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CHARACTERIZATION OF NEURONS IN THE VISCERAL GANGLIA OF THE GREEN-LIPPED MUSSEL (*Perna canaliculus*) USING ANTIBODIES RAISED AGAINST NEUROPEPTIDES AND NEUROTRANSMITTERS

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Abstract

Central neurons in the visceral ganglia of both male and female Green lipped mussel, *Perna canaliculus* were characterized by immunohistochemical techniques. We used mollusc antibodies raised against neuropeptides and neurotansmitters known to control reproduction and spawning. Anti-ELH and anti-APGWamide showed very strong immunoreactivity in small type of neurons. Anti-5-HT and anti-DA immunoreactivity was mostly in large type of neurons. The labelled neurons are consistent with descriptions of neurosecretory cells implicated in the control of reproduction and spawning on the basis of earlier histological staining techniques used in this species. The use of selective immunological markers for peptides and amines appears to be a promising tool for further Characterization of neurosecretory cells. To isolate and characterise neuropeptides and other biologically active materials involved in the control of reproduction in *Perna canaliculus.*

Key words : Characterization, Visceral ganglia, Green lipped mussel, Antibodies, Neuropeptides

Introduction

Classical staining techniques are recognised methods to identify neurosecretory cells, with a limited ability to describe the functional properties of those identified neurosecretory cells. To overcome this limitation, a number of studies have been done using immuno-cytochemistry to characterise neurosecretory materials in the bivalve central nervous system (Stefano and Martin, 1983; Kobayashi dn Muneoka, 1990; Candelario-Martinez *et al*., 1993; Croll *et al*., 1993; Kerkhoven *et al*., 1993). The presence of neurotransmitters and numerous neuropeptides in the nervous system of those bivalves are revealed by these studies. However, knowledge neuropeptides and neurotransmitters in the green-lipped mussel (*Perna canaliculus*) lags far behind that of other bivalves and gastropod molluscs.

The presence of APGWamide-like immunoreactivity has been demonstrated within central neurons of the scallop *Pecten maximus* (Jegou *et al*., 1993). Indeed, APGWamide is involved in myoactive and copulatory behaviour (Li *et al.,* 1992; De Lange *et al.,* 1997a), and it has effects on central neurons involved in control of egg-laying behaviour and metabolism (Croll *et al.,* 1991). APGWamide has been isolated from ganglia of the prosobranch *Fusinus ferrugineus* (Kuroki *et al*., 1990) and the ganglia of the African giant snail *Achatina fulica* (Lui *et al*., 1991). The high degree of homology of

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certain domains in the preprohormones not only suggests conserved functionality throughout gastropod evolution but also suggests that antibodies raised against some of these domains might serve as markers for the identification of neurones which produce ovulation hormones in other species (Croll *et al.,* 1993). The positions of identified cells in several species together with previous histochemical and ultrastructural studies (Roubos and Van Den Ven, 1987) support the hypothesis of homologous neurons. (Theunis *et al.,* 1990) detected peptides immunoreactive to antisera specifically directed against caudo-dorsal cell hormone (CDCH) and caudo-dorsal cell protein (α-CDCP or β-CDCP), in the central nervous system of *Sarcophaga bullata* (Diptera), *Leptinotarsa decemlineata* (Coleoptera), *Locusta migratoria* and *Periplaneta americana* (Orthoptera)). Further investigations indicate that the egg-laying preprohormone is relatively conserved across a wide range of molluscan classes (Nambu and Scheller, 1986). Using this antibody, as well as in antibody raised against CDCH, it has also been shown that neurons in the bivalves *Mytilus edulis*, *Mya arenaria* and *Placopecten magellanicus* contain a similar vitellogenic factor (Croll *et al.,* 1993). These selective immunological markers, therefore, suggest that related peptides may be involved in the egg laying of various gastropods and bivalve molluscs (Cummuns *et al*., 2000).

Therefore, this study was designed with an attempt to locate and identify the neurons containing neurotransmitters or egg-laying hormones in the green-lipped mussel using immunohistochemistry. In the present study, these deficiencies are addressed by providing a detailed description of the distribution of serotonin (5-HT), dopamine (DA), APGWamide, and egg-laying hormone (ELH) within the visceral ganglia of the green-lipped mussel, *P. canaliculus.*

Materials and Methods

Collection of mussels, fixation and dissection of ganglia

The green-lipped mussels, *Perna canaliculus,* were collected from an exposed rocky shore at Purihurihu Point, near Blueskin Bay, in the South Island of New Zealand. Collection of ganglia of both sexes for immunohistochemistry was done shortly after transporting the mussels to the laboratory of the Department of Physiology at the University of Otago, Dunedin, New Zealand. The visceral ganglia were collected from both sexes. Individual tissues were placed gently in the bottom of an aluminium foil boat containing pre-cooled Tissue-Tek™ O.C.T. compound and then the foil boat was filled with O.C.T. compound. The tissue was snap frozen by partial immersion of the foil boat into isopentane cooled in liquid nitrogen. Individual tissues were preserved at –70°C for sectioning.

Antibodies used for immunohistochemistry

Four antisera were used in this study, all produced in rabbits: (i) Anti-ELH was raised against a synthetic peptide representing the N-terminal fragment (ISINQDLKAITDML) from the egg laying hormone of *Aplysia*. This antibody was produced by G. T. Nagle and J. E. Blankenship (University of Texas Medical Branch), and its Characterization and specificity were described by Ram *et al*., (1998), (ii) Anti-APGWamide (CHEMICON International, Inc. 28835 Single oak Drive, Temecula, CA 92590), (iii) Anti-Dopamine (CHEMICON International, Inc. 28835 Single Oak Drive, Temecula, CA 92590), and (iv) Anti-Serotonin was obtained from Dept. of Zoology, University of Otago, Dunedin, New Zealand. The unlabelled goat anti-rabbit secondary antibody was obtained from Cappel Research Products (Durham, North Carolina) and the peroxidase-antiperoxidase complex employing rabbit antibodies was obtained from Sigma Chemical Co. (Mississauga, Ontario).

Immunocytochemistry protocol

Serial sections (two sets - one for experimental and another for control) were cut at 10 µm in a cryostat at -18°C and approximately 8-10 sections were mounted on each slide for immunohistochemistry. The dried sections were fixed for 10 minutes in freshly prepared 4% paraformaldehyde and were washed in PBS. Primary antiserum were then applied and left overnight at 4°C. Antiserum dilutions of between 1:400 and 1:100 were used in an immunodiluent (ID) solution of 2% normal goat serum (Sigma Chemical Co.) and 0.2% Triton X-100 (Sigma Chemical Co.) in PBS.

Next day, secondary antibody was added to all slides after washing in PBS and was left for an hour at room temperature. The secondary antiserum was diluted 1:200 in ID. After another several washes in PBS the slides were kept for another one-hour incubation in peroxidase-antiperoxidase diluted 1:400 in ID. After incubation, slides were washed off again in PBS and were developed for 2-3 minutes using diaminobenzidine (DAB)- hydrogen peroxide. Slides were dehydrated in graded ethanols washed in xylene, and mounted in DPX. One set of serial sections from each ganglion was processed as described above, with the elimination of the incubation in primary antibody as a negative control. Slides were viewed through an Olympus BX50 Microscope and photographed digitally.

Results

The location of neurons containing different neuropeptides and neurotransmitters in the visceral ganglia of the green-lipped mussel, *Perna canaliculus*, was examined immunohistochemically and is shown in Fig. 1. A substantial number of neurons and nerve fibres were labelled with anti-ELH, anti-APGWamide, anti-5-HT and anti-DA in the visceral ganglia. These immunoreactive neurons are presented in Fig. 2.

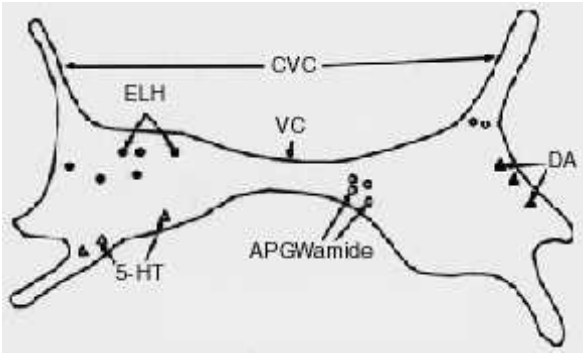

Fig. 1. Schematic representations of anti-ELH immunoreactivity (black circles) and anti-5-HT immunoreactivity (white triangles) on the left side and anti-APGWamide immunoreactivity (white circles) and anti-DA immunoreactivity (black triangles) on the right side in the visceral ganglia of *Perna canaliculus.* All descriptions are bilaterally symmetric in the ganglia. VC, visceral commissure; CVC, cerebral-visceral connective.

The periphery of the visceral ganglia of both sexes showed anti-ELH immunoreactivity like the cerebral and pedal ganglia. The immunoreactivity was observed only in small cells (Fig. 2A), and these cells were located mostly in the cortex of the visceral ganglia. Immunoreactive nerve fibres were found throughout the ganglia.

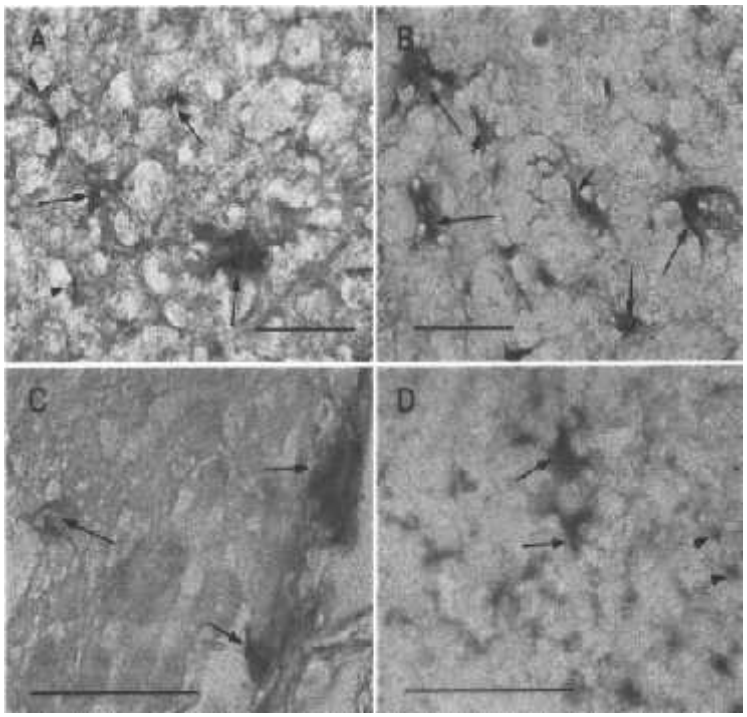

Fig. 2. Immunoreactivity in the visceral ganglia of *Perna canaliculus*

(A) Small cells (arrows) and nerve fibres (arrowheads) showing immunoreactivity to anti-ELH in a spawned male. Scale bar 20 µm.

(B) Cell (long arrow) showing immunoreactivity to anti-APGWamide along with long axonal profile (arrowheads) to the neuropile region in a ripe female. Scale bar 20 µm.

(C) Large cell (long arrows), the periphery of the small cell (short arrows) and nerve fibres (arrowheads) showing immunoreactivity to anti-5-HT to anti-5-HT in a spawned male. Scale bar 20 µm.

(D) A few large cells (long arrows), periphery of the nucleus and nerve fibres (arrow heads) showing immunoreactivity to anti-DA. Scale bar 20 µm.

The anti-APGWamide immunoreactive small cells were scattered between larger cells near the periphery of the visceral ganglia. Sometimes cells were projecting their axonal profiles towards the neuropile region. Many small cells were found to show immunoreactivity but only at the periphery of the cell body and the immuno-positive fibres were distributed widely throughout the visceral ganglia (Fig. 2A). A few immunoreactive neurons were found near the commissure of the visceral ganglia.

A few neurons, and the peripheral connective sheath of the ganglia, peripheral layer of the cell body and nerve fibres revealed moderate immunoreactivity with antibodies raised against 5-HT. Anti-5HT produced immunoreactivity both in small and large type cells, and was located near the periphery of the ganglia. A few small cells produced moderate staining intensity. A few large cells showed weak immunoreactivity, whereas the axonal profiles of a few large cells, the peripheral layer of the cell bodies and the nuclei produced strong immunoreactivity in the visceral ganglia (Fig. 2C). Numerous

immunoreactive fibres were revealed in the neuropile of the visceral ganglia. Only a few large cells and the nerve fibres produced weak immunoreactivity against anti- DA in this ganglion (Fig. 2).

Discussion

The present study presents the first immunocytochemical description of various monoamines and neuropeptides in the central nervous system of *Perna canaliculus*, which might be involved in controlling reproduction. Antibodies raised against ELH, APGWamide, 5-HT and DA stained relatively large numbers of cell bodies, fibres, axons and connective sheaths in the visceral ganglia of *P. canaliculus*.

It is possible that endogenous peptides unrelated to reproduction cross-reacted with the antibodies used in this study. The results of several studies, however, support the hypothesis that some of the labelled neurons contain peptides homologous to those involved in gastropod ovulation. First, the antibodies have already been shown to be highly specific for ovulation related peptides in other molluscs (Theunis *et al*., 1990; Van Minnen *et al.,* 1992); they apparently do not react with any of the numerous other well characterized and evolutionarily conserved peptides within the gastropods (Kerkhoven *et al*., 1991) and bivalves (Stefano and Martin, 1983; Vitellaro-Zuccarello and DeBasi, 1988). Second, antibodies raised against both ELH and APGWamide labelled in a few cells and the fibres in the same positions in cerebral, pedal and visceral ganglia of bivalves. Such findings are consistent with the possibility that the immunoreactive peptides are synthesised within a single preprohormone, as occurs in gastropods (Croll *et al.,* 1993). However, it must be noted that labelling in all regions was not co-localised, thus suggesting that immunoreactive peptides are not necessarily synthesised together by every cell. Finally, the several immunoreactive cells in this study are very similar to those described as possible neurosecretory cells involved in bivalve reproduction (Illanes-Bucher, 1979; Mahmud and Mladenov, 1998).

In the present study, some of the neurons in the visceral ganglia were labelled with anti-ELH, anti-APGWamide, anti-5HT and anti DA. According to the size of the immunoreactive neurons in these ganglia, there were two distinct groups. Small cells were mostly located near the periphery of the ganglia with a few in the neuropile region. Large cells were mostly located between the peripheral edge of the ganglia and neuropile region. The small cells exhibited strong immunoreactivity with both anti-ELH and anti-APGWamide in all three ganglia of *P. canaliculus*. The relationships between neurosecretory cells and gonad state were observed in the green-lipped mussel (Mahmud and Mladenov, 2000). Similar patterns were observed in other studies in bivalves and gastropods. Neurosecretory staining in 'a' cell in *Mytilus edulis* was reported to correlate with the reproductive cycle (Lubet and Mathieu, 1982). They also demonstrated the gonadotropin action of the cerebral ganglia in *M. edulis;* germ cell proliferation and the reinitiation of meiosis in males, previtellogenesis and vitellogenesis in females are stimulated by products from visceral ganglia. The studies in gastropods by Hahn (1990) in *Haliotis discus hannai* and by Upatham *et al*. (1998) in *Haliotis asinina* established that the secretion from certain cells in the ganglia of *Haliotis* spp. are correlated with vitellogenesis, gametogenesis or spawning. The injection of ganglionic homogenates caused spawning in green-lipped mussel. Therefore, the labelling of small cells by both anti-ELH and anti-APGWamide in the visceral ganglia of the green-lipped mussel, *Perna canaliculus,* is strong evidence for the presence of ovulation and reproduction hormones.

The anti-5-HT and anti-DA immunoreactive neurons were lightly stained and located in well-defined locations in the visceral ganglia in *P. canaliculus* (Fig.1). The neurons perhaps correspond to cell types 'C' and 'D' as shown in the previous study (Mahmud and Mladenov, 1998). The labelled cell by both anti-5HT and anti-DA indicates the presence of neurotransmitters/monoamine(s) in these cells. The presence of 5-HT and DA-like substances has also been previously reported in *M. edulis* (Aramant *et al*., 1981; Mathieu and Van Minnen, 1989). Although, the neurosecretory cell types 'C' and 'D' did not show any substantial changes in colour intensity with changes in gonad development and spawning (Mahmud and Mladenov, 2000), the presence of neurotransmitters 5-HT and DA in these cells indicate that they might have other modulating or physiological functions in this species which need to be evaluated.

While the present study was based upon the hypothesis that peptides controlling reproduction might be evolutionarily conserved between gastropods and bivalves, it must also be considered that spawning and external fertilisation of bivalves are very different from in gastropods in terms of copulation and subsequent egg-laying behaviour (Croll *et al*., 1993).

Therefore, even though related peptides might be involved in reproduction within both taxa, details of their distribution and mechanisms of actions are bound to vary. Their abundance should be investigated seasonally and correlated with stage of reproduction in order to determine which processes or mechanisms they are involved in. In the present study, samples from both mature and spawned mussels showed immuno-reactivity. The sampling protocol (small number of samples) in the present study does not allow assigning such physiological roles to these cells. An elaborate investigation using immunohistochemistry with samples (ganglia) collected for the entire reproductive cycle up to the completion of spawning would reveal the exact role of the immunoreactive products. However, results indicate that the ganglia of this mussel contain substances antigenically similar to peptides known to control reproduction in other molluscs. Thus, the present study lays the foundation for a promising new avenue of research into the neuroendocrine control of reproduction in the green-lipped mussel, *P. canaliculus.*

The present immunocytochemical study identifies unique population of cells containing neuropeptides and neurotransmitters, which are the likely candidates responsible for different aspects of reproduction and spawning in *P. canaliculus.* Although, the labelling of cells with anti-ELH, anti-APGWamide, anti-5HT and anti-DA does not necessarily confirm any physiological functions at this stage but it does indicate the presence of a preprohormone with ovulation factors and neurotransmitters.

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