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Neurons characterization in the cerebral ganglia of the green-lipped mussel, *Perna canaliculus*, using antibodies raised against neuropeptides and neurotransmitters involved in gastropod egg-laying behaviour and bivalve reproduction and spawning

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Abstract
Immunohistochemical techniques were used to characterise central neurons in the cerebral ganglia of both male and female *Perna canaliculus*. 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 characterisation of neurosecretory cells, and to isolate and characterise neuropeptides and other biologically active materials involved in the control of reproduction in *Perna canaliculus*.

Key words: *Perna canaliculus*. Antibodies, Immunohistochemistry

Introduction

Demonstration of the existence of neurosecretory cells has been mainly through histological work. As far as mussels are concerned, a number of descriptive studies of the different types of neurosecretory cells have been published (Illanes-Bucher 1979). A number of more recent studies have been done using immunocytochemistry to characterise neurosecretory materials in the bivalve central nervous system (Stefano and Martin 1983, Croll *et al.* 1993, Kerkhoven *et al.*1993). These studies indicate the presence of neurotransmitters and numerous neuropeptides in the nervous system of

those bivalves. However, knowledge neurotransmitters and neuropeptides in the green-lipped mussel lag 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). APGWamide is well studied in gastropod molluscs, where it appears to play an important role in the control of male reproduction. APGWamide may also function as a neurotransmitter within the central nervous system of *Lymnaea stagnalis,* inhibiting the activity of certain neuroendocrine cells (Croll *et al.* 1991) and co-ordinating the activity of different populations of penial motorneurons. Indeed, APGWamide is involved in myoactive and copulatory behaviour (Croll *et al.* 1991, De Lange *et al.* 1997), 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).

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). Physiological functions in mussels are controlled by the central nervous system (Mathiei *et al.* 1990).

To date, no study has attempted to locate and identify the neurons containing neurotransmitters or egg-laying hormones in the green-lipped mussel, *P. canaliculus* 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 cerebral ganglia of the green-lipped mussel, *P. canaliculus.* The immunocytochemical trials have been carried out to tentatively identify neurosecretory cells involved in reproduction in bivalve molluscs to examine whether the results can be generalised across the class for a better understanding of mussel reproduction and provide a basis for further research.

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 cerebral 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 immuno-histochemistry

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 characterisation 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 localisation of neurons containing different neuropeptides and neurotransmitters in the cerebral ganglia of the green-lipped mussel, *Perna canaliculus,*

were examined imuno-histochemically. The tentative locations of these immunoreactive neurons are shown in Fig. 1.

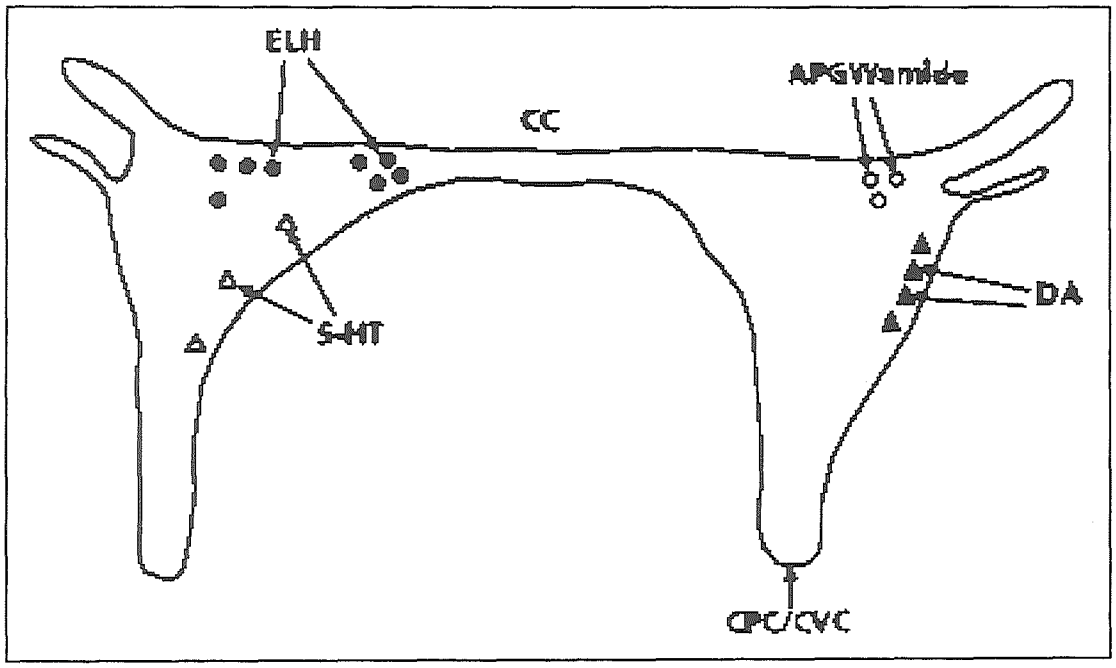

Fig. 1. Schematic representations of anti-ELH immunoreactivity (black circles) and anti-5-HT immunoreactivity (white triangles) on the left side and anti-APGWamide immunoreactivity (white circles) and anti-DA immunoreactivity (black triangles) on the right side in the cerebral ganglia of *Perna canaliculus*.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

The immunoreactive neurons and fibres throughout the cerebral ganglia labelled with anti-ELH, anti-APGWamide, anti-5-HT and anti-DA are presented in Fig. 2. Antibodies raised against anti-ELH labelled neurons and fibres throughout the cerebral ganglia of the females (Fig. 2A). The immunoreactivity was observed only in small cells. Numerous immunoreactive fibres were also present in the cerebral ganglia. The large neurons and their pigments/granules (Fig. 2B) revealed no immunoreactivity above the levels of control slides (Fig. 2C). The small cells also showed positive immunoreactivity and stained strongly with anti-APGWamide. The immunoreactive cells were mostly found along the cortex (Fig. 2D). Anti-APGWamide produced positive immunoreactivity in both sexes. Large numbers of immunoreactive fibres were found throughout the cerebral ganglia.

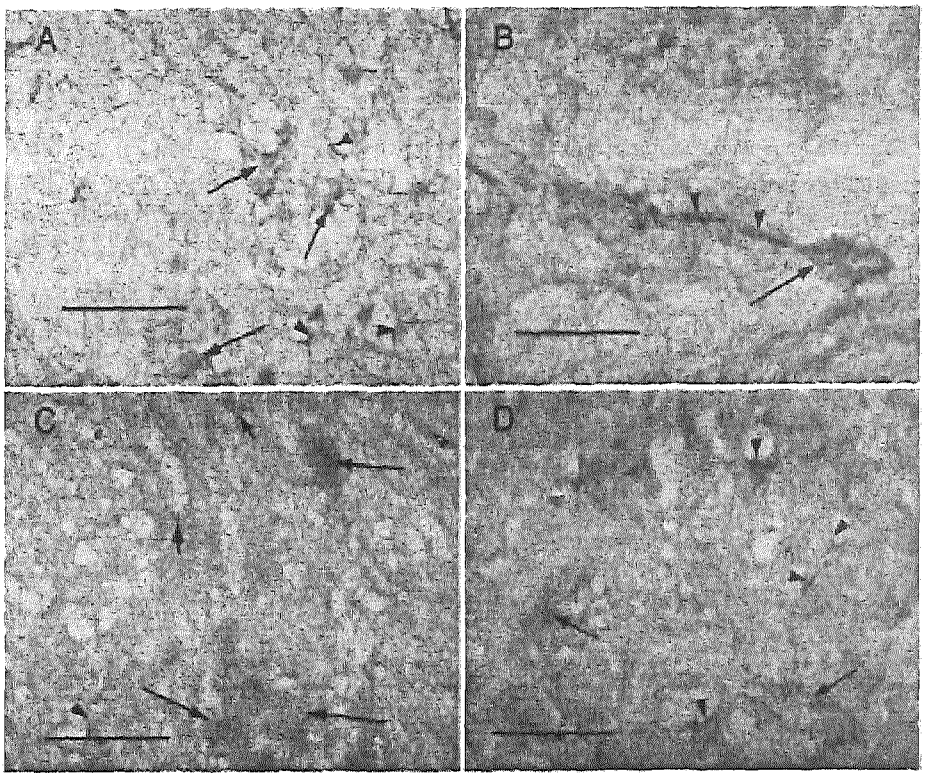

Fig. 2. Immunoreactivity in the cerebral ganglia of *Perna canaliculus.*

(A) Several small cells (long arrows) and nerve fibres (arrowheads) showing strong immunoreactivity to anti-ELH in ripe female. Scale bar 20 μm.

(B) Several small cells (long arrows), nerve fibres (short arrows) showing positive immunoreactivity to anti-APGWamide in spawned female. Scale bar 20 μm.

(C) A few large cells (long arrows) showing positive immunoreactivity to anti-5-HT in ripe female. Scale bar 50 μm.

(D) Large cells (long arrows) and nerve fibres (short arrows) showing positive immunoreactivity to anti-DA in a spawned female mussel. Scale bar 50 μm.

Mostly large cells, connective tissue sheath and numerous immunoreactive-fibres were revealed in the neuropile of the cerebral ganglia with antibodies raised against anti-5-HT. The immunoreactive cells were located near the periphery of the ganglia. Only a few of the large cells were found with strong immunoreactivity (Fig. 2E). A few small cells showed positive immunoreactivity with anti-5-HT. Mostly the peripheries of these cells were found with immuno-reaction.

Discussion

This study perhaps the first of its kind describes 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 cerebral ganglia of *P. canaliculus*.

In the present study, some of the neurons in the cerebral ganglia were labelled with anti-ELH, anti-APGWamide, anti-5HT and anti DA (Figure 1). 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 (Mathieu and Van Minnen 1989). 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 cerebral ganglia of the green-lipped mussel, *Perna canaliculus*, is strong evidence for the presence of ovulation and reproduction hormone(s).

The anti-5-HT and anti-DA immunoreactive neurons were lightly stained and located in well-defined locations in the cerebral ganglia in *P. canaliculus* (Fig. 1). These 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 1998). 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.

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 (Fig. 2). 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 immuno-histochemistry with

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