

A novel neuropeptide, Hym-355, positively regulates neuron differentiation in *Hydra*

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SUMMARY

During the course of a systematic screening of peptide signaling molecules in *Hydra* a novel peptide, Hym-355 (FPQSFLPRG-NH₂), was identified. A cDNA encoding the peptide was isolated and characterized. Using both in situ hybridization and immunohistochemistry, Hym-355 was shown to be expressed in neurons and hence is a neuropeptide. The peptide was shown to specifically enhance neuron differentiation throughout the animal by inducing interstitial cells to enter the neuron pathway.

Further, co-treatment with a PW peptide, which inhibits neuron differentiation, nullified the effects of both peptides, suggesting that they act in an antagonistic manner. This effect is discussed in terms of a feedback mechanism for maintaining the steady state neuron population in *Hydra*.

Key words: Neuropeptide, Neuron differentiation, Positive feedback signal, *Hydra*

INTRODUCTION

Hydra has one of the most primitive nervous systems among metazoans. Neurons are connected to one another, forming a nerve net throughout the animal. The nerve net is composed of two morphologically distinguishable cell types, ganglion cells and sensory cells (David, 1973). Subsets of neurons have been characterized by the presence of different neuropeptides or by specific antigens recognized by monoclonal antibodies (Grimmelikhuijzen et al., 1982a,b; Dunne et al., 1985; Koizumi et al., 1988).

The differentiation pathway of neurons in *Hydra* has been well worked out at the cellular level. Neurons arise by differentiation from multipotent stem cells among the interstitial cells, which are uniformly distributed along the body column, but absent in the head and foot (David and Gierer, 1974; Yaross and Bode, 1978; David and Plotnick, 1980). The stem cells also give rise to two other classes of somatic cells, the nematocytes (David and Gierer, 1974) and gland cells (Schmidt and David, 1986; Bode et al., 1987) as well as germ line cells (Bosch and David, 1987). After entering the neuron differentiation pathway, about half of these neuron precursor cells migrate to their sites of differentiation, in the

head and foot (Heimfeld and Bode, 1984; Fujisawa, 1989; Teragawa and Bode, 1990, 1995; Technau and Holstein, 1996; Hager and David, 1997). Once in an extremity, the neuron precursor cell undergoes one, or occasionally two to three, rounds of cell division, before differentiating into neurons (Heimfeld and Bode, 1986; Bode et al., 1990; Hager and David, 1997). The other half of the neuron precursor cells do not migrate, but complete differentiation and are integrated into the nerve net.

Due to the tissue dynamics of *Hydra*, the nerve net is also in a dynamic state. The epithelial cells of the body column in both tissue layers are continuously in the mitotic cycle (Campbell, 1967a; David and Campbell, 1972). As the tissue grows, it is displaced apically into the head from the upper part of the body column, and basally into developing buds or onto the foot from the lower part of the column (Campbell, 1967b, 1973). Epithelial tissue displaced into the head moves onto, and along, the tentacles and is eventually sloughed at the tentacle tips. A similar process occurs at the basal end. Thus, the epithelial cell populations are in a steady state of production and loss. The nerve net is in a similar steady state of production and loss of neurons. Neurons, which reside among the epithelial cells, are continuously displaced towards

an extremity, and are eventually lost along with their epithelial neighbors. To balance this loss, neurons arise continuously by differentiation from the stem cells of the interstitial cell lineage all along the body column (David and Gierer, 1974). In this way the nerve net is maintained (Bode et al., 1990)

The mechanisms underlying this steady state are unknown. However, there is some data suggesting the involvement of a feedback mechanism to maintain the homeostasis of the neuron population (Yaross and Bode, 1978; Sugiyama and Fujisawa, 1979; Bosch et al., 1991). For example, when the tissue of the body column is devoid of neurons, the rate of neuron differentiation is higher than in normal tissue (Bosch et al., 1991).

Although the mechanisms for maintaining the steady state of nerve net are not understood, a few of the molecules involved have been identified. One, the head activator, which stimulates neuron differentiation and cell proliferation has been known for a long time (Schaller, 1976a,b; Schaller and Bodenmueller, 1981). Recently, we have initiated a systematic effort to isolate peptide signaling molecules from *Hydra* (Takahashi et al., 1997). As part of this effort, we have identified a group of peptides, the PW family, which inhibit neuron differentiation (Takahashi et al., 1997). The PW family consists of four members which share a common L(I)PW motif at the C terminus (Takahashi et al., 1997). In the present study we have identified a novel neuropeptide, Hym-355, which enhances neuron differentiation. Here, we describe the isolation and characterization of the peptide and the gene encoding the peptide as well as an analysis of its role in neuron differentiation. Finally we will propose that this peptide is a positive signal involved in a feedback mechanism for maintaining the homeostasis of a neuron population in a steady state.

MATERIALS AND METHODS

Animals and culture conditions

The 105 strain of *Hydra magnipapillata* was used for most experiments, and cultured as described previously (Sugiyama and Fujisawa, 1977). For some experiments 'epithelial *Hydra*', which are devoid of all cells of the interstitial cell lineage except for gland cells (Marcum and Campbell, 1978; Sugiyama and Fujisawa, 1978) and which have been maintained by hand feeding for more than 4 years, were used.

Extraction and purification of Hym-355

Extraction and non-targeted purification of *Hydra* peptides was carried out as described previously with small modifications (Takahashi et al., 1997). About 150 g of frozen *Hydra* tissue was boiled in 1.5 l of 5% acetic acid for 5 minutes and homogenized. The homogenate was centrifuged at 16,000 *g* for 40 minutes at 4°C and the precipitate was homogenized and centrifuged again. The first and second supernatants were pooled and concentrated by rotary evaporation. To this solution, 1/10 volume of 1 N HCl was added, mixed and centrifuged as described above. The supernatant containing peptides was applied to C-18 cartridges (Mega Bond-Elut, Varian). After washing the cartridges with 10% methanol, the retained material was eluted with 60% methanol. The eluent (RM60) was then fractionated in a C-18 reverse-phase HPLC column (ODP-50, Asahipak; 6 mm × 250 mm) with a linear gradient of 0%-50% acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA) (pH 2.2) at a flow rate of 1 ml/minute (Fig. 1A). The chromatography was monitored at 220 nm. The fractions eluted between 5% and 37% ACN

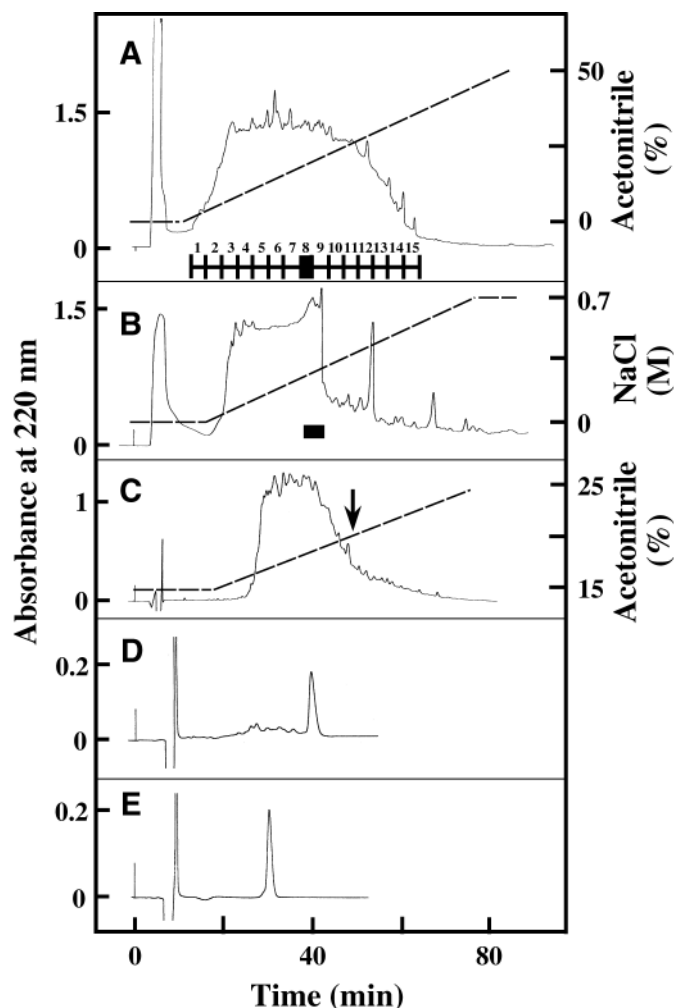


Fig. 1. Five steps in the purification of Hym-355. (A) Step 1: C-18 reverse-phase HPLC. The fractions were divided into 15 groups, as indicated on the elution profile. (B) Step 2: cation-exchange HPLC of group 8 from A. The fraction indicated by the bar was subjected to further purification. (C) Step 3: C-18 reverse-phase HPLC. The peak indicated with the arrow was used in the next step. (D) Step 4: HPLC with an isocratic elution of 20% ACN. (E) Step 5: HPLC with an isocratic elution of 21% ACN. See the text for details.

were divided into 15 groups. Group 8, eluted at approximately 20% ACN, was selected and peptides present in this group were systematically purified.

Hym-355, which was present in group 8, was purified using HPLC in the following steps. Cation-exchange HPLC (SP-5PW, Tosoh; 7.5 mm × 75 mm) was carried out with a 70-minute linear gradient of 0-0.7 M NaCl in 10 mM phosphate buffer (PB) (pH 7.2) at a flow rate of 0.5 ml/minute (Fig. 1B). The fractions eluted at about 0.25 M NaCl were subsequently subjected to C-18 reverse-phase HPLC (ODS-80TM, Tosoh; 4.6 mm × 150 mm) with a 75-minute linear gradient of 15%-30% ACN in 0.1% TFA at a flow rate of 0.5 ml/minute (Fig. 1C). The fractions eluted at around 20% ACN were subjected to the next HPLC (ODS-80TM) with an isocratic elution of 20% ACN at a flow rate of 0.3 ml/minute (Fig. 1D). Finally, the fraction was purified as a single peak (Hym-355) on the same column with an isocratic elution of 21% ACN in 0.1% TFA (Fig. 1E).

Structure analysis and peptide synthesis

The amino acid sequence of Hym-355 was determined with an

automated gas-phase sequencer (PPSQ-10, Shimadzu). Peptides were synthesized using a standard solid-phase method (PSSM-8, Shimadzu), followed by TFA-anisole cleavage and HPLC purification. The structure of each synthetic peptide was confirmed with amino acid sequencing and HPLC analyses.

Isolation of the gene encoding the peptide Hym-355

A nested 3'RACE method was used to isolate a cDNA encoding Hym-355. Total RNA was extracted from polyps starved for 48 hours with an AGPC method (Chomczynski and Sacchi, 1987). This RNA was used as a template for first strand cDNA synthesis together with an oligo-dT primer anchored with an M13 sequence (5'-CAGCAC-TGACCCTTTTGT₁₅-3') and AMV reverse transcriptase (Boehringer Mannheim). The M13 sequence was used as a downstream primer in the next two PCRs. The first PCR was carried out using the first strand cDNA, a degenerate primer (Primer-1: 5'-TTTCNCAAT-CNTTCTNCC-3', which corresponds to amino acids 1-7 of Hym-355), a downstream primer and *Taq* DNA polymerase (Boehringer Mannheim) for 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 2 minutes. For the second PCR, another degenerate primer (Primer-2: 5'-CA(A/G)TCNTT(C/T)(C/T)-TNCN-3', corresponding to amino acids 3-9), a downstream primer and one tenth of the first PCR mixture as a template were used. The amplified DNA was separated on a 1.5% agarose gel and three DNA fragments between 120 and 280 bp were detected. All fragments were isolated and purified using a GENECLEAN II (BIO 101) and cloned into the pCR 2.1 plasmid (Invitrogen). After sequencing the clones, one of the bands was shown to contain cDNA that encoded the Hym-355 precursor. This cDNA was used to screen a *Hydra* cDNA library constructed in Uni-ZAPII (Stratagene) (Yum et al., 1998a). 3.6×10^5 clones were screened, yielding a single clone that contained a full-length cDNA.

Northern blot analysis

Poly(A)⁺ RNA (3 µg) was fractionated on a formaldehyde-agarose gel and blotted to Hybond N⁺ nylon membrane (Amersham). The cDNA insert of the positive clone was labeled with [α -³²P]dCTP (Amersham) by using a random primer labeling kit (Takara), and hybridized to the blot according to standard procedures (Sambrook et al., 1988). The membrane was exposed overnight to an imaging plate and the image was analyzed with BAS-2000 (Fuji film).

In situ hybridization

Digoxigenin (DIG)-labeled antisense and sense riboprobes were prepared using the full-length cDNA containing a Hym-355 precursor sequence according to the manufacturer's method (Boehringer Mannheim). Whole-mount in situ hybridization was carried out as described by Grens et al. (1996).

Antibody production and determination of antibody specificity

An antiserum against the amidated peptide, Hym-355 (FPQSFLPRGa = FPQSFLPRG-NH₂), was produced using the procedure described by Yum et al. (1998b) with two modifications. (1) A synthetic peptide of Cys-Hym-355 (CFPQSFLPRGa) was conjugated to keyhole limpet hemocyanin (KLH) via succinimidyl m-maleimidebenzoate (MBS; Peeters et al., 1989). (2) Japanese White rabbits were used for immunization.

Competitive ELISA was carried out to examine the specificity of anti-Hym-355 antibody according to Yum et al. (1998b). The competitor peptides used were Cys-Hym-355, Hym-355, SFLPRGa, FLPRGa, LPRGa, PRGa, RGA, a non-amidated version of Hym-355 (FPQSFLPRG) and an unrelated amidated peptide, Hym-54 (GPMTGWLW) (Takahashi et al., 1997). The first five peptides competed strongly with the immobilized Cys-Hym-355 peptide, and all to a similar degree. The rest of peptides exhibited essentially no competition. Thus, the anti-Hym-355 antibody specifically recognized LPRGa.

Immunohistochemistry

For tissue localization of Hym-355, indirect immunofluorescence staining was carried out using the anti-Hym-355 antiserum (1/1000 dilution) on whole mounts of *Hydra*. Animals were fixed with 10% neutral buffered formalin (Wako Chemical, Japan) for 30 minutes at room temperature. Binding of the antiserum was visualized with biotinylated anti-rabbit Igs (Amersham, 1/100 dilution) and fluorescein isothiocyanate (FITC)-conjugated to streptavidin (Amersham, 1/25 dilution).

Indirect immunofluorescence staining with the NV1 monoclonal antibody was carried out as described by Hobmayer et al. (1990) on newly detached polyps, and analyzed with a confocal microscope. Briefly, the procedure was following. Animals were fixed with Lavdowsky's fixative (ethanol:formalin:acetic acid:water, 50:10:4:40) overnight at 4°C. After washing in PBS/0.1% BSA/0.02% NaN₃ for 30 minutes, animals were incubated in 50 ml of the monoclonal antibody, NV1, for 12 hours. They were then further incubated for 6 hours in 50 ml of FITC-conjugated goat anti-mouse immunoglobulin IgG/IgM (TAGO, California) diluted 1/20 in PBS/BSA/NaN₃ for 2 hours. Animals were then washed in PBS and mounted in buffered glycerin.

Biological assays

Cell proliferation, cell differentiation and myoactivity assays were carried out as described previously (Takahashi et al., 1997; Yum et al., 1998b). Briefly, the cell proliferation analysis was as follows. 10 young polyps detached from parents during the previous 24 hours (stage I polyps) were incubated with Hym-355 (10⁻⁶ M) for 48 hours. The peptide solution was renewed once at 24 hours. They were pulse-labeled with 2 mM BrdU (Sigma) for 1 hour from hour 47 to hour 48 of the peptide treatment, and then immediately macerated into a suspension of single cells (David, 1973) to score for labeled cells. The cell types of the macerated cells were readily identified by their morphology as described by David (1973).

To assay cell differentiation, 10 stage-I polyps were pulse-labeled for 1 hour with 2 mM BrdU (Sigma) in the presence of Hym-355 (10⁻⁶ M). Thereafter, the gastric cavity was flushed thoroughly with the culture solution, and the animals were transferred to fresh culture solution containing the same concentration of Hym-355. 24 hours later, the peptide solution was replaced with a freshly prepared one. At various times after labeling, samples of polyps were macerated into a solution of single cells, and the fraction of BrdU-labeled cells for each of four cell types was determined as described previously (Takahashi et al., 1997). The four types of cells were epithelial cells, single and pairs of large interstitial cells, dividing nematoblasts (nests of four interstitial cells) and nerve cells (David, 1973). Control animals were processed similarly except for the peptide treatment.

The rate of head or foot regeneration was examined as follows. For head regeneration, the upper one quarter of a stage-I polyp was removed and the animal allowed to regenerate in the presence or absence of Hym-355 (10⁻⁶ M). The number of tentacles produced was a measure of head regeneration. For foot regeneration, the lowest quarter of the body column was removed and the regeneration of the foot assayed in terms of the ability to trap air bubbles.

RESULTS

Isolation of the Hym-355 peptide and determination of its structure

Peptides were extracted from 150 g of *Hydra* tissue and purified with a series of HPLC as described in Materials and Methods. Fig. 1 shows the elution profile of each purification step. After 5 steps of purification a single peak designated as Hym-355 was obtained (Fig. 1E).

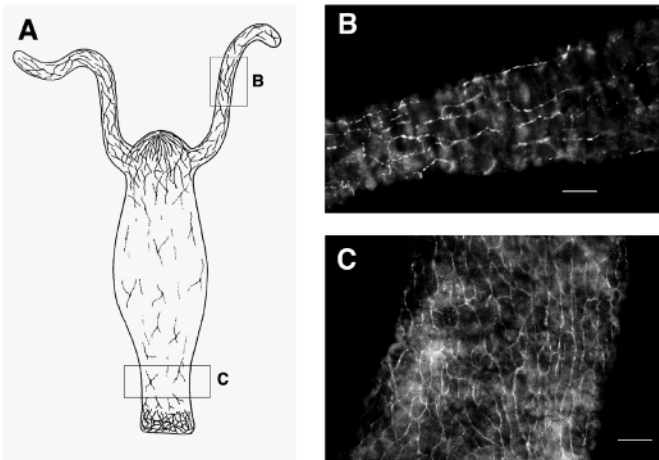


Fig. 5. Immunohistochemical staining of a normal polyp by using an anti-Hym-355 antiserum. (A) An illustration of the overall staining pattern. (B) Stained ganglion cells in a part of the tentacle indicated by the square B in A. (C) Stained ganglion cells in the peduncle region within square C in A. Bars, 100 μ m.

recognized only neurons, 'epithelial animals', which are *Hydra* devoid of all neurons, were also stained with the antibody. No cells in these animals were stained (data not shown). The regional distribution of labeled neurons (see Fig. 5A) indicates that large numbers of labeled neurons were found in the head and foot regions, but fewer in the body column. This is in agreement with the results obtained with in situ hybridization.

Thus, both the immunolocalization of the Hym-355 peptide and the expression pattern of the Hym-355 gene indicate that the gene is expressed in neurons. In both cases the distribution pattern is similar to the overall regional distribution of neurons in *Hydra* (Bode et al., 1973), although the gene does not appear to be expressed in all neurons.

Hym-355 has no effect on muscle contraction, head and foot regeneration, or on cell proliferation

In order to determine the function of Hym-355, a series of biological assays were carried out (see Materials and Methods). Several peptides are known to affect muscle contraction in *Hydra* and other cnidarians (Takahashi et al., 1997; Yum et al., 1998b; Grimmelikhuijzen et al., 1996). Hym-355 has been shown to weakly promote the contraction of an isolated retractor muscle of the sea anemone, *Anthopleura fuscoviridis* (T. Takahashi et al., unpublished observations), but no effect on muscle contraction in *Hydra* was observed (data not shown). Other peptides such as the head activator and Hym-346 /pedibin have been shown to affect the rate of head regeneration (Schaller, 1973) and foot regeneration (Hoffmeister, 1996; Grens et al., 1999), respectively. Treatment of animals with Hym-355 following head or foot removal indicated that the peptide had no effect on the rate of regeneration of either extremity (data not shown).

Finally, the peptide has no effect on cell proliferation. The epithelial cells of both layers of the body column: the large interstitial cells, which include the multipotent stem cells and the early committed cells of the interstitial cell lineage, and gland cells, although some of them arise from interstitial cells (Schmidt and David, 1986; Bode et al., 1987), are constantly

Table 1. Effect of Hym-355 on cell proliferation

Hym-355 (M)	Labelling index (%)			
	Epithelial cells	Interstitial cells ^a	Nematoblasts (4s) ^b	Gland cells
0	25.5 \pm 3.2	50.5 \pm 2.8	75.3 \pm 7.4	15.9 \pm 0.3
10 ⁻⁶	26.1 \pm 1.7	54.6 \pm 3.2	77.9 \pm 7.7	17.4 \pm 3.9

Values are means \pm s.d. For each cell type 3 samples of 10 animals each were analyzed for both treated and controls.

^aInterstitial cells consisted of single and pairs of large interstitial cells.

^bNematoblasts in a cluster of 4 cells.

in the mitotic cycle (David and Campbell, 1972; Campbell and David, 1974). To determine if the peptide affects their proliferation, animals were treated with Hym-355 for 48 hours and pulse-labelled with BrdU for an hour. Thereafter, the samples were macerated into suspensions of fixed single cells (David, 1973), and analyzed. As shown in Table 1, the peptide had no effect on the labeling index, indicating that it probably has no effect on the cell division rate of these proliferating cell populations.

Hym-355 enhances neuron differentiation

The effects of the peptide on nematocyte and neuron differentiation were examined. Animals were pulse-labeled with BrdU for 1 hour in the presence of the peptide and then chased for 47 hours in the peptide solution. At 48 hours, samples of animals were macerated and analyzed for nematocyte and neuron differentiation.

Interstitial cells entering the nematocyte differentiation pathway undergo 2-5 cell divisions before forming the nematocyst (David and Gierer, 1974). After two divisions, the four resulting interstitial cells form a cluster, termed 4s, which are the first cells that are recognizable as being unique to the nematocyte pathway (David and Gierer, 1974). These clusters arise about 2 days after an interstitial cell enters the nematocyte pathway. Since epithelial cells were unaffected by the Hym-355, the ratio of 4s to epithelial cells is a good measure of the effect of the peptide on nematocyte differentiation. As shown in Table 2, the ratio was the same as for controls, suggesting the peptide did not affect the number of stem cells entering the nematocyte pathway.

In contrast, Hym-355 had a pronounced effect on neuron differentiation. A stem cell entering the neuron pathway undergoes one or more cell divisions and these cells then undergo neuron differentiation (Bode et al., 1990; Hagar and

Table 2. Effect of Hym-355 on neuron and nematocyte differentiation

Hym-355 (M)	Labeling index (%) Nv	Ratio to epithelial cells	
		4s	Nv ^a
0	20.2 \pm 3.3	0.048 \pm 0.012	0.036 \pm 0.007
10 ⁻⁶	28.7 \pm 1.7**	0.047 \pm 0.015	0.050 \pm 0.008*

Values means \pm s.d. For each cell type and for each type of measurement the sample size was 3-6 of 10 animals each for both treated and controls.

4s, clusters of 4 nematoblasts; Nv, neurons.

^aRatio of labeled neurons to total epithelial cells.

**Statistically significant difference from control at the 99.9% level and * at the 90% level, Student's *t*-test.

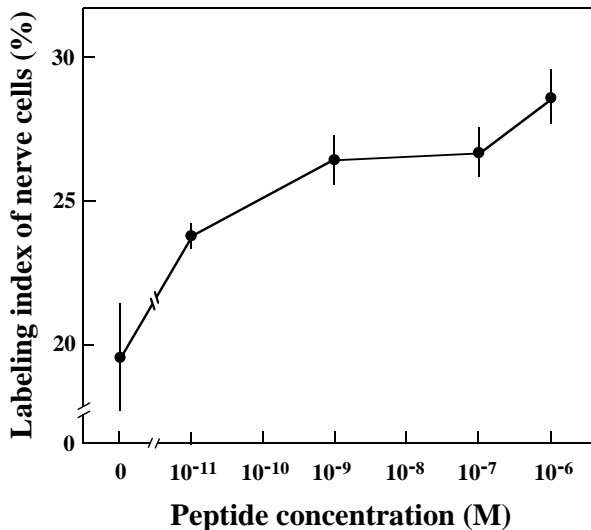


Fig. 6. Effect of different concentrations of Hym-355 on the rate of neuron differentiation. 10 animals were used per sample, and each point represents an average of 3-6 independent experiments.

David, 1997). Most of the interstitial cells entering the pathway have traversed it by 48 hours. The results (Table 2) clearly show that treatment with the peptide increased the fraction of labeled neurons, and hence the rate of neuron production (the number of neurons produced by a polyp per unit time) by about 1.5-fold. This is also reflected in the 1.5 \times increase in the density of newly formed neurons (labeled neurons/epithelial cells) observed 48 hours (Table 2). Further, the effect of Hym-355 on neuron differentiation was concentration-dependent (Fig. 6).

To gain a more precise understanding as to where in the neuron differentiation pathway, Hym-355 was acting, animals were pulse-labeled for 1 hour with BrdU and chased for 47 hours in the peptide solution. Periodically thereafter the labeling index of the neurons was measured. As shown in Fig. 7, the labeling index rose more rapidly in the treated animals than in controls. Of interest here is that for both controls and peptide-treated animals, the first labeled neurons appeared about 10 hours after labeling. This 10-hour lag is consistent with the time required for completing neuron differentiation from the final S phase as described previously (David and Gierer, 1974; Berking, 1979). Thus, the peptide does not increase the rate of traverse of the final differentiation phase. Instead, it apparently affects early stages of neuron differentiation and increases the rate of neuron differentiation by increasing the number of precursor cells or shortening of the S-phase in differentiation intermediates.

To determine if Hym-355 affects neuron differentiation throughout the animal or, conversely, only in specific regions, the initial experiment was modified. Animals were pulse-labeled with BrdU, treated for 48 hours with the peptide, and then divided into four axial regions (1-4) of equal length. The first region included the head, while the fourth region included the foot. The data in Table 3 indicates that Hym-355 affects neuron differentiation throughout the animal.

One last issue addressed was the type of neuron affected by the peptide. Most of the neurons in the nerve net of *Hydra* are ganglion cells (Kinnamon and Westfall, 1981; Westfall et al.,

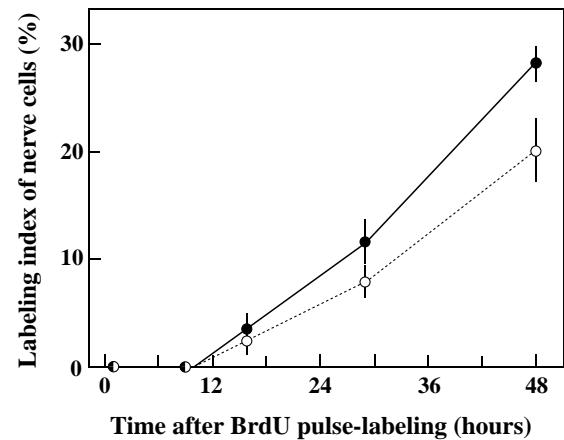


Fig. 7. Effect of Hym-355 on the labeling kinetics of nerve cells. Filled circles represent treated animals, while open circles are controls. 10 animals were used per sample, and each point represents an average of 3-6 independent experiments. Vertical bars represent the standard deviation of the mean. Labeling indices of treated samples at 29 hours and 48 hours, respectively, show statistically significant difference from control at the 99.5% and 99.9% levels by the Student *t*-test.

1983). As these are found throughout the animal, it is highly likely that the measured increase in the rate of neuron differentiation is a reflection of an increased rate of ganglion cell formation. Sensory cells are found primarily in the tentacles and the basal disk (Dunne et al., 1985; Koizumi and Bode, 1991). To determine if the peptide also affected differentiation of sensory neurons, treated animals were assayed with the monoclonal antibody, NV1, which specifically recognizes an antigen of the sensory cells of the tentacles (Hobmayer et al., 1990). As shown in Table 4, with increasing time of treatment with the peptide, the number of NV1⁺ neurons increased compared to controls. Hence, the peptide also affects the rate of sensory cell differentiation.

A PW peptide interferes with the enhancement of neuron differentiation by Hym-355

The fact that Hym-355 is produced in neurons and also enhances neuron differentiation suggests a positive feedback loop. To maintain the nerve net in steady state one would expect a countervailing influence. This could be provided by the PW peptides, which inhibit neuron differentiation in *Hydra* (Takahashi et al., 1997). It is, therefore, possible that PW

Table 3. Effect of Hym-355 on neuron differentiation in different body regions

Hym-355 (M)	Labeling index (%) of neurons in body region			
	Region 1	Region 2	Region 3	Region 4
0	17.8 \pm 1.1	17.2 \pm 1.0	10.4 \pm 3.6	14.9 \pm 1.9
10 ⁻⁶	28.8 \pm 1.5*	24.8 \pm 4.7*	20.8 \pm 0.4*	19.9 \pm 1.5*

Each region contains one quarter of the length of the animal. Region 1 includes the head, while region 4 includes the foot.

Values means \pm s.d. 3 samples of 10 animals each were analyzed for both treated and controls.

*Statistically significant difference from control at the 99.9% level, Student's *t*-test.

Table 4. Effect of Hym-355 on the differentiation of NVI⁺ neurons in the tentacles

Hym-355 (M)	Length of treatment (days)		
	2	4	7
0	356±24	359±20	388±50
10 ⁻⁶	355±28	426±56*	457±50*

Values are number of NVI⁺ neurons in the tentacles per polyp (means ± s.d.). 10 animals each were used for both treated and controls.

*Statistically significant difference from the control at the 99.5% level at 4 days and at the 99.0% level at 7 days, Student's *t*-test.

Table 5. Effect of cotreatment with Hym-355 and Hym-33H on neuron differentiation

Peptide (10 ⁻⁶ M)	Labeling index of neurons (%)
None	17.0±0.9
Hym-355	21.9±2.0*
Hym-33H	13.1±0.8*
Hym-335 + Hm-33H	16.7±0.7

Values means ± s.d. 3 samples of 10 animals each were analyzed for both treated and controls.

*Statistically significant difference from control at the 99.9% level, Student's *t*-test.

peptides and Hym-355 interact with each other in neuron differentiation. To examine this possibility, polyps were cotreated with Hym-355 and the PW peptide, Hym-33H (AALPW), each at 10⁻⁶ M, and the effect on neuron differentiation was examined as before. As shown in Table 5, Hym-355 increased, while Hym-33H decreased the rate of neuron differentiation as expected. However, treatment with both peptides simultaneously resulted in a control level of neuron differentiation, indicating that the two peptides canceled out each other's effect on neuron differentiation.

DISCUSSION

Hym-355 is a neuropeptide involved in neuron differentiation

The primary structure of Hym-355 was determined as FPQSFLPRGa. Muneoka et al. (1994) have proposed to group the peptides that have a PRXamide sequence at their carboxyl termini as PRXamide peptides. These are further divided into three sub-groups: (1) pheromone biosynthesis activating neuropeptide (PBAN) (Raina et al., 1989) and related peptides, (2) small cardioactive peptides (SCPs) (Morris et al., 1982; Lloyd et al., 1987) and (3) antho-RPamide (Carstensen et al., 1992) and related peptides. Members of the first sub-group have quite different functions, while peptides in the other two sub-groups all affect myoactivity. Hym-355 shares some homology with members of the last group: LPPGPLRPamide from sea anemone, *Anthopleura elegantissima* (Carstensen et al., 1992), AAPLPRLamide from echiuran, *Urechis unicinctus* (Ikeda et al., 1993) and QPPLPRYamide and pQPPLPRYamide from snail, *Helix pomatia* (Minakata et al., 1993). Although no myoactivity of Hym-355 has been found in *Hydra*, it weakly

elicited contraction of the retractor muscle of sea-anemone, *A. fuscoviridis* (T. Takahashi et al., unpublished).

That Hym-355 is a neuropeptide present in a sub-population of neurons is supported by the following observations. (1) In situ hybridization analysis of the gene encoding a Hym-355 precursor sequence showed that the gene was expressed in neurons that were heavily concentrated in the head and foot regions (Fig. 4). (2) Indirect immunofluorescence staining by using an anti-Hym-355 antiserum (Fig. 5) showed that Hym-355 is localized in ganglion cells but not in sensory cells.

The role of Hym-355 appears to be one of inducing interstitial stem cells or neuron precursor cells to undergo neuron differentiation. There are several pieces of evidence for this view. (1) Treatment with the peptide increased the labeling index of neurons above the level found in controls (Fig. 7, Table 2) as well as increasing the ratio of labeled neurons to epithelial cells (Table 2). The rise in the ratio indicates that the increase in the labeling index was not due to an increase in the rate of turnover of the neuron population. Were that the case, the ratio would have remained constant. (2) Treatment with Hym-355 also caused a rise in the NVI⁺ sensory cells. Since these cells are known to differentiate directly from interstitial cells (Hobmayer et al., 1990), the increase in their number following treatment with Hym-355 also indicates the peptide affects the entrance of stem cells into neuron differentiation pathways. (3) Finally, Hym-355 had no effect on the traverse of the last stage of neuron differentiation.

Control of neuron differentiation

Neuron differentiation occurs within a very dynamic context. In the body column the epithelial cells are continuously increasing in number as they are constantly in the mitotic cycle (David and Campbell, 1972). To maintain the neuron density in the body column, stem cells among the interstitial cells constantly enter the neuron differentiation pathway to produce new neurons (David and Gierer, 1974). Many of the newly formed neurons are intercalated into the net in the body column, while more than half of the differentiation intermediates migrate apically into the head or basally into the foot and differentiate, thereby accounting for the higher neuron densities in the extremities (Heimfeld and Bode, 1984; Fujisawa, 1989; Teragawa and Bode, 1990, 1995; Technau and Holstein, 1996; Hager and David, 1997). As noted previously, feedback mechanisms have been implicated in the homeostasis of this neuron density (Yaross and Bode, 1978; Sugiyama and Fujisawa, 1979; Bosch et al., 1991).

Recently we have found that the short term treatment with PW peptides inhibits neuron differentiation in *Hydra* tissue (Takahashi et al., 1997). In this study, we have shown that the neuropeptide Hym-355 enhances neuron differentiation, probably by affecting an early stage in the pathway (Fig. 7). Further, Hym-33H, one of the four PW peptides, and Hym-355 act in an antagonistic manner as cotreatment with both peptides nullified both the enhancement and inhibition of neuron differentiation (Table 5). These observations suggesting the involvement of these peptides in a feedback loop controlling neuron differentiation have been incorporated into the model shown in Fig. 8.

According to this model, Hym-355 produced by neurons increases the rate of neuron differentiation by acting early in the pathway. To control this positive feedback loop, epithelial

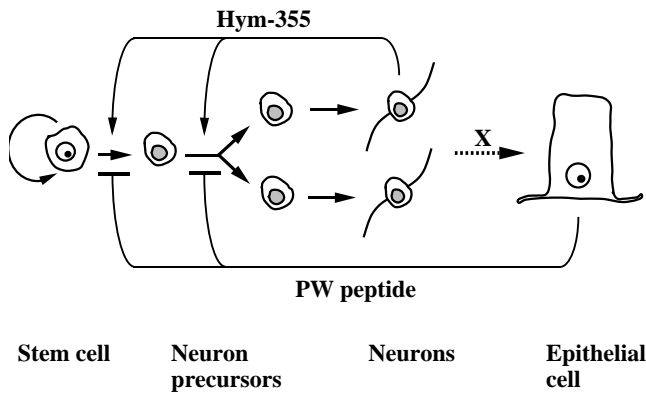


Fig. 8. A feedback model for the control of neuron differentiation.

cells produce PW peptides (O. Koizumi et al., unpublished) that block either stem cell commitment or traverse of the nerve cell differentiation pathway. Thus, the two peptides balance one another's effects either as part of a single feedback loop, or by acting on different parts of the differentiation pathway.

However, the feedback mechanism is incomplete in this form. Were the PW peptide continuously produced and released by epithelial cells, then the introduction of a few interstitial cells into tissue devoid of neurons would never lead to neuron differentiation because the relative concentration of the neuron inhibitor, the PW peptide, would be too high. Yet, interstitial cells do produce neurons under these conditions (Bosch et al., 1991). Thus, there must be an additional mechanism affecting the PW peptides. One possibility, as shown by the dotted arrow in Fig. 8, is that the mature neurons control the production and/or release of PW peptides in the epithelial cells. Then the amount of PW peptide released would be proportional to the neuron density. Since there could be local fluctuations in nerve cell density along the body column, we postulate that the third factor operates either by cell-cell contact or short-range diffusion, over a few cell diameter lengths.

This three-part mechanism would permit adjustments in the rate of nerve cell production to maintain the nerve cell density in the adult over the long term. For example, should the neuron density decline precipitously, then the level of PW would eventually be so low as to be ineffective. Subsequently, the auto-catalytic part of the feedback loop involving Hym-355 would take over leading to a rise in nerve cell density. Consistent with this idea, O. Koizumi et al. (unpublished) have shown that long-term treatment with a PW peptide initially causes a drop in nerve cell density, but later a recovery occurs.

One other aspect of the nerve net needs to be addressed. The neuron density, and hence the rate of neuron differentiation, is 6-10 \times higher in the head and 3-4 \times higher in the foot than in the body column (Bode et al., 1973). In large measure the higher rates in the extremities are due to the migration of neuron differentiation intermediates from the body column into both extremities (Heimfeld and Bode, 1984; Fujisawa, 1989; Teragawa and Bode, 1990, 1995; Technau and Holstein, 1996; Hager and David, 1997). The described feedback loop (Fig. 8) would result in a uniform neuron density throughout the animal. The differences in density may be explained in two possible ways. Since the density of neurons producing Hym-

355 is much higher in the head and foot than in the body column (see Figs 4, 5), the higher level of Hym-355 could result in a higher rate of nerve cell differentiation. Another possibility is that additional factors are involved. The head activator, an 11-amino acid peptide, is found in high concentration in the head, but in decreasing amounts down the column (Schaller and Gierer, 1973; Schaller and Bodenmueller, 1981). It may specifically enhance the effects of Hym-355 on nerve cell differentiation in the head.

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