

Polyps, peptides and patterning

Thomas C.G. Bosch^{1*} and Toshitaka Fujisawa²

Summary

Peptides serve as important signalling molecules in development and differentiation in the simple metazoan *Hydra*. A systematic approach (*The Hydra Peptide Project*) has revealed that *Hydra* contains several hundreds of peptide signalling molecules, some of which are neuropeptides and others emanate from epithelial cells. These peptides control biological processes as diverse as muscle contraction, neuron differentiation, and the positional value gradient. Signal peptides cause changes in cell behaviour by controlling target genes such as matrix metalloproteases. The abundance of peptides in *Hydra* raises the question of whether, in early metazoan evolution, cell–cell communication was based mainly on these small molecules rather than on the growth-factor-like cytokines that control differentiation and development in higher animals. *BioEssays* 23:420–427, 2001.

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Introduction

The tiny polyp, *Hydra*, is an abundant but inconspicuous inhabitant of freshwater ponds and lakes. In 1744, Abraham Trembley was the first to introduce *Hydra* to the academic circles in France and Switzerland. By demonstrating that this simple animal has the astonishing capacity to completely regenerate missing parts of the body,⁽¹⁾ he refuted the pre-formation theorists who were very influential at that time. In 1909, nearly 200 years later, embryologist Ethel Browne used *Hydra* to demonstrate for the first time that distinct regions of the body can act as organiser and induce neighbouring tissue to differentiate.⁽²⁾ Similar lateral transplantation experiments were done 15 years later by Hans Spemann and Hilde Mangold in *Triturus*.⁽³⁾ The third time that *Hydra* had a major impact on the formulation of biological concepts was when Alan Turing, in 1952, and a number of notable developmental biologists thereafter, used *Hydra* to support concepts of graded distribution of positional information along the body axis.^(4–6)

Despite the major impact that *Hydra* undoubtedly had on developmental biology research, its importance as experimental system remained limited because the molecules and biochemical mechanisms that control pattern formation and position-dependent cell differentiation were not known. In the last few years, hydra is experiencing a striking renaissance as a model organism for two reasons. First, the successful application of molecular techniques to hydra promises insights into the molecular circuitry controlling shape and form. Breakthroughs of the past decade include the identification of a plethora of transcription factor genes^(7–12) and generation of loss-of-function phenotypes by RNA interference⁽¹³⁾ or antisense thio-oligo nucleotides.⁽¹⁴⁾ Second, in the emerging field of evolutionary developmental biology, or “evo-devo”, recent molecular analyses reveal common themes in axial patterning in protostome invertebrates and deuterostomes. For a full insight into the evolution of body plans, however, studies outside the Bilateria in more basal metazoan groups are required.⁽¹⁵⁾ *Hydra* as a member of the most basal eumetazoan phylum Cnidaria is an attractive model to trace the evolutionary conservation of mammalian developmental pathways.

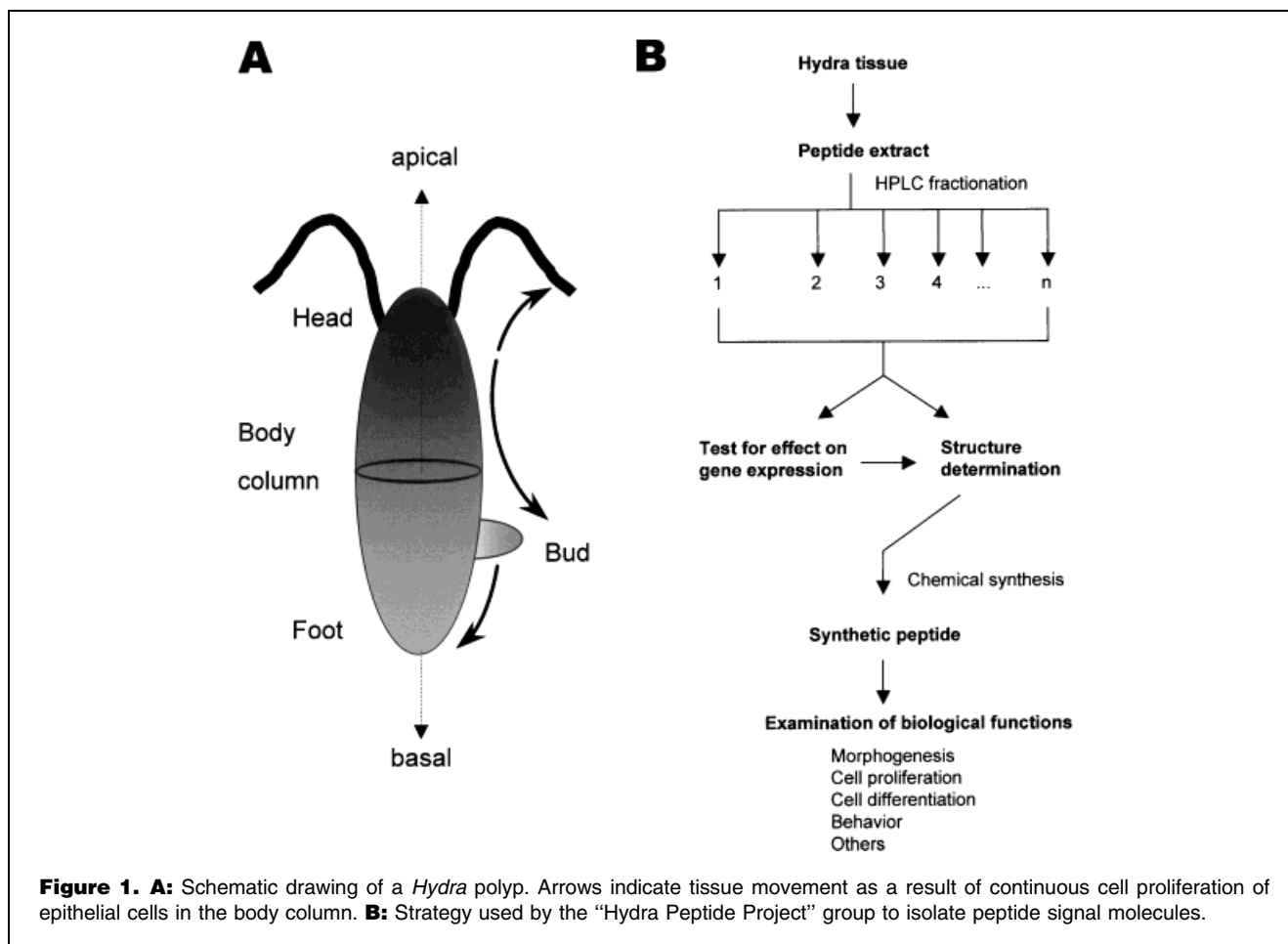
Hydra has a simple body plan with a head and tentacles at one end and a foot at the opposite end of a hollow, two-layered gastric column (Fig. 1A). The two cell layers, the ectoderm and endoderm, are separated by an extracellular matrix, the mesoglea. There is no mesoderm. The cells of *Hydra* either belong to the epithelial cell lineage, the major component of both ectoderm and endoderm, or to the interstitial cell lineage, which is mostly localised in the interstitial space between ectodermal epithelial cells. The interstitial cells consist of multipotent stem cells and precursor cells, which differentiate into neurons, cnidocytes, gland cells and, during sexual differentiation, gametes.⁽¹⁶⁾ Interstitial stem cells are uniformly distributed along the body column and are absent in head and foot tissue. After entering the differentiation pathway, some of the interstitial precursor cells (e.g. neuron precursor cells) migrate to their sites of differentiation. The epithelial cells continuously proliferate with a doubling time of about 3.5 days.⁽¹⁷⁾ The population of interstitial cells doubles at this same rate, although some individual cells multiply faster.⁽¹⁸⁾ The continuous cell proliferation in an animal of fixed size results in continuous tissue displacement towards the extremities (see Fig. 1A). Cells in *Hydra*, therefore, continuously change their position in the body and differentiate according to their position into head- or foot-specific cells. The long-known and remarkable capacity to regenerate missing

¹Zoological Institute, Christian-Albrechts-University Kiel, Germany.

²National Institute of Genetics, Mishima, Shizuoka 411-8540, Japan.

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*Correspondence to: Thomas C.G. Bosch, Zoological Institute, Christian-Albrechts-University, Olshausenstrasse 40, 24098 Kiel, Germany. E-mail: tbosch@zoologie.uni.kiel.de



body parts is at least partially due to this continuous production of all cell types.

What molecules control axis formation in this simple animal, and how are they localised in space and time? About 25 years ago, Chica Schaller characterised factors in extracts from *Hydra* tissue that enhanced head formation, cell division and nerve differentiation.⁽¹⁹⁾ The active component in these extracts was identified as the 11-amino acid neuropeptide “head activator” (Table 1).^(20,21) Additional support for the view that small peptides might serve as important signaling molecules in *Hydra* came from two independent experimental approaches. First, Cornelis Grimmelikhujzen and others demonstrated that *Hydra* (and related cnidarians) contain numerous neuropeptides.⁽²²⁾ Second, screening *Hydra* cells that are undergoing de novo axis formation for differentially expressed RNAs⁽²³⁾ resulted in the isolation of the cognate gene for the 12-amino-acid peptide HEADY (Table 1). Preliminary observations indicate that in *Hydra* HEADY is involved in early specification of apical fate.⁽²³⁾

The small size of peptides makes them ideal molecules for travelling with ease in the interepithelial space in *Hydra* to exert

the long-range effects necessary for the establishment of morphogenetic gradients. To systematically identify and characterise them, a remarkable joint effort of Japanese, American and German laboratories termed “The Hydra Peptide Project” was initiated in 1993 by Drs. Tsutomu Sugiyama, Yoshiro Muneoka, Osamu Koizumi and others. As members of this international enterprise we report here the goals, the experimental approach and the results obtained so far. We will show that this effort has not only identified a number of interesting and novel peptides, but that it also has set an example that laboratories with different types of expertise and localised in different parts of the world can successfully cooperate to solve an intriguing problem: how does *Hydra* make a head on one side and a foot on the other side of the gastric column?

The Hydra peptide project

The goal of the peptide project is to isolate and identify all the peptides that play a role in the control of position-dependent differentiation in *Hydra*. Since a polyp consists of only about 100,000 cells and 10 µg protein, the availability of sufficient

Table 1. *Hydra* peptides involved in cell and/or axis differentiation

Peptide name	Peptide structure	Proposed function	Gene	Expression/localization	References
Head activator	pEPPGGSKYILF	Head activation, mitogen, enhancement of neuron differentiation	n.d.	Neurons	19,20
HEADY	FHTMILLDTQSPa	Apical fate determination	AF188478	Endodermal epithelial cells of developing head	23
Hym-323	KWVQGKPTGEVKQIKF	Foot activation	AB040074	Both epithelial layers except basal disk and tentacles	37
Hym-346/Pedibin	AGEDVSHELEEKEKALANHS(E)	Foot activation	AB030084	Endodermal epithelial cells of the tentacle base and peduncle	36,45
Pedin/Hym-330 PW family	EELRPEVLDPVS(E)	Foot activation	n.d.	Endodermal epithelial cells (UP)	36
Hym33H	AALPW	Inhibition of neuron differentiation	n.d.	Ectodermal epithelial cells (UP)	26
Hym-35	EPSAAIPW				
Hym-37	SPGLPW				
Hym-310	DPSALPW				
Hym-355	FPQSFLPRGa	Enhancement of neuron differentiation	AB025945	Neurons in the head and foot, and less abundant in the body	26

n.d., not determined; up, unpublished.

amount of tissue to carry out a quantitative study of the peptides was the first formidable logistic challenge. M. Hatta (Mishima) mastered that problem by developing a mass culturing system that allowed the production of 2.5 kg of *Hydra* tissue. Our strategy for isolating signal peptides from *Hydra* extract is shown in Fig. 1B. Peptides were extracted and purified with successive steps of HPLC. Three out of 15 fractions have been characterised so far. Each fraction was found to contain a number of peptides. Signalling peptides were identified by their effect on the gene expression profile of *Hydra*, which was determined by Differential Display (DD-) PCR. About 1/10 of each peptidic fraction was used for structural determination by the automated Edman degradation method and/or the tandem mass spectrometry. Based on the sequence, chemical synthesis of the peptide was carried out by the automated solid-phase procedure. Finally the identity of the synthetic to the native peptide was confirmed by co-chromatography using HPLC.

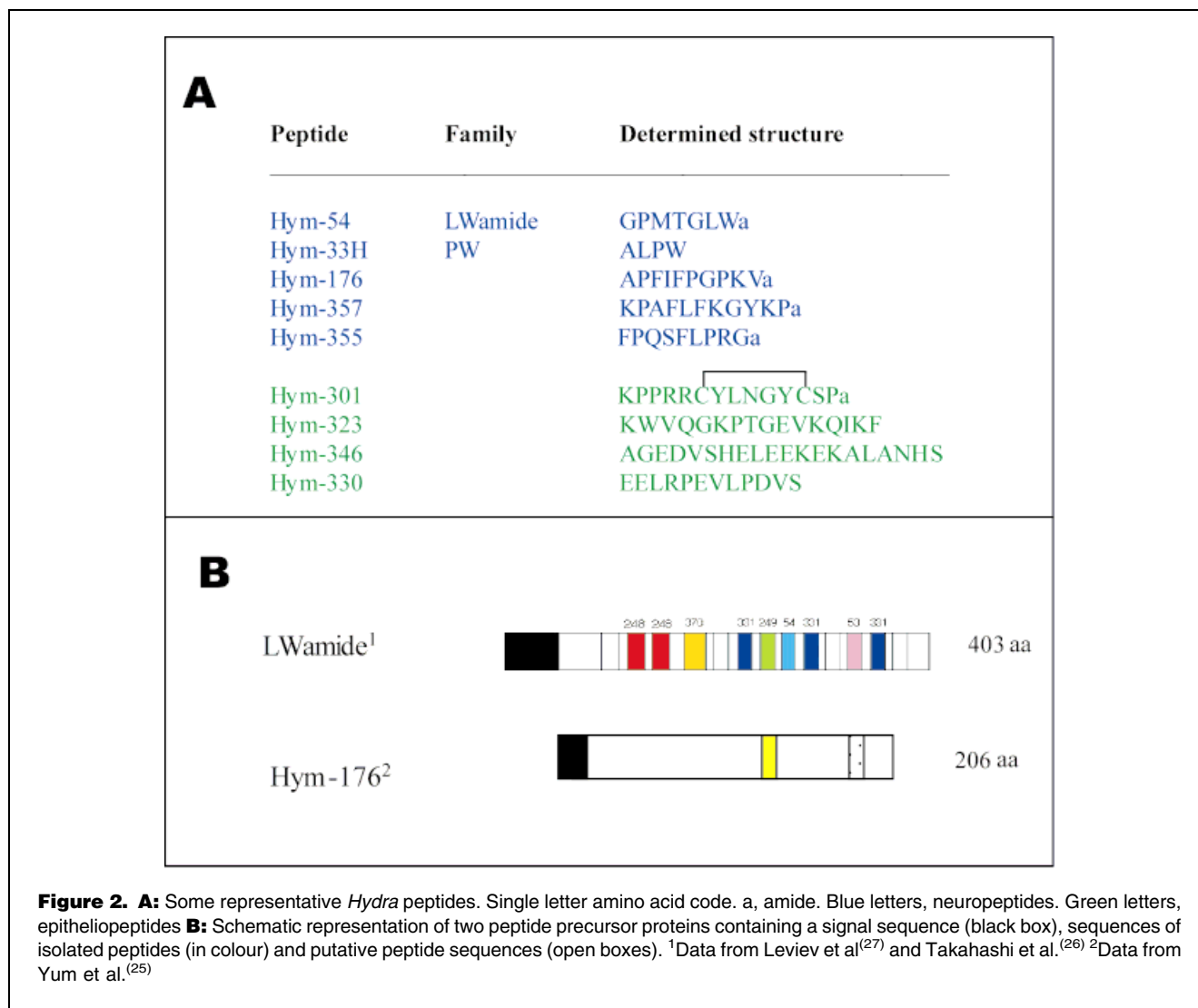
So far a total of 823 peptides have been purified to homogeneity and the sequences of 259 peptides tentatively determined. The final structures of 40 peptides have been determined. While some of the peptides were already known from the work of others or related to peptides described previously, many novel peptides have been identified. Some peptides were found to be members of peptide families. For example, the PW family consists of four members (Hym-33H, Hym-35, Hym-37, Hym-310) of five to eight amino acid residues, with Leu (or Ile)-Pro-Trp at the C terminus.⁽²⁴⁾ Members of a family cause identical changes in gene expression and appear to perform the same functions in *Hydra*. Fig. 2A summarises the structures of some of the peptides isolated so far.

The genes encoding a number of *Hydra* peptides have been cloned by us^(25,26) and others^(27,28) (Table 1). These genes are typical preprohormone genes. The deduced proteins have a hydrophobic signal sequence at their N terminus for translocation across the rough endoplasmic reticulum membrane. While some of the precursor proteins contain multiple copies of immature peptide sequences, other immature peptide sequences are localised as single copies on one common precursor protein (Fig. 2B).

The immature peptide sequences are usually preceded by acidic residues (Asp, Asn) followed by X-Pro sequences and C terminally flanked by dibasic (Lys-Arg) processing sites.^(22,29) Compared with neuropeptide precursors in vertebrates, the processing of *Hydra* preprohormones at acidic residues is highly unusual. The processing steps of the peptide precursors are not yet fully understood. They may include unusual processing sites and, therefore, hitherto unknown processing enzymes. Dipeptidyl aminopeptidase (DPAP) has been suggested to be one of the enzymes cleaving at the C-terminal side of the acidic residues.⁽²²⁾

In order to function as signal molecules travelling in the interepithelial space to their target cells, *Hydra* peptides have to be fairly stable molecules protected against degradation. Stabilisation is achieved by protecting groups at both the N- and C-terminal ends. Peptides Hym-176⁽³⁰⁾ and Hym-355,⁽²⁶⁾ for example, are protected against degrading enzymes by X-Pro sequence at the N terminus and amidation at the C terminus.

Since peptides were initially isolated without involving any biological assays (Fig. 1B), some totally unexpected functions have been demonstrated for the isolated peptides. Peptides have been found to serve in a variety of biological processes as



diverse as metamorphosis induction in *Hydractinia*⁽²⁴⁾ and muscle contraction in *Hydra*.^(24,30) Below we focus on two additional aspects of peptide function in *Hydra*: interstitial cell differentiation and positional information.

Two antagonistic peptides control neuron differentiation

The nervous system in *Hydra* is a simple nerve net that extends throughout the animal with high densities of neurons in the head and foot region (Fig 3A). In order to maintain a constant number of neurons and their correct regional densities while the cells are constantly dividing and being lost, *Hydra* neurons differentiate continuously from the multipotent stem cells among the interstitial cells and intercalate into the nerve net at a rate appropriate to the rate of epithelial cell division.⁽³¹⁾ Results of the Hydra Peptide Project have shown

that two groups of peptides are involved in coordinating this process.^(24,26)

When animals are exposed to peptides of the PW family for 2 days and to BrdU for a period of 1 hour at the beginning of the experiment, some of the proliferating interstitial cells that incorporated BrdU during the initial 1-hour labelling period, later entered into the differentiation pathways and appear as BrdU-labelled nematoblasts or BrdU-labelled neurons. In the presence of the PW peptides, Hym-33H (AALPW) and Hym-310 (DPSALPW) at 10^{-5} and 10^{-6} M, the labelling index of nerve cells, but not of any other cell types, significantly decreased indicating that these peptides interfered with nerve cell differentiation. Since the PW peptides are produced by epithelial cells (Koizumi et al., unpublished), these results strengthen the view that the cellular environment provides cues that are instrumental in interstitial stem cell decision

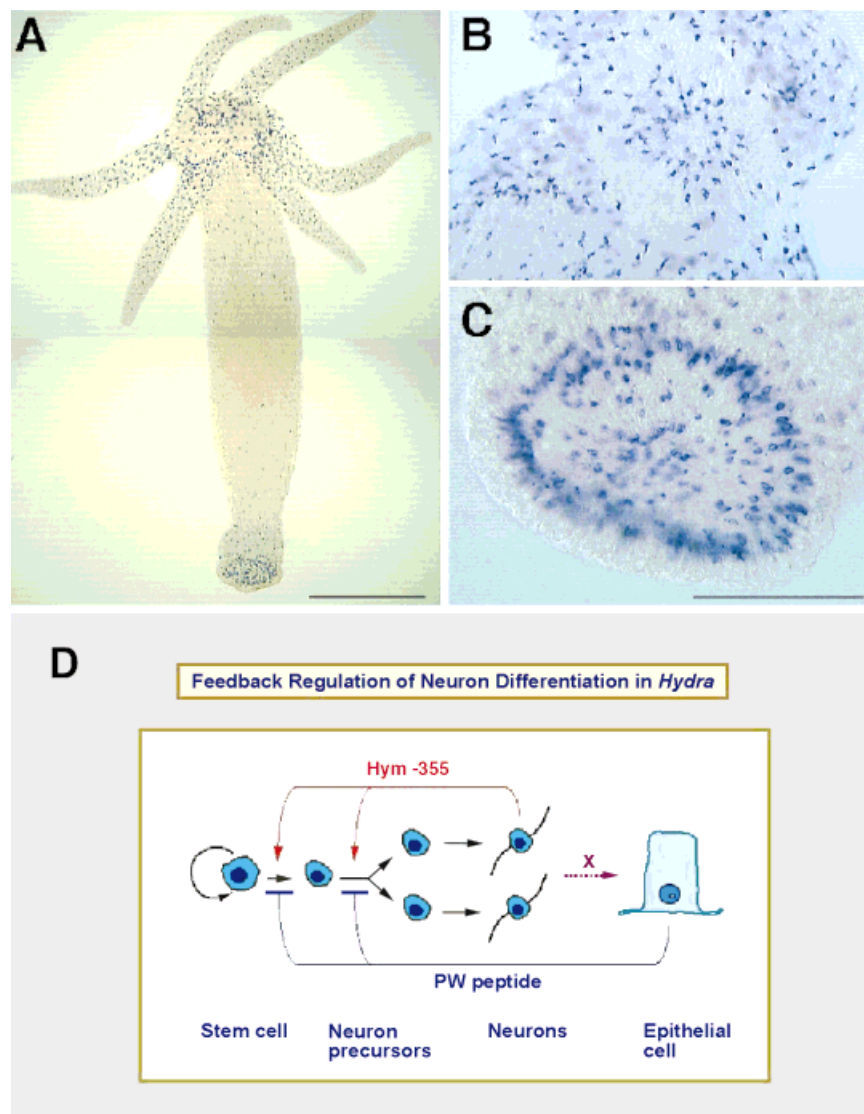


Figure 3. Peptides control neuron differentiation in *Hydra*. **A–C:** Expression pattern of peptide Hym-355 as determined by in situ hybridisation. The Hym-355 gene is expressed in neurons throughout the body. **A:** whole animal; **B:** head region; **C:** basal disk. **D:** A feedback model for the control of neuron differentiation. Hym-355 produced by neurons increases the rate of neuron differentiation. To control this positive feedback loop, epithelial cells produce PW peptides that block the nerve cell differentiation pathway. From Takahashi et al.⁽²⁶⁾

making.⁽³²⁾ The same experimental approach indicated that the peptide Hym-355 (FPQSFLPRGa) acts in the opposite direction and induces interstitial cells to undergo neuron differentiation. Treatment with the peptide Hym-355 increased the labelling index of neurons as well as the ratio of labelled neurons to epithelial cells.⁽²⁶⁾ In contrast to Hym-33H, Hym-355 is a neuropeptide produced by a subpopulation of ganglion cells concentrated in the head and foot region (Fig. 3A–C). Simultaneous treatment of polyps with peptides Hym-33H and Hym-355 resulted in normal level of neuron differentiation. Thus, both *Hydra* peptides are involved in control of neuron

differentiation but act antagonistically. To correlate the effects of the PW peptides and Hym-355 on neuron differentiation, we have proposed a model in which these peptides are involved in a feedback loop controlling the differentiation of interstitial cells into neurons (Fig. 3D).

Peptides encoding positional information

A recurring theme in embryonic development is the use of spatially localised signals to inform cells of their relative positions within the embryo. In *Hydra*, there is good evidence that patterning is governed by a positional value gradient that is

maximal in the head and decreases down the body column towards the foot.^(5,6,33,34) What are these diffusible factors that form the proposed self-regulatory, interacting gradients of signals along the apical-basal body axis? As summarised below, key signals are conveyed by peptides Hym-346 and Hym-323.

Hym-346 has been identified in extract of *Hydra magnipillata*⁽²⁴⁾ and plays a role in the positional value gradient acting as morphogenetic signal for foot differentiation.⁽³⁵⁾ A closely related peptide, pedibin was identified previously from *Hydra vulgaris*.⁽³⁶⁾ Both peptides not only accelerate foot regeneration but also cause an increase in the foot activation potential in gastric tissue by lowering the positional value gradient. This became first evident by studying genes that are restricted in their expression to *Hydra* tissue of low positional value.⁽³⁵⁾ For example, expression of homeobox gene *CnNK-2* in control polyps is localised in the basal region, which has a low positional value.⁽⁹⁾ Conversely, in polyps treated with 10^{-6} M Hym-346 for 15 days, the *CnNK-2* expression is greatly extended towards the gastric region. Thus, the peptide appears to cause a decrease in positional value of gastric tissue. The role of Hym-346 as a signal factor affecting the positional value gradient was confirmed in lateral grafting experiments. Using this experimental approach, the morphogenetic potential of tissue can be tested without interference from cell proliferation effects. Polyps were continuously incubated in 10^{-6} M Hym-346 peptide for 6 days and then used as donor tissue in homotopic gastric transplantation. Small pieces of donor gastric tissue were transplanted into the same gastric position in untreated host polyps. The fraction of transplants forming structures specific for low positional values (feet) was significantly higher in peptide-treated samples compared to untreated controls. Moreover, regeneration of peptide-treated aggregates resulted in a large increase of feet (low positional value) and sharp reduction in the number of heads (high positional value) formed.⁽³⁵⁾ Thus, the pedibin/Hym346 peptides appear to lower the positional value gradient along the body column and favour foot formation.

Transplantation and regeneration experiments indicate that another peptide, Hym-323, which shares no structural similarity to Hym-346 (Table 1) also plays an important role in *Hydra* foot formation.⁽³⁷⁾ The gene encoding Hym-323 is expressed in epithelial cells along the body column, but not in foot and head tissue. Since tissue treated with the peptide has an enhanced capacity to form a foot, the peptide affects the patterning processes involved in foot formation. Immunofluorescence studies showed that the peptide is found in epithelial cells in all regions of the polyp except the foot. It seems likely, therefore, that upon initiation of foot formation, the stored Hym-323 peptide is released from the epithelial cells and induces differentiation of basal disk cells of the foot.⁽³⁷⁾

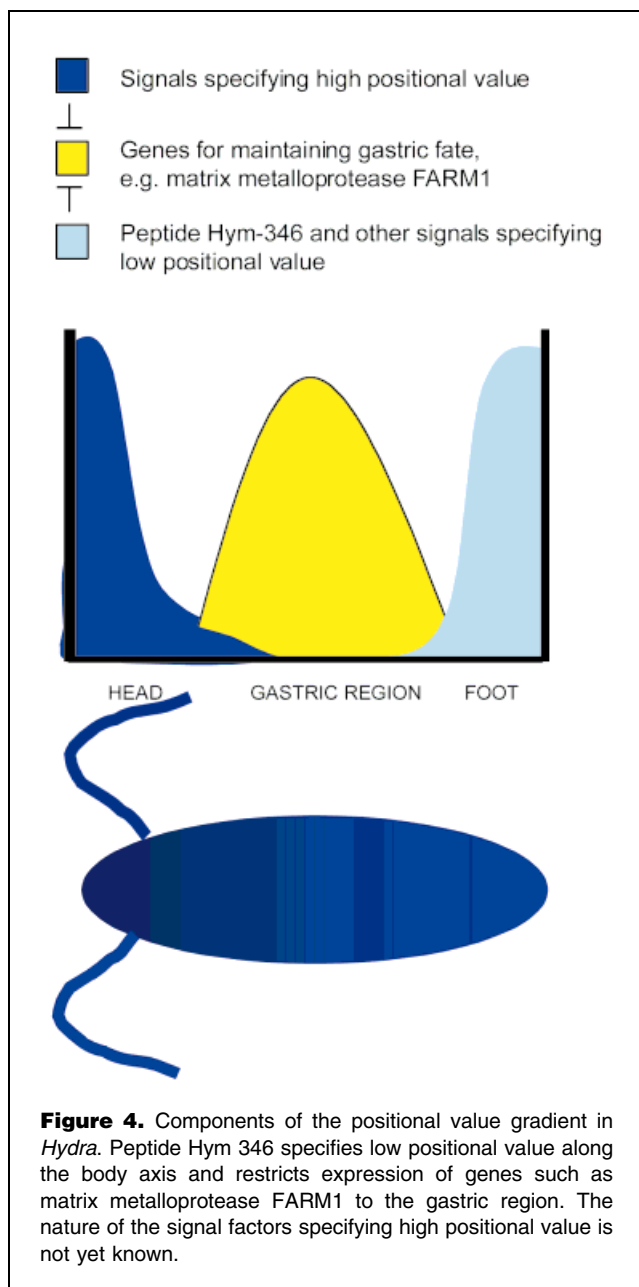
A target gene of the peptide Hym-346 modifies the extracellular matrix

How is the positional value gradient translated into changes in cell behaviour? To determine how the “foot activator” peptide Hym-346 might affect differentiation of gastric cells into foot cells, we screened for transcripts selectively activated or repressed by the peptide. Using DD-PCR,⁽³⁸⁾ we have identified and characterised an astacin matrix metalloprotease, termed Farm1 (foot activator responsive matrix metalloprotease).⁽³⁹⁾ Farm1 is expressed in epithelial cells of the gastric region and absent in apical and basal tissue. Incubation of polyps in peptides Hym-346/pedibin causes immediate downregulation of Farm1 expression. The above-mentioned peptide Hym-323, which also enhances foot formation in hydra, downregulates Farm1 expression. Treatment of polyps with the ectopic foot-inducing agent LiCl also decreased the level of Farm1 transcripts. Thus, metalloprotease Farm1 is a transcriptional target of positional signals specifying foot differentiation. Since differentiation of foot-specific cells is inversely correlated to the presence of Farm1, Farm1 activity appears to be involved in maintenance of gastric fate (Fig. 4).

What role does a metalloprotease play in patterning the basal end of a *Hydra* polyp? The foot of *Hydra* consists of a peduncle (stalk) and a basal disk of ectodermal cells secreting adhesive substances. Foot formation involves extensive tissue remodelling, occurs in the absence of cell division as a morphallactic process, and requires an intact extracellular matrix, which represents an important guide affecting cell behaviour in *Hydra*.^(40,41) Most likely, the proteolytic activity of the matrix metalloprotease Farm1 participates in modelling a microenvironment in *Hydra* that determines whether epithelial cells have presumptive gastric or foot fate.

Open questions and conclusion

Although the goal of the Peptide Project, the understanding of the molecular basis of morphogenesis in *Hydra*, has not yet been achieved, the project has led to a greater understanding of the biology of position-dependent cell differentiation in this simple animal. It is now clear that (i) peptides are abundant with the phylum Cnidaria, (ii) they play multiple roles in cell communication, cell differentiation and patterning in *Hydra*, and (iii) they are produced either as epitheliopeptides such as PW peptides or neuropeptides such as Hym-176 and Hym-355 (Table 1). Since they are so widely used in so many different biological processes, the question arises whether *Hydra* also uses the classical growth factors and cytokines which in higher organisms control cell communication and differentiation. The recent observation that, in addition to peptides, members of the *Wnt* gene family encoding secreted glycoproteins are expressed during *Hydra* axial patterning⁽⁴²⁾ may provide the answer: despite their basal position in phylogeny Cnidaria appear to use a surprisingly large



spectrum of molecules for transmission of developmental signals.

Much remains to be learned about peptides in *Hydra*. But now that we know that they exist in such abundance and variety, one can think of developing new techniques and strategies to allow functional insights. How are the peptides activated? What molecules act genetically upstream of, e.g. Hym-346? Is there a direct interaction between peptides with foot-activator activity and peptides, such as HEADY,⁽²³⁾ which are involved in specifying apical fate? Which signal transduction pathway mediates expression of the genes? Of particular

interest would be the identification of proteins that mediate the peptide actions. In higher animals, peptides bind to G-protein-coupled (7-transmembrane) receptors. For the head activator peptide, a large, multiple-domain transmembrane protein was recently reported as “head activator”-binding protein.⁽⁴³⁾ Interestingly, this transmembrane protein has no similarity to a G-protein-coupled receptor but is an evolutionary conserved LDL-related receptor. Since the receptors for the peptides isolated in the Hydra Peptide Project have not yet been identified, the question of whether *Hydra* peptide receptors are evolutionary related to the hormone receptors from higher animals remains open.

Current research is focussed on adult polyps in which patterning processes are continuously active. Are the peptides shown to be involved in maintaining the apical–basal body axis in polyps also active during early embryogenesis and, therefore, directly involved in axis formation?

Another interesting aspect concerns the question of whether *Hydra* peptides have their counterparts in higher animals or whether they are restricted to this basal metazoan group. The head activator peptide was originally isolated from *Hydra*, but later an identical form was identified in mammalian hypothalamus and intestine acting as mitogenic factor for neural cells.⁽⁴⁴⁾ Interestingly, immunohistochemical studies using antibodies highly specific to particular domains of our neuropeptides showed that subsets of neurons were immunopositive in all the animals or tissues tested so far including mammalian brains. It is intriguing to ask what tasks these peptides may have acquired in the course of metazoan evolution.

Irrespective of finding counterparts of *Hydra*'s developmental control mechanisms in higher organisms, the Hydra Peptide Project has shown that this simple animal, which you can find in nearly every single pond in your neighbourhood, has evolved astonishingly complex biochemical mechanisms to control development and differentiation.

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