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Localization of the SALMFamide neuropeptides in the starfish *Marthasterias glacialis*

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In echinoderms, the SALMFamide neuropeptides sharing the SxL/FxFamide motif seem widespread throughout the phylum and may be important signalling molecules that mediate various physiological functions. Recent identification of S1 and its analogues, MagS3 and MagS4, along with the S2 analogue, MagS2 from the starfish *Marthasterias glacialis*, indicated that SALMFamides in the class Asteroidea are more diverse than previously thought. Further, isolation of the neuropeptides from the radial nerve cord and studies on pharmacological actions of the neuropeptides on the cardiac stomach warrant studies on the tissue distributions of these peptides in both the nervous and digestive systems. In the present study, antisera raised against an S1 analogue, KYSALMFamide, and an S2 analogue, KYSGLTFamide, were used to localize the distribution patterns of the S1- and S2-like immunoreactivities (S1-IR/S2-IR) in the nervous and digestive systems of the starfish. In the nervous system, cell bodies in the ectoneural part were immunostained for both S1 and S2 peptides, while in the digestive system, the basi-epithelial plexus and mucosal cell bodies were immunoreactive. These immunocytochemical data support the notion that the SALMFamides may play a neuroendocrine role in mediating feeding behaviour of the starfish. Further studies including identification of peptide binding sites and differential expression pattern of mRNAs encoding the peptides are required to elucidate their physiological functions.

Keywords: SALMFamides; echinoderms; radial nerve cord; cardiac stomach

Introduction

It is well established that neuropeptides are involved in a wide range of physiological functions such as cellular metabolism, growth, reproduction, and the regulation of muscle activity (Scharrer 1987; Thorndyke 1989). In invertebrates, numerous neuropeptide families have been studied with regard to their molecular structures, biological functions, and biosynthetic pathways (Thorndyke and Goldsworthy 1988). However, less attention was paid to the echinoderms for the physiological roles of neuropeptides until the new family of neuropeptides from the starfish, *Asterias rubens*, was identified (Elphick et al. 1991a). Since the first identification of SALMFamides, the octapeptide GFNSALMFamide (S1) and the dodecapeptide SGPYSFNSGLTFamide (S2), 16 neuropeptides have been either biochemically isolated or deduced using the genomic database from the three echinoderm Classes Asteroidea, Holothuroidea and Echinoidea, as summarized in Figure 1 (Elphick et al. 1991a, 1991b; Diaz-Miranda et al. 1992; Ohtani et al. 1999; Elphick and Thorndyke 2005; Yun et al. 2007). The majority of SALMFamides that have been identified share the C-terminal motif SxL/FxFamide, with a few exceptions.

The identified molecular structures of SALMFamides in echinoderms indicated that even closely related species possess species-specific neuropeptides with some of the motif shared. For example, *M. glacialis* contained SALMFamide 1 (S1) identical to that from *A. rubens* and other structurally related peptides, MagS3 and MagS4, while they possess an S2 analogue with two residues replaced (Yun et al. 2007). Following the biochemical identification of four SALMFamides from *M. glacialis*, their pharmacological actions were investigated using synthetic neuropeptides, revealing that MagS2 and MagS3 could elicit relaxation of KCl-contracted cardiac stomach of the starfish (Yun et al. 2007). In the earlier studies, it was shown that both S1 and S2 are able to cause relaxation of starfish muscle preparations of the cardiac stomach, tube foot, and apical muscle (Melarange et al. 1999). Therefore, it was speculated that SALMFamide neuropeptides may have a general function as inhibitory neurotranmitters in the nervous and neuromuscular systems of starfish (Yun et al. 2007).

In echinoderms, immunocytochemical investigations were made possible based on the peptide structure first identified from *A. rubens* (Elphick et al. 1991a). The immunocytochemical studies revealed that SALMFamide-like immunoreactivity is widely distributed in the larval and adult nervous systems of the starfish *A. rubens* (Moore and Thorndyke 1993; Moss et al. 1994; Elphick et al. 1995; Newman et al. 1995a, 1995b), yielding new insights into the molecular neuroarchitecture of the starfish nervous system.

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However, no immunocytochemical investigation on the tissue distribution of SALMFamide-related peptides in *M. glacialis* has been reported. In the present study, using antisera against S1 (KYSALMFamide) and S2 (KYSGLTFamide) analogues, we report immunocytochemical localization of SALMFamides on the nervous and digestive systems of *M. glacialis*.

Material and methods

Fixation and embedding

Specimens of *M. glacialis* were collected at Dunstaffnage Marine Lab., Oban. For the radial nerve cord preparations, the arms of the specimen were cut into pieces of 0.5–1 cm and fixed in Bouin's fluid (Sigma, Dorset, UK) or 4% paraformaldehyde (BDH, Leicestershire, UK) in filtered seawater. Fixation was carried out for 4–6 hours at 4°C. Tissues dissected after fixation were dehydrated through an alcohol series and cleared in xylene (BDH) for 15 minutes. The tissue was then placed in molten paraffin wax with a melting point of 58°C and was vacuum embedded for 30 minutes. The wax was replaced (30 minutes each) 2–3 times to ensure that all xylene was removed. The tissue was then transferred and embedded in fresh molten wax. For stomach preparations, dissected cardiac stomach was fixed in the same fixatives and processed as above.

Immunocytochemistry

The resulting tissue blocks were mounted on wooden blocks and 8 μm sections cut. The sections were mounted on clean poly-L-lysine (Sigma) coated glass slides (BDH) and dried on a hot plate at 37°C. The slides were then dried in an oven at 37°C for 6–12 hours then de-waxed and rehydrated to phosphate-buffered saline (PBS). Sections were incubated for 1 hour in blocking solution containing 5% normal goat serum in PBS to block non-specific binding. After washing, the sections were incubated overnight with primary antisera, BL VI (1:200) for S1 and NEV VII (1:200) for S2. BL VI and NEV VII antisera were raised at the Thorndyke laboratory, and characterization of them was described elsewhere (Potton 1997). After three washes in PBS, the sections were incubated for 1 hour in biotinylated goat anti-rabbit antiserum (Vector Labs, Peterborough, UK) 1:200 in PBS. The controls for immunocytochemistry (ICC) throughout these studies were by omission of the primary antisera and preabsorption of the primary antisera with S1 and S2 analogues. BL VI and NEV VII antisera were incubated overnight at 4°C with 10 nmol/ml of the peptides, KYSALMFamide and KYSGLTFamide, respectively, and then treated as primary antibodies for control tests.

Visualization

For permanent preparations, the sections were processed using the ABC reagent (Vector labs) according to the manufacturer's instructions. Immunolabelling was visualized with 0.04% diaminobenzidine (DAB, Vector Labs) and 0.01% hydrogen peroxide in Tris-HCl at pH 7.6. After incubation for 3–10 minutes, the reaction was stopped by washing in water. The developed sections were dehydrated and mounted using DPX (BDH). For fluorescent preparations, sections were incubated with Fluorescein Avidin D (Vector Labs) for 1 hour (1:100 in PBS). Finally, the sections were washed, mounted in Vectashield (Vector Labs) and sealed using nail varnish.

Peptide	Sequence	Source	Ref.
S1	Gly-Phe-Asn-**Ser**-Ala-**Leu**-Met-**Phe**-NH_2	*M. glacialis, A. rubens*	1, 2
S2	Ser-Gly-Pro-Tyr-Ser-Phe-Asn-**Ser**-Gly-**Leu**-Thr-**Phe**-NH_2	*A. rubens*	2
MagS2	Ser-Gly-Pro-Tyr-Ser-Met-Thr-**Ser**-Gly-**Leu**-Thr-**Phe**-NH_2	*M. glacialis*	3
MagS3	Ala-Tyr-His-**Ser**-Ala-**Leu**-Pro-**Phe**-NH_2	*M. glacialis*	3
MagS4	Ala-Tyr-Gln-*Thr*-Gly-*Leu*-Met-*Phe*-NH_2	*M. glacialis*	3
GFSKLYFa	Gly-Phe-**Ser**-Lys-**Leu**-Tyr-**Phe**-NH_2	*H. glaberrima*	4
SGYSVLYFa	Ser-Gly-Tyr-**Ser**-Val-**Leu**-Tyr-**Phe**-NH_2	*H. glaberrima*	4
GYSPFMFa	Gly-Tyr-**Ser**-Pro-**Phe**-Met-**Phe**-NH_2	*S. japonicus*	5
FKSPFMFa	Phe-Lys-**Ser**-Pro-**Phe**-Met-**Phe**-NH_2	*S. japonicus*	5
SpurS1	Pro-Pro-Val-Thr-Thr-Arg-**Ser**-Lys-**Phe**-Thr-**Phe**-NH_2	*S. purpuratus*	6
SpurS2	Asp-Ala-Tyr-**Ser**-Ala-**Phe**-Ser-**Phe**-NH_2	*S. purpuratus*	6
SpurS3	Gla-Met-**Ser**-Ala-**Phe**-Ser-**Phe**-NH_2	*S. purpuratus*	6
SpurS4	Ala-Gln-*Pro*-Ser-*Phe*-Ala-*Phe*-NH_2	*S. purpuratus*	6
SpurS5	Gly-Leu-Met-*Pro*-Ser-*Phe*-Ala-*Phe*-NH_2	*S. purpuratus*	6
SpurS6	Pro-His-Gly-Gly-**Ser**-Ala-**Phe**-Val-**Phe**-NH_2	*S. purpuratus*	6
SpurS7	Gly-Asp-*Leu*-Ala-*Phe*-Ala-*Phe*-NH_2	*S. purpuratus*	6

Figure 1. Comparative alignment of sequences of the SALMFamide neuropeptides from ehcinoderms. The majority of the SALMFamides share the C-terminal motif, SxL/FxFamide (bold and underlined; where *x* is variable). Note that there are some SALMFamide peptides whose Ser residue is replaced by a threonine, proline or leucine residue (italicized). References: 1. Elphick et al. 1991b; 2. Elphick et al. 1991a; 3. Yun et al. 2007; 4. Diaz-Miranda et al. 1992; 5. Ohtani et al. 1999; 6. Elphick and Thorndyke 2005.

The slides were viewed using a Zeiss Axioplan microscope (Carl Zeiss, Göttingen, Germany) with fluorescent attachments.

Results

Localization of S1- and S2- like immunoreactivity in the nervous system

Both S1- and S2-like immunoreactivities (S1- and S2-IRs) were localized to the ectoneural nerve plexus of the RNC. Most staining was concentrated in epithelial cell bodies (Figure 2), while no SALMFa-IR was detected in the neuropile of the ectoneural part. No immunostaining was found in the hyponeural nerve plexus. A dramatic attenuation of S1- and S2-IRs was observed with the preabsorption of the primary antisera with the peptides KYSALMFamide and KYSGLTFamide at a concentration of 10 nmol/ml (data not shown). Five starfish were dissected and several portions of the RNC were fixed and processed. Over 20 slides for each animal were visualized to find similar distribution patterns with S1- and S2-IRs in the RNC.

Localization of S1- and S2-like immunoreactivity in the cardiac stomach.

Both S1- and S2-IRs were localized throughout the cardiac stomach associated with the basi-epithelial nerve plexus and prominent mucosal cell bodies (Figures 3 and 4). The positive S1- and S2-like immunoreactivities attenuated dramatically on preabsorption with 10 nmol/ml of KYSALMFamide and KYSGLTFamide while the omission of the primary antisera completely abolished staining. Examination of the basi-epithelial nerve plexus revealed that S1- and S2-like peptides to be principally localized to an intricate meshwork of longitudinally and circularly oriented nerve fibres. The positive fibres were distributed throughout the neuropile closely apposed to the connective tissue layer, which had the appearance of a continuous, well-defined boundary. In addition, a number of spindle-shaped perikarya were scattered amongst the mucosal cells (Figures 3 and 4). Five starfish were dissected and several portions of the cardiac stomach were fixed and processed. Over 20 slides for each animal were visualized to find similar distribution patterns with S1- and S2-IRs in the cardiac stomach.

Discussion

In this study, an immunocytochemical investigation was performed in both the nervous (radial nerve cord) and digestive (cardiac stomach) systems of *M. glacialis.* Antisera, BL VI and NEV VII raised against S1 and S2 analogues, KYSALMFamide and KYSGLTFamide, respectively, were successfully employed to map the localization of S1- and S2-like peptides in *M. glacialis.* Although no attempts were made to localize the S1- and S2-IRs in other systems, the existence of the peptide-IR appears to be certain because the previous studies demonstrated that SALMFamide-IR was widespread throughout tissues such as apical muscle, tube feet and the digestive system of *A. rubens* by immunocytochemistry (Elphick et al. 1989; Moore and Thorndyke 1993; Newman et al. 1995a, 1995b) and by radioimmunoassays (Elphick et al. 1995).

Contrary to the studies on the localization of the peptides in *A. rubens*, interpretation of the S1- and S2-IRs pattern in this study is more complicated since two additional S1-related peptides, MagS3 and MagS4, have been isolated and characterized from *M. glacialis* (Yun et al. 2007). S1-IR could be attributed to either S1, MagS3 or MagS4. Sequence homology in the C-terminal region between the three S1-related peptides, a region believed to be crucial for antigen-antibody reaction, makes it difficult to raise any probes totally specific for each peptide. However, it is clear that S2-IR can be attributed to the S2-related peptide, MagS2.

S1/S2-IR in the nervous system

As illustrated in Figure 2, the ectoneural tissue consists of the supporting cells including the epithelial cells and sensory cells and the supporting fibres. The presence of S1- and S2-IRs identified in this study was concentrated in the epithelial cell bodies of the ectoneural system in the RNC of *M. glacialis.* No staining was found in the supporting fibres and neuropile. These results are slightly different from the studies on *A. rubens*, where an additional widespread localization of S1- and S2-IRs to the fibre systems of nerve cord as well as to the epithelial cell bodies was demonstrated (Moore and Thorndyke 1993; Newman et al. 1995a). However, in an earlier study, Unger (1960) described the supporting cells filled with secretory granules as neurosecretory cells in *M. glacialis*, which may explain the positive staining in the cell bodies. At present, it is not certain what makes this distribution pattern of SALMFamide-IR different between *A. rubens* and *M. glacialis* but this may represent different features of the neuronal anatomy between the two species.

For a putative function of S1 and S2 neuropeptides, it was speculated that they may be involved in the conduction of sensory information (Newman et al. 1995a) but no firm evidence by physiological study is available so far due to the difficulties associated with the small size of neurones in starfish. However, the recent revelation that these peptides can cause the relaxation of

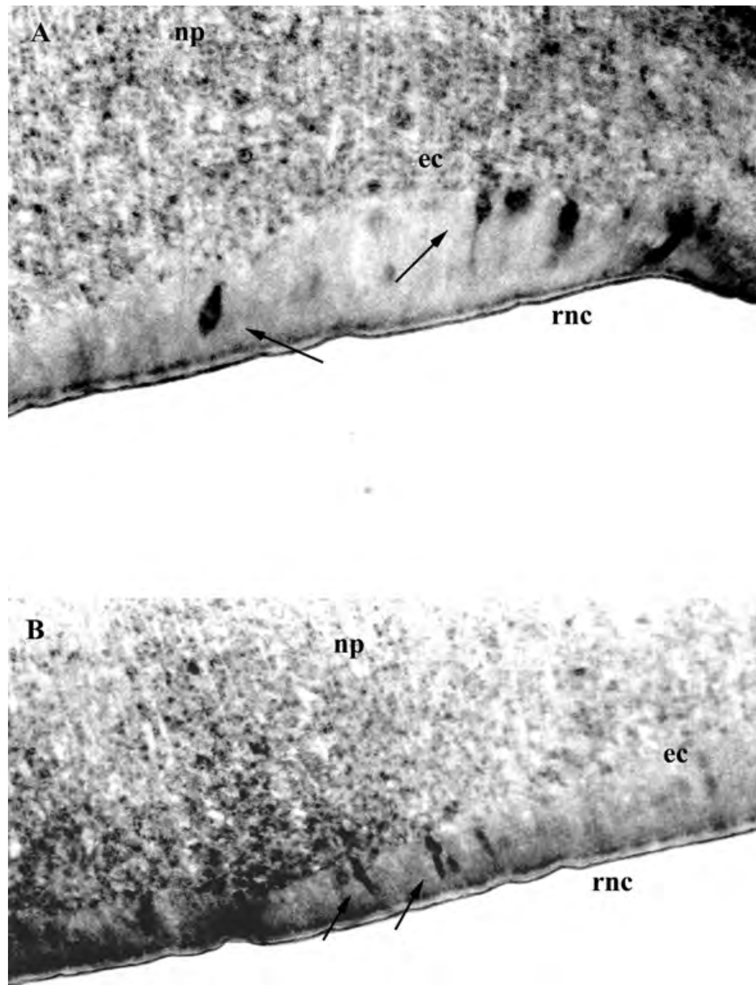

Figure 2. SALMFamide-like immunoreactivity in the nervous system of *M. glacialis*. S1- and S2-IR were localized in the ectoneural epithelial cell bodies (arrows) of the radial nerve cord. (A) S1-like immunoreactivity. (B) S2-like immunoreactivity. rnc, radial nerve cord; ec, ectoneural; np, neuropile. Magnification ×800.

the starfish stomach (Elphick et al. 1995; Melarange et al. 1999; Potton 1997; Yun et al. 2007) prompted a speculation that they may be involved in co-ordinating behaviour of the starfish. The SALMFamide-like material was also detected by radioimmunoassays designed to recognize S1 and S2 peptides and these were used to monitor the isolation of novel SALMFamide peptides from *M. glacialis* (Yun et al. 2007).

S1/S2-IR in the digestive system (cardiac stomach)

S1- and S2-IRs were detected extensively throughout the cardiac stomach and were localized predominantly to the basi-epithelial nerve plexus and some mucosal cell bodies. These results are in line with those obtained from the studies on *A. rubens* (Moore and Thorndyke 1993; Newman et al. 1995b) and others, and also on *M. glacialis* (Martinez et al. 1993). In the latter study, S1-IR was clearly demonstrated in basi-epithelial and mucosal cell bodies, which can be postulated as endocrine cells. In a study on the digestive system of *M. glacialis*, Martinez et al. (1989) described a diffuse endocrine system in the epithelium of the pyloric caeca, which consists of endocrine cells connected to the basi-epithelial plexus. Although there has been controversy on whether the gut mucosal cell bodies represent endocrine cells or whether they are neuronal perikarya serving the basi-epithelial plexus (Moore and Thorndyke 1993), it can be argued that cells associated with the basi-epithelial plexus could be the release site for S1 and S2 peptides. Furthermore, Cobb and Raymond (1979) suggested that the extensive neuronal network underlying the gut epithelium is involved in the innervation of various secretory cells. The presence of S1

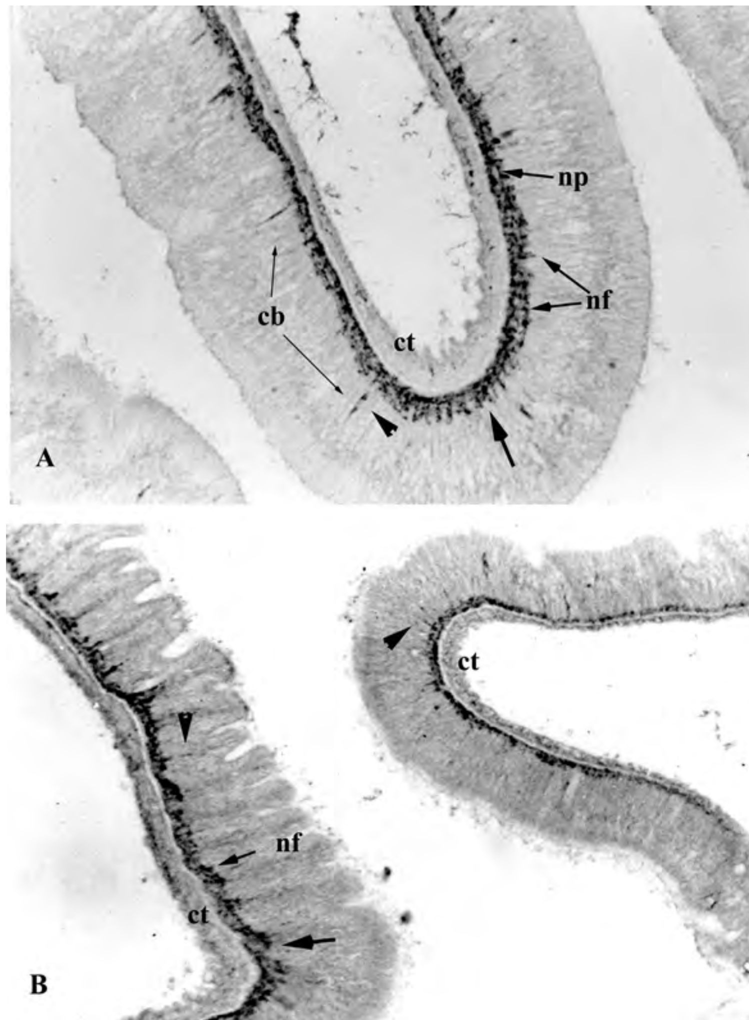

Figure 3. SALMFamide-like immunoreactivity in the cardiac stomach of *M. glacialis* (DAB). S1- and S2-IR were localized throughout the cardiac stomach associated with the basi-epithelial nerve plexus (arrows) and mucosal cell bodies (arrowheads). (A). S1-like immunoreactivity ($\times$200). (B). S2-like immunoreactivity ($\times$200). cb, cell bodies; ct, connective tissue; nf, nerve fibres; np, basi-epithelial nerve plexus.

and S2 in these nerve fibres and endocrine-like mucosal cells provides a firm basis for speculation on its role in the control of digestive activity.

An earlier study described the pharmacological investigation of the effects of novel neuropeptides isolated from *M. glacialis* on the cardiac muscles of *A. rubens* (Melarange et al. 1999: Elphick and Thorndyke 2005). The extensive distribution pattern of S1- and S2-IRs in the cardiac stomach indicates that they may be involved in neurotransmission or neuro-modulation associated with stomach mobility. In addition, the data obtained in this study, combined with a previous study that identified novel SALMFamides in *M. glacialis* (Yun et al. 2007), support the putative function of the SALMFamides as relaxing agents, which has been fully described in *A. rubens* (Elphick et al. 1995; Potton 1997; Melarange et al. 1999) and *M. glacialis* (Yun et al. 2007).

In summary, the existence of SALMFamide-like immunoreactivity was clearly demonstrated in the nervous system as well as in the digestive system of *M. glacialis.* In the nervous system, cell bodies in the ectoneural part were immunostained for both S1 and S2 peptides, while in the digestive system the basi-epithelial plexus and mucosal cell bodies were immunoreactive. This immunocytochemical evidence indicates that the SALMFamides may have a role as neurotransmitters or neuromodulators in

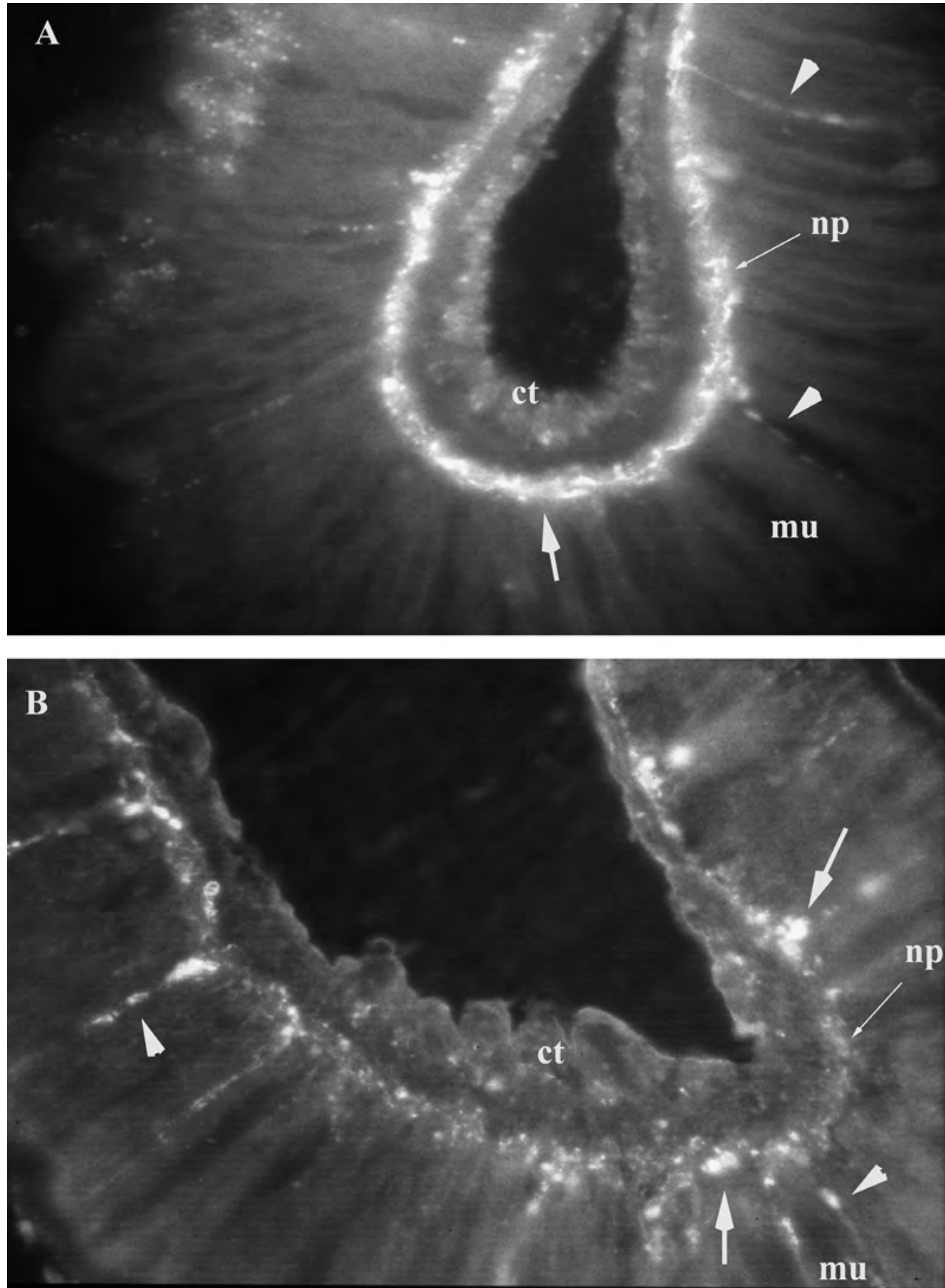

Figure 4. SALMFamide-like immunoreactivity in the cardiac stomach of *M. glacialis* (fluorescent). S1- and S2-IR were localized throughout the cardiac stomach associated with the basi-epithelial nerve plexus (arrows) and mucosal cell bodies (arrowheads). (A). S1-like immunoreactivity ($\times 400$). (B). S2-like immunoreactivity ($\times 400$). ct, connective tissue; mu, mucosal cells; np, basi-epithelial nerve plexus.

co-ordination of behaviour of the starfish. However, in future more studies including identification of peptide binding sites, differential expression pattern of mRNAs encoding the peptides, and some physiological investigations need to be carried out to designate their functions more accurately.

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References

Cobb JLS, Raymond AM. 1979. The basiepithelial nerve plexus of the viscera and coelom of eleutherozoan Echinodermata. Cell Tissue Res. 202:155–163.

Diaz-Miranda L, Price DA, Greenberg MJ, Lee TD, Doble KE, Garcia-Arraras JE. 1992. Characterization of two novel neuropeptides from the sea cucumber *Holothuria glaberrima*. Biol. Bull. 182:241–247.

Elphick MR, Emson RH, Thorndyke MC. 1989. FMRFamide-like immunoreactivity in the nervous system of the starfish *Asterias rubens*. Biol Bull. 177:141–145.

Elphick MR, Price DA, Lee TD, Thorndyke MC. 1991a. The SALMFamides: a new family of neuropeptides isolated from an echinoderm. Proc R Soc Lond B. 243:121–127.

Elphick MR, Reeve JR, Burke RD, Thorndyke MC. 1991b. Isolation of the neuropeptide SALMFamide-1 from starfish using a new antiserum. Peptides. 12:455–459.

Elphick MR, Newman SJ, Thorndyke MC. 1995. Distribution and action of SALMFamide neuropeptides in the starfish *Asterias rubens*. J Exp Biol. 198:2519–2525.

Elphick MR, Thorndyke MC. 2005. Molecular characterization of SALMFamides neuropeptides in sea urchins. J Exp Biol. 208:4273–4282.

Martinez A, Villaro AC, Sesma P. 1989. Microscopic study of the pyloric caeca of the starfish *Marthasterias glacialis* (Echinodermata): Finding of endocrine cells. J Morphol. 202:151–164.

Martinez A, Lopez J, Montuenga LM, Sesma P. 1993. Regulatory peptides in gut endocrine cells and nerves in the starfish *Marthasterias glacialis*. Cell Tissue Res. 271:375–380.

Melarange R, Potton DJ, Thorndyke MC, Elphick MR. 1999. SALMFamide neuropeptides cause relaxation and eversion of the cardiac stomach in starfish. Proc R Soc B. 266:1785–1789.

Moore SJ, Thorndyke MC. 1993. Immunocytochemical mapping of the novel echinoderm neuropeptide SALMFamide 1 (S1) in the starfish *Asterias rubens*. Cell Tissue Res. 274:605–618.

Moss C, Burke RD, Thorndyke MC. 1994. Immunolocalizaation of the neuropeptide S1 and serotonin in the larvae off the starfish *Pisaster ochraceus* and *Asterias rubens*. J Mar Biol Assoc UK. 74:61–71.

Newman SJ, Elphick MR, Thorndyke MC. 1995a. Tissue distribution of the SALMFamide neuropeptides S1 and S2 in the starfish *Asterias rubens* using novel monoclonal and polyclonal antibodies. I. Nervous and locomotory systems. Proc R Soc Lond B. 261:139–145.

Newman SJ, Elphick MR, Thorndyke MC. 1995b. Tissue distribution of the SALMFamide neuropeptides S1 and S2 in the starfish *Asterias rubens* using novel monoclonal and polyclonal antibodies. II. Digestive system. Proc R Soc Lond B. 261:187–192.

Ohtani M, Iwakoshi E, Muneoka Y, Minakata H, Nomoto K. 1999. Isolation and characterization of bioactive peptides from the sea cucumber *Stichopus japonisus*. In: Shimonish Y, editor. Peptide science – present and future. London: Kluwer Academic. p. 419–420.

Potton DJ. 1997. Neuroendocrine control of feeding in the European starfish *Asterias rubens* [PhD thesis]. Royal Holloway, University of London.

Scharrer B. 1987. Neurosecretion: Beginnings and new directions in neuropeptide research. Annu Rev Neurosci. 10:1–17.

Thorndyke MC. 1989. Peptides in invertebrates. In: Holmgren S, editor. The comparative physiology of regulatory peptides. London: Chapman & Hall. p. 203–228.

Thorndyke MC, Goldsworthy GJ. 1988. Neurohormones in invertebrates. Cambridge: Cambridge University Press.

Unger H. 1960. Neurohormone bei Seesternen (Marthasterias glacialis). Symposia Biol Hungarica. 1:203–207.

Yun SS, Thorndyke MC, Elphick MR. 2007. Identification of novel SALMFamide neuropeptides in the starfish *Marthasterias glacialis*. Comp Biochem Physiol A. 147:536–542.