Int. J. Dev. Biol. 47: 439-450 (2003)

Original Article

The role of α-amidated neuropeptides in hydroid development - LWamides and metamorphosis in *Hydractinia echinata*

GÜNTER PLICKERT*,[1], EVA SCHETTER[2], NICOLE VERHEY-VAN-WIJK[3], JÖRG SCHLOSSHERR[4], MARLIS STEINBÜCHEL[5] and MARTIN GAJEWSKI[6]

[1]Institut für Zoologie der Universität zu Köln, Köln, Germany, [2]Köln, Germany, [3]Max-Planck-Institut für Molekulare Genetik, Berlin, Germany, [4]Qiagen GmbH, Hilden, Germany, [5]Köln, Germany and [6]Institut für Genetik der Universität zu Köln, Germany

ABSTRACT Peptides are increasingly attracting attention as primary signals in the control of development. Even though a large number of peptides have been characterized in cnidarians, little experimental evidence addresses their endogenous role. The life cycle of *Hydractinia echinata* includes metamorphosis from planula larva into the adult stage of the polyp. This process of stage conversion includes internal signalling, which controls cell cycle activity, cell differentiation, cell death and proportion-controlled morphogenesis. LWamide peptides are considered to be part of the control system. We implemented methods to silence gene activity by dsRNAi in *Hydractinia* and show a substantial knock-down of *LWamide* gene activity. In addition, LWamide function was knocked-out pharmacologically by targeting the biosynthesis of amidated peptides and thus preventing functional LWamides. Here we show that extinction of bioactive LWamides from planulae causes loss of metamorphosis competence, a deficiency which can be rescued by synthetic LWamide peptides. Thus, it is shown that LWamides are indispensable and act by conveying outer metamorphosis stimuli to target cells within the animal. Considering non-availability of genetic analysis and the so-far limited success in expressing transgenes in hydroids, gene functions are difficult to analyse in hydroids. The approach as outlined here is suitable for functional analysis of genes encoding amidated peptides in hydroids.

KEY WORDS: *neuropeptide, amidation, LWamides, dsRNA-mediated interference (RNAi), Hydractinia*

Introduction

During the development of multicellular organisms, cells communicate via sectreted or membrane-resident signals to ensure coordinated behaviour and appropriate spatiotemporal patterns of differentiation. Most known signalling molecules belong to some few well conserved protein families They usually consist of more than 50 amino acid residues. In *Hydra*, the major proteins of the Wnt/Frizzled pathway have been identified as cloned cDNAs (Hobmayer *et al.*, 2000) showing that conservation extends even to the most basic metazoan phylum Cnidaria. A great variety of amidated peptides has been isolated from Cnidaria in native form or as deduced amino acid sequence from cloned cDNAs (Grimmelikhuijzen *et al.*, 1989; Grimmelikhuijzen *et al.*, 1994). Initially, peptides were assayed primarily for a possible function in neurotransmission as they had been attributed a role in physiological processes (McFarlane *et al.*, 1991). Since the isolation of the *hydra* peptide "head activator", however, peptides are discussed also as primary signals in hydroid development (Schaller and Bodenmüller, 1981). Pursueing this concept, Takahashi and collaborators discovered many different peptides in *Hydra* (Takahashi *et al.*, 1997). In recent studies amidated peptides were proposed to participate in basal and apical fate specification and to constitute signals in cell differentiation in *Hydra* (Hoffmeister, 1996; Lohmann and Bosch, 2000; Takahashi *et al.*, 2000).

In the colonial hydroid *Hydractinia*, the complex process of planula metamorphosis into a primary polyp has been suggested to be under control of peptides (Leitz *et al.*, 1994; Leitz, 1997; Schmich *et al.*, 1998). Anterior fragments of planulae of *Hydractinia* induced to metamorphosis convey a metamorphosis stimulus to non-induced posterior fragments if combined by transplantation (Schwoerer-Böhning *et al.*, 1990). This internal signal could be

Abbreviations used in this paper: DDC, diethyldithiocarbamate; dsRNA, double stranded RNA; He-LWamide, Hydractinia echinata-Leu-Trp-NH_2; hpf, hours post-fertilization; PHM, petidylglycine α-hydroxylating mono-oxygenase; RFamide, Arg-Phe-NH_2.

*Address correspondence to: Prof. Dr. Günter Plickert. Inst. Zoology, Weyertal 119, D-50923 Köln, Germany. Fax: +49-221-470-6928. e-mail: g.plickert@uni-koeln.de

0214-6282/2003/$25.00

Printed in Spain
www.ijdb.ehu.es

substituted by a peptide isolated from the anthozoan *Anthopleura elegantissima*, pEQPGLWamide, termed therefore Metamorphosin A (Leitz *et al.*, 1994). By cDNA cloning, two LWamide-peptides, He-LWamide I and He-LWamide II were then described in *Hydractinia*. At least 9 further LWamide peptides were identified by cDNA-cloning from the anthozoans *Actinia equina* and *Anemonia sulcata* showing that LWamides constitute an entire novel family of peptides (Gajewski, 1995, Gajewski *et al.*, 1996). It has been proposed that LWamides act as internal signals carrying the information of a perceived exogenous metamorphosis stimulus from the anterior part of a *Hydractinia* planula to its posterior body as central key molecules of metamorphosis control (Leitz, 1997). This has been doubted by others based on the observation that metamorphosis does not depend on an existing anterior end which include the LWamide-secreting cells (Berking and Walther, 1994; Walther *et al.*, 1996). Moreover, LWamides are not only expressed in anterior neurosensory cells of the planula where they could function in the proposed way to signal metamorphosis activation but also in endodermal cells of the polyp stage (Gajewski *et al.*, 1996). LWamides occur in polyps of sea anemones (Leitz *et al.*, 1994; Leviev and Grimmelikhuijzen, 1995) where they must have different functions. LWamides have been isolated from *Hydra*, which does not even include a larva in its life cycle (Leviev *et al.*, 1997). LWamides must be considered to be multifunctional. It is not evident, per se, from the inducing capacity of the peptide whether or not LWamides are indeed essential in metamorphosis.

To address this question and, in general, what function other peptides may have, we implemented a gene knock-out procedure by microinjection of dsRNA or on a pharmacological basis. Since C-terminal amidation is the rate-limiting step in peptide biosynthesis (Eipper *et al.*, 1993) and likely essential for receptor binding and activation (rev. by Kulathila *et al.*, 1999) this final step of processing was targeted. The results show that LWamides are required as indispensible control molecules in metamorphosis.

Results

Neuropeptide precursors are expressed individually during development of Hydractinia echinata*; kinetics fit a possible role of LWamides during metamorphosis*

LWamide neurosecretory and RFamide immunoreactive nerve cells occur both in the anterior part of developing and mature larvae (Plickert, 1989; Leitz and Lay, 1995; Gajewski *et al.*, 1996). Only

TABLE 1

EXPRESSION OF NEUROPEPTIDE PRECURSORS DURING EMBRYONIC DEVELOPMENT OF *HYDRACTINIA ECHINATA*

Time after fertilization (h)	No. of animals analysed	No. of cells/animal expressing precursor mRNA ±SD	Relative intensity of hybridization signal +, ++, +++, ++++ *
a) Expression of LWamide precursor			
20	24	0	
24	54	1 ± 2	++
28	51	17 ±14	++++
48	50	18 ± 9	++
72	9	6 ± 5	+
b) Expression of multiple neuropeptide precursor			
20	14	0	
28	23	0 ± 4	++
30	19	3 ± 4	++
32	32	4 ± 4	+++
34	37	15 ± 9	++++
49	20	42 ±16	++++
72	20	50 ±12	++++

*Arbitrary units of staining intensity after chromogenic substrate reaction from +: staining detectable in the cytoplasm but too faint to negatively contrast the nucleus of the cell to ++++: maximum staining intensity observed - the shape of nucleus of the labelled cells is obscured by label.

LWamides are expected to be involved in metamorphosis control since RFamides did not induce metamorphosis (Gajewski *et al.*, 1996 and unpblished results). The *Hydractinia* RFamide pEWLKGRFamide has the same structure as Pol-RFamide II from the hydromedusa *Polyorchis penicillatus* (Schmutzler *et al.*, 1994). It is encoded in a multiple precursor (multiple neuropeptide precursor) together with three further peptides, He-RYamide, He-RNamide and He-C-terminal peptide (Gajewski *et al.*, 1998). The function of these peptides is unknown. To discover periods of requirement for LWamides and the peptides of the multiple precursor expression kinetics were studied during embryogenesis and metamorphosis. The data obtained were used for proper timing of the inhibition studies.

During embryogenesis (at 18°C), LWamide expression commences 24 h after fertilization and thus 4 h earlier than the expression of the multiple neuropeptide-precursor. Inferred from the number of cells detectable by *in situ* hybridization (ISH) and from the apparent amount of precursor mRNA in these cells, LWamide expression was most pronounced 28h-32 h after onset of embryogenesis. Expression decreased to weak levels in mature larvae of 72 h age (Fig. 1).

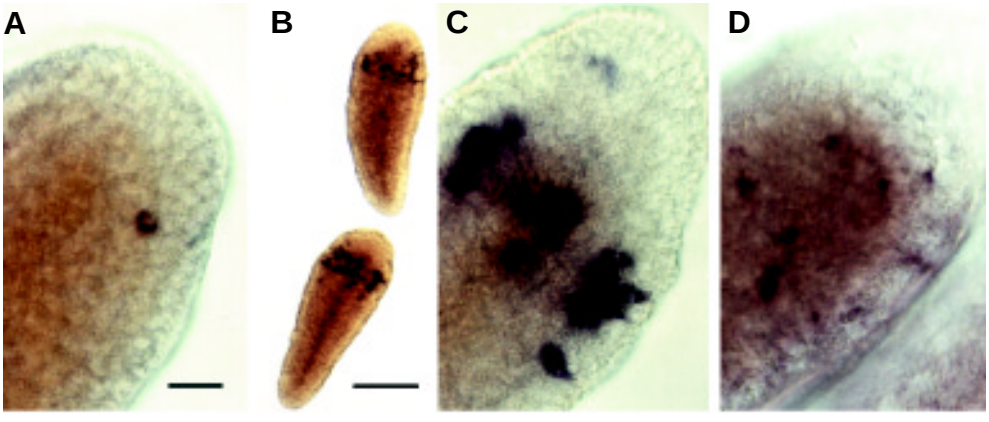

Fig. 1. Expression kinetics of *He-LWamides* during embryonic development of *Hydractinia echinata*. *At 18°C, expression becomes detectable in single cells by means of* in situ *hybridization 24 h post fertilization* **(A)**. *Expression yields maximum levels 28 h* **(B,C)** *to 35 hpf and decreases to weak levels in mature planulae at 72 hpf* **(D)**. *The sense probe did not produce any hybridization signal. Bar in A: 20 µm; same magnification as in C and D. Bar in B,100 µm. Staining intensity as referred to in Table 1 is ++ in A, ++++ in B,C and + in D.*

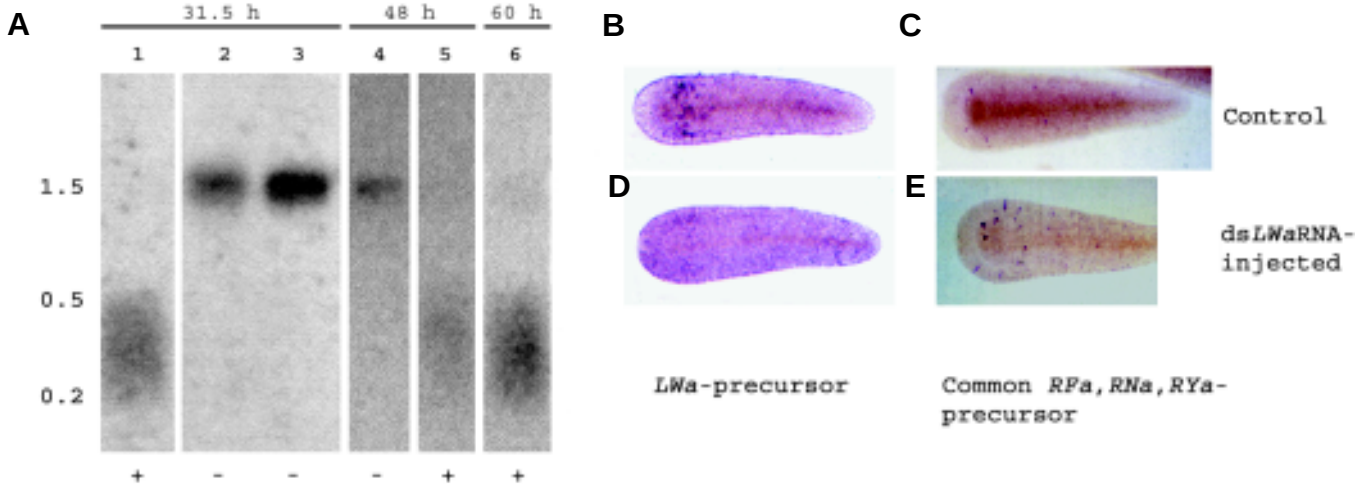

Fig. 2. Analysis of dsRNA interference in *Hydractinia echinata*. *Fertilized eggs were injected with 7x10⁸ molecules of dsRNA transcribed from the 5´-terminal 506 bp of* Hydractinia *LWamide precursor cDNA including the UTR and part of the protein coding region.* **(A)** *For Northern analysis, total RNAs were prepared from 77 (1), 154 (2), 231 (3) and 117 (4,5,6) embryos at the indicated times after fertilization and hybridized to an LWamide precursor cDNA probe.* LWamide *mRNA of 1500 nt size is obvious in samples from non-injected embryos (-), while it is absent in injected embryos (+) at 31.5 h (lane 1) and 48 h (lane 5). Transcripts reappear again as a in samples from 60 h embryos (lane 6, faint band). DsRNA originally sized 506 bp, reappears in samples from injected animals in original size and degraded down to 200 bp. Expression of* He-LWamide *precursor mRNA* **(B,D)** *and of mRNA of the multiple precursor for the* Hydractinia *RFamide (=Pol-RFamide II), He-RY-amide, and of He-RNamide* **(C,E)**. In situ *hybridization was performed in preplanulae 34 h post fertilization.*

Multiple neuropeptide precursor expression, in contrast, increased steadily during embryogenesis and subsequently was maintained at elevated levels in mature (72 h) and ageing larvae (Table 1, Fig. 4). As expression of the two precursors is clearly different during embryonic development it also differs during metamorphosis. A common feature, however, was obvious. Both genes transiently arrested expression in the passing larva stage and recommenced activity in the developing primary polyp. Neither cells expressing LWamides nor cells expressing the multiple neuropeptide precursor could be detected by ISH 6.5 – 8 h after removal of CsCl (referred to as onset of metamorphosis, Table 2). As soon as metamorphosis started, RFamide nerve cells began to change shape and became fragmented. They obviously were removed by cell death. Cell numbers of RFamide cells and precursor mRNA abundance were observed to decrease steadily from the very beginning of metamorphosis. Remarkably different from that, LWamide expression first increased 3-4 h after onset of metamorphosis before eventually ceasing down to non-detectable levels (Table 2). Gene re-activation in the developing polyp was different, too. Both genes are expressed in ectodermal cells of the larva stage. In the nascent polyp stage LWamides are expressed in endodermal cells while cells expressing the multiple neuropeptide precurser are in the ectoderm. Expression of the multiple neuropeptide precursor recommenced by 15- 16 h after onset of metamorphosisand. LWamide precursor mRNA became clearly detectable already 3 to 4 h earlier. Though both precursors are initially expressed in the same spatial area of the larva, expression kinetics substantially diverge later on.

Transcripts for LWamide precursor protein are knocked-down by dsLWamide RNA - dsRNAi is specific and efficient but transient

DsRNA was prepared from two different parts of the LWamide cDNA. 5´LWamide dsRNA was derived from cDNA spanning the 5´untranslated region and part of the protein coding region (Gajewski *et al.*, 1996) This 5´part was also used to generate a probe for Northern analysis. DsRNA was also prepared from a fragment of the cDNA flanked by two *Sst*I-restriction sites (*Sst* I-LWamide dsRNA) encoding almost all propeptide units of the precursor. Both dsRNA-preparations were used for metamorphosis experiments. To evaluate the effect of RNAi in treated specimens by *in situ* hybridization a probe was generated from the 3´part of the cDNA. It did not hybridize to injected 5- or *Sst* I-LWamide dsRNA.

TABLE 2

EXPRESSION OF NEUROPEPTIDE PRECURSORS DURING METAMORPHOSIS OF *HYDRACTINIA ECHINATA* [a]

	Multiple neuropeptide precursor		LWamide precursor	
Time (h) after onset of metamorphosis, inducer (CsCl)	No. of animals analysed	Cells/animal expressing precursor mRNA±SD	No. of animals analysed	Cells/animal expressing precursor mRNA±SD
Experiment I				
0	12	18 ± 8	19	4 ± 5
3.5	20	15 ± 8	20	12 ±11
6.5	20	0 ± 1	20	1 ± 2
10	15	0 ± 1	18	1 ± 1
13	9	0 ± 1	11	8 ± 5
15.5	19	9 ± 6	12	15 ± 7
Experiment II				
2	22	23 ±10	27	5 ± 5
4	19	10 ± 7	23	10 ± 8
6	34	6 ± 5	35	3 ± 5
9	20	0	16	0 ± 0
11	14	1 ± 1	11	3 ± 4
13	6	1 ± 1	4	7 ± 6
16	13	10 ± 6	12	18 ±14

a) Data sets of two independent experiments are shown. Expression analysis of both neuropeptide precursors was performed in parallel in animals of the same batch.

Northern Analysis. DsRNA interference is effective in knocking-down LWamide gene activity. Total RNA from injected embryos of 31.5 h age did not contain detectable quantities of endogenous LWamide transcripts even though expression of the gene is most active just about that time (Table 1). The effect was, however, transient. While in RNA-preparations from injected 48 h embryos endogenous LWamide transcripts were not detectable expression started to recover in embryos of 60 h age as indicated by a faint signal in the Northern blot (Fig. 2A, Lane 6). The observed re-appearance of endogeous transcripts was not due to a possibly effect-limiting half-life of the dsRNA. DsRNA could still be detected 60 hours after injection in RNA preparations from injected embryos (Fig. 2A). Its size distribution indicates partial degradation of the material but also presence of yet undegraded, originally sized dsRNA.

ISH Analysis. Knock down of expression was obvious on the level of individual cells, too. In 39 out of 44 injected embryos expression levels were quenched down to background level (Fig. 2D). In 5 embryos, LWamide-expressing cells could be identified and counted since expression levels were above background. Compared to normal expression in not-injected specimens levels were reduced substantially. In uninjected control animals 21±6 cells were detected. Only half the normal numbers of LWamide cells per larva (11 ±1) were observed in these five specimens. Numbers of cells expressing the multiple neuropeptide precursor were, in contrast, not different in LWamide dsRNAi-treated and non-treated specimens (Fig. 2C). Also the amount of endogenous mRNA of the precursor was not affected visibly (Fig. 2E). The effect of LWamide dsRNAi is highly specific to transcripts of the corresponding gene.

LWamide dsRNA interference reduces metamorphosis rates in Hydractinia

DsRNAi-treated animals were tested for metamorphosis competence by treatment with CsCl (28 mM, 3 h). Inducibility was probed 44.5 h or 48 h after fertilization. Significantly reduced metamorphosis was observed as a consequence of dsRNAi by *Sst* I-LWamide dsRNA when induction was done at 44.5 h. In contrast, if animals prepared by the same dsRNA injection were tested for competence only 3.5 h later no difference as compared to the non-injected control group was observed (Table 3). A third batch of animals was prepared by injection of 5´LWamide dsRNA and analysed for metamorphosis competence also 48 h after fertilization/ dsRNA injection. 24 h after induction the numbers of undoubtedly identifiable metamorphosed animals were significantly different (as, respectively, were the numbers of non-metamorphosed larvae). This initial difference, however, did not persist (analysis 48 h after induction) since most of the animals not stageable at 24 h after induction progressed in development the following 24 h and completed metamorphosis. Accordingly, the percentage of animals with uncertain stage identity, i.e., animals within the process of transformation was different in the experimental groups in which dsRNAi was effective (Table 3). Reduction of LWamide transcripts due to dsRNAi obviously causes rates of metamorphosis to decrease but apparently causes also the process of metamorphosis to slow down.

The hydroid α-amidating enzyme as a target for functional knock-out of amidated peptides

Extracts of Hydractinia echinata contain α–amidating enzyme activity. Artificial peptidylglycine substrate dansyl-D-Tyr-Val-Gly when added to extracts and supplemented with the required co-factors is converted into the corresponding dansyl-dipeptide amide dansyl-D-Tyr-Val-NH_2 (Fig. 3 A,B). Both enzymes, peptidylglycine α-hydroxylating monooxygenase (PHM) and peptidyl-alpha-hydroxylating lyase (PAL) known to catalyze amidation must therefore be contained in the extracts. Vertebrate PHM requires copper (II) ion for activity. Copper chelators, such as Diethyl-dithiocarbamate (DDC) and its disulfide Tetra-ethyl-thiuram-disulfide (TETD or Disulfiram or antabuse) reversibly inhibit

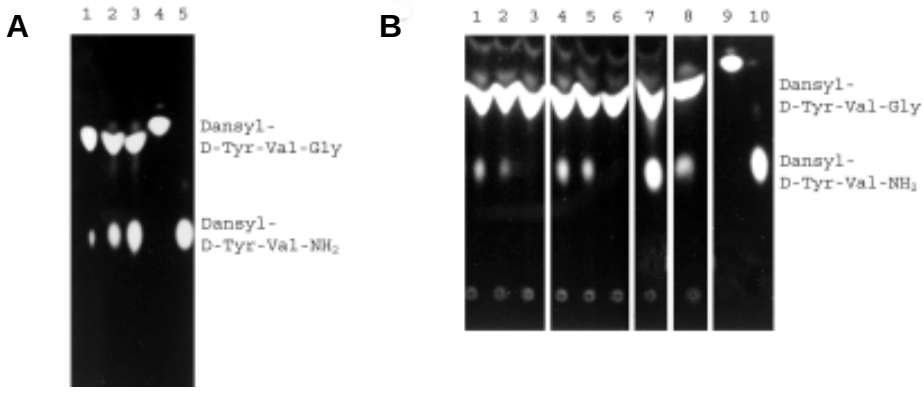

Fig. 3. Evidence for hydroid α-amidating enzyme. (A) *Crude extracts from adult polyps of* Hydractinia echinata *were assayed for amidating activity by analysing conversion of the artificial, stable peptidylglycine substrate dansyl-D-Tyr-Val-Gly into its corresponding amidated product dansyl-D-Tyr-Valamide (according to Bendig, 1986) after 1.5 h (1) or 17 h (2) at 22°C. 10 nM each of dansylated substrate standard (4) and product standard (5) were co-analysed as a reference. A second reaction sample having proceeded for 17 h was supplemented with 5 nM dansyl-D-Tyr-Val amide as an internal standard that co-localizes with the expected dansyl-D-Tyr-Val amide (lane 3).* **(B)** *Conversion of substrate peptide is inhibited by Diethyldithiocarbamate (DDC) 2 μM (1,4), 20 μM (2,5) or 200 μM (3,6) The amount o f product is decreased as compared to reactions without DDC (8). DDC inhibition is compensated for by copper added to the reaction (0.5 μM $CuSO_4$) (4,5,6). Even 200 μM DDC did not inhibit amidation when copper was added in surplus (1 mM $CuSO_4$) (lane 7). Note that despite the presence of the inhibitor, even more product than in the control reaction (8) formed due to copper supplementation. 10 nM each of dansylated substrate standard (9) and product standard (10) were co-analysed as a reference.*

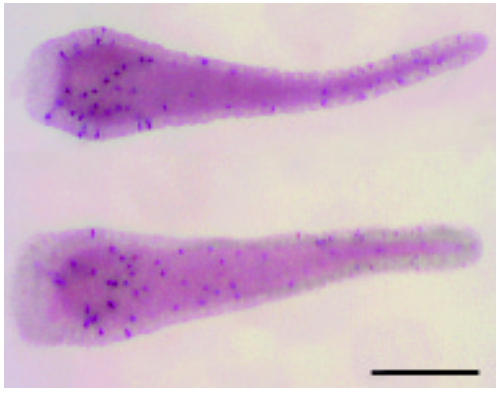

Fig. 4. Expression of multiple neuropeptide precursor for *Hydractinia* RFamide (Pol-RFamide II), He-RNamide and He-RYamide in planula larvae of *Hydractinia echinata*. *72 h after fertilization, approximately 55/65 cells in which the gene is active are detected per individual by means of* in situ *hybridization. Note the gradient of labelling intensity indicating maximum gene activity in anterior nerve cells (left) as compared with cells in the larva tail (right). The bar corresponds to 100 μm.*

amidation by preventing hydroxylation as the rate-limiting step of the reaction (Mains *et al.*, 1986). As vertebrate PHM the coelenterate enzyme can be inhibited *in vitro* by the copper chelating agent DDC. The observed inhibition is reverted if copper is added in surplus to the reaction (Fig. 3B, Lane 7)

In vivo *inhibition of hydroid α-amidating enzyme prevents formation of immunoreactive RFamide but not expression of its precursor gene - evidence for post-transcriptional control of neuropeptide biosynthesis*

Different known inhibitors of PHM were used to inhibit cnidarian PHM *in vivo*. Inhibition was assayed by an antibody specific for the amidated moiety RFamide. Since this antibody does neither react with Arg-Phe-COOH nor with Arg-Phe-X (Grimmelikhuijzen and Graff, 1986) effective inhibition of amidation is expected to result in loss of RFamide immunoreactivity. Inhibitor treatment was started 20 h after fertilization and thus preceding the onset of multiple neuropeptide precursor gene activity (Table 1). Planulae were analysed 72 h after fertilization or later. Treatment with TETD and DDC effectively prevented RFamide immunoreactivity while at the same time mRNA-expression of the precursor was not altered significantly (Table 4). In single of the TETD-treated specimens, very little residual immunoreactivity was observed in nerve processes in the frontal-most part of the nerve net. This was restricted to the region where in untreated animals nerve cell processes immunoreactive to RFamide antibody occur in the highest density and displaying the strongest immunoreactivity (Plickert, 1989).

4-phenyl-3-butenoic acid known to inhibit *in vivo* amidation in vertebrates (Bradbury *et al.*, 1990; Mueller *et al.*, 1999) was less effective in hydroids than DDC and TETD. Concentrations greater than 300 μM were necessary to inhibit amidation significantly and to reduce numbers of RFamide immunoreactive cells from control level (10 cells/animal) to 3-4 cells. To quench RFamide immunoreactivity completely even mM concentrations had to be applied (data not shown).

Comparison of *in situ* hybridization data and data from immunocytochemistry analysis in sibling specimens indicated important features of RFamide expression. As a rule, the number of ISH-positive cells was approximately three to five times the number of immunoreactive cells. Even though cell numbers considerably varied from animal to animal and in particular between different embryo batches the difference between ISH and ICC data was striking. By *in situ* hybridization, 30-95 cells per larva were observed in untreated animals and at 72-76 h after fertilization (data from 22 experiments comprising 460 larvae). In contrast, only 3-35 cells per animal were observed by immunocytochemistry (data from 35 experiments including 480 individual animals). Thus, the majority of nerve cells expressing the multiple neuropeptide precursor gene does not contain detectable quantities of amidated and thus bioactive peptide. Expression control by transcription is tuned by at least one further post-transcriptional mechanism of control. The intensity of the *in situ* hybridization signal was observed to vary along the anterior-posterior axis of the larva indicat-

TABLE 3

dsLW AMIDE RNA-INTERFERENCE AND METAMORPHOSIS INDUCTION

	***Sst* I-LWamide dsRNA**				**5´LWamide dsRNA**	
	Metamorphosis induction (28 mM CsCl, 3 h) at					
	44.5 h post fertilization		48 h post fertilization		48 h post fertilization	
	Control	Injected	Control	Injected	Control	Injected
% metamorphosis	76 (80)	25* (31*)	82 (91)	72 (82)	67 (72)	40* (59)
% larvae	20 (17)	58* (56*)	10 (7)	19(14)	29 (24)	40* (33)
% uncertain stage identity	4 (3)	17* (13)	8 (2)	9 (4)	5 (4)	20* (9)
No. of animals investigated	557 (551)	116 (113)	762 (760)	154 (154)	490 (479)	127 (126)

Analysis 24 h (48 h) after induction of metamorphosis. *) Significantly different from control value at 5% level (χ^2 analysis).

TABLE 4

MULTIPLE NEUROPEPTIDE PRECURSOR mRNA-EXPRESSION AND RFAMIDE-IMMUNOREACTIVITY AFTER TREATMENT WITH TETD OR DDC[a]

Type and time of analysis	**Treatment (20 h after fertilization until time of analysis)**						
	control,sea water	**control, 0.02% EtOH**	**0.5 μM TETD**	**1.0 μM TETD**	**2.0 μM TETD**	**20 μM DDC**	**50 μM DDC**
			Experiment I				
ISH, 72 h	51±15	56±13	47±12	31±13	30±11	54±14	58±10
ICC, 72 h	13± 7	14± 6	0± 0	0± 0	0	0	0
			Experiment II				
ISH, 76 h	57±13	51± 8	53±17	48±12	42±13	64±18	54±13
ICC, 76 h	12± 5	6± 5	0± 1	1± 1	2± 3	0± 0	0
ICC,138 h	11± 5	5± 2	1± 2	0± 0	0	0	0

a) Average number of cells/animal detected by *in situ* hybridization (ISH) or by immunocytochemistry (ICC) ± standard deviation. Note that for *in situ* hybridization a probe was used that detects precusor mRNA while the antibody used for immunocytochemistry is specific for carboxyamidated Arg-Phe. For every mean value 20 specimens were analysed. 0±0 means that one or some very few of the specimens contained single cells with detectable amounts of immunoreactive RFamide. 0 indicates that in none of the 20 specimens any RFamide cells were observed. Experiment I and II are independent experiments performed with two different batches of embryos.

ing different contents of multiple neuropeptide precursor mRNA in individual nerve cells. The gene thus shows graded activity from an anterior maximum to a posterior minimum (Fig. 4). As the number of RFamide cells detected by *in situ* hybridization was not changed significantly by TETD or DDC treatment (Table 4), this graded pattern of gene activity was not either.

In sum, coelenterate PHM requires copper as do the enzymes from vertebrates or from higher invertebrates. It can be functionally knocked out by copper chelators as assayed by an amidation-specific antibody. *In vivo* inhibition resulted in a complete loss of RFamide while the treatment did not alter expression of its multiple neuropeptide precursor on the mRNA level. Under normal conditions, the majority of the cells that express the precursor gene in terms of mRNA do not appear as immunoreactive cells.

Pharmacological knock out of PHM prevents inducibility by bacteria or CsCl - the affected mechanism is reversible and requires copper

Among the various natural and artificial inducers of metamorphosis in *Hydractinia echinata*. are bacteria (Müller, 1973; Leitz and Wagner, 1993). Routinely used very potent among monovalent cations and other artificial inducers is CsCl (Müller *et al.*, 1977). Numerous developmental studies on metamorphosis and pattern formation are based on metamorphosis induction by CsCl. By the LWamides peptides with inducing capacity were discovered (Leitz *et al.*, 1994). They occur as endogenous He-LWamides in *Hydractinia* and thus may be involved in the internal control of this morphogenetic process (Gajewski *et al.*, 1996; Schmich *et al.*, 1998). By inhibiting PHM and therefore synthesis of endogenous bioactive peptide amides including LWamides these three modes of induction were investigated with respect to requirement for peptide amides.

The internal pathways the inducers CsCl and bacteria (*Pseudoalteromonas espeijana*) activate and which eventually result in the transformation of larva into adult tissues are highly susceptible to TETD-treatment. A concentration of 2 μM TETD (which almost completely prevented the biosynthesis of immunoreactive RFamide) inhibited also metamorphosis induction. As long as the amidating enzyme was under inhibition neither bacteria nor low concentrations of CsCl were able to trigger metamorphosis even if the inducers were continuously present for 24 h. (Table 5). In untreated larvae, higher concentrations of CsCl i.e., 96 or 112 mM usually very reliably trigger metamorphosis by a short-term treatment for only 3 hours. Nevertheless, 2 μM TETD prevented induction by 96 mM CsCl almost completely, too. In these experiments the inhibitor was applied from 20 h after fertilization through induction and until 3 h after the inducing treatment. The internal mechanism of CsCl induction is obviously substantially affected by TETD. In contrast, He-LWamide stimulated metamorphosis in TETD-treated animals with comparable effectiveness as in the control group. Exogenously applied He-LWamide II thus substituted or by-passed an internal signal normally provoked by bacteria or CsCl but disabled by the inhibitor. Though not in the focus of this study, it should be mentioned that He-LWamide II-induced metamorphosis stages are different in shape compared to those provoked by CsCl-induction. The peptide appeared to trigger primarily morphogenesis in posterior regions of the planula since often hypostome-bearing semi-metamorphosed primary polyps formed. This was observed, too, but in lower frequency when normal larvae not treated with inhibitor of amidation were induced by LWamides.

TABLE 5

METAMORPHOSIS INDUCTION BY BACTERIA, CsCl OR HE-LWAMIDE II AND EFFECT OF TETD

Pre-treatment 20-72 h	Treatment	Post-treatment	% Metamorphosis after		No. of animals analysed
			24 h	48 h	
Experiment I: 24 h induction by *Pseudoalteromonas espeijana* or 9 mM CsCl					
EtOH, 0.05%	Bacteria, EtOH, 0.05%	+ EtOH, 0.05% 24 h	n.d. [a]	89	37
TETD, 1 μM	Bacteria +TETD, 1 μM	TETD, 1 μM 24 h	n.d.	3	35
TETD, 2 μM	Bacteria, +TETD, 2 μM	TETD, 2 μM 24 h	n.d.	0	58
EtOH, 0.05%	CsCl, 9 mM +EtOH, 0.05%	EtOH, 0.05% 24 h	96	96	51
TETD, 1 μM	CsCl, 9 mM + TETD, 1 μM	TETD, 1 μM 24 h	3	65	47
TETD, 2 μM	CsCl, 9 mM + TETD, 2 μM	TETD, 2 μM 24 h	0	3	69
Experiment II: 3 h induction by 96 mM CsCl or 10 μM He-LWamide II					
EtOH, 0.05%	CsCl, 96 mM +EtOH, 0.05%	EtOH, 0.05% 3 h	92	93	430
TETD, 2 μM	CsCl, 96 mM + TETD, 2 μM	TETD, 2 μM 3 h	0	0	36
EtOH, 0.05%	He-LWamide II, 10 μM, +EtOH, 0.05%	EtOH, 0.05% 3 h	19	36*	42
TETD, 2 μM	He-LWamide II, 10 μM +TETD, 2 μM	TETD, 2 μM 3 h	21	21*	39

a) n.d.: not determined. *) Values are not significantly different.

Metamorphosis is released from inhibition as soon as amidating enzyme is allowed to recover

TETD and DDC are known to inhibit PHM by hindering reversibly the interaction of the active centre of the enzyme with copper ion (Mains *et al.*, 1986). The compounds were therefore expected to be neutralized by supplementing surplus copper. This was indeed observed *in vitro* for DDC (Fig. 3) and *in vivo* (see below). Inhibition of metamorphosis should, in addition, be reversible. It was, therefore tested whether or not the inactivated metamorphosis mechanism recovers from inhibition after removal of the inhibitor. Planulae were reared in 2 μM TETD from a time point (20 h) preceding the very start of LWamide-precursor expression (24 h, Table 1) until 72 h. They were either treated in continueous presence of inhibitor by CsCl or were released from TETD-treatment by a 3 h wash in sea water prior to CsCl incubation. While continuation of the TETD-treatment during the CsCl incubation and for further 3 h significantly reduced the rate of metamorphosis, 100% of the animals released from inhibition underwent metamorphosis. In parallel the effectiveness of He-LWamide II was compared in continueously TETD-treated animals with those released from inhibition prior to peptide treatment. The rates observed in both compared groups were not significantly different (data not shown).

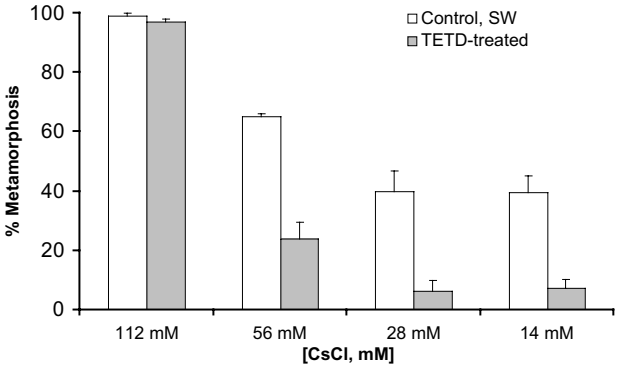

Fig. 5. (Left) Metamorphosis induction by 3 h CsCl treatment – effect of TETD present during and for 3 h after induction. *Mean values and standard deviation from three single experiments including 180-220 larvae each.*

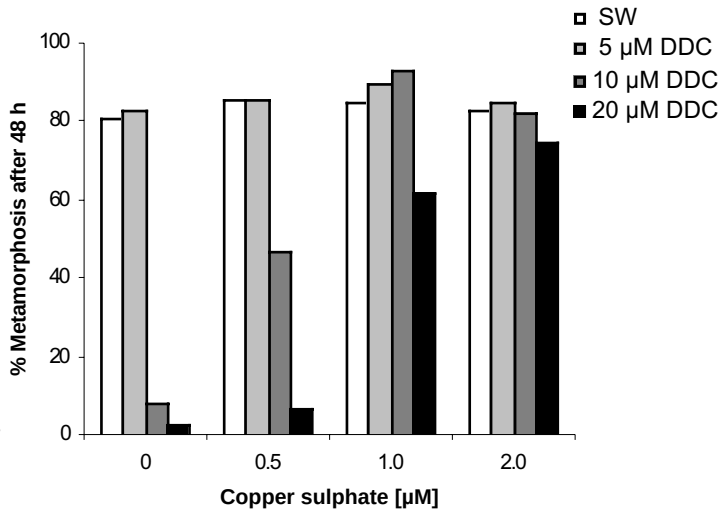

Fig. 6. (Right) Compensation for metamorphosis inhibition of DDC by copper *Planula larvae of 72 h age were induced to metamorphosis by continous low-dose treatment with CsCl (5 mM) for 48 h without or with additional supplement of copper (0.5, 1.0, or 2.0 µM copper sulphate). They were treated with 5, 10, or 20 µM DDC. For each value in the Figure, 180- 221 animals were scored. From 684 larvae kept in sea water without inducer, 3 (0.5%) underwent spontaneous metamorphosis.*

Metamorphosis induction is susceptible to inhibitors of PHM also during and after CsCl-treatment

Three hours after release from inhibitor pre-treatment the internal metamorphosis control was observed to function normally. The reason for that could be that recovered enzyme activity resumes so far blocked amidation. As an alternative, recovered enzyme could function by amidating propeptides which were de novo synthesized as a consequence of metamorphosis induction. If the latter is true, susceptibility for inhibitor has to be expected in so far untreated animals just at the time when LWamide gene activity resumes after onset of metamorphosis induction (Table 2). Animals that developed normally without inhibitor pre-treatment were therefore CsCl-induced in sea water or in sea water supplemented with 2µM TETD. Inhibitor treatment was continued for three further hours after removal of CsCl. Metamorphosis rates were indeed significantly reduced after application of low and moderate CsCl-concentrations (14, 28, 56 mM) but not changed after treatment with 112 mM CsCl (Fig. 5). The susceptibility for inhibitor in normally developed larvae was tested by a different experiment based on continous low-dose tratment. CsCl at a concentration of 5 mM has no visible toxic effect and can be left in the incubation set up for continuous treatment. Larvae do not synchronously react to this treatment but acquire the induced state after various individual incubation times. 48 hours after begin of continuous treatment, 81% of the larvae had started or finished metamorphosis (Fig. 6). While co-incubation with 5 µM DDC during the CsCl-treatment did not reduce this rate both concentrations, 10 or 20 µM DDC, almost completely prevented metamorphosis induction. Addition of copper (sulphate) compensated for the inhibitor effect. As a rule, higher concentrations of inhibitor required more copper for compensation (Fig. 6). Copper by itself (2.0 µM maximum) did not have any visible effect on viability nor, on the effectiveness by which metamorphosis was stimulated by low-dose and long-term treatment. Zinc sulphate applied in equal molar concentrations, in contrast, did not compensate for the inhibitory effect of DDC (data not shown).

Inhibitor treatment prevents inducibility by knocking out endogenous He-LWamide function

We addressed the question whether or not the observed loss of inducability is indeed due to absence of amidated He-LWamides by analysing treated animals immunocytochemically for presence of amidated He-LWamides. For this, a large batch of embryos was treated with 20 µM DDC or 50 µM DDC from 22 h post fertilization and subjected to several experiments 50 hours later. The material was checked in parallel for nerve cells immunopositive to amidation-sensitive anti LWamide antibody, by *in situ* hybridization for the numbers of cells showing He-LWamide precursor mRNAs and for inducibility of metamorphosis by CsCl or by synthetic He-LWamide II. A fraction of the specimens was again analysed for multiple neuropeptide precursor mRNA expression by *in situ* hybridization. From a second batch, total RNA was isolated from untreated control animals and from animals treated with 20 and 50 µM DDC,

TABLE 6

METAMORPHOSIS INDUCIBILITY AFTER TREATMENT WITH DDC

Inhibitor treatment	**[a]Induction of metamorphosis by treatment for 3 h with**			
	CsCl			He-LWamide II
	14 mM	28 mM	56 mM	20 µM
None (sea water)				
No. of animals	202	151	135	205
% metamorphosis 24 h	50	86	98	70
% metamorphosis 84 h	n.d.	n.d.	n.d.	98
20 µM DDC				
No. of animals	180	202	198	120
% metamorphosis 24 h	0	0	0	98
% metamorphosis 84 h	0	0	0	98
50 µM DDC				
No. of animals	205	199	220	119
% metamorphosis 24 h	0	0	0	100
% metamorphosis 84 h	5	5	4	100

a) Treatment from 22 h after fertilization until metamorphosis induction at 72 h, during induction and for further 3 h after removal of inducer.

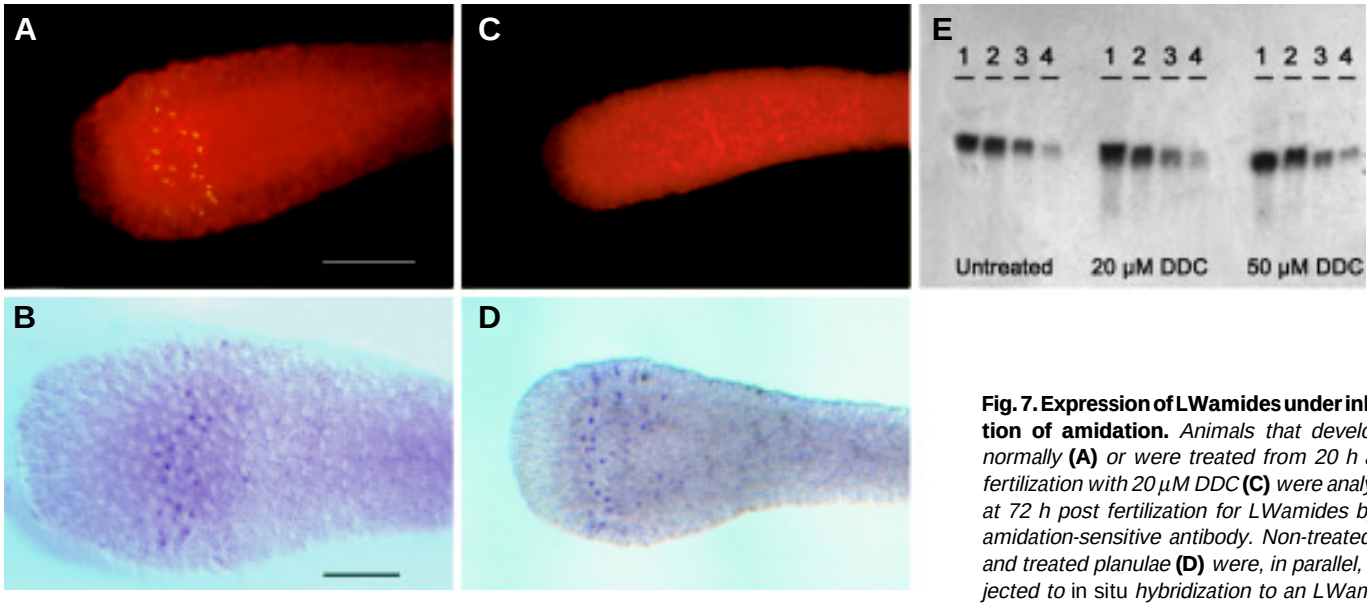

Fig. 7. Expression of LWamides under inhibition of amidation. *Animals that developed normally* **(A)** *or were treated from 20 h after fertilization with 20 µM DDC* **(C)** *were analysed at 72 h post fertilization for LWamides by an amidation-sensitive antibody. Non-treated* **(B)** *and treated planulae* **(D)** *were, in parallel, subjected to* in situ *hybridization to an LWamide-precursor anti-sense probe. Bars in A and B correspond to 50 µm.* **(E)** *Northern analysis of LWamide-precursor mRNA (1500 nt) in RNA preparations from untreated and treated (20 µM, 50 µM DDC) animals. 50, 25, 10 or, 5 µg of total RNA were loaded to lanes 1 to 4, respectively.*

respectively. Equal amounts of these RNAs were analysed by Northern blotting for the abundance of transcripts of the He-LWamide genes.

In embryos treated by DDC, no immunoreactivity to amidation sensitive anti LWamide was observed at all, even in the group treated by the lower dose 20µM (48 specimens analysed, Fig. 7C). Control larvae contained 20-40 LWamide immunopositive nerve cells in the typical belt-like arrangement in the anterior larva (Fig. 7A). *In situ* hybridization of sibling specimens showed precursor-expressing cells in untreated (Fig. 7B) as well as in treated animals (Fig. 7D). Numbers of cells expressing precursor mRNA were not different in these experimental groups. The same result was again observed after ISH analysis of multiple neuropeptide precursor expression (see also Table 4). That the treatment obviously did not affect LWamide gene expression was also confirmed by Northern analysis. Even 50µM DDC did not cause changes in the abundance of He-LWamide precursor transcripts in total RNA-preparations (Fig. 7E). Inducibility was tested in animals from the same batch of embryos used for molecular analysis by incubation with 14, 28, and 56 mM CsCl and, by incubation with He-LWamide II. DDC-treatment (20and 50 µM) was continued during the 3 h-induction period and for further three hours after removal of CsCl or peptide. In this experiment, even 56 mM CsCl could not induce treated larvae to undergo metamorphosis while treatment with 20 µM He-LWamide (3 h) was sufficient to induce 98-100% metamorphosis (Table 6).

Discussion

In Hydractinia*, dsRNAi is effective in modulating LWamide gene activity and function*

DsRNAs prepared from the coding region of the LWamide precursor gene and injected into the fertilized egg was observed to deplete endogenous LWamide transcripts. The effect was most pronounced when LWamides were expressed at highest levels. As reported for other systems including *Hydra* (Lohmann *et al.*, 1999), dsRNAi silenced genes in *Hydractinia* in a sequence-specific manner. *In situ* hybridization revealed substantial reduction of LWamide transcripts in nerve cells of dsRNA-injected animals. This caused complete loss of detectability of LWamide nerve cells while cells expressing the multiple neuropeptide precursor for He-RF/RN/RYamides showed up in normal numbers. Thus, dsRNAi is a very efficient means to knock-down and thus functionally analyse gene activity in *Hydractinia* and, generally, in all hydroids with accessible embryonic development. As observed by Northern analysis and also with respect to its biological effect in metamorphosis, the knockdown of the neuropeptide precursor gene is transient and expected to be ultimately reversible. Similar observations on transiency of the silencing effect of RNAi in *Hydra* were interpreted as being due to dilution of initially electroporated dsRNA material from steadily proliferating cells (Lohmann and Bosch, 2000). In *Hydractinia*, transiency is not likely due to a mere depletion of originally injected dsRNA. In preparations showing re-appearance of endogenous transcripts substantial quantities of dsRNA in its original size are still present. The dsRNA silencing mechanism as based on a type III RNAse termed Dicer and on RISC (RNA-induced silencing complex) has been observed in several organisms from fungi to mammals indicating evolutionary conservation (Hannon, 2002). According to known properties of the silencing machinery it cannot be rationalized how such a mechanism in *Hydractinia* could become unfunctional as long as dsRNA is present in a cell and, as long as the silencing signal amplifies cell autonomously to sustain the process. Nevertheless, RNAi ceased in nerve cells forcing the conclusion that LWamide dsRNA is obviously absent from these cells of the embryo while still present in others. Second, systemic RNAi by spreading of the silencing signal from cell to cell as occurring in C. elegans and plants (Hannon, 2002) cannot be expected for *Hydractinia*, at least, not for silencing genes active in nerve cells. It is remarkable, that also in C.elegans neurons are resistant to systemic RNAi due to the fact that neurons in C. elegans lack the *sid*-1 gene product, a transmembrane protein

proposed to be the transporter of the silencing signal into the cells (Winston *et al.*, 2002).

***Coelenterates contain a copper-dependent α-amidating enzyme which can be inhibited* in vitro *and* in vivo**

Carboxamidated peptides from Coelenterates have been proposed to be processed from their respective precursor proteins as glycine-extended propeptides and then modified by C-terminal amidation (Grimmelikhuijzen *et al.*, 1994). A cDNA encoding a PHM was cloned in a cnidarian, the sea anemone, *Anthopleura elegantissima* (Hauser *et al.*, 1997). As expected, hydroids contain α-amidating enzyme as the artificial glycine extended substrate dansyl-D-Tyr-Val-Gly (Bradbury *et al.*, 1982; Bendig, 1986) was converted into peptide amide in extracts of *Hydractinia* tissue. *In vitro* amidation was reversibly inhibited by copper depletion in the reaction assay. As vertebrate PHM the hydroid enzyme(s) require copper, too.

That copper depletion is suitable to inhibit amidation *in vivo* was assayed by anti RFamide antibody, 146II (from C. Grimmelikhuijzen) that was reported to be amidation sensitive (Grimmelikhuijzen and Graff, 1986). In a parallel experimental approach, a specific amidation-sensitive antibody to LWamides (Gajewski *et al.*, 1996) was used to screen for processed LWamides. Absence of RFamide or LWamide immunoreactivity after TETD or DDC-treatment showed, therefore, absence of the corresponding peptides. Inhibitor treatment obviously did neither substantially affect mRNA-expression of the *Hydractinia* RFamide peptide-precursor nor of the LWamide precursor. In principle, any of the steps of biosynthesis from translation through processing and up to amidation could have been affected. Since no enzymes involved in processing and modification except PHM are known to be targets of DDC or TETD and considering the results of the *in vitro* study we must interpret the absence of RFamide/ LWamide immunoreactivity as a consequence of *in vivo* inhibition of PHM.

Amidated peptides are regulated at a post-transcriptional level

Comparison of ISH and ICC data revealed striking differences. 3 to 5 times more cells were detected by ISH than by ICC. Cells contain mRNA for peptide precursor protein but no detectable amounts of immunoreactive peptides. Two explanations are possible. The amount of antigen in the majority of the cells is below the detection limit for ICC while at the same time the mRNA is detected by ISH. If correct, then bioactive peptide is present even though not detectable. Alternatively, immunonegative cells may contain mRNA, translated preproprotein and even partially processed precursor but virtually no peptide amide. In the latter case, gene activity would not be controlled exclusively on the transcriptional level but on translational level or by the post-translational modification machinery. To consider amidation as an essential mechanism in determining the amount of bioactive peptide one has to expect that the relative amount of glycine extended propeptide is substantial as compared to its respective peptide amide. This has indeed been observed in other animals but also many examples for balanced quantities of glycine-extended and amidated peptide have been reported (reviewed in Bradbury and Smyth, 1991).

Nerve cell differentiation has been extensively studied by use of the 146 II anti RFamide antibody (e.g., Plickert, 1989; Koizumi *et al.*, 1992). From the absence of RFamide immunoreactivity the absence of RFamide nerve cells was inferred in these and many other articles. Some of the conclusions prompted by the ICC results in these papers may need reconsideration in the light of the results reported here. For instance, conversion of a non-RFamide cell type into a RFamide nerve cell may be reinterpreted as due to simply modifying translation activity or, amidation in a true RFamide neurosecretory cell.

A treatment that prevents amidation also interferes with metamorphosis control - role of endogenous He-LWamides

He-LWamides occur in *Hydractinia* planulae and trigger metamorphosis (Gajewski *et al.*, 1996; Schmich *et al.*, 1998). The results of this study showed that inhibitor treatment when timed with respect to gene expression kinetics interfered inducibility. The effectiveness by which CsCl and bacteria induce metamorphosis was reduced. Amidation is a structural prerequisite for receptor binding and activation (Kulathila, 1999). Non-amidated LWpeptides had indeed no effect as an inducer in *Hydractinia* larvae (Schmich *et al.*, 1998). The results must, therefore, be interpreted as an inhibitor effect on the maturation of bioactive LWamides. To prove a knock-out of LWamide gene function, one further requirement must however be met: bioactive LWamide supplemented from outside should rescue the deficiency. This was observed: synthetic He-LWamide II induced metamorphosis in inhibitor-treated and untreated larvae with comparable effectiveness. He-LWamide I was also effective while neither He-RNamide, He-RYamide nor the RFamide of *Hydractinia* (=Pol-RFamide II) had any inductive capacity (unpublished results). An endogenous signal naturally provoked by bacteria or by artificial inducers and prevented by inhibitor treatment is thus substituted by exogenously applied He-LWamides. We are forced to conclude that this endogenous signal is substantiated by He-LWamides. These peptides are indispensable elements in controlling stage transition from larva to polyp.

It is an open question what information is signalled by LWamides to the target. Since LWamides induce metamorphosis the peptides could function in the induction process itself. Alternatively, or in addition, later events of metamorphosis comprising tissue contraction, morphogenetic movements, cell cycle reinitiation or pattern formation (Plickert *et al.*, 1988; Berking, 1998) could be controlled. Two results of this study and other observations strongly suggest that LWamides control development downstream of the induction process. 3-4 hours after the end of induction treatment mRNA-expression of the LWamide precursor transiently increased. We are forced to interpret this as indicating requirement for newly synthesized LWamides just at that time. Expression of the multiple neuropeptide precursor was regulated down at the same time. The increase in LWamide gene expression is therefore not just reactivation of neuropeptide precursor activity but specific up-regulation. LWamides are obviously required after metamorphosis induction while the peptides of the multiple neuropeptide precursor may not. Strong support for requirement beyond the phase of induction came from the observation that so-far untreated animals are susceptible for inhibitors of amidation. The subsequent processes of tissue reorganization and cell control thus require peptide processing, at least, functionality of the amidation machinery. The finding is remarkable since these animals are expected to store large quantities of LWamides (Leitz and Lay, 1995; Schmich *et al.*, 1998). Inhibitors of amidation can only prevent or limit modification if peptides are de novo processed. Thus the results favour a biphasic (at least!) mode of of LWamide release. One release occurs from sources of stored

peptide at the beginning of induction, a second one starts after the end of induction and releases de novo synthesized peptide. In this context it is of interest that metamorphosis induction was observed to be still reversible after a 3 h CsCL induction (Walther *et al.*, 1996). Release of stored LWamides as proposed to convert the outer primary inducing signal into an internal signal (Leitz, 1997) may not suffice to irreversibly trigger metamorphosis. Secondary signals released as a consequence of induction must be expected to control morph conversion. The newly synthesized LWamides could constitute one of these signals.

The observed activity increase in LWamide gene expression after onset of metamorphosis in terms of increased mRNA must be taken into account as outlined before. The expression status on mRNA-level, however, was not directly proportional to the amount of the quantity of processed peptide. This became impressingly obvious from a direct comparison of immunoreactivity to RFamide and abundance of mRNA for its precursor protein. It must, therefore, be concluded that the generation of peptide signals, at least of amidated neuropeptides is controlled or fine-tuned post-transcriptionally. Finally, the absolute content of LWamides in a larva may not be crucial for inducibility, at all, as long as minimum quantities are available for secretion. LWamide RNAi resulted in extensive loss of endogenous LWamide precursor transcripts. Despite that, silencing of LWamide function as phenotypically displayed by complete loss of competence for metamorphosis induction was not achieved.

Conclusion and Outlook

A functional knock-out of LWamides in the larva of *Hydractinia echinata* causes loss of competence for metamorphosis stimulation by otherwise effective bacteria or by CsCl. LWamides added to these animals from outside, however, trigger transformation of larva into polyp tissue. These peptides are essential in metamorphosis control. Two experimental approaches though aiming at different molecules gained similar results. DsRNAi knocking down the mRNA for the precursor of LWamides reduced metamorphosis rates. Pharmacological inactivation of peptidylglycine-hydroxylating monooxygenase resulted in even more pronounced depletion of inducibility. Thus, for the first time, the function of a hydroid gene has been elucidated by means of a knock-out/rescue approach. The latter approach of pharmacological interference with amidation has been used in other systems successfully, too. The developmental role of amidated pituitary adenylyl cyclase-activating polypeptide was studied in olfactory neurogenesis of mice (Hansel *et al.*, 2001). PHM mutants were studied in *Drosophila*. By rescue-experiments it was shown that C-terminal amidation is essentially required for transition between developmental stages (Jiang *et al.*, 2000). Signalling by amidated peptides will probably turn out to be important not only by controlling cell differentiation and body plan conversion as shown in this study but as a general way to control development. In hydroids, functional analysis of peptide genes by blocking amidation and probing the peptide of interest is a promising way to shed light on essential functions of peptide amides in development. Work of this kind is in progress.

Materials and Methods

Animal culture and induction of metamorphosis

Colonies of *Hydractinia echinata* were maintained as described (Plickert, 1989) with minor modifications. Animals were kept at 15°C, fed five times a week with a mixed diet of three day old brine shrimp larvae and grinded sea food mix (Cod, shrimps and shellfish). Embryos were reared at 18°C. All experiments were done at 18°C. Metamorphosis induction by CsCl were performed as published (Müller and Buchal, 1973). He-LWamide II (Bachem) was dissolved in de-aerated bi-distilled water at 10 mmol/l and kept as a stock solution at –20°C. From this stock, aliquots were diluted in sea water at the due final concentration. Incubations was performed in a volume of 1 or 2 ml in small glass-dishes. In order to compare peptide-driven induction with induction by CsCl, the incubation period was limited to 3 h.

Molecular techniques

Nucleic acid isolation and Northern blot analysis were carried out according to standard procedures. A radioactive probe was generated by PCR-labelling from the 5´portion (nt 1- 467) of the cDNA encoding the He-LWamide precursor (Gajewski, 1995; Genbank accession number 89735). Digoxygenine-labelled sense and antisense RNA probes for *in situ* hybridization (ISH) were generated from cDNA fragments cloned in pBS KSII or SKII by using T7 or T3 RNA-polymerase. Probes were prepared from the entire cDNA of the multiple neuropeptide/RNamide/RYamide precursor (Gajewski *et al.*, 1998; genbank accession number X97413) hydrolysed to 150 nt average size and from the 3´portion of the LWamide precursor (nt 1218-1444). ISH was performed as published elsewhere (Gajewski *et al.*, 1996; Plickert *et al.*, 1997).

RNAi, dsRNA-preparation

DsRNA was synthesized from either nt 1-467 (5-LWamide dsRNA) or, using the *Sst*I fragment (nt 468-1217, *Sst*I-dsRNA) of the LWamide cDNA as a template. *In vitro* transcription was performed by using the "RiboMaxTM Large Scale RNA Systems" (Promega) according to the manufacturers protocol. After template DNA removal by DNAse and subsequent purification by acidic phenol extraction, the single stranded RNAs were hybridised. For that, equimolar quantities of ssRNAs were mixed and adjusted to 2% Polyethyleneglycole 6000-8000, 20 mM Potassium phosphate (pH 7.5), 3 mM Potassium citrate (pH 7.5) (RNA-hybridization buffer). The reaction was incubated for 15 minutes at 75°C in a water bath (volume 5 l) in order to linearize the RNAs. For slow hybridization, the water bath was switched off and allowed to cool to room temperature during 3 hours. The preparation was checked for complete hybridization by RNAse A incubation and, by non-denaturing gel separation of ssRNA and dsRNA followed by Northern blot analysis.

Injection

Fertilized eggs and cleavage stages up to the four-cell stage were pressure-injected by using a PV 820 PicoPump (World Precision Instruments). Embryos were immobilized in micro-grooves. These were produced by scratching the bottom of the wells of Terasaki plates with a fine needle. Via micromanipulated injection capillaries (Science Products, GB120F-P), 270 pl were delivered to each zygote. DsRNA was injected in hybridization buffer; $7x10^8$ (5´-LWamide dsRNA) or, $8.5x10^8$ (*Sst* I-dsRNA) molecules were transferred with each injection. Viability was 90% after injection; embryos developed normally.

Inhibitor treatment

Inhibitors of PHM were prediluted either in ethanol (TETD) or, in distilled water (DDC), and adjusted to the final concentration in sea water. Solvent controls were included using the final ethanol concentration in the experimental set-up.

Assay for PHM activity

α-amidating activity was assayed according to published procedures (Bendig, 1986) with minor modification. Crude extracts were prepared from starved *Hydractinia* gastrozooids by high speed dispersion of tissue (Ultra Turrax) in ice-cold buffer (25 mM PIPES, pH 6.8), 100 mM NaCl, 0.1% Triton X-100, 100u/ml Catalase, 1 mM Phenylmethylsulfonylfluoride).

Extracts were centrifuged and supernatants were immediately assayed for amidating activity. For that, extract corresponding to 5 µg of total protein was set up as a 50 µl- reaction in 25 mM PIPES (pH 6.8), 100 mM NaCl, 0.1% TRITON X-100, and containing 1 mM ascorbate, 61 mM KI, 5000 U Catalase, 1 mM Phenylmethylsulfonylfluoride (PMSF). The reaction was supplemented with 10 nmol/l of dansylated substrate-peptide, allowed to proceed at room temperature for 1.5 or 22 hours and analysed by thin layer chromatography for product of amidating enzyme. Artificial glycine-extended substrate N-terminally protected to enzymatic degradation (by a D-amino acid) was used (Bendig, 1986). After dansylation dansyl-peptides were separated from dansylchloride and from unlabelled peptide by liquid chromatography on Sep-Pak C18 cartridges (Waters). For analysing *in vitro* amidation, separation of dansylated substrate (dansyl-D-Tyr-Val-Gly) from its amidated product (dansyl-D-Tyr-Val $CONH_2$ was done on high-performance thin layer chromatography plates (RP-18, $F_{254}S$, Merck). Dansylated substrate peptide (dansyl-D-Tyr-Val-Gly) as well as dansylated product peptide (dansyl-D-Tyr-Val-NH_2) were included as standards. Further assay conditions, in particular, concentration of copper sulphate added to the reaction are indicated in the result section.

Immunocytochemistry

Detection and counting of nerve cells expressing neuropeptides was performed as published (Plickert, 1989). In order to detect RFamide-like immunoreactivity, the amidation-sensitive anti-RFamide antibody 146II from C.J.P. Grimmelikhuijzen was used. For LWamide immunocytochemistry, the amidation-sensitive antibody 1676 IIp was used (prepared by T. Leitz, outlined in Gajewski *et al.*, 1996).

Statistical analysis

Differences were analysed for significance by chi-square-analysis or by Student's *t*- test. Only when it is not obvious from the data, significance or non-significance is mentioned in the tables (eg., differences of 90% vs. 0% are not commented further).

Acknowledgements

We thank Cok Grimmelikhuijzen and Thomas Leitz for providing antisera to RFamide and LWamide, respectively and Stefan Berking and Klaus Herrmann for critical reading of the manuscript.

References

BENDIG, M.M. (1986). Post-translational processing in Xenopus oocytes includes carboxyl-terminal amidation. *J. Biol. Chem.* 261: 11935 - 11937.

BERKING, S. and WALTHER, M. (1994). Control of metamorphosis in the hydroid *Hydractinia*. In *Perspectives in Comparative Endocrinology* (Eds. DAVEY, K. G., PETER, R. E. and TOBE, S. S.). National Research Council of Canada, Ottawa, 381 - 388.

BERKING, S. (1998). Hydrozoa Metamorphosis and Pattern Formation. Current Topics in *Dev. Biol.* 38: 81 - 131.

BRADBURY, A.F., FINNIE, M.D.A. and SMYTH, D.G. (1982). Mechanism of C-terminal amide formation by pituitary enzymes. *Nature* 298: 686 - 688.

BRADBURY, A.F., MISTRY,J.,ROOS, B.A. and SMYTH, D.G. (1990). 4-Phenyl-3-butenoic acid, an *in vivo* inhibitor of peptidylglycine hydroxylase (peptide amidating enzyme). *Eur. J Biochem.* 189: 363 - 368.

BRADBURY, A.F. and SMYTH, D.G. (1991). Peptide amidation. Review. *Trends Biochem Sci.* 3: 112 - 115.

EIPPER, B.A., MILGRAM, S.L., HUSTEN, E.J., YUN, H.Y. and MAINS, R.E. (1993). Peptidylglycine α-amidating monooxygenase: A multifunctional protein with catalytic, processing and routing domains. Review. *Protein Sci.* 4: 489 - 497.

GAJEWSKI, M. (1995). Submission of cDNA sequences for He-LWamide, Ae-LWamide and As-LWamide to Genbank database, accession numbers X89734, X89735, X89736, respectively.

GAJEWSKI, M., LEITZ, T., SCHLOSSHERR, J. and PLICKERT, G. (1996). LWamides from Cnidaria constitute a novel family of neuropeptides with morphogenetic activity. Roux´s Arch. *Dev. Biol.* 205: 203 - 242.

GAJEWSKI, M., SCHMUTZLER, C. and PLICKERT, G. (1998). Structure of neuropeptide precursors in Cnidaria. In *Trends in Comparative Endocrinology and Neurobiology* (Ed. VAUDRY, H.). *Ann. N.Y. Acad Sci* 839: 311 - 315.

GRIMMELIKHUIJZEN, C. J. P. and GRAFF, D. (1986). Isolation of pyroGlu-Gly-Arg-Phe-NH2 (Antho-RFamide), a neuropeptide from sea anemones. *Proc Natl Acad Sci, USA,* 83: 9817 - 9821.

GRIMMELIKHUIJZEN, C.J.P., GRAFF, D. and MCFARLANE, I.D. (1989). Neurones and neuropeptides in coelenterates. *Arch. Histol. Cytol.* Suppl. 52: 265 - 278.

GRIMMELIKHUIJZEN, C.J.P., DARMER, D., SCHMUTZLER, C., REINSCHEID, R.K. and CARSTENSEN, K. (1994). Biosynthesis of neuropeptides in the Cnidaria: new discoveries of old principles. *Perspectives Comp. Endocrinol.* 97 - 108.

HANNON, G. J. (2002). RNA interference. Review. *Nature.* 418: 244 - 251.

HANSEL, D.E., MAY, V., EIPPER, B.A. and RONETT, G.V. (2001). Pituitary Adenyl Cyclase-activating peptides and amidation in olfactory neurogenesis and neuronal survival *in vitro*. *J. Neurosci.* 21: 4625 - 4636.

HAUSER, F., WILLIAMSON, M. and GRIMMELIKHUIJZEN, C.J.P. (1997). Molecular cloning of a peptidylglycine alpha-hydroxylating monooxygenase from sea anemones. *Biochem. Biophys. Res. Commun.* 241: 509 - 512.

HOBMAYER, B., RENTZSCH, F., KUHN, K., HAPPEL, C.M., VON LAUE, C.C., SNYDER, P., ROTHBACHER, U. and HOLSTEIN, T.W. (2000). WNT signalling molecules act in axis formation in the diploblastic metazoan *Hydra*. *Nature* 407: 186 - 189.

HOFFMEISTER, S.A.H. (1996). Isolation and characterization of two new morphogenetically active peptides from *Hydra* vulgaris. *Development* 122: 1941 - 1948.

JIANG, N., KOLHEKAR, A.S., JACOBS, P.S., MAINS, R.E., EIPPER, B.A. and TAGHERT, P.H. (2000). PHM is required for normal developmental transitions and for biosynthesis of secretory peptides in Drosophila. *Dev. Biol.* 226: 118 - 136.

KOIZUMI, O., ITAZAWA, M., MIZUMOTO, H., MINOBE, S., JAVOIS, L.C., GRIMMELIKHUIJZEN, C.J.P. and BODE, H.R. (1992). Nerve ring of the hypostome in *hydra*. I. Its structure, development, and maintenance. *J. Comp. Neurol.* 326: 7 - 21.

KULATHILA, R., MERKLER, K.A. and MERKLER, D.J. (1999). Enzymatic formation of C-terminal amides. *Nat. Prod. Rep.* 16: 145 - 154.

LEITZ, T. and WAGNER, T. (1993). The marine bacterium Alteromonas espeijana induces metamorphosis of the hydroid *Hydractinia echinata*. *Mar Biol.* 115: 173 - 178.

LEITZ, T., MORAND, K. and MANN, M. (1994). Metamorphosin A, a novel peptide controlling development of the lower metazoan, *Hydractinia echinata*. *Dev. Biol.* 163: 440 - 446.

LEITZ, T. and LAY, M. (1995). Metamorphosin A is a neuropeptide. Roux´s Arch. *Dev. Biol.* 204: 276 - 279.

LEITZ, T. (1997). Induction of settlement and metamorphosis of Cnidarian larvae: Signals and signal transduction. *Invertebrate Reprod. Dev.* 31: 109 - 122.

LEVIEV, I. and GRIMMELIKHUIJZEN, C.J.P. (1995). Molecular cloning of a preprohormone from sea anemones containing numerous copies of a metamorphosis-inducing neuropeptide: A likely role for dipeptidyl aminopeptidase in neuropeptide precursor processing. *Proc. Natl. Acad. Sci. USA* 92: 11647 - 11651.

LEVIEV, I., WILLIAMSON, M. and GRIMMELIKHUIJZEN, C.J.P. (1997). Molecular cloning of a preprohormone from *Hydra magnipapillata* containing multiple copies of Hydra-LWamide (Leu-Trp-NH2) neuropeptides: evidence for processing at Ser and Asn residues. *J Neurochem.* 68: 1319 - 1325.

LOHMANN, J. U., ENDL, I. and BOSCH, T.C.G. (1999). Silencing of developmental genes in Hydra. *Dev. Biol.* 213: 1 - 4.

LOHMANN, J. U. and BOSCH, T.C.G. (2000). The novel peptide HEADY specifies apical fate in a simple radially symmetric metazoan. *Genes. Dev.* 14: 2771 - 2777.

MAINS, R. E., PARK, L. P. and EIPPER, B. A. (1986). Inhibition of peptide amidation by Disulfiram and Diethyldithiocarbamate. *J. Biol. Chemistry* 261: 11938 - 11941.

MCFARLANE, I. D, ANDERSON, P. A. and GRIMMELIKHUIJZEN, C.J.P. (1991). Effects of three anthozoan neuropeptides, Antho-RWamide I, Antho-RWamide II and Antho-RFamide, on slow muscles from sea anemones. *J. Exp. Biol.* 156: 419-431.

MUELLER, G.P., DRISCOLL, W.J. and EIPPER, B.A. (1999). *In vivo* inhibition of peptidylglycine-alpha-hydroxylating monooxygenase by 4-phenyl-3-butenoic acid. *Pharmacol. Exp. Ther.* 90: 1331 - 1336.

MÜLLER, W.A. (1973). Induction of metamorphosis by bacteria and ions in the planulae of Hydractinia echinata; an approach to the mode of action. *Publ. Seto Mar. Biol. Lab.* 20: 195 - 208.

MÜLLER, W.A. and BUCHAL, G. (1973). Metamorphoseinduktion bei Planulalarven. II. Induktion durch monovalente Kationen: Die Bedeutung des Gibbs-Donnan-Verhältnisses und der Na^+/K^+-ATPase. *Wilhelm Roux´s Arch.* 173: 122 - 135.

MÜLLER, W.A., MITZE, A., WICKHORST, J.P. and MEIER-MENGE, H.M. (1977). Polar morphogenesis in early hydroid development: Action of caesium, of neurotransmitters and of an intrinsic head activator on pattern formation. *Roux's Arch. Dev. Biol.* 182: 311 - 328.

PLICKERT, G., KROIHER, M. and MUNCK, A. (1988). Cell proliferation and early differentiation during embryonic development and metamorphosis of *Hydractinia echinata*. *Development* 103: 795 - 803.

PLICKERT, G. (1989). Proportion Altering Factor (PAF) stimulates nerve cell formation in *Hydractinia echinata*. *Cell Diff. Dev.* 26: 19 - 28.

PLICKERT, G., GAJEWSKI, M., GEHRKE, G., GAUSEPOHL, H., SCHLOSSHERR, J. and IBRAHIM, H. (1997). Automated insitu detection (AISD) of biomolecules. *Dev. Genes Evol.* 207: 362 - 367.

SCHALLER, H. C. and BODENMÜLLER, H. (1981). Isolation and amino acid sequence of a morphogenic peptide in *hydra*. *Proc. Natl. Acad. Sci. USA* 78: 7000 - 7004.

SCHMICH, J., TREPEL, S. and LEITZ, T. (1998). The role of GLWamides in metamorphosis of *Hydractinia echinata*. *Dev. Genes Evol.* 208: 267 - 273.

SCHMUTZLER, C., DIEKHOFF, D. and GRIMMELIKHUIJZEN, C.J.P. (1994). The primary structure of the Pol-RFamide neuropeptide precursor protein from the hydromedusa *Polyorchis penicillatus* indicates a novel processing proteinase activity. *Biochem J.* 299 (Pt 2): 431 - 436.

SCHWOERER-BÖHNING, B., KROIHER, M. and MÜLLER, W.A. (1990). Signal transmission and covert prepattern in the metamorphosis of *Hydractinia echinata*. Roux´s Arch. *Dev. Biol.* 198: 245 - 251.

TAKAHASHI, T., MUNEOKA, Y., LOHMANN, J., LOPEZ de HARO, M. S., SOLLEDER, G., BOSCH, T.C.G., DAVID, C.N., BODE, H.R., KOIZUMI, O., SHIMIZU, H., HATTA, M., FUJISAWA, T. and SUGIYAMA, T. (1997). Systematic isolation of peptide signal molecules regulating development in *hydra*: LWamide and PW families. *Proc. Natl. Acad. Sci.* 94: 1241 - 1246.

TAKAHASHI, T., KOIZUMI, O., ARIURA, Y., ROMANOVITCH, A., BOSCH, T. C. G., KOBAYAKAWA, Y., MOHRI, S., BODE, H. R., YUM, S., HATTA, M. and FUJISAWA, T. (2000). A novel neuropeptide, Hym-355, positively regulates neuron differentiation in *Hydra*. *Development*. 127: 997 - 1005.

WALTHER, M., ULRICH, R., KROIHER, M. and BERKING, S. (1996). Metamorphosis and pattern formation in *Hydractinia echinata*, a colonial hydroid. *Int. J. Dev. Biol.* 40: 313 - 322.

WINSTON, W.M., MOLODOWITCH, C., HUNTER, C.P. (2002). Systemic RNAi in C. elegans requires the putative transmembrane protein SID-1. *Science*. 295: 2456 - 2459.

Received: January 2003
Reviewed by Referees: February 2003
Modified by Authors and Accepted for Publication: July 2003