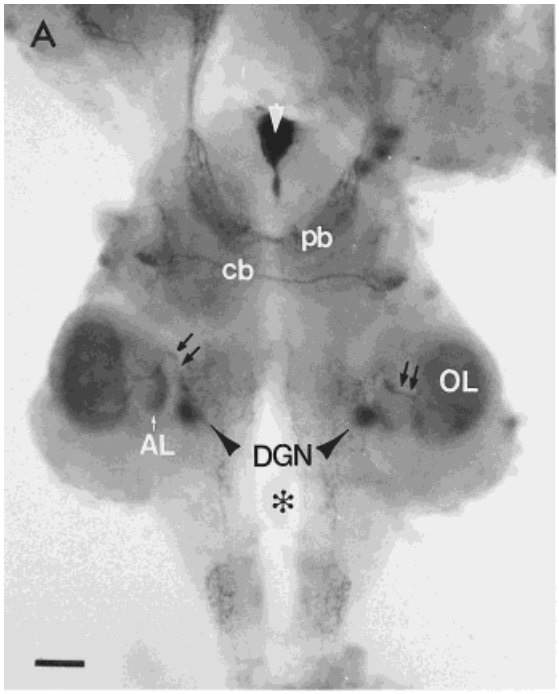
Analyses of Serotonin Levels by HPLC. To confirm the immunocytochemical studies and quantify the reduction in serotonin after toxin injection, HPLC analyses were performed on whole embryos (normal, control, and 5,7-DHT). In an experiment spanning E55–77% (Fig. 7), serotonin was depleted by ~90%. When the mean values for serotonin concentration of the normal and control animals were compared, no significant differences were found. The results shown in Figure 7 represent the same experimental group illustrated in the immunocytochemical blind ratings of Figure 6(A). Another HPLC experiment used embryos from the longitudinal study that demonstrated consistent depletion for more than 1 month after toxin treatment (first injection at E68% → sacrifice at E80% → sacrifice at E99%) [see immunocytochemical ratings, Fig. 6(B)]. The HPLC analysis, performed at E80% confirmed ~90% depletion of the first study. HPLC also was performed for the second longitudinal study where embryos were injected only twice (first injection at E80% → sacrifice at E82% → sacrifice at E92% → sacrifice at postembryonic stage IV) [Fig. 6(C)]. Roughly 75% depletion was demonstrated by HPLC at E92%.

In the longitudinal experiments, as well as the individual studies, pairing of immunocytochemical assessment with HPLC analyses demonstrated that the results interpreted by these two methods were consistent and that the sensitivity of immunocytochemical methods was adequate to discriminate relative differences in serotonin levels between the 5,7-DHT embryos and the normal/control animals. Serotonin levels were drastically reduced in toxin-treated embryos despite growth-related increases in serotonin levels during development (Beltz et al., 1990).

Does Serotonin Reduction Alter Olfactory and Accessory Lobe Differentiation?

One anatomic indication of brain maturity is the breadth of the deutocerebrum relative to the rest of the brain. While younger brains have a relatively elongate appearance, progressively older brains become broader, owing primarily to the growth of the olfactory and accessory lobes (Helluy et al., 1995). In the current experiments, histological observations revealed that the brains of toxin-treated animals could generally be identified based on a less mature brain shape, which was due largely to the smaller size of the accessory lobes in the serotonin-depleted embryos. However, the eye-index staging measurements at the times of dissection did not indicate a significant difference in general development among the three groups. In addition, during normal development, we saw a rotation of the deutocerebral cell body clusters relative to the olfactory and accessory lobes (Beltz et al., 1992). The toxin-treated brains also showed a more immature condition in terms of the positions of these cell clusters.

Morphometric Analyses of Accessory and Olfactory Lobe Volumes. In embryos first injected at E38% and sacrificed at E55%, the olfactory lobes were reduced in volume by ~20% compared to these regions in brains of control embryos; the accessory lobes were reduced in volume by ~50% compared to accessory lobes in the brains of control embryos [Fig. 8(A,B)]. The antenna II neuropils, which are located in the tritocerebrum just posterior to the accessory lobes, were used as a control region to test for generalized effects of the toxin treatments; these neuropils do not receive an early serotonergic input by immunocytochemical criteria, but do have a rate of growth similar to the olfactory and accessory lobes during mid-embryonic life (Fig. 9). Because the antenna II neuropils are located in close proximity to the accessory lobes, their access to 5,7-DHT should be similar. Nevertheless, the histological appearance and volumes of the antenna II neu-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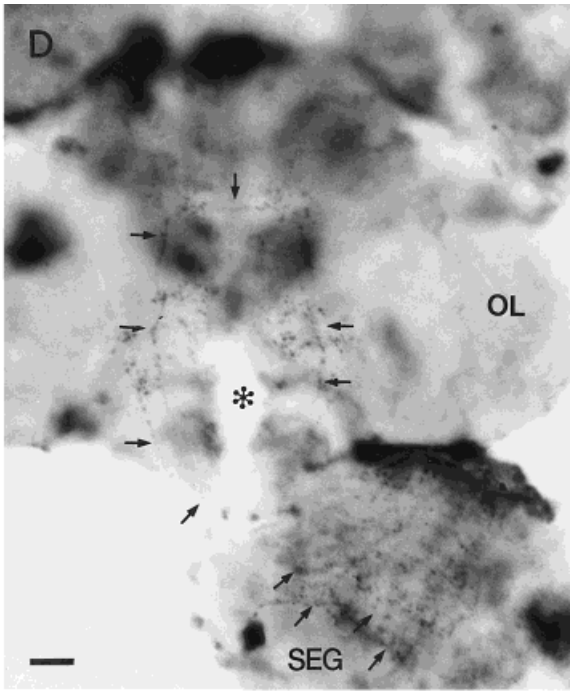
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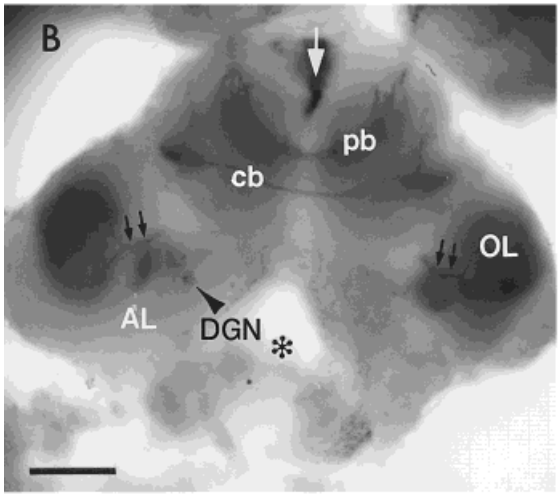
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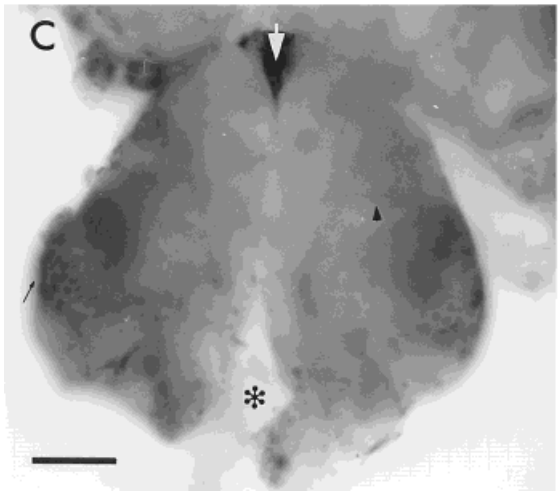
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ropils were unaffected by the toxin, suggesting that only embryonic serotonergic targets were affected by 5,7-DHT treatment [Fig. 8(C)].

Emergence of Accessory Lobes and Formation of Olfactory Glomeruli. The accessory lobe anlagen and columnar glomeruli in the olfactory lobes are first apparent in histological sections at about E40–45%. To test whether these developmental events are dependent upon serotonergic mechanisms, embryos were injected with 5,7-DHT beginning at E38% and sacrificed 3 days after the fourth injection, at E55%. Immunocytochemical assays confirmed the depletion of serotonin in the toxin-treated embryos. Histological assessments of these brains showed that the accessory lobes emerged and olfactory glomeruli formed as expected during this period, despite the reduction in serotonin levels [Fig. 10(A–C)]. Olfactory glomeruli appeared to be normal for this stage of development in terms of number, size, and general organization, as assessed at the light-microscopic level [Fig. 10(C)].

In contrast to the precocious development of the olfactory lobe glomeruli during mid-embryonic life, the spherical glomeruli of the accessory lobes begin to form during the transition from the first to second larval stage. When embryos were treated twice with 5,7-DHT prior to the formative period for these glomeruli (first injection at E80% → sacrifice at fourth postembryonic stage), accessory lobe glomeruli nevertheless formed as expected [Fig. 10(D)]. Preliminary observations of their number and size suggest that these parameters are in the normal range for this time period (Helluy et al., 1995, 1996).

DISCUSSION

The targets of the DGNs, the olfactory and accessory lobes, have contrasting patterns of growth and differentiation during embryogenesis. The olfactory lobes first appear as small outgrowths of neuropil by E10%, and the typical glomerular elements form at E40–45%, coincident with the ingrowth of the chemosensory afferents from the antennulae. The ingrowth of the sensory neurons triggers the formation of glomeruli in the olfactory areas in several organisms (*Manduca sexta*: Oland and Tolbert, 1987; *Cherax destructor*: Sandeman and Sandeman, 1990), and this feature appears to be related to an intrinsic ability of these sensory neurons to ''glomerulize'' tissues (Graziedei and Graziedei, 1986). The accessory lobes emerge later in development than the olfactory lobes, by ~E40–45%, coincident with glomerular formation in the olfactory lobes and also with the ingrowth of the serotonergic fibers of the DGNs to the deutocerebrum. Glomeruli do not form in the accessory lobes until the transition from the first to second larval stage. Because the emergence of the accessory lobes coincides with serotonin appearance in the DGN fibers projecting to that region, the growth and differentiation of the accessory lobes proceed in the constant presence of serotonin. This correlation led us to question whether this was merely a coincidence of timing, or whether there might be a causative relationship between the emergence of the accessory lobes and the onset of serotonin immunoreactivity in the deutocerebrum. In the present study, we tested whether the depletion of serotonin by the selective toxin 5,7-DHT alters the development of deutocerebral regions, and especially of the accessory lobes whose differentiation is so intimately linked temporally with ingrowth of serotonergic axons.

Use of 5,7-DHT to Deplete Serotonin Levels

5,7-Dihydroxytryptamine, a neurotoxic analog of serotonin, is a useful tool for studying the anatomy

Figure 5 (A) In whole-mount preparations of normal embryonic brains at E55%, several structures appear moderately or intensely stained for 5-HT: protocerebral bridge (pb), central body (cb), olfactory (OL) lobes, the emerging accessory lobes (AL), and paired dorsal giant neurons (DGNs), each with a single bifurcating ipsilateral projection (double arrows) to the AL and OL. The naupliar eye located dorsal to the midsection of the embryonic brain (white arrow) is in focus for 4(A–C), indicating approximately the same depth of focus within the tissues. Anterior structures are toward the top. (B) The brains from control embryos that were injected with vehicle solution show staining patterns for serotonin that are similar to the brains of normal embryos. (C) In the 5,7-DHT–injected brains (first injected at E38% → sacrificed at E55%), there was little or no detectable serotonin labeling. The arrowhead points to faint and partial staining in the cb of the right hemibrain. No additional specific staining was found within the brain. An arrow points to the edge of the OL which contains background staining. (D) The neural staining patterns for SCP_b (first injected at E75% → sacrificed at E99%) are similar for normal, control, and 5,7-DHT embryos. Labeling in a 5,7-DHT brain is shown. Arrows point to regions of punctate staining in the brain and subesophageal ganglion (SEG). Asterisks mark the esophagus in each photo. Scale bars = 50 μm.

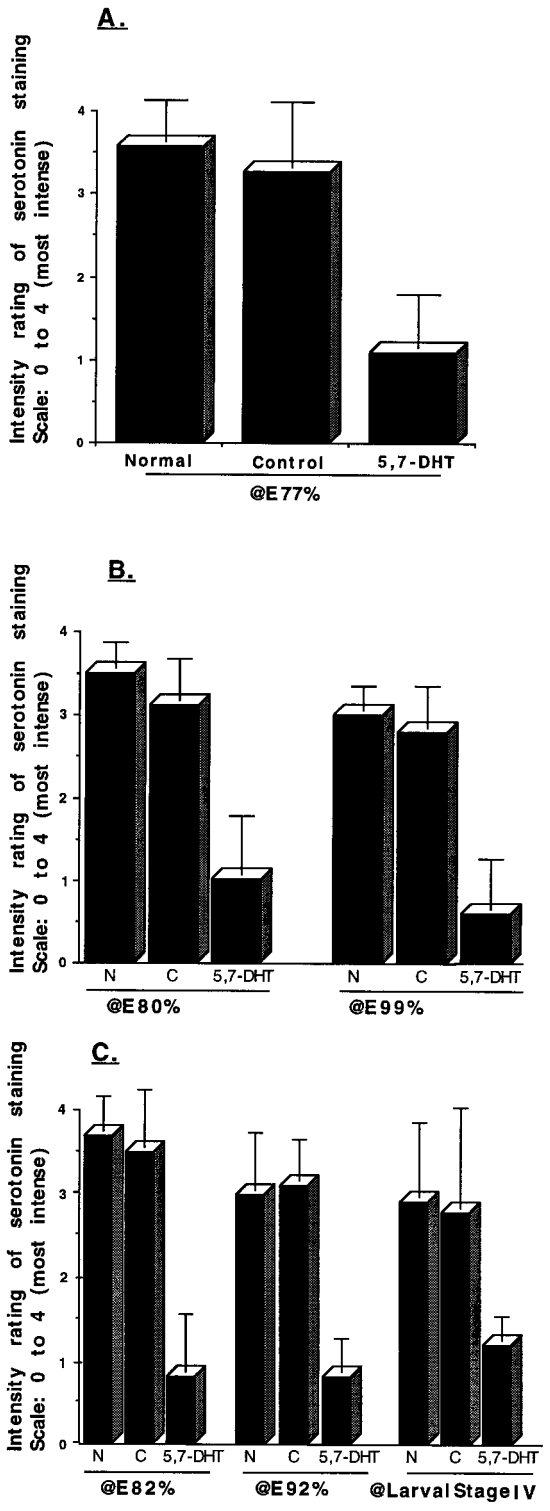

Figure 6 Serotonin depletion in immunocytochemical preparations from normal (N), control (C), and 5,7-DHT injected embryos at various stages of development was evaluated. Blind ratings are shown of 6 nerve cords/group by a minimum of three individuals. (A) Blind ratings for one experiment; embryos were first injected at E55% → sacrificed at E77%. Normal and control groups were rated as having moderate to intense staining. Ratings for toxin-treated embryos were described as zero to faint. One-way analysis of variance revealed a significant difference among these groups [ANOVA: $F(2, 42) = 65.3$; $p < 0.001$]. (B) In a longitudinal study, samples were taken at E80% and again at E99%, 1 month later (four injections given, initial injection at E68%). Significant differences among the three groups were revealed using ANOVAs [at E80%, $F(2, 33) = 78.7$; $p \leq 0.001$; and at E99%, $F(2, 51) = 127.5$; $p \leq 0.001$]. (C) In a second longitudinal study, embryos were given two 5,7-DHT injections 1 week apart. These samples also show a significant reduction in serotonin immunolabeling which lasts throughout the embryonic stages tested [sacrificed at E82%, ANOVA: $F(2, 51) = 129.5$; $p \leq 0.001$; 4 weeks later at E92%, ANOVA: $F(2, 33) = 70.45$; $p \leq 0.001$] and persisted through postembryonic stage IV (ANOVA: $F(2, 12) = 12.99$; $p \leq 0.001$].

and physiology of serotonergic neurons. In vertebrates, 5,7-DHT acts as a selective toxin via a mechanism involving intraneuronal oxidation by monoamine oxidase (Baumgarten and Björklund, 1976; Klemm et al., 1979; Wolf and Bobik, 1988), although its specific actions are unclear (Klemm and Baumgarten, 1978; Klemm et al., 1979). The result of 5,7-DHT injections in rats is unequivocal: Within 2 weeks after injection, there is a profound decrease in brain serotonin levels and the serotonergic neurons begin to degenerate (Björklund et al., 1975; Smale et al., 1990). In the invertebrates, where monoamine oxidase does not appear to be present, catabolism of monoamines is achieved primarily by *N*-acetylation (Evans, 1980; Slowley and Downer, 1984; Trimmer, 1985), although oxidative deamination and other mechanisms have been proposed in some species. The toxic action is attributable to the fact that 5,7-DHT competitively inhibits the uptake of serotonin and simultaneously reduces the synthesis of serotonin (Cook and Orchard, 1993). In many mature invertebrate species, the effects of 5,7-DHT are transient. Serotonin levels are temporarily reduced, but the serotonergic neurons are not destroyed and can recover their function (Lent and Dickinson, 1984; Gadotti et al., 1986). Although *Helisoma* embryos recover serotonergic function after treatment with the toxin (Goldberg and Kater, 1989), the effects of 5,7-DHT are more dramatic and permanent in leech embryos, where serotonergic neurons are destroyed (Glover and Kramer, 1982).

5,7-dihydroxytryptamine has been used in prior studies of the adult and juvenile lobster nervous system (Livingstone et al., 1981; Beltz and Kravitz, 1983). These studies demonstrated that the toxin

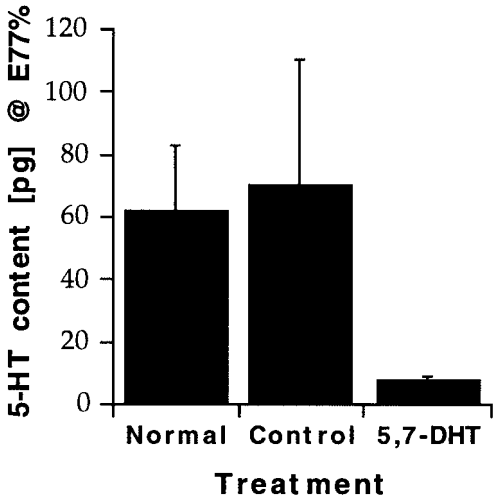

Figure 7 High-performance liquid chromatographic analyses. Bar chart of serotonin content for the three experimental groups after the standard 5,7-DHT injection protocol, for embryos first injected at E55% and sacrificed at E77%. Each value represents the mean number of picograms per group (using individual eggs) ± the standard error. When the mean values of the normals and controls were compared, there were no significant differences; 5,7-DHT–treated embryos were ~90% depleted. A one-way ANOVA revealed that there were significant differences among the three groups [Welch ANOVA: $F(2, 7.511) = 3D14.491$, $0.0001 < p = D < 0.01$].

has dramatic effects on serotonin content in the nervous system, while the levels of octopamine and its rate of synthesis are not changed. In the current experiments, the effectiveness of 5,7-DHT in depleting serotonin and the permanence of the depletion were examined in lobster embryos. The standard protocol of one toxin injection each week for 4 consecutive weeks eliminated or severely reduced serotonin immunostaining in the developing nervous system (Fig. 5–7). HPLC studies demonstrated that this decrease in staining represents a reduction in serotonin content of ~90%, measured in tissue samples from animals sacrificed 3–4 days after a 5,7-DHT injection. After such a treatment, the embryos are viable and progress developmentally; however, their mortality rate, particularly near the time of hatching, is high compared to normal and control embryos. If, however, the embryos are ''hatched'' by hand, the larvae that emerge survive. The problems induced by the toxin during hatching therefore are likely to relate to the reduced levels of serotonin, rather than to a generalized toxic effect. When toxin was injected for only 2 weeks, the mortality rate improved and serotonin was reduced—however, to a lesser degree (~75%) [Fig. 6(C)]. Although we have not performed time-course studies of the onset of serotonin depletion, we do know that once the ~90% depletion is achieved within a matter of a few days after a series of at least three toxin treatments, the depletion is long-lasting, persisting for at least 2 months after the final toxin injection. We have not tested lobsters after a more extended period following toxin treatment, and therefore, it may be that the depletion is even more protracted or permanent. Figure 6(B,C) suggests that this could well be the case, since the reduced levels are relatively stable throughout the postinjection period tested. The multiple 5,7-DHT injections used in these studies therefore provide a sensitive and long-lasting means of reducing serotonin levels in lobster embryos. The fact that the relative intensity of immunostaining correlates well with levels of serotonin measured by HPLC also suggests that immunocytochemical methods can be effectively used to assess relative levels of the amines in neural tissues.

In addition to prior studies in the lobster where it was demonstrated that 5,7-DHT does not decrease octopamine levels (Livingstone et al., 1981; Beltz and Kravitz, 1983), we have assessed immunostaining for SCP_b which resides in the same deutocerebral regions as serotonin. Since SCP_b labeling was not affected in the brain or ventral nerve cord, we interpret this as an additional finding in favor of the selective effects of the toxin on serotonergic neurons in the lobster, that argues against broad toxic influences of this substance when delivered by the protocol described.

Does Serotonin Depletion by 5,7-DHT Alter the Growth or Differentiation of Neuropil Regions in the Lobster Brain?

Our data suggest that although many developmental processes proceed normally following introduction of 5,7-DHT (i.e., formation of glomeruli in both olfactory and accessory lobes, the emergence of the accessory lobes), the growth of specific serotonergic target regions, the olfactory and accessory lobes, is altered by 5,7-DHT [Fig. 8(A,B)]. The antenna II neuropils, which reside just posterior to the accessory lobes in the tritocerebrum, should have the same access to toxin as the other lobes under study, but do not normally have a serotonergic input during mid-embryonic life. Their growth rate also is similar to the accessory lobes during mid-embryonic life (Fig. 9). Therefore, we used these areas as control regions. According to our hypothesis related to serotonergic effects during development, we would not expect the growth or differentiation of these areas to be altered by toxin treatment. Indeed, the growth

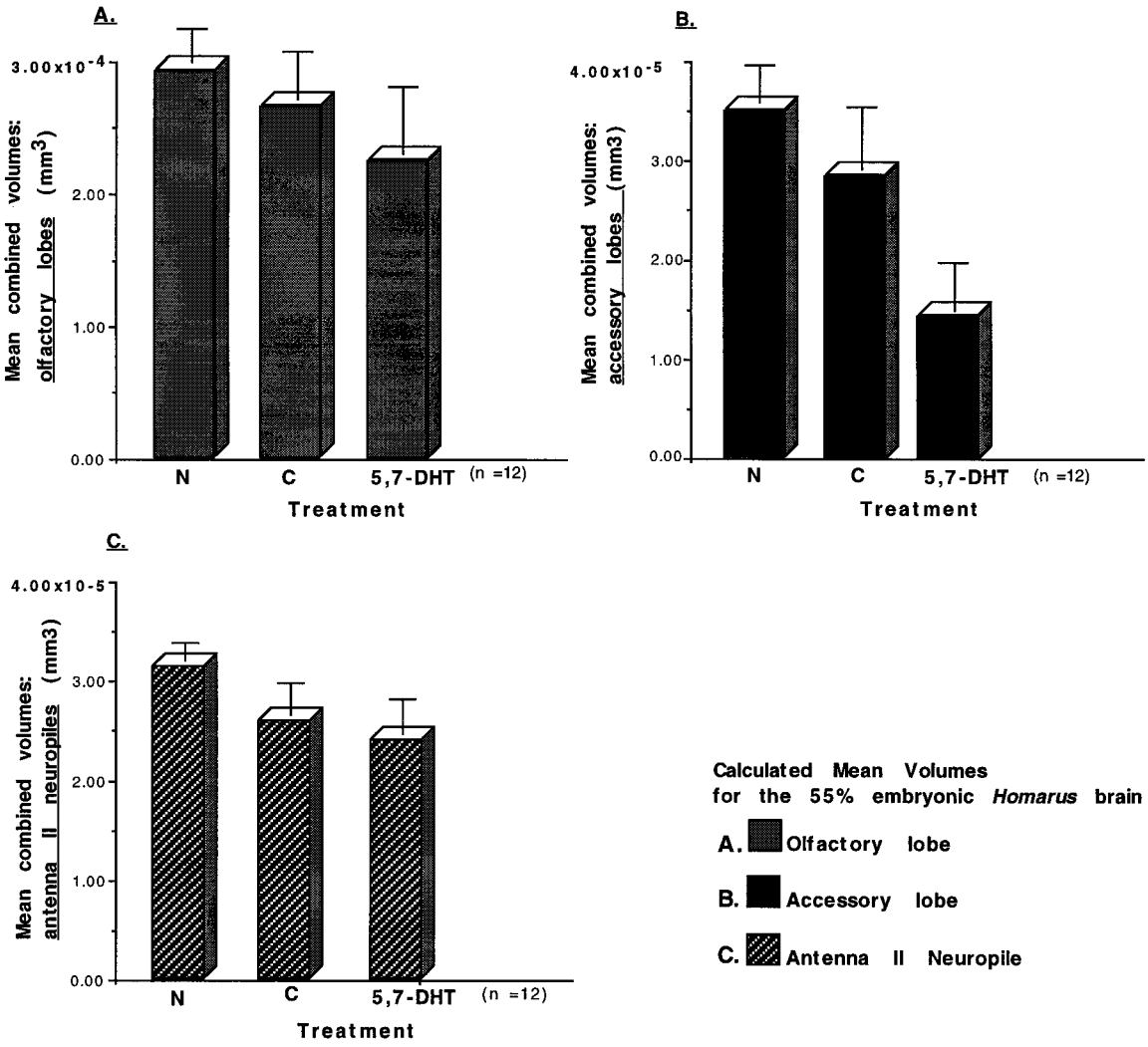

Figure 8 Morphometric analyses: mean volumes for olfactory, accessory, and antenna II neuropils showing the effects of serotonin depletion by 5,7-DHT (first injection at E38%, sacrificed at E55%). In the 5,7-DHT embryos, the volumes of the olfactory (A) and accessory (B) lobes were significantly different (reduced) compared to the normal and control groups as revealed by ANOVAs: OL: $F(2, 33) = 9.5$, $p = < 0.001$; AL: $F(2, 33) = 56.2$, $p \leq 0.001$. There was a particularly strong effect on the accessory lobes of the toxin-treated embryos (B), which were reduced to half the volume of accessory lobes in the control group. Control and 5,7-DHT mean volumes of the antenna II neuropils (C) were not significantly different from one another; however, there was a significant statistical difference in the experimental and control groups versus the normal group [ANOVA: $F(2, 33) = 18.8$, $p \leq 0.001$]. The mean volumes of the olfactory and accessory lobes, and antenna II neuropils in the control groups, are consistently smaller than these regions in the normal group. N = normal; C = control; 5,7-DHT = toxin-treated group.

of the antenna II neuropils is not altered by the introduction of 5,7-DHT [Fig. 8(C)].

The data in Figure 8 also indicate that the process of transferring the eggs to the injection apparatus and introduction of the vehicle solution had a slight adverse effect on growth of all the brain regions being examined, since the sizes of lobes of sham-injected control embryos were significantly smaller than those of normal embryos. It is also clear in this figure that the growth of the olfactory lobes was affected less severely by 5,7-DHT than was the growth of the accessory lobes. This may be explained by the fact that the olfactory lobes began to develop much earlier than the accessory lobes, and

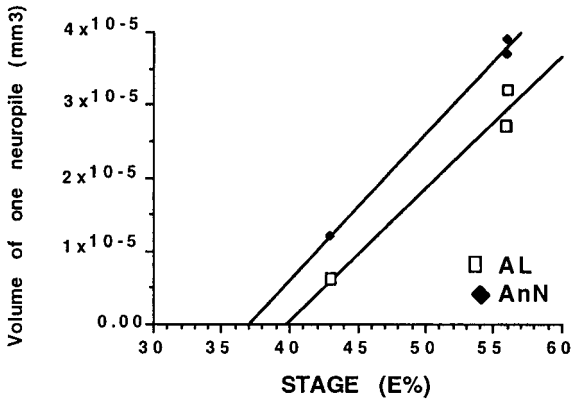

Figure 9 Normal growth curves of accessory and antenna II neuropils. The antenna II neuropils (filled diamonds) and accessory lobes (open squares) emerged during the same period in development and grew at similar rates. Each data point represents the average of the left and right neuropils of one embryo.

therefore, the percentage effect on their final volume would not be as great as for a region just beginning to emerge at the time of serotonin reduction. Second, since the olfactory lobes develop early on in the absence of serotonin, it may be that the olfactory lobes are less influenced by the amine than the accessory lobes, which in normal embryos develop in the constant presence of serotonergic input from the DGNs.

In this study, serotonin depletion by 5,7-DHT impeded the growth of serotonergic target regions. Therefore, the presence of serotonin should promote the growth of these areas. Serotonin has, in fact, been found to promote neurite extension *in vitro* of at least one type of neuron from the antennal lobe of *Manduca sexta*. The antennal lobes of the moth are functionally equivalent to the olfactory lobes of the lobster, and this effect on cultured neurons is consistent with our findings on the growth of lobster neurons *in vivo*. This is, however, in contrast to the inhibition of neurite outgrowth that was observed after serotonin treatment of identified *Helisoma* neurons *in vitro* (Haydon et al., 1984). As a consequence of this inhibition of neurite extension, serotonin regulated both the development of arborizations and the formation of connectivity. The effects of serotonin on *Helisoma* neurons *in vivo*, however, are less clear. Embryos treated embryonically with 5,7-DHT experienced a transient 43% reduction in serotonin levels (Goldberg and Kater, 1989). Identified neurons from these toxin-treated embryos showed aberrations in neuronal morphology, neuronal dye coupling, and strength of electrical synaptic connections; however, neurite elongation was not specifically examined. The variety of outgrowth inhibition/promotion effects seen as a consequence of *in vitro* and *in vivo* treatments in different species may not, however, suggest opposite mechanisms of action. It may be that the specific type of effect seen in serotonergic target neurons could be related to the stage of development or to the degree of serotonin depletion. The level of depletion achieved in our studies was greater, and the length of the depletion period much longer, than in other studies. Direct comparisons between experiments performed under diverse conditions and using different species may therefore be misleading.

The finding that serotonin depletion does not alter the timing of glomerulus formation in the olfactory or accessory lobes also is corroborated by studies in *M. sexta*. A pair of large serotonergic neurons is present in the moth brain, each of which densely innervates the contralateral antennal lobe (Sun et al., 1993). Surgical interruption of the input from this neuron should effectively deplete serotonin in the antennal lobes. However, even in the apparent absence of serotonin, glomeruli formed in the antennal lobes in response to sensory neuron ingrowth, and the lobes appeared to be histologically normal (Oland et al., 1995). These data therefore suggest that serotonin is not likely to play an important role in the formation of glomeruli that occurs in the lobster olfactory lobes during mid-embryonic life, nor in the accessory lobes during larval development. The primary trigger for glomerular formation in the olfactory lobes, as in the antennal lobes, is likely to be the ingrowth of the sensory neurons from the antennules. In the lobster, the ingrowth of antennular fibers to the olfactory lobes is, in fact, correlated with glomerular formation (Helluy et al., 1996). The trigger(s) for formation of the accessory lobe glomeruli is not known; however, we have proposed that the timing of this event during larval life hints that functional validation of inputs to this region may be necessary before glomerularization is possible (Helluy et al., 1996). This idea might be likened to the situation in the vertebrate visual cortex, for instance, where the pattern of binocular activity in retinal ganglion cells promotes the organization of ocular dominance columns. To progress in our understanding of the controls over this process in the accessory lobes, we require a clearer understanding of the inputs and functions of this region (Sandeman et al., 1995; Wachowiak and Ache, 1996).

Conclusions

This panel of studies in the lobster indicates that the effects of 5,7-DHT are confined to serotonergic target regions and that serotonin may be influencing

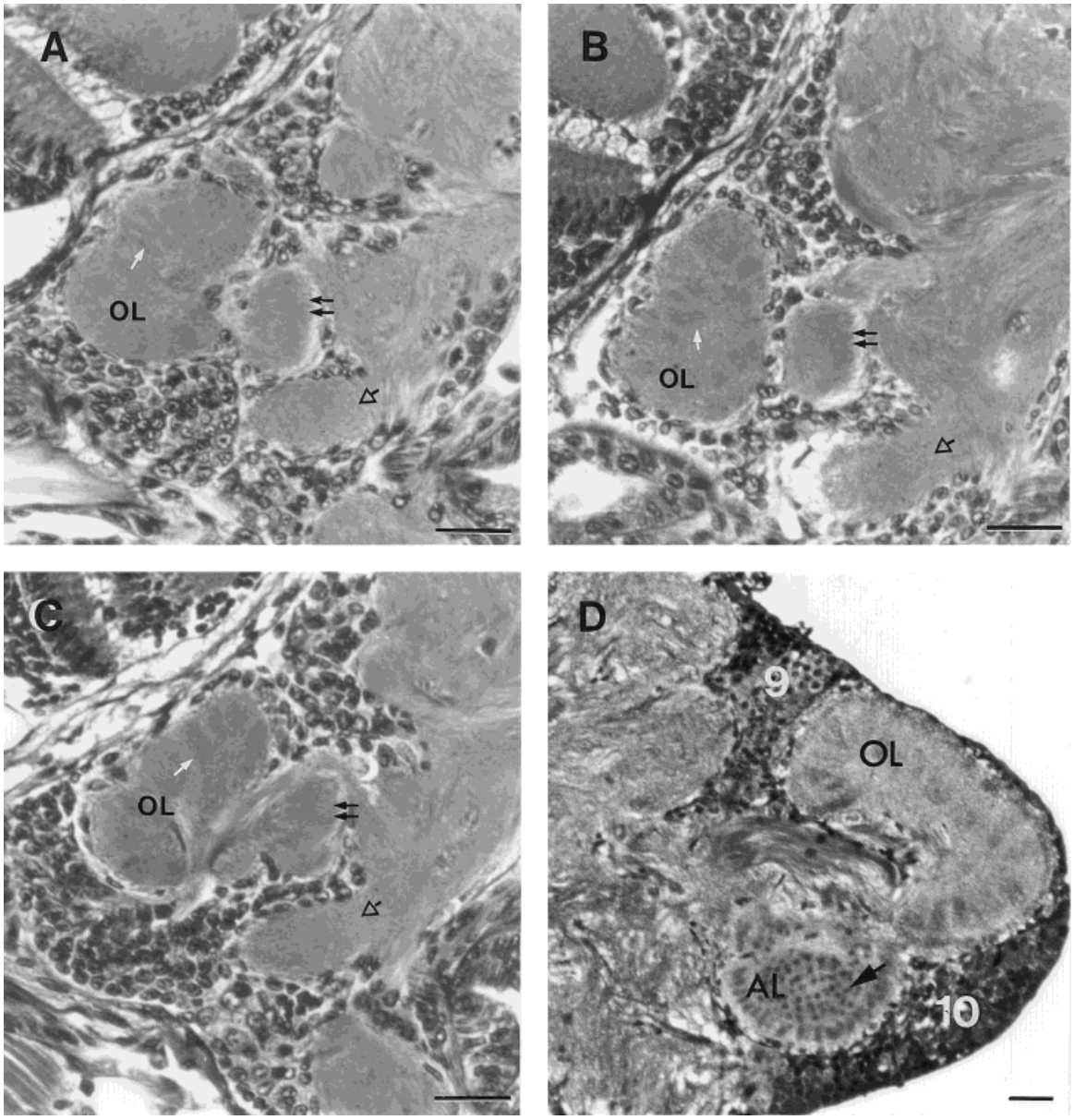

Figure 10 Toluidine blue–stained horizontal semi-thin sections (4 μm) of hemibrains from normal (A), control (B), and toxin-treated (C) embryos at E55% revealed the emerging accessory lobes (double arrows) and the formation of glomeruli in the olfactory lobes (white arrows) in all embryos irrespective of treatment. The antenna II neuropil anlagen (open arrows) were posterior to the accessory lobes. (D) In postembryonic stage IV brains, formation of glomeruli (arrow) in the accessory lobes (ALs) occurred after just 2 weekly toxin treatments (first injection at E80%), although the immunocytochemical results showed that serotonin depletion persists throughout this developmental period. Cell clusters 9 and 10 are labeled as well as the olfactory lobe (OL). Anterior is toward the top. Note that (A–C) show left hemibrains and (D) shows a right hemibrain. Scale bars = 100 μm.

the growth of deutocerebral neuropil areas during embryogenesis. These studies are unique in examining the *in vivo* effects of long-term serotonin depletion on the assembly and growth of higher-order synaptic regions, the accessory lobes. The effects on the olfactory lobes parallel those seen on antennal lobe neurons of the moth *in vitro* (Mercer et al., 1996). Prior studies in insects and mollusks examined the influence of serotonin or serotonin reduction on the development of primary sensory areas (the antennal/

olfactory regions) (Oland et al., 1995), or on axonal outgrowth of specific identified neurons (Goldberg and Kater, 1989; Mercer et al., 1996).

The cellular locus of the growth retardation effects in lobster embryos remains to be defined. We are currently testing the effect of 5,7-DHT on the growth and differentiation of the DGNs during embryonic development. It may be that these neurons can self-regulate their growth, as demonstrated for embryonic neurons in *Helisoma* (Diefenbach et al., 1995). Our preliminary findings suggest that the DGNs may, in fact, be stunted by toxin treatment. Since the DGNs send massive inputs to both the olfactory and accessory lobes, a reduction in growth and/or branching of the DGNs could at least partly explain the effects that we see after 5,7-DHT injection. In addition, the specific effects of 5,7-DHT on the input and projection neurons innervating these lobes are being investigated.

Our studies also are significant because the serotonin depletion induced in these embryos is long-term: For at least 2 months after the final injection of 5,7-DHT, serotonin levels are still reduced by ~90% according to HPLC analyses, and immunostaining for serotonin is virtually absent. We are currently testing longer intervals following toxin treatment, to extrapolate these findings to much later periods in lobster development and maturation. One goal is to rear serotonin-deficient juvenile lobsters, raised throughout embryonic and larval life with reduced serotonin, to look at influences on behavior. Serotonin is known to be involved in dominance and aggression in the lobster (Livingstone et al., 1980; Huber et al., 1997), as well as in a long list of other organisms, including mammals (Steklis et al., 1986; Coccaro, 1992; Winberg et al., 1992). It may be feasible, using chemical treatment early in embryonic life, to cause a permanent suppression of serotonin synthesis. It will then be possible to study aggressive behaviors and dominance status of juvenile lobsters that have never had normal serotonin levels.

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