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Expression of prohormone convertase 2 and the generation of neuropeptides in the developing nervous system of the gastropod *Haliotis*

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ABSTRACT Prohormone convertase 2 (PC2) belongs to a family of enzymes involved in the proteolytic maturation of neuropeptide precursors into mature peptides that act as neurotransmitters, neuromodulators or neurohormones. Here we show that a gene encoding a PC2-like enzyme *(HasPC2)* is expressed during larval development and in the adult ganglia of the vetigastropod *Haliotis asinina*. *HasPC2* exhibits high sequence identity to other gastropod PC2s and thus is likely to function in peptide processing. Analysis of *HasPC2* expression indicates that it is activated early in nervous system development. During trochophore and early veliger larval stages, *HasPC2* is expressed in the vicinity of the forming ganglia of the central nervous system and parts of the putative peripheral nervous system. Later in larval development, at the time the veliger becomes competent to interact with the external environment and initiate metamorphosis, *HasPC2* expression largely restricts to cells of the major ganglia and their commissures. Profiling of veliger larvae by bioinformatic approaches suggests the expression of a variety of peptides. Direct MALDI-MS-based peptide profiling of juvenile *Haliotis* cerebral ganglia (brain) reveals an abundance of neuropeptides, including FMRFamide-related peptides and APGWamide, compatible with PC2 functioning in neuropeptide processing in these regions. These results are consistent with PC2 regulating neuropeptide generation in the earliest functioning of the gastropod nervous system.

KEY WORDS: *prohormone convertase, abalone, neuropeptide, development*

Neuropeptides are widespread in the animal kingdom and are often involved in the control of critical functions throughout the life cycle. The regulation of development by peptidergic hormones is a common feature of multicellular animals and has been studied in great detail in arthropods and vertebrates. Strikingly, investigations reveal that insects are as well or even better supplied with neuropeptides than mammals. In insects, neuropeptides regulate not only the functioning of endocrine glands, but also a wide range of physiological and developmental processes (Reumer *et al.*, 2008). Although a complete developmental peptidome analysis has not been established for other major invertebrate groups, such as the molluscs, numerous immunolocalization studies suggest that neuropeptides are equally important for their development. For instance, in gastropods FMRFamide and related peptides are amongst the earliest neurotransmitters expressed within the larval nervous system (Croll and Voronezhskaya, 1996; Dickinson *et al.*, 1999; Dickinson *et al.*, 2000; Haszprunar *et al.*, 2002; Dickinson and Croll, 2003; Croll, 2006). In the adult stages of these same animals, neuropeptides play a number of roles as local neurotransmitters and neuromodulators and as circulating hormones (Chase, 2002).

Peptide hormones and neuropeptides are often generated from larger precursor proteins via a complex series of post-translational modifications. One critical modification is proteolytic

Abbreviations used in this paper: DIG, digoxigenin; MALDI-MS, matrix-assisted laser desorption/ionization MS; PBS, phosphate-buffered solution; PC, prohormone convertase; RACE, rapid amplification of cDNA ends; WMISH, whole-mount in situ hybridization.

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processing to release one or more biologically active products. As a result, extensive research has focussed on characterising the enzymes responsible for this post-translational modification, in particular the prohormone convertases (PCs). Vertebrate and invertebrate PCs have been reported to cleave precursor proteins such as proglucagon (Rouille *et al.*, 1997), proopiomelanocortin (Korner *et al.*, 1991) and proinsulin (Davidson *et al.*, 1988). Presently, several vertebrate PCs have been characterised, including PC1 (same as PC3), PC2, PC4 and furin, each of which has a distinctive tissue distribution (Seidah *et al.*, 1990; Bloomquist *et al.*, 1991; Smeekens *et al.*, 1991; Braks *et al.*, 1992). These convertases are structurally related to bacterial subtilisins and KEX2 (Julius *et al.*, 1984), and typically cleave proproteins at pairs of basic amino acids, although cleavage at monobasic sites is also known (Hwang *et al.*, 2000b).

Homologues of PCs have also been reported in the pulmonate mollusc, *Lymnaea stagnalis* (Smit *et al.*, 1992) and the marine opisthobranch, *Aplysia californica* (Nagle *et al.*, 1995), as well as numerous other invertebrates, including the sheep blowfly, *Lucilia cuprina* (Mentrup *et al.*, 1999) and the fruitfly *Drosophila melanogaster* (Hwang *et al.*, 2000a). *L. stagnalis* possesses a PC2 gene that is exclusively expressed in the neuroendocrine system and encodes two alternatively-spliced transcripts of 3.0 and 4.8 kb (Smit *et al.*, 1992). In *A. californica*, PC2 cDNA was first identified from the abdominal ganglia (Chun *et al.*, 1994), which is known to produce large amounts of egg-laying peptides derived from the processing of a larger preprohormone (Fisher *et al.*, 1988).

In this study, we report the identification of a PC gene from the vetigastropods *Haliotis asinina* and *Haliotis rubra* (abalone), which appear to be orthologues of other gastropod PC2 genes. We have investigated the expression of this gene during the development of *H. asinina*, whose development and neuroendocrinology is relatively well-characterized (O'Brien and Degnan, 2000; Hinman and Degnan, 2002; Page, 2006). Whole mount *in situ* hybridization revealed a widespread expression pattern of the transcript during larval development and in the juvenile ganglia, largely restricted to nerve cells of the central and peripheral nervous systems. Bioinformatic and MALDI-MS molecular mass profiling of *H. asinina* allowed for detection of a large number of putative peptides, including FMRFamide, which is compatible with PC2 generating neuropeptides during development and growth.

Results

Characterisation of the abalone PC2 *genes*

Sequencing of PCR products, generated with degenerate PC2 oligonucleotide primers, from *H. rubra* neural and *H. asinina* larval

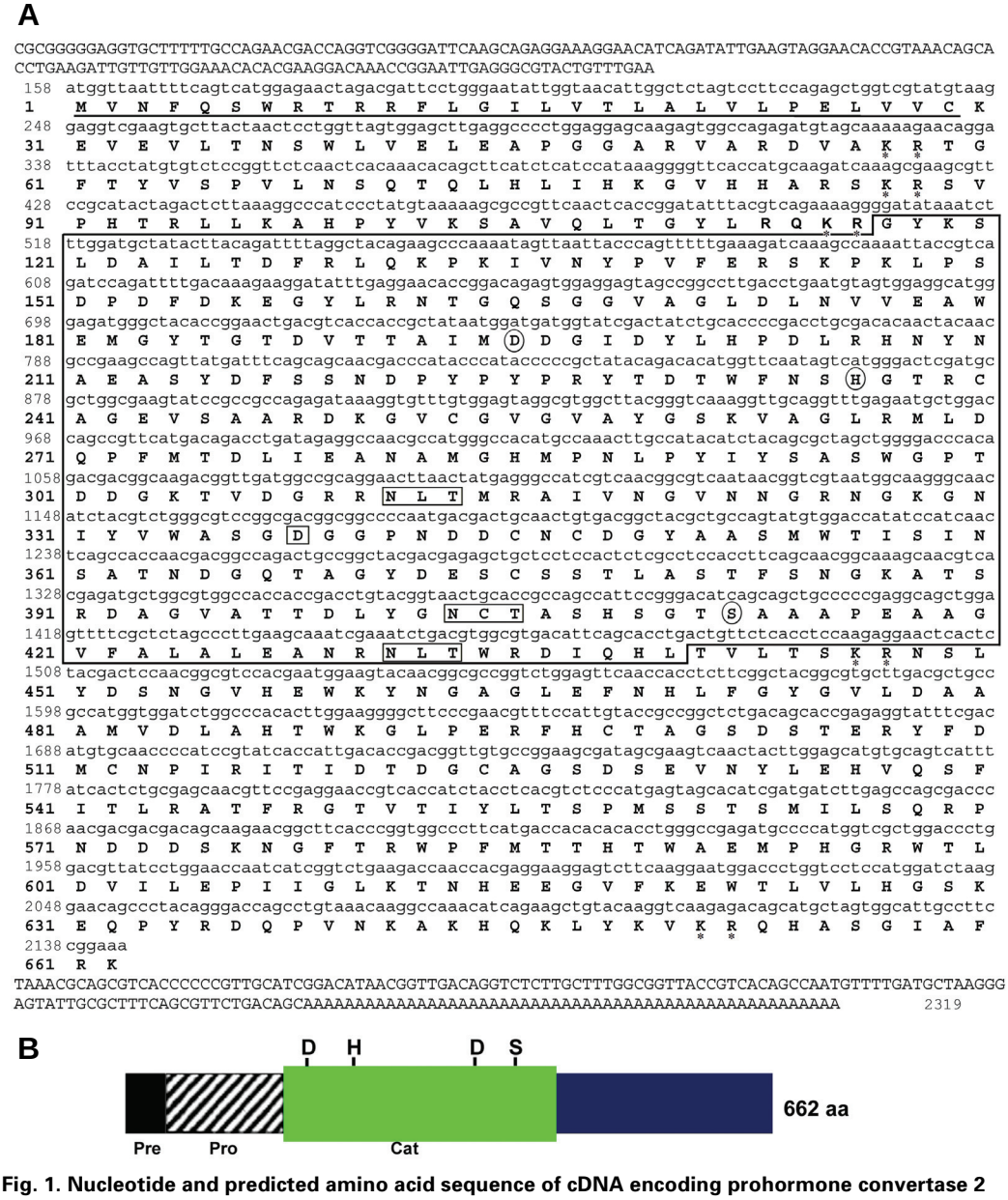


Fig. 1. Nucleotide and predicted amino acid sequence of cDNA encoding prohormone convertase 2 in *H. asinina*. (A) HasPC2 *consists of 2,319 bp encoding a precursor protein of 662 amino acids. Underline, predicted signal sequence; boxed, catalytic region and putative N-glycosylation sites. The Asp, His and Ser residues forming the catalytic triad are circled and the Asp residue stabilizing the oxyanion is boxed. Dibasic cleavage sites are identified with an asterisk.* **(B)** *Schematic representation of HasPC2 precursor protein showing the arrangement of encoded signal (pre), pro domain and catalytic region.*

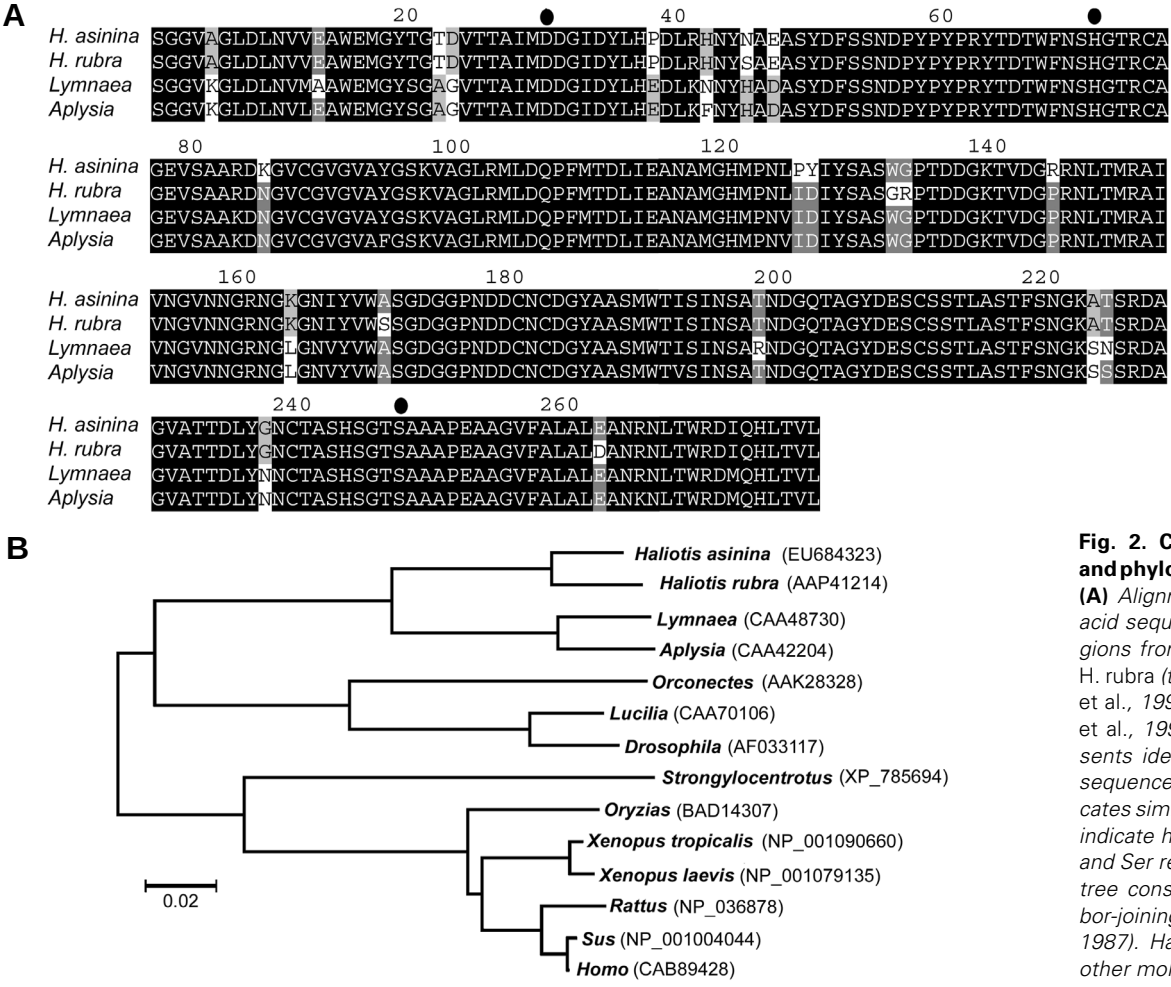


Fig. 2. Comparative alignment and phylogenetic analysis of PC2. **(A)** *Alignment of predicted amino acid sequence of PC2 catalytic regions from* H. asinina *(this study),* H. rubra *(this study),* Aplysia *(Nagle* et al.*, 1995), and* Lymnaea *(Kellett* et al.*, 1994). Black shading represents identical residues in all the sequences and grey shading indicates similar residues. Black circles indicate highly conserved Asp, His and Ser residues.* **(B)** *Phylogenetic tree constructed using the neighbor-joining method (Saitou and Nei, 1987). HasPC2 is most similar to other molluscan PC2's.*

cDNAs, confirmed that PC2 is expressed in these two species of abalone. In *H. asinina*, RACE led to a full-length cDNA of 2319 bp (*HasPC2*; GenBank accession number EU684323), which encodes a 662 amino acid precursor (Fig. 1A). The domain architecture of HasPC2 is identical to other PC2s, containing pre (signal), pro and catalytic domains (Fig. 1B). On both sides of the catalytic domain are two putative proteolytic cleavage sites (with a consensus sequence Lys-Arg). Both sites are likely to be used sequentially for autocatalytic activation and folding of the enzyme.

In *H. rubra*, RT-PCR led to a partial-length cDNA of 1,194 bp (*HrubPC2*, GenBank accession number AY237916), which encodes a 398 amino acid precursor. The resulting deduced amino acid sequences of both *H. asinina* and *H. rubra* catalytic regions were compared with molluscan homologues and showed significant sequence identity with *A. californica* (Nagle *et al.*, 1995) and *L. stagnalis* (Smit *et al.*, 1992) PC2s (Fig. 2A). A phylogenetic analysis clearly places the identified abalone genes within the PC2 class of enzyme, and most similar to *Lymnaea* and *Aplysia* homologues (Fig. 2B). Predicted subtilisin-related serine protease residues - D_{195}, H_{236} and S_{410} - were present within the abalone PC2s, and there were three conserved consensus sequences for putative N-linked glycosylation at NLT_{311}, NCT_{403} and NLT_{431}. As well, the abalone PC2s contain conserved multiple dibasic residue sequences, KR. The end of the catalytic region is likely to be directly after the L_{440}.

Larval expression of H. asinina *PC2 by RT-PCR*

RT-PCR was performed to identify *HasPC2* expression during larval development (Fig. 3). The *Has*PC2 gene is expressed continuously throughout development as *HasPC2* was present from 7 hours post-fertilization (hpf) to 24 hpf. The *HasPC2* amplicon (242 bp) is also obtained from a cDNA preparation of mixed larvae at various stages in development and the adult pooled central nervous system (CNS). The control, in which no cDNA template was used (blank), was negative.

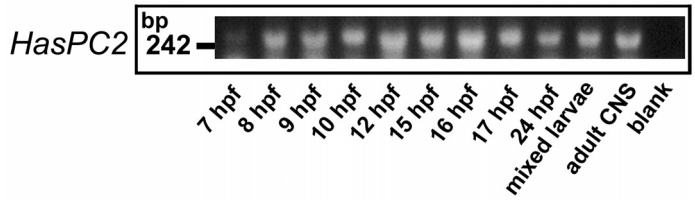


Fig. 3. Temporal expression of *Has*PC2 during larval development. *RT-PCR using specific primers was performed to identify PC2 expression at stages 7 hpf to 24 hpf, in mixed larvae and adult pooled CNS.* Has*PC2 is present at all stages examined. Blank corresponds to a negative control where the PCR reaction contained no cDNA. hpf, hours post-fertilization.*

In situ *hybridisation analysis of* HasPC2 *expression during larval development*

A 1021-bp digoxigenin-labeled antisense riboprobe was used to examine *HasPC2* expression in whole mount larval preparations during development. *HasPC2* transcripts were detected in the vicinity of the developing central and peripheral nervous systems in all stages of larval development, from newly hatched trochophores to competent veligers (96 hpf). In the 9 hpf trochophore larva, cells in the region of the apical tuft expressed *HasPC2* (Fig. 4 A,B). In 12 hpf and 14 hpf pretorsional veliger larvae and 17 hpf posttorsional veliger larvae, localised expression of *HasPC2* was detected in the head, foot, velum, and later, in the mantle (Fig. 4 C-H). Expression was restricted largely to the areas of the developing cerebral, pleural, pedal, esophageal and branchial ganglia, with the cerebral ganglia staining most intensely. Distinct expression was also observed in the region of the ganglia and commissures of 96 hpf competent larvae (Fig. 5).

TABLE 1

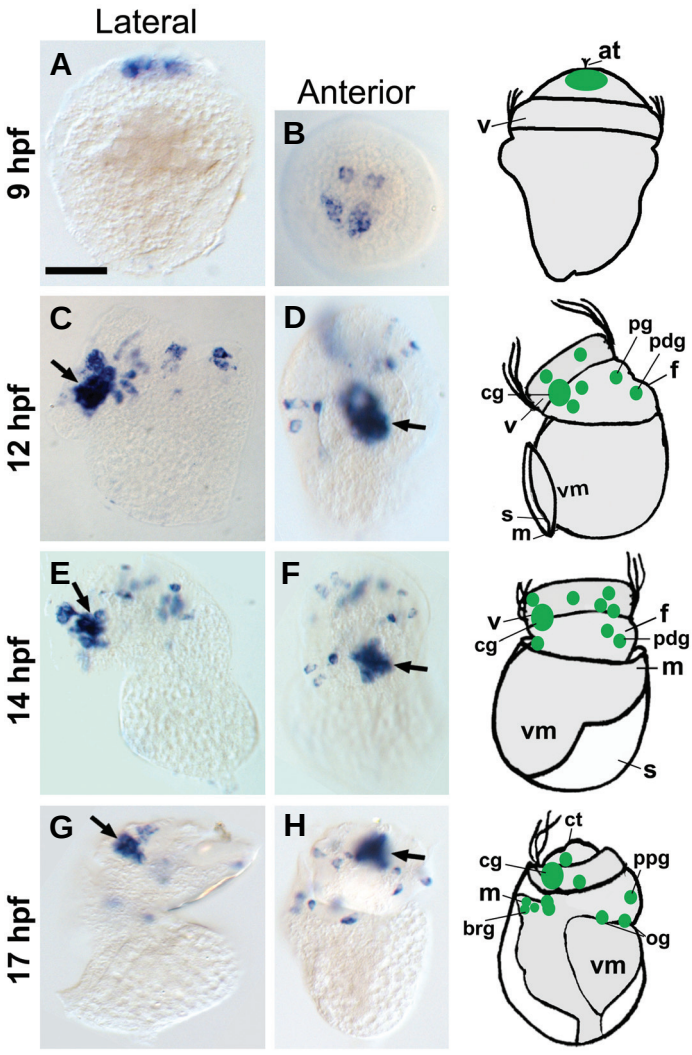
PEPTIDE DISTRIBUTION IN CEREBRAL AND PLEURO-PEDAL GANGLIA

Peptide	Expected MW (Da)	Observed MW (Da)	Ganglion region identified
FMRFamide	598.7	598.8	CC, LG1, LG2, PGM, RG1, RG2
FLRFamide	580.7	580.9	CC, LG1, LG2, PGM, RG1, RG2
QFYRIamide	724.9	724.9	LG1, PGM, RG1, RG2
pQFYRIamide	707.8	707.8	LG1, RG1, RG2
APGWamide	428.2	428.9	CC, LG1, LG2, PGM, RG1, RG2

CC, cerebral commissure; LG, left cerebral ganglion; RG, right cerebral ganglion; PGM, pleuro-pedal ganglion middle.

Direct MALDI-MS-based peptide profiling of juvenile H. asinina *ganglia*

Direct MALDI-MS-based profiling of neurons and nerves allows the determination of many of the mature processed peptides present within a sample. As shown in Fig. 6A, neuronal samples were derived from areas of the juvenile abalone cerebral and pleuro-pedal ganglion. MALDI-MS of a sub-section of the right ganglion (RG1) reveals the presence of several previously characterized molluscan peptides including APGWamide (428 Da),



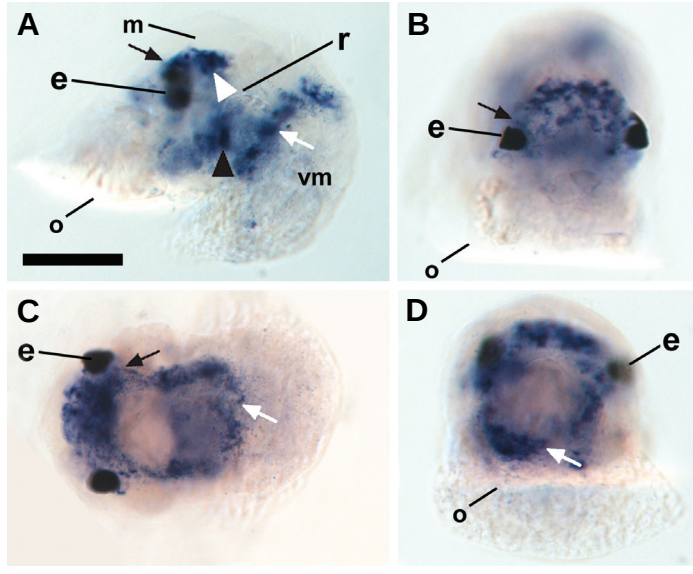


Fig. 4 (Left). WMISH showing spatial expression of *HasPC2* during larval development. (A,B) *Lateral and anterior, respectively, 9 hpf trochophore larva showing* HasPC2 *expression in the apical tuft.* **(C-H)** *12, 14 and 17 hpf larvae showing* HasPC2 *expression in areas of the cerebral (arrow), pedal, visceral, esophageal, branchial ganglia, and developing foot. Right schematics: Corresponding summary (lateral view) of spatial expression of* HasPC2 *from 9 hpf to 17 hpf. Areas represented by solid green circles were consistently stained. cg, cerebral ganglia; pg, pleural ganglia; pdg, pedal ganglia; ppg, fused pleuropedal ganglia; og, esophageal ganglia; brg, branchial ganglia; at, apical tuft; s, shell; v, velum; vm, visceral mass; f, foot; m, mantle; ct, cephalic tentacle. Scale bar, (A) 50 μm (same magnification in all panels).*

Fig (Right). 5. WMISH showing spatial expression of *HasPC2* in 96 hpf competent larvae. *Representative micrographs showing* HasPC2 *expression in the regions of developing ganglia from the lateral* **(A)**, *anterior* **(B)**, *dorsal* **(C)** *and posterior* **(D)** *views. Expression is most prominent in or near cerebral ganglia (black arrows), pleuro-pedal ganglia (black arrow heads), putative brachial ganglia (white arrow head) and developing loop of visceral cells (white arrows). vm, visceral mass; r, radula; e, eye; m, mantle; o, operculum. Scale bar: (A) 50 μm (same magnification in all panels).*

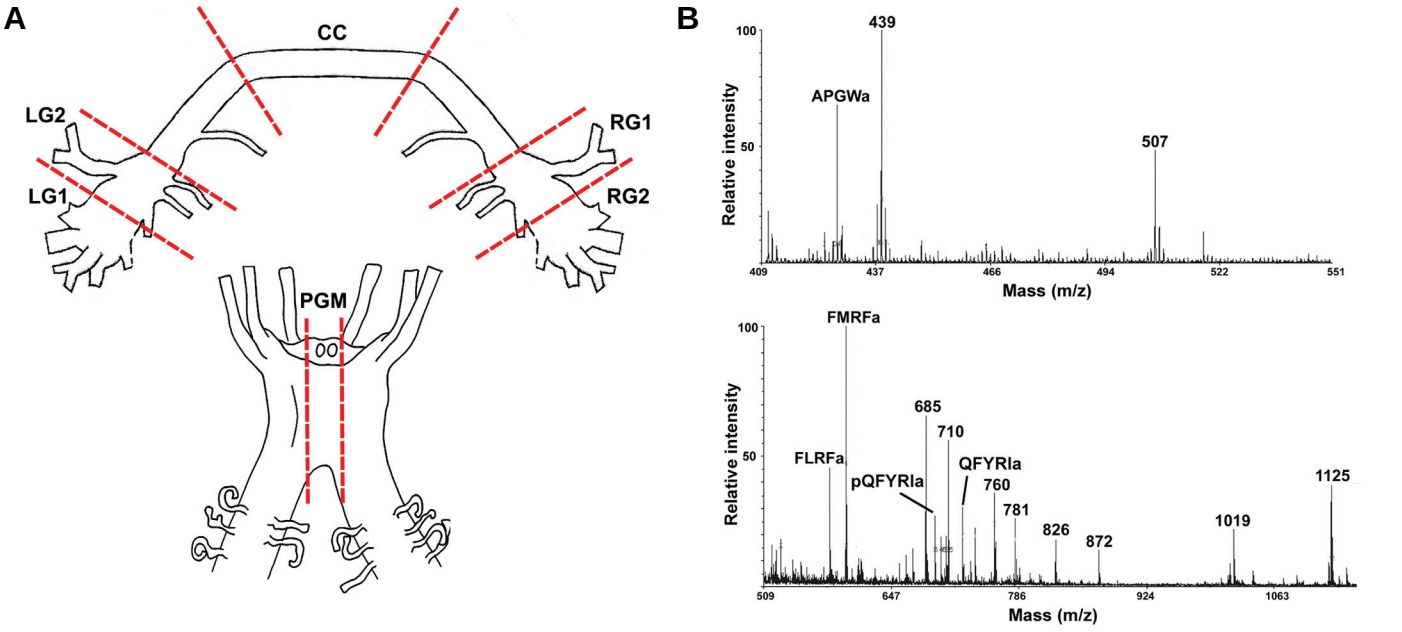


Fig. 6. *H. asinina* ganglia preparation and MALDI-MS analysis. (A) *Cerebral ganglia and pleuro-pedal ganglia were dissected and segments removed (shown by dashed lines). Sub-sections of LG (left cerebral ganglion), CC (cerebral commissue), RG (right cerebral ganglion) and pleuro-pedal ganglia middle (PGM) were spotted onto a MALDI sample plate.* **(B)** *Representative mass spectrums obtained from a sub-section of RG1. Mass peaks that match previously characterized molluscan peptides are labelled with peptide name.*

FLRFamide (580 Da), FMRFamide (598 Da), pQFYRIamide (707 Da), and QFYRIamide (725 Da) (Table 1; Fig. 6B). APGWamide, FLRFamide, and FMRFamide, were detected in all regions examined. pQFYRIamide and QFYRIamide were in the left and right cerebral and pleuro-pedal ganglion, but were not detected in cerebral commissure. Additional unassigned mass peaks likely represent other important neuropeptides processed from precursor proteins.

The Neuropred processing prediction web tool was applied to previously published EST sequences (Jackson and Degnan, 2006) to predict *H. asinina* larval peptides that may be released from precursor proteins following convertase activity. Table 2 lists peptide sequences, including post-translational modifications (Amide, amidation; Ac-, acetylation; p-, pyroglutamation) and average masses obtained. Precursors are predicted to be processed at defined basic cleavage sites and subsequent post-translational modifications added to generate peptides listed. Enzymes are expected to remove the N-terminal Arg, and those peptides ending in Gly are likely to be amidated, resulting in the final predicted average masses observed. The processing predictions imply that all precursors would be directed through the secretory pathway for extracellular release.

Discussion

This study shows that haliotids possess a *PC2* gene. Structurally, PCs contain defined regulatory regions including a prosegment, a catalytic domain, a structural domain (P domain), and a highly variable C-terminal domain. The catalytic domain is characterised by the presence of a region similar to the subtilisin-related serine proteases, including active site residues aspartic acid, asparagine, histidine and serine. All conserved subtilisin

TABLE 2

PEPTIDES PREDICTED TO BE GENERATED AND RELEASED FROM DEVELOPING ABALONE LARVAE

Clone*	Sequence (amino acid) + post-translational modification	MW (average)	Probability extracellular**
EST 36	[Ac-]GADDCILLAGPCG[Amide]	1245.43	67%
EST 48	[p-]EQELVTLL[Amide]	925.09	67%
EST 54	[p-]QFPGGGYRNMRFRGQGINYGPGPGIGS[Amide]	2823.14	33%
EST 63	[p-]QIGLYNRYNRFGLLN	1824.07	56%
EST 153	[p-]ETDQVSMRNI[Amide]	1173.31	67%
EST 1372	[Ac-]ASFPINISDTTAGDIVFAQSGSDITVSLKYSVGATPFADNGNVVYAC	4826.32	56%
EST 1395	[Ac-]AVLHYDPLEITSRLESEMDDLMKLVMKPHTLS	3755.37	56%
EST 1482	[p-]QPAPNELPDKL	1204.3	56%
	[Ac-]SDSVFYTSTDGQNGKGISFMATRHSFPSADLSLSFCQLMVL[Amide]	4488.04	
EST 2823	[p-]QHIPLYPCETCVIEAFSD	2048.31	44%
EST 3345	DIGHSLTNVFQSLGHSLMHQLDTTGVDLLNAGINA[Amide]	3689.12	67%

* (Jackson and Degnan, 2006). ** Based on precursor signal sequence (SignalP 3.0 and PSORTII)

sites are present in HasPC2. There is a high degree of sequence identity within this domain, thus reflecting the functional importance of these residues in the catalytic activity of the enzyme.

Apart from the high degree of conservation for the convertase gene sequences, there are several additional common features, which are important for convertase maturation and activity, including the presence of putative post-translational modification sites. Post-translational modifications known to occur in PCs (Seidah *et al.*, 1993) include tyrosine sulfation and phosphorylation, and N-linked glycosylation, which prevents PC2 degradation in the endoplasmic reticulum (Benjannet *et al.*, 1993). From the sequences analysed, it appears that abalone PC2 is also a glycoprotein. In HasPC2, there are three conserved consensus sequences for N-linked glycosylation; although based on analysis of vertebrate PC2, it has been suggested that only one site, NCT_{403}, is used (Smit *et al.*, 1992). Multiple basic sites within PC are also required for co- and post-translational processing.

Analysis of a range of larval developmental stages and the juvenile brain ganglia of *H. asinina* reveals that the *HasPC2* gene is consistently expressed in the vicinity of ganglia and putative sensory cells. It has been shown that PC genes in other invertebrates have a neuroendocrine-specific expression pattern (Smit *et al.*, 1992; Nagle *et al.*, 1995). For instance, the *Lymnaea PC2* mRNA is found exclusively in the central nervous system (Smit *et al.*, 1992). *HasPC2* transcripts are also present in the nervous system (i.e. cerebral ganglia and pleuro-pedal ganglia). Within these ganglia, *HasPC2* mRNA appears to be expressed in a cell-specific manner from the earliest formation of the nervous system (Hinman *et al.*, 2003). In fact, the cells labelled in the periphery (foot and mantle) may also represent sensory cells (O'Brien and Degnan, 2002a; O'Brien and Degnan, 2002b). For example, peripheral sensory cells appear to be abundant in the foot of developing molluscs (Dickinson *et al.*, 1999), and some of these cells have been shown to contain FMRFamide-like peptides (Dickinson and Croll, 2003; Croll, 2006). These observations suggest that HasPC2 has a general role in processing neuropeptides such as FMRFamide and related peptides throughout the life cycle of haliotids and possibly other gastropods. In all other animals studied, FMRFamide and related peptides are produced by proteolytic cleavage from a precursor protein with subsequent amidation of the individual peptides (Kellett *et al.*, 1994; Favrel *et al.*, 1998).

This is the first study to show a convertase is expressed early in molluscan development. While FMRFamide-like immunoreactivity has been reported widely in early larval stages of gastropod molluscs (Croll and Voronezhskaya, 1996; Dickinson *et al.*, 2000; Haszprunar *et al.*, 2002; Dickinson and Croll, 2003; Croll, 2006), neither the transcript for FMRFamide encoding genes nor the enzymes necessary for post-translational processing of the precursor protein have previously been demonstrated in such early larval stages. The results from this study suggest that HasPC2 is contributing to the proteolytic activity necessary for the synthesis of FMRFamide and related peptides. Further supporting a role for HasPC2 in the generation of neuropeptides during larval development, is the bioinformatic detection of a range of peptides from a larvae EST set (Jackson and Degnan, 2006). This analysis combined with direct MALDI-MS-based profiling of a range of neuropeptides in the juvenile brain (e.g. FMRFamide, FLRFamide, QFYRIamide) is consistent with the requirement for convertase activity early in the abalone's life. Our direct demonstration of PC2 expression in the adult nervous system, when combined with immunocytochemical evidence (Chansela *et al.*, 2008), and our own findings in juvenile abalone of FMRFamide-related peptides and APGWamide, both of which are processed from larger precursor proteins, suggests that HasPC2 is functioning from the earliest stages of development onwards. Previous studies have additionally suggested the presence of other neuropeptides such as egg-laying hormone peptides (Saitongdee *et al.*, 2005) in adult abalone, expanding the potential role of HasPC2 to a range of physiological processes.

We conclude that HasPC2 is likely to be important throughout abalone ontogeny for successful neuropeptide activation necessary for regulation of ongoing physiological functions and possibly even the control of the large-scale remodelling that the nervous system undergoes during larval development. The expression pattern is consistent with the notion that genes encoding prohormone convertases are active where specific cleavages of precursor proteins are known to occur. This information will be of value in further investigations to determine the roles played by the various peptides released by prohormone convertases.

Materials and Methods

Animals

Gravid *H. asinina* were collected from Heron Island Reef, Great Barrier Reef, Australia. Gametes were taken from a natural *H. asinina* spawning, and the resultant eggs, embryos and larvae were reared at 24°C in"10" µm-filtered sea water. Larvae of different developmental stages and one whole juvenile were prepared for either (1) total RNA extraction using the TriReagent (Molecular Research Centre), following the manufacturer's instructions, or (2) fixed in 4% paraformaldehyde for 30 min, followed by serial dehydration in ethanol. The developmental stages of *H. asinina* have been described previously (Hinman *et al.*, 2003). Adult female *H. rubra* were collected from Port Phillip Bay (Victoria, Australia) under a Fisheries research permit (97/R/049A). Total RNA was extracted from cerebral ganglia using Trizol (Invitrogen) following the manufacturer's instructions.

Isolation and characterisation of abalone PC2 *cDNAs*

Conserved portions of the *H. asinina* and *H. rubra* PC2 were amplified from cDNA reverse transcribed from RNAs isolated from larvae and cerebral ganglia, respectively. Degenerate oligonucleotide primers were AbPC2(1) 5′-AGCMATTATGGAYGAYGGWATYGA-3′;
AbPC2(2), 5′-CCANGTYTGNGTNGTCATYAANGG-3′, or as previously described (Selvamani *et al.*, 1997). The full-length *H. asinina PC2* (*HasPC2*) was isolated by RACE of same stage cDNA using a set of gene-specific primers (available upon request). All RT-PCR products were size fractionated by gel electrophoresis, cloned into a pGEM-T vector (Promega), sequenced, and consensus sequences created in Sequencher*t* 3.1.1 (Gene Codes Corporation). Nucleotide sequences belonging to the targeted class were identified using the BLASTx algorithm to search against the National Center for Biotechnology Information (NCBI) database.

Phylogenetic analysis

Conceptual amino acid translations of the *HasPC2* was aligned using Clustal X 1.64b (Thompson *et al.*, 1997) to a selection of protein sequences that closely matched during the BLAST analysis and represented all the known families of PC2. Alignments were edited visually in the Sequence Alignment Program Se-Al v1.d1 (Rambaut *et al.*, 1996) and regions of uncertain alignment were removed. Phylogenetic trees were

constructed using MEGA3 software (Kumar *et al.*, 2004) with 1000 bootstrap trials using the neighbor-joining method (Saitou and Nei, 1987) and presented with a cutoff bootstrapping value of 50.

Temporal expression during* H. asinina *larval development

H. asinina cDNAs were generated from collected larvae at stages 7 h, 8 h, 9 h, 10 h, 12 h, 15 h, 16 h, 17 h and 24 h post-fertilization, from a mixed larval preparation, and from adult pooled CNS total RNA. For PCR, 1 µl cDNA was used for amplification using the following sequence-specific primers derived from *H. asinina* PC2 to produce a 242-bp product –
PC2-sense 5′- ACCAGCCGTTCATGACAGACC -3′,
PC2-antisense 5′- TCGTTGGTGGCTGAGTTGATG -3′.

PCR reactions had a final concentration of 1x PCR Buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.5 µM of sense and antisense primer, 0.25 units of *Taq* polymerase, and water to a final volume of 25 µl. As a negative control, the PCR reaction contained no cDNA. PCR consisted of 36 cycles, including 94°C/2 min denaturation, 62°C/1 min annealing and 72°C/1 min extension. Following PCR, 10 µl of amplification product was fractionated on a 2% agarose gel and visualized by ethidium bromide staining.

Probes and whole mount* in situ *hybridization (WMISH)

A 1021-bp amplification fragment was obtained spanning nucleotides 599-1619 of the *Has*PC2 cDNA following PCR with the sense primer 5'-TTCAACTCACCGGATATTTACG-3' and the antisense primer 5'-TTGGAGGTGAGAACAGTCAGG-3'. The amplicon was cloned into the transcription vector pGEM-T. After plasmid isolation and amplification of the template DNA using M13 forward and reverse primers, *in vitro* transcription reactions were carried out in the presence of digoxigenin-UTP (DIG RNA Labeling Kit, Roche), with SP6 polymerase for antisense probes. The template was degraded with RNase-free DNase (Roche Molecular Biochemical). The DIG-labeled riboprobes were purified by ethanol and lithium chloride precipitation and stored at -80°C in RNase-free water until use for *in situ* hybridization.

WMISH was performed using DIG-labeled riboprobes with modifications according to (Hinman *et al.*, 2000). Fixed larvae (9 hours post-fertilization, 12 hpf, 14 hpf, 17 hpf and 96 hpf) were rehydrated into phosphate-buffered solution (PBS) with 0.1% Tween20. After Proteinase K treatment (20 µg/ml in PBS plus 0.1% Tween20 at 37°C for 10–20 min) specimens were pre-hybridized 5 h in 50% formamide, 5x sodium saline citrate (SSC), 5 mM EDTA, 1% Denhardt's solution, 100 µg/ml heparin, 100 µg/ml tRNA, 0.1% Tween20 at 55°C. Hybridization was performed using the same solution and by adding 200 ng/ml DIG-labeled riboprobe overnight at 55°C. Specimens were subsequently washed at 55°C twice in 50% formamide, 4xSSC, 0.1% Tween20, then twice in 50% formamide, 2xSSC 0.1% Tween20, then twice in 50% formamide, 1xSSC, 0.1% Tween20, for 15 min each, and then stepped into 0.1 M maleic acid, pH 7.5, 0.15 M NaCl, 0.1% Tween20. Antibody incubation for detection procedure was also performed overnight, followed by several washing steps (Shain and Zuber, 1996). Staining reactions were done by using Nitro Blue Tetrazolium/5-Bromo-4-Chloro-3- Indolyl Phosphate in glycerol. For documentation, specimens were dehydrated by stepwise ethanol changes, cleared in benzyl benzoate: benzyl alcohol (2:1 v/v) and mounted in 70% glycerol.

Direct MALDI-MS-based peptide profiling of ganglia

Cerebral ganglia, including commissure, and pleuro-pedal ganglia were removed from a juvenile (14 mth) abalone after injection of 390 mM $MgCl_2$. Segments were cut into left cerebral ganglion 1 and 2 (LG1, LG2), right cerebral ganglion 1 and 2 (RG1, RG2), cerebral commissure (CC) and pleuro-pedal ganglia middle (PGM). The method of direct MALDI-MS followed that described by Floyd *et al*. (Floyd *et al.*, 1999), with modifications. Salts were eliminated by washing in an aqueous MALDI matrix solution, 20 mg/ml of 2,5-dihydroxybenzoic acid (Sigma) in 30% acetonitrile/0.1% trifluoroacetic acid. Fine tweezers and needles were used to desheath and isolate (<1 mm) sections from each segment. Each subsection was then placed onto a MALDI-MS sample plate containing 0.5 µl of matrix solution. After drying at ambient temperature, samples were analyzed immediately. MALDI-TOF mass spectrometry was performed on a Voyager-DE STR Biospectrometry workstation (Applied Biosystems, Australia), equipped with a N_2 laser and pulsed ion extraction accessory. The instrument was calibrated using a standard peptide mixture (Sigma). Final spectra resulted from 500 shots, recorded in the reflectron mode within a mass range from m/z 400 to m/z 1200.

Prediction of larval precursor processing

General rules for precursor protein cleavage recognition sites have been proposed (Southey *et al.*, 2006b). The NeuroPred processing prediction web tool (http://neuroproteomics.scs.uiuc.edu/neuropred.html) (Southey *et al.*, 2006a) was used to predict cleavage sites at basic amino acid locations, average masses and post-translational modifications to selected abalone larval EST precursor sequences (Jackson and Degnan, 2006). Signal sequence predictions were performed by SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP) (Bendtsen *et al.*, 2004) and cellular pathway prediction determined by PSORTII (http://psort.ims.u-tokyo.ac.jp/form2.html) (Nakai and Horton, 1999).

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References

BENDTSEN, J., NIELSEN, H., VON HEIJNE, G. and BRUNAK, S. (2004). Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* 340: 783–795.

BENJANNET, S., RONDEAU, N., PAQUET, L., BOUDREAULT, A., LAZURE, C., CHRETIEN, M. and SEIDAH, N.G. (1993). Comparative biosynthesis, covalent post-translational modifications and efficiency of prosegment cleavage of the prohormone convertases PC1 and PC2: glycosylation, sulphation and identification of the intracellular site of prosegment cleavage of PC1 and PC2. *Biochem J* 294: 735-743.

BLOOMQUIST, B.T., EIPPER, B.A. and MAINS, R.E. (1991). Prohormone-converting enzymes: regulation and evaluation of function using antisense RNA. *Mol Endocrinol* 5: 2014-2024.

BRAKS, J.A., GULDEMOND, K.C., VAN RIEL, M.C., COENEN, A.J. and MARTENS, G.J. (1992). Structure and expression of *Xenopus* prohormone convertase PC2. *FEBS Lett* 305: 45-50.

CHANSELA, P., SAITONGDEE, P., STEWART, P., SOONKLANG, N., STEWART, M., SUPHAMUNGMEE, W., POOMTONG, T. and SOBHON, P. (2008). Existence of APGWamide in the testis and its induction of spermiation in *Haliotis asinina* Linnaeus. *Aquaculture* 279: 142-149.

CHASE, R.B. (2002). *Behavior and its neural control in gastropod molluscs*. Oxford University Press US.

CHUN, J.Y., KORNER, J., KREINER, T., SCHELLER, R.H. and AXEL, R. (1994). The function and differential sorting of a family of *Aplysia* prohormone processing enzymes. *Neuron* 12: 831-844.

CROLL, R.P. (2006). Development of embryonic and larval cells containing serotonin, catecholamines, and FMRFamide-related peptides in the gastropod mollusc *Phestilla sibogae*. *Biol Bull* 211: 232-247.

CROLL, R.P. and VORONEZHSKAYA, E.E. (1996). Early elements in gastropod neurogenesis. *Dev Biol* 173: 344-347.

DAVIDSON, H.W., RHODES, C.J. and HUTTON, J.C. (1988). Intraorganellar calcium and pH control proinsulin cleavage in the pancreatic beta cell via two distinct site-specific endopeptidases. *Nature* 333: 93-96.

DICKINSON, A.J., CROLL, R.P. and VORONEZHSKAYA, E.E. (2000). Development of embryonic cells containing serotonin, catecholamines, and FMRFamide-

related peptides in *Aplysia californica*. *Biol Bull* 199: 305-315.

DICKINSON, A.J.G. and CROLL, R.P. (2003). Development of the larval nervous system of the gastropod *Ilyanassa obsoleta*. *J Comp Neurol* 466: 197-218.

DICKINSON, A.J.G., NASON, J. and CROLL, R.P. (1999). Histochemical localization of FMRFamide, serotonin and catecholamine in embryonic *Crepidula fornicata* (Prosobranchia: Gastropoda). *Zoomorphology* 119: 49-62.

FAVREL, P., LELONG, C. and MATHIEU, M. (1998). Structure of the cDNA encoding the precursor for the neuropeptide FMRFamide in the bivalve mollusc *Mytilus edulis*. *Neuroreport* 9: 2961-2965.

FISHER, J.M., SOSSIN, W., NEWCOMB, R. and SCHELLER, R.H. (1988). Multiple neuropeptides derived from a common precursor are differentially packaged and transported. *Cell* 54: 813-822.

FLOYD, P.D., LI, L., MOROZ, T.P. and SWEEDLER, J.V. (1999). Characterization of peptides from *Aplysia* using microbore liquid chromatography with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry guided purification. *J Chromatogr A* 830: 105-113.

HASZPRUNAR, G., FRIEDRICH, S., WANNINGER, A. and RUTHENSTEINER, B. (2002). Fine structure and immunocytochemistry of a new chemosensory system in the Chiton larva (Mollusca: Polyplacophora). *J Morphol* 251: 210-218.

HINMAN, V.F., BECKER, E. and DEGNAN, B.M. (2000). Neuroectodermal and endodermal expression of the ascidian Cdx gene is separated by metamorphosis. *Dev Genes Evol* 210: 212-216.

HINMAN, V.F. and DEGNAN, B.M. (2002). Mox homeobox expression in muscle lineage of the gastropod *Haliotis asinina*: evidence for a conserved role in bilaterian myogenesis. *Dev Genes Evol* 212: 141-144.

HINMAN, V.F., O'BRIEN, E.K., RICHARDS, G.S. and DEGNAN, B.M. (2003). Expression of anterior Hox genes during larval development of the gastropod *Haliotis asinina*. *Evol Dev* 5: 508-521.

HWANG, J.R., SIEKHAUS, D.E., FULLER, R.S., TAGHERT, P.H. and LINDBERG, I. (2000a). Interaction of *Drosophila melanogaster* prohormone convertase 2 and 7B2. Insect cell-specific processing and secretion. *J Biol Chem* 275: 17886-17893.

HWANG, S.R., NG, S.M., STEINECKERT, B., SEIDAH, N.G. and HOOK, V.Y. (2000b). Molecular cloning demonstrates structural features of homologous bovine prohormone convertases 1 and 2. *DNA Cell Biol* 19: 409-419.

JACKSON, D.J. and DEGNAN, B.M. (2006). Expressed sequence tag analysis of genes expressed during development of the tropical abalone *Haliotis asinina*. *J Shellfish Res* 25: 225-231.

JULIUS, D., BRAKE, A., BLAIR, L., KUNISAWA, R. and THORNER, J. (1984). Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro-alpha-factor. *Cell* 37: 1075-1089.

KELLETT, E., SAUNDERS, S.E., LI, K.W., STADDON, J.W., BENJAMIN, P.R. and BURKE, J.F. (1994). Genomic organization of the FMRFamide gene in *Lymnaea*: multiple exons encoding novel neuropeptides. *J Neurosci* 14: 6564-6570.

KORNER, J., CHUN, J., HARTER, D. and AXEL, R. (1991). Isolation and functional expression of a mammalian prohormone processing enzyme, murine prohormone convertase 1. *Proc Natl Acad Sci USA* 88: 6834-6838.

KUMAR, S., TAMURA, K. and NEI, M. (2004). MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* 5: 150-163.

MENTRUP, B., LONDERSHAUSEN, M., SPINDLER, K. and WEIDEMANN, W. (1999). Isolation and characterization of insect PC2-like prohormone convertase cDNA. *Insect Mol Biol* 8: 305-310.

NAGLE, G.T., GARCIA, A.T., KNOCK, S.L., GORHAM, E.L., VAN HEUMEN, W.R. and KUROSKY, A. (1995). Molecular cloning, cDNA sequence, and localization of a prohormone convertase (PC2) from the *Aplysia* atrial gland. *DNA Cell Biol* 14: 145-154.

NAKAI, K. and HORTON, P. (1999). PSORT: a program for detecting the sorting signals of proteins and predicting their subcellular localization. *Trends Biochem Sci* 24: 34-35.

O'BRIEN, E.K. and DEGNAN, B.M. (2000). Expression of POU, Sox, and Pax genes in the brain ganglia of the tropical abalone *Haliotis asinina*. *Mar Biotechnol (NY)* 2: 545-557.

O'BRIEN, E.K. and DEGNAN, B.M. (2002a). Developmental expression of a class IV POU gene in the gastropod *Haliotis asinina* supports a conserved role in sensory cell development in bilaterians. *Dev Genes Evol* 212: 394-398.

O'BRIEN, E.K. and DEGNAN, B.M. (2002b). Pleiotropic developmental expression of *Has*POU-III, a class III POU gene, in the gastropod *Haliotis asinina*. *Mech Dev* 114: 129-132.

PAGE, L.R. (2006). Early differentiating neuron in larval abalone (*Haliotis kamtschatkana*) reveals the relationship between ontogenetic torsion and crossing of the pleurovisceral nerve cords. *Evol Dev* 8: 458-467.

RAMBAUT, A., GRASSLY, N., NEE, S. and HARVEY, P. (1996). Bi-De: an application for simulating phylogenetic processes. *Comput Appl Biosci* 12: 469-471.

REUMER, A., VAN LOY, T., CLYNEN, E. and SCHOOFS, L. (2008). How functional genomics and genetics complements insect endocrinology. *Gen Comp Endocrinol* 155: 22-30.

ROUILLE, Y., KANTENGWA, S., IRMINGER, J.C. and HALBAN, P.A. (1997). Role of the prohormone convertase PC3 in the processing of proglucagon to glucagon-like peptide 1. *J Biol Chem* 272: 32810-32816.

SAITONGDEE, P., APISAWETAKAN, S., ANUNRUANG, N., POOMTHONG, T., HANNA, P. and SOBHON, P. (2005). Egg-laying-hormone immunoreactivity in the neural ganglia and ovary of *Haliotis asinina* Linnaeus. *Invert Neurosci*. 5: 165-172.

SAITOU, N. and NEI, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4: 406-425.

SEIDAH, N.G., DAY, R. and CHRETIEN, M. (1993). The family of pro-hormone and pro-protein convertases. *Biochem Soc Trans* 21 (Pt 3): 685-691.

SEIDAH, N.G., GASPAR, L., MION, P., MARCINKIEWICZ, M., MBIKAY, M. and CHRETIEN, M. (1990). cDNA sequence of two distinct pituitary proteins homologous to Kex2 and furin gene products: tissue-specific mRNAs encoding candidates for pro-hormone processing proteinases. *DNA Cell Biol* 9: 789.

SELVAMANI, M.J.P., COUNIHAN, R.T., VAN HEUMEN, W.R.A., JEBREEN, E.J., PRESTON, N.P. and DEGNAN, B.M. (1997). Regulation of reproduction in a tropical abalone *Haliotis asinina*: expression of a prohormone convertase gene in the central nervous system. In *Proceedings of the Australian Coral Reef Society 75th Anniversary*, (ed. GREENWOOD, J. G. and HALL, N. J.), pp. 119-124. Heron Island School of Marine Science, University of Queensland.

SHAIN, D.H. and ZUBER, M.X. (1996). Sodium dodecyl sulfate (SDS)-based whole-mount *in situ* hybridization of *Xenopus laevis* embryos. *J Biochem Biophys Methods* 31: 185-188.

SMEEKENS, S.P., AVRUCH, A.S., LAMENDOLA, J., CHAN, S.J. and STEINER, D.F. (1991). Identification of a cDNA encoding a second putative prohormone convertase related to PC2 in AtT20 cells and islets of Langerhans. *Proc Natl Acad Sci USA* 88: 340-344.

SMIT, A.B., SPIJKER, S. and GERAERTS, W.P. (1992). Molluscan putative prohormone convertases: structural diversity in the central nervous system of *Lymnaea stagnalis*. *FEBS Lett* 312: 213-218.

SOUTHEY, B., AMARE, A., ZIMMERMAN, T., RODRIGUEZ-ZAS, S. and SWEEDLER, J. (2006a). NeuroPred: a tool to predict cleavage sites in neuropeptide precursors and provide the masses of the resulting peptides. *Nucl Acids Res* 34: 267–272.

SOUTHEY, B., RODRIGUEZ-ZAS, S. and SWEEDLER, J. (2006b). Prediction of neuropeptide prohormone cleavages with application to RFamides. *Peptides* 27: 1087-1098.

THOMPSON, J.D., GIBSON, T.J., PLEWNIAK, F., JEANMOUGIN, F. and HIGGINS, D.G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl Acids Res* 25: 4876-4882.

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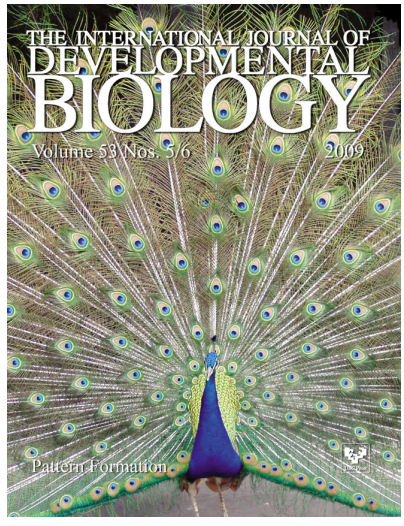

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