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ORIGINAL ARTICLE

Jürgen Schmich · Stefan Trepel · Thomas Leitz

The role of GLWamides in metamorphosis of *Hydractinia echinata*

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Abstract The metamorphosis of many marine invertebrate larvae is induced by environmental signals. Upon reception of the cues, internal signals have to be set in motion to convey information to all cells of the larvae. For hydrozoan larvae it was hypothesised that ectodermal neurosensory cells at the anterior part are those cells receptive of the inducer. Recently, it was shown that novel peptides with a common GLWamide terminus are found in Cnidaria. These peptides are located in a specific subset of the anterior sensory cells. It was hypothesised that the neuropeptides represent an internal signal coordinating the metamorphic process. In the current study we present further evidence for this hypothesis. Induction of metamorphosis is very specific for the GLWamide terminus and amidation is essential. The potency to metamorphose is strongly correlated with the presence of GLWamide-immunoreactive cell bodies. Our data fit our hypothesis about a very important role of GLWamides in the initiation of the morphogenetic processes very well.

Key words Neuropeptides · Nervous system · Hydrozoa · Metamorphosin

Introduction

The metamorphosis of many marine invertebrates is different from the metamorphosis of insects or amphibians with respect to the mode of induction. Marine invertebrate larvae often depend on exogenous chemical cues to start the metamorphic events. The information carried by these external signals has to be transmitted into the interior of the larvae and subsequently between the various cells of the animal. Many attempts had been made but definitive identifications of the internal metamorphic signals had not been achieved (Pawlik 1992 for review). Recently, metamorphosin A (MMA=pEQPGLWamide), a peptide inducing metamorphosis of the planulae of the marine hydrozoan *Hydractinia echinata* was isolated from the anthozoan *Anthopleura elegantissima* (Leitz et al. 1994; Leitz and Lay 1995). Subsequently, in the search for the *Hydractinia* peptides, He-LWamide I and II could be predicted from the cDNA of the precursor protein of *H. echinata* (Gajewski et al. 1996). This showed that MMA was the prototype of a novel family of neuropeptides, the GLWamides (Leitz 1998a for review). These peptides are synthesised in ectodermal sensory cells of the planulae. The cell bodies are located in a belt-like fashion in the anterior part of the larvae. Their fibres extend along the entire mesoglea (Leitz and Lay 1995). It was hypothesised that the planula larvae of *H. echinata* regulate metamorphic events by using the GLWamide(s) as internal coordinative chemical signal. In *H. echinata*, the external metamorphic signal is provided by bacteria (Müller 1973; Leitz and Wagner 1993). We hypothesise that this hitherto unknown chemical signal is perceived by the sensory cells which respond by subsequently releasing the GLWamide(s) (Leitz 1997, 1998b for reviews). This hypothesis was questioned by Berking and coworkers (Berking and Walther 1994; Walther et al. 1996) on the basis of their studies with partially metamorphosed specimens. Their statement was as follows: The metamorphosis of the planulae of *H. echinata* is not in every case complete. Infrequently, anteriorly metamorphosed specimens are observed. There is a prepattern in the larvae in that the anterior parts develop to the basal stolonal regions of the primary polyps and correspondingly the posterior parts develop to the apical hypostomal regions (Müller et al. 1977; Schwoerer-Böhning et al. 1990). Therefore, anteriorly metamorphosed animals adhere to the substratum with their stolons and have a posterior larval part. Since these so-called “mosaics” (Berking 1991) develop stolons typical for polyp tissue, Berking and coworkers concluded that they lack anterior larval tissue including the sensory cells. However, these anteriorly metamorphosed larvae

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J. Schmich · S. Trepel · T. Leitz (✉)
Zoologisches Institut der Universität, Abteilung Physiologie,
Im Neuenheimer Feld 230, D-69120 Heidelberg, Germany

are able to undergo further posterior metamorphosis when treated with an inducer (Berking 1991), leading Berking and coworkers to the conclusion that obviously larval posterior tissue can sense the inductive signal by itself (reviewed in Berking 1998). Since anteriorly metamorphosed larvae should not contain GLWamides but are able to undergo further posterior metamorphosis, GLWamides should not be causally involved in metamorphosis. In the present study we refute these arguments and present further evidence for a major role of GLWamides as internal signals during metamorphosis of *H. echinata*. Experiments were also performed to elucidate the appearance of GLWamides during embryonic development and regeneration in parallel with the potency to undergo metamorphosis.

Materials and methods

Animals

Colonies of *H. echinata* were maintained and embryos were reared as described previously (Leitz and Wagner 1993). Metamorphosis bioassays with peptides were done as described (Leitz et al. 1994). To produce anteriorly metamorphosed specimens, larvae were incubated with 100 µM dioctanoylglycerol/10 µM cycloheximid for 3 h (Kroiher et al. 1991). Settlement of the larvae on coverslips in the petri dishes facilitated subsequent microscopy of the metamorphosed specimens.

For regeneration experiments, larvae were transversally cut at about one third of their length (measured from the anterior pole). After the time periods specified in the results section, regenerating animals were either fixed for immunohistochemistry or treated with 116 mM CsCl to check for their potency to undergo metamorphosis.

Peptides

Non-commercially available peptides were synthesised and high-performance liquid chromatography (HPLC)-purified by Richard Jacob in the protein and peptide group of the European Molecular Biological Laboratory (EMBL, Heidelberg, Germany). MMA (Leitz et al. 1994) and He-LWamide II (Gajewski et al. 1996) were from Bachem (Heidelberg, Germany).

Immunohistochemistry

H. echinata embryos, larvae and polyps were anaesthetised by incubating them for 30 min in a solution of 200 mM $MgCl_2$ in 50% artificial sea water. Subsequently, the anaesthetic solution was replaced two times by 4% paraformaldehyde in 100 mM sodium phosphate buffer, pH 7.2, followed by an overnight fixation at 4°C. The immunohistochemical procedure was as described (Schmich et al. 1998) using the anti-GLWamide antibody 1676IIIp. Controls were performed by (1) omission of the anti-GLWamide serum, and (2) by using antiserum preadsorbed with He-LWamide II coupled to Hi-Trap NHS-activated beads (Pharmacia, Freiburg, Germany) according to the instructions of the manufacturer. No staining was observed in either control. Additionally, the specificity of the primary antibodies was tested by enzyme linked immunosorbent assay (ELISA) or competitive enzyme immunoassay (EIA) and found to be exclusive for GLWamides (Schmich et al. 1998).

Miscellaneous

Percentages of metamorphosis are presented in the figures together with the respective 95% confidence intervals on the basis of a binomial distribution (Sachs 1992).

Results

Specificity of induction for the GLWamide terminus

Metamorphosis was dose-dependently induced by synthetic He-LWamide II (Fig. 1). The dose response curve strongly resembles the curves obtained with other GLWamides (Leitz et al. 1994; Gajewski et al. 1996). We found that the non-amidated analogue of He-LWamide II was completely inactive (Fig. 1). All GLWamide peptides tested to date by us and others were effective in inducing metamorphosis (Table 1) whereas other biologically active non-GLWamides had no inducing activity (Table 1; Gajewski et al. 1996). The results demonstrate the specificity of metamorphosis induction for GLWamides and the necessity for amidation.

Appearance of GLWamide immunoreactivity (IR) during embryonic development

The first signals detected by immunohistochemistry appeared 36 h post fertilisation (hpf; Fig. 2A). They were very small and were not assignable to a specific cell morphology. Instead, the appearance of the signals suggests the existence of immunoreactive varicosities in otherwise non-staining fibres, a situation typical for neuropeptide synthesising cells (Sossin et al. 1989; Ogaki et al. 1996). *H. echinata*-GLWamides are synthesised like other neuropeptides from a proprotein (Gajewski et al. 1996). The processing of proproteins to their amidated products occurs mainly during axonal transport of dense

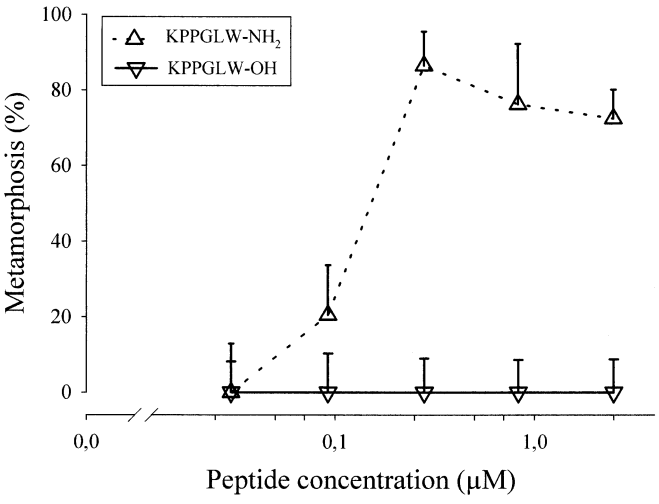
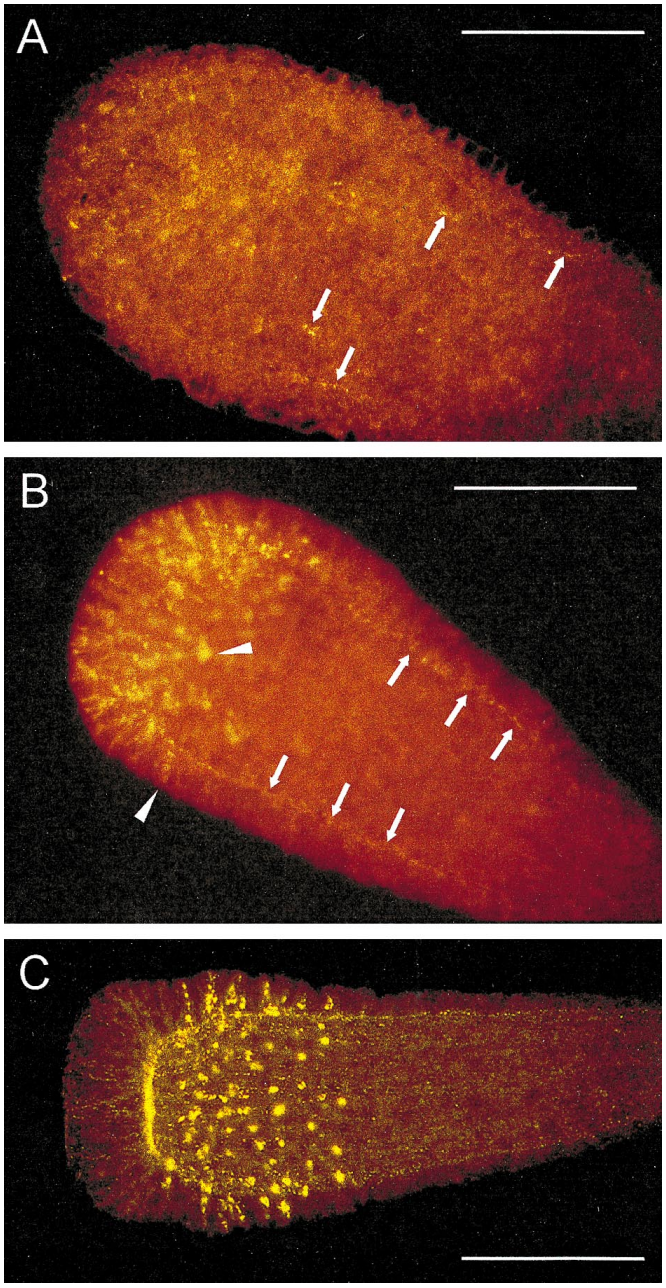


Fig. 1 Metamorphosis bioassays with synthetic He-LWamide II (KPPGLW-NH_2) and its non-amidated analogue. *Error bars* represent 95% confidence limits on the basis of a binomial distribution

Table 1 List of peptides tested for metamorphosis-inducing activity in *Hydractinia* spp. Other biologically active non GLWamide peptides tested were ineffective (see Gajewski et al. 1996)

Name of peptide	Amino acid sequence	Induction of metamorphosis
Metamorphosin A	pE–Q–P–G–L–W · NH_2	+[1]
	E–Q–P–G–L–W · NH_2	+[1]
	P–P–G–L–W · NH_2	+[2]
	A–K–P–P–G–L–W · NH_2	+[2]
Hym-53[3] (Hydra-LWamide IV[6])	N–P–Y–P–G–L–W · NH_2	+[3]
Hym-54[3] (Hydra-LWamide V[6])	G–P–M–T–G–L–W · NH_2	+[3]
Hym-248[3] (Hydra-LWamide II[6])	E–P–L–P–I–G–L–W · NH_2	+[3]
Hym-249[3] (Hydra-LWamide III[6])	K–P–I–P–G–L–W · NH_2	+[3]
Hym-331[3] (Hydra-LWamide I[6])	G–P–P–P–G–L–W · NH_2	+[3]
Hym-38[3] (Hydra-LWamide I[6])	G–P–P–P–G–L–W · NH_2	+[3]
	C–A–A–P–P–G–L–W · NH_2	+[4]
	Biotin–C_6–K–P–P–G–L–W · NH_2	+[4]
He-LWamide II	K–P–P–G–L–W · NH_2	+[5]
	K–P–P–G–L–W · OH	–[5]

P=Hydroxyproline
Data and terms are from [1] Leitz et al. (1994), [2] Gajewski et al. (1996), [3] Takahashi et al. (1997), [4] this study (data not shown), [5] this study (Fig. 1), [6] terms given by Leviev et al. (1997)



core vesicles (Sossin et al. 1989) such that most of the amidated immunoreactive material is localised in the fibres. Therefore, the GLWamide-immunoreactivity (-IR) is first confined to the fibres and is detected in the varicosities containing the highest amounts of material.

At 42 hpf the first GLWamide-IR cell bodies are visible (Fig. 2B). The fully developed, mature larva displays up to 40 GLWamide-IR sensory cells (Fig. 2C). This sequence of GLWamide appearance during development correlates with the established potency of differently aged larvae to metamorphose. At 30 hpf all embryos are not able to undergo metamorphosis. Beyond 48.5 hpf more than 70% of the larvae undergo metamorphosis upon induction with the artificial inducer CsCl. A plateau of 98–100% metamorphoses is reached for larvae older than 58 h (Plickert et al. 1988).

Appearance of GLWamide immunoreactivity during regeneration of the anterior part

Upon transversal sectioning of the larvae, posterior fragments are not able to react to either the natural or artificial stimuli (Müller et al. 1977; Schwoerer-Böhning et al. 1990). The larvae regain the potency to metamorphose with increasing time for regeneration (Schwoerer-Böhning et al. 1990; Fig. 3A). Upon transversal sectioning at about one third the length of the larva, GLWamide-IR pericarya are lacking from the posterior fragment. These fragments do not undergo metamorphosis when stimulated directly after cutting (Fig. 3A). Regenerates quickly redevelop GLWamide-IR pericarya (Fig. 3B). As apparent from Fig. 3, the reappearance of the new GLWamide-IR pericarya in the regenerating posterior fragment strongly correlates with the potency of

Fig. 2A–C Appearance of GLWamide-immunoreactivity during embryonic development. **A** Thirty-six hours post fertilisation (hpf), **B** 42 hpf, **C** 72 hpf planulae. The posterior part of the larvae is not shown in order to enlarge the anterior part where the cell bodies are found. *Arrows* denote immunoreactivities in fibres, *arrowheads* denote the positions of two immunoreactive cell bodies (*Bars* 150 µm)

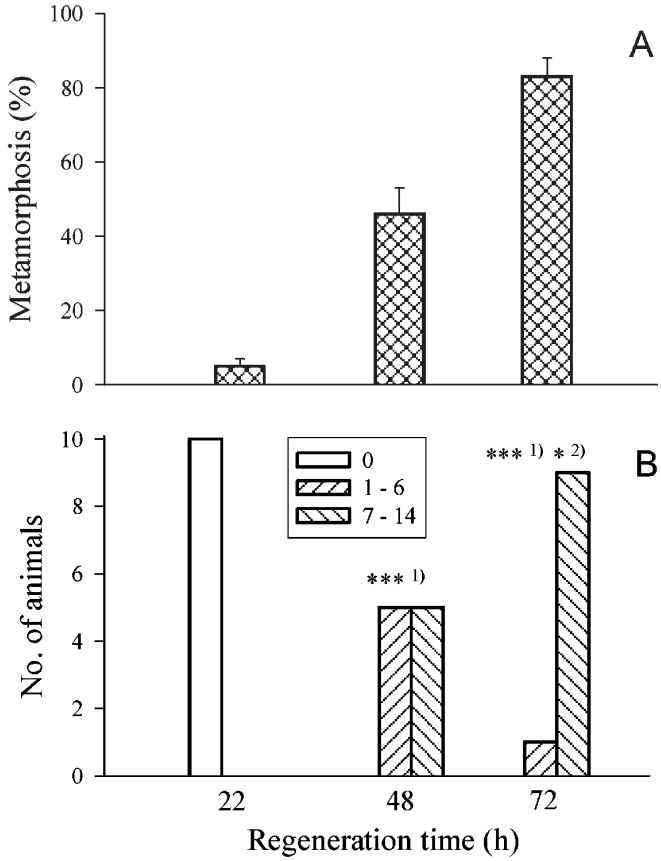


Fig. 3A, B Induction of metamorphosis in regenerating posterior fragments of *Hydractinia echinata* larvae. **A** Percentages of metamorphoses of regenerates treated for 3 h with 116 mM CsCl. *Error bars* represent 95% confidence limits on the basis of a binomial distribution. **B** Number of regenerated animals with either 0, 1–6 or 7–14 GLWamide-immunoreactive pericarya at different times after cutting. The significance of the difference between 22 h[1)] and 48 h[2)] is shown with * $P<0.05$ and *** $P<0.001$ (Student's t-test). All larvae used for the generation of the data in **A** and **B** were from the same batch

the respective animals to metamorphose. Twenty-two hours after cutting, the regenerates still lack GLWamide-IR pericarya and only a few animals are able to undergo metamorphosis. At later stages of regeneration the number of GLWamide-IR cell bodies increases and progressively more larvae undergo metamorphosis upon induction with CsCl.

Disappearance of GLWamide immunoreactivity during metamorphosis and reappearance in primary polyps

During the metamorphic process, the GLWamide-IR in sensory cells gradually disappears (Fig. 4) up to 8 h after the addition of an inducer, when no GLWamide-IR is found any more. We do not know if this is due to death of the cells or to discontinued GLWamide synthesis in otherwise unchanged cells. Subsequently, GLWamide-IR

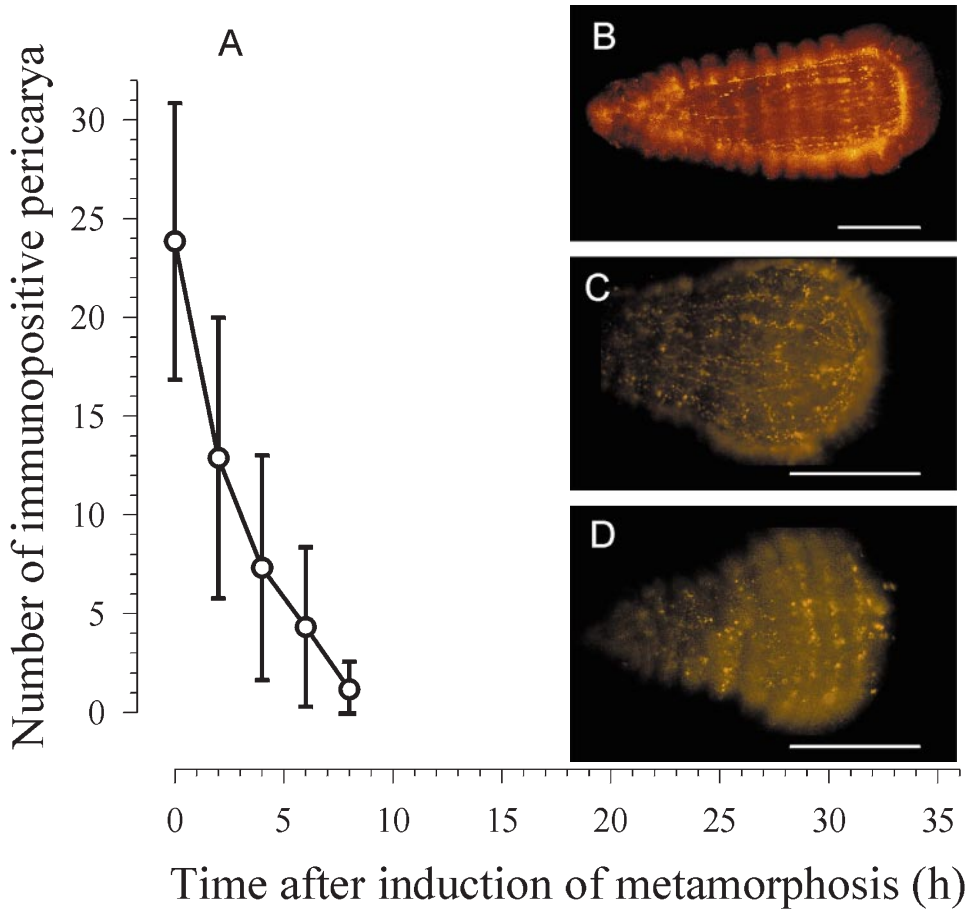


Fig. 4A–D Disappearance of GLWamide-immunoreactivity in anterior ectodermal sensory cells during metamorphosis. **A** Number of GLWamide-immunoreactive pericarya in metamorphosing larvae of *H. echinata* (mean±SD). **B** Immunohistochemical specimens at 2 h, **C** 4 h and **D** 6 h after induction of metamorphosis. For a non-metamorphosed larva compare Fig. 2C (*Bars* 100 µm)

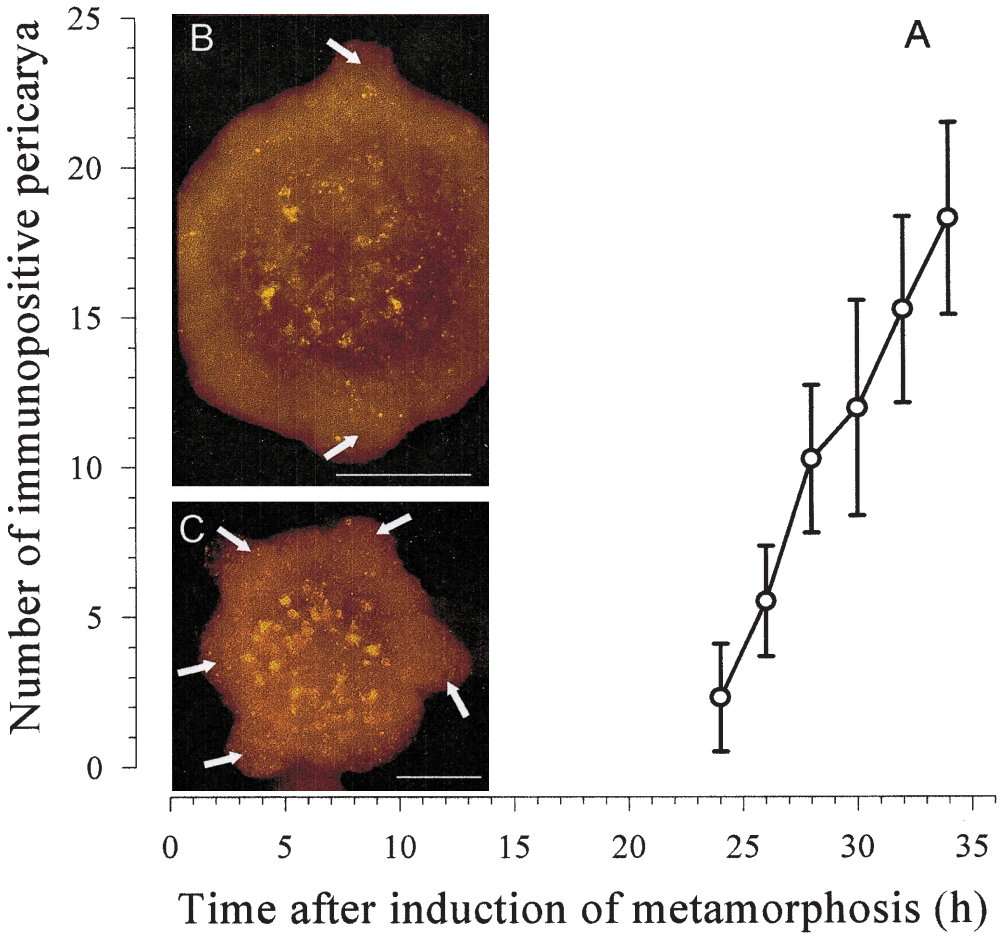


Fig. 5A–C Reappearance of GLWamide-immunoreactivity in endodermal hypostomal cells of the primary polyp. **A** Number of GLWamide-immunoreactive pericarya in primary polyps of *H. echinata* (mean±SD). **B** 32 h and **C** 34 h after induction of metamorphosis Apical views representing optical cross sections through the hypostomal region. *Arrows* denote the tentacle buds (*Bars* 100 µm)

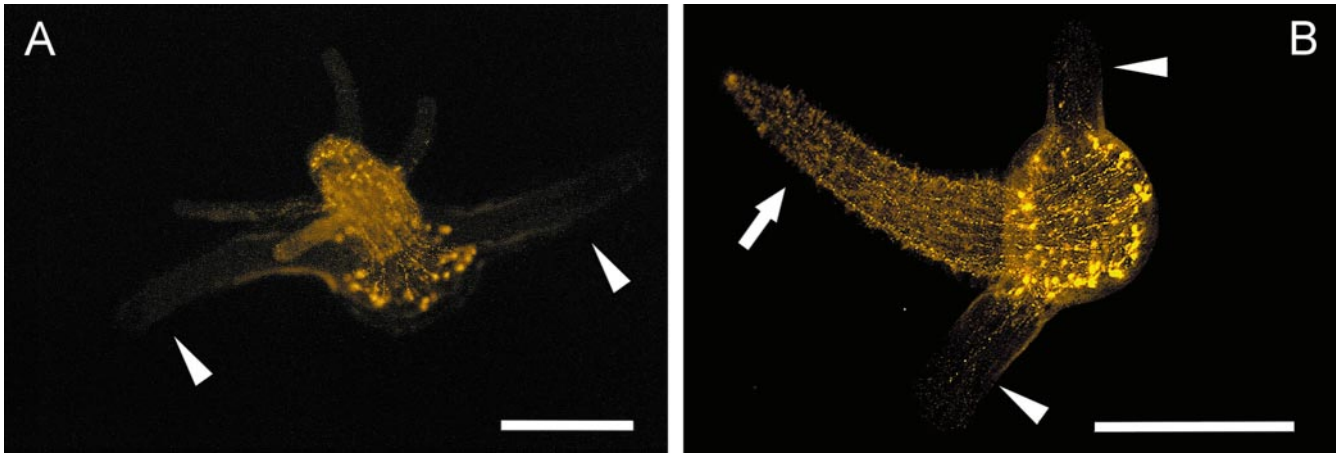


Fig. 6A, B GLWamide-immunoreactivity in sensory cells of primary polyps (**A**) and anteriorly metamorphosed specimens (**B**). Note: these GLWamide-immunoreactive sensory cells were thought to be a larval characteristic, but are now found in all anteriorly metamorphosed specimens and in very rare cases also in primary polyps (**A**). *Arrowheads* denote stolonal polyp tissue, the *arrow* in **B** denotes the larval posterior (*Bars* 200 µm)

gradually builds up in endodermal hypostome cells of the primary polyp (Fig. 5), which obviously are not identical to the cells stained in the larva (Gajewski et al. 1996; Schmich et. al. 1998). No cells resembling larval sensory cells were found in the vast majority of primary polyps. However, on very rare occasions, larval sensory cells were found in the basal plate region of primary polyps (Fig. 6A).

Presence of GLWamide immunoreactivity in anteriorly metamorphosed specimens

Anteriorly metamorphosed specimens bear GLWamide-IR sensory cells in their anterior part, which morphologically appears as a polyp basal plate (Fig. 6B). These

cells are not distinct from the GLWamide-IR cells in normal larvae. Their fibres also extend along the entire mesoglea. We never observed anteriorly metamorphosed specimens without GLWamide-IR sensory cells. Instead, on very rare occasions (<1%) we found primary polyps displaying GLWamide-IR sensory cells in their basal plate (Fig. 6A). They were nevertheless morphologically undiscernable from all other primary polyps.

Discussion

Internal metamorphic signals have long been sought for but definitive identifications had not been achieved. Schwoerer-Böhning et al. (1990) showed that posterior fragments of *Hydractinia echinata*, in contrast to anterior fragments, do not undergo metamorphosis when treated with inducers. Untreated posterior fragments grafted on to stimulated anterior fragments underwent metamorphosis. Since these pioneering experiments it was reasonable to assume that in larvae of *H. echinata* an internal signal is used to coordinate the metamorphic events. This signal should be produced by cells of the anterior part and be transmitted to most or all of the other cells to convey the information (Leitz 1997, 1998b for review). By using a biossay with posterior fragments, Leitz et al. (1994) succeeded in isolating an active compound from the heterologous source, *Anthopleura elegantissima*. It was identified as a neuropeptide with a so far unknown COOH-terminus, namely GLWamide. In search for the *H. echinata* autologous peptide, Gajewski et al. (1996) deduced the amino acid sequence of two closely related GLWamide peptides, He-LWamide I and II, from the cDNA structure of the precursor protein. Additional GLWamides were found in other cnidarian species either by peptide isolation or by precursor cDNA cloning and sequencing (Leviev and Grimmelikhuijzen 1995; Gajewski et al. 1996; Leviev et al. 1997; Takahashi et al. 1997).

As apparent from the present and other studies (Table 1) all synthetic GLWamides tested until now induce metamorphosis of *Hydractinia* sp. larvae. The specificity for induction is very high since the non-amidated analogue of He-LWamide II did not induce metamorphosis. Although the percentages of metamorphosis are variable depending on the larval batch, it can be concluded that the NH_2-terminus of the molecule does not have any influence on induction. Even an NH_2-terminally biotinylated analogue induced metamorphosis with the same efficiency (Table 1).

In larvae it was found that GLWamide-IR is confined to ectodermal sensory cells with pericarya in the anterior region and fibres along the mesoglea (Leitz and Lay 1995; Gajewski et al. 1996). Sensory cells were previously hypothesised to transmit the metamophic stimulus through the larva (Plickert 1990; Schwoerer-Böhning et al. 1990; Leitz 1993). The GLWamide-IR pattern nicely fits the proposition of GLWamides as the internal signal released from sensory cells (Leitz et al. 1994; Leitz and Lay 1995).

Berking and coworkers questioned this hypothesis on the basis of their studies with anteriorly metamorphosed specimens (Berking and Walther 1994; Walther et al. 1996). These animals should not have GLWamide-IR neurons but are able to undergo additional posterior metamorphosis (see introduction). We now demonstrate GLWamide-IR sensory cells in the anteriorly metamorphosed larval part (Fig. 6B). This fits our hypothesis of stimulation of these cells as a very early event during metamorphosis. Accordingly, the anteriorly metamorphosed specimens undergo metamorphosis because, upon induction, GLWamides are released from their sensory cells and lead to the metamorphic events in the remaining posterior larval part of the mosaics.

The sensory cells in the larval anterior part were previously thought to be characteristic for larval tissue. The existence of these cells in metamorphosed tissue and even in morphologically normally developed primary polyps (Fig. 6A) shines new light on the plasticity of metamorphic events. Although primary polyps with persistent larval sensory cells are very rare, they nevertheless do exist. This raises the questions whether it is purely malregulation or whether these cells serve any special function in those “abnormal” animals. Is there no exact control mechanism for loss or persistence of cells? What determines the normal outcome of a primary polyp? There could be signals for down-regulation of the sensory cells in “normal” metamorphosis. Since all anteriorly metamorphosed larvae bear larval sensory cells it would be reasonable to assume that these signals originate from an intact polyp head region. These signals could have been lost or changed in the “abnormal” specimens. On the other hand, metamorphosis at least in hydrozoans could also be a very plastic event resulting in a wide range of individuals differing with respect to their cellular composition. It would be interesting to find out how these differently organised individuals are able to build morphologically undistinguishable colonies. Unfortunately, live primary polyps bearing larval sensory GLWamide-IR cells are not distinguishable from their “normal” counterparts and are therefore not suitable for experimental manipulations.

The presence of GLWamide-IR pericarya is a prerequisite for induction of metamorphosis. It was found that embryos having not yet developed GLWamide immunoreactivity are not able to undergo metamorphosis, whereas the full potency to metamorphose is reached when GLWamide-IR is maximal (Fig. 2). Likewise, posterior fragments lacking GLWamide-IR pericarya do not undergo metamorphosis, whereas upon regeneration of GLWamide-IR pericarya they are able to metamorphose (Fig. 3).

We have presented evidence for an important role of GLWamides in metamorphosis of *H. echinata*. Synthetic GLWamides induce metamorphosis of planulae dose-dependently. With respect to peptides the induction is specific for GLWamides. Even non-amidated analogues are ineffective. The presence of GLWamide-IR sensory cells is a prerequisite for induction. Taken together, these re-

sults strengthen our hypothesis about the release of GLWamides as a reaction to the bacterial inducer. According to this hypothesis the bacterial inducer should bind to the sensory cells stimulating the PI-cycle and protein kinase C (Schneider and Leitz 1994; Leitz 1997). Protein kinase C is known as an important regulator of secretion (Majewski et al. 1997); therefore it may be directly involved in the release of the GLWamides. This release is dependent on functional pericarya but may occur either at nerve endings or all along the fibres at varicosities. The GLWamides could either interact with all cells of the larva or, more likely, with a specific subset(s) of the cellular inventory. Studies shall be performed to identify the bacterial inducer and its targets as well as the targets of the GLWamides and their mechanism of action in order to elucidate the cellular interaction network that is turned on during the induction of metamorphosis.

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