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NGFFFamide and echinotocin: structurally unrelated myoactive neuropeptides derived from neurophysin-containing precursors in sea urchins

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SUMMARY

The myoactive neuropeptide NGIWYamide was originally isolated from the holothurian (sea cucumber) *Apostichopus japonicus* but there is evidence that NGIWYamide-like peptides also occur in other echinoderms. Here we report the discovery of a gene in the sea urchin *Strongylocentrotus purpuratus* that encodes two copies of an NGIWYamide-like peptide: Asn-Gly-Phe-Phe-Phe-(NH_2) or NGFFFamide. Interestingly, the C-terminal region of the NGFFFamide precursor shares sequence similarity with neurophysins, carrier proteins hitherto uniquely associated with precursors of vasopressin/oxytocin-like neuropeptides. Thus, the NGFFFamide precursor is the first neurophysin-containing neuropeptide precursor to be discovered that does not contain a vasopressin/oxytocin-like peptide. However, it remains to be determined whether neurophysin acts as a carrier protein for NGFFFamide. The *S. purpuratus* genome also contains a gene encoding a precursor comprising a neurophysin polypeptide and 'echinotocin' (CFISNCPKGamide) – the first vasopressin/oxytocin-like peptide to be identified in an echinoderm. Therefore, in *S. purpuratus* there are two genes encoding precursors that have a neurophysin domain but which encode neuropeptides that are structurally unrelated. Furthermore, both NGFFFamide and echinotocin cause contraction of tube foot and oesophagus preparations from the sea urchin *Echinus esculentus*, consistent with the myoactivity of NGIWYamide in sea cucumbers and the myoactivity of vasopressin/oxytocin-like peptides in other animal phyla. Presumably the NGFFFamide precursor acquired its neurophysin domain following partial or complete duplication of a gene encoding a vasopressin/oxytocin-like peptide, but it remains to be determined when in evolutionary history this occurred.

Key words: NGFFFamide, echinotocin, vasopressin, oxytocin, vasotocin, neurophysin, NGIWYamide, echinoderm, *Strongylocentrotus purpuratus*, *Echinus esculentus*, neuropeptide, nematocin, *Caenorhabditis elegans*.

INTRODUCTION

Neuropeptide signalling molecules have been identified throughout the animal kingdom and are involved in the regulation of a variety of physiological processes, acting as neurotransmitters, neuromodulators or neurohormones (Greenberg and Price, 1983; Grimmelikhuijzen et al., 1999; Hoyle, 1999; O'Shea and Schaffer, 1985; Strand, 1999). However, relatively little is known about neuropeptide structure and function in the phylum Echinodermata (e.g. sea urchins, starfish, sea cucumbers). New opportunities to identify and characterize echinoderm neuropeptides have emerged recently with the sequencing of the genome of the sea urchin *Strongylocentrotus purpuratus* (Order Echinoida; Family Strongylocentrotidae) (Burke et al., 2006; Sodergren et al., 2006). Moreover, there are a number of reasons why analysis of neuropeptides in echinoderms is of interest.

Adult echinoderms are unique in the animal kingdom in having a pentaradial morphological organization, which is both evolutionarily and developmentally derived from bilateral symmetry (Burke et al., 2006). It is of particular interest, therefore, to determine how neuropeptides participate in the neural coordination of physiology and behaviour in the context of a pentaradial bauplan. Furthermore, analysis of neuropeptide expression provides a useful approach for investigation of the changes in neuroarchitecture that accompany the transition from bilaterally symmetrical larvae to radially symmetrical adult echinoderms (Byrne and Cisternas, 2002).

As deuterostomian invertebrates, echinoderms occupy an interesting phylogenetic position in the animal kingdom because, together with hemichordates and xenoturbellids, they form a sister clade to the chordates (Bourlat et al., 2006; Bromham and Degnan, 1999; Dunn et al., 2008). Comparative analysis of echinoderms and chordates therefore provides a basis for identifying synapomorphies shared within the deuterostome clade as well as characters that differentiate echinoderms from chordates.

Echinoderms have many unusual biological properties, which include remarkable powers of autotomy and regeneration and the ability to rapidly and reversibly change the mechanical state of their body wall and/or body wall-associated appendages (Byrne, 2001; Patruno et al., 2001; Thorndyke et al., 2001; Wilkie, 2001; Wilkie, 2005). Neuropeptides have been implicated as potential regulators of these processes (Birenheide et al., 1998; Mladenov et al., 1989; Tamori et al., 2007) but more detailed investigation of the role of neuropeptides in these and other aspects of echinoderm biology is needed.

The first neuropeptides to be identified in echinoderms were a family of peptides known as SALMFamides, which have a characteristic C-terminal motif, S*x*(L/F)*x*Famide (where *x* is variable). The prototypes for this family, S1 (GFNSALMFamide) and S2 (SGPYSFNSGLTFamide), were both isolated from the starfish *Asterias rubens* and *Asterias forbesi* (Elphick et al., 1991a; Elphick et al., 1991b). Subsequently, members of the SALMFamide family have been identified in sea cucumbers, including GFSKLYFamide and SGYSVLYFamide from *Holothuria glaberrima* (Díaz-Miranda et al., 1992). Pharmacological studies have revealed that SALMFamide neuropeptides cause relaxation of

muscle preparations in starfish and sea cucumbers (Díaz-Miranda and García-Arrarás, 1995; Elphick et al., 1995; Elphick et al., 1991a; Melarange and Elphick, 2003; Melarange et al., 1999) and SALMFamides may have a general role as muscle relaxants throughout the phylum Echinodermata (Elphick and Melarange, 2001). Furthermore, evidence of other physiological roles of SALMFamides in echinoderms has been reported, including modulation of luminescence in brittle stars (De Bremaeker et al., 1999) and regulation of neurohormone (gonad-stimulating substance) secretion in starfish (Mita et al., 2004).

Sequencing of the genome of the sea urchin *S. purpuratus* facilitated identification of a gene encoding SALMFamides, the first neuropeptide precursor gene to be characterized in an echinoderm (Elphick and Thorndyke, 2005). The *S. purpuratus* SALMFamide gene comprises two protein-coding exons: the first exon encodes an N-terminal signal peptide and the second exon encodes seven putative SALMFamide neuropeptides known as SpurS1–SpurS7 (Elphick and Thorndyke, 2005). Discovery of this gene is of interest because it has revealed an unprecedented diversity of SALMFamides in an echinoderm species. Moreover, identification of the SALMFamide gene in *S. purpuratus* has paved the way for the identification of other neuropeptide genes in this species.

SALMFamide neuropeptides were originally isolated from starfish and sea cucumbers because of their cross-reactivity with antibodies to molluscan FMRFamide-related peptides (Díaz-Miranda et al., 1992; Elphick et al., 1991a). Subsequently, Iwakoshi and colleagues used a different strategy for the isolation and identification of echinoderm neuropeptides (Iwakoshi et al., 1995). Radial longitudinal muscle and intestinal preparations from the sea cucumber *Apostichopus japonicus* were used to test for the presence of myoactive peptides in body wall extracts of the same species (Iwakoshi et al., 1995; Ohtani et al., 1999). Amongst the peptides identified were two members of the SALMFamide family (GYSPFMFamide and FKSPFMFamide) and, consistent with previous pharmacological tests with SALMFamides, both peptides caused relaxation of muscle preparations (Ohtani et al., 1999). Many of the other peptides identified had indirect effects on muscle contractility, either potentiating or inhibiting electrically evoked contractions. However, one of the peptides identified (NGIWYamide) was found to cause contraction of the muscle preparations tested (Iwakoshi et al., 1995; Ohtani et al., 1999).

The physiological roles of NGIWYamide in holothurians have been investigated in detail by testing the effects of NGIWYamide on longitudinal body wall muscle, tentacles and intestine from *Apostichopus japonicus* (Inoue et al., 1999). NGIWYamide caused contraction of body wall muscle and tentacle preparations, consistent with the effects of NGIWYamide originally observed by Iwakoshi and colleagues (Iwakoshi et al., 1995). However, NGIWYamide also caused inhibition of the spontaneous rhythmic contractile activity of intestine preparations. Using antibodies to NGIWYamide to analyse the distribution of this peptide in *Apostichopus japonicus*, abundant NGIWYamide immunoreactivity was observed in the radial nerve cords and circumoral nerve ring, localized in neuronal cell bodies and their processes. In addition, and consistent with the pharmacological effects of NGIWYamide, NGIWYamide immunoreactivity was detected in the innervation of body wall dermis, intestine, tentacles and tube feet (Inoue et al., 1999).

More recently, Saha and colleagues tested the effects of NGIWYamide on tube foot preparations from the starfish species *Asterina pectinifera* and found that the peptide causes contraction (Saha et al., 2006). Furthermore, antibodies to NGIWYamide revealed the presence of NGIWYamide-like immunoreactivity in the radial nerve cords and tube foot innervation in *Asterina pectinifera*. Collectively, these data indicate that NGIWYamide-related peptides may occur throughout the phylum Echinodermata and may have a general role in neural regulation of muscle contraction in echinoderms. However, to test these hypotheses it will be necessary to identify NGIWYamide-related peptides in other echinoderms apart from sea cucumbers. Therefore, building on a successful strategy that led to the identification of a SALMFamide gene in the sea urchin *S. purpuratus*, here we have investigated the occurrence of a gene encoding an NGIWYamide-related peptide in this species.

MATERIALS AND METHODS

Analysis of *S. purpuratus* genome and cDNA sequence data

A search for a gene encoding an NGIWYamide-like peptide in the genome of the sea urchin *S. purpuratus* Stimpson 1857 was initiated in January 2005 employing the Basic Local Alignment Search Tool [BLAST (Altschul et al., 1990)] facility available on the Baylor College of Medicine Human Genome Sequencing Center website (http://www.hgsc.bcm.tmc.edu/blast/blast.cgi?organism=Spurpuratus). The strategy used was similar to that employed previously to identify a gene encoding novel SALMFamide neuropeptides in *S. purpuratus* (Elphick and Thorndyke, 2005). Thus, the query sequence comprised three copies of the sequence NGIWY separated by the sequence GKR, with the glycine (G) residues putative substrates for C-terminal amidation and the lysine–arginine (KR) dipeptide sequences putative cleavage sites for endopeptidases (i.e. NGIWYGKRNGIWYGKRNGIWYG). Using this approach, a contig containing a DNA sequence encoding two copies of a putative NGIWYamide-like peptide (NGFFFamide) was identified.

The full-length sequence of the putative NGFFFamide precursor protein was determined by analysis of *S. purpuratus* genome and cDNA sequence data using resources available on the Baylor College of Medicine Human Genome Sequencing Center Sea Urchin Genome Project website (http://www.hgsc.bcm.tmc.edu/projects/seaurchin) and the NCBI Sea Urchin Genome Resources website (http://www.ncbi.nlm.nih.gov/genome/guide/sea_urchin/). As described in detail in the Results section, determination of the full-length sequence of the putative NGFFFamide precursor revealed that it shares sequence similarity with the precursor of a vasopressin/oxytocin-like peptide in *S. purpuratus*, which we have named ‘echinotocin’.

Comparison of the sequences of the NGFFFamide precursor, the echinotocin precursor and precursors of vasopressin/oxytocin-like peptides in other species was performed using ClustalX for multiple sequence alignment and NJ plot for construction of trees with bootstrap analysis (Saitou and Nei, 1987; Thompson et al., 1997).

In vitro pharmacology

The pharmacological activity of NGFFFamide and echinotocin was investigated by testing the effects of these peptides on *in vitro* preparations of tube feet and oesophagus from specimens of the sea urchin *Echinus esculentus* L. (Order Echinoida; Family Echinidae), which were collected off the coast of Ayrshire in Scotland, transported to QMUL and maintained in a seawater aquarium at about 11°C. NGFFFamide and echinotocin were custom synthesized by the Advanced Biotechnology Centre at Imperial College London. Echinotocin (CFISNCPKGamide) was synthesized with a disulphide bridge between the cysteine residues, consistent with the occurrence of a disulphide bridge in other members of the vasopressin/oxytocin neuropeptide family (De Bree and Burbach, 1998; Light and Du Vigneaud, 1958).

Tube foot preparations were obtained from specimens of *E. esculentus* by severing extended tube feet. Silk ligatures were tied around each end of the severed tube foot and one of the ligatures was attached to a glass rod. The preparation was then suspended in a 20 ml bath containing aerated seawater at 11°C and the second ligature was attached to an isometric force transducer (Harvard Apparatus, Edenbridge, Kent, UK). Likewise, oesophageal preparations were set up using approximately 1.5 cm sections of oesophagus. Once set up, tube foot and oesophageal preparations were allowed to equilibrate until a stable resting tension was obtained. The effects of NGFFFamide and echinotocin on tube foot and oesophageal preparations were examined by applying the peptides to achieve bath concentrations within the range 10^{-11} to 10^{-6} mol l^{-1}. Additionally, NGFFFamide and echinotocin at a concentration of 3×10^{-6} mol l^{-1} were tested consecutively on tube foot and oesophagus preparations to enable direct comparison of their efficacy.

After dissection of tube foot and oesophagus preparations, sea urchins were anaesthetized in seawater containing 0.1 mol l^{-1} magnesium chloride.

RESULTS

Identification of a gene encoding the neuropeptide NGFFFamide in *S. purpuratus*

Analysis of *S. purpuratus* genomic sequence data using the tBLASTn method with the query NGIWYGKR-NGIWYGKRNGIWYG resulted in the identification of a 6296 base contig (21522) containing a sequence of 54 bases encoding an amino acid sequence (KRNGFFFGKRNGFFFGKR) that comprises two copies of the peptide sequence NGFFFG separated and flanked by putative dibasic cleavage sites (KR). Thus, endopeptidase-mediated cleavage at the dibasic cleavage sites followed by C-terminal amidation mediated by peptidylglycine α-amidating monooxygenase could give rise to two copies of the NGIWYamide-like peptide Asn-Gly-Phe-Phe-Phe-(NH_2) or NGFFFamide.

Analysis of the most recent assembly of *S. purpuratus* genome sequence data (version 2.1) revealed that the KRNGFFFGKRNGFFFGKR sequence is located within scaffold 54273. Furthermore, BLAST analysis of expressed sequence tag (EST) data obtained from a *S. purpuratus* radial nerve cDNA library revealed that the KRNGFFFGKRNGFFFGKR sequence is encoded by a cDNA (RNSP-5L15) for which both 5′ EST (EC439145; GI: 109403168) and 3′ EST (EC438106; GI: 109402129) data are available. The RNSP-5L15 cDNA encodes a protein comprising 266 amino acid residues and, as expected for a neuropeptide precursor, analysis of this protein sequence using SignalP 3.0 [www.cbs.dtu.dk/services/SignalP (Bendtsen et al., 2004)] predicts an N-terminal signal peptide (Fig. 1). Following the predicted 26 amino acid residue signal peptide there is a 114 amino acid residue sequence, which is then followed by the 18 residue sequence KRNGFFFGKRNGFFFGKR, comprising two copies of the putative NGFFFamide neuropeptide separated and flanked by potential dibasic cleavage sites (KR). On the C-terminal side of the NGFFFamide-encoding region of the precursor is a 108 amino acid residue sequence that contains 14 cysteine residues. Moreover, submission of the putative NGFFFamide precursor as a BLASTp query against the GenBank non-redundant database revealed that the C-terminal region of the protein shares a high level of sequence

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1                                                                  aaacttga
9     ttcgatcgcggggagcataacaccaggagagatttttgatgttaagagagtggacaaaac
69    acagcatttgatcattggttagtggaactaggtggaagagaattcaatggttcgcctcgc
129   tggaaacaacagtggagaataggcacgagctatacgtggagaaaaggttttcgttagatt
189   taggtgcctcgagaaaaGAtcgcaggtcgattggtttaagggaaggtaccgaaggaaaag
249   atgggctacgagagacggatactgcggacgttattgtcgatcttaatagtactagcatca
        M  G  Y  E  R  R  I  L  R  T  L  L  S  I  L  I  V  L  A  S    20
309   ttcgtcacagtctatggagaaagagatagcaactttatgcaacagaaGCaattcaggaat
        F  V  T  V  Y  G  E  R  D  S  N  F  M  Q  Q  K  Q  F  R  N    40
369   atagtaccgtcaccgcttatccaaaaatggcgggaaaatcgaatgggtcccgcggaggag
        I  V  P  S  P  L  I  Q  K  W  R  E  N  R  M  G  P  A  E  E    60
429   aagactagcaacgaacagtggagggacgagctccttagtaaccttagaaacgtgcttaga
        K  T  S  N  E  Q  W  R  D  E  L  L  S  N  L  R  N  V  L  R    80
489   aaacacaacgcatcaccatcatcacgaagcagagacagaaccgacatcacagcctacggc
        K  H  N  A  S  P  S  S  R  S  R  D  R  T  D  I  T  A  Y  G   100
549   ctccaagaacctatgcagcagcttcctgcagacgtaacggccgatcagttgttcatacta
        L  Q  E  P  M  Q  Q  L  P  A  D  V  T  A  D  Q  L  F  I  L   120
609   gagggcgctgtaaactcacccagggaaaactacgaggaggaaacgcccattgacgaggat
        E  G  A  V  N  S  P  R  E  N  Y  E  E  E  T  P  I  D  E  D   140
669   aagagaaacggatttttcttcggtaaacgtaacggatttttcttcgggaagaggtcggat
        K  R  N  G  F  F  F  G  K  R  N  G  F  F  F  G  K  R  S  D   160
729   agcgatgcctcatcaaccaagatggacgatgacagactacccaaatacgaatcatcagga
        S  D  A  S  S  T  K  M  D  D  D  R  L  P  K  Y  E  S  S  G   180
789   tcatttgataaGTgcagaccatgcggtccaggccggcagggacgatgcgtaatggtgggt
        S  F  D  K  C  R  P  C  G  P  G  R  Q  G  R  C  V  M  V  G   200
849   acatgttgtagtcccctattcggctgctacctattcacacccgaagccgcagcgtgtatg
        T  C  C  S  P  L  F  G  C  Y  L  F  T  P  E  A  A  A  C  M   220
909   acagaagatgtgtccccgtgtcaactcaatgcgccttcctgtggccttgcaggaaagtgt
        T  E  D  V  S  P  C  Q  L  N  A  P  S  C  G  L  A  G  K  C   240
969   gtagccgatgggatttgctgttctgccgcagagggcgcctgccaccttgacccgacctgt
        V  A  D  G  I  C  C  S  A  A  E  G  A  C  H  L  D  P  T  C   260
1029  acgtcgatgtcattaaattaatttttggacgcattggtgtatttttcaaatcaatatcatg
        T  S  M  S  L  N  *                                          266
1089  gtgtacagtttttctgaccctgtaatttcctctagctccgaaaataatcttcttactcg
1149  accgttccatttacctttttctttggtcgaattcattaggaacgtgcgcttacaaaatgt
1209  tgtgtaattgtactatcaactcctggttgttatgagcatttattcgctattgagattgtt
1269  gtatttccgtactcaaacttgaatccaacgcgct
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Fig. 1. The *Strongylocentrotus purpuratus* NGFFFamide precursor. The sequence of a cDNA (lowercase, 1302 bases) encoding the NGFFFamide precursor protein (bold uppercase, 266 amino acid residues) is shown. The DNA sequence was derived from genomic sequence data but EST data were used to determine the length of 5′ and 3′ non-coding regions and the positions of introns. The positions of introns in the gene encoding the NGFFFamide precursor are shown by highlighting the pairs of bases (bold and underline) in the cDNA sequence that are interrupted by an intron in the corresponding genomic sequence. The predicted signal peptide is shown in blue and the two copies of the NGFFFG sequence are shown in red, interrupted and flanked by putative dibasic cleavage sites (KR) shown in green. The C-terminal neurophysin-like region of the precursor is shown in purple with the 14 cysteine residues underlined. The asterisk shows the position of the stop codon.

identity with neurophysins, proteins that form the C-terminal region of precursors for the neuropeptide hormones vasopressin, oxytocin and vasotocin. For example, residues 181–260 of the NGFFFamide precursor share 46% sequence identity with residues 37–116 of the chicken vasotocin precursor.

Comparison of the RNSP-5L15 cDNA sequence with the *S. purpuratus* genome sequence revealed that the 266 amino acid NGFFFamide precursor is encoded by a gene comprising four exons (Fig. 1). Exon 1 is a 206 base 5′ non-coding sequence, which is separated from exon 2 by an intron comprising 37,141 bases. Exon 2 (150 bases) consists of a 5′ non-coding region (42 bases) followed by 118 bases that encode the N-terminal signal peptide and the first 10 amino acid residues of the 114 residue polypeptide that separates the signal peptide from the NGFFFamide-encoding region. A short second intron (715 bases) is followed by exon 3, which comprises 444 bases encoding the remaining 104 residues of the 114 residue polypeptide, two copies of the NGFFFG sequence separated and flanked by putative dibasic cleavage sites (Lys–Arg) and then a 26 residue sequence. The third intron comprises 16,420 bases and is followed by exon 4 (502 bases), which comprises 246 bases encoding a neurophysin-like sequence followed by a stop codon and a 253 base 3′ non-coding sequence.

In the sea urchin genome project, annotation of *S. purpuratus* genome sequence data was facilitated by production of a list of genes predicted by the GLEAN3 gene prediction algorithm (Elsik et al., 2007; Sodergren et al., 2006). Interestingly, the NGFFFamide precursor gene was one of a number of genes that were not predicted by the GLEAN3 algorithm. Therefore, we manually annotated this gene as part of the sea urchin genome project annotation process and the NGFFFamide gene has been assigned the official ID number SPU_030074 (see http://www.spbase.org/SpBase/search/viewAnnoGeneInfo.php?spu_id=SPU_030074 for further details).

Identification of a gene encoding a vasopressin/oxytocin-like peptide (‘echinotocin’) in *S. purpuratus*

Our discovery that the C-terminal region of the putative NGFFFamide precursor contains a polypeptide sequence similar to neurophysins that occur in precursors of the peptide hormones vasopressin and oxytocin prompted us to investigate the occurrence of a gene or genes encoding vasopressin/oxytocin-like peptides in *S. purpuratus*. To do this the human vasopressin precursor sequence was submitted as a BLASTp query against putative *S. purpuratus* proteins predicted by the gene prediction algorithm GLEAN3 (Elsik et al., 2007; Sodergren et al., 2006). The protein with the highest level of sequence identity with the query sequence was a putative 225 amino acid residue protein (GLEAN3_06899). Analysis of the sequence of this protein revealed that residues 87–98 comprised a vasopressin/oxytocin-like peptide sequence (CFISNCPKG) followed by a potential substrate for C-terminal amidation (G) and a putative dibasic cleavage site (KR). Moreover, the C-terminal region of the protein contained a neurophysin-like sequence. However, the N-terminal part of the protein sequence (residues 1–86) did not share sequence similarity with vasopressin and oxytocin precursors. Furthermore, analysis of the protein sequence using SignalP 3.0 revealed that a predicted N-terminal signal peptide was located between residues 61 and 86 of the putative 225 residue protein. This suggested that inclusion of the N-terminal 60 residues of the 225 residue protein, as predicted by GLEAN3, is likely to be erroneous. Thus, it appears that in *S. purpuratus* there is a 165 residue vasopressin/oxytocin-like precursor protein, which comprises a 26 residue N-terminal signal peptide, a putative vasopressin/oxytocin-like peptide (CFISNCPKGamide), which we have named ‘echinotocin’, and a neurophysin-like protein (Fig. 2).

A large number of *S. purpuratus* ESTs have been deposited in the GenBank database but cDNA/EST data have as yet not been obtained for the echinotocin precursor. Therefore, the sequence shown in Fig. 2 is derived from the GLEAN3 prediction (06899). The predicted echinotocin precursor gene comprises 3 exons, with the first exon (138 bases) encoding the N-terminal signal peptide, echinotocin and the N-terminal region of neurophysin. Exons 1 and 2 are separated by an intron comprising 24,141 bases. The second exon (208 bases) encodes the core of the neurophysin protein and is followed by an intron comprising 2379 bases. The third exon (152 bases) encodes the C-terminal region of the neurophysin protein and is followed by a stop codon (Fig. 2). This gene has been assigned the official gene ID number SPU_006899 (see http://www.spbase.org/SpBase/search/viewAnnoGeneInfo.php?spu_id=SPU_006899).

Comparison of the NGFFFamide precursor, the echinotocin precursor and precursors of vasopressin/oxytocin-like peptides in other species

To facilitate comparison of the sequences of the NGFFFamide precursor and the echinotocin precursor and comparison of these sea urchin precursors with precursors of vasopressin/oxytocin-like peptides in other species, ClustalX was used to generate a multiple sequence alignment (Fig. 3). This revealed that whilst the 14 cysteine residues that are characteristic of neurophysins are conserved in both the echinotocin precursor and the NGFFFamide precursor, there is variation in the length of the peptide sequences

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1    atgatgtccgtcaagagtatagtcacctgtttgtttctgtcattggttctggctctatgg
      M  M  S  V  K  S  I  V  T  C  L  F  L  S  L  V  L  A  L  W   20
61   atagggggggagcttcgcctgttttatctccaactgcccaaaggggggtaaaagatcaaat
      I  G  G  S  F  A  C  F  I  S  N  C  P  K  G  G  K  R  S  N   40
121  tctcgtccacttagacagtgcctcgaatgcggaccaggcggcgtaggaaggtgcatgggc
      S  R  P  L  R  Q  C  L  E  C  G  P  G  G  V  G  R  C  M  G   60
181  ccagggatctgctgcggaccaacgattggctgtcacatcaacacacaacacacactgtcc
      P  G  I  C  C  G  P  T  I  G  C  H  I  N  T  Q  H  T  L  S   80
241  tgtatgcgagaaaacgagatctcaacgccatgtgaactcccaggaaacccttgtcagact
      C  M  R  E  N  E  I  S  T  P  C  E  L  P  G  N  P  C  Q  T  100
301  gtcccaagtggtacatgtggagcaatgggtgtatgctgcaatagtaattcttgttcagaa
      V  P  S  G  T  C  G  A  M  G  V  C  C  N  S  N  S  C  S  E  120
361  gatgcatcctgtctgatgattgaagaggatgactccctcaaaagatttgagcagatgagt
      D  A  S  C  L  M  I  E  E  D  D  S  L  K  R  F  E  Q  M  S  140
421  cgagaggaaaacggttcgacgaggaaagacctccgggttaaacttctcgatttacttttg
      R  E  E  N  G  S  T  R  K  D  L  R  V  K  L  L  D  L  L  L  160
481  aacatgcaggatcaataa
      N  M  Q  D  Q  *                                            165
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Fig. 2. The *S. purpuratus* echinotocin precursor. The nucleotide sequence (lowercase) encoding the echinotocin precursor is shown, as predicted by the GLEAN3 gene prediction algorithm, with the corresponding protein sequence (165 residues) shown in bold uppercase. The positions of introns in the gene encoding the echinotocin precursor are shown by highlighting the pairs of bases (bold and underline) in the sequence that are interrupted by an intron. The predicted signal peptide is shown in blue, the echinotocin sequence (CFISNCPKGG) is shown in red followed by a putative dibasic cleavage site (KR) shown in green. The neurophysin-like region of the precursor is shown in purple with the 14 cysteine residues underlined. The asterisk shows the position of the stop codon.

between some of the conserved cysteine residues. For example, between cysteines 7 and 8 in the NGFFFamide-associated neurophysin there are seven residues, whereas in the neurophysins associated with echinotocin and with vasopressin/oxytocin-like peptides in other species there are nine residues. Conversely, there are six residues between cysteines 12 and 13 in the NGFFFamide-associated neurophysin, whereas in the neurophysins associated with echinotocin and with vasopressin/oxytocin-like peptides in other species there are only four residues.

To assess the overall similarity of the NGFFFamide- and echinotocin-associated neurophysins, a neighbour-joining tree was generated based on a ClustalX alignment of the sequences of the neurophysin domains from the NGFFFamide precursor, the echinotocin precursor and precursors of other vasopressin/oxytocin-like peptides (Fig. 4). This revealed that, based on sequence similarity, NGFFFamide neurophysin is not more closely related to the echinotocin neurophysin than to neurophysins associated with vasopressin/oxytocin-like peptides in other phyla.

NGFFFamide and echinotocin cause contraction of sea urchin tube foot and oesophagus preparations

Both NGFFFamide (Fig. 5A,B) and echinotocin (Fig. 5C,D) caused contraction of tube foot and oesophagus preparations from the sea urchin *E. esculentus*. Comparison of the effects of NGFFFamide and echinotocin suggested that the magnitude of the NGFFFamide-induced contraction was larger than the echinotocin-induced

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NGFFFamide      MGYERRILRTLLSILIVLASFVTVYGERDSNFMQQKQFRNIVPSPLIQKWRENRMGPAEEKTSNEQWRDELLSNLRNVLR 80

NGFFFamide      KHNASPSSRSRDRTDITAYGLQEPMQQLPADVTADQLFILEGAVNSPRENYEEETPIDEDKRNGFFFGKRNGFFFGKRSD 160
Echinotocin     --------------------MMSVKSI---VTCLFLSLVLALWIGGSFACFISNCPKG-----------------GKRSN 40
Vasopressin     ----------------------------MPDTMLPACFLGL-LAFSSACYFQNCPRG-----------------GKR-- 31
Oxytocin        ----------------------------MAGPSLACCLLGL-LALTSACYIQNCPLG-----------------GKR-- 31
Vasotocin       --------------------------MARCAPLTLAVSVLSL-VLISSACYIQNCPRG-----------------GKR-- 34
SOP             ----------------------MTSSHPKRLNQLICCVVIMSYVTVQGCYISDCPNS-----------------RFWST 40
Lys-conopressin ---------------------MMSSLCG---MPLTYLLTAAVLSLSLTDACFIRNCPKG-----------------GKRSL 40
Cephalotocin    --------------------MSQNCFAIVQLLFVLFTVCSLFIATTDGCYFRNCPIG-----------------GKRAT 42
Annetocin       -----------------MACTKKSANMKLRKSLTVTAFLLFVNLSLSSACFVRNCPTG-----------------GKRSV 46
Inotocin        ----------------------------MSTIITSIILLVLSESLVSGCLITNCPRG-----------------GKRSK 34
Nematocin       ----------------------MGSSP-------ILLVLAISIGLASACFLNSCPYR-----------------RYGRT 33

                                      1  2             3     45     6           7           8
NGFFFamide      SDASSTKMDDDRLPKYESSGSFDKCRPCGPGRQGR-----CVMVGTCCSPLFGCYLFTP-EAAACMTEDVSP----CQLN 230
Echinotocin     --------------SRP----LRQCLECGPGGVGR-----CMGPGICCGPTIGCHINTQ-HTLSCMRENEIS--TPCELP 94
Vasopressin     --------------AMSDLELRQCLPCGPGGKGR-----CFGPSICCADELGCFVGTA-EALRCQEENYLP--SPCQSG 88
Oxytocin        --------------AAPDLDVRKCLPCGPGGKGR-----CFGPNICCAEELGCFVGTA-EALRCQEENYLP--SPCQSG 88
Vasotocin       --------------DLTD-SVRQCLPCGPGGQGR-----CFGPRICCGEAMGCRLGGP-DVAICRAERLMP--SPCESR 90
SOP             G-----KREPTREKQTRSGPPIRKCPPCGLRGTGQ-----CFSSRMCCTPALGCVIGENEITEPCRYESRIP--VECASA 108
Lys-conopressin ---------------DTG-MVTSRECMKCGPGGTGQ-----CVGPSICCGQDFGCHVGTA-EAAVCQQENDSS--TPCLVK 97
Cephalotocin    -------------PMSEQGSNQKCMSCGPNGEGQ-----CVGSNICCHKD-GCIIGT--LAKECNEENEST--TACSVK 98
Annetocin       -------------LLSPLQPARQCMPCGATVGGRSVLGVCVSENTCCVAHLGCFVNTE-ESKVCALENHLS--TPCRLE 109
Inotocin        -------------FAISENAVKPCVSCGPGQSGQ-----CFGPSICCG-PFGCLVGTP-ETLRCQREGFFHEREPCIAG 93
Nematocin       ----------------------IRCSSCGIENEGV-----CISEGRCCT------NEECFMSTECSYSAVCP--ELFCKI 78

                   9              10    11 12    13    14
NGFFFamide      APSCG----------LAGKCVADGICCSAAEGACHLDPTCTSMSLN-------------------------------- 266
Echinotocin     GNPCQTVP--------SGTCGAMGVCCNSNS--CSEDASCLMIEEDDSLKRFEQMSREENGSTRKDLRVKLLDLLLNMQD 164
Vasopressin     QKACGS----------GGRCAAFGVCCNDES--CVTEPECREVFHRRAR--ASDR---SNATQLDGPAGALLLRLVQLAG 151
Oxytocin        QKACGS----------GGRCAVLGLCCSPDG--CHADPAC----DAEAT--FSQR------------------------ 125
Vasotocin       GEPCGH----------GGKCGAPGLCCSSES--CAEDASCG--WEGGDS--PGERPFPHSALRLQSPAAEAMLELINSNS 154
SOP             GPTCMRKDREKGNVQSMGVCAADGLCCNADG--CTYHHECLLAEKDPSDSMAPLATIRSSL-------------------- 167
Lys-conopressin GEACGSRD--------AGNCVADGICCDSES--CAVNDRCRDLD-------------GNAQANRGDLIQLIHKLLKVRD 153
Cephalotocin    GVPCGTDG--------QGRCVADGVCCDESS--CFTTDRCDREN-------------HRSMA--------MQKLLEIRD 146
Annetocin       GPPCGSDG--------QDVCAVEGICCAGQN--CRYDAQC------------------------------------- 139
Inotocin        SAPCRKN---------TGRCAFDGICCSQDS--CHADKSCAS--DDKSP--IDLYTLINYQAELAGDK------------ 146
Nematocin       G-------------HHPGYCMKKGYCCTQGG--CQTSAMC------------------------------------- 103

NGFFFamide      ------------ 266
Echinotocin     Q----------- 165
Vasopressin     APEPFEPAQPDAY 164
Oxytocin        ------------ 125
Vasotocin       LRD--------- 157
SOP             ------------ 167
Lys-conopressin ---YD-------- 155
Cephalotocin    GIYYKK------- 152
Annetocin       ------------ 139
Inotocin        ------------ 146
Nematocin       ------------ 103
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Fig. 3. ClustalX multiple alignment of the sequences of the *S. purpuratus* NGFFFamide precursor, the *S. purpuratus* echinotocin precursor and precursors of vasopressin/oxytocin-like peptides in other species. Signal peptides are shown in blue, neuropeptides are shown in red, dibasic cleavage sites are shown in green and neurophysin-like domains are shown in purple. The conserved cysteine residues in the neurophysin-like domains are underlined and numbered 1–14. The precursors of vasopressin/oxytocin-like peptides from other species include precursors of human vasopressin (Mohr et al., 1985), human oxytocin (Mohr et al., 1985), vasotocin from the lamprey *Lethenteron japonicum* (Suzuki et al., 1995), an oxytocin-like peptide (SOP) from the urochordate *Styela plicata* (Ukena et al., 2008), Lys-conopressin from the mollusc *Lymnaea stagnalis* (Van Kesteren et al., 1992), cephalotocin from the mollusc *Octopus vulgaris* (Reich, 1992), annetocin from the annelid *Eisenia foetida* (Oumi et al., 1994) and inotocin from the arthropod (insect) *Tribolium castaneum* (Aikins et al., 2008; Stafflinger et al., 2008). Also included is a precursor from the nematode *Caenorhabditis elegans* (GenBank: NP_001033548, GI:86564869) that has not been reported previously in the literature; this precursor contains an unusual putative vasopressin/oxytocin-like peptide comprising just eight residues (CFLNSCPY), which we have named ‘nematocin’.

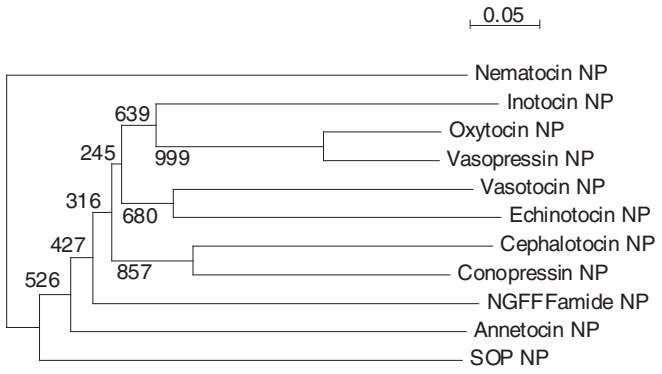

Fig. 4. Neighbour-joining tree (with bootstrap values) based on a ClustalX multiple alignment of neurophysin (NP) sequences, incorporating residues from the first to the fourteenth conserved cysteines. The tree shows that the neurophysin domain of the *S. purpuratus* NGFFFamide precursor does not have a higher level of overall sequence similarity with the neurophysin domain of the *S. purpuratus* echinotocin precursor than with the neurophysins from other species.

contraction of both tube foot and oesophagus preparations. Thus, the mean force of contraction induced by 3×10^{-6} mol l^{-1} NGFFFamide on tube feet was 1.35±0.21 mN (±s.e.m.; N=3), whereas the mean force of contraction induced by 3×10^{-6} mol l^{-1} echinotocin on tube feet was 0.81±0.42 mN (±s.e.m.; N=3). Similarly, the mean force of contraction induced by 3×10^{-6} mol l^{-1} NGFFFamide on oesophagus was 1.47±0.23 mN (±s.e.m.; N=3), whereas the mean force of contraction induced by 3×10^{-6} mol l^{-1} echinotocin on oesophagus 0.71±0.01 mN (±s.e.m.; N=3). However, statistical analysis of these data using a t-test did not reveal significant differences in the magnitudes of contraction induced by NGFFFamide and echinotocin.

NGFFFamide caused dose-dependent contraction of oesophagus preparations at concentrations ranging from 10^{-11} to 10^{-6} mol l^{-1} (Fig. 5E). With tube foot preparations, dose-dependent contractile effects were only observed with higher concentrations of NGFFFamide within the range 10^{-8} to 10^{-6} mol l^{-1} (Fig. 5E). These data indicate that NGFFFamide is more potent as a contractant of oesophagus than as a contractant of tube feet.

Echinotocin caused dose-dependent contraction of tube foot (10^{-8} to 10^{-6} mol l^{-1}) and oesophagus (10^{-9} to 10^{-7} mol l^{-1}) preparations (Fig. 5F).

DISCUSSION

NGFFFamide: a novel myoactive neuropeptide in sea urchins

We have identified a gene in the sea urchin *S. purpuratus* that encodes a novel myoactive neuropeptide Asn-Gly-Phe-Phe-Phe-(NH_2) or NGFFFamide, which we pronounce 'negfamide'. NGFFFamide was identified on account of its sequence similarity with NGIWYamide, a myoactive neuropeptide in holothurians (sea cucumbers) (Iwakoshi et al., 1995; Ohtani et al., 1999). NGIWYamide-like immunoreactive peptides also occur in starfish (Saha et al., 2006) and therefore NGFFFamide and NGIWYamide may be members of a family of neuropeptides that occur throughout the phylum Echinodermata. A cDNA encoding the NGFFFamide precursor protein was identified in a cDNA library generated from *S. purpuratus* radial nerve tissue, demonstrating that the NGFFFamide gene is expressed in the sea urchin nervous system and indicating that NGFFFamide may function as a neuropeptide.

To investigate the physiological roles of NGFFFamide, the pharmacological effects of synthetic NGFFFamide on *in vitro* preparations of tube feet and oesophagus from the sea urchin *E. esculentus* were examined. NGFFFamide caused contraction of *Echinus* tube foot and oesophagus preparations, consistent with the contracting action of NGIWYamide on sea cucumber body wall muscle and tentacle preparations and starfish tube foot preparations (Inoue et al., 1999; Saha et al., 2006). Thus, it appears that members of the NGIWYamide/NGFFFamide neuropeptide family typically cause muscle contraction in echinoderms. Further studies are now required to investigate the mechanisms by which NGIWYamide and NGFFFamide affect muscle activity in sea cucumbers and sea urchins, respectively. One scenario would be direct interaction with receptor proteins expressed by muscle cells; an alternative possibility is that these peptides act indirectly by stimulating the release of myoactive factors from nerves or other cell types.

The NGFFFamide precursor contains a neurophysin domain

The discovery of a new family of myoactive neuropeptides in echinoderms is of interest with respect to the neurobiology and physiology of these animals. However, perhaps of more general interest is our discovery that the NGFFFamide precursor, in addition to encoding two copies of the NGFFFamide peptide, also comprises a polypeptide that shares a high level of sequence identity with neurophysins, a family of proteins that are derived from the precursors of vasopressin/oxytocin-type neuropeptides. Neurophysins act as carrier proteins, which are important for packaging, processing and protection of vasopressin/oxytocin-type neuropeptides (De Bree, 2000; De Bree and Burbach, 1998; Legros and Geenen, 1996). Hitherto neurophysins have been uniquely associated with vasopressin/oxytocin-type neuropeptides and to the best of our knowledge the NGFFFamide precursor is the first to be discovered comprising neurophysin and a neuropeptide that is not a member of the vasopressin/oxytocin family of peptides.

Echinotocin: a vasopressin/oxytocin-like peptide in sea urchins

The vasopressin/oxytocin neuropeptide family has a widespread phylogenetic distribution indicative of an ancestry that dates back at least as far as the common ancestor of bilaterian animals. Accordingly, vasopressin/oxytocin-like peptides have been identified in vertebrates (Hoyle, 1999; Urano et al., 1992), protostomian invertebrates (Cruz et al., 1987; Oumi et al., 1994; Proux et al., 1987; Reich, 1992; Van Kesteren et al., 1992) and most recently in two deuterostomian invertebrates, the urochordates *Ciona intestinalis* and *Styela plicata* (Kawada et al., 2008; Ukena et al., 2008). However, vasopressin/oxytocin like peptides have thus far not been identified in any echinoderm species. Against this background and our discovery that the sea urchin NGFFFamide precursor contains a neurophysin domain, it was of interest to investigate the occurrence of a gene encoding a vasopressin/oxytocin-like peptide in sea urchins. BLAST analysis of *S. purpuratus* genomic sequence data using the human vasopressin precursor as a query enabled identification of a gene encoding a peptide (CFISNCPKGamide) that is a member of the vasopressin/oxytocin-type neuropeptide family and which we have named 'echinotocin'. Likewise, if vasopressin/oxytocin-like peptides are identified in other echinoderm species, we suggest that these are collectively known as 'echinotocins'.

Comparison of the sequence of echinotocin with other members of the vasopressin/oxytocin neuropeptide family reveals that residues Cys^1 and Cys^6 in echinotocin are conserved throughout the family

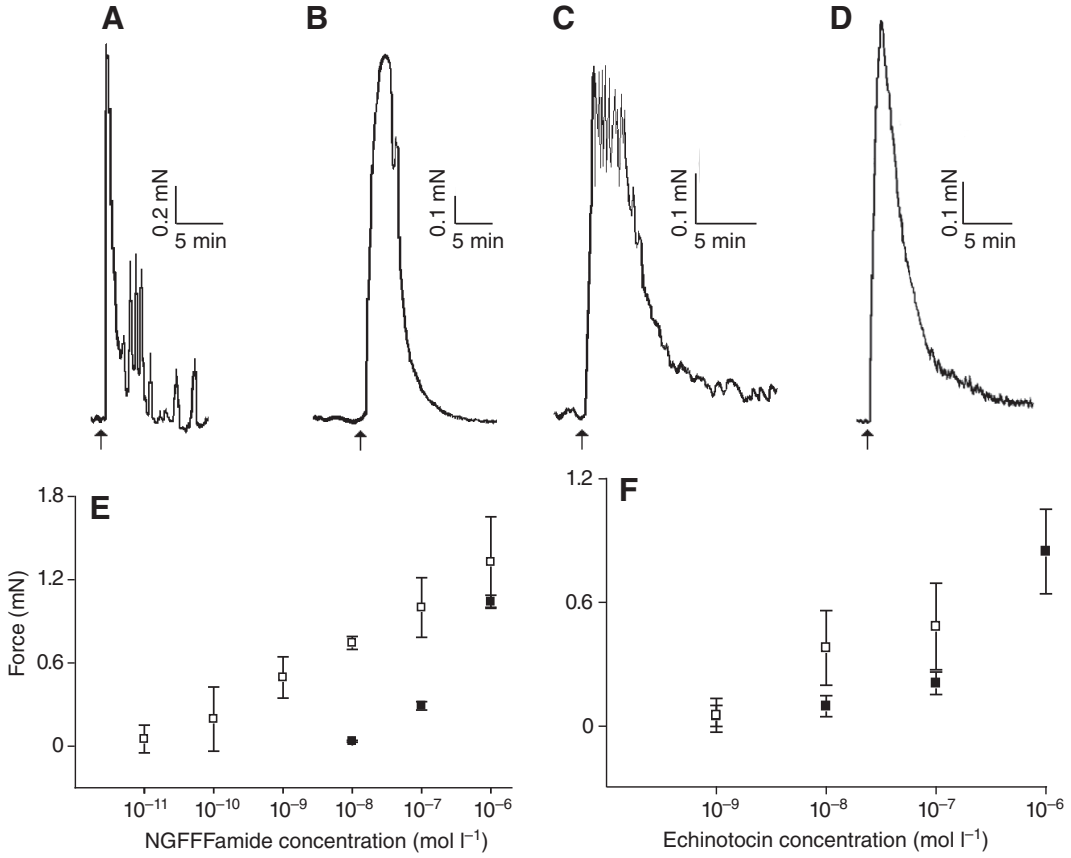

Fig. 5. NGFFFamide and echinotocin cause contraction of sea urchin tube foot and oesophagus preparations. Representative traces show that application (arrows) of NGFFFamide (3×10^{-6} mol l^{-1}; A,B) and echinotocin (3×10^{-6} mol l^{-1}; C,D) causes contraction of oesophagus (A,C) and of tube foot (B,D) preparations from the sea urchin *Echinus esculentus*. (E) Graph showing the dose-dependent effect of NGFFFamide on tube foot (filled squares) and oesophagus (open squares) preparations. Data points are mean values (N=4) with bars showing s.e.m. (F) Graph showing the dose-dependent effect of echinotocin on tube foot (filled squares) and oesophagus (open squares) preparations. Data points are mean values (N=4) with bars showing s.e.m.

(Fig. 6). This is not surprising because in vasopressin/oxytocin-like peptides these two residues form a disulphide bridge, conferring a cyclic conformation that is important for the biological activity of these peptides (Hruby et al., 1990; Sawyer, 1977). Other residues in the echinotocin sequence are shared with some of the known vasopressin/oxytocin-like peptides. Thus, the C-terminal amidated glycine residue and residues Asn^5 and Pro^7 in echinotocin are also features of most vasopressin/oxytocin-like peptides, with notable exceptions being two vasopressin/oxytocin-like peptides identified in urochordates (Kawada et al., 2008; Ukena et al., 2008) and a putative neuropeptide (CFLNSCPY or 'nematocin') in the nematode *Caenorhabditis elegans* (NP_001033548; GI:86564869). Residues 2 and 3 in echinotocin are phenylalanine and isoleucine, respectively, which is consistent with the occurrence of hydrophobic residues (Phe, Tyr, Leu or Ile) in these positions in other vasopressin/oxytocin-like peptides. Finally, the presence of a basic amino acid residue (Lys) at position 8 in echinotocin confers similarity with vasopressin, which has an arginine residue at this position, whereas oxytocin has a leucine residue in this position (Fig. 6).

Structure of the echinotocin precursor and organization of the echinotocin gene

The predicted structure of the echinotocin precursor protein is consistent with precursors of vasopressin/oxytocin-like peptides in vertebrates and in other invertebrates (De Bree and Burbach, 1998; Hoyle, 1999). Thus, the echinotocin sequence is preceded by an N-terminal signal peptide and followed by a C-terminal neurophysin-like domain (Fig. 3). A signal peptide, vasopressin/oxytocin-like neuropeptide and neurophysin occur in all of the known precursor proteins for vasopressin/oxytocin-like neuropeptides in both vertebrates and invertebrates (De Bree and Burbach, 1998; Hoyle, 1999). However, there is variability in the length of the C-terminal polypeptide sequence following the highly conserved neurophysin

Peptide	Sequence	Source	Ref.
Echinotocin	**Cys**-Phe-Ile-Ser-Asn-**Cys**-Pro-Lys-Gly-NH_2	*S. purpuratus* (Echinodermata)	1
Vasopressin	**Cys**-Tyr-Phe-Gln-Asn-**Cys**-Pro-Arg-Gly-NH_2	*Homo sapiens* (Chordata)	2
Oxytocin	**Cys**-Tyr-Ile-Gln-Asn-**Cys**-Pro-Leu-Gly-NH_2	*Homo sapiens* (Chordata)	2
Vasotocin	**Cys**-Tyr-Ile-Gln-Asn-**Cys**-Pro-Arg-Gly-NH_2	*Lethenteron japonicum* (Chordata)	3
Ciona-VP	**Cys**-Phe-Phe-Arg-Asp-**Cys**-Ser-Asn-Met-Asp-Trp-Tyr-Arg	*Ciona intestinalis* (Chordata)	4
Styela-OP	**Cys**-Tyr-Ile-Ser-Asp-**Cys**-Pro-Asn-Ser-Arg-Phe-Trp-Ser-Thr-NH_2	*Styela plicata* (Chordata)	5
Conopressin	**Cys**-Phe-Ile-Arg-Asn-**Cys**-Pro-Lys-Gly-NH_2	*Lymnaea stagnalis* (Mollusca)	6
Cephalotocin	**Cys**-Tyr-Phe-Arg-Asn-**Cys**-Pro-Ile-Gly-NH_2	*Octopus vulgaris* (Mollusca)	7
Annetocin	**Cys**-Phe-Val-Arg-Asn-**Cys**-Pro-Thr-Gly-NH_2	*Eisenia foetida* (Annelida)	8
Inotocin	**Cys**-Leu-Ile-Thr-Asn-**Cys**-Pro-Arg-Gly-NH_2	*Locusta migratoria* (Arthropoda)	9–11
Nematocin	**Cys**-Phe-Leu-Asn-Ser-**Cys**-Pro-Tyr	*Caenorhabditis elegans* (Nematoda)	12

Fig. 6. Comparative alignment of the amino acid sequence of echinotocin with the sequences of vasopressin, oxytocin, vasotocin and vasopressin/oxytocin-like peptides identified in other invertebrate species. Cysteine residues, which are conserved in all of the peptides, are shown in bold. References: [1]present study; [2](Light and Du Vigneaud, 1958); [3](Suzuki et al., 1995); [4](Kawada et al., 2008); [5](Ukena et al., 2008); [6](Van Kesteren et al., 1992); [7](Reich, 1992); [8](Oumi et al., 1994); [9](Proux et al., 1987); [10](Aikins et al., 2008); [11](Stafflinger et al., 2008); [12]GenBank NP_001033548, GI:86564869.

domain. For example, in the oxytocin, annetocin and nematocin precursors it is very short (nine residues) or absent (Fig. 3), whereas in the vasopressin precursor there is a 39 amino acid residue peptide, which is known as copeptin. Moreover, following cleavage at a monobasic site separating it from neurophysin, copeptin is co-secreted with vasopressin. Three notable characteristics of copeptin are that it is preceded by a monobasic cleavage site, it is glycosylated at a site (N^6-X-T^8) located near its N-terminus and it has a conserved hydrophobic LLLRLV sequence comprising residues 17–22 (De Bree and Burbach, 1998). Interestingly, the C-terminal region of the echinotocin precursor has some of these features; it has a putative glycosylation site (NGS) that aligns with the glycosylation site in the vasopressin precursor (NAT) and it has a hydrophobic sequence (LLDLLL) that aligns with the LLLRLV sequence in the vasopressin precursor (Fig. 3). There is also a potential dibasic cleavage site (KR) at residues 134 and 135 in the echinotocin precursor, which if utilized *in vivo* would liberate a 30 amino acid residue copeptin-like molecule.

Interestingly, glycosylation of copeptin is a characteristic hitherto uniquely associated with vasopressin precursors (De Bree and Burbach, 1998). Therefore, the presence of a putative glycosylation site in the C-terminal region of the echinotocin precursor is intriguing and worthy of further investigation to assess whether it is glycosylated *in vivo* in sea urchins. Measurement of serum levels of copeptin can be used as a biomarker for several clinical conditions in humans (Katan et al., 2008) but little is known about the physiological relevance of this molecule. It has been postulated that copeptin may act as a modulator of excitatory neurotransmission in the brain (Van den Hooff et al., 1990) and as a prolactin-releasing factor (Nagy et al., 1988), but further studies are required (Hyde et al., 1989). Comparative studies on the echinotocin-associated copeptin-like peptide in sea urchins may provide new insights on this issue.

The predicted echinotocin precursor protein is encoded by three exons, which is consistent with the structural organization of genes encoding vasopressin/oxytocin-like peptides in other animals (De Bree and Burbach, 1998; Hoyle, 1999). The first and third exons of genes encoding precursors of vasopressin/oxytocin-like peptides also have 5′ and 3′ non-coding sequences, respectively (Ivell and Richter, 1984), and it is likely, therefore, that the echinotocin gene is similar in this respect. The positions of introns interrupting the coding sequence are conserved between the echinotocin gene and other genes encoding vasopressin/oxytocin-like peptides. Thus, the first intron is located between the codons for residues Gln^{46} and Cys^{47} in the echinotocin precursor and the second intron interrupts the codon for residue Asn^{116} (Fig. 2); introns are located at equivalent positions in genes encoding precursors of vasopressin/oxytocin-like peptides in mammals (Ivell and Richter, 1984) and in the gastropod mollusc *Lymnaea stagnalis* (Van Kesteren et al., 1995). Thus, the conserved positioning of the two introns in genes encoding vasopressin/oxytocin-like precursors presumably dates back to the common ancestor of all bilaterian animals. However, this feature appears to have been secondarily lost in some lineages because, for example, genes encoding vasopressin/oxytocin-like peptides (cephalotocin and octopressin) in the mollusc *Octopus vulgaris* lack introns (Kanda et al., 2003).

Physiological roles of echinotocin in sea urchins

Analysis of the *in vitro* pharmacological effects of synthetic echinotocin on *Echinus* tube feet and oesophagus revealed that, like NGFFFamide, it causes contraction. However, as with NGFFFamide, the mechanisms by which echinotocin causes muscle contraction in sea urchins remain to be determined. We can, however, speculate on the molecular identity of a receptor that may mediate the effects of echinotocin because, as part of a genome-wide annotation of genes associated with nervous system function, we have identified a gene (SPU_021290) encoding a G-protein-coupled receptor in *S. purpuratus* that is an orthologue of vasopressin/oxytocin receptors (Burke et al., 2006) (see also http://www.spbase.org/SpBase/search/viewAnnoGeneInfo.php?spu_id=SPU_021290).

The myoactivity of echinotocin is consistent with the effects of vasopressin/oxytocin-like peptides in other animals. For example, in mammals vasopressin regulates blood pressure by causing vasoconstriction. However, perhaps the most well known physiological role of vasopressin is in osmoregulation, acting as an anti-diuretic hormone (Sawyer, 1977). Interestingly, a gene encoding a vasopressin/oxytocin-like peptide (*Styela* oxytocin-related peptide or SOP) was recently identified in an invertebrate chordate, the sea-squirt *Styela plicata* (Ukena et al., 2008). Analysis of the expression of the SOP gene in the cerebral ganglion of *Styela* revealed that it is upregulated when animals are exposed to dilute (60%) seawater, which also causes closure of their inhalant and exhalant siphons. Furthermore, SOP causes contraction of *in vitro* preparations of inhalant and exhalant siphons from *Styela*. Ukena and colleagues (Ukena et al., 2008) conclude that SOP acts to prevent the influx of dilute seawater in *Styela*, suggesting an evolutionarily ancient role for vasopressin/oxytocin-like peptides in osmoregulation. It is possible, therefore, that the contractile effect of echinotocin on tube feet *in vitro* is indicative of a similar role in sea urchins, with retraction of tube feet reducing water influx in hypo-osmotic conditions.

Oxytoxin causes uterine contraction and stimulates lactation in mammals (Sawyer, 1977) and evidence of an evolutionarily conserved role for vasopressin/oxytocin-like peptides in reproductive physiology has emerged from studies on invertebrates. For example, the molluscan peptide conopressin causes contraction of the vas deferens in the pond snail *Lymnaea stagnalis* (Van Kesteren et al., 1992) and the annelid peptide annetocin induces egg-laying behaviour in earthworms (Oumi et al., 1996). Moreover, a recent study suggests that neurons releasing oxytocin/vasopressin-like peptides are an evolutionarily ancient neuronal population with dual photosensory–neurosecretory properties coordinating reproduction with light cycles (Tessmar-Raible et al., 2007). Consistent with this hypothesis, neurons releasing a vasopressin/oxytocin-like peptide in the insect *Locusta migratoria* are more active in the dark than in the light and this activity is regulated by extraocular photoreceptors (Thompson and Bacon, 1991). Interestingly, genes encoding orthologues of mammalian retinal transcription factors are expressed in sea urchin tube feet, suggesting that these organs have a photosensory function (Burke et al., 2006). Therefore, the contractile effect of echinotocin on tube feet *in vitro* may be a manifestation of an *in vivo* role in mediating photosensory regulation of physiological processes in sea urchins.

In addition to the peripheral actions of oxytocin and vasopressin in mammals and other vertebrates, there is growing evidence of roles in the central nervous system (CNS) associated with reproductive behaviour and social behaviour/cognition. For example, there is evidence that oxytocin has important roles in maternal–infant bonding, pair bonding and social interaction, whilst differences in vasopressin receptor expression in the brain are associated with monogamy *versus* polygamy in vole species (Caldwell et al., 2008; Donaldson and Young, 2008; Israel et al., 2008; Winslow et al., 1993). The evolutionary origins of these CNS-mediated actions of

vasopressin and oxytocin are unknown; discovery of vasopressin/oxytocin-type peptides in deuterostomian invertebrates provides new opportunities to address this issue.

The role of neurophysins as carrier proteins for vasopressin/oxytocin-like peptides.

Neurophysins are required to facilitate endopeptidase-mediated cleavage of vasopressin/oxytocin-like peptides from precursor proteins and for binding and transport of the biologically active peptides in secretory granules from neuronal somata to axonal terminals (De Bree, 2000; De Bree and Burbach, 1998). There are 14 highly conserved cysteine residues in neurophysins (see Fig. 3), which form seven intramolecular disulphide bridges (De Bree and Burbach, 1998). Furthermore, neurophysins form dimers and binding of vasopressin/oxytocin-like peptides favours dimerization (Nicolas et al., 1978). The interaction of vasopressin/oxytocin-like peptides with neurophysins was one of the first ligand–protein interactions to be analysed (Acher et al., 1958) and more recently it has been investigated in detail using NMR spectroscopy and X-ray crystallography (Chen et al., 1991; Sardana and Breslow, 1984; Wu et al., 2001). The first three amino acids in the N-terminal part of vasopressin (Cys-Tyr-Phe) and oxytocin (Cys-Tyr-Ile) are the residues that are most important for binding to neurophysin (De Bree and Burbach, 1998) and the corresponding residues in echinotocin are structurally identical or similar (Cys-Phe-Ile). The strongest interaction is a salt bridge between the αNH_3^+ group of the N-terminal cysteine residue and the γCOO^- group of Glu^{47} in the oxytocin/vasopressin neurophysins and both of these residues are conserved in the echinotocin precursor. The aromatic side-chain of residue Tyr^2 in oxytoxin and vasopressin is located in a pocket formed by the disulphide bridges Cys^{10}-Cys^{54} and Cys^{21}-Cys^{44}, the Cys^{21}-Phe-Gly-Pro^{24} backbone, and the side-chains of Pro^{24}, Glu^{47} and Asn^{48}. By comparison, echinotocin has a residue (Phe^2) with an aromatic side-chain and all but one of the residues in neurophysin that form a pocket for the aromatic side-chain of Tyr^2 in oxytoxin and vasopressin are conserved in the echinotocin precursor sequence, the exception being Phe^{22}, which is a methionine residue in the sea urchin sequence. Other evolutionarily conserved characteristics of vasopressin and oxytocin that are important for binding to neurophysin are the disulphide bridge between Cys^1 and Cys^6, the peptide backbone between residues 2 and 3 and the side-chain of residue 3 (De Bree and Burbach, 1998). Based on these similarities, it is likely that echinotocin interacts with the neurophysin domain of the echinotocin precursor.

Does the neurophysin encoded by the NGFFFamide gene act as a carrier protein for NGFFFamide?

By analogy with the role of neurophysins as carrier proteins for vasopressin/oxytocin-like peptides, the neurophysin domain in the NGFFFamide precursor may likewise act as a carrier protein for NGFFFamide. Consistent with this notion, several residues that are involved in binding of vasopressin/oxytocin-like peptides (see above) are conserved in the NGFFFamide neurophysin, including the residues corresponding to the cysteines at positions 10, 21, 44 and 54 and the glutamate at position 47 in the vasopressin/oyxtocin neurophysins. There are, however, some interesting differences between the NGFFFamide-associated neurophysin and neurophysins associated with echinotocin and other vasopressin/oxytocin-like peptides. Thus, between cysteines 7 and 8 in the NGFFFamide-associated neurophysin there are seven residues, whereas in the neurophysins associated with echinotocin and with vasopressin/oxytocin-like peptides in other species there are nine residues. Furthermore, there are six residues between cysteines 12 and 13 in the NGFFFamide-associated neurophysin, whereas in the neurophysins associated with echinotocin and with vasopressin/oxytocin-like peptides in other species there are only four residues. Unusual structural features such as these may facilitate binding of NGFFFamide by its associated neurophysin. However, experimental investigation of an interaction of NGFFFamide with neurophysin, which was beyond the scope of this study, will be required to address these issues.

The evolutionary origin of the neurophysin domain in the NGFFFamide precursor

Genes encoding precursors for vasopressin/oxytocin-like peptides with an associated neurophysin domain have been identified throughout the animal kingdom (De Bree and Burbach, 1998) and the echinotocin gene reported here is a new member of this gene family. This widespread phylogenetic distribution indicates that the evolutionary origin of the vasopressin/oxytocin family of neuropeptide precursors dates back at least as far as the common ancestor of all bilaterian animals. The NGFFFamide precursor is the first protein to be identified that has a neurophysin domain without an associated vasopressin/oxytocin-like peptide and therefore it is of interest to explore the evolutionary origin of this novel protein.

The occurrence of the neurophysin domain in the *S. purpuratus* NGFFFamide precursor is presumably a consequence of duplication and transposition of DNA encoding the precursor, or part of the precursor (i.e. the neurophysin domain), of a vasopressin/oxytocin-like peptide in an ancestor of *S. purpuratus*. Consistent with this notion, the NGFFFamide and echinotocin genes both have an intron preceding the codon encoding the first cysteine residue of their neurophysin domains. In the echinotocin gene and in most genes encoding vasopressin/oxytocin-like peptides there is also a second intron that interrupts the neurophysin-encoding sequence. The NGFFFamide neurophysin, however, is encoded by a single exon (exon 4). Thus, if the neurophysin domain of the NGFFFamide precursor originated as a consequence of complete or partial duplication of a gene encoding a vasopressin/oxytocin-like peptide, then the second intron that interrupts the neurophysin coding sequence must have been lost subsequently. There is a precedent for this, however, because, as discussed above, the two genes encoding vasopressin/oxytocin-like peptides in *Octopus vulgaris* both lack introns (Kanda et al., 2003).

Based on sequence similarity, NGFFFamide neurophysin is not more closely related to the echinotocin neurophysin than to neurophysins associated with vasopressin/oxytocin-like peptides in other phyla. Moreover, the echinotocin neurophysin shares more similarity with the neurophysin associated with lamprey vasotocin than it does with the NGFFFamide-associated neurophysin (see Fig. 4). This suggests that, with respect to a putative common ancestral sequence, the NGFFFamide-associated neurophysin is more divergent than the neurophysin associated with echinotocin. Furthermore, this feature of the NGFFFamide-associated neurophysin may be related to accommodation of NGFFFamide as a binding partner.

Determination of the timing of the duplication event that gave rise to the occurrence of a neurophysin domain in the *S. purpuratus* NGFFFamide precursor will be facilitated if genes encoding precursors for NGFFFamide-like peptides with a neurophysin domain are identified in other echinoderms. BLAST analysis of genome sequence data obtained for the sea urchin *Allocentrotus fragilis* (http://www.hgsc.bcm.tmc.edu/blast.hgsc?organism=15)

reveals the presence of an exon encoding a neurophysin domain that is identical to residues 185–266 of the *S. purpuratus* NGFFFamide precursor. Thus, this feature is not unique to *S. purpuratus* but also occurs in other sea urchins. More interesting would be to determine whether the NGIWYamide precursor protein in the holothurian *Apostichopus japonicus* also has a neurophysin domain. If it does not, this would suggest that the neurophysin domain in the NGFFFamide precursor originated in an echinoid ancestor of *S. purpuratus*. If the NGIWYamide precursor does have a neurophysin domain, this would suggest that it originated prior to the common ancestor of echinoids and holothurians. It is possible that precursors comprising NGFFFamide/NGIWYamide-like peptides together with a neurophysin domain occur throughout the phylum Echinodermata and even in closely related phyla such as the Hemichordata and the Xenoturbellida (see Bourlat et al., 2006). Further investigation of this issue will be possible when genome sequences are determined for other echinoderm species and for hemichordate and xenoturbellid species.

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