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Distribution of dopamine-like immunoreactivity suggests a role for dopamine in the courtship display behavior of the blue crab, *Callinectes sapidus*

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Abstract. Injection of dopamine initiates a posture in the blue crab, *Callinectes sapidus*, identical to courtship display behavior of the male crab. The threshold for proctolin-induced rhythmic components of courtship display is lowered in preparations when dopamine is co-applied with proctolin. To elucidate the anatomical substrate of this behavior, immunocytochemistry was used to map dopamine-immunoreactive neurons. Courtship display is sex-specific, and dependent on the hormonal, developmental, and seasonal state of the animal. We compared the distribution of dopamine-like immunoreactivity between adults and juveniles of both sexes across seasons, with hormonal alteration, and with the distribution of proctolin-like immunoreactivity. Dopamine-like immunoreactivity was found throughout the nervous system in identical patterns between the sexes and hormonal states. Differences were found between juveniles and adults that are not obviously correlated with the development of behavior. Two areas of staining were of interest: neurites that longitudinally traverse and terminate in the posterior ventral nerve cord, and a neuron in the esophageal ganglion that has projections to the pericardial organ. The results do not suggest that proctolin-like and dopamine-like immunoreactivity co-localize, but in the subesophageal ganglion there was a region of close proximity.

Key words: Dopamine – Invertebrate CNS – Immunocytochemistry – Biogenic amine – *Callinectes sapidus* (Crustacea)

Introduction

Dopamine has been identified chemically in several invertebrate systems (Cooke and Goldstone 1970; Kushner and Maynard 1977; Barker et al. 1979) and localized with histochemistry in a few others (Maynard and Welsh 1959; Siwicki et al. 1987; Cournil et al. 1994). More recently, functional relationships between the presence of dopamine and regulation of behaviors in invertebrate animals have been shown. In jellyfish, dopamine has effects on swimming motor neurons (Anctil 1989; Chung and Spencer 1991). Identified dopamine-like immunoreactive (DIR) cells in the lobster (*Homarus gammarus*) play a role in initiating swimmeret beating (Barthe et al. 1989). In the shore crab (*Carcinus maenas*), dopamine is correlated with increased activity in the movements of the scaphognathite (Berlind 1977) and was shown to cause increases in bursting in motor neurons involved in ventilation (Rajashekhar and Wilkens 1992). In several species of crustaceans, dopamine has excitatory effects on the cardiac ganglion and on the stomatogastric system (for reviews see Beltz and Kravitz 1986; Beltz 1988; Harris-Warrick et al. 1988; Cournil et al. 1994). When injected into freely moving blue crabs (*Callinectes sapidus*), dopamine and the peptide proctolin produce several behaviors that are often seen within a known context – the courtship display (CD) behavior of the male blue crab (Wood et al. 1995). Demonstration of an anatomical substrate for the modulation of CD by dopamine is an important step in revealing the actions of these neuromodulators in the production of the CD behavior.

Male CD behavior is initiated naturally by a pheromone released by the female crab (Gleeson 1980). Hormonal alteration of the male via termination of blood

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Abbreviations: CD, courtship display; *DIR,* dopamine-like immunoreactivity; *dopamine Ab,* anti-dopamine serum; *Ab,* antibody; *THIR,* tyrosine hydroxylase-like immunoreactivity; *PO,* pericardial organ; *re,* retina; *tm,* terminal medulla; *on,* olfactory neuropil; *pt,* protocerebral tract; *sg,* sinus gland

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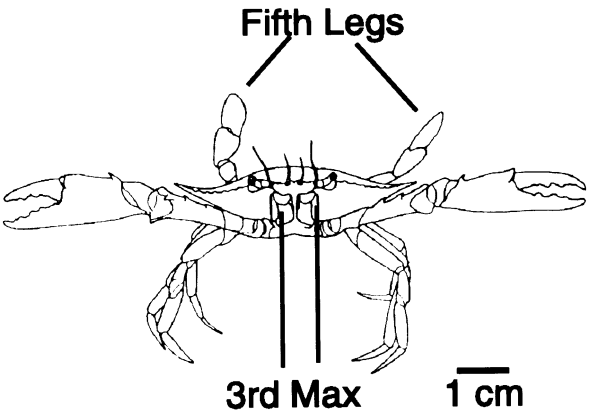

Fig. 1. The courtship display posture of the male blue crab (see text). *Scale bar:* 1 cm

flow to the eyestalk can lead to spontaneous production of CD (Gleeson et al. 1987). During courtship the male crab assumes a stationary posture with the walking legs fully extended and the chelae extended laterally (Teytaud 1971); this posture is seen in agonistic contexts as well (Jackowski 1974). Rhythmic behaviors associated with CD consist of the extension of the third maxillipeds with the flagella of the maxillae (and probably the scaphognathite) beating rhythmically to produce a 'stream' of water currents moving anteriorly away from the mouthparts (Wood et al. 1995), and the most characteristic feature of the CD behavior – the rhythmic waving of the fifth legs anterodorsally above the carapace (Teytaud 1971; Wood and Derby 1995; Fig. 1).

Behavioral experiments have shown that injection of dopamine into freely-moving blue crabs evokes an 'aggressive' posture identical to that produced during CD. Similarly, proctolin injected into crabs leads to rhythmic waving of the swimming legs as in CD behavior. Injected proctolin has differential effects on CD behavior depending upon the sex, season, and the reproductive developmental status of the animal (Wood et al. 1995). Our primary interests in this system are the mechanisms underlying neuromodulation of the fifth leg waving behavior.

In reduced preparations, it was demonstrated that dopamine perfused jointly with proctolin lowers the threshold for proctolin-induced rhythmic waving of the fifth legs (Wood 1995). As a first step in identifying neurons that might contain dopamine, and therefore, play a role in modulating both components of CD behavior, we have localized DIR in the central nervous system of the blue crab in whole-mount tissues. Because there is a joint effect of proctolin and dopamine, we have compared the distribution of DIR with that previously established for proctolin-like immunoreactivity (Wood 1993; Wood et al. 1996) to identify cells that might potentially modulate CD behavior by releasing both substances. It was also of interest to determine whether there are DIR-positive neurons with intersegmental projections through the ventral nerve cord. Interneurons have been identified in both crayfish (Larimer and Jellies 1983) and lobsters (Harris-Warrick 1985) that are involved in 'commanding' abdominal postures in these animals. The axons of these neurons descend the esophageal connectives and project into the thoracic and abdominal ganglia of lobsters or crayfish. Neuromodulatory interneurons containing serotonin have been described in the lobster that appear to release serotonin into circulation as a pathway for modulation of behavior (Beltz and Kravitz 1983; Kravitz 1988). Neurons displaying DIR that have projections into ventral nerve cord of the crab or that release dopamine into the circulation near the ventral nerve cord are of special interest as potential effectors or modulators of CD behavior.

Hormones or other humoral factors have been shown to contribute to the acquisition of cellular neurotransmitter phenotype in other model systems (Eränkö et al. 1972; Fukada 1980). Since juvenile crabs must undergo changes in reproductive physiology before adult sexual behavior may be expressed, it is possible that hormonal changes associated with maturation might contribute to concomitant changes in behavior by virtue of changes in the transmitter content of particular neurons in the blue crab. This possibility was examined also by comparing animals that were hormonally altered by eyestalk ligation with unligated males. Since the behavioral responses to dopamine and proctolin have a seasonal component, it was hypothesized that seasonal changes in the expression of dopamine may underlie the decreased tendency to produce CD behaviors. The potential for seasonal differences in the detection of DIR staining was examined across development, gender, and hormonally altered groups.

Materials and methods

Animals

Blue crabs, *Callinectes sapidus*, were obtained from the Whitney Laboratory, St. Augustine, Fla., USA, and from the University of Georgia Marine Institute, Sapelo Island, Ga., USA. Animals were maintained in laboratory aquaria with recirculating artificial seawater at 20° C. Specimens were judged to be juvenile (reproductively immature), adult male, or female, based on features of external morphology (Pyle and Cronin 1950; Haefner 1988).

Immunocytochemistry: dissection and fixation

Central nervous systems were dissected from animals and pinned out in Sylgard-coated dishes under physiological saline (Mulloney and Selverston 1974; modified as by Hamilton and Ache 1983). Crabs were anesthetized by cooling on ice for 10–20 min before dissection. Tissues to be stained with anti-dopamine serum (dopamine Ab) were fixed with 2% glutaraldehyde with 1% sodium meta-bisulfite in phosphate-buffered saline (0.462 M NaCl, 0.016 M KCl, 0.1 M Na_2PO_4, pH 7.6) at 4° C overnight. Tissues to be stained with anti-tyrosine hydroxylase serum were fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). The pH of the fixative solution was more critical to the quality of the DIR staining than to tyrosine hydroxylase-like immunoreactivity (THIR). No DIR staining was seen when the pH was below 7.4, and staining was most distinct when pH was 7.6–7.8. It was also critical in fixation of tissues to be stained with dopamine Ab that the fixative contain 1% sodium meta-bisulfite: DIR staining was not observed when sodium meta-bisulfite was omitted from incubating solutions. Double-labelling experiments with the dopamine and proctolin Abs were not successful due to necessary differ-

Table 1

Animal size	Ab	Fixation	Rinses	1° Ab	2° Ab	ABC
Below 2 cm	DA	5–9 h	4 over 6 h	36 h (1: 500)	18 h (1:100)	12 h
2–5 cm	DA	10–12 h	6 over 8 h	48 h (1:200)	18 h (1:100)	16 h
Above 5 cm	DA	14–16 h	6 over 8 h	72 h (1:200)	24 h (1:100)	24 h
Below 2 cm	TH	6 h	4 over 6 h	36 h (1:500)	18 h (1:100)	12 h
2–5 cm	TH	6 h	6 over 8 h	48 h (1:300)	18 h (1:100)	16 h
Above 5 cm	TH	10–16 h	6 over 8 h	48 h (1:300)	24 h (1:100)	24 h
Sectioned	DA/TH	na	3 over 1.5 h	4 h (1:500)	1 h (1:200)	1 h

DA, monoclonal Ab for dopamine; TH, polyclonal Ab for tyrosine hydroxylase

ences in the fixation procedures such as the addition of glutaraldehyde with sodium meta-bisulfite. The duration of fixation was necessarily different between animals of varying sizes because the length of fixation proved to be a critical variable in the quality of DIR staining (Table 1). The duration of fixation was not as critical a variable for THIR staining, but animals below 5 cm in width were not fixed for longer than 6 h. Fixative was injected into the eyestalks of all animals before beginning dissection to improve the integrity of the tissue. After fixation, tissues were rinsed during desheathing with phosphate-buffered saline (see Table 1) and Triton X-100 (0.5%; with 1% sodium meta-bisulfite for dopamine Ab tissues). The desheathed CNS was processed for immunocytochemistry staining as whole mounts. Tissues to be sectioned were not desheathed and were cut with a cryostat into 32 µm sections. Before sectioning, the tissues were cryo-protected by incubation in phosphate-buffered saline with successively increasing concentrations of sucrose (10%, 20%, 30%; Beltz and Burd 1989).

Antisera and control experiments

A monoclonal dopamine Ab (conjugated to bovine serum albumin with glutaraldehyde from rat, Eugene Tech. International, Allendale, N.J., USA) and a polyclonal tyrosine hydroxylase Ab (from rabbit, Chemicon International, Temecula, Calif., USA) were used in these experiments. Dopamine Ab was tested for specificity by pre-incubation with synthetic dopamine (Sigma; 0.1 mg of dopamine per ml of Ab; Beltz and Burd 1989). In addition, dopamine Ab preadsorbed by octopamine, L-DOPA, tyrosine, norepinephrine, epinephrine, or BSA did not decrease or alter the pattern of staining from that seen when dopamine Ab alone was used. Initial staining attempts revealed no excessive background staining once titration controls for primary antibody concentrations were completed (Table 1). Injection of animals with colchicine solution (0.015 mg/g body weight) did not improve staining appreciably. Staining of tissues with secondary antibodies alone (for use with dopamine Ab, biotinylated goat anti-rat (IgG), Jackson Laboratories; for use with tyrosine hydroxylase Ab, biotinylated goat anti-rabbit (IgG), Jackson Laboratories) and with avidin-biotin complex (ABC, Vectastain kit by Vector Laboratories) alone was performed. Rinses of tissues before and during antibody incubation included 0.5% Triton X-100, and 5% normal goat serum.

Ten CNS whole-mounts were stained for each of the groups examined in this study: adult males, adult females, juveniles less than 2 cm in width, juveniles between 2 and 5 cm in width, and juveniles larger than 5 cm in width. Four adult nervous systems were sectioned and used as titration controls for dilution series of the primary antibodies. These sectioned tissues also served as a precaution against the possibility that antibodies might not fully penetrate the thickest tissues such as the average adult crab ventral nerve cord (up to several hundred micrometers in thickness). The term ventral nerve cord is used here to signify the fused segments of the subesophageal, thoracic, and abdominal ganglia.

Immunocytochemistry protocols

A three-step avidin-biotin-peroxidase labelling method was used. The protocols for juvenile and adult animals were necessarily different because of thickness differences in nervous tissues (Table 1). This method was adapted from Beltz and Burd (1989) and was identical for both primary antisera except where noted. The protocol consisted of incubation of tissues in phosphate-buffered saline with 0.5% Triton X-100 with 10% normal goat serum (and 1% sodium meta-bisulfite for dopamine Ab) for 1–2 h before incubation in primary antibodies. Primary antibody was diluted with phosphate-buffered saline with 0.5% Triton X-100 and 5% normal goat serum (and 1% sodium meta-bisulfite for dopamine Ab) and final dilution depended on the animal size (Table 1). After the entire incubation protocol with antibody was completed, whole-mount tissues were then rinsed in phosphate-buffered saline two times over 15 min and incubated with diaminobenzidine [DAB (Vector); used at 0.005%] for 30 min before reaction with H_2O_2 (0.003%) for 8–15 min. Sectioned tissues were not pre-incubated with DAB but were incubated in DAB/H_2O_2 solution for 1–3 min. All tissues were dehydrated in an ethanol series and cleared in either xylene or methyl salicylate. The whole-mount tissues were mounted on depression slides in Permount and photographed using an Olympus microscope. A camera lucida system was used for preparing tracings of tissues. Anatomical nomenclature used to identify groups of cell bodies and regions of neuropil in the brain and optic ganglia is from Sandeman et al. (1992), for the ventral nerve cord from Maynard (1961).

Results

Staining with both the dopamine Ab and the tyrosine hydroxylase Ab was found throughout the nervous system of both adult and juvenile blue crabs, and the staining was consistent and reproducible across many preparations. The staining pattern exhibited by the tyrosine hydroxylase Ab was almost identical to that seen with dopamine Ab. The number of cell bodies and projecting fibers, and their positions relative to identified cell body clusters or neuropils were identical, with the exception that longer neurites stained in THIR experiments tended not to stain along their entire length with the consistency that DIR fibers did. The staining pattern for both antibodies was bilaterally symmetrical within the nervous system with a few exceptions of unpaired cell bodies that consistently stained (see sections below). The specificity controls showed that staining with the dopamine Ab was blocked by dopamine preadsorption of the primary antibody and not blocked by BSA preadsorption. Application of secondary antibodies or ABC solution alone resulted in preparations with no patterned staining and very low background.

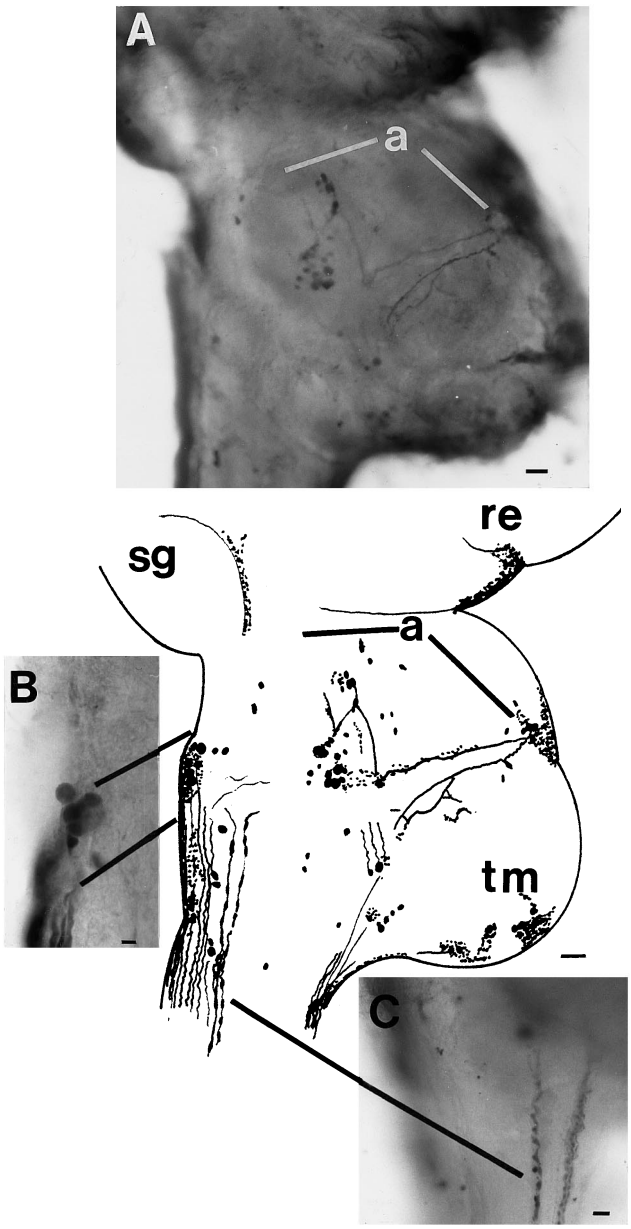

Fig. 2A–C. DIR staining of the eyestalk ganglia of a male blue crab. *re*, Retina; *tm*, terminal medulla; *sg*, sinus gland. **A** Dorsal view of a whole-mount eyestalk preparation featuring stained cell bodies and neurites in the *tm* (indicated by *a* in micrograph and in camera lucida drawing of the ganglia). Camera lucida tracing of all staining (DIR/THIR) observed in the eyestalk. The *right side* of the drawing represents the lateral region of the eyestalk; *a* refers to area labelled in both drawing and micrograph. **B** Higher magnification view of cell bodies on the medial edge of the *tm* with neurites projecting posteriorly into the optic tract. **C** Neurites from the medial region of the optic tract showing 'beaded' staining pattern in these axons. *Scale bars:* **A** 50 μm. *Scale, camera lucida:* 50 μm, **B** 10 μm, **C** 10 μm

The brain: optic ganglia and lateral protocerebrum

DIR and THIR were seen in several regions of the optic ganglia and lateral protocerebrum: in the lamina, the optic tract, internal medulla and terminal medulla (Fig. 2A, B). A diffuse cluster or band of small cell bodies (less than 8 μm in diameter) was present in the lamina (Fig. 2B). The majority of cells that stained were in the internal and terminal medullae with some neurites staining in neuropil regions and others projecting into the optic tract (Fig. 2A, B). There was a range of 20 to 26 axons that stained positively for DIR in the optic tract (Fig. 2B). It could not be strictly determined which of these axons were associated with cell bodies in the terminal medulla but at least some of these projections appeared to descend the optic tract as determined by the degree of branching seen as they enter the brain. On the medial margin of the medullary regions, posterior to the sinus gland, a cluster of 7 to 10 cell bodies appeared to have unbranching projections toward the optic tract (Fig. 2B). These apparently descending axons projected on the medial margin through the optic tract, perhaps as part of the protocerebral tract (Fig. 2A, B). This group of axons were among those that projected into the anterior medial protocerebral neuropil in the ventral protocerebrum (Fig. 3E). Those DIR fibers that appeared to originate in the lateral region of the terminal medulla or those that were visualized near the midline of the optic tract (Fig. 2C) projected across the brain dorsally and posteriorly to those originating on the medial edge (Fig. 3A, D). The projections from the lateral and midline regions of the optic tract stained densely and the stain appeared to be 'beaded' along their length (Fig. 2C).

The brain: median protocerebrum, deutocerebrum, and tritocerebrum

As mentioned above, there were fibers that stained on the medial margin of the optic tract that terminated in the neuropil of the most anterior regions of the brain as viewed from the ventral side (Fig. 3E). The staining of these fibers was often very intense, and because of the density of the stain, they were difficult to resolve individually. Fibers in the lateral regions of the optic tract that projected into the brain appeared to terminate in a region close to or within the olfactory globular tract. Fine varicosities apparent in the staining of this region indicate potential synaptic terminals (Fig. 3A, D).

Several pairs of cell bodies in the ventral clusters stained for DIR/THIR. One cell stained on the posterior border of each anterior cell body cluster. Three DIR/THIR cell bodies were located in the ventral paired medial cluster, and one cell in each of the ventral paired posterior clusters (Fig. 3B, D). From viewing the dorsal side of the brain, one pair of cell bodies stained with DIR/THIR were probably part of the dorsal posterior cluster (Fig. 3C, D). Two pairs of stained fibers apparently ascend the lateral dorsal region of each of the esophageal connectives and terminate in a neuropil region where the connectives meet the brain (Fig. 3C, D). Another apparent 'pair' of stained fibers located in the esophageal connectives were in fact a single projection that originated from a DIR cell body in the esophageal ganglion (the L-cell, see Fig. 4B, C). This stained projection passes anteriorly through the ipsilateral connective to the tritocerebrum where it turned posteriorly and returned to the esophageal ganglion (Fig. 4A, B, D).

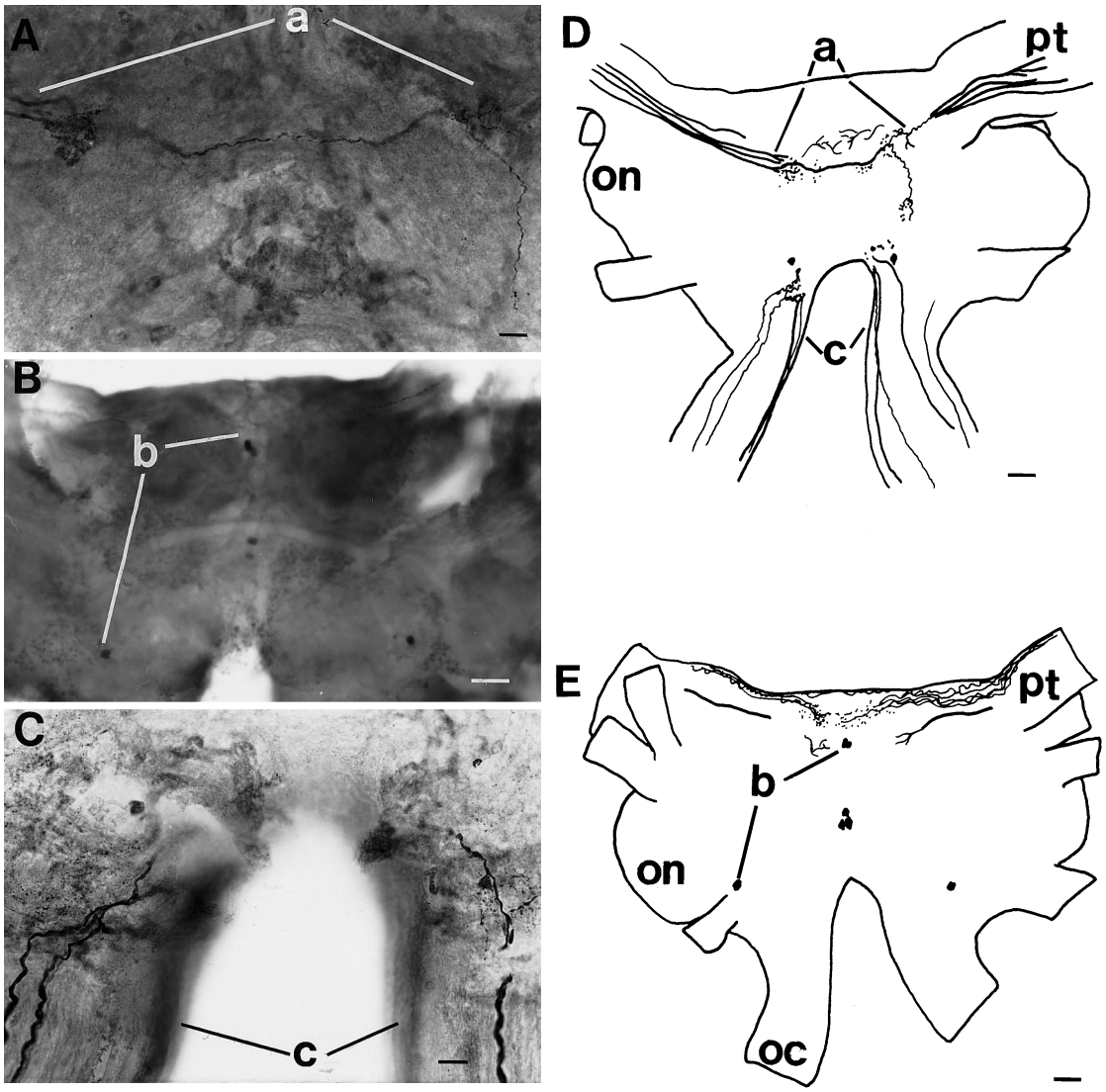

Fig. 3A–E. Staining in the brain of a female blue crab. *on*, Olfactory neuropil; *pt*, protocerebral tract or optic tract; *oc*, esophageal connectives. **A** DIR fibers apparently descending the optic tract and terminating in the brain. *Scale bar:* 100 μm. **B** DIR cell bodies that stain in the ventral regions of the brain. *Scale bar:* 100 μm. **C** DIR in dorsal cell bodies and in the *oc*. *Scale bar:* 100 μm. **D** Camera lucida tracing of DIR/THIR in brain of the male crab, dorsal view. *a* Area shown in panel **A**; *c* area shown in panel **C**. **E** Camera lucida tracing of DIR/THIR in brain of the male crab, ventral view. *b*, Area shown in panel **B**. *Scale bars:* **D, E** 250 μm

The ventral nerve cord

With the exception of the esophageal ganglion, the ganglia of the ventral nerve cord are fused together but occupy distinct segmental regions (Maynard 1961; Wood 1993). Of the six to eight pairs of fibers that were stained in the esophageal connectives, three or four pairs projected as far as the most posterior segment (Fig. 5A, C). One pair of these projections appeared to terminate in a region very close to a large cluster of identified neurosecretory cells (C-cells; see Maynard 1961) that displayed proctolin-like immunoreactivity (Wood 1993; Wood et al. 1996; Fig. 5B). Numerous DIR fibers traversed the neuropil of the third thoracic segment (Fig. 5A, D). Near the opening in the ganglion where the sternal artery attaches to the sheath, numerous fibers appeared to partially encircle the sternal opening in a plane that is ventral to the fibers that project from the esophageal connectives (Fig. 5C, D).

In the ventral cell body clusters associated with each segment, at least one pair of cell bodies stained in each segment from the first subesophageal through all of the thoracic segments (Fig. 6A, B). These cells are all of the same approximate size (15- to 20-μm diameter) and relative location within each segment (more medial than lateral within each cluster of cells). In the third and fifth thoracic segments there were two pairs of cells that displayed DIR (Fig. 6A, B). There were no DIR-positive neurons in the areas that correspond to the abdominal segments.

Comparison of DIR staining between seasonal, developmental, gender, and eyestalk ligated groups of animals

There were no differences between the staining patterns of adult males and females. Juveniles in the smallest size

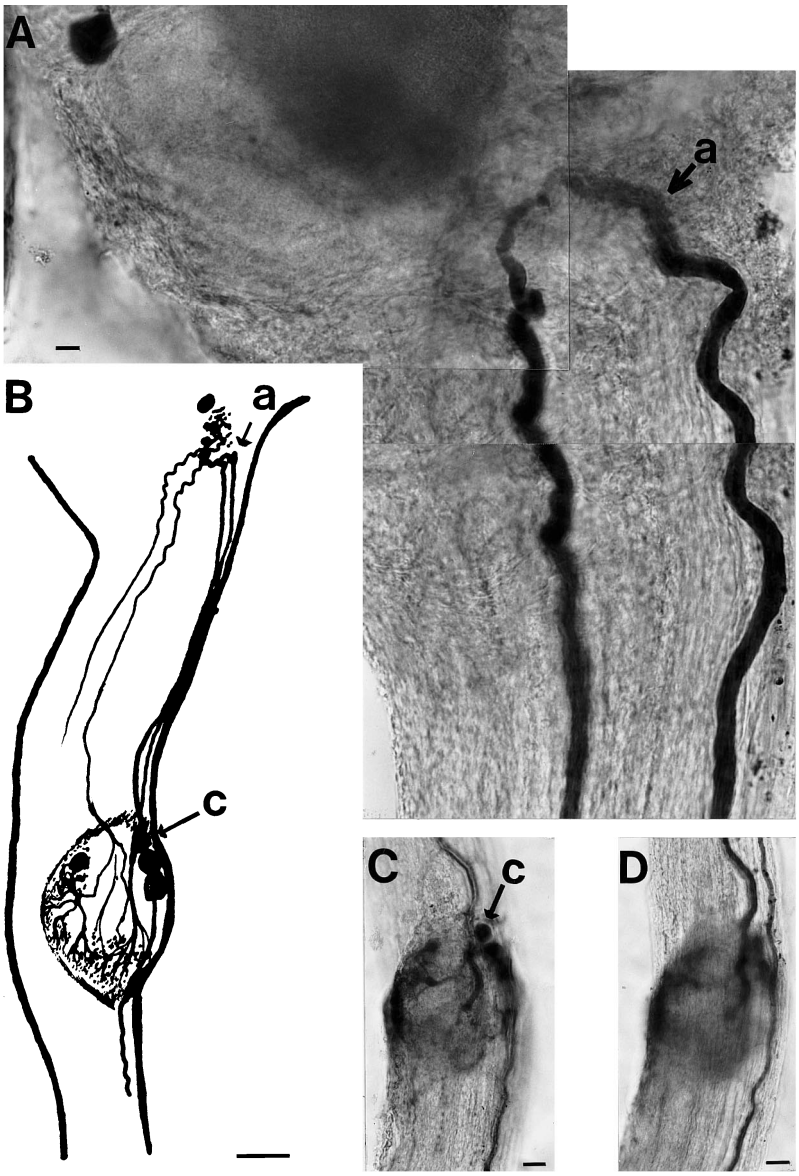

Fig. 4A–D. DIR in the esophageal ganglion and in the esophageal connectives (oc). **A** Cell body in the brain and the looping projection (indicated by *a* with *arrow*) from the L-cell in the esophageal ganglion. *Scale bar:* 10 μm. **B** Camera lucida tracing of all stained cells and neurites in the esophageal ganglion and in the esophageal connectives. *a* Area indicated by *a* in panel **A**; *c*, L-cell shown in panel **C**. *Scale bar:* 100 μm. **C** The esophageal ganglion with the L-cell indicated by *c* with an *arrow*. *Scale bar:* 50 μm. **D** The esophageal ganglion with a projection from the L-cell that exits the esophageal ganglion in an anterior direction then returns in a posterior direction past the esophageal ganglion with a likely termination in the PO. *Scale bar:* 50 μm

range differed from adults only in one general region of the nervous system: an average of 16 fewer cell bodies in various clusters in the terminal medulla were absent in the very smallest of both male and female juveniles. There were typically fewer fibers (8–12) in the anterior protocerebral tract that display DIR/THIR in the juvenile. There was no difference between male and female juvenile animals. There also were no differences anywhere in the nervous systems of male animals that were eyestalk ligated (n=8) versus those males that were not ligated, and no consistent differences were seen between animals across seasons (adults n=10, juveniles n=10, for each season for 3 years).

Discussion

The consistent patterns of staining by a monoclonal antibody directed against dopamine and an antiserum directed against the enzyme necessary for catecholamine synthesis, tyrosine hydroxylase, provide evidence for the presence of dopamine in the nervous system of the blue crab. The fact that staining was blocked by preadsorption controls and not by preadsorption with catecholamines other than dopamine suggests that the dopamine Ab binding is specific to a substance with a structural similarity to dopamine. This result was further strengthened by the fact that titration controls (see Methods) performed on both sectioned and whole-mount tissues showed no staining of any areas below a definite primary antibody threshold concentration.

The fact that our results depended upon two very well-characterized antibodies decreases the likelihood that there was cross-reactivity with other similar antigens. It is, of course, always possible that an antigen with structural similarity to dopamine might stain in some regions of the nervous system. Final assessment of the presence of dopamine in any particular cell in the

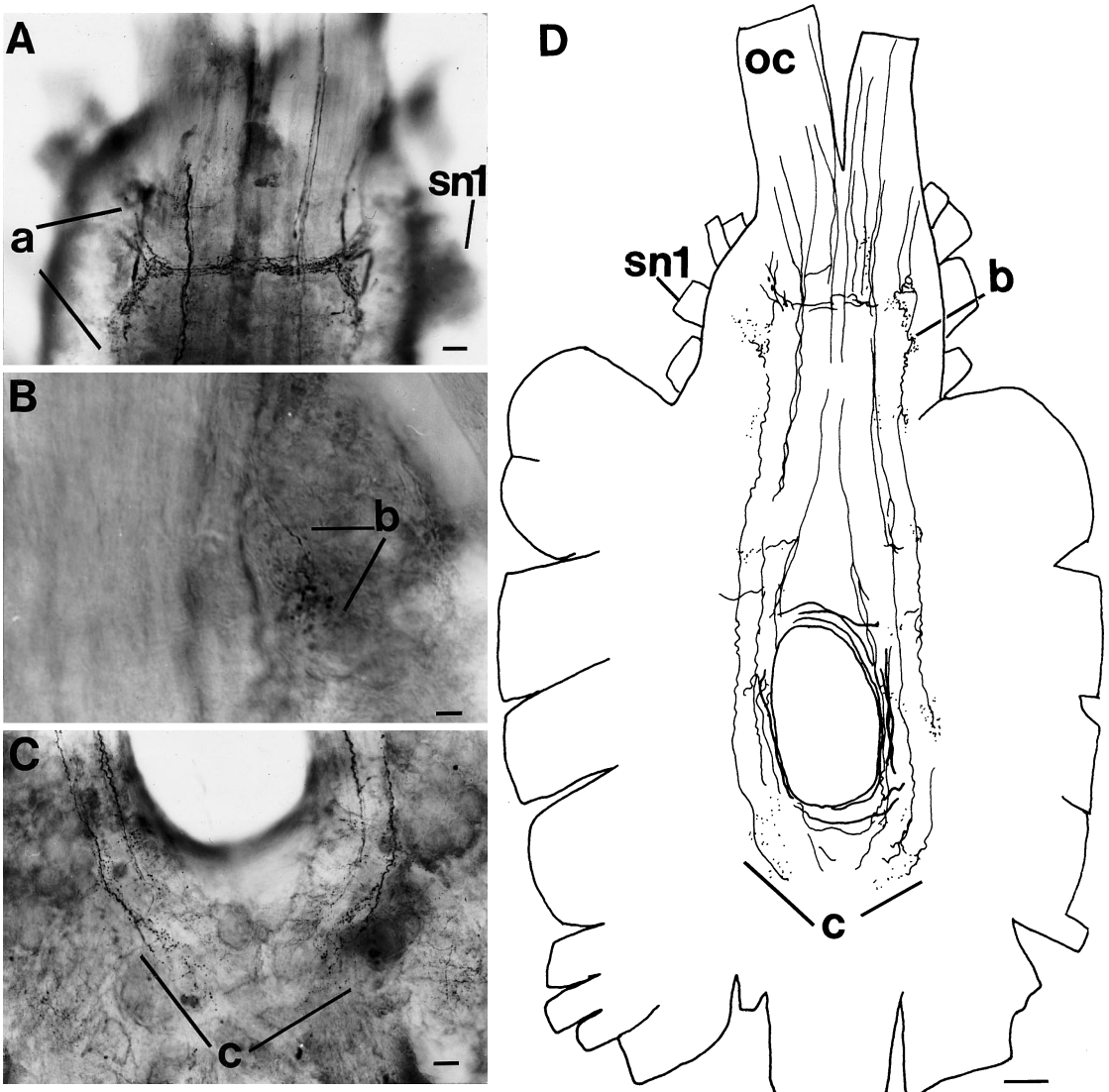

Fig. 5A–D. Dorsal view of DIR in the ventral nerve cord of the blue crab. *oc*, Esophageal connective; sn_1, the first nerve of the fused subesophageal ganglia. **A** DIR fibers that descend the *oc*. This photograph is represented in the upper one-third of panel **D**. Neurites indicated by *a* are those in panel **D. B** DIR-stained fiber that has apparent terminals that are very near the *sn1*. The neurosecretory C-cells many of which display proctolin-like immunoreactivity are located near this area and have axons that project through the *sn1*. Label *b* indicates an area of possible synaptic terminals (see panel D for low magnification view of same region labelled as *b*). **C** DIR fibers surrounding the opening in the ganglion for the sternal artery. This photograph is represented in the lower one-third of panel **D**; neurites in panel **C** are labelled as *c* in panel **D. D** Camera lucida tracing of a dorsal view of whole-mount ventral nerve cord stained for DIR/THIR. *Scale bars:* **A** 200 μm, **B** 20 μm, **C** 100 μm, **D** 200 μm

blue crab CNS remains to be defined by chemical analysis. However, the existence of authentic dopamine in other decapod crustaceans is very likely in at least two areas of the neurohormonal system: an identified dopaminergic cell in the esophageal ganglion (the L-Cell; Kushner and Maynard 1977), and the pericardial organ (PO) have both been shown to display aminergic histofluorescence, to synthesize and release dopamine (Maynard and Welsh 1959) and to contain an amine chemically indistinguishable from dopamine (Cooke and Goldstone 1970; Barker et al. 1979; Kushner and Barker 1983; Cournil et al. 1984). The dopaminergic L-cell in the esophageal ganglion has very distinctive morphology: the L-cell has a neurite in the esophageal connective that projects to the posterior tritocerebrum and reverses direction to return to the esophageal ganglion. A cell with identical morphology and relative location stained in the blue crab using both the monoclonal dopamine Ab and the tyrosine hydroxylase Ab. The L-cell identified in the lobster (*Homarus americanus*) has been shown to contain proctolin as well as dopamine (Siwicki et al. 1987) but this was not the case in *Callinectes* where the L-cell does not stain for proctolin (Wood 1993; Wood et al. 1996). It was reported in another lobster (*Homarus gammarus*) that the projection of the L-cell posteriorly past the esophageal ganglion (or commissural ganglion) was not verified by DIR staining. This projection is clearly evident in the blue crab (Fig. 4D).

The presence of THIR indicates that the first step necessary for synthesis of L-DOPA from tyrosine may

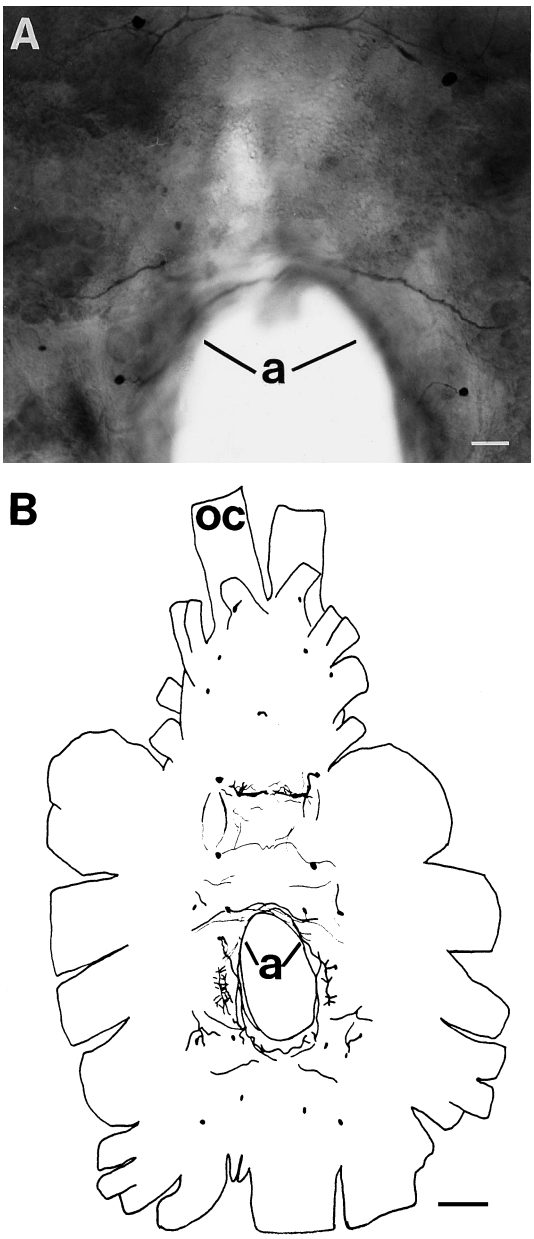

Fig. 6A, B. Ventral view of ventral nerve cord DIR staining. *oc*, Esophageal connectives. **A** Ventral view of stained ganglion showing paired cells in two thoracic segments anterior to the sternal artery opening. *a* Opening for the sternal artery in both panels **A** and **B**. **B** Camera lucida tracing of whole-mount ventral nerve cord stained with DIR/THIR. *Scale bars:* **A** 100 μm, **B** 300 μm

take place in the region that stains. The fact that identical staining patterns occur with both antibodies indicates that conversion of L-DOPA to dopamine likely occurs. These results do not directly indicate whether norepinephrine or epinephrine are then synthesized from dopamine; however, if norepinephrine was synthesized from dopamine, then dopamine concentrations would be lower in those locations and the probability of achieving identical staining for dopamine and tyrosine hydroxylase would be decreased. Moreover, it is unlikely that dopamine is converted to norepinephrine because norepinephrine or epinephrine have not been found in crustaceans (Barker et al. 1979). There was no apparent cross-reactivity of the dopamine Ab with octopamine, and it has been determined in lobster that the octopamine synthetic pathway diverges from the dopamine pathway at the first step, as tyramine β-hydroxylase is the first enzyme to convert tyrosine to tyramine (Wallace 1976). For this reason we do not believe that DIR-staining regions necessarily contain octopamine.

While dopamine has been localized and has demonstrated physiological effects in some particular ganglia, the presence of dopamine in some other crustaceans (the lobster *Homarus americanus*, or the crayfish *Procambarus clarkii*) appears not to be widely distributed as it is in *Callinectes*. The L-cell (Siwicki et al. 1987) and the stomatogastric ganglia (Harris-Warrick et al. 1988) are the only reported locations for dopamine in the lobster *Homarus americanus*. A study conducted for the related species *Homarus gammarus* found a wider distribution of DIR over most segments of the nervous system (Cournil et al. 1994). Other related species of crabs may well show a CNS distribution of dopamine similar to the blue crab but immunocytochemical studies of other species have not been reported.

In the crab, cell bodies in the lamina, external medulla, internal medulla, and terminal medulla stained intensely for DIR/THIR. In the adult, fibers were stained in the optic tract, some of which appeared to be more specifically in the protocerebral tract. These fibers appeared generally to project through the eyestalk but the origin of these projections was not resolved. The staining patterns for adults and juveniles were consistent within each size grouping, but there were some differences in the average number of cells that stained and the intensity of staining between the juvenile and adult animals. The terminal medulla had fewer DIR cell bodies and the protocerebral tract had fewer projections in juveniles versus adults. These differences had no relationship to the sex of the animal. It is possible that the levels of dopamine or tyrosine hydroxylase in the very young juvenile brain were insufficient to be detected by immunocytochemistry. In the developing lobster, proctolin-like immunoreactivity appears at a later time in development than does serotonin-like immunoreactivity (Beltz et al. 1990). It is also possible that dopamine in this region of the nervous system appears late in development, but there is no correlation between the appearance of dopamine in these regions of the nervous system and the response of crabs to injected dopamine with the behaviors that we have studied.

Given our interest in the modulation of these behaviors as they are performed in the context of CD behavior of male blue crabs, any DIR cells that have projections into the posterior portion of the ventral nerve cord or the potential to release dopamine into the circulation near the ventral nerve cord are of special interest as potential effectors or modulators of CD behavior. Previous studies with blue crabs have identified a large cell in the esophageal ganglion that has a projecting neurite in the esophageal connectives and projections to the PO (Kushner and Maynard 1977), where presumably dopamine may be released into the circulation to modulate the nervous

system. We have located a neurite showing DIR in the esophageal connectives with a morphology that was very similar to the identified dopamine cell that has a projection into the PO. Identified interneurons descending the esophageal connectives in other crustaceans play a role in regulation of postures (lobsters, Harris-Warrick 1985; crayfish, Larimer and Jellies 1983). The fibers that stain for DIR and that appear to descend the esophageal connectives are by virtue of their anatomical location candidate interneurons for involvement in the process of initiation of the CD-like posture.

Injected proctolin and dopamine are correlated with initiation of the CD behavior in freely moving animals (Wood et al. 1995). Proctolin initiates rhythmic CD behavior in both the freely moving animal (Wood et al. 1995) and in a reduced preparation, especially when coupled with stimulation of descending projections in the esophageal connectives (Wood 1995). Dopamine was shown to decrease the threshold concentration of proctolin needed for initiation of rhythmic waving in the reduced preparation (Wood 1995). Whether dopamine and proctolin are acting as neurohormones or more locally as transmitters, this result implies that the target tissues involved increase their sensitivity to proctolin in the presence of dopamine. If proctolin and dopamine are acting as local transmitters or modulators, then areas of the ganglion where proctolin-like immunoreactivity and DIR might be in close proximity would be of interest for further investigation. Co-localization of proctolin-like and dopamine-like immunoreactivity was not technically possible (see Methods). There was no DIR staining in neurites or cell bodies that appeared to be in position to be both DIR and proctolin-like immunoreactivity containing cells. The subesophageal region of the ventral nerve cord showed the presence of a DIR neurite that appeared to have terminals very near cells that display proctolin-like immunoreactivity (Wood 1993; Wood et al. 1996). These proctolin-like immunoreactive cells have been previously identified by Maynard (1961) as the C-cells and were shown to have projections to the PO. The C-cells display patterned bursting activity coincident with initiation of CD in a reduced preparation, suggesting further that these cells might play a role in neuromodulation of CD (Wood 1995).

Dopamine has been suspected to function as a neurohormone because of its presence in the POs of several decapod crustacean species (for review, see Beltz and Kravitz 1986) and because of the cardioacceleratory effects of dopamine released from the PO onto the cardiac ganglion of the crab (Miller et al. 1984). An example of a neurohormonal function for dopamine is seen in the stomatogastric system of crustaceans where dopamine appears to play a role in the reconfiguration of the apparently multifunctional stomatogastric circuitry (Flamm and Harris-Warrick 1986; Harris-Warrick and Johnson 1989).

Previous investigators have suggested that when a neuroactive substance functions as a neurohormone by release into the general circulation, the point of synthesis may not always be coincident with areas of accumulation of the product (Barker et al. 1979). In the case of the distribution of THIR/DIR within the cell bodies of the crab CNS that were investigated here, it generally appears that the synthesis of dopamine may be coincident with the site of release. There were no sites in the CNS that would allow for release of dopamine into the general circulation other than the L-cell which has been described to have axonal projections to the PO in crustacean species. The identification of a posterior projection of the L-cell toward the ventral nerve cord suggests that in the blue crab there may be a dopaminergic projection from the esophageal ganglion to the PO.

Collectively, these data suggest several possibilities that might link the currently known anatomical localization of dopamine with the effects of dopamine on CD behavior. (1) The effects associated with dopamine may occur by synaptic release of dopamine from descending fibers in the esophageal connectives. Release of dopamine from this site could modulate release of proctolin (for example, at least one descending DIR fiber appears to synapse near the proctolinergic C-cells). Alternatively, dopamine could modulate sensitivity of neurons to proctolin. (2) Dopamine may be released by descending fibers that may be more directly involved in initiation of postural components of CD by acting as a neurotransmitter for ‘command’ elements in the motor system. (3) Dopamine may be released from the PO and act as a circulating neurohormone, thus influencing the C-cells or neurons of the pattern generating circuitry of the fifth legs and resulting in modulation of the action of proctolin on rhythmic leg waving (again by either increasing release or by increasing tissue sensitivity to proctolin). It is important to note that these hypotheses concerning the role of dopamine in CD behavior are not mutually exclusive; dopamine could be acting through any or all of these pathways, and could act as both a classical transmitter and a neurohormone during the course of typical CD behavior.

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