

Further characterization of the PW peptide family that inhibits neuron differentiation in *Hydra*

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Abstract From an evolutionary point of view, *Hydra* has one of the most primitive nervous systems among metazoans. Two different groups of peptides that affect neuron differentiation were identified in a systematic screening of peptide signaling molecules in *Hydra*. Within the first group of peptides, a neuropeptide, Hym-355, was previously shown to positively regulate neuron differentiation. The second group of peptides encompasses the PW family of peptides that negatively regulate neuron differentiation. In this study, we identified the gene encoding PW peptide preprohormone. Moreover, we made the antibody that specifically recognizes

LPW. In situ hybridization and immunohistochemical analyses showed that the PW peptides and the gene encoding them were expressed in ectodermal epithelial cells throughout the body except for the basal disk. The PW peptides are produced by epithelial cells and are therefore termed “epithelipeptides.” Together with Hym-355, the PW family peptides mediate communication between neurons and epithelial cells and thereby maintain a specific density of neurons in *Hydra*.

Keywords *Hydra* · Neuron differentiation · PW peptides · Epithelipeptide · Hormone

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Introduction

The freshwater cnidarian *Hydra* has one of the most primitive nervous systems among metazoans. Within this system, neurons are connected to one another forming a nerve-net throughout the entire body. The nerve-net is composed of two morphologically distinguishable cell types, ganglion cells and sensory cells (David 1973). The nerve-net is not simply a uniform and diffuse structure, but has a high concentration of neurons in the head and foot regions (Bode et al. 1973; Koizumi et al. 1988). Neurons can be subtyped by the expression of specific neuropeptides and by specific antigens that can be recognized by monoclonal antibodies (Grimmelikhuijzen et al. 1982a, b; Dunne et al. 1985; Koizumi et al. 1988; Yum et al. 1998; Takahashi et al. 2000; Hansen et al. 2002; Hayakawa et al. 2004, 2007).

Neurons in *Hydra* are differentiated from multipotent interstitial stem cells that are distributed throughout the body column except two extremities (David and Gierer 1974). The stem cells give rise to two other somatic cell types, nematocytes (David and Gierer 1974), and gland cells (Schmidt and David 1986; Bode et al. 1987) as well as germ cells (Bosch and David 1987). After entering the neuron differentiation pathway, about a half of the neuron precursor cells migrate toward the head or the foot (Heimfeld and Bode 1984; Fujisawa 1989; Teragawa and Bode 1990, 1995; Technau and Holstein 1996; Hager and David 1997). The remaining half of the neuron precursors do not migrate, but completely differentiate and are integrated into the nerve-net of the body column.

Hydra tissue is in a dynamic state and is constantly undergoing renewal as a result of continuous growth and differentiation (Campbell 1967a, b; 1973). The nerve-net is also in a steady state of production and loss of neurons. To compensate for the loss, new neurons arise continuously by differentiation from multipotent interstitial stem cells (David and Gierer 1974). In this way, the homeostasis of a neuron population is maintained (Bode et al. 1990). Although the underlying mechanisms for the homeostasis are not well understood, we have identified two groups of peptides that affect neuron differentiation through a systematic screening of peptide signaling molecules from *Hydra* (Takahashi et al. 1997, 2000). The neuropeptide, Hym-355, positively regulates neuron differentiation, while the PW peptide family negatively regulates neuron differentiation. Originally, 4 PW peptides that share a common C-terminal LPW or IPW motif were identified (Takahashi et al. 1997). These two groups of peptides counteract to each other. The actions of these molecules have been incorporated into a feedback model to explain the homeostasis of a neuron population (Takahashi et al. 2000).

In the present study, we report further characterization of the PW peptide family, including identification of the gene

encoding these peptides, its expression pattern, immunohistochemical localization of the PW peptides, an additional function of the peptides and identification of a new member of the PW peptide family (Hym-1397; TPALHW) that is encoded in the precursor protein.

Materials and methods

Hydra and culture conditions

The wild-type strain (105) of *Hydra magnipapillata* (Sugiyama and Fujisawa 1977) and the epithelial *Hydra* strain derived from 105 by colchicine treatment (Campbell 1976) were used. The wild-type *Hydra* was cultured as described previously (Sugiyama and Fujisawa 1977). The epithelial *Hydra* was cultured according to the protocols of Nishimiya-Fujisawa and Sugiyama (1993). The epithelial *Hydra* consists only of ectodermal and endodermal epithelial cells and lacks all cells of the interstitial stem cell lineage with the exception of gland cells (Marcum and Campbell 1978; Sugiyama and Fujisawa 1978).

Peptide purification and sequencing

Systematic purification of the peptides and their amino acid sequencing was carried out as described previously (Takahashi et al. 1997; 2000).

Peptide synthesis

Chemical synthesis of peptides was carried out using a standard solid-phase method (PSSM-8, Shimadzu), followed by TFA-anisole cleavage and high-performance liquid chromatography (HPLC) purification. The structure of the synthetic peptides was confirmed by amino acid sequencing with an automated gas-phase sequencer (PPSQ-10, Shimadzu) and FAB mass spectrometry.

Identification of the gene encoding the PW peptides

All *Hydra* EST sequences were downloaded from DDBJ database/Genbank and translated in six frames using custom perl script. The Hym-37 sequence (SPGLPW) was searched against the translated EST database, and the longest clone containing Hym-37 sequence was selected. Primers corresponding to the 5' and 3' ends of this clone were used to amplify the entire clone for sequencing. Reverse transcriptase-polymerase chain reaction and nucleotide sequencing were performed as described previously (Takahashi et al. 2000). For analyzing the genomic organization of the gene, the entire cDNA sequence was blasted against *Hydra* whole genome shotgun (WGS) sequences using MegaBLAST (NCBI).

For searching the PW peptide gene-related gene, entire WGS sequences were downloaded and translated in six frames. The translated cDNA as well as PW peptide sequences flanked by Lys or dibasic amino acids were used as queries to blast against the translated genome database.

In situ hybridization

Digoxigenin-labeled antisense and sense probes were synthesized from a fragment of PW cDNA precursor sequence (nucleotide positions; 795–1624) using an RNA in vitro transcription kit (Roche). Whole-mount in situ hybridization (WISH) was carried out as described previously (Grens et al. 1996). Samples were hybridized for 48 h using a probe concentration of 0.75 ng/μl and then stained with BM-purple (Roche) at 37°C for 20 min in the dark. Thereafter, they were rinsed, incubated in 100% ethanol and subsequently mounted in Euparal (Asco Laboratories).

Antibody production and determination of its specificity

An antiserum against Hym-33H was produced as described by Yum et al. (1998) with the following modifications: (1) A synthetic peptide corresponding to Cys-Hym-33H (CAALPW) was conjugated to keyhole limpet hemocyanin (KLH) using an *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) coupling method (Peerters et al. 1989). (2) Three New Zealand white rabbits and three Japanese white rabbits were immunized. In all cases, antibodies with equally high titers were obtained.

A competitive enzyme-linked immunosorbent assay (ELISA) was carried out to examine the specificity of the anti-Hym-33H antibody. The Cys-Hym-33H peptide, or Cys-Hym-33H conjugated to KLH, was used as the immobilized antigen in competitive ELISA (Takahashi et al. 2003). Both coating plates gave similar results. The anti-Hym-33H antibody was diluted to 1/5,000 with blocking solution (phosphate-buffered saline containing 0.1% bovine serum albumin). The competitive peptides used were Cys-Hym-33H, Hym-33H, Hym-37 (SPGLPW), Hym-37amide (SPGLPW–NH₂), an unrelated amidated peptide Hym-54 (GPMTGLW–NH₂), and Hym-54–OH (GPMTGLW). The first three peptides strongly competed with the immobilized peptide, while the latter three showed poor or no competition indicating high specificity of the antibody to LPW (data not shown).

Immunohistochemistry

Indirect immunofluorescent staining was carried out on whole mounts of polyps using the anti-Hym-33H antibody (1:1,000 dilution) as described previously (Minobe et al.

1995). Samples were observed under a fluorescence microscope (Nikon) or a confocal microscope (Olympus).

Peptide treatment

Hydra was treated with the PW peptide Hym-33H (AALPW) at a concentration of 10⁻⁶ M in the culture solution as described previously (Takahashi et al. 1997). The culture solution with or without the peptide was replaced daily with a freshly prepared one.

BrdU labeling and detection

Polyps were placed in 2 mM BrdU (Aldrich) in culture solution with or without peptide. The same solution was then immediately injected into the gastric cavity through the mouth using fine-tipped polyethylene tubing. The animals were incubated in the solution for a total of 1 h of pulse labeling. Polyps were sampled immediately after pulse labeling or were washed thoroughly and chased in solution containing no BrdU for varying periods of time before sampling. Polyps were not fed over the entire period. For whole mount observations, the polyps were relaxed in 2% urethane and fixed in 70% ethanol. Fixed animals were processed for immunostaining with an anti-BrdU monoclonal antibody (Becton-Dickinson) followed by a fluorescein isothiocyanate-labeled rabbit anti-mouse IgG antibody (Amersham). For observation of dissociated cells, a previously described maceration method (David 1973) was employed. In brief, the macerated cells were spread onto a gelatin-coated glass slide, air dried, and processed for immunostaining with an anti-BrdU monoclonal antibody and an ABC kit (Vector) according to the manufacturer's recommended protocol.

For region-specific observation, each polyp was bisected at the end of the experiment into three regions along the body axis: the distal one-fourth, middle two-fourths, and proximal one-fourth fractions of the body column (from the hypostome to the basal disk). The distal one-fourth region contains the tentacles, a hypostome, and the upper gastric region, and the proximal one-fourth region contains both the peduncle and the basal disk.

Results

Isolation and expression of the gene encoding the PW peptides

The recent collection of *Hydra* ESTs in the database (DDBJ/Genbank) enabled us to quickly identify the gene that encodes the PW peptides. We first searched for the presence of the Hym-37 sequence in the translated *Hydra*

EST database. A number of clones were found that contained one or two copies of the Hym-37 as well as other PW peptide sequences identified previously (Hym-33H, Hym-35, and Hym-310; see Table 1). These peptide sequences were flanked by N-terminal Lys or Asn and C-terminal dibasic amino acids. A few clones were selected and used as queries against the database to connect the clones in a single sequence. One continuous sequence that presumably covers the entire coding sequence was obtained. The clones that correspond to the 5' and 3' ends of the entire sequence have been deposited in the NCBI database as DN244215 and CO745640, respectively. Primers were designed so that the entire sequence could be amplified and the nucleotide sequence determined. The obtained nucleotide sequence (DDBJ Accession no., AB459520), and the deduced amino acid sequence of the precursor protein are shown in supplementary Fig. S1.

The precursor protein contained an N-terminal signal sequence predicted by SignalP and the sequences of seven species of PW peptides including three putative PW peptides (EPSAALPW, IPALPW, and TRTLPW). There were three copies of Hym-37 (SPGLPW) and one copy each of Hym-33H (AALPW), Hym-35 (EPSAAIPW), Hym-310 (DPSALPW), and putative peptide sequences within the precursor protein. The precursor also contained a new species of peptide with the sequence TPALHW (Hym-1397). This TPALHW peptide was recently identified over the course of a non-targeted screen of *Hydra* peptides (the *Hydra* peptide project; Takahashi et al. 1997; Fujisawa 2008). The sequences of these peptides are summarized in Table 1. All peptides in the precursor protein are flanked by a processing site of C-terminal dibasic amino acids. An N-terminal Lys residue is present in all peptides except for AALPW (Hym-33H) that is preceded by an Ile, and IPALPW, EPSAAIPW and one copy of Hym-37 that are preceded by an Asn. Since the Ile that precedes AALPW is itself preceded by an Asn, and since Asn has been

suggested to function as a processing site in *Hydra* (Leviev et al. 1997), AALPW could be a naturally or artificially occurring derivative of IAALPW. A second possibility is that AALPW might be generated from the putative PW peptide EPSAALPW by cleavage at its Ser residue, as Ser has also been suggested as a cleavage site for the generation of cnidarian neuropeptides (Leviev et al. 1997).

The genomic structure of *PW* was analyzed as described in the “Materials and methods” section. The *PW* consisted of three exons with two introns of 115 and 23 nucleotides. *PW*-related genes were also searched in the translated WGS database as described in the “Materials and methods” section. However, no such genes were detected suggesting that the *Hydra* genome contains a single copy of *PW* but no *PW*-related genes.

To determine the cell type in which the gene encoding the PW peptides is expressed, WISH was carried out. The body wall of hydra consists of two epithelial layers, the ectoderm and endoderm, separated by a basement membrane called the mesoglea. As shown in Fig. 1a–c, the gene is clearly expressed in the ectoderm throughout the entire body with the exception of the basal disk. Epithelial *Hydra* that lacks cells of the interstitial stem cell lineage including neurons was also used for WISH. As shown in Fig. 1e, the gene was expressed in the ectodermal epithelial cells confirming the result obtained in the intact animals. In order to examine the gene expression in endoderm, a stained sample was transversely sectioned (Fig. 1d). The endoderm was completely free of a signal, whereas the ectoderm was stained blue. The result demonstrated the specific expression of *PW* in the ectodermal epithelial cells.

Localization of the PW peptide in the ectodermal epithelial cells

Tissue localization of the PW peptide was examined by indirect immunofluorescent staining using the anti-Hym-33H antiserum on whole mounts of *Hydra*. As shown in Fig. 2a–c, the ectodermal epithelial cells throughout the entire body were stained with the exception of the basal disk. Immunostaining patterns of the PW peptide on epithelial *Hydra* were very similar to that obtained with normal polyps (Fig. 2d–f). The result showed that the peptides localize in the ectodermal epithelial cells. In order to examine if the peptides localize in endodermal epithelial cells, confocal micrographs on the side of body column were prepared. As shown in Fig. 2h, the intense staining in the apical part of ectodermal epithelial cells was observed but not in the endodermal epithelial cells. Bright stains in irregular sizes in the endoderm presumably represent an artifact. The result is in good agreement with the WISH data. Subcellular localization of the peptides in the

Table 1 Established and putative PW peptides that could be released from the PW peptide precursor protein

Copies	Name	Structure
1	Hym-33H	AALPW ^a
1	Hym-35	EPSAAIPW ^a
1	Putative PW peptide 1	EPSAALPW
3	Hym-37	SPGLPW ^a
1	Hym-310	DPSALPW ^a
1	Hym-1397	TPALHW
1	Putative PW peptide 2	IPALPW
1	Putative PW peptide 3	TRTLPW

^a Takahashi et al. (1997)

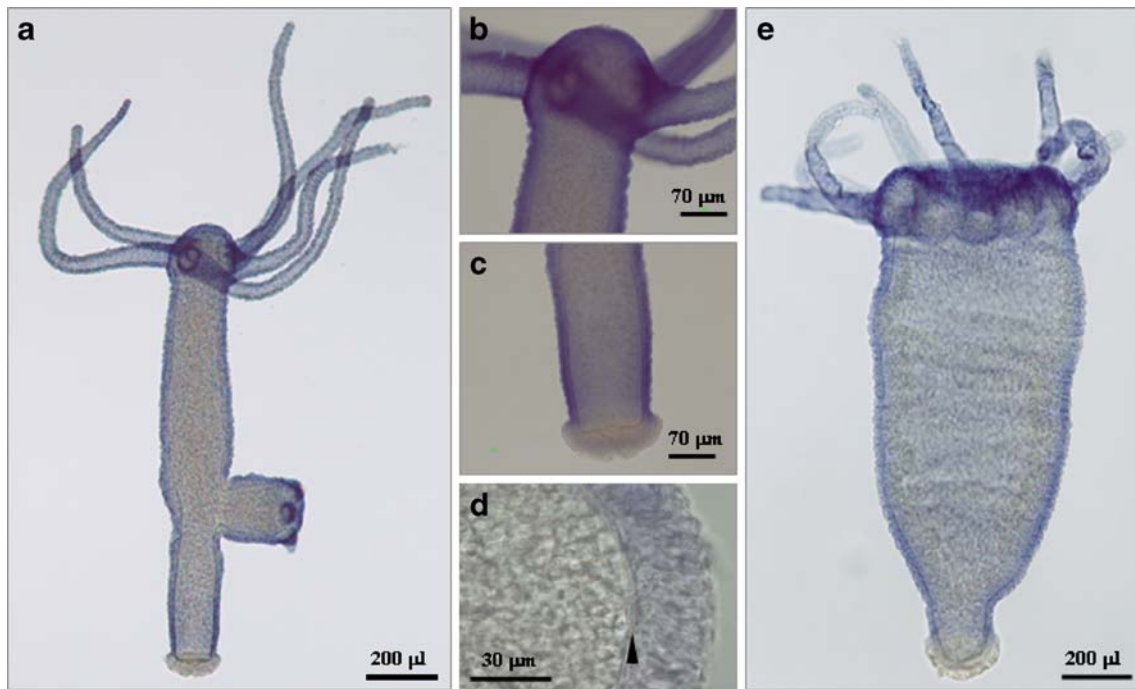


Fig. 1 Expression pattern of *PW* in a normal polyp and in an epithelial *Hydra*. **a** Whole normal hydra. **b** Enlargement of the head region. **c** Enlargement of the foot region. **d** Enlargement of transverse

section of body column. An *arrowhead* indicates a basement membrane, mesoglea. Ectoderm is on the *right* of mesoglea and endoderm on the *left*. **e** Whole epithelial *Hydra*

ectodermal epithelial cell is not clear at the moment, but it appears that they localize in discrete dots under the apical cell membrane (also see Fig. 2c, f for dotted stains). The nature of these dots will be discussed later.

No signal was observed when the antibody was pre-absorbed with an excess of the Hym-33H peptide, or when the animals were fixed with 70% ethanol which usually washes peptide antigens out of tissues (Harafuji et al. 2001; data not shown). The results suggest that the antibody specifically recognizes the *PW* peptides and that the *PW* epitope is present only in the ectodermal epithelial cells being consistent with the WISH result.

Hym-33H affects the early stages of neuron differentiation

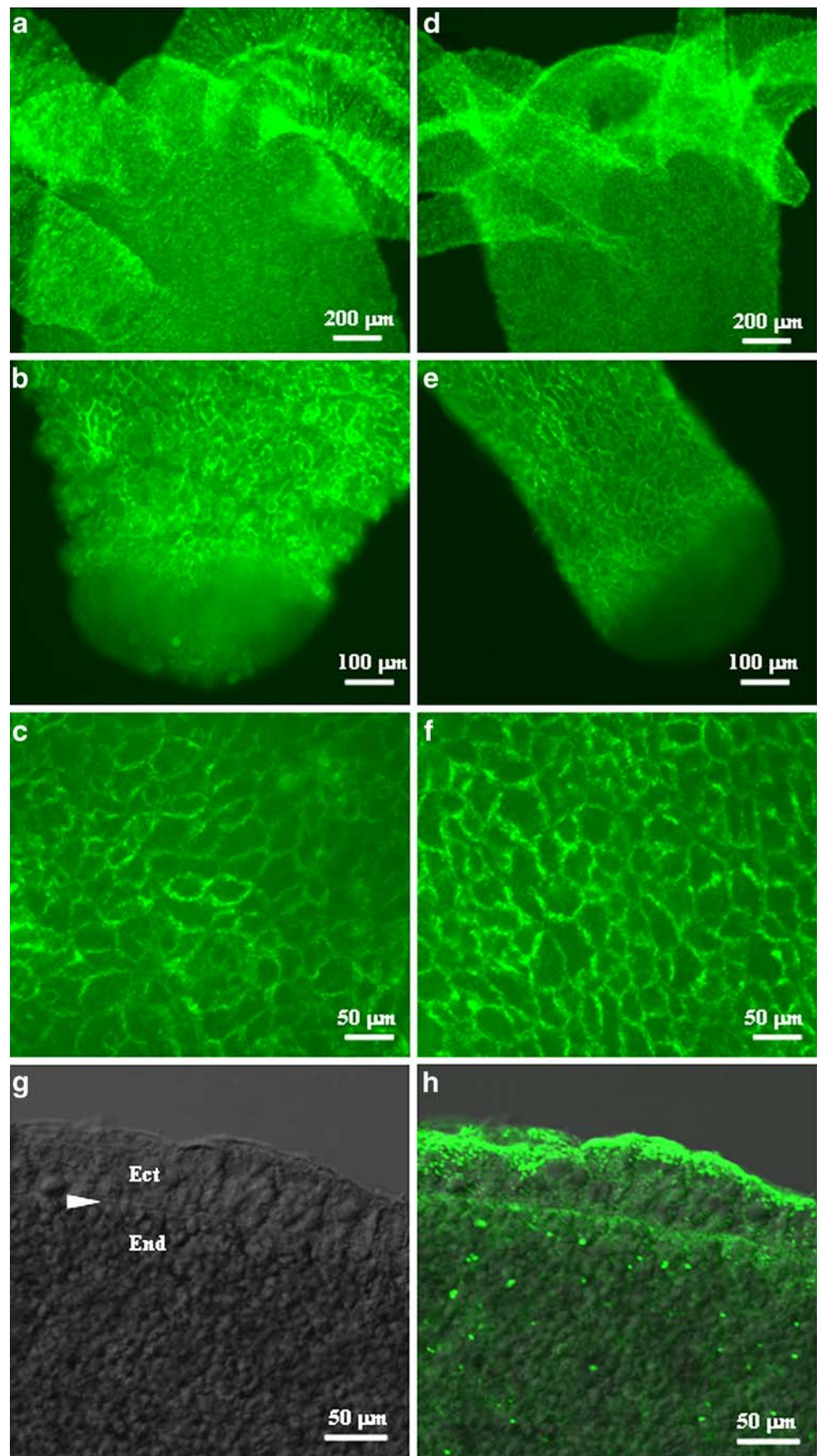
Differentiation of interstitial stem cells into neurons involves several sequential events, including commitment to differentiation, migration of committed precursors to the site of differentiation, final mitosis, and overt differentiation into neurons. To examine at what point during the differentiation process Hym-33H affects, we pulse-labeled polyps with BrdU (2 mM) for 1 h and chased for varying periods of time in the presence or absence of the peptide. At the end of the chase, the animals were macerated, and the number of labeled neurons was scored.

As shown in Fig. 3, labeled neurons appeared about 10 h after labeling, and the number of labeled neurons continued

to increase thereafter. No difference was observed in the kinetics of neuron differentiation between control and treated animals. Thus, the peptide did not alter the rate at which the final differentiation phase occurred. Instead, the peptide appears to decrease the number of precursor cells by inhibiting the commitment or migration of precursor cells or by lengthening the S-phase of the final cell cycle of the precursors.

To further investigate this effect of the peptide, a polyp was pulse-labeled for 1 h, and the lower two thirds of its body was axially grafted to the upper one third of a non-labeled polyp (see Fig. 4 for the experimental scheme). The grafted polyps were treated for 2 days with Hym-33H. At the end of the peptide treatment, the head was isolated by amputating at the subtentacular position, and the number of BrdU-labeled small single or paired nuclei in the hypostome area was scored in whole mounts. Neuron precursors that are generally considered to be single, or a pair of small interstitial cells have smaller nuclei (ca 7 µm) compared to large interstitial cells (ca 11 µm). The number of labeled nuclei detected in the hypostome was much smaller in the treated animals than in the non-treated control (Table 2). Since neuron precursors appear to migrate immediately after commitment (Hager and David 1997), it is not easy to differentiate commitment and migration. However, the results indicate that the peptide inhibited the early stages of neuron differentiation.

Fig. 2 Immunohistochemical staining of a normal and an epithelial *Hydra* with an anti-Hym-33H antiserum. For both hydra, a signal was detected in ectodermal epithelial cells throughout the body with the exception of the basal disk. **a** Head region and **b** foot region of a normal hydra. **c** Enlargement of stained ectodermal epithelial cells in a part of the body column of a normal hydra. **d** Head region and **e** foot region of an epithelial hydra. **f** Enlargement of stained ectodermal epithelial cells in a part of the body column of an epithelial hydra. **g** Differential interference contrast image of a part of the body column. Ectodermal (*Ect*) and endodermal (*End*) epithelial layers separated by the mesoglea (*arrowhead*). **h** Confocal image of (**g**)



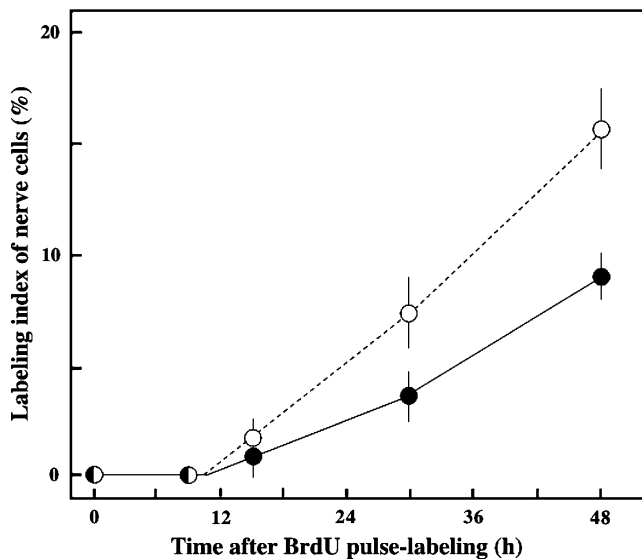


Fig. 3 Effect of Hym-33H on the labeling kinetics of neurons. *Filled circles* represent treated animals, while *open circles* represent the control. *Vertical bars* represent SD of the mean. Labeling indices of samples treated for 30 or 48 h were statistically different from the control at the 99.5% and 99.9% significance levels as assessed by the Student *t* test

Inhibition of neuron differentiation by Hym-33H throughout the body

We have previously shown that PW peptides inhibit neuron differentiation in the entire body of normal polyps (Takahashi et al. 1997). In this study, we examined the effect of PW peptides on neuron differentiation in more detail.

To examine whether nerve cells distributed throughout the body column are affected by peptide treatment or whether the effect of the peptide is restricted to a particular region along the body column, we pulse-labeled newly detached young polyps (stage 1) with 2 mM BrdU for 1 h in the presence of Hym-33H at a concentration of 10^{-6} M.

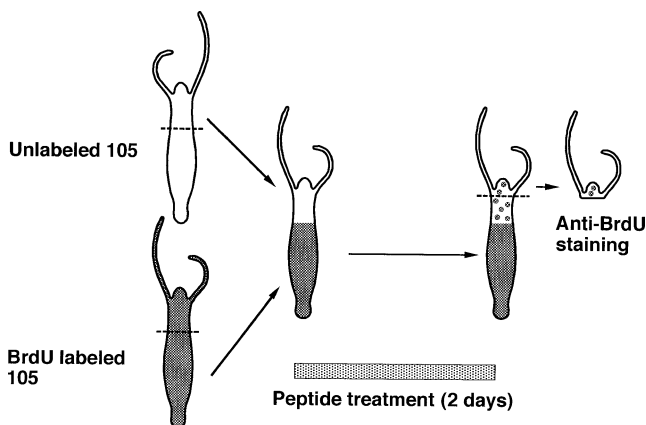


Fig. 4 Outline of the transplantation experiment designed to examine the effect of Hym-33H on the stage of neuron differentiation

Table 2 Effect of Hym-33H on the migration of BrdU positive cells from the lower two thirds of the body into the hypostome of *Hydra*

Hym-33H	Number of BrdU positive cells in the hypostome
0	244.75±36.58
10^{-6} M	179.67±10.27 ^a

Values are the mean±SD. The sample size was 20 animals for the Hym-33H treated group and 15 animals for the control group

^a Statistically significant difference from control at the 99.5% level using the Student *t* test

Animals were washed thoroughly and further cultured for 47 h without BrdU but with the peptide. The culture solution was replaced once at 24 h with fresh solution containing the same concentration of the peptide. At 48 h, each animal was divided into three parts, apical one fourth, middle two fourths, and basal one fourth of the body column, and ten pieces each were macerated together and processed for scoring BrdU-labeled cells in macerated preparations (see “Materials and methods” section). For control, animals were processed similarly without peptide treatment. Table 3 shows the labeling index of nerve cells in each region in the presence or absence of peptide treatment. No difference was observed in the labeling indices of epithelial cells, single and paired large interstitial cells, a nest of four nematoblasts (the first cell type in the nematocyte differentiation pathway), and gland cells between control and peptide-treated samples. Table 3 includes only the labeling index of epithelial cells in a whole body. In all three body regions, peptide treatment induced significant decreases in the labeling index of neurons. The lack of effect of the peptide on other cell types is consistent with previous observations (Takahashi et al. 1997). These results indicate that the Hym-33H peptide affects neuron differentiation in all three body regions of *Hydra*.

Effect of long-term treatment with Hym-33H on neuron differentiation

Since short-term treatment with the peptide for 2–3 days significantly reduced neuron differentiation (Takahashi et al. 1997), a more significant reduction in the total number of neurons was expected with longer-term treatment. Newly detached young polyps were treated with Hym-33H (10^{-6} M) for 7 days with daily change of the peptide solution. On day 7, each polyp was cut into three parts as described above, and ten pieces each were macerated together to score for the neuron density (the number of neurons compared to the number of epithelial cells). As shown in Table 4, unexpected results were obtained. The neuron density in the entire hydra was the same in the presence or absence of Hym-33H. Only the distal one-fourth region of the treated hydra still showed a small reduction in neuron density, but

Table 3 Inhibition of neuron differentiation throughout the body column by Hym-33H

Hym-33H	Labeling index (%)				
	Neurons				Epithelial cells
	Apical 1/4	Middle 2/4	Basal 1/4	Whole	Whole
0	16.3±3.0	14.8±1.2	15.6±3.2	15.8±1.8	21.0±2.0
10 ⁻⁶ M	9.7±1.0 ^a	8.6±0.2 ^a	8.8±2.4 ^a	9.0±1.0 ^a	21.4±1.8

Values are the mean±SD. For all treatments, the experiment was carried out in triplicate using ten animals per group

^a Statistically significant difference from that of the control at 99.5% using the Student *t* test

this decrease was not statistically significant. These results suggest that the decrease in the density of the neuron population induced by Hym-33H is counteracted by a feedback signal.

Discussion

PW peptides are epitheliopeptides

The following three lines of evidence indicate that PW peptides are synthesized and localized in epithelial cells:

1. A gene encoding all known PW peptides was identified (supplementary Fig. S1). No other PW peptide genes or PW peptide gene-related genes were found in the *Hydra* genome sequence, suggesting that the PW gene is a single copy gene in the genome. The sequence of the precursor protein, deduced from the cDNA sequence, contained an N-terminal signal sequence, six PW peptides, three putative PW peptides, and one HW peptide (supplementary Fig. S1 and Table 1). Essentially, all of the peptide sequences were flanked by processing sites at both ends. However, none of these peptide sequences terminated in a Gly residue before dibasic amino acids at their C-termini. Since a Gly residue functions as an amide donor, the lack of C-terminal Gly indicates that these peptides are not amidated. This lack of amidation is in contrast to neuropeptides, as all of the known neuropeptides in

Hydra are C-terminally amidated. The expression of the PW gene exclusively in ectodermal epithelial cells throughout the body except the basal disk was shown by WISH (Fig. 1).

2. Immunostaining of whole mounts of hydra using an anti-Hym-33H antiserum showed that the epitope was localized in ectodermal epithelial cells (Fig. 2). The antigen used to produce this antibody was a peptide with the sequence CAALPW that was conjugated to KLH via an N-terminal Cys residue. Since there is a probability that the AALPW sequence could occur in proteins, it was unclear whether the antibody specifically recognizes the peptide. To confirm the specific binding of the antibody to the peptide, two different methods were employed (data not shown): First, the antibody was pre-absorbed with an excess of the Hym-33H peptide (AALPW); second, short peptides but not proteins were washed out of the tissue by using ethanol as a fixative (Harafuji et al. 2001). Both treatments eliminated the immunofluorescent signal from the tissue indicating that this signal is specific for peptide recognition.

The epitope appears to be present in dots in the apical submembrane part of the cells (Fig. 2h). These dotted structure may be secretory vesicles in the ectodermal epithelial cells. A similar observation has been made recently by Khalturin et al. (2008). They show that the fusion protein of Hym-301 peptide precursor and GFP (Hym-301 is an epitheliopeptide, Takahashi et al. 2005) is localized in secretory vesicle-like structures in the apical part of ectodermal epithelial cells. Also, an electron microscopic study shows that ectodermal epithelial cells contain secretory vesicle-like structures under the cell membrane (Javois et al. 1986; Noda, personal communication). Therefore, our observation might suggest that the PW peptides are stored in such vesicles and released when neurons are present in the vicinity possibly inhibiting new neuron differentiation nearby.

3. Analysis of peptide extracts from elutriated epithelial cells or epithelial *Hydra* by HPLC indicated that specific fractions eluted from C-18 reverse-phase column (ODS-80TM, Tosoh) exhibited immunoreac-

Table 4 Effect of long-term (7 days) treatment with Hym-33H on neuron density

Hym-33H	Number of neurons/number of epithelial cells by region			
	Apical 1/4	Middle 2/4	Basal 1/4	Whole
0	0.31±0.02	0.09±0.03	0.15±0.02	0.18±0.02
10 ⁻⁶ M	0.25±0.03	0.09±0.01	0.19±0.01	0.17±0.01

Values are the mean±SD. For all treatments, the experiment was carried out in quadruplicate using ten animals per group

tivity to an anti-Hym-33H antibody (data not shown). These fractions coincided with those in which synthetic PW peptides were eluted.

From these results, we conclude that PW peptides are “epitheliopeptides” produced from ectodermal epithelial cells throughout the body column except for the basal disk.

Regulation of neuron differentiation in *Hydra*

Hydra tissue is in a dynamic state and is constantly undergoing renewal as a result of continuous growth and differentiation of epithelial cells and interstitial stem cells. Nevertheless, neuron density is maintained at a constant level suggesting a homeostatic mechanism that monitors and controls the level of neurons. It has been suggested that interstitial cells or their derivatives control the level of interstitial cells and neurons possibly through a negative feedback mechanism (Sugiyama and Fujisawa 1979). In our previous studies, we showed that PW peptides inhibit neuron differentiation and suggested that these peptides counteract the effect of a neuropeptide Hym-355 that enhances neuron differentiation to maintain homeostasis of a neuron population in the *Hydra* body (Takahashi et al. 1997; 2000).

Treatment with the PW peptide for 2–3 days inhibits 40% of neuron differentiation compared to the control (Takahashi et al. 1997; Table 3). If neuron differentiation is continuously inhibited at this rate, a longer treatment may quickly wipe out the entire neuron population. However, such an event did not occur; rather, the neuron density recovered after 1 week of Hym-33H treatment (Table 4). This might be interpreted that the recovery involves a negative feedback signal of a PW peptide family emanated from neurons and counteracted by some positive signal. However, as shown in this study, PW peptides are localized in ectodermal epithelial cells, not in neurons.

These findings suggest the existence of a cross-talk between epithelial cells and neurons in regulating the neuron density in *Hydra* tissue. Epithelial cells must somehow sense the local neuron density and subsequently either emit or halt the emission of an inhibitory signal. When interstitial cells are introduced into an epithelial polyp, the rate of nerve cell differentiation initially increases and then returns to a normal level when enough neurons are produced (Fujisawa 1989). If epithelial cells continuously emitted PW peptides, an inhibitory signal, neuron differentiation would not take place at an increased rate. Thus, our observations suggest the following scenario: (1) The inhibitory signal is not emitted until neurons are produced. (2) Thus, new neuron differentiation takes place. (3) Only epithelial cells that have interacted with neurons emit the inhibitory signal. (4) The signal acts in a short

range to inhibit neuron differentiation only in the vicinity of interaction. We have previously reported that the positive signal is most likely the neuropeptide, Hym-355 and that Hym-33H and Hym-355 counteract to each other (Takahashi et al. 2000). Based on these results, we have proposed a feedback model to explain the regulation of neuron differentiation in *Hydra* (Takahashi et al. 2000).

PW peptides inhibit commitment of neuron precursors and/or their migration

The PW peptide family appears to be redundant in function, with four peptides of the family (Hym-33H, Hym-35, Hym-37, and Hym-310) evoking the same alteration in gene expression patterns in *Hydra* when analyzed by differential display PCR (Takahashi et al. 1997; Liang and Pardee 1992) and equally inhibiting neuron differentiation (Takahashi et al. 1997). However, the need for multiple peptides for the same signal is currently unclear.

Neurons differentiate continuously from multipotent stem cells that are located among the interstitial cells along the entire *Hydra* body column. Thus, new neurons are constantly intercalated into the nerve-net at a rate appropriate to the rate of epithelial cell division. In this way, individual subsets of neurons, as well as the total number of neurons, are maintained. In this study, we showed that the PW peptide inhibited neuron differentiation at early stages of the pathway, possibly at the stage of commitment to differentiation and/or at the stage of migration to the site of differentiation (the head and foot) that occurs immediately after commitment (Hager and David 1997). This was concluded by the following results: (1) Hym-33H has no effect on the traverse through the last stage of neuron differentiation (Fig. 3); and (2) the number of precursors that migrated to the head region decreases upon Hym-33H treatment (Table 2). These results strongly support that PW peptides are a negative signal involved in homeostatic maintenance of a neuron population.

An additional aspect of the control of neuron differentiation is the maintenance of higher densities of neurons in the head and foot compared to the body column. A higher rate of neuron differentiation takes place in the extremities because a greater number of stem cells enter the neuron pathway and because neuron precursors migrate from the body column to the extremities (Hager and David 1997). As we saw in this study, Hym-33H inhibited neuron differentiation uniformly along the body column (Table 3). Thus, some additional mechanisms are required for generating an elevated level of neuron densities in the head and foot. At the moment, no good explanations are available, but underway are several lines of study to uncover factors that are involved in the regional regulation of neuron differentiation.

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