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Characterisation of neurons in the pedal 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
To characterise central neurons in the pedal ganglia of both male and female green lipped mussel, *Perna canaliculus* immunohistochemical techniques were used. Mollusc antibodies were used 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, Neurons

Introduction

The existence of neurosecretory cells has been demonstrated mainly through histological work. The application of the Gomori methods, based on the reactions with protein-bound cysteine, appeared to be rather specific for neurosecretory cells. As far as mussels are concerned, a number of descriptive studies of the different types of neurosecretory cells have been published (Illanes-Bucher 1979). Although these classical staining techniques are a recognised method to identify neurosecretory cells, they have a limited ability to describe the functional properties of those identified neurosecretory cells. To overcome this limitation, a number of more recent studies have been done using

immunocytochemistry to characterise neurosecretory materials in the bivalve central nervous system (Croll *et al.* 1993). These studies indicate the presence of neurotransmitters and numerous neuropeptides in the nervous system of those bivalves. However, knowledge of neurotransmitters and neuropeptides in the green-lipped mussel 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). 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 (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). Neurosecretory cells controlling egg-laying have been identified and well characterised in the pulmonate, *Lymnaea stagnalia,* and the opisthobranch, *Aplysia californica.* The ovulation hormones in both *Lymnaea* and *Aplysia* are formed within larger preprohormones which produce a number of additional neuropeptides, each of which may regulate particular aspects of ovulation and egg-laying behaviour (Brussaard *et al.* 1990). The preprohormones in *Lymnaea* and *Aplysia* have similar structural organisations despite the fact that the lineage of the two genera is thought to have diverged about 350 million years ago (Moore and Pitrat 1960). 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. The peptides immunoreactive to antisera specifically directed against caudo-dorsal cell hormone (CDCH) and caudo-dorsal cell protein (α-CDCP or β-CDCP) have been detected in the central nervous system of *Sarcophaga bullata* (Diptera), *Leptinotarsa decemlineata* (Coleoptera), *Locusta migratoria* and *Periplaneta americana* (Orthoptera) (Theunis *et al.* 1990).

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).

So far information no studies have been attempted to date to locate and identify the neurons containing neurotransmitters or egg-laying hormones in the green-lipped mussel using immuno-histochemistry. 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 pedal ganglia of the green-lipped mussel, *Perna 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.

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 pedal 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 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 Deptartment 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 pedal ganglia of the green-lipped mussel, *Perna canaliculus*, was examined imunohistochemically and is shown in Fig. 1 . The labelled neurons and fibres produced by anti-ELH, anti-APGWamide, anti-5-HT and anti-DA in the pedal ganglia are shown in Fig 2.

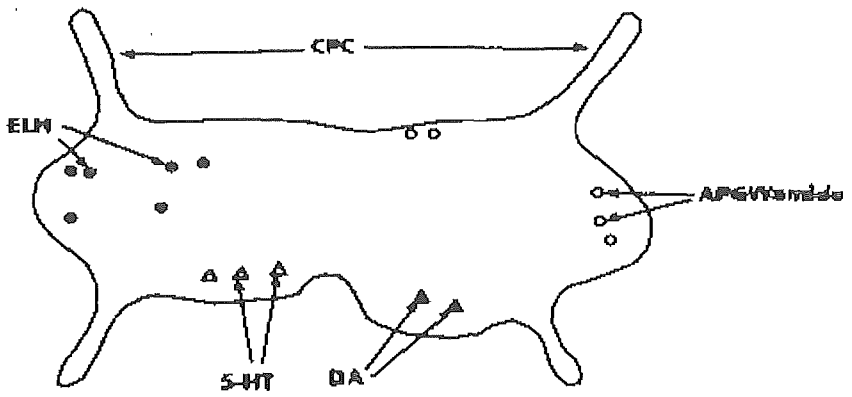

Fig. 1. Schematic representations of anti-ELH immunoreactivity (black circles) and anti-5-HT immunoreactivity (white triangles) on the left side and anti-APGWamide immunoreactivity (white circles) and anti-DA immunoreactivity (black triangles) on the right side in the pedal ganglia of *Perna canaliculus*.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

In the present study, immunocytochemical trials were carried out with antibodies raised against neurotransmitters and peptides including serotonin (5-HT), dopamine (DA), Ala-Pro-Gly-Trp-NH_2 (APGWamide) and egg-laying hormone (ELH), substances that have biological action in other molluscan species. The localisation of neurons containing serotonin in the central nervous system and the gonad of the scallop, *Patinopecten yessoensis,* was examined immunohisto-chemically (Matsutani and Nomura 1984) and these authors suggested that 5-HT be involved in the mechanism of spawning in *Patinopecten yessoensis.*

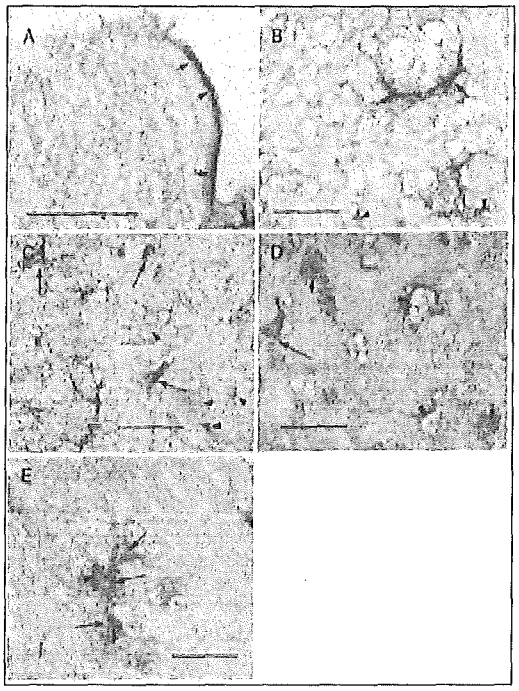

Fig. 2. Immunoreactivity in the pedal ganglia of green lipped mussel, *Perna canaliculus.* (A) Periphery of the ganglia (arrows) showing strong immunoreactivity to anti-ELH in a ripe female. Scale bar 50 μm. (B) Small cells (arrows), cell periphery and nerve fibres (arrowheads) showing immunoreactivity to anti-ELH in a spawned female. Scale bar 20 μm. (C) Several small cells (long arrows), nerve fibres (arrowheads) showing positive immunoreactivity to anti-APGWamide in a ripe male. Scale bar 20 μm. (D) Large cells (long arrow), the cell periphery of the cell body (arrowheads) and inclusion materials (short arrows) showing immunoreactivity to anti-5-HT in a spawned female. Scale bar 20 μm. (E) A few large cells (long arrows) and the inclusion materials (arrowheads) showing immunoreactivity to anti-DA in a spawned male. Scale bar 20 μm.

Antibodies raised against anti-ELH produced positive immunoreactivity in a few small cells, the connective sheath and in fibres in the pedal ganglia. The periphery of the pedal ganglia produced very strong anti-ELH immunoreactivity (Fig. 2A). Only the small cells showed immunoreactivity (Fig. 2B). These cells were not concentrated within any single region of the pedal ganglia but rather a few weakly stained cells were scattered throughout.

The size and location of anti-APGWamide labelled cells were similar to that of anti–ELH labelled cells in the pedal ganglia. Anti-APGWamide immunoreactive cell bodies were located along the peripheral margin and in the neuropile region (Fig. 2C). The pedal ganglia also contained numerous immunoreactive fibres throughout the ganglia. The larger cells revealed no immunoreactivity (Fig. 2D).

Anti-5HT produced light immunoreactivity throughout the pedal ganglia. A few large neurons and fibres in the neuropile revealed moderate immunoreactivity in the pedal ganglia of males (Fig. 2E). They were located in the cortex near the periphery of

the ganglia. Only the periphery of a few small cells showed positive immunoreactivity in the pedal ganglia. Nerve fibres were found to produce light immunoreactivity.

The peripheral sheath of the pedal ganglia and a few neurons produced anti-DA immunoreactivity. Large cells with different shapes and sizes were found with different staining intensities. The periphery of a few large cells produced strong immunoreactivity (Fig. 2F). These neurons were randomly scattered throughout the pedal ganglia.

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 pedal 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 characterised and evolutionarily conserved peptides within the gastropods (e.g., Van Minnen and Schallig 1990, Kerkhoven *et al.* 1993) 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 pedal 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 cerebral, pedal and 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*. 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 (Illanes-Bucher and Lubet 1980, Lubet and Mathieu 1978, 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 pedal 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 pedal 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* (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 1998), 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. However, results indicate that the ganglia of this mussel contain substances antigenically similar to peptides known to control reproduction in other molluscs. 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. Further experimentation is needed to identify different hormones/amines in these cells and to assign their exact role in the reproductive functions of the green-lipped mussel, *P. canaliculus.*

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