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Source: Zoological Science, 13(4) : 577-585

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.13.577

ZOOLOGICAL SCIENCE **13**: 577–585 (1996)

Extensive Distribution of Galanin-Immunoreactive Neuronal Systems in a Fresh Water Snail, *Indoplanorbis exustus*, and Partial Coexistence with Choline Acetyltransferase-like Immunoreactivity

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ABSTRACT—Immunohistochemical techniques were employed to investigate the distribution of galanin-like immunoreactivity in the central nervous system (CNS) of the snail, *Indoplanorbis exustus*, and immunoreactivity with an antibody against choline acetyltransferase (CAT) in galanin-immunoreactive somata was also examined. Galanin-immunoreactive (Gal-IR) cells were observed in all ganglia. They were classified into large (50 μm), medium (21 μm), and small (13 μm)-sized neurons and small Gal-IR cells dominated over large and medium Gal-IR cells in number. Gal-IR cells were most abundant in the cerebral ganglion. Several immunoreactive somata were seen in the parietal, visceral, and pedal ganglia, and only a few Gal-IR cells in the pleural and buccal ganglia. Densely arranged Gal-IR fiber bundles were observed in the cerebro-pleural, parieto-pleural and bucco-cerebral connectives. Of the peripheral nerves, the pallial and pharyngeal nerves contained more numerous Gal-IR fibers than other peripheral nerves. By Western blot analysis, the galanin antibody detected approximately 6.1 kDa band, suggesting a pre-form or a long form of galanin. Some Gal-IR cells in the cerebral ganglion and a few in the pleural and parietal ganglia were also immunoreactive for CAT. These results indicate that a distinct neuronal system with galanin or galanin-like peptide is present in the CNS of the snail, and suggest that a part of the galanin neuronal system is cholinergic. The peptide may function as a neurotransmitter/neuromodulator and/or neurohormone in the snail.

INTRODUCTION

Galanin is a biologically active peptide consisting of 29 amino acid residues and was originally isolated from the small intestine of pigs (Tatemoto *et al.*, 1983). Relatively minor variations in amino acid sequences are known among various species of vertebrates (rat, Vrontakis *et al.*, 1987; Kaplan *et al.*, 1988: bovine, Rökaeus and Carlquist, 1988: chicken, Norberg *et al.*, 1991). Human galanin appears to be unique, comprising 30 amino acids possessing an additional nonamidated serine residue as its C-terminus (Evans and Shine, 1991; Bersani *et al.*, 1991; Schmidt *et al.*, 1991). In invertebrates, galanin was biochemically studied only in blowfly (*Phormia terraenovae*), and its galanin appears to be highly alkaline in nature but similar in size to those of vertebrates (Lundquist *et al.*, 1991).

The neuroanatomical distribution of galanin has been well documented in the rat (Skofitsch and Jacobowitz, 1985; Melander *et al.*, 1986), primates (Kordower and Mufson, 1990), opossum (Elmquist *et al.*, 1992), amphibians (Olivereau and Olivereau, 1992), and fish (Olivereau and Olivereau, 1991; Yamamoto *et al.*, 1992). Galanin-immunoreactive (Gal-IR) somata and fibers were widely distributed in the vertebrate central nervous system (CNS), especially in the hypothalamus, Gal-IR fibers being densely located in the median eminence. These profiles suggest that galanin may act as a neurotransmitter, a neuromodulator, or a neurohumoral peptide (Hökfelt *et al.*, 1987; Bartfai *et al.*, 1993). It is of interest that galanin coexists with various neurotransmitter markers and bioactive peptides, including cholinergic markers in the basal forebrain (Melander *et al.*, 1985; Melander and Staines, 1986), tyrosine hydroxylase in the arcuate nucleus (Everitt *et al.*, 1986), adenosine deaminase in the tuberomammillary nucleus (Staines *et al.*, 1986), luteinizing hormone releasing hormone in the diagonal band of Broca (Merchenthaler *et al.*, 1991), dopamine in the median eminence (Nordström *et al.*, 1987), and vasopressin in the supraoptic and paraventricular nuclei (Rökaeus *et al.*, 1988; Gaymann and Martin, 1989; Skofitsch *et al.*, 1989).

The snail CNS has been shown to be a good model for neurobiological studies because of its simplicity and readily accessible localization of neurons (Boer and van Minnen, 1988). However, only *Bulla gouldiana*, a marine gastropod, was studied in regard to the distribution of galanin-like immunoreactivity in the neuronal system (Roberts *et al.*, 1989). This prompted us to investigate in detail galanin-containing

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neuronal systems in a fresh water snail, *Indoplanorbis exustus*, which has long hair-like tentacles and this morphological characteristic may offer good opportunities for analyzing the tentacle-withdrawal reflex. In fact, a closely related species, *Planorbarius* (*Planorbis*) *corneus*, has been utilized to study an experimental-learning paradigm involving the tentacle-withdrawal reflex (Biral *et al.*, 1978a, b). In the present study, we examined galanin-like immunoreactivity in the CNS of the snail and demonstrated its colocalization with choline acetyltransferase (CAT)-like immunoreactivity in a subpopulation of Gal-IR cells of the cerebral ganglion. Furthermore, we analyzed extracts of the CNS by Western blots.

MATERIALS AND METHODS

Indoplanorbis exustus were anesthetized with ethyl m-aminobenzoate methanesulfonate (Nacalai Tesque, Inc., Kyoto, Japan) and the CNS were carefully dissected out with the aid of a stereoscopic microscope. The CNS of six animals were removed with a part of the pharynx and fixed for 16 hr in Bouin-Hollande solution at room temperature. After washing in distilled water, the tissue was dehydrated and wax-embedded according to the conventional method. Horizontal sections were cut at a thickness of 5 μm, and serial sections at intervals of 20 μm were mounted onto slides for galanin immunohistochemistry. Consecutive sections were used for comparison of galanin and CAT immunoreactivities.

Sections on slides were dewaxed in xylene and gradually hydrated with graded alcohol solutions. The sections were then washed overnight in 0.1 M sodium phosphate buffer containing 0.9 % saline (PBS) at pH 7.4 and incubated for 48 hr at 4°C with rabbit anti-rat galanin serum (Peptide Institute, Inc., Osaka, Japan) diluted in PBS containing 0.05% Triton X-100 (PBST) at 1:2,000. After washing in PBST, the sections were incubated for 1 hr at room temperature with biotinylated anti-rabbit IgG (Vector Laboratories, Berlingame, USA) diluted 1:100 in PBST. The sections were then washed again in PBST and incubated for 1 hr at room temperature with avidin-biotin-horseradish peroxidase complex (Vector Laboratories) diluted 1:100 in PBST. After final wash in PBST, the sections were reacted with a 0.05 M Tris-HCl buffer solution (pH 7.4) containing 0.02% 3,3'-diaminobenzidine hydrochloride (DAB) and 0.005% hydrogen peroxide, before being dehydrated, cleared in xylene, and coverslipped with Malinol (Muto Pure Chemicals. Ltd., Tokyo, Japan). Alternate sections were immunohistochemically stained with anti-galanin and goat anti-human placental CAT antibodies (1:5,000; Chemicon International Inc., Temecula, USA). Immunohistochemical controls were carried out by omission of the antiserum in the first incubation, or by incubation with the normal rabbit or the goat serum, or with antiserum preabsorbed with synthetic rat galanin (10 μg/ml; Peptide Institute, Inc.) as the primary antibody. Some adjacent sections were stained with Gomori's paraldehyde-fuchsin. Some immunohistochemically-stained sections were lightly counterstained for 1 min with 0.05% thionine solution buffered with 0.02 M acetic buffer at pH 4.0 for observation of general histology.

For block-staining, the CNS was fixed for 24 hr in PB (pH 6.9) containing 4% paraformaldehyde and 0.2% picric acid and kept for 1 week in PBS containing 0.3% Triton X-100 with several changes of the solution. Then the tissue was incubated for 48 hr at 4°C with anti-galanin serum. After washing with PBST, the tissue was incubated for 24 hr at 4°C with dichlorotriazinyl aminofluorescence-conjugated anti-rabbit IgG (1:100; Chemicon International Inc.), and cleared in anti-fading medium (Valnes and Brandtzaeg, 1985).

For Western blots, CNSs from 20 snails (about 28 mg wet weight) were homogenized in 140 μl of 0.05 M Tris-HCl buffer (pH 7.4) containing 140 units of aprotinin (a protease inhibitor) with a microhomogenizer at 4°C. Then the homogenates were centrifuged at 10,000 xg for 1 hr at 4°C. Equivalent volume of Laemmli's sample buffer (Laemmli, 1970) was added to the supernatant. These samples were analyzed by sodium dodecyl sulfate-polyacrilamid gel (15%) electrophoresis (SDS-PAGE). After SDS-PAGE, separated proteins were transferred to Clear Blot Membrane-p (Atto Corporation, Tokyo, Japan) and probed with anti-rat galanin antibody (1:2,000) after blocking non-specific protein binding with PBS containing 3% bovine serum albumin (BSA). Probed membranes were then incubated with biotinylated anti-rabbit IgG (1:1,000) and avidin-biotin-horseradish peroxidase complex (1:1,000) diluted in PBS containing 3% BSA, and finally reacted with DAB solution containing 0.005% hydrogen peroxide and 0.6% ammonium nickel sulfate for 5-10 min. Rat galanin (Peptide Institute, Inc.) and molecular marker proteins (cytochrome *c*, monomer to hexamer; Oriental East Co., Ltd., Osaka, Japan) were also electrophoresed simultaneously.

RESULTS AND DISCUSSION

General anatomy of the CNS

For general neuroanatomy of the snail, we referred to the description of *Planorbarius corneus* (Sonetti *et al.*, 1990) and *Lymnaea stagnalis* (Bonga, 1970). The CNS of the snail, *Indoplanorbis exustus*, was observed to be composed of five paired ganglia (cerebral, pleural, parietal, pedal, and buccal ganglia) and an unpaired visceral ganglion, being basically same to the CNS of *Planorbarius corneus* (Sonetti *et al.*, 1990). The dorsal body was located anterodorsally on the cerebral commissure in a single mass, not in two masses as in *Lymnaea*. The cerebral ganglion was large and lunar in shape with a small lateral lobe, while the pleural and buccal ganglia were relatively small and round. The left pleural ganglion was situated under the left parietal ganglion, and right pleural ganglion was formed into ring-shape with the cerebral, visceral, and left parietal ganglia. The left parietal ganglion was larger than the right one, in contrast with the larger right parietal ganglion in *Lymnaea*. The pedal and visceral ganglia were relatively large and slightly flattened.

Immunoreactive somata

Gal-IR cells were seen in all ganglia (Fig. 1). Their somata were round, ovoid, or sometimes triangular in shape. Almost all Gal-IR neurons were unipolar with a process directing to the center of the ganglion. They were classified into three types based on their size; i.e., small (7.5-17.5 μm; mean, 12.7 μm in diameter), medium (18.7-25.0 μm; mean, 21.1 μm in diameter), and large (38.4-63.7 μm; mean, 50.3 μm in diameter) neurons. Large and medium Gal-IR cells were a minor population and were located on the peripheral region of the ganglion. On the other hand, small Gal-IR cells were abundant and tended to be situated in the inner area of the ganglion.

Gal-IR cells were seen most abundantly in the cerebral ganglion (Figs. 1 and 2A-C). The cerebral ganglion contained lateral, medial, and anterior Gal-IR cell groups. A few medium and many small Gal-IR cells were seen in each cell group, while only one large Gal-IR cell was observed in the medial Gal-IR cell group of the ganglion in each side (Fig. 1). A few

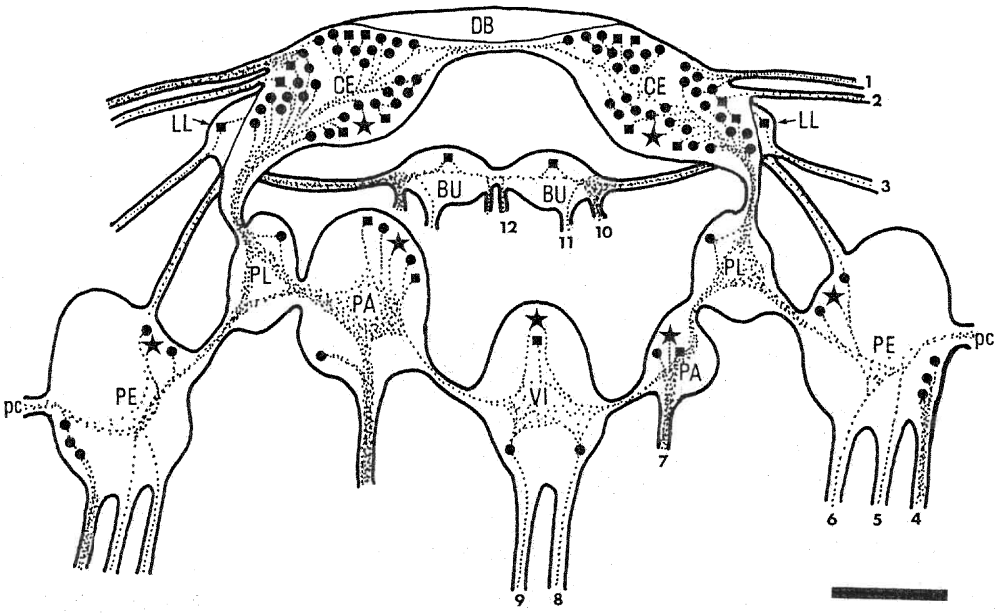

Fig. 1. Schematic diagram of the dorsal view of the CNS of the snail, *Indoplanorbis exustus* showing the localization of Gal-IR cells and fibers. Asterisks, squares, and circles represent large, medium and small Gal-IR cells, respectively. The pedal ganglion (PE) is dislocated for presentation. Abbreviations: BU, buccal ganglion; CE, cerebral ganglion; DB, dorsal body; LL, lateral lobe of the cerebral ganglion; PA, parietal ganglion; pc, pedal commissure; PL, pleural ganglion; VI, visceral ganglion; 1, tentacular nerve (n); 2, frontal lip n; 3, median lip n; 4, inferior pedal n; 5, median pedal n; 6, superior pedal n; 7, pallial n; 8, intestinal n; 9, anal n; 10, pharyngeal n; 11, gastric n; 12, radular n. Scale bar: about 200 μm.

medium Gal-IR cells were seen in the lateral lobe (Fig. 1). One large Gal-IR cell was seen in the medial portion of the left and right parietal ganglia (Figs. 1 and 3A). A few medium and small Gal-IR cells were located in the left parietal ganglion, while the right ganglion contained fewer of both medium and small Gal-IR cells (Figs. 1 and 3A, B). Just a few small Gal-IR cells were seen in the pleural ganglion. In the pedal ganglion, one large and several small Gal-IR cells were seen (Figs. 1 and 3C, D). One large, a few medium and small Gal-IR cells were observed in the visceral ganglion (Figs. 1 and 4C). In the buccal ganglion, a few medium Gal-IR cells were detected on both sides (Figs. 1 and 5C) but no large and small Gal-IR cells were found in this ganglion.

One large Gal-IR cell was observed in each cerebral, parietal, pedal, and visceral ganglion. The number of these cells in individual ganglion could not be underestimated, because the size of large Gal-IR cells was approximately 50 μm and we counted the cell number in 5 μm-thick sections at intervals of 20 μm.

Immunoreactive fibers

Fine Gal-IR fibers were seen in the centrally located neuropil in the each ganglion and in almost all peripheral nerves. In the intercerebral commissure, a few Gal-IR fiber bundles were seen (Figs. 1, 2A and 4A). The tentacular and lip nerves from the cerebral ganglion contained massive Gal-IR fibers (Figs. 1 and 4A). Most immunoreactive fibers from the medial Gal-IR cell group of the cerebral ganglion traveled to the ipsilateral parietal ganglion through the pleural ganglion (Figs. 1, 3A and 4A). Many Gal-IR fibers were observed in the pallial nerve from the parietal ganglion (Figs. 1, 3B and 4A). Relatively abundant Gal-IR fibers were seen in the intestinal nerve from the visceral ganglion (Fig. 4C). A few Gal-IR fibers were seen in the pedal commissure, and thick fiber bundles were seen in the neuropil of the pedal ganglion (Fig. 4A). Among three main nerves from the pedal ganglion, the inferior pedal nerve contained most abundant Gal-IR fibers (Figs. 1 and 4B). A considerable number of immunoreactive fibers were seen in the bucco-cerebral connective nerve (Fig. 1), and the pharyngeal nerve from the buccal ganglion contained many Gal-IR fibers (Fig. 5).

These immunostaining profiles were abolished by preabsorption of antiserum with synthetic rat galanin, and no immunoreactivity was observed in the sections processed with normal rabbit serum or with omission of the primary antibody (data not shown).

Many bioactive peptides discovered originally in the mammalian organs were also present in the CNS of gastropods (Boer *et al.*, 1979; Schot *et al.*, 1981; Roberts *et al.*, 1989; Sonetti *et al.*, 1990; Kasuya *et al.*, 1991; Honma *et al.*, 1994). Furthermore, FMRFamide, one of bioactive peptides first isolated from a clam *Macrocallista nimbosa* (Price and Greenberg, 1977), was immunohistochemically demonstrated to be expressed in the gastropoda CNS in a significant population of cells (*Lymnaea*, Schot and Boer, 1982; Schot *et al.*, 1984: *Aplysia*, Schaefer *et al.*, 1985). Compared with the relative number of FMRFamide-immunoreactive cells in these species, the Gal-IR cell population is minor in *Indoplanorbis*, but still is a distinct neuronal system. A considerable number of Gal-IR neurons and fibers were found in the CNS of

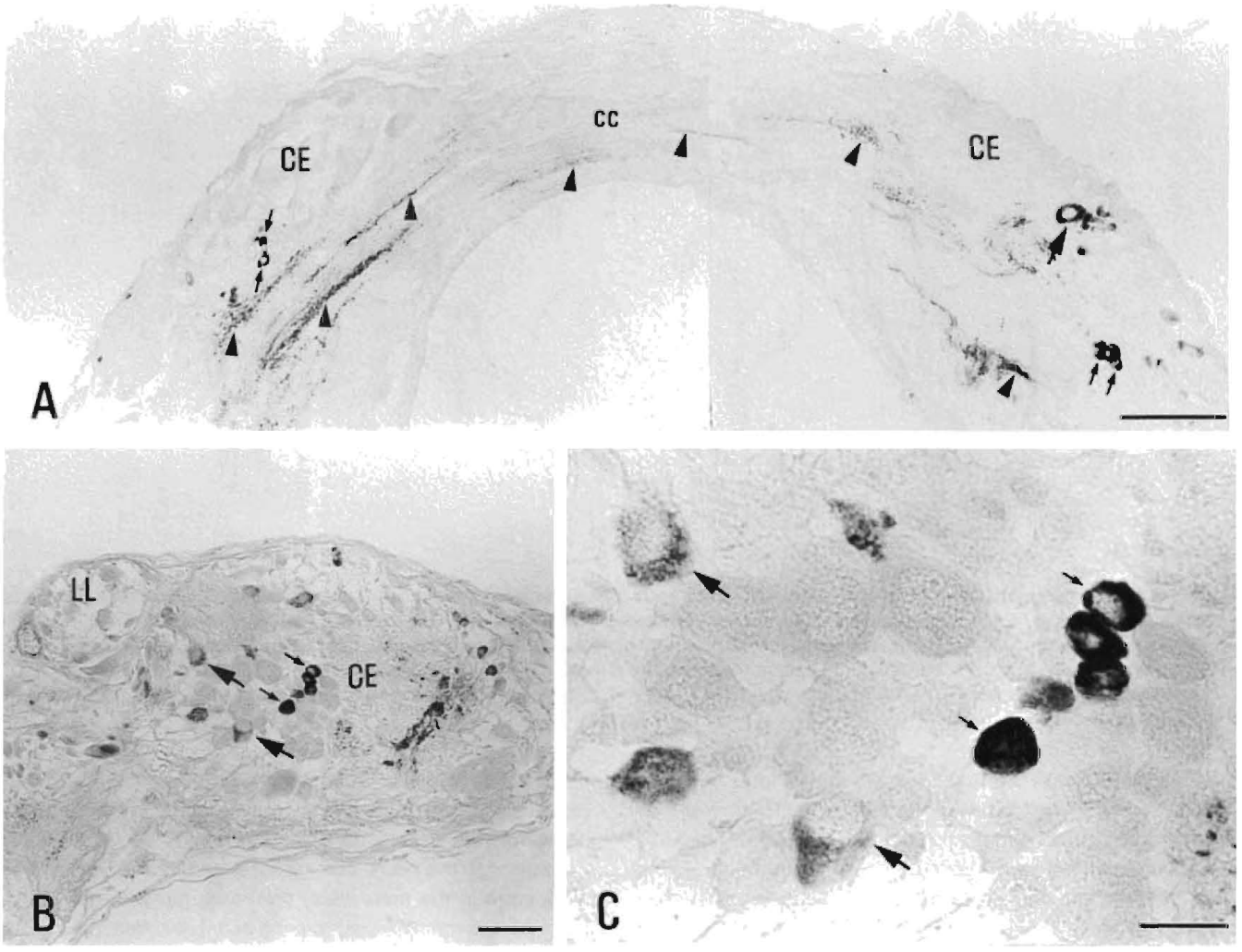

Fig. 2. Photomicrographs showing Gal-IR somata and fibers in the cerebral ganglion. A: Photomontage showing the anterior Gal-IR cell group of cerebral ganglion (CE) and fibers in the cerebral commissure (cc). A large arrow, small arrows, and arrowheads indicate a medium Gal-IR cell, small Gal-IR cells and Gal-IR fibers, respectively. B,C: Low (B) and high (C) magnifications showing Gal-IR cells in the lateral Gal-IR cell group of the cerebral ganglion (CE). Weakly stained medium neurons (large arrows) are located in the peripheral region of the ganglion and intensely stained small neurons (small arrows) are relatively centrally situated. No large Gal-IR cell is located in this area. B and C are lightly counterstained with thionine. Abbreviation: LL, lateral lobe. Scale bars: A, 100 μm; B, 50 μm; C, 20 μm.

Indoplanorbis exustus. On the other hand, in the CNS of *Bulla gouldiana*, a marine gastropod, a few Gal-IR neurons, but almost no Gal-IR fibers, were detected only in the cerebral ganglion (Roberts *et al.*, 1989). This discrepancy may have been caused by different antiserum used, species difference and/or different habitats of these two species; *Indoplanorbis exustus* is a fresh water snail and *Bulla gouldiana* a marine gastropod. The antiserum used here was against rat galanin, but Roberts *et al.* (1989) used an antiserum against porcine galanin. Between these two kinds of galanin, three among 29 amino acid residues are different (Tatemoto *et al.*, 1983; Vrontakis *et al.*, 1987).

Western blot analysis

The anti-rat galanin antibody used here detected approximately 6.1 kDa band by Western blots of the supernatant from the homogenates of the snail CNS (Fig. 6). This size was larger than that of rat galanin itself (3.2 kDa). This suggests that snail galanin may have a larger size than those of other higher animals. Alternately the main form of galanin in the snail CNS is a precursor. In the extracts of adrenal gland, the presence of large forms of galanin, as well as the usual molecular size, was reported in mammals (Bauer *et al.*, 1986).

Comparison with CAT-like immunoreactivity

CAT-immunoreactive (CAT-IR) cells and fibers were also widely distributed in the CNS. CAT-like immunoreactivity was seen in medium and small cells based on our classification, and no large CAT-IR cells were detected in the present study. CAT-IR cells were relatively aboundant in the cerebral, left parietal, visceral, and pedal ganglia. Only a few CAT-IR cells were seen in the pleural and right parietal ganglia. No CAT-IR cells were found in the buccal ganglion. By comparison with

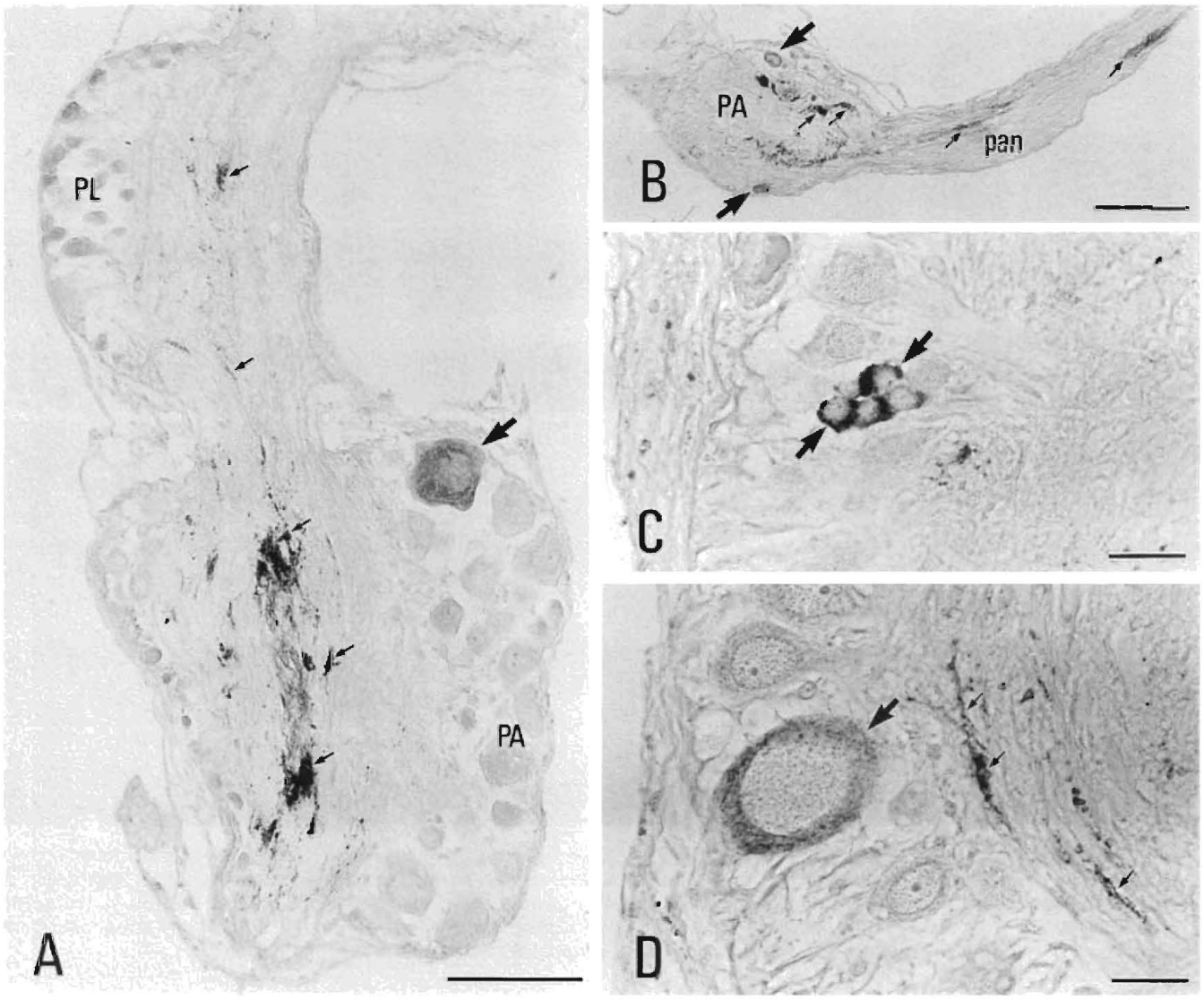

Fig. 3. Photomicrographs showing Gal-IR cells and fibers in the left pleural, parietal (A,B), and pedal (C, D) ganglia. A: Gal-IR fibers (small arrows) in the neuropil of the pleural (PL) and parietal (PA) ganglia and one large Gal-IR cell (large arrow). B: Two medium Gal-IR cells (large arrows) in the parietal ganglion (PA) and immunoreactive fiber bundles (small arrows) in the neuropil of the ganglion and the pallial nerve (pan). In these two planes, no small Gal-IR cells are seen. C: Small Gal-IR cells (arrows) in the pedal gnalgion. D: One large Gal-IR cell (large arrow) in the pedal ganglion and immunoreactive fibers (small arrwos) in the centrally located neuropil. Scale bars: A, B, 100 μm; C, D, 20 μm.

Gal-IR cells, CAT-IR cells were observed more frequently than Gal-IR cells in the cerebral ganglion (Fig. 7A, B), and about 20% of CAT-IR cells were also immunopositive for galanin (Fig. 7C, D). That ratio corresponded to about 25% of Gal-IR cells in the cerebral ganglion. However, in the pedal and visceral ganglion, no CAT-IR cells showing galanin-like immunoreactivity were found. In the pleural and parietal ganglia, only a few co-existing cells were seen. In general, CAT-IR fibers were distributed more densely than Gal-IR fibers in the neuropil of all ganglia except in the buccal ganglion. Although CAT-IR fibers existed in all main peripheral nerves, especially many CAT-IR fibers were seen in the three pedal nerves. No CAT immunoreactivity was observed in the sections processed with normal goat serum or with omission of the primary antibody (data not shown).

The antiserum used here is against human placental CAT. Although the cross-reactivity of the antiserum with rat CAT has been reported (Shiromani *et al.*, 1990), no data are available showing cross-reactivity with snail CAT. Therefore, results obtained with this serum should be cautiously interpreted. CAT and acetylcholine esterase activities have been biochemically demonstrated in the CNS of the snail *Helix aspersa* (Emson and Kerkut, 1971) and leech *Hirudo medicinalis* (Sargent, 1977; Wallace, 1981). It may be reasonable to consider that CAT-IR neurons of *Indoplanorbis* are cholinergic. Several bioactive peptides (myomodulin, buccalin, and FMRFamide) existing in the cholinergic system have been demonstrated to modulate effects of acetylcholine

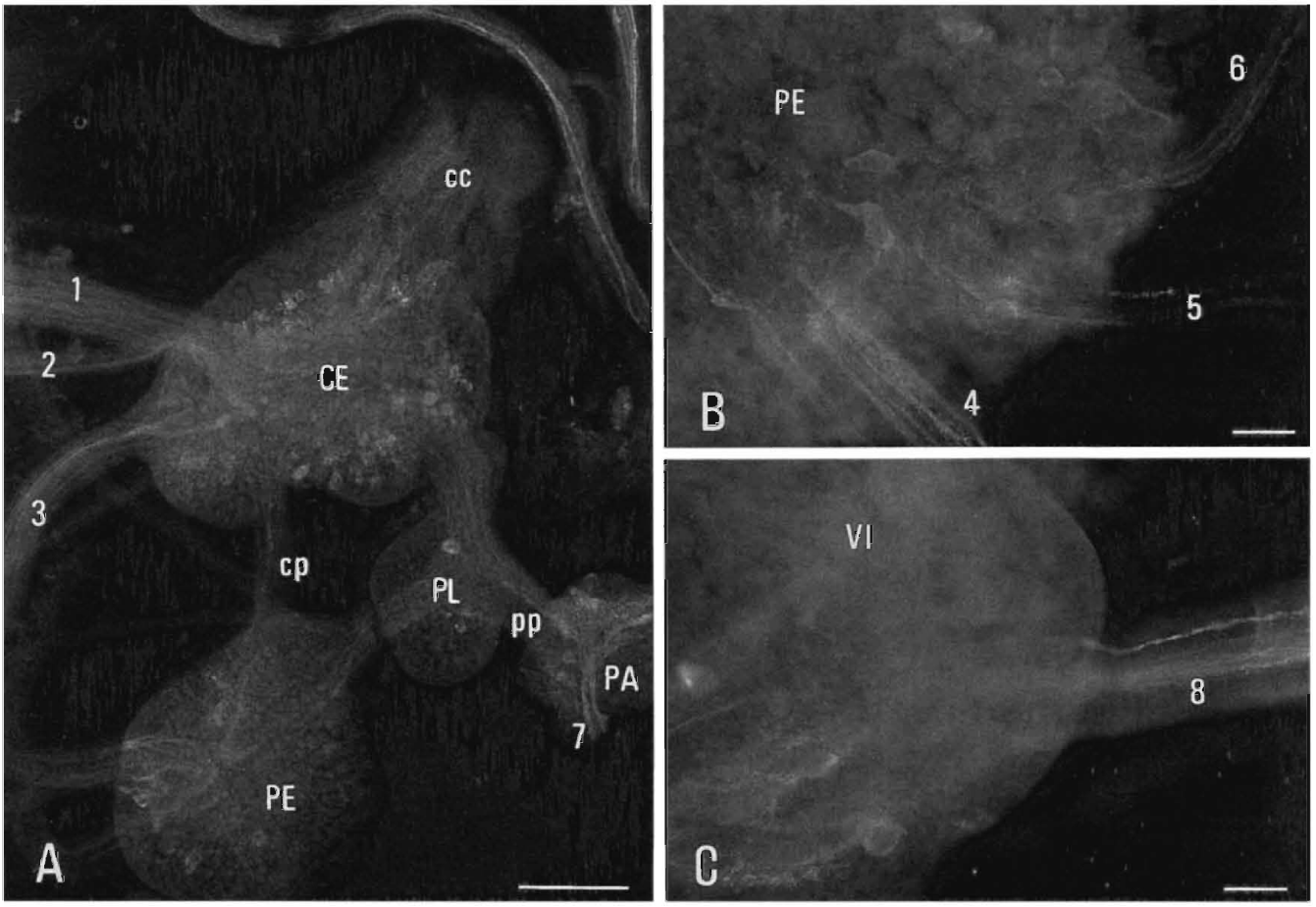

Fig. 4. Fluorescent micrographs showing Gal-IR cells and fibers in the whole-mount CNS of the snail. A: Ventral view of the left cerebral (CE), pleural (PL), pedal (PE), and parietal (PA) ganglia, and main peripheral nerves (1, tentacular nerve (n); 2, frontal lip n; 3, median lip n; 7, pallial n). Abbreviations: cc, cerebral commissure; cp, cerebro-pedal connective; pp, parieto-pleural connective. B: Fluorescent micrograph showing Gal-IR fibers in the inferior (4), median (5), and superior (6) pedal nerves from the pedal ganglion (PE). C: Fluorescent micrograph showing Gal-IR fibers in the intestinal nerve (8) from the visceral ganglion (VI). Scale bars: A, 200 μm; B, C, 50 μm.

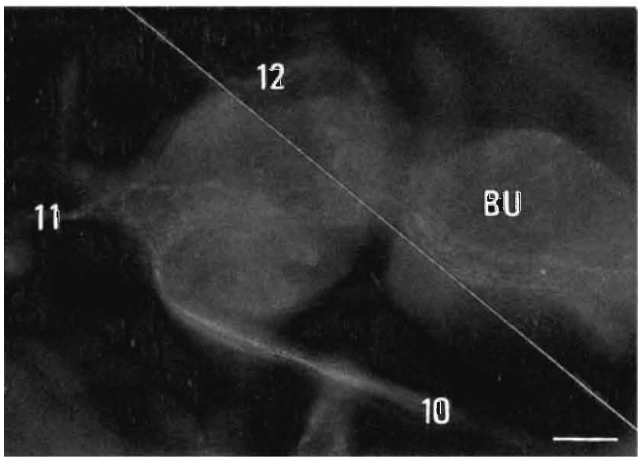

Fig. 5. Photomontage showing galanin-like immunoreactivity in the buccal ganglion (BU) and pharyngeal (10), gastric (11), and radular (12) nerves. Note dense Gal-IR fibers in the pharyngeal nerve. Scale bar, 50 μm.

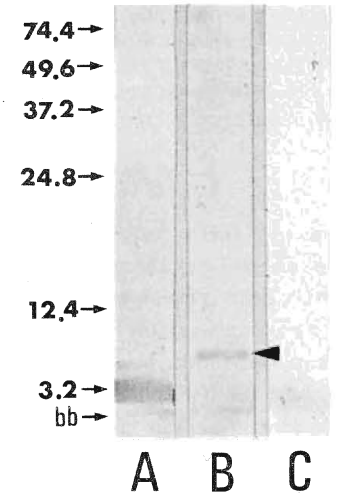

Fig. 6. Western blots of extracts from the snail CNS. Left numbers indicate molecular weights of marker proteins and rat galanin (3.2) by kDa. bb, the level of brilliant blue. A, rat galanin; B, supernatant stained with anti-galanin antibody, arrowhead indicates a 6.1 kDa band; C, supernatant stained with normal rabbit serum diluted 1:1,000.

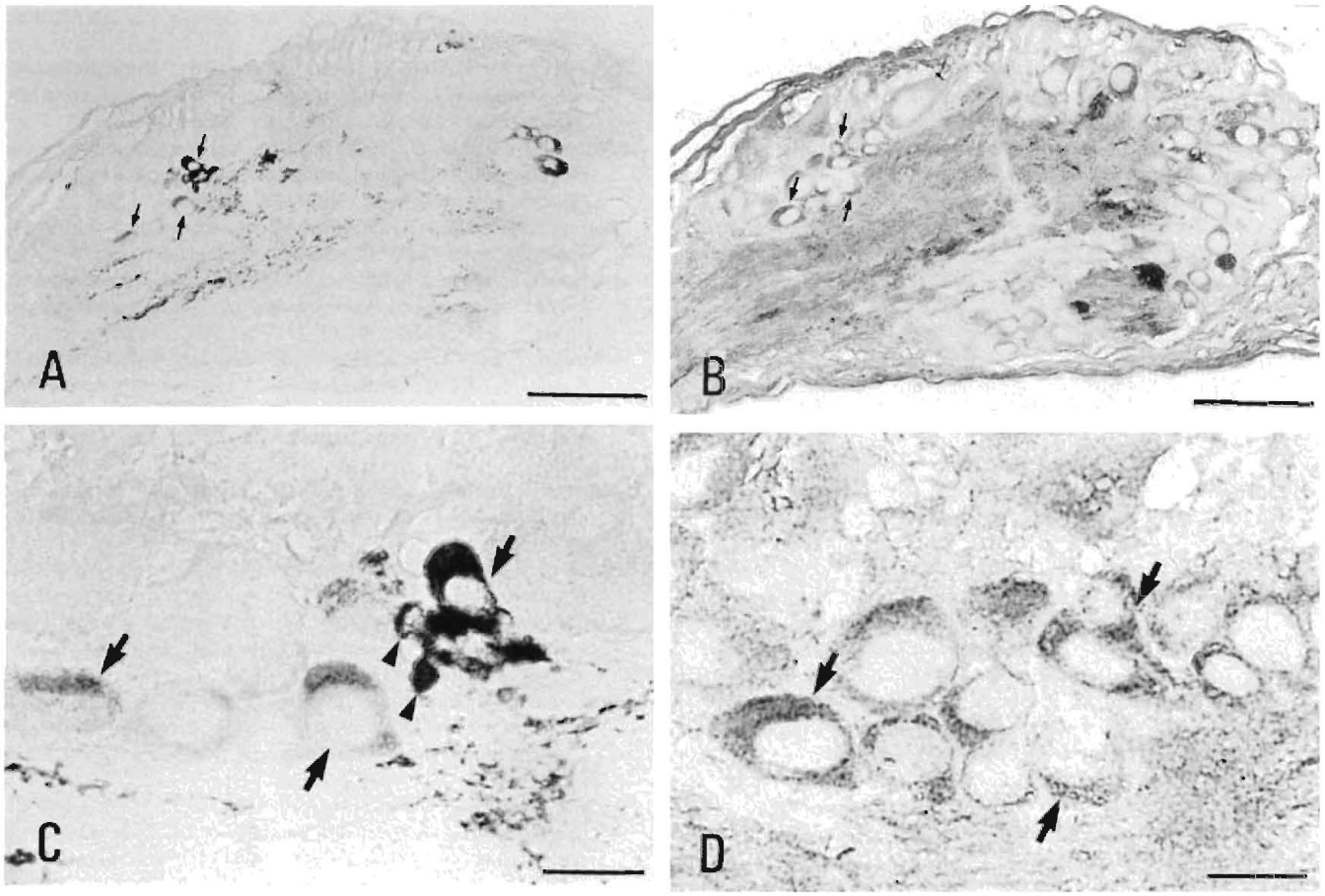

Fig. 7. Photomicrographs of consecutive sections showing colocalization of galanin- (A, C) and CAT-like immunoreactivities (B, D) in the cerebral ganglion. A-D: Low (A, B) and high (C,D) magnification photographs showing that some of medium Gal-IR cells (arrows in A,C) are also immunopositive with an anti-CAT antibody (arrows in B, D). Small Gal-IR cells (arrowheads in C) are difficult to recognize in the adjacent section D. Scale bars: A, B, 100 μm; C, D, 20 μm.

in the terminal area of the *Aplysia* CNS (Cropper *et al.*, 1987, 1988) and in the *Busycon* heart (Smith and Hill, 1987). In *Indoplanorbis*, CAT-IR cells were medium and small in size, and some of the medium-sized cells showed galanin-like immunoreactivity. Galanin in CAT-IR cells possibly modulates cholinergic functions. In this context, it has been suggested in the rat that galanin creates functional diversity of cholinergic signaling in relation to higher brain functions such as memory and learning (Melander and Staines, 1986). In the *Aplysia*, excellent analyses have been achieved about memory and learning which are composed of two different levels, namely, habituation and sensitization (Kandel, 1977). In the gill-withdrawal reflex, short-time habituation involves a transient decrease in synaptic efficacy, and long-term habituation produces a more prolonged change leading to a functional disruption between terminals of sensory neurons and motorneurons located in the abdominal ganglion. Serotoninergic metacerebral cells in the head sensitize the reflex by means of an increase in cAMP. Similar mechanisms seem to underlie the feeding-behavior and heart rate control. It is very interesting to study whether neurons showing both galanin and CAT immunoreactivities in the snail cerebral ganglion related to memory and learning.

The presence of Gal-IR neurons with and without CAT immunoreactivity suggests the possibility that some Gal-IR neurons contain other neurotransmitter markers such as tyrosine hydroxylase or adenosine deaminase as observed in the rat brain as cited in Introduction. Furthermore, the large Gal-IR cell in the parietal ganglion was stained with paraldehyde-fuchsin stain, but the large Gal-IR cells in the cerebral and pedal ganglia were not stained (data not shown). This suggests that subpopulation of Gal-IR cells belong to the classical neurosecretory cells. Such partial overlap of other bioactive peptide-containing cells with classical neurosecretory cells was also reported in other snails (Schot *et al.*, 1981; Sonetti *et al.*, 1990). Thus, the present study suggests the functional diversity of galanin in the snail. Further studies are required to clarify whether galanin in these cells acts as neurohormone or neurotransmitter/neuromodulator. Gal-IR neurons are possibly good candidates to colocalize a number of other signal molecules and to analyze physiological and pharmacological functions of galanin.

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(Received February 8, 1996 / Accepted April 16, 1996)