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ScienceDirect
General and Comparative Endocrinology 155 (2008) 526–533
GENERAL AND COMPARATIVE ENDOCRINOLOGY
www.elsevier.com/locate/ygcen

Short Communication

Identification of A-type allatostatins possessing −Y*X*FGI/Vamide carboxy-termini from the nervous system of the copepod crustacean *Calanus finmarchicus*

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Received 9 July 2007; revised 23 August 2007; accepted 12 September 2007
Available online 19 September 2007

Abstract

The copepod crustacean *Calanus finmarchicus* plays a critical role in the ecology of the Gulf of Maine and other regions of the North Atlantic. To increase our understanding of the physiology of this species, a normalized, whole organism cDNA library was constructed, and expressed sequence tags (ESTs) of the clones were generated. Among these ESTs was one with homology to known cDNAs encoding prepro-A-type allatostatins (A-type ASTs), a well-known family of arthropod peptides that regulate juvenile hormone production in insects. Sequence analysis of the clone from which the EST was generated, with subsequent translation of its open reading frame, showed it to encode five novel A-type ASTs, whose mature structures were predicted to be APYGFGIamide, pE/EPYGFGIamide, ALYGFGIa mide, pE/EPYNFGIamide, and pQ/QPYNFGVamide. Each of the peptides is present as a single copy within the prepro-hormone with the exception of APYGFGIamide, which is present in three copies. Surprisingly, the organization of the *Calanus* prepro-A-type AST, specifically the number of encoded A-type peptides, is more similar to those of insects than it is to the known decapod crustacean prepro-hormones. Moreover, the *Calanus* A-type ASTs possess isoleucine or valine residues at their carboxy (C)-termini rather than leucine, which is present in most other family members. Wholemount immunohistochemistry suggests that six pairs of somata produce the native *Calanus* A-type ASTs: five in the protocerebrum and one in the suboesophageal region. To the best of our knowledge, our report is the first characterization of a neuropeptidergic system in a copepod, the first identification of A-type ASTs from a non-decapod crustacean, the first report of crustacean A-type ASTs possessing isoleucine C-terminal residues, and the first report from any species of an A-type peptide possessing a valine C-terminal residue.

Keywords: *Calanus finmarchicus*; Allatostatin (AST); Expressed sequence tag (EST); cDNA; Neuropeptide; Neurohormone; Central nervous system (CNS); Immunohistochemistry

1. Introduction

In insects, juvenile hormone (JH), a sesquiterpenoid produced by the corpora allata, is responsible for maintaining juvenile characteristics during development and promoting reproductive function in adults (for review see, Nijhout, 1994; Riddiford, 1994; Wyatt and Davey, 1996).

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0016-6480/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygcen.2007.09.002

In the insect corpora allata, JH synthesis is regulated, at least in part, by innervation from protocerebral neurosecretory cells, which contain and release peptides possessing the carboxy (C)-terminal motif −Y*X*FGL/Iamide (where *X* is a variable amino acid), commonly referred to as A-type allatostatins (A-type ASTs). A large number of A-type ASTs have been identified in insects, and functional studies have implicated these peptides in a number of physiological processes in addition to their role in the regulation of JH (for review see, Stay and Tobe, 2007).

While most work on the A-type ASTs has been done in insects, A-type peptides have also been identified from decapod crustaceans, here all possessing −Y*X*FGLamide C-termini (Duve et al., 1997, 2002; Dircksen et al., 1999; Huybrechts et al., 2003; Fu et al., 2005; Yasuda-Kamatani and Yasuda, 2006; Yin et al., 2006). While few functional studies of A-type ASTs have been conducted in decapods, one role that has been suggested is the regulation of sesquiterpenoid production, i.e., regulation of methyl farnesoate synthesis by the mandibular organ (Kwok et al., 2005).

Given the putatively ubiquitous role of A-type ASTs in the regulation of sesquiterpenoid biosynthesis, and thus their regulation of development, growth and reproduction, it is interesting that nothing is known about this peptide family in any primitive crustaceans, e.g., copepods, despite their ecological significance as food items for commercially important fish species and critically endangered marine mammals (e.g., Murison and Gaskin, 1989). In fact, little is known in general about hormonal control in these animals. To help facilitate gene-based studies of copepod biology, expressed sequence tags (ESTs) have recently been produced from a normalized, whole organism, cDNA library constructed for *Calanus finmarchicus*, a copepod species of considerable importance in the Gulf of Maine and other North Atlantic regions. Here, we utilized this resource to identify a *C. finmarchicus* cDNA encoding A-type ASTs and then used immunohistochemistry to map the distribution of its putative gene products. Collectively, the data that follow are the first complete characterization of a neuropeptidergic system from a copepod, the first identification of A-type ASTs from a non-decapod crustacean, the first report of crustacean A-type peptides possessing isoleucine C-termini, and the first report from any species of an A-type AST possessing a valine C-terminus. Moreover, our data now position us for molecular and physiological investigations of the roles played by A-type ASTs in the developmental maturation and reproduction of this ecologically important species.

2. Materials and methods

2.1. Animals

Adult and last-stage (C5) copepodid *C. finmarchicus* were collected in mid-June using oblique net hauls through the upper 100 m of water near Mount Desert Rock in the Gulf of Maine (44°25.7′N, 66°11.8′W). For the construction of the *Calanus* cDNA library, RNA was isolated from animals within 3 days of their capture. For immunohistochemistry, animals were maintained at densities of approximately 10 individuals per liter in jars of filtered seawater at 5–9 °C and were fed three times a week on a diet of *Tetraselmis* sp. paste and live *Isochrysis* sp. Both sexes were used in our study, but females predominated.

2.2. Calanus cDNA library construction and EST submission

The methods used for construction and normalization of the *C. finmarchicus* cDNA library were essentially identical to those used for the generation of the American lobster *Homarus americanus* and green crab *Carcinus maenas* libraries described in detail in a previous report (Towle and Smith, 2006). In brief, total RNA was extracted from whole *C. finmarchicus* (both C5 copepodids and adults), checked for quality, and then prepared for the construction and normalization of the cDNA library (pCMV Sport 6.1 vector) by Invitrogen Corporation (Carlsbad, CA). Plasmids were isolated and inserts were single-pass sequenced from their 5′ end using SP6 primer (5′-ATTTAGGTGACACTATAG-3′). Sequence traces were processed for submission to dbEST (NCBI; Bethesda, MD) using the trace2dbest component of PartiGene (University of Edinburgh, Edinburgh, Scotland, UK). Before submission, all ESTs were subjected to blastx analysis (Altschul et al., 1997) and annotated accordingly.

2.3. Sequence analysis of the Calanus prepro-A-type allatostatin cDNA

For sequencing of the *C. finmarchicus* prepro-A-type allatostatin cDNA, a sample of *Escherichia coli* possessing the insert-containing vector was cultured overnight in LB-medium at 37 °C, the cDNA-containing plasmid isolated using a Purelink™ Quick Plasmid Miniprep kit (Invitrogen), and the insert sequenced on an ABI 3100 16-capillary sequencer (Applied Biosystems, Foster City, CA) using vector-specific (5′-GTA AAACGACGGCCAGT-3′ and 5′-CAGGAAACAGCTATGAC-3′) and insert-specific (5′-GGAGACCTTATGTTAAAGATATGTGG CAAT-3′ and 5′-GCTGGCAGCACAGGTGAAATA-3′) sequencing primers (Integrated DNA Technologies, Coralville, IA). The resulting sequence trace files were analyzed using Chromas 2.31 (Technelysium Pty Ltd., Tewantin, Queensland, Australia), and the high quality nucleotide sequences were aligned using SeqMan 2.6 (DNASTAR, Madison, WI).

2.4. Nucleotide translation and structural analysis of the deduced amino acid sequence

The full-length nucleotide sequence of the *Calanus* prepro-A-type allatostatin cDNA was translated using the Translate tool of ExPASy (Swiss Institute of Bioinformatics, Basel, Switzerland; http://www.expasy.ch/tools/dna.html). Signal peptide cleavage analysis was done via SignalP 3.0, using both Neural Networks and Hidden Markov Models algorithms (Center for Biological Sequence Analysis, Technical University of Denmark, Lyngby, Denmark; http://www.cbs.dtu.de/services/SignalP/; Bendtsen et al., 2004). Pro-hormone cleavage sites were predicted based on the information presented in Veenstra (2000). Prediction of the sulfation state of tyrosine residues was done using the online program Sulfinator (Swiss Institute of Bioinformatics; http://www.expasy.org/tools/sulfinator/; Monigatti et al., 2002). Prediction of other post-translational modifications (i.e., cyclization of amino [N]-terminal glutamine and glutamic acid residues and C-terminal amidations) was based on homology to known A-type allatostatins.

2.5. Wholemount immunohistochemistry

Immunoprocessing was done as wholemounts. Specifically, *C. finmarchicus* were pinned ventral side down in a Sylgard-lined Petri dish containing filtered seawater and the dorsal cuticle of the prosome, as well as the digestive tract overlying the ventral nerve cord (VNC), were removed by microdissection. Animals were then fixed for 12–24 h at 4 °C in a solution of 4% paraformaldehyde in 0.1 M sodium phosphate (*P*) buffer (pH 7.4).

After fixation, tissues were rinsed five times over at least 1.5 h in a solution of *P* containing 0.3% Triton X-100 (*P-Triton*) and then incubated for approximately 70 h in a 1:100 dilution (in *P-Triton*) of a mouse monoclonal antibody generated against the cockroach *Diploptera punctata* A-type AST APSGAQRLYGFGLamide conjugated to BSA via glutaraldehyde (Stay et al., 1992; Woodhead et al., 1992). After incubation in primary antibody, tissues were again rinsed five times over at least 1.5 h in *P-Triton* and then incubated overnight in a 1:300 dilution (in *P-Triton*) of donkey anti-mouse immunoglobulin G labeled with either Rhodamine Red-X (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; catalog #715-295-151) or Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA; catalog #A-21202). After secondary antibody incubation, preparations were rinsed five times over at least 1.5 h in *P* and then mounted between a glass microscope slide and coverslip using Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA; catalog #H-1000). Incubations in both primary and secondary antibody were conducted at 4 °C, while all rinses were done at room temperature (approximately 18 °C). Secondary antibody incubation was conducted in the dark, as was all subsequent processing.

After immunolabeling, preparations were viewed and digital images were collected using an Olympus Fluoview 300 laser scanning confocal microscope system (Olympus America Inc., Melville, NY). This system was equipped with an Olympus IX70 microscope and a krypton/argon mixed gas laser. Imaging was done using Olympus UPlanApo 10× 0.4NA dry, UPlanApo 20× 0.7NA dry, UplanApo 40× 0.85NA dry and PlanApo 60× 1.4NA oil immersion objective lenses, standard Olympus-supplied FITC (barrier filters 510 and 550 nm) and TRITC (barrier filters 585 and 610 nm) filter sets, and manufacturer-supplied software.

2.6. Preadsorption controls

To confirm the specificity of A-type AST labeling, the primary antibody was preadsorbed with 10^{-5} M APSGAQRLYGFGLamide (Bachem AG, King of Prussia, PA; catalog #H-8065) for two hours at room temperature prior to its application to tissue.

3. Results and discussion

3.1. Identification and sequence analysis of a C. finmarchicus A-type AST cDNA

Using the blastx algorithm (i.e., translated nucleotide sequence versus protein sequence), a *C. finmarchicus* EST (Accession No. EL965876) with homology to a crayfish *Procambarus clarkii* cDNA encoding an A-type AST-containing prepro-hormone (Accession No. AB106899; Yasuda-Kamatani and Yasuda, 2006) was identified. Using a combination of vector- and insert-specific forward and reverse sequencing primers, a 1385 base pair (bp), putative full-length cDNA (clone CF_WO0_50f12) was sequenced (Accession No. EU000307). As Fig. 1 illustrates, this full-length clone consists of a 17 bp 5′-untranslated region (UTR), a 423 bp open-reading frame (ORF), as well as a 945 bp 3′-UTR containing two AATAAA polyadenylation signal sequences located 14 and 846 bp upstream of a 14 bp poly-A tail.

3.2. Analysis of the deduced amino acid sequence of the C. finmarchicus prepro-hormone and prediction of mature A-type ASTs and precursor-related peptides

Translation of the ORF of cDNA clone CF_WO0_50f12 predicted a 140 amino acid prepro-hormone (Fig. 1). SignalP 3.0 analysis (Bendtsen et al., 2004) of this amino acid sequence, using both Neural Networks and Hidden Markov Models algorithms, identified the first 15 amino acids of the prepro-hormone as a signal peptide, with a cleavage site between Gly^{15} and Ala^{16} (Fig. 1). Processing at these residues by a signal peptidase would yield a 125 amino acid pro-hormone, which, by homology to insect sequences (Veenstra, 2000), contains nine Lys-Arg pro-hormone convertase processing sites. Enzymatic action at these sites is predicted to liberate 10 peptides: ARPYNGQQGTMLANLAFN DNNSNGLDYEIGEDGPDTDIDVSKR, EPYGFGIG KR, APYGFGIGKR, ALYGFGIGKR, APYGFGIGKR, APYGFGIGKR, EPYNFGIGKR, SQMWGKR, QPYNF GVGKR, and GMLAL (listed in their order within the pro-hormone sequence). With the exception of APYGFGIGKR, which is encoded three times, all are present as single copies within the pro-hormone. In eight of the ten peptides, carboxypeptidase action would expose a C-terminal glycine residue, which likely serves as a target for α-amidation by peptidyl-amidating monooxygenase. Action by this enzyme on these glycine residues would result in the amidation of the carboxy-termini of these peptides, yielding EPYGFGIamide, APYGFGIamide (three copies), ALYGFGIamide, EPYNFGIamide, SQMWamide, and QPYNFGVamide. With the exception of isoleucine or valine for leucine substitutions at the C-terminal amino acid, seven of these peptides contain the −YXFGLamide consensus sequence characteristic of most A-type AST family members (Stay and Tobe, 2007). Based on homology to other crustacean A-type ASTs (e.g., Fu et al., 2005), it is possible that the amino (N)-terminal glutamic acid or glutamine residues in EPYGFGIamide, EPYNFGIamide, and QPYNFGVamide undergo enzymatic or spontaneous cyclization, resulting in the formation of pyro-residues (pE or pQ) in their mature forms. The peptides SQMWamide and GMLAL are not predicted to undergo any additional post-translational modifications and, based on structure, do not fit into any known peptide family. Sulfation of tyrosine 27 was predicted by the software program Sulfinator in ARPYNGQQGTMLANLAFNDN NSNGLDYEIGEDGPDTDIDVS, which, like SQMWamide and GMLAL, does not fit into any known peptide family. Thus, the mature peptides predicted from the *C. finmarchicus* prepro-A-type allatostatin precursor are the novel A-type ASTs pE/EPYGFGIamide (Calfi-A-AST Ia/b), APYGFGIamide (Calfi-A-AST II), ALYGFGIamide (Calfi-A-AST III), pE/EPYNFGIamide (Calfi-A-AST IVa/b), and pQ/QPYNFGVamide (Calfi-A-AST Va/b), as well as the precursor-related peptides ARPYNGQQGTMLAN-LAFNDNNSNGLDY$_{(SO_3H)}$EIGEDGPDTDIDVS (Calfi-A-APRP I), SQMWamide (Calfi-A-APRP II), and GMLAL (Calfi-A-APRP III). These peptides are the first putative peptide hormones identified from *C. finmarchicus*, as well as the first crustacean A-type ASTs exhibiting the isoleucine for leucine C-terminal motif (i.e., Calfi-A-AST I-IV), and the first description from any species of an A-type peptide possessing a valine for leucine C-terminus (i.e., Calfi-A-AST V).

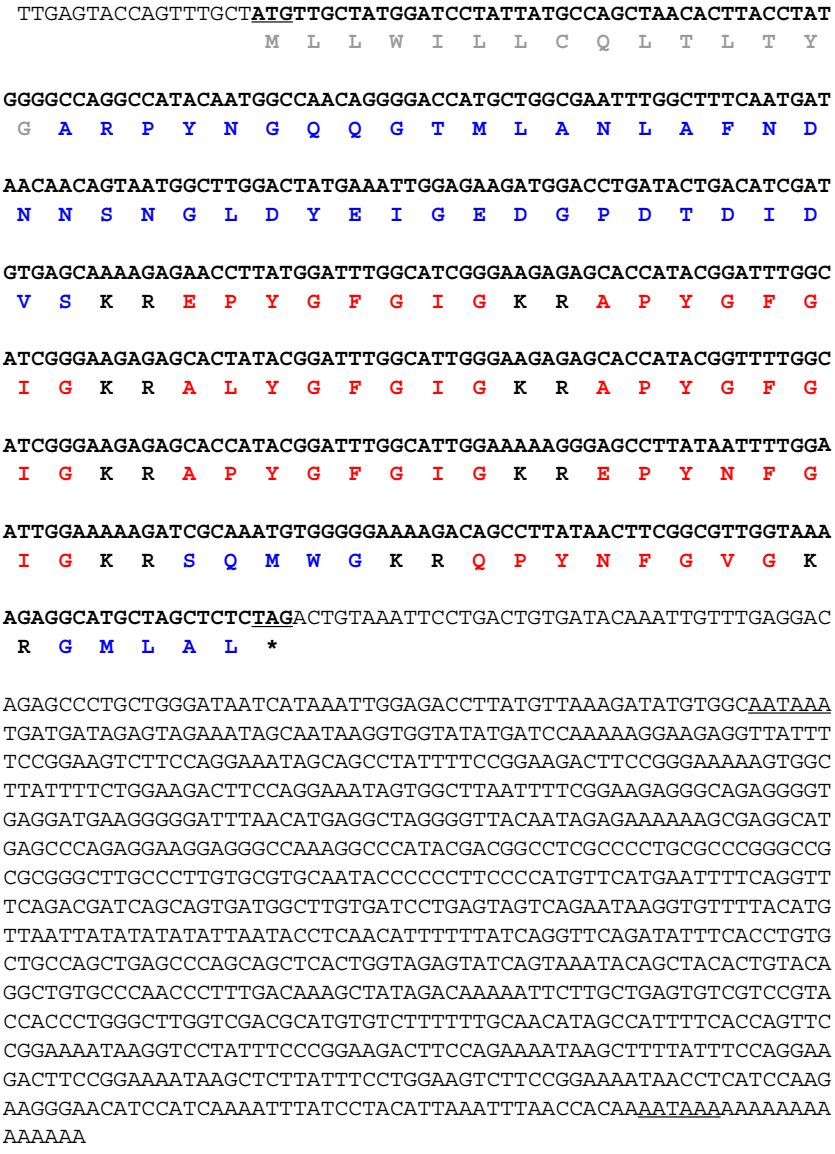

Fig. 1. Nucleotide and deduced amino acid sequences of *Calanus finmarchicus* prepro-A-type allatostatin (Accession No. EU000307). Within the coding region of the cDNA (bold font), both the start (ATG) and stop (TAG) codons are underlined, as are the two polyadenylation signal sequences (AATAAA) present in the 3′-UTR. The predicted amino acid sequence of the prepro-hormone signal peptide is shown in gray. Predicted cleavage sites within the prepro-hormone are shown in black. The amino acid sequences of the *Calanus* A-type allatostatins, including the C-terminal glycine residues that serve as targets for α-amidation, are shown in red. The amino acid sequences of three putative *Calanus* precursor-related peptides (including the C-terminal glycine residue of Calfi-APRP II) are shown in blue. Within the amino acid sequence of the prepro-hormone, the position of the stop codon is denoted with an asterisk.

3.3. Comparison of the Calanus A-type AST precursor with those of decapod crustaceans and insects

A-type AST precursors have been identified from a number of insect species (e.g., Ding et al., 1995; Vanden Broeck et al., 1996; Davey et al., 1999; Bowser and Tobe, 2007). In contrast, only two crustacean (decapod) prepro-A-type ASTs had been cloned prior to our study, i.e., those of the crayfish *P. clarkii* and the freshwater prawn *Macrobrachium rosenbergii* (Yasuda-Kamatani and Yasuda, 2006; Yin et al., 2006). Comparison of the organization of these prepro-hormones shows a striking difference between the insect and decapod proteins, specifically the number of encoded A-type peptides, with the insect prepro-hormones possessing relatively few copies (<15) and the decapod precursors containing large numbers of A-type ASTs (approximately 30). This difference between insects and decapods is further supported by biochemical and mass spectrometric studies in which the maximum number of A-type ASTs found in an insect is less than half that of the maximum number of peptides thus far reported from a decapod, i.e., 14 from the cockroach *Periplaneta americana* versus over 40 from the tiger prawn *Penaeus monodon* (Ding et al., 1995; Duve et al., 2002). Surprisingly, our finding

of seven A-type peptides encoded in the *Calanus* prepro-hormone groups it with the insect-type precursors rather than those of decapod species. Thus, as additional work is done on members of other crustacean taxa, particularly other primitive groups, it will be interesting to see whether their prepro-A-type ASTs encode few or many members of this peptide family. Likewise, as the prepro-hormones encoding other peptides are characterized from *Calanus*, it will be interesting to see if they too are insect-like in their organization.

3.4. Is the −YXFGIamide C-terminus limited to copepod A-type ASTs?

As described in Section 1, numerous A-type ASTs have been identified from insects and crustaceans. The vast majority of these peptides have been characterized as containing the C-terminal motif −YXFGLamide; however, in cockroaches, a subset of the native peptides were found to exhibit an isoleucine for leucine C-terminal substitution (Donly et al., 1993; Belles et al., 1999). Here, using molecular methods, we show that all the A-type ASTs of *C. finmarchicus* possess −Y*X*FGI/Vamide C-termini. While it is too early to say whether these motifs are common or rare in arthropods, when taken collectively with the data from cockroaches (Donly et al., 1993; Belles et al., 1999), our data raise a cautionary note to the assumption that A-type peptides must possess the sequence −YXFGLamide. This is especially important for the identification of A-type ASTs via mass spectrometry, as the majority of the mass spectral methods commonly used to identify arthropod peptides (e.g., matrix assisted laser desorption/ionization Fourier transform mass spectrometry [MALDI-FTMS] or electrospray ionization quadrupole time-of-flight tandem mass spectrometry [ESI-Q-TOF MS/MS]) can not distinguish between leucine and isoleucine residues, as they possess identical masses. Thus, if characterized by MALDI-FTMS or ESI-Q-TOF MS/MS, all of the *Calanus* peptides, save Calfi-A-AST Va/b, would likely have been assigned a C-terminal leucine residue, based on homology to known crustacean family members. Given the plethora of insect and crustacean A-type ASTs that have been identified solely by mass spectrometry (e.g., Huybrechts et al., 2003; Fu et al., 2005), it will be interesting to see if the currently assigned sequences withstand future molecular characterizations.

3.5. Distribution of A-type ASTs in C. finmarchicus

In both insects and decapod crustaceans, A-type ASTs have been localized to neurons (Stay and Tobe, 2007). In at least insects, A-type ASTs have also been found in midgut epithelial endocrine cells (e.g., Sarkar et al., 2003; Davey et al., 2005). As whole *Calanus* were used to produce the library from which our cDNA clone was obtained, the origin(s)/tissue distribution of this transcript, and its encoded peptides, were unknown. To address this question, we mapped the distribution of A-type AST-like immunoreactivity in *C. finmarchicus* using an antibody previously employed for immunohistochemical mappings in both insects and decapod crustaceans (e.g., Stay et al., 1992; Yoon and Stay, 1995; Pulver and Marder, 2002; Fu et al., 2005; Messinger et al., 2005; Yagi et al., 2005).

In *C. finmarchicus*, the only A-type AST-like immunopositive neuronal structures were up to six pairs of neuronal somata, a cluster of up to 5 pairs located in the protocerebrum and a single pair in the suboesophageal region, as well as axonal processes putatively emanating from these cell bodies ($n = 9$ preparations; Fig. 2). In the protocerebral cluster ($PC_{A\text{-}AST}$ in Fig. 2A), between 2 and 5 pairs of somata with diameters between 10 and 15 μm were visible in the different preparations. These cells sent axons into an intensely stained protocerebral neuropil that appeared to correspond to the region in which processes of other neuromodulator-containing neurons terminate (Hartline and Beltz, 2006; Hartline and Christie, 2007). Somata were arrayed around the neuropil anteriorly and laterally at distances up to 100 μm. One feature seen in the better-labeled protocerebral preparations was a bilaterally symmetric, dumb-bell shaped region of neuropil 50–70 μm across near the posterior edge of the neuropil, oriented perpendicular to the longitudinal axis (arrow in Fig. 2A). This structure may be homologous to the central complexes of insect and decapod species, which possess similar shapes and exhibit A-type AST-like labeling (e.g., Utting et al., 2000).

The two A-type AST-immunopositive suboesophageal somata ($MX_{A\text{-}AST}$ in Fig. 2A) were approximately 15 μm in diameter and were located 75–100 μm from the midline and 150–200 μm posterior to the mandibles, near the exit of the maxillary nerves (Lowe, 1935). Each soma sent a fine neurite (<0.5 μm, arrows in Fig. 2B) into the ipsilateral VNC, where it bifurcated into large (2–3 μm) branches running anteriorly and posteriorly in the VNC. After 100–150 μm, the ascending branch gave rise to a fine side branch that traveled laterally for 50–90 μm before turning posteriad for 30–50 μm and giving rise to bleb-like endings that formed a putative neurosecretory complex, named here the mandibular release complex (mrc in Fig. 2). The anterior branch ascended an additional 250–300 μm, projecting into the tritocerebrum, before turning laterally and terminating in a series of branches and blebs, named here the tritocerebral release complex (trc in Fig. 2A). The posterior branch extended 200–300 μm, gradually diminishing in diameter and terminating in the second thoracic segment. From the level of the mrc to the posterior termination, the axon gave rise to numerous (>30–40) blebs of 1 μm diameter or more along its length or on small side branches, giving the appearance of an extensive release complex some 300–400 μm long. Whether or not any of the putative neuroendocrine structures labeled by the A-type AST antibody are homologous to those of insect and/or decapod species remains unknown, though interestingly the decapod pericardial organ is known to be a rich source of A-type peptides (Fu et al., 2005).

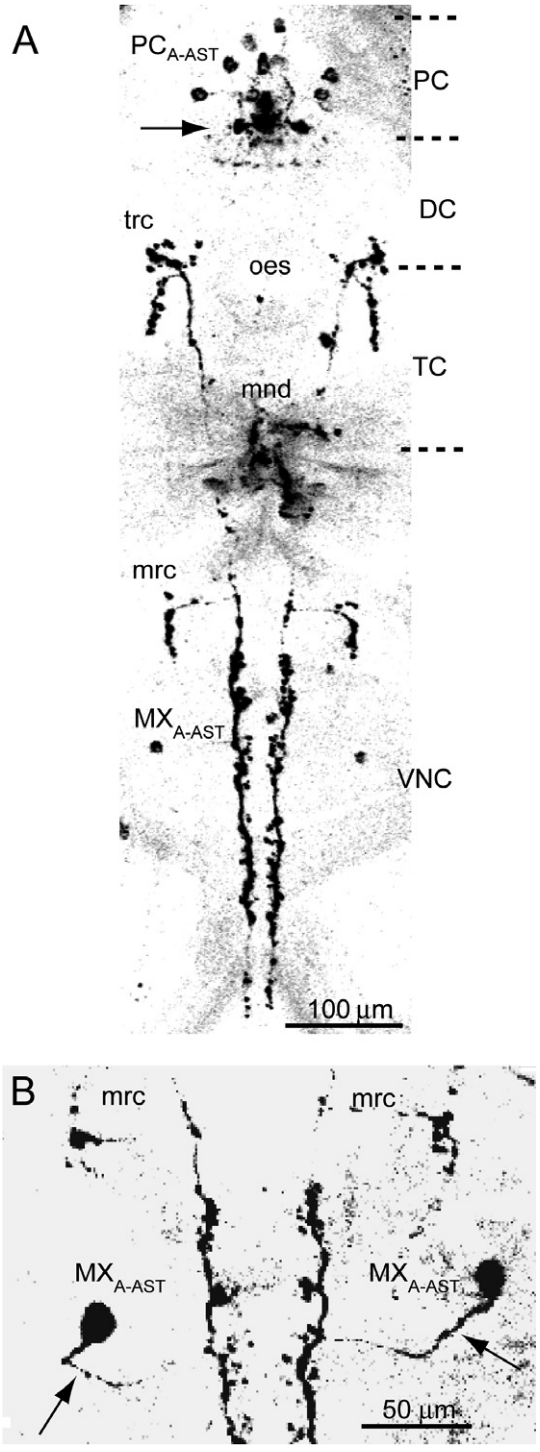

Fig. 2. AST-like immunoreactivity of *Calanus finmarchicus* nervous system. (A) A-type AST immunoreactivity in the central nervous system from anterior protocerebrum to the posterior end of the cephalic segments. Arrow indicates dumb-bell shaped neuropil region in protocerebrum. (B) High magnification image of the axonal projections emanating from the immunopositive suboesophageal soma pair (arrows). Abbreviations: DC, deutocerebrum; mnd, mandible; mrc, mandibular release complex; MX_{A-AST}, maxillary A-type allatostatin immunopositive soma; oes, oesophagous; PC, protocerebrum; PC_{A-AST}, A-type allatostatin immunopositive protocerebral cell bodies; TC, tritocerebrum; trc, tritocerebral release complex; VNC, ventral nerve cord. Scale bars 100 µm in (A) and 50 µm in (B).

The antibody used to map the distribution of A-type ASTs in *C. finmarchicus* has been shown to be highly specific for members of this peptide family (Stay et al., 1992; Woodhead et al., 1992). Moreover, this antibody has been used extensively to map the distribution of A-type peptides in both insects and decapods (e.g., Stay et al., 1992; Woodhead et al., 1992; Pulver and Marder, 2002; Fu et al., 2005). Based on these factors, and because all A-type AST-like staining in the *Calanus* CNS was abolished by preadsorption of the primary antibody with the peptide antigen used for its generation (10^{-5} M final concentration of peptide used; $n = 3$ preparations), we feel confident that the A-type AST labeling reported here is a true reflection of the presence of A-type peptides in the immunopositive profiles.

3.6. Conclusions and future directions

In this report we have used molecular methods to identify and characterize a prepro-A-type AST precursor from the copepod *C. finmarchicus*. Moreover, we have mapped the distribution of the products of this transcript via immunohistochemistry using an antibody to an insect A-type AST in the CNS of this species. The native *Calanus* ASTs were all found to differ from those previously identified in decapod species in that they possessed C-terminal isoleucine (Calfi-A-AST Ia/b, Calfi-A-AST II, Calfi-A-AST III, and Calfi-A-AST IVa/b) or valine (Calfi-A-AST Va/b) residues, rather than the conventional C-terminal leucine, the former substitution previously reported only for a subset of cockroach A-type peptides (Donly et al., 1993; Belles et al., 1999) and the latter reported here for the first time. The functional consequence(s) of these amino acid substitutions are unknown; however, the identification of these peptides will now allow for investigation of this question. The discovery of "unconventional" A-type peptides in *Calanus* also provides a foundation for future investigations of the structure of A-type peptides in other, as yet unstudied, arthropod taxa, and thus the potential for a better understanding of the evolution of this peptide family. This line of investigation is of particular importance given that the organization of the *Calanus* prepro-hormone appears to be much more like that of insects than that of decapod crustaceans, though the number of species for which comparisons can be made, particularly for the decapods, remains small. Finally, the identification of the native *C. finmarchicus* A-type ASTs now allows for molecular and physiological investigations of their role(s) in the regulation of developmental maturation and reproduction in *Calanus*, two areas of particular importance for this species, given the critical role it plays in the health of North Atlantic ecosystems.

Acknowledgments

Andrew Peterson (College of the Atlantic) is thanked for providing us access to the R/V Indigo, which was used for collecting the animals used in this study. Dr. Petra Lenz,

Benjamin Burpee, and Elizabeth Mitchell are thanked for their help in the collection and husbandry of the animals used for this study. Maria Voznesensky is thanked for extracting the mRNA used to produce the normalized cDNA library. Dr. Barbara Stay (University of Iowa) is thanked for providing us with the A-type AST antibody. Dr. William Stegeman (Jackson ImmunoResearch Laboratories, Inc.) is thanked for his generous contribution of many of the reagents used in our immunohistochemistry. Financial support for this work was provided by: NIH Grant No. P20 RR-016463 (from the INBRE Program of the National Center for Research Resources), NSF Grant Number DBI-0139190, NSF Grant No. OCE-0451376 (to Petra Lenz and D.K.H) and a Mount Desert Island Biological Laboratory New Investigator Award (Salisbury Cove Research Fund provided by the Thomas H. Maren Foundation; to A.E.C.).

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