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 HYPOTHESIZED, 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 A. rubens REVEALED A PRECURSOR PROTEIN COMPRISING TWO COPIES OF A NOVEL NG PEPTIDE, NGFFYamide, WHICH WAS CONFIRMED BY MASS SPECTROMETRY. A NOTABLE 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 INTO 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).
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 NGFWNamidase 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 – is derived from a 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. Therefore, we tested the effects of the sea urchin neuropeptide NGFFFamide on in vitro cardiac stomach preparations from the starfish A. rubens and found that it causes contraction (R. Melarange and M.R.E., unpublished data). Thus, the aim of this study was to determine the molecular identity of the NG peptide(s) in the starfish A. rubens and to investigate a potential physiological role in regulation of cardiac stomach retraction.

MATERIALS AND METHODS
Animals and chemicals
Starfish (Asterias rubens Linnaeus 1758) were collected at low tide from the Thanet coast (Kent, UK) and transported to Queen Mary University of London (QMUL), where they were maintained in a seawater aquarium at ~11°C and fed with mussels (Mytilus edulis). Synthetic neuropeptides were custom synthesized by Peptide Protein Research (Bishops Waltham, Hampshire, UK).

Sequencing and analysis of A. rubens nerve cord transcriptome
Radial nerve cords (~30mg) dissected from a male adult specimen of A. rubens were used for RNA isolation (Total RNA Isolation System, Promega, Southampton, UK). Library preparation (TruSeqv2 kit, Illumina, Little Chesterford, Essex, UK) was performed at the QMUL Genome Centre and sequencing was performed on an Illumina HiSeq platform at the National Institute for Medical Research (Mill Hill, London, UK), with cBot used to generate clusters. Raw sequence data was assembled using SOAPdenovo-Trans v1.0 (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 1000bp) were then set up for BLAST analysis using SequenceServer (http://www.sequenceserver.com/), which is freely available to academic users (A. Priyam, B. J. Woodcroft and Y. Wurm, in preparation).

NanoLC-ESI-MS/MS
Radial nerve cords were dissected from five specimens of A. 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 (11,300 g in a MiniSpin centrifuge, Eppendorf, Stevenage, UK) 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, USA) and MassLynx v4.0 service pack 4 software (Waters Corporation, Milford, MA, USA).

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×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⁻¹ for 1 min, after which the fluidic flow path included the analytical capillary column (HSS T3 75 μm×150 mm, 1.8 μm particle size, 100 Å pore size, Waters Corporation). A linear gradient of 5–40% mobile phase B over 45 min was utilized 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°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 A. rubens and set up in a 20 ml organ bath as described previously (Elphick et al., 1991; Melarange et al., 1999). Cardiac stomach contraction was recorded using an isotonics 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 pmol l⁻¹ to 1 μmol l⁻¹.

In vivo pharmacology
Ten specimens of A. rubens, which had been withheld from a food supply for 1 week, were placed in a glass tank containing 2% magnesium chloride (MgCl₂) 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 A. rubens, typically within a period of ~30 min (M.R.E., unpublished observations). Hamilton 75N 5 μl syringes (Sigma-Aldrich, St Louis, MO, USA) 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 10 μl distilled water (control) and video recorded for 4 min. The same animals were then injected with 10 μl of 100 nmol l⁻¹ 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
NGFFYamide: novel starfish neuropeptide

RESULTS

Identification of a transcript in A. 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 A. rubens, the S. purpuratus NGFFYamide precursor transcript has been deposited in the GenBank database and assigned accession number KC977457. The sequence of the 1268 bases) encoding the NGFFYamide precursor protein with a C-terminal neurophysin domain and two copies of a novel NG peptide NGFFFamide is shown in blue, interrupted and flanked by putative dibasic cleavage sites (KR), and a putative signal peptide [as predicted by SignalP 3.0 (Bendtsen et al., 2004)]. This protein is the precursor of two copies of the amino acid sequence Asn-Gly-Phe-Phe-Tyr-Gly (NGFFYG) flanked by putative dibasic cleavage sites (KR) and a C-terminal domain comprising 239 amino acids (Fig. 1). Thus, subjecting the NGFFYamide precursor protein with a C-terminal neurophysin domain and two copies of NGFFFamide 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 NG peptide NGFFYamide. The sequence of the 1268 bases) encoding the NGFFYamide precursor protein with a C-terminal neurophysin domain and two copies of NGFFFamide is shown in blue, interrupted and flanked by putative dibasic cleavage sites (KR), and a putative signal peptide [as predicted by SignalP 3.0 (Bendtsen et al., 2004)]. This protein is the precursor of two copies of the amino acid sequence Asn-Gly-Phe-Phe-Tyr-Gly (NGFFYG) flanked by putative dibasic cleavage sites (KR) and a C-terminal domain comprising 239 amino acids (Fig. 1). Thus, subjecting the NGFFYamide precursor protein with a C-terminal neurophysin domain and two copies of NGFFFamide 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 NG peptide NGFFYamide. The sequence of the 1268 bases) encoding the NGFFYamide precursor protein with a C-terminal neurophysin domain and two copies of NGFFFamide is shown in blue, interrupted and flanked by putative dibasic cleavage sites (KR), and a putative signal peptide [as predicted by SignalP 3.0 (Bendtsen et al., 2004)]. This protein is the precursor of two copies of the amino acid sequence Asn-Gly-Phe-Phe-Tyr-Gly (NGFFYG) flanked by putative dibasic cleavage sites (KR) and a C-terminal domain comprising 239 amino acids (Fig. 1). Thus, subjecting the NGFFYamide precursor protein with a C-terminal neurophysin domain and two copies of NGFFFamide 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 NG peptide NGFFYamide.

NGFFYamide is a potent stimulator of cardiac stomach contraction in vitro

Analysis of the in vitro effect of NGFFYamide on cardiac stomach preparations from A. rubens revealed that it caused dose-dependent contraction at concentrations ranging from 30 pmol l−1 to 1 nmol l−1, with maximal efficacy at 100 nmol l−1 (Fig. 3). 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 NGFFYamide and NGFFFamide data using a random intercept linear mixed effects model (Bates and Sarkar, 2007) revealed a significant difference in the effect of NGFFYamide and NGFFFamide 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% MgCl₂. Injection of NGFFYamide (10 μl of 100 nmol l−1) 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. Fig. 4B shows data from 10 experiments, with the mean area of cardiac stomach everted at 20 s intervals during a 220 s recording period following peptide injection at time 0 (T₀) expressed as a percentage of the area everted at T₀. Importantly, in a control experiment in which starfish were injected with water, no retraction was observed.

The results show that NGFFYamide is a novel neuropeptide in starfish, with the potential to trigger retraction of the cardiac stomach in vivo. The peptide is encoded by a novel transcript in A. rubens, and its expression in the radial nerve cord suggests a role in neuronal function.
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 *A. 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 *A. 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 sea cucumber NGIWYamide precursor probably reflects conservation of features of 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 adjacent in the genome of *B. florideae* (Mirabeau and Joly, 2013; Putnam et al., 2008; M.R.E., unpublished observations). 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 *B. florideae* 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 contractions of starfish cardiac stomach preparations at concentrations ranging from 30 pmol l$^{-1}$ to 1 μmol l$^{-1}$, with a maximal efficacy at 100 pmol l$^{-1}$. 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 NGFFFamide but not on the C-
terminal phenylalanine (F) residue in NGFFYamide. 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 A. 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 into physiological mechanisms underlying the unusual feeding behaviour of starfish. Thus, cardiac stomach eversion and retraction that occur during feeding in starfish appear 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 whether 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 NGFFYamide 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 nmol l\(^{-1}\) (present study), whilst at this concentration the relaxing effect of S1 or S2 was, respectively, only ~25% or ~50% of the effect at the highest concentration tested (10 \(\mu\)mol l\(^{-1}\)) (Melarange et al., 1999). Accordingly, 100 \(\mu\)l of 1 nmol l\(^{-1}\) 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 \(\mu\)l of 100 nmol l\(^{-1}\) NGFFYamide (present study). However, these apparent differences in potency may not be physiologically relevant. Recently, it was discovered that in the starfish *Asterias rubens* (*Ar*) has a structure similar to that of the NGFFYamide 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 in the 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 human neuropeptide S precursor, with arrangement in accordance with animal phylogeny.

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**AUTHOR CONTRIBUTIONS**

D.C.S., M.R.P. and M.R.E. discovered the NGFFYamide precursor transcript; S.E.S., J.H.S., D.C.S. and M.R.E. performed the mass spectrometry; and R.E.D., D.C.S. and M.R.E. performed the in vitro and in vivo pharmacology. All authors contributed to writing or editing of the manuscript.

**COMPETING INTERESTS**

No competing interests declared.

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Figure S1. A. Theoretical fragment ions produced in an MS/MS experiment from NGFFYamide peptide resulting from collisionally induced decomposition, producing predominantly b, immonium (i) and y ion series. Fragment ions in black were observed from both the synthetic NGFFYamide peptide (figure S1b below) and from NGFFYamide peptide present in the Asterias rubens radial nerve cord (figure 2). Fragment ions in red were not identified from the synthetic peptide or from the extract. B. Deconvoluted monoisotopic, singly charged spectrum derived from MS/MS analysis of synthetic NGFFYamide peptide, 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 NGFFYamide peptide present in Asterias rubens radial nerve cord extract is shown in figure 2.