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REGULAR ARTICLE

Jürgen Schmich · Rüdiger Rudolf · Stefan Trepel
Thomas Leitz

Immunohistochemical studies of GLWamides in Cnidaria

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Abstract GLWamides are a recently described, novel family of neuropeptides in Cnidaria. Antibodies specific for the GLWamide terminus have been raised and used to evaluate the occurrence and localisation of immunopositive material in various Cnidaria in order to determine whether GLWamides are present and to obtain a first impression of the possible regulatory role of these neuropeptides. GLWamide immunoreactivity has been found in all species tested and is not confined to distinct life stages but is present during most of the life cycle of the Cnidaria. Additionally, GLWamides are expressed by different nerve cells at different life stages. GLWamide-immunoreactive cells constitute a subset of the neural equipment. Overall our data suggest that GLWamides generally occur in the nervous system of Cnidaria and that these peptides are multifunctional. Putative functions other than the control of development include the regulation of nematocyst discharge, muscle contraction and the regulation of gastric function.

Key words Neuropeptides · Nervous system · Immunoreactivity · Coelenterates · Hydrozoa · Anthozoa

Abbreviations *AKH* Adipokinetic hormone, *ECH* erythrophore-concentrating hormone, *EIA* enzyme immunoassay, *ELISA* enzyme-linked immunosorbent assay, *GLWamides* glycine-leucine-tryptophane·amides, *IR* immunoreactive, *KLH* keyhole limpet hemocyanine, *Lom-AKH Locusta migratoria* adipokinetic hormone, *NHS* N-hydroxysuccinimide, *PBS* phosphate-buffered saline, *Pea-CAH Periplaneta americana* cardioacceleratory hormone, *RFamides* arginine-phenylalanine amides

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J. Schmich · R. Rudolf · S. Trepel · T. Leitz (✉)
Zoologisches Institut der Universität, Abteilung Physiologie,
Im Neuenheimer Feld 230, D-69120 Heidelberg, Germany
Tel.: +49 6221 54 5663 (Office) 6482 (Lab);
Fax: +49 6221 54–4913;
e-mail: Leitz@sirius.mgen.uni-heidelberg.de

Introduction

Neuropeptides are potent regulatory molecules in the animal kingdom. Those animals thought to be closest to the ancestors with the first nervous system are the Cnidaria. The basic plan of their nervous system is a nerve net with nerve plexuses or nerve tracts. At the ultrastructural level, many cnidarian neurons show combined characteristics of motor, sensory and neurosecretory neurons and interneurons (Grimmelikhuijzen and Westfall 1995). The primitive nervous system of the Cnidaria is strongly peptidergic (for a review, see Grimmelikhuijzen et al. 1996). Most cnidarian neuropeptides known so far have been classified into different families according to their identical C-terminal amino acid sequences (e.g. RFamides, RPamides, etc.). Recently, metamorphosin A (pEQPGLWamide), a peptide inducing metamorphosis of planulae of the marine hydrozoan *Hydractinia echinata*, has been isolated from the anthozoan *Anthopleura elegantissima* (Leitz et al. 1994; Leitz and Lay 1995). This compound has turned out to be the prototype of a novel neuropeptide family, the GLWamides. Subsequently, two additional GLWamides have been predicted from the cDNA of the GLWamide precursor protein of *H. echinata* (Gajewski et al. 1996). Various other GLWamides have been found either by identification of the preproproteins or by purification and sequencing of the peptides in three anthozoans and in *Hydra magnipapillata* (Gajewski et al. 1996; Leviev and Grimmelikhuijzen 1995; Leviev et al. 1997; Takahashi et al. 1997; for a review, see Leitz 1998a). It has been hypothesised that planula larvae of *H. echinata* regulate metamorphic events by using GLWamide(s) as internal coordinative signals (for recent reviews, see Leitz 1997, 1998b).

However, because GLWamides have been found in life stages of *Hydractinia* other than planulae and even in species without larval stages, it must be concluded that GLWamides serve some additional functions. As a first step to investigate further the appearance of these peptides throughout the Cnidaria, we have generated antibodies against a GLWamide coupled to keyhole limpet hemo-

cyanine (KLH) via the N-terminus (Gajewski et al. 1996). In the present study, we demonstrate the specificity of these antibodies for the GLWamide residue by competitive enzyme immunoassay (EIA) and show the usefulness of the antibody for evaluating GLWamide immunoreactivity in the nervous system of Cnidaria. We have used various hydrozoan species to obtain a comparative view of the distribution of GLWamide-immunoreactive (IR) cells.

Materials and methods

Animals

Antipathes subpinnata and *Halocordyle disticha* were obtained by Scuba diving from various locations around Sicily (Italy). Medusae of *Podocoryne carnea* were provided by Prof. Günter Plickert (University of Cologne, Germany). *Hydractinia echinata* and *Hydra* spp. were maintained as described previously (Leitz and Wagner 1993; Sugiyama and Fujisawa 1977).

Peptides and antibody

GLWamides were synthesised and purified by high pressure liquid chromatography by Richard Jacob in the Protein and Peptide Group of the European Molecular Biological Laboratory. Substance P and bombesin were purchased from Boehringer Ingelheim Bioproducts (Heidelberg, Germany) and erythrophore-concentrating hormone (ECH) and *Locusta migratoria* adipokinetic hormone II (Lom-AKH II) were from Bachem (Heidelberg, Germany). Pro^1-Pea-CAH-I was a kind gift from M. Eckert (University of Jena, Germany). Hydra-RFamide I was kindly sent by C. J. P. Grimmelikhuijzen (University of Copenhagen, Denmark).

Antisera were raised in New Zealand White rabbits, which were immunised with CAAPPGLWamide conjugated via succinimidyl *m*-maleimidobenzoate to KLH. Boosters were performed 14, 28 and 56 days after the first immunisation. The immunisation was carried out by Eurogentec (Seraing, Belgium). For immunohistochemistry, it was essential to preadsorb the antisera with KLH (1 mg/ml) followed by centrifugation. The preadsorbed sera were stored in aliquots at –80°C. Antiserum 1676IIIp from the final bleed, two weeks after the last boost, yielded the best signal-to-noise ratio and was therefore used in this study.

Immunoassays

In order to test the specificity of the antibody, enzyme immunoassays (EIAs) were performed. The competitive assay type was used to test N-terminally blocked peptides, which cannot be assayed in direct ELISAs. The assays were performed in N-hydroxysuccinimide(NHS)-amine-binding 96-well immunoplates (Costar, Bodenheim, Germany). The wells were incubated with 50 µl 350 nM PPG-LW-NH_2 in PBS (pH 7.4) overnight at 4°C. All subsequent incubation and blocking steps were performed at 37°C. The wells were washed three times with 0.05% Tween 20 in PBS (PBST) and blocked for 20 min with 10% horse serum in PBST (blocking buffer). Standard peptides or samples in 25 µl blocking buffer were added, followed immediately by 25 µl antiserum 1676IIIp diluted 1:1000 with blocking buffer. After a 2-h incubation at 37°C, the wells were washed three times with PBST and subsequently incubated for 2 h with 100 µl alkaline-phosphatase-conjugated goat anti-rabbit-IgG (Dianova, Hamburg, Germany) diluted 1:1000 in blocking buffer. After the wells had been washed twice with blocking buffer and twice with substrate buffer (10 mM diethanolamin, 0.5 mM $MgCl_2$, pH 9.5), colour development was performed by a 30-min incubation with 100 µl substrate buffer containing 1 mg/ml p-nitrophenyl phosphate (Sigma, Deisenhofen, Germany). The plates were read in a Dynatech MR 5000 reader and data were evaluated with the BioLinx program (Dynatech) by using the sigmoidal curve fit option.

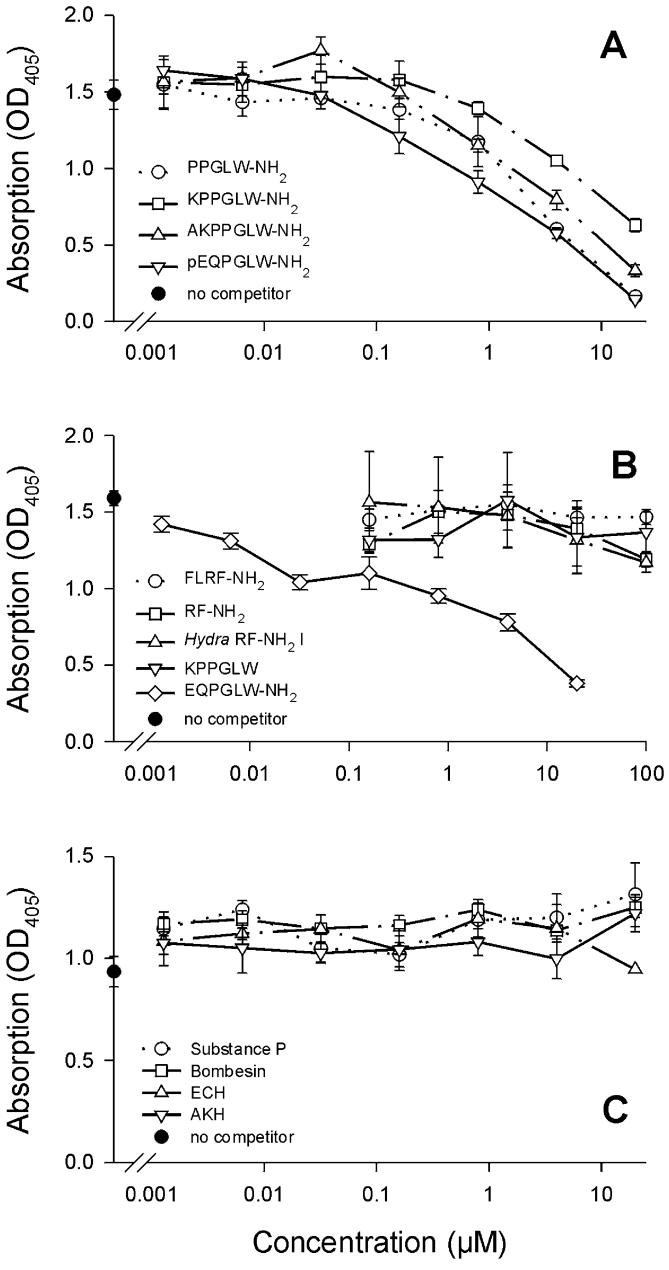

Fig. 1A–C Competition of various GLWamides and related peptides with PPGLWamide at the antibody 1676IIIp. **A–C** Results from different immunoplates

For direct ELISAs, the wells were coated with various concentrations of non-N-terminally blocked peptides. Washing and blocking steps were performed as in the competitive assay.

In situ hybridisation and immunohistochemistry

The identity of the riboprobe and the procedure for in situ hybridisation were as described in Gajewski et al. (1996). For immunohistochemistry, *Hydra* spp. were anaesthetised by adding 2% urethane

step by step to the medium. Subsequently, the anaesthetic solution was replaced by fixative. This method relaxed the animals, especially their tentacles. Some specimens of marine species were anaesthetised in a solution of 200 mM $MgCl_2$ in 50% artificial seawater for 30 min. The animals were fixed in freshly prepared 4% paraformaldehyde in 100 mM phosphate buffer, pH 7.2, overnight at 4°C. Some specimens were stored in phosphate-buffered saline pH 7.2 (PBS) for several days. After being washed twice with 100 mM sodium phosphate buffer, pH 7.2, and four times with 400 mM glycine pH 7.2, all specimens were preincubated for at least 2×15 min in 10% (v/v) horse serum, 0.5% (v/v) Triton X-100, 0.1% (w/v) sodium azide in PBS (blocking buffer). Subsequently, incubation with antiserum 1676IIIp in blocking buffer was performed for 1 h at room temperature with moderate shaking. The antiserum was used at dilutions of 1:300–1:500. Washing and blocking steps were performed as described above. Subsequently, the specimens were incubated for 1 h with Cy3-conjugated goat anti-rabbit IgG antibody (Dianova, Hamburg, Germany) diluted 1:500 in blocking buffer. They were washed twice with blocking buffer and twice with PBS before being embedded in Mowiol/1,4-diazabicyclo-(2,2,2) octane. Controls were performed by omission of the anti-GLWamide serum or by incubation with antiserum preadsorbed with KPPGLWamide coupled via the NH_2-groups to Hi-Trap NHS-activated beads (Pharmacia, Freiburg, Germany). Both procedures abolished all staining. The specificity of the primary antibody was also tested by ELISA or competitive EIA (see above). The fluorochrome was detected with a Zeiss IM35 microscope equipped with the filter combinations HQ 535/50; SN ×6806; Q565LP; SN c5863; HQ 610/75; SN m6063. Photographs were taken on Ektachrome 320 T or Ilford Delta 400 films.

Some specimens were evaluated by using a TCS4 confocal laser scanning microscope and SCANware software (Leitz-Leica, Heidelberg, Germany). Plates were arranged by digital processing of the images by the software packages CorelDraw 8.0 and Powerpoint 7.0 for Windows NT.

Results and discussion

The specificity of the antibody used for immunohistochemistry was evaluated by competitive EIA. Various peptides were tested, including previously described GLWamides (Leitz et al. 1994; Gajewski et al. 1996). As apparent from the competition curves in Fig. 1, only GLWamides competed with PPGLWamide (Fig. 1A,B). Substance P (a GLMamide), bombesin (a HLMamide), crustacean ECH (a PGWamide), and Lom-AKH II (a AGWamide) did not compete (Fig. 1B,C). Various RFamides were also tested because of their abundance in the Cnidarian nervous system. These peptides showed no cross-reactivity with PPGLWamide. Pro^1-Pea-CAH-I (a PNWamide) did not cross-react in a direct ELISA (data not shown), like all peptides with a non-amidated COOH-terminus. The peptide KPPGLW was not a competitor (Fig. 1B), whereas KPPGLWamide competed strongly (Fig. 1A), showing that the amide terminus is essential for recognition by the antibody. Additionally, the antiserum did not cross-react with KPPGLW in a direct ELISA, whereas the amide derivative KPPGLWamide interacted significantly (Fig. 2). The cross reactivity data are summarised in Table 1.

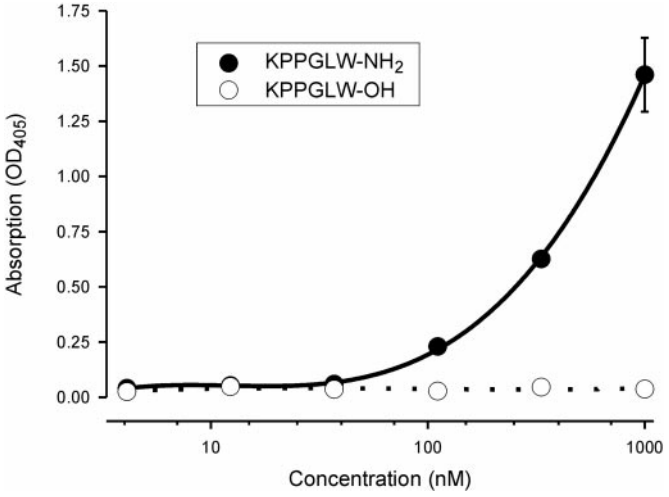

Fig. 2 Direct ELISA comparing the binding of antibody 1676IIIp to He-LWamide II (KPPGLWamide) and its non-amidated derivative

Thus, we have shown the high specificity of the anti-GLWamide antibody used. Additionally, immunohistochemical staining is abolished by absorption of the antiserum with KPPGLWamide coupled to Hi-Trap beads. GLWamides have previously been identified by peptide sequencing and precursor cDNA cloning from various hydrozoan and anthozoan species (for a review, see Leitz 1998a). With respect to the NH_2-terminal identity of the peptide sequences and the estab-

Table 1 Peptides used for ELISA and competitive EIA. Amino acid residues identical to those in the GLWamides are in *boxes* (*Lom-AKH Locusta migratoria* adipokinetic hormone, *ECH* erythrophore-concentrating hormone, *Pea-CAH Periplaneta americana* cardioacceleratory hormone)

Name of peptide	Amino acid sequence	Immunoreactivity[a]
	P-P-G-L-W·NH_2	+
Metamorphosin A	pE-Q-P-G-L-W·NH_2	+
	E-Q-P-G-L-W·NH_2	+
	A-K-P-P-G-L-W·NH_2	+
	Biotin-C_6H_{12}-K-P-P-G-L-W·NH_2	+
	C-A-A-P-P-G-L-W·NH_2	+
He-LWamide II	K-P-P-G-L-W·NH_2	+
	K-P-P-G-L-W·OH	–
Substance P	R-P-K-P-Q-Q-F-F-G-L-M·NH_2	–
Bombesin	pE-Q-R-L-G-N-Q-W-A-V-G-H-L-M·NH_2	–
Lom-AKH II	pE-L-N-F-S-A-G-W·NH_2	–
ECH	pE-L-N-F-S-P-G-W·NH_2	–
Pro^1-Pea-CAH I	P-V-N-F-S-P-N-W·NH_2	–

[a] Immunoreactivity was tested for all peptides in the competitive EIA and for non-N-terminally blocked peptides in the ELISA

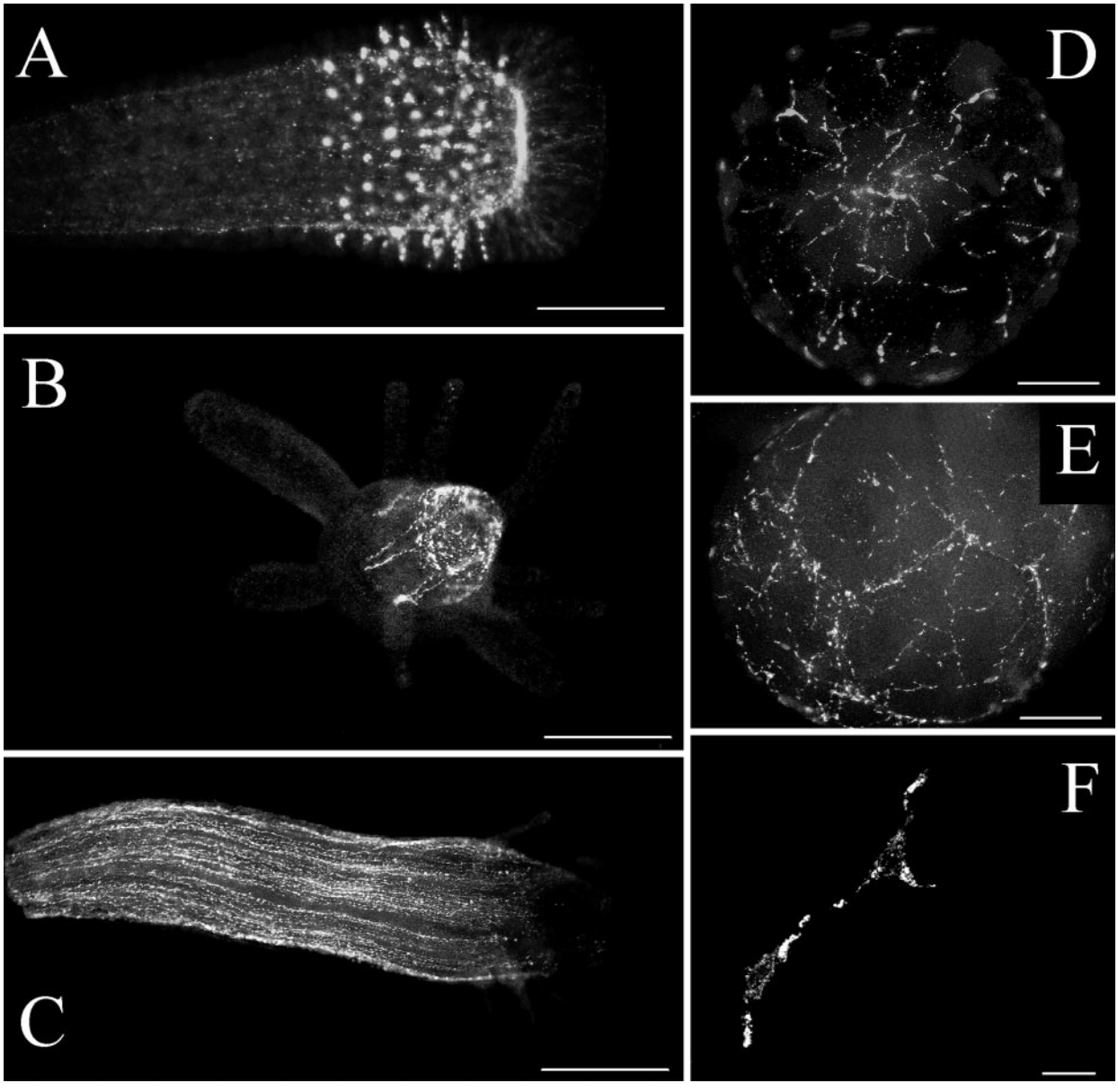

Fig. 3A–F GLWamide immunoreactivity in *Hydractinia echinata.* **A** Anterior region of a larva showing the belt-like arrangement of the perikarya of sensory cells. **B** Primary polyp, 36 h after induction of metamorphosis. GLWamide immunoreactivity is found (in addition to the endodermal cells shown in Fig. 4) in ectodermal fibres projecting between the tentacles down towards the stolonal region. **C** Gasterozoid showing a dense logitudinal arrangement of IR fibres. **D** Male gonophore. **E** Female gonophore. **F** Close-up of the female gonophore showing one bi- and one multipolar cell. *Bars* 100 µm in **A**, 250 µm in **B**, 450 µm in **C,** 200 µm in **D**, 150 µm in **E**, 10 µm in **F**

lished specificity of the antibody, the probability of any cross-reactivity of our antibody with a closely related but presently unknown compound is very low. We can therefore conclude that the results from the immunohistochemical experiments observed in the various studied species are valid.

GLWamide immunoreactivity appeared in all species under investigation. In planula larvae of the marine colonial hydroid *Hydractinia echinata*, immunopositive ectodermal sensory cells were found, the perikarya of which were arranged in a belt-like fashion at the subpolar anterior region (Fig. 3A). The fibres of these cells projected along the mesoglea to the posterior part of the animals. Weis et al. (1985) have presented electron-microscopical data demonstrating sensory cells with cell bodies in the posterior part of *H. echinata* larvae. Such cells have not been detected by using the anti-GLWamide antibody. In contrast to *H. echinata*, ectodermal GLWamide-immunopositive perikarya of sensory cells were scattered over the body columns of larvae of *Phialidium gregarium*, *Anthopleura elegantissima*, and *Agaricia humilis* and in the buds of *Cassiopea andromeda* (not shown).

GLWamide immunoreactivity first appeared in endodermal cells of the hypostome about 24 h after the induction of metamorphosis in primary polyps of *H. echinata* (Fig. 4A,B). Previously, these cells had been identified

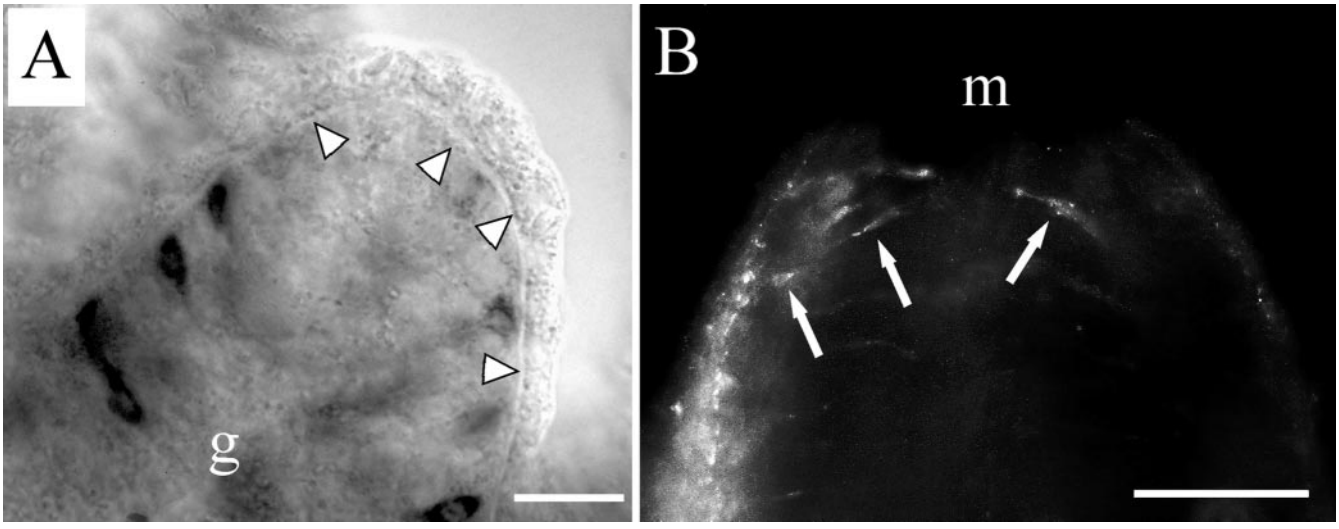

Fig. 4A, B GLWamides in the hypostome of primary polyps of *Hydractinia echinata*. **A** In situ hybridisation showing the localisation of RNA for the precursor molecule of the GLWamides. Note the endodermal location of the cells situated with their bases at the mesoglea (*arrowheads*). **B** GLWamide immunohistochemistry showing cellular branches or fibres (*arrows*) extending from the perikarya to the gastric cavity. Confocal laser scanning microscopical image focussed on the endodermal fibres (*g* gastric cavity, *m* mouth). *Bars* 40 µm in **A**, 80 µm in **B**

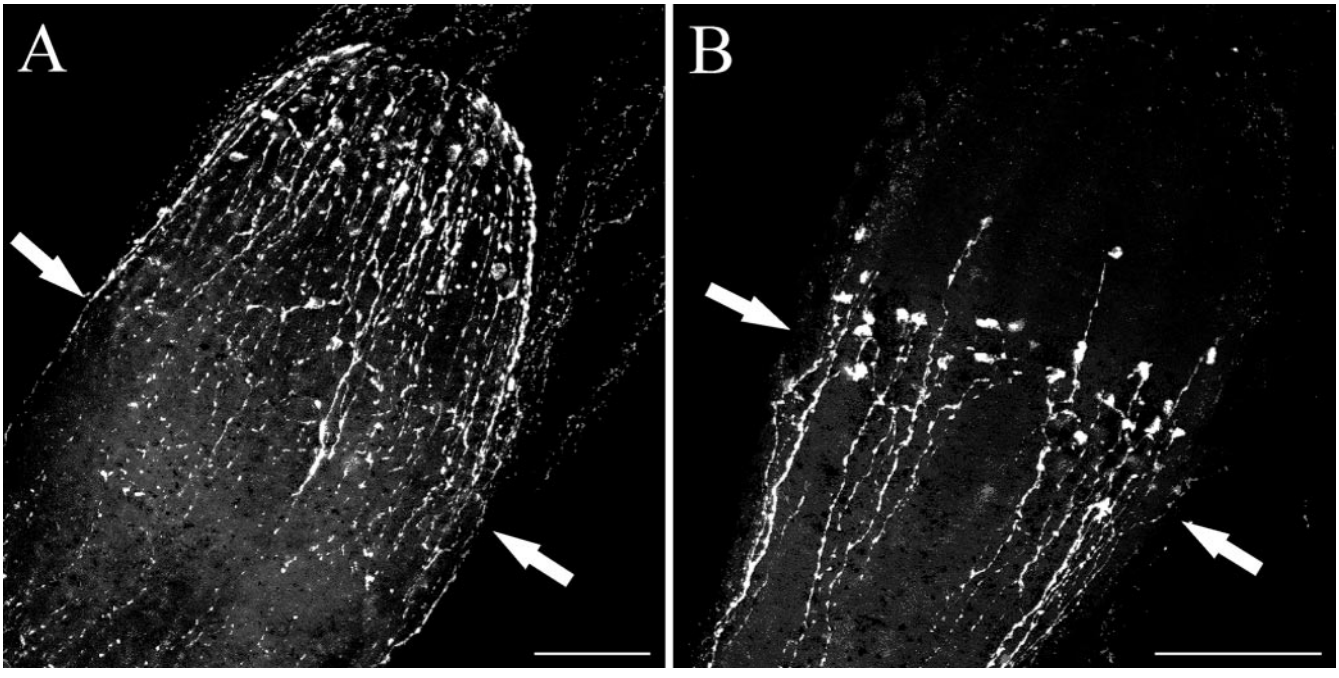

Fig. 5A, B Neuropeptide immunoreactivity in gasterozoids of young colonies of *Hydractinia echinata*. **A** RFamide immunoreactivity, **B** GLWamide immunoreactivity. Two different specimens are shown. Note the different location of the immunoreactive cells showing that RFamide-IR and GLWamide-IR cells constitute different subsets of neurons (*arrows* tentacle insertion zone). *Bars* 50 µm in **A**, 100 µm in **B**

by in situ hybridisation with a probe corresponding to a part of the GLWamide precursor (Gajewski et al. 1996; see also Fig. 4A). Whereas only the pyramidal structure of the perikarya with their bases located at the mesoglea could be shown by in situ hybridisation, immunohistochemistry demonstrated that these cells bore short fibres extending perpendicularly to the body axis and towards the gastric cavity (Fig. 4B). In gasterozoids, these cells were found not only in the hypostomal region but also in the whole head region until a position immediately below the tentacle ring (not shown).

As primary polyps matured, they developed GLWamide immunoreactivity in ectodermal neuronal fibres extending from the area between the tentacles down towards the gastric region (Fig. 3B). The perikarya of these sensory cells were not visible initially but could be detected later in the subhypostomal region of the ectoderm. With in-

creasing age of the polyp, the IR fibres disappeared from the hypostomal region. In adult gasterozoids, GLWamide immunoreactivity was localised in ectodermal, presumably sensory cells, the perikarya of which extended perpendicular to the mesoglea. These perikarya could be demonstrated by in situ hybridisation with a probe representing a part of the GLWamide precursor (Gajewksi et al. 1996). In our immunohistochemical studies, fibres of these cells extended longitudinally along the mesoglea defining a close net of strongly immunopositive fibres in the gastric region (Fig. 3C). Similar nerve nets were found in dactylozoids and gonozoids (not shown).

Bi- and multipolar neurons were stained in the gonophores of *H. echinata* (Fig. 3D–F). The gonophores bear several layers of ecto- and endoderm (Hertwig and Hündgen 1984). Oocytes in female gonophores are surrounded by four layers of different cell types with three layers of mesogleae as separating structures. Male gonophores are built differently. An ectodermal and endodermal layer, separated by mesoglea 1, are followed by mesoglea 2 and a further ectodermal structure and form a testicle-like structure. Confocal imaging revealed that the GLWamide-immunopositive neurons were localised along mesoglea 1 between the first ectodermal and gastrodermal structures in both male and female gonophores and additionally along mesoglea 3 in female gonophores. In contrast, RFamide-IR neurons had previously been found covering "the surface" of both male and female gonophores (Grimmelikhuijzen 1985).

Nerve cells other than those described here have been detected in *Hydractinia* species by electron microscopy (Stokes 1974; Tsuneki and Kobayashi 1977; Weis et al. 1985). Additionally, the RFamide-IR nervous system has been described extensively by Grimmelikhuijzen (1985). RFamide- and GLWamide-positive neurons occur as different subsets of the entire nervous system not only in the gonophores, but also in gasterozoids of young colonies, where RFamide-IR and GLWamide-IR cells are differently located. Whereas the perikarya of RFamide-IR cells are distributed mainly in the hypostomal region above the tentacle ring, the perikarya of GLWamide-IR cells are prominent in the more basal region between and just above the tentacle ring (Fig. 5A,B). Therefore, it can be concluded that the GLWamide-IR nerve cells represent only a subset of the neural equipment. This may apply to all Cnidarian species.

H. echinata bears gonozoids that develop the gonophores. These structures are called (crypto)medusoids because they are sessile medusae (Hertwig and Hündgen 1984). We have found bi- and multipolar neurons in the

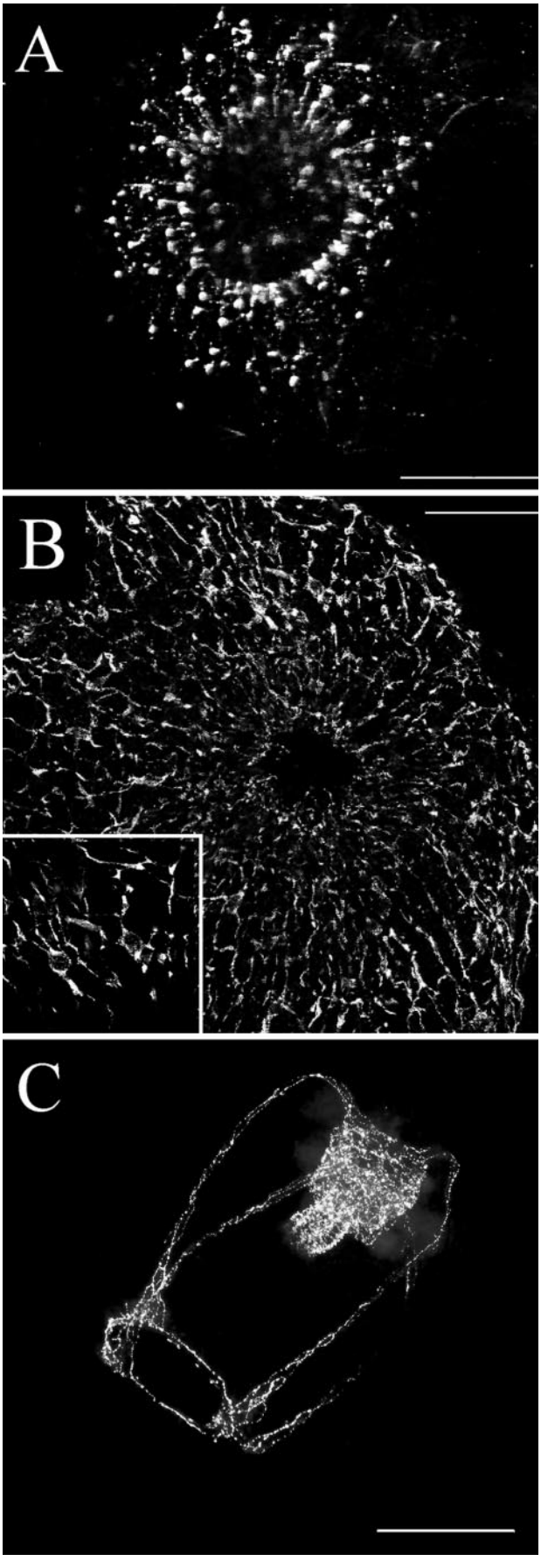

Fig. 6A–C GLWamide immunoreactivity in various cnidarian species. **A** *Antipathes subpinnata*. Hypostome. Ectodermal GLWamide-positive nerve cells form a concentric plexus in the hypostome. **B** *Hydra magnipapillata*. Hypostomal region with a dense plexus of ganglionic cells. Close-up of perikarya of multipolar cells in the *inset*. **C** *Podocoryne carnea.* Medusa. A dense manubrial nerve plexus and fibres in the ring and radial channels are GLWamide-immunopositive. *Bars* 100 µm in **A**, 50 µm in **B**, 600 µm in **C**

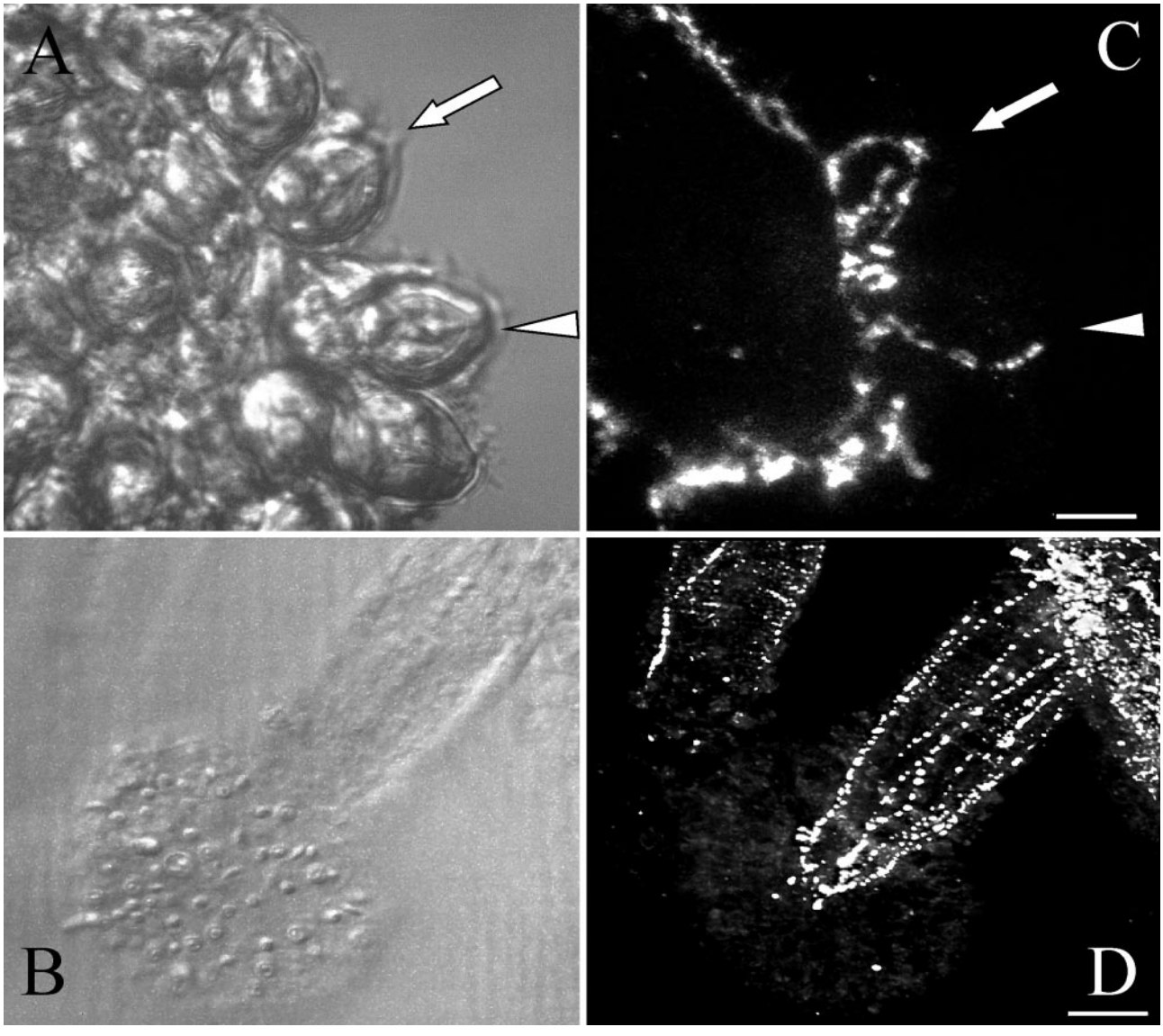

Fig. 7 GLWamide immunoreactivity in neurons associated with nematocytes in the filiform tentacles of *Hydra magnipapillata* (**C**) and in the capitate tentacles of *Halocordyle disticha* (**D**). **A, B** The corresponding differential interference-contrast images (*arrows* indicate corresponding positions in the respective micrographs). *Bars* 10 μm in **A, C**, 40 μm in **B, D**

gonophores of *H. echinata*. In order to search for homologous GLWamide-IR structures in a medusa, we investigated the closely related *Podocoryne carnea*. We found no immunoreactivity in the gonads of the medusa but GLWamide immunoreactivity in perikarya of the manubrium and in fibres of the manubrium and of the ring and radial channels (Fig. 6C). Cryptomedusoids lack ring and radial channels and therefore no homologous IR structures were to be expected in the gonophore. However, one part of the male gonophore, the spadix, which is the homolog of the manubrium (Hertwig and Hündgen 1984), exhibited no IR cells. No cells morphologically similar to the bi- and multipolar cells of *H. echinata* were found in the medusa of *P. carnea*. With respect to the close homology of the cryptomedusoid of *H. echinata* to the medusa of *P. carnea*, it was astonishing to find this entirely different GLWamide-IR cellular inventory.

The immunostaining of adult *Hydra vulgaris* was identical to the staining pattern in *H. magnipapillata,* revealing that, in *Hydra* spp., GLWamides are synthesised predominantly in ectodermal ganglionic cells. A dense plexus of these cells was found in the hypostomal region (Fig. 6B) and in the foot (not shown). GLWamide-IR ganglionic cells were also scattered in the gastric region and in the tentacles (not shown). This is different from our previous findings with an antibody generated by a slightly different method (Leitz and Lay 1995). In our previous study, only fibres in the tentacles and the foot were stained. This might have been because of the lower affinity of the antibody compared with the affinity of the antibody used in the present study. IR material is thought to be more concentrated in the fibres than in the perikarya. Therefore, a lower affinity antibody would recognize only

the fibres and not the perikarya. In the previous study, the GLWamide-IR cells of the gastric region were also not stained. This indicates that the amount of IR GLWamide is lower in these cells than in the cells of the distal body portions. As in the previous study, GLWamide-IR dendrites were also found in close contact to the battery cells. On rare occasions, GLWamide-positive fibres extended along the capsule of the stenoteles (Fig. 7A,C). For a comparison with the filiform tentacles of *Hydra* spp., we examined the capitate tentacles of *Halocordyle disticha,* a species with very prominent cnidocyst-containing structures in these tentacles. In the capitate tentacles, GLWamide-IR material was found in varicose fibres projecting to the nematocytes (Fig. 7B,D).

The distribution and appearance of the GLWamide-IR nerve net varied considerably with the genus. Whereas in *Hydra* spp., the nerve net was composed of ganglionic cells with the highest density in the head and foot region and the lowest density in the gastric region, in *H. echinata*, it was composed of sensory cells with their highest density in the region below the head down to the stolons, which contained no labelled cells. These data show that, although all species exhibit GLWamide-IR neural elements, the distribution of these elements can be very different. A comparison of the hypostomal region of the anthozoan *Antipathes subpinnata* with the hypostomal region of the hydrozoan *Hydra magnipapillata* (Fig. 6) indicates that their GLWamide-IR cellular inventory is similar. Whether the great variability in the distribution of the GLWamide-IR neural elements is attributable to different functions of the GLWamides in the respective species remains to be clarified.

We have described GLWamide expression for several species and various life stages. What putative roles as endogenous factor are implied by these data? First, as described above, the staining patterns in larvae suggest a role for GLWamides in metamorphosis. According to this hypothesis, GLWamides should serve as internal signals released after the reception of environmental chemical cues by the sensory cells (reviewed in Leitz 1997, 1998a). We have found GLWamide-IR sensory cells in planula larvae of *Phialidium gregarium*, *Anthopleura elegantissima*, and *Agaricia humilis*, and in actinulae of *Tubularia mesembryanthemum* (not shown). Indeed, synthetic GLWamides induce the metamorphosis of planulae of *Phialidium gregarium* (McCauley 1997) and *Agaricia humilis* (J. Budzik and D. Morse, personal communication). GLWamides might be used in the larvae for integrative purposes during the metamorphic process (Schmich et al. 1998). Neuropeptides of the RFamide family have also been localised in sensory cells of Cnidarian larvae and buds (Brumwell and Martin 1997; Martin 1992; Plickert 1989) but whether GLWamides and RFamides are colocalized and what role RFamides play during metamorphosis remain to be established.

One function of GLWamides could be the regulation of contraction status. Application of GLWamides in *H. echinata* leads to the contraction of the hypostome and tentacles or even of the whole polyp (own unpublished observations). It is also known from *Hydra magnipapillata* and *Anthopleura fuscoviridis* that exogenous GLWamides can influence the contraction status (Takahashi et al. 1997). Our immunohistochemical results make it tempting to speculate that GLWamides also regulate gonophore function, e.g. contraction during spawning. The spawning of ripe gonophores is independent of the integrity of the gonozoid but is dependent on the dark-light cycle (Ballard 1942). The appearance of GLWamides primarily in ripe gonophores could mean that an autonomous regulation circuit involves GLWamides, such that the nerve cells release the peptides after the perception of light. This hypothesis has previously been proposed for the RFamides by Grimmelikhuijzen (1985). RFamides have also been suggested to play a role as transmitters at neuromuscular junctions (for a review, see Spencer 1991). It remains to be clarified whether both peptide families have similar or identical functions in the gonophore or whether the differential distribution of the peptides reflects different functions.

The presence of immunopositive fibres projecting to and located nearby nematocytes of *Hydra* spp. and *Halocordyle disticha* leads to the conclusion that GLWamides play a role in the regulation of the discharge of nematocysts. On one hand, the results of Aerne et al. (1991) suggest that nematocyst discharge is independent of the presence of nerve cells. On the other hand, the colocalisation of fibres and cnidocytes and the results of Lawonn et al. (1996), who have found a 25% elevation of cathode-directed discharge of *Hydra* stenoteles by incubation with a GLWamide, strongly indicate that GLWamides have a role as neuromodulators in the regulation of nematocyst discharge. Since the probability of discharge induction is modulated by the nutritional state of the animal (Lawonn et al. 1996), GLWamides might directly or indirectly mediate the effect of starvation on the probability of discharge.

Nerve cells displaying GLWamide immunoreactivity clearly constitute a subset of the Cnidarian neural equipment. The various cell types and locations of GLWamide-IR cells indicate that these neuropeptides are multifunctional in the hydrozoa. GLWamides might serve as neurotransmitters, neuromodulators or neurohormones. Several functions may be exhibited in one species. To investigate these functions adequately, studies should be performed to identify and localise the receptor for GLWamides to determine which cells and which subcellular structures are addressed by these neuropeptides.

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