RESEARCH ARTICLE

Discovery of a novel neurophysin-associated neuropeptide that triggers cardiac stomach contraction and retraction in starfish

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SUMMARY

Feeding in starfish is a remarkable process in which the cardiac stomach is everted over prey and then retracted when prey tissue has been resorbed. Previous studies have revealed that SALMFamide-type neuropeptides trigger cardiac stomach relaxation and eversion in the starfish *Asterias rubens*. We hypothesised, therefore, that a counteracting neuropeptide system controls cardiac stomach contraction and retraction. Members of the NG peptide family cause muscle contraction in other echinoderms (e.g. NGFFFamide in sea urchins and NGIWYamide in sea cucumbers), so we investigated NG peptides as candidate regulators of cardiac stomach retraction in starfish. Generation and analysis of neural transcriptome sequence data from *Asterias rubens* revealed a precursor protein comprising two copies of a novel NG peptide, NGFFYamide, which was confirmed by mass spectrometry. A noteworthy feature of the NGFFYamide precursor is a C-terminal neurophysin domain, indicative of a common ancestry with vasopressin/oxytocin-type neuropeptide precursors. Interestingly, in precursors of other NG peptides the neurophysin domain has been retained (e.g. NGFFFamide) or lost (e.g. NGIWYamide and human neuropeptide S) and its functional significance remains to be determined. Investigation of the pharmacological actions of NGFFYamide in starfish revealed that it is a potent stimulator of cardiac stomach contraction *in vitro* and that it triggers cardiac stomach retraction *in vivo*. Thus, discovery of NGFFYamide provides a novel insight on neural regulation of cardiac stomach retraction as well as a rationale for chemically based strategies to control starfish that feed on economically important shellfish (e.g. mussels) or protected marine fauna (e.g. coral).

Keywords: NG peptides; NGFFYamide; neurophysin; starfish; echinoderm; *Asterias rubens*.

INTRODUCTION

Feeding in many starfish species, including the common European starfish *Asterias rubens*, involves eversion of the cardiac stomach through a narrow oral opening over the digestible parts of prey. This remarkable feeding mechanism enables starfish to feed on relatively large prey (e.g. mussels) as tissue is partially digested externally and then transported internally to the pyloric caecae, where digestion and absorption is completed. When the soft tissue of prey has been entirely resorbed, the cardiac stomach is retracted back into the central disk region of the starfish body (Anderson, 1954).

Experimental studies on *Asterias rubens* have revealed that cardiac stomach eversion is triggered by injection of the starfish SALMFamide neuropeptides S1 and S2. Furthermore, consistent with these *in vivo* effects of SALMFamides, S1 and S2 cause dose-dependent relaxation of cardiac stomach preparations *in vitro* (Elphick et al., 1995; Elphick et al., 1991; Melarange et al., 1999; Newman et al., 1995a; Newman et al., 1995b). Thus, neural control of cardiac stomach eversion in starfish appears to be mediated, at least in part, by the release of neuropeptides (SALMFamides) that cause muscle relaxation. We hypothesize, therefore, that a counteracting neuropeptide(s) that causes muscle contraction may mediate neural control of cardiac stomach retraction in starfish.

Muscle preparations from the sea cucumber *Apostichopus japonicu*s have been used as bioassays to screen for myoactive neuropeptides in echinoderms (Elphick, 2012; Inoue et al., 1999; Iwakoshi et al., 1995; Ohtani et al., 1999). Two SALMFamide-type neuropeptides were identified as muscle relaxants and the pentapeptide Asn-Gly-Ile-Trp-Tyr-NH_2 (NGIWYamide) was identified as a muscle contractant. Furthermore, subsequent studies have revealed that NGIWYamide also causes contraction of tube foot preparations from the starfish *Asterina pectinifera* and consistent with this finding NGIWYamide-like immunoreactivity was detected in the starfish nervous system (Saha et al., 2006). However, the molecular identity of NGIWYamide-like peptide(s) in *Asterina pectinifera* or in other starfish species has been not determined.

Facilitated by genome sequencing (Burke et al., 2006; Sodergren et al., 2006), an NGIWYamide-like neuropeptide was recently identified in the sea urchin *Strongylocentrotus purpuratus*. The sea urchin peptide has the amino acid sequence Asn-Gly-Phe-Phe-Phe-NH_2 (NGFFFamide) and, consistent with the myoactivity of NGIWYamide, NGFFFamide causes

contraction of tube foot and oesophagus preparations from the sea urchin *Echinus esculentus* (Elphick and Rowe, 2009). An interesting feature of the precursor protein that NGFFFamide is derived from is that it contains a neurophysin domain, a polypeptide hitherto thought to be uniquely associated with precursors of vasopressin/oxytocin-type neuropeptides and that is required for biosynthesis of these neuropeptides (De Bree, 2000; De Bree and Burbach, 1998). Furthermore, NGFFFamide belongs to a family of neuropeptides in deuterostomian invertebrates that have an Asn-Gly motif ("NG peptides") and that are typically derived from neurophysin-containing precursors (Elphick, 2010). These include NGFYNamide and NGFWNamide in the hemichordate *Saccoglossus kowalevskii* and SFRNGVamide in the cephalochordate *Branchiostoma floridae*. Interestingly, however, the prototype of the NG peptide family – the sea cucumber neuropeptide NGIWYamide – is derived from a precursor protein that lacks a neurophysin domain (Elphick, 2012).

The discovery and functional characterisation of the NG peptide family in echinoderms and other deuterostomian invertebrates provided a rationale for investigation of NG peptides as potential regulators of cardiac stomach retraction in starfish. To address this issue, we tested the effects of the sea urchin neuropeptide NGFFFamide on *in vitro* cardiac stomach preparations from the starfish *Asterias rubens* and found that it causes contraction (R. Melarange & M.R. Elphick, unpublished data). Thus, the aim of this study was to determine the molecular identity of the NG peptide(s) in the starfish *Asterias rubens* and to investigate a potential physiological role in regulation of cardiac stomach retraction.

MATERIAL AND METHODS

Animals and chemicals

Starfish (*Asterias rubens*) were collected at low tide from the Thanet coast (Kent, UK) and transported to Queen Mary, University of London, where they were maintained in a seawater aquarium at approximately 11°C and fed with mussels (*Mytilus edulis*). Synthetic neuropeptides were custom synthesised by Peptide Protein Research Ltd (Bishops Waltham, Hampshire, UK).

Sequencing and analysis of *Asterias rubens* nerve cord transcriptome

Radial nerve cords (~30 mg) dissected from a male adult specimen of *Asterias rubens* were used for RNA isolation (Total RNA Isolation System, Promega). Library preparation (TruSeqv2 kit, Illumina) was performed at the QMUL Genome Centre and sequencing was performed on an Illumina HiSeq platform at NIMR (Mill Hill), with cBot used to generate clusters. Raw sequence data was assembled using SOAPdenovo-Trans version 1.0 (http://soap.genomics.org.cn/SOAPdenovo-Trans.html), a short-read assembly method developed by the Beijing Genomics Institute (Li et al., 2008). Contigs were assembled from reads with an overlap greater than 31 bp, which were then mapped back to the raw reads. The 326,816 contigs generated (with 16,316 over 1000 bp) were then set up for BLAST analysis using SequenceServer (http://www.sequenceserver.com/), which is freely available to academic users (Priyam et al., in prep).

NanoLC-ESI-MS/MS mass spectrometry

Radial nerve cords were dissected from five specimens of *Asterias rubens* using a method described previously (Chaet, 1964) and neuropeptides were extracted in 1 ml 80% acetone on ice (Elphick et al., 1991). After removal of the acetone by evaporation using nitrogen, the aqueous fraction was centrifuged (13,000 rpm in MiniSpin® (Eppendorf) centrifuge) for 10 min and the supernatant frozen at -80°C. The acetone extract was thawed and filtered through a 0.22 µm Costar® Spin-X® centrifuge tube filter to remove particulates. Then the extract was analysed by means of nanoflow liquid chromatography with electrospray ionisation quadrupole time-of-flight tandem mass spectrometry (nanoLC-ESI-MS/MS) using a nanoAcquity UPLC system coupled to

a Q-TOF Ultima Global mass spectrometer (Waters Corporation, Milford, MA) and MassLynx v4.0 service pack 4 software.

The mobile phases used for the chromatographic separation were: 0.1% aqueous formic acid (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). An aliquot containing 5 µl of the nerve extract was applied to a trapping column (Symmetry C18 180 µm x 20 mm, 5 µm particle size, 100 Å pore size, Waters Corporation) using 99.9% mobile phase A at a flow rate of 15 µl/min for 1 min, after which the fluidic flow path included the analytical capillary column (HSS T3 75 µm x 150 mm, 1.8 µm particle size, 100 Å pore size, Waters Corporation) and a linear gradient of 5–40% mobile phase B over 45 min was utilised with a total run time of 60 min.

The nanoflow ESI source conditions were as follows: capillary voltage 3.5 kV, sample cone voltage 25 V with a source temperature of 80^{o}C. A data dependent acquisition was performed that would trigger an MS/MS scan on any singly charged peptide having a *mass/charge* (*m/z*) ratio of 646.2989, or a doubly charged peptide of *m/*z 323.6534. A tolerance of 150 mDa was allowed on the precursor *m/z*. MS/MS spectra, obtained from data dependent acquisition, were processed using MassLynx software. Spectra were combined and processed using the MaxEnt 3 algorithm to generate singly charged, monoisotopic spectra for interpretation and manual validation.

In vitro **pharmacology**

Cardiac stomachs were dissected from specimens of *Asterias rubens* and set up in a 20 ml organ bath as described previously (Elphick et al., 1995; Melarange et al., 1999). Cardiac stomach contraction was recorded using an isotonic transducer (Harvard, Edenbridge, Kent, UK; 0.5 g load) linked to a Goerz SE 120 chart recorder (Recorderlab, Sutton, Surrey, UK). Stock solutions of synthetic neuropeptides tested were prepared in distilled water and added to the organ bath to achieve final concentrations ranging from 30 pM to 1 µM.

In vivo **pharmacology**

Ten specimens of *Asterias rubens*, which had been withheld from a food supply for one week, were placed in a glass tank containing 2% magnesium chloride ($MgCl_2$) dissolved in seawater, which acts as a muscle relaxant in marine invertebrates (Mayer, 1909). This treatment

conveniently and reproducibly causes eversion of the cardiac stomach in *Asterias rubens*, typically within a period of ~ 30 min (M.R. Elphick, unpublished observations). Hamilton® 75N 5 µl syringes (Sigma-Aldrich®) were used to inject test compounds into the perivisceral coelom of animals at two sites in the aboral body wall of the arms proximal to the junctions with the central disk region. Care was taken to inject test agents into the perivisceral coelom and not into the cardiac stomach. Animals were first injected with a total of 10 µl distilled water (control) and video recorded for 4 min. The same animals were then injected with 10 µl of 100 nM peptide (a concentration selected based on results from *in vitro* pharmacology) and video recorded for 4 min. Static images from video recordings were captured at 20 s intervals from the time of injection. Then the 2D area of everted cardiac stomach was measured from the images using Image J software (NIH, USA; http://rsb.info.nih.gov/ij/) and normalised as a percentage of the area of cardiac stomach everted at the time of injection.

RESULTS

Identification of a transcript in *Asterias rubens* encoding a precursor protein with a C-terminal neurophysin domain and two copies of the putative novel NG peptide NGFFYamide

To search for a transcript encoding an NG peptide in the starfish *Asterias rubens*, the *Strongylocentrotus purpuratus* NGFFFamide precursor protein sequence (Elphick and Rowe, 2009) was submitted as a query in a tBLASTn search of *Asterias rubens* radial nerve cord transcriptome sequence data. The top hit was contig 1104160 (1268 bp), which encodes a 239 residue protein comprising a 23-residue N-terminal signal peptide (as predicted by SignalP 3.0; (Bendtsen et al., 2004)), two copies of the amino acid sequence Asn-Gly-Phe-Phe-Tyr-Gly (NGFFYG) flanked by putative dibasic cleavage sites (KR) and a 100-residue C-terminal neurophysin domain (Fig. 1). Thus, subject to conversion of the C-terminal glycine to an amide (Bradbury et al., 1982), this protein is the precursor of two copies of a novel putative NG peptide: NGFFYamide. The sequence of the 1268 bp NGFFYamide precursor transcript has been deposited in the GenBank database and assigned accession number KC977457.

Confirmation that NGFFYamide is present in *Asterias rubens*

Synthetic NGFFYamide peptide was analysed using nanoLC-ESI-MS/MS mass spectrometry and eluted at a retention time of 30.3 min with the singly charged species observed at a mass-to-charge ratio (*m/z*) of 646.3. Analysis of *Asterias rubens* radial nerve cord extract under identical conditions revealed that a single charged peptide with a *m/z* of 646.3 eluted at a similar retention time to synthetic NGFFYamide. Both peptides were subjected to MS/MS during the experiment and the resulting deconvoluted, singly charged, monoisotopic spectra were compared, confirming the presence of NGFFYamide in the radial nerve cord extract (Fig. 2 and Fig. S1).

NGFFYamide is a potent stimulator of cardiac stomach contraction *in vitro*

Analysis of the *in vitro* effect of NGFFYamide on cardiac stomach preparations from *Asterias rubens* revealed that it caused dose-dependent contraction at concentrations ranging from 30 pM to 1 µM, with maximal efficacy at 100 nM (Fig. 3A,B). The sea urchin NG peptide NGFFFamide also caused dose-dependent contraction of cardiac stomach preparations but with lower efficacy

and potency than NGFFYamide (Fig. 3A). Accordingly, comparison of the NGFFFamide and NGFFYamide data using a random intercept linear mixed effects model (Bates and Sarkar, 2007) revealed a significant difference in the effect of NGFFFamide and NGFFYamide on cardiac stomach contraction, irrespective of concentration ($p < 0.001$).

NGFFYamide triggers cardiac stomach retraction *in vivo*

To investigate the effects of NGFFYamide *in vivo*, the peptide was tested on starfish in which cardiac stomach eversion had been induced by immersion in seawater containing 2% $MgCl_2$. Injection of NGFFYamide (10 µl of 100 nM) into the perivisceral coelom of the central disk region triggered retraction of the cardiac stomach (Fig. 4A), consistent with the contracting action of NGFFYamide *in vitro*. NGFFYamide triggered cardiac stomach retraction in all experiments but with variability in the rate and extent of retraction. The graph in figure 4B shows data from ten experiments, with the mean area of cardiac stomach everted at 20 s intervals during a 220 s recording period following peptide injection at T_0 expressed as a percentage of the area everted at T_0. Importantly, in a control experiment in which starfish were injected with water no retraction of the cardiac stomach was observed. Accordingly, comparison of control (water) and treatment (NGFFYamide) data using a random intercept linear mixed effects model (Bates and Sarkar, 2007) revealed a significant difference in cardiac stomach retraction between the control (water) and treatment (NGFFYamide) ($p < 0.001$).

DISCUSSION

Discovery of NGFFYamide, a novel neurophysin-associated NG peptide in starfish

We report here the discovery of NGFFYamide, a neuropeptide in the starfish *Asterias rubens*. NGFFYamide is a novel member of a family of "NG peptides" that have been identified in deuterostomes (Elphick, 2010). The NGFFYamide precursor contains an N-terminal signal peptide, two copies of the sequence NGFFYG in tandem flanked by dibasic cleavage sites (KR) and a C-terminal neurophysin domain (Fig. 1). Comparison of the NGFFYamide precursor with NG peptide precursors in other echinoderms reveals similarity with the sea urchin NGFFFamide precursor (Elphick and Rowe, 2009), which has two copies of the sequence NGFFFG in tandem and a C-terminal neurophysin domain (Fig. 5B). This contrasts with the NGIWYamide precursor in the sea cucumber *Apostichopus japonicus*, which lacks a C-terminal neurophysin domain and contains five copies of the sequence NGIWYG (Elphick, 2012). The similarity of the NGFFYamide precursor and NGFFFamide precursor probably reflects conservation of features of a common ancestral precursor. Furthermore, taking into account that sea urchins and sea cucumbers belong to sister classes within the phylum Echinodermata (Pisani et al., 2012), we conclude that the lack of a neurophysin domain in the *Apostichopus japonicus* NGIWYamide precursor is a derived characteristic. Evidence in support of this conclusion is provided by comparison of the echinoderm NG peptide precursors with NG peptide precursors in other deuterostomian invertebrates. Thus, NG peptide precursors in the hemichordate *Saccoglossus kowalevskii* and the cephalochordate *Branchiostoma floridae* both have a C-terminal neurophysin domain (Fig. 5B and (Elphick, 2010)).

The discovery that the starfish neuropeptide NGFFYamide and other NG peptides are derived from precursors that contain a neurophysin domain provides an insight on the evolutionary origin of these peptides. The only other proteins known to contain a neurophysin domain are precursors of vasopressin/oxytocin-type neuropeptides (De Bree, 2000; De Bree and Burbach, 1998). Therefore, NG peptide precursors and vasopressin/oxytocin-type precursors probably originated by duplication of a gene encoding a common ancestral precursor protein. In support of this hypothesis, genes encoding the vasopressin/oxytocin-type precursor (Brafl-84802) and the NG peptide precursor (Brafl-84803) are located adjacently in the genome of *Branchiostoma floridae* (M.R. Elphick, unpublished observations; (Mirabeau and Joly, 2013;

Putnam et al., 2008)). Because the neurophysin domain is required for biosynthesis of vasopressin/oxytocin-type neuropeptides (De Bree, 2000; De Bree and Burbach, 1998), the conservation of this domain in the NGFFYamide precursor and the majority of other identified NG peptide precursors suggests that neurophysin may be similarly required for biosynthesis of these neuropeptides. However, the absence of a neurophysin domain in the sea cucumber NGIWYamide precursor suggests that the neurophysin domain is dispensable.

Precursor proteins comprising NG peptides with a neurophysin domain have not been discovered in vertebrates. However, the NG peptide precursor in the cephalochordate *Branchiostoma floridae* comprises two copies of a putative neuropeptide (SFRNGVamide) that is identical to the N-terminal region of neuropeptide S (Fig. 5A), an anxiolytic neuropeptide in mammals and other vertebrates (Elphick, 2010; Xu et al., 2004). This suggests a common evolutionary ancestry of neuropeptide S precursors found in vertebrates and NG peptide precursors in deuterostomian invertebrates. Furthermore, the absence of a neurophysin domain in neuropeptide S precursors (Fig. 5B) may be further evidence that neurophysins are dispensable for biosynthesis of NG peptide-type neuropeptides. In conclusion, it remains unclear why the neurophysin domain has been lost in some NG peptide type precursors and retained in others. Discovery of the neurophysin-containing NGFFYamide precursor in starfish provides a new experimental system in which the functional significance of conservation of the neurophysin domain could be investigated.

NGFFYamide: a regulator of cardiac stomach retraction in starfish

Analysis of the *in vitro* pharmacological effects of NGFFYamide revealed that it causes dose-dependent contraction of starfish cardiac stomach preparations at concentrations ranging from 30 pM to 1 µM, with a maximal efficacy at 100 nM. The sea urchin NG peptide NGFFFamide also causes dose-dependent contraction of cardiac stomach preparations but with lower efficacy and potency than NGFFYamide (Fig. 3). Interestingly, the difference in the potency and efficacy of NGFFYamide and NGFFFamide can be attributed to a single hydroxyl group (OH), which is present on the C-terminal tyrosine (Y) residue in NGFFYamide but not on the C-terminal phenylalanine (F) residue in NGFFFamide. Therefore, this OH group is probably important for activation of the as yet unidentified NGFFYamide receptor(s).

Importantly, analysis of the *in vivo* pharmacological effects of NGFFYamide revealed

that it triggers retraction of the everted cardiac stomach in *Asterias rubens* (Fig. 4). Accordingly, endogenous release of NGFFYamide may mediate neural control of cardiac stomach retraction in starfish. This is of interest because it provides a new insight on physiological mechanisms underlying the unusual feeding behaviour of starfish. Thus, cardiac stomach eversion and retraction that occurs during feeding in starfish appears to be controlled by counteracting neuropeptide systems, with SALMFamide neuropeptides triggering stomach eversion (Melarange et al., 1999) and NGFFYamide triggering stomach retraction. Previous studies have revealed that the SALMFamides S1 and S2 are synthesized by neurons intrinsic to the cardiac stomach (Newman et al., 1995a; Newman et al., 1995b) and therefore it will be of interest to determine if NGFFYamide-expressing neurons are similarly located in the cardiac stomach. Additionally, identification of receptors that mediate the effects of NGFFYamide and SALMFamides would facilitate investigation of the mechanisms by which these peptides exert their counteracting effects on the cardiac stomach in starfish.

It is noteworthy that NGGFYamide is much more potent than the SALMFamides S1 and S2, both *in vitro* and *in vivo*. Thus, the maximal contracting effect of NGFFYamide *in vitro* was observed at 100 nM (this study), whilst at this concentration the relaxing effect of S1 or S2 was, respectively, only ~25% and ~50% of the effect at the highest concentration tested (10 µM) (Melarange et al., 1999). Accordingly, 100 µl of 1 mM S1 or S2 induced stomach eversion *in vivo* within a period of up to 30 min (Melarange et al., 1999), whilst stomach retraction within a period of up to 4 min was triggered by only 10 µl of 100 nM NGFYYamide (this study). However, these apparent differences in potency may not be physiologically relevant. Recently, it was discovered that in the starfish *Patiria miniata* S1 and an S2-like peptide are derived from precursor proteins that comprise fourteen other putative SALMFamides (Elphick et al., 2013). Likewise, we have identified neural transcripts encoding the S1 and S2 precursors in *Asterias rubens* and have found that the S1 precursor contains six other putative SALMFamides and the S2 precursor contains seven other putative SALMFamides (D.C. Semmens, M.R. Pancholi and M.R. Elphick, unpublished data). Therefore, for a physiologically relevant comparison to be made it will be necessary to compare the effect of NGFFYamide with the effects of “cocktails” of S1 precursor-derived SALMFamides and/or S2 precursor-derived SALMFamides.

Discovery of neuropeptides that trigger cardiac stomach eversion or retraction in starfish is of interest from economic and environmental perspectives. The feeding behaviour of starfish

species such as *Asterias rubens* has an economic impact due to predation on shellfish that are harvested as foodstuffs (Aguera et al., 2012; Dare, 1982; Dolmer, 1998; Magnesen and Redmond, 2012). Furthermore, other starfish species such as the crown-of-thorns starfish *Acanthaster planci* feed on reef-building corals and periodic increases in the population density of this species causes massive destruction of Pacific reef tracts (De'ath et al., 2012; Kayal et al., 2012; Timmers et al., 2012). Identification of neuropeptides that trigger cardiac stomach eversion (SALMFamides) or retraction (NGFFYamide) may provide a basis for development of non-peptidic small molecule agonists or antagonists that mimic or block the effects of SALMFamides or NGFFYamide, which could be used for chemical control of starfish feeding.

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AUTHOR CONTRIBUTIONS

Discovery of NGFFYamide precursor transcript (DCS, MRP, MRE); Mass spectrometry (SES, JHS, DCS, MRE); *In vitro* and *in vivo* pharmacology (RED, DCS, MRE). All authors contributed to writing or editing of the manuscript.

COMPETING INTERESTS

No competing interests declared.

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FIGURE LEGENDS

Fig. 1. *Asterias rubens* NGFFYamide precursor. The DNA sequence of a transcript (contig 1104160; lowercase, 1268 bases) encoding the NGFFYamide precursor protein (uppercase, 239 amino acid residues) is shown. The predicted signal peptide of the precursor protein is shown in blue, the two copies of NGFFYamide are highlighted in red, interrupted and flanked by putative dibasic cleavage sites (KR), which are shown in green. The C-terminal region of the protein comprises a neurophysin domain, with 14 cysteine residues (underlined) that are a characteristic and conserved feature of neurophysins (purple). The asterisk shows the position of the stop codon.

Fig. 2. Mass spectrometric confirmation that NGFFYamide is present in an acetone extract of radial nerve cords from *Asterias rubens*. The deconvoluted monoisotopic, singly charged spectrum derived from MS/MS data is shown, with the b series of fragment ions annotated (b2, b3, b4). Also labeled are two fragment ions from the y series (y1, y2), immonium ions from phenylalanine (F) and tyrosine (Y) and the precursor ion (NGFFFamide; 646.31). A complementary spectrum derived from MS/MS analysis of synthetic NGFFYamide peptide is shown in supplementary figure S1.

Fig. 3. NGFFYamide is a potent stimulator of starfish cardiac stomach contraction *in vitro.* (A) Representative recordings from a single cardiac stomach preparation showing the dose-dependent effect of NGFFYamide. NGFFYamide causes cardiac stomach contraction when applied (upward pointing arrowheads), an effect that is reversed by washing (downward pointing arrowheads). (B) Dose-response curves comparing the effects of NGFFYamide (filled circles) and NGFFFamide (filled squares) in causing cardiac stomach contraction. Effects of both peptides are normalized to the maximal effect observed with NGFFYamide in each experiment, with mean values (± s.e.m.) from eight experiments shown.

Fig. 4. NGFFYamide triggers cardiac stomach retraction in starfish (A) Photographs from an experiment showing that injection of NGFFYamide (10 µl 100 nM) causes retraction of the cardiac stomach. At time 0 the fully everted cardiac stomach and the needles of the syringes used

for peptide injection can be seen. At 60 s, 120 s and 180 s after injection of NGFFYamide the area of cardiac stomach everted (marked by white dots) is progressively reduced. (B) Graph comparing experiments where starfish were first injected with vehicle (filled circles; 10 µl distilled water) and then injected with NGFFYamide (filled squares; 10 µl of 100 nM NGFFYamide). The area of cardiac stomach everted (in 2D) at each time point (0 – 220 s) is normalized to the area of cardiac stomach everted at T_0, with means (± s.e.m.) from ten experiments shown.

Fig. 5. NG peptides and NG peptide precursors A. Comparison of the sequence of NGFFYamide with the sequences of related "NG peptides" that share a common NG motif (highlighted in yellow), with arrangement in accordance with animal phylogeny. B. Comparison of the NGFFYamide precursor with NG peptide precursors in other deuterostomian invertebrates and the human neuropeptide S precursor, with arrangement in accordance with animal phylogeny. N-terminal signal peptides are shown in blue, NG peptides are shown in red, cleavage sites are shown in green and C-terminal neurophysin domains are shown in purple. The NGFFYamide precursor in the starfish *Asterias rubens* (Ar) has a similar structure to the NGFFFamide precursor in the sea urchin *Strongylocentrotus purpuratus* (Sp) with two NG peptides in tandem and a C-terminal neurophysin domain; this probably reflects conservation of the features of a common ancestral precursor. In contrast, the NGIWYamide precursor in the sea cucumber *Apostichopus japonicus* (Aj) has what appears to be a derived precursor structure comprising five copies of NGIWYamide without a C-terminal neurophysin domain. The NG peptide precursor in the hemichordate *Saccoglossus kowalevskii* (Sk), which contains five copies of NGFWNamide and one copy of NGFYNamide, and the SFRNGVamide precursor cephalochordate *Branchiostoma floridae* (Bf) both have a C-terminal neurophysin domain, indicating that this is an ancestral characteristic of NG peptide precursors in deuterostomes, but the number and positions of NG peptide copies is variable. Vertebrate (e.g. human) precursors of neuropeptide S, which shares 100% N-terminal sequence identity with the *Branchiostoma* NG peptide SFRNGVamide, do not have a C-terminal neurophysin domain, indicating loss of this character in the vertebrate lineage.

```
1     ctacacgcagtgattgcacggtaatgcagcgtgacgtagccacgaggaggcgtaactttc
61    tcgttgcgaacagactactagcgcaccggggctgtgcgattattgtttccaacacgaggt
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181   tcgagaagagcttgagttactttacctggcgctcaggtgggaattcattttctatcagca
241   agaacactccttagtttacaatcaattacaagtggaatatcgctcatttggaaacatcaa
301   caagattttgacgaactaggaggggtgtcggtgggacgtgggggatctaagctggatatg
                                                              M     1
361   accatgggcagcaggtcgttattagtgacaattgtgatcacagtagtcatacccagcatc
       T   M   G   S   R   S   L   L   V   T   I   V   I   T   V   V   I   P   S   I    21
421   tgggcaggtgcaatagctggggctcaaacacaaaagattcgtcgtgaaagtcgagaatct
       W   A   G   A   I   A   G   A   Q   T   Q   K   I   R   R   E   S   R   E   S    41
481   ggcaagtactggccaaactccgtgggtatctcagaccaacagctacggcaactcctagca
       G   K   Y   W   P   N   S   V   G   I   S   D   Q   Q   L   R   Q   L   L   A    61
541   cactctctggcggactcgtacagtacgtcaggggcaagtcacatacggggaggagacggg
       H   S   L   A   D   S   Y   S   T   S   G   A   S   H   I   R   G   G   D   G    81
601   gatgcagggtatatatacgatagtcgagatcaggtcgatgacacggggacgaacgaggag
       D   A   G   Y   I   Y   D   S   R   D   Q   V   D   D   T   G   T   N   E   E   101
661   gaaggggaacgcgtaatcgggagcgaggttacatcgagagactcgaaccccggtacaagc
       E   G   E   R   V   I   G   S   E   V   T   S   R   D   S   N   P   G   T   S   121
721   aagagaaatgggttcttctatggcaaaagaaatgggttctttatggaaagagatcagcg
       K   R   N   G   F   F   Y   G   K   R   N   G   F   F   Y   G   K   R   S   A   141
781   tcaacccctggcaatgcaaatgaagtaactcaatgcatcccgtgtgggcctcaaaacaac
       S   T   P   G   N   A   N   E   V   T   Q   C   I   P   C   G   P   Q   N   N   161
841   ggccagtgcgtcatgtttggtacatgttgcagctatgaactaggtggctgctttttcctg
       G   Q   C   V   M   F   G   T   C   C   S   Y   E   L   G   G   C   F   F   L   181
901   acagaggaggcccttccctgtgtgacgtcaaaatcgtcatcattatgtgagctgagcgga
       T   E   E   A   L   P   C   V   T   S   K   S   S   S   L   C   E   L   S   G   201
961   ttgccgtgcggtgacgagggatatggaaggtgcgtggcagactctgtctgttgtctgccg
       L   P   C   G   D   E   G   Y   G   R   C   V   A   D   S   V   C   C   L   P   221
1021  caagagggctcttgtcatattaacgcagaatgtggaggcaagatgacatttcaataggac
       Q   E   G   S   C   H   I   N   A   E   C   G   G   K   M   T   F   Q   *       239
1081  ttgcattatgcggactttaaattatttataaagggataggaaaaggtggttaatatctgt
1141  attttgaaaagggtaataaaatttaaggttgtttgagaaaagggacacgaatgttatttt
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1261  taactgtt
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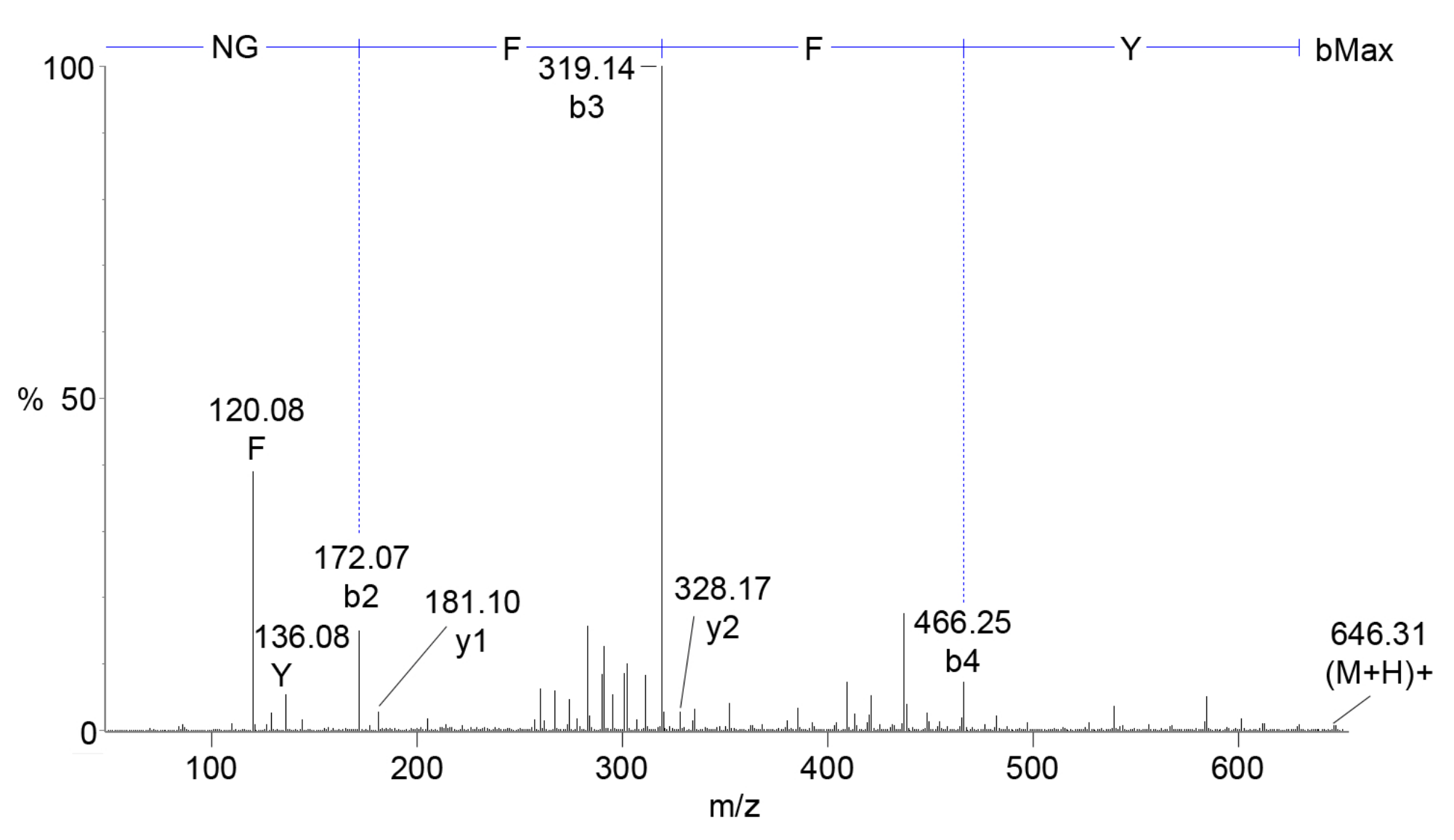
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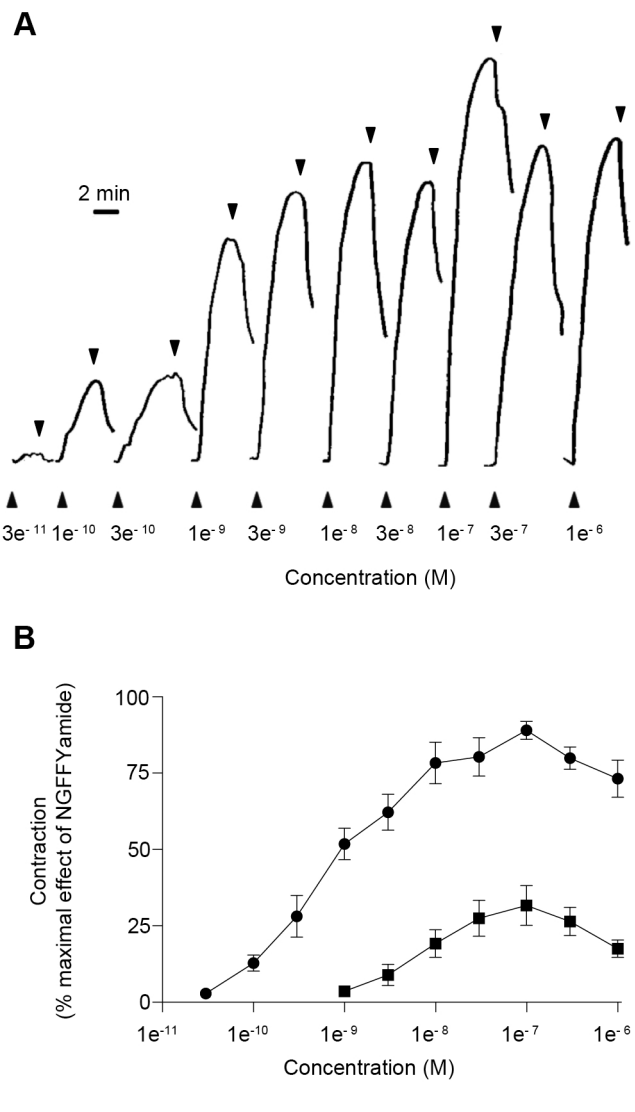
m/z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1e-6
Concentration (M)
B
Contraction
(% maximal effect of NGFFYamide)
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50
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0
1e-11
1e-10
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1e-8
1e-7
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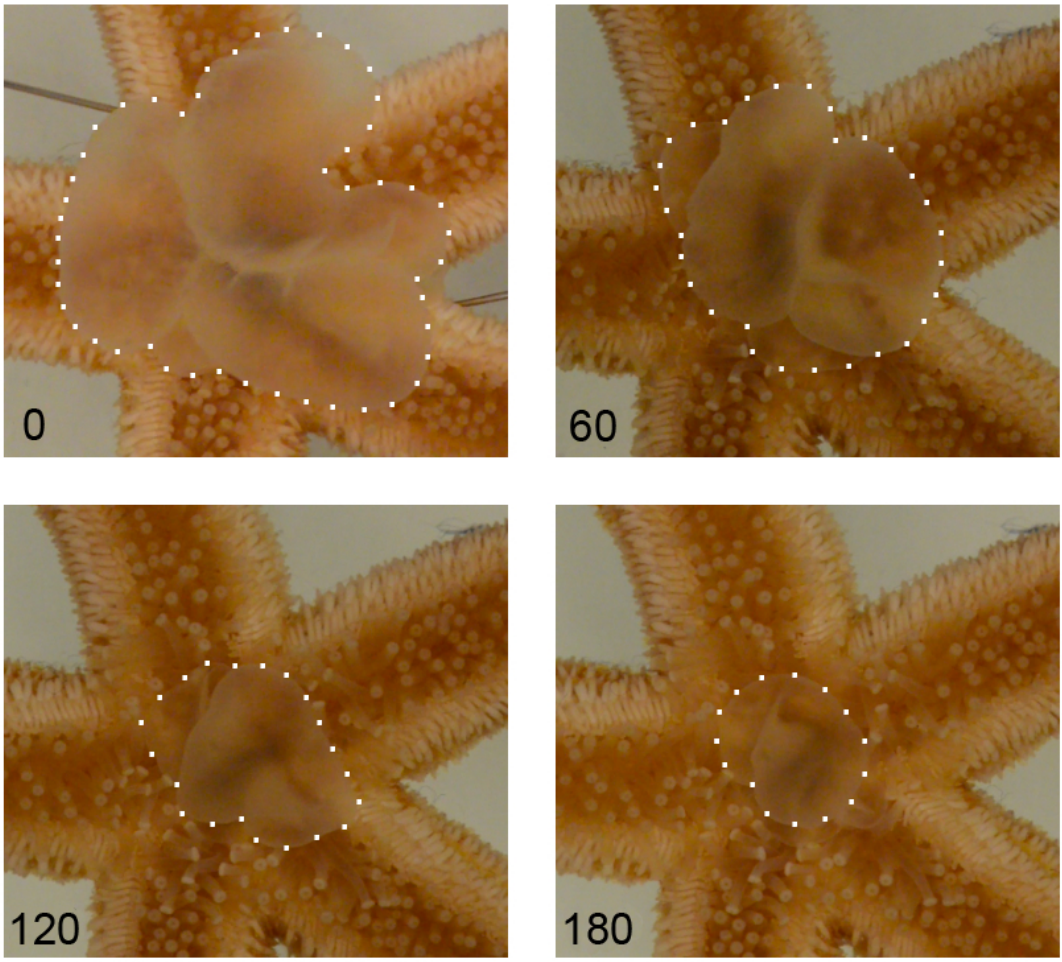
Concentration (M)

A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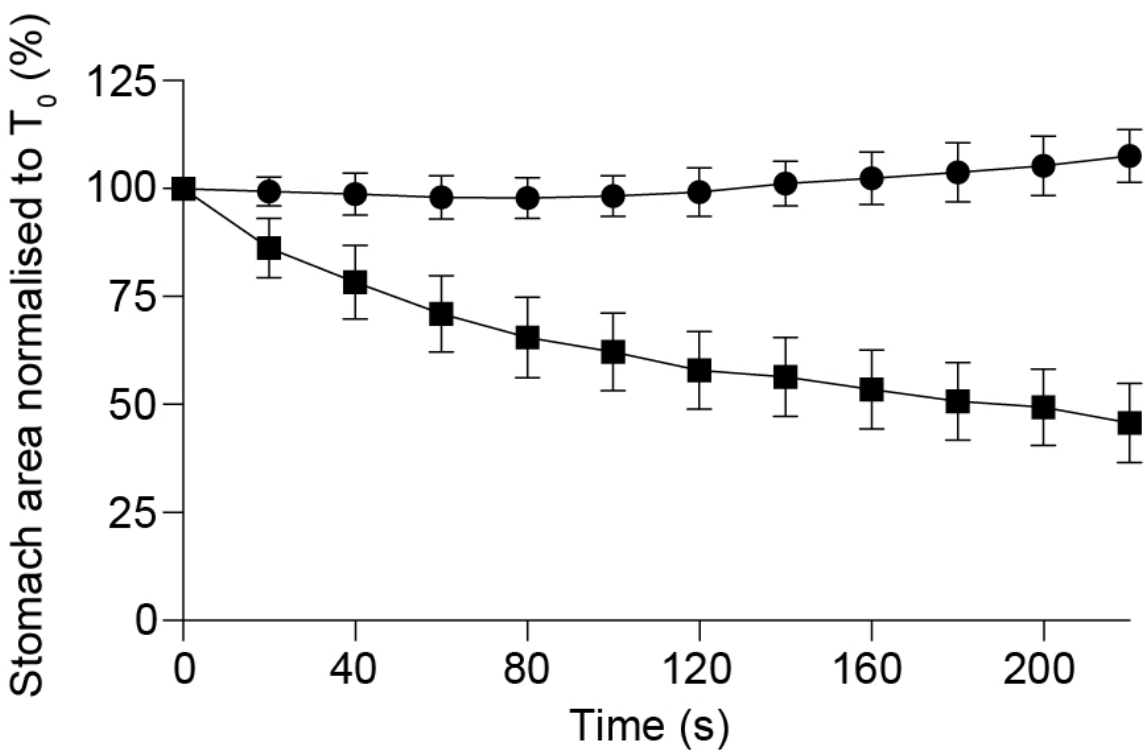
B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0
0
40
80
120
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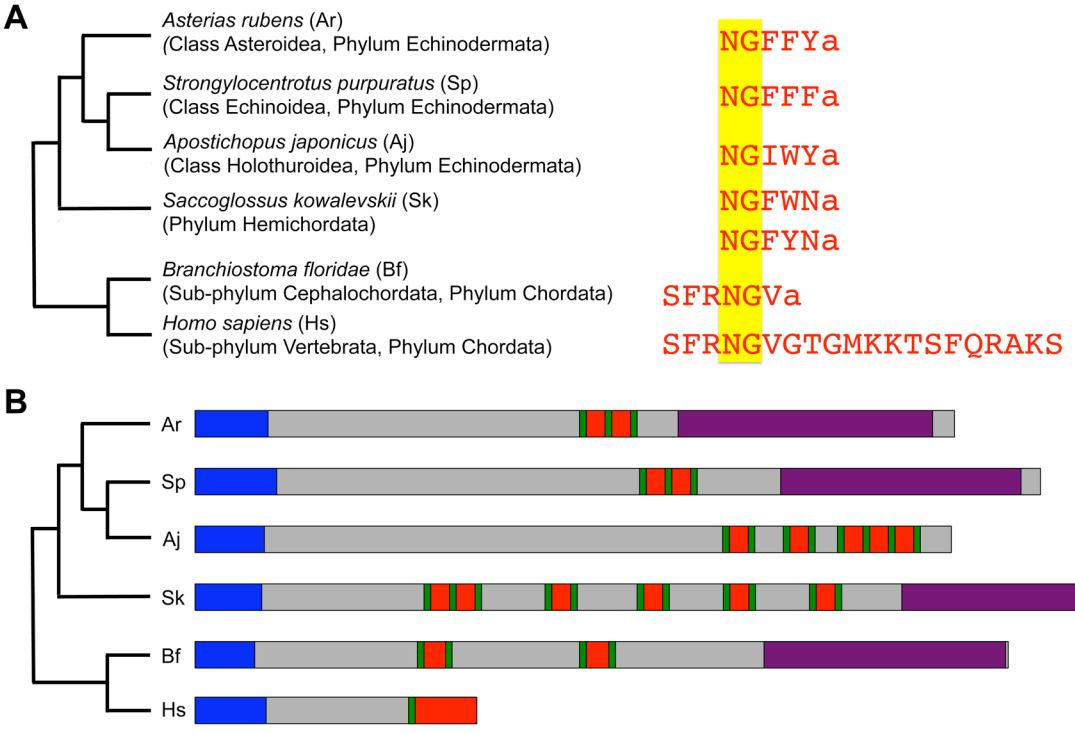
200
Time (s)


A
Asterias rubens (Ar)
(Class Asteroidea, Phylum Echinodermata)
Strongylocentrotus purpuratus (Sp)
(Class Echinoidea, Phylum Echinodermata)
Apostichopus japonicus (Aj)
(Class Holothuroidea, Phylum Echinodermata)
Saccoglossus kowalevskii (Sk)
(Phylum Hemichordata)
Branchiostoma floridae (Bf)
(Sub-phylum Cephalochordata, Phylum Chordata)
Homo sapiens (Hs)
(Sub-phylum Vertebrata, Phylum Chordata)
NGFFYa
NGFFFa
NGIWYa
NGFWNa
NGFYNa
SFRNGVa
SFRNGVGTGMKKTSFQRAKS
B
Ar
Sp
Aj
Sk
Bf
Hs