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REGULAR ARTICLE

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A new case of neuropeptide coexpression (RGamide and LWamides) in *Hydra*, found by whole-mount, two-color double-labeling in situ hybridization

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Abstract The freshwater polyp *Hydra* has a primitive nervous system that expresses at least six different neuropeptide genes: (1) three genes, coding for the preprohormones-A, -B, and -C that each gives rise to a variety of peptides with the C-terminal sequence Arg-Phe-NH_2 (the Hydra-RFamides); (2) one gene, coding for a preprohormone that gives rise to five peptides with the C-terminal sequence Leu-Trp-NH_2 (the Hydra-LWamides); (3) one gene, coding for a preprohormone that produces a peptide with the C-terminal sequence Lys-Val-NH_2 (Hydra-KVamide, also called Hym-176); and (4) one gene, coding for a preprohormone that gives rise to a peptide with the C-terminal sequence Arg-Gly NH_2 (Hydra-RGamide, also called Hym-355). In a previous paper, we described that a population of neurons in the peduncle (a region just above the foot) of *Hydra* coexpresses the preprohormone-A and KVamide genes, whereas neurons in the other regions only express either the preprohormone-A, -B, -C, LWamide, or the KVamide genes. Here, we investigated the RGamide gene expression, using whole-mount, two-color double-labeling in situ hybridization, and found that neurons in the basal disk (foot), gastric region, hypostome (a region around the mouth), and tentacles coexpress this gene together with the LWamide gene. A small population of neurons in the hypostome and upper gastric region expresses only the LWamide gene. No coexpression of the RGamide gene with any of the other neuropeptide genes was observed. This is the second example of coexpression of two neuropeptide genes in cnidarians. It demonstrates that many neurons in the primitive nervous systems of cnidarians use combinations ("cocktails") of neuropeptides for their signaling. It also shows that *Hydra* has at least seven neurochemically different populations of neurons.

Keywords Neurohormone · Colocalization · Evolution · Metazoa · *Hydra magnipapillata* (Cnidaria)

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Introduction

Cnidarians are primitive multicellular animals such as *Hydra*, corals, sea anemones, and jellyfishes, and are phylogenetically located near the roots of metazoan evolution. Cnidarians, or animals belonging to a closely related ancestor phylum, have "invented" a large number of mechanisms and systems that are essential features of higher metazoans such as body axes, pattern formation, and the presence of a nervous system.

In their primitive nervous systems, cnidarians use peptides as transmitters, suggesting that neuropeptides were the first signal molecules used by early nervous systems (Grimmelikhuijzen et al. 1996). All neuropeptides isolated from cnidarians so far (about 40) have a C-terminal amide group, but also their N-termini contain various protecting groups (such as <Glu residues and X-Pro-Pro sequences), which we still find in many neuropeptides from higher organisms (Grimmelikhuijzen et al. 1996). The cnidarian neuropeptides are stored in neurosecretory vesicles (Koizumi et al. 1989; Westfall and Grimmelikhuijzen 1993; Westfall et al. 1995) and have excitatory or inhibitory actions when tested on intact animals, isolated muscle preparations, or isolated muscle cells (McFarlane et al. 1987, 1991, 1992, 1993; McFarlane and Grimmelikhuijzen 1991; Yum et al. 1998a), indicating that these substances are neurotransmitters or neuromodulators at neuromuscular or neuroneuronal synapses.

We have also clarified the biosyntheses of most of the cnidarian neuropeptides that we isolated and found that they are synthesized as preprohormones that can contain up to 38 neuropeptide copies (Darmer et al. 1991; Reinscheid and Grimmelikhuijzen 1994; Leviev and

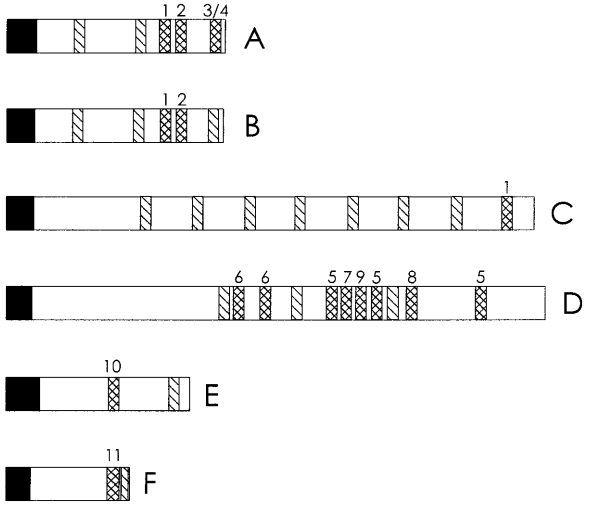


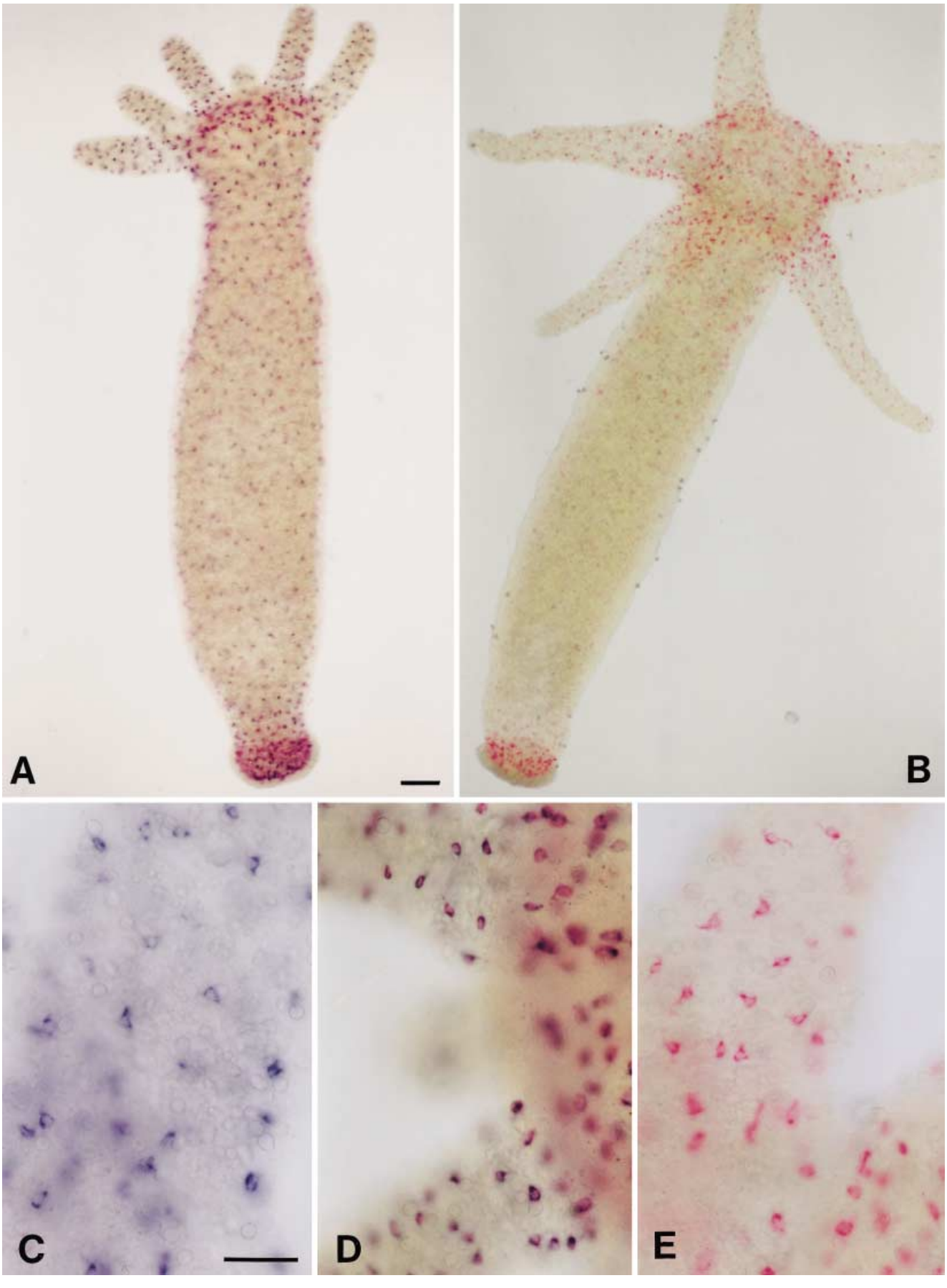
Fig. 1A–F Schematic representation of five preprohormones in *Hydra*. **A** Preprohormone-A, containing a signal sequence (*black box*), one unprocessed sequence of Hydra-RFamide I (*hatched* and marked by *1*), Hydra-RFamide II (*2*), Hydra-RFamide III/IV (*3, 4*), and several putative neuropeptide sequences (*hatched, without numbers*). **B** Preprohormone-B, containing a signal sequence (*black*) and one unprocessed sequence of Hydra-RFamide I (*1*), Hydra-RFamide II (*2*), and several putative neuropeptide sequences (*hatched, without numbers*). **C** Preprohormone-C, containing a signal sequence (*black*), one copy of unprocessed Hydra-RFamide I (*1*), and seven putative neuropeptide sequences (*hatched, without numbers*). **D** The Hydra-LWamide precursor, containing a signal sequence (*black*), eight unprocessed sequences of the isolated peptides Hydra-LWamide I-V (*hatched* and marked by *5–9*), and three putative neuropeptide sequences (*hatched, without numbers*). **E** The Hydra-KVamide precursor, containing a signal sequence (*black*), one copy of unprocessed Hydra-KVamide (*hatched* and marked by *10*), and another copy of a putative neuropeptide (*hatched, without number*). **F** The Hydra-RGamide precursor, containing a signal sequence (*black*) and one copy of unprocessed Hydra-RGamide (*11*), together with a putative neuropeptide sequence (*hatched, without number*). *1* = <Glu-Trp-Leu-Gly-Arg-Phe-NH_2 (Hydra-RFamide I); *2* = <Glu-Trp-Phe-Asn-Gly-Arg-Phe-NH_2 (Hydra-RFamide II); *3/4* = Lys-Pro-His-Leu-Arg-Gly-Arg-Phe-NH_2/His-Leu-Arg-Phe-NH_2 (Hydra-RFamide III/IV); *5* = Gly ProPro-Pro-Gly-Leu-Trp-NH_2 (Hydra-LWamide I, also called Hym-331); *6* = Glu-Pro-Leu-Pro-Ile-Gly-Leu-Trp-NH_2 (Hydra-LWamide II/Hym-248); *7* = Lys-Pro-Ile-Pro-Gly-Leu-Trp-NH_2 (Hydra-LWamide III/Hym-249); *8* = Asn-Pro-Tyr-Pro-Gly-Leu-Trp-NH_2 (Hydra-LWamide IV/Hym-53); *9* = Gly-Pro-Met-Thr-Gly-Leu-Trp-NH_2 (Hydra-LWamide V/Hym-54); *10* = Ala-Pro-Phe-Ile-Phe-Pro-Gly-Pro-Lys-Val-NH_2 (Hydra-KVamide/Hym-176); *11* = Phe-Pro-Gln-Ser-Phe-Leu-Pro-Arg-Gly-NH_2 (Hydra-RGamide/Hym-355). (The data for **A–C** are from Moosler et al. 1996 and Darmer et al. 1998; those for **D** from Leviev et al. 1997 and Takahashi et al. 1997; those for **E** from Yum et al. 1998a, 1998b; and those for **F** from Takahashi et al. 2000)

Grimmelikhuijzen 1995; Grimmelikhuijzen et al. 1996; Leviev et al. 1997). The cnidarian preprohormones have normal dibasic and monobasic processing sites for endoproteolytic cleavage but, in addition, other more unusual processing sites such as acidic residues (Darmer et al. 1991; Reinscheid and Grimmelikhuijzen 1994; Leviev and Grimmelikhuijzen 1995; Grimmelikhuijzen et al. 1996; Leviev et al. 1997). At the C-terminal side of each immature cnidarian neuropeptide sequence, Gly residues occur that in higher animals are known to be converted into a C-terminal amide group by the use of two enzymes, peptidyl α-hydroxylating monooxygenase (PHM), and peptidyl-α-hydroxyglycine α-amidating lyase (PAL; Eipper et al. 1992). We have now cloned several PHM and PAL enzymes from sea anemones which are closely related to the mammalian enzymes (Hauser et al. 1997; Williamson et al. 2000; M. Williamson, unpublished). Furthermore, many introns in these cnidarian PHM and PAL genes occur in the same positions and have the same intron phasing as in the mammalian genes, showing that the cnidarian α-amidating enzymes are not only structurally, but also evolutionarily related to their mammalian counterparts (Williamson et al. 2000). All these data, therefore, show that many mechanisms that we find in the peptidergic neurons from higher organisms have already been invented by cnidarians or their closely related ancestors.

Of all cnidarians, *Hydra* has been investigated most intensively, because it is a useful model to study developmental processes such as pattern formation and regeneration (Gierer and Meinhardt 1972; Gierer 1977; Meinhardt 1993, 1996). Neuropeptides probably do not play a role in the basic pattern formation of *Hydra*, because nerve-free *Hydra* mutants and phenotypic variants have been produced that only consist of epithelial cells, but that still have the same overall form and regeneration properties as a normal *Hydra* (Campbell 1976; Sugiyama and Fujisawa 1978). Neuropeptides, however, could play an important role in the differentiation of stem cells (also called interstitial or I-cells) into their various product cells, such as nerve cells and gametes (Grimmelikhuijzen et al. 1996; Takahashi et al. 2000).

So far, six neuropeptide genes have been cloned from *Hydra* and their corresponding peptides have been isolated (Moosler et al. 1996; Leviev et al. 1997; Takahashi et al. 1997; Darmer et al. 1998; Yum et al. 1998a, 1998b; Takahashi et al. 2000). These genes are: (1) the preprohormone-A, -B, and -C genes that each code for a differ-

Fig. 2A–E Whole-mount in situ hybridization of *Hydra* with cRNA probes, coding for either the Hydra-RGamide or Hydra-LWamide preprohormone. **A** Two-color double-labeling in situ hybridization of a whole *Hydra*, using a probe coding for the Hydra-RGamide preprohormone (stained with BM purple) and a probe coding for the Hydra-LWamide preprohormone (stained with Fast Red). The resulting color in the neurons is a nuance of violet (clearly different from **B**, **C**), indicating that the two preprohormone genes are coexpressed. Note that these neurons are mainly present in the head and in the foot. **B** The same hybridization as in **A**, but the staining with BM purple was omitted. The red staining (Fast Red) shows neurons expressing the Hydra-LWamide gene. **C** In situ hybridization of the tentacle region, using a probe coding for Hydra-RGamide, followed by staining with BM purple (blue neurons are seen). **D** Two-color double-labeling in situ hybridization of the same region, using a probe coding for the Hydra-RGamide preprohormone (stained with BM purple) and a probe coding for the Hydra-LWamide preprohormone (stained with Fast Red). The stained neurons are all violet (a color that is clearly different from **C**, **E**), showing that they coexpress the two preprohormone genes. **E** In situ hybridization of neurons of the same region, using a probe that codes for the Hydra-LWamide preprohormone (stained with Fast Red). *Bars* **A**, **B** 100 μm; **C**–**E** 50 μm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ent preprohormone, containing various neuropeptides with the C-terminal sequence Arg-Phe-NH_2 (the Hydra-RFamides; Fig. 1A–C); (2) one gene that codes for a preprohormone that contains various neuropeptides with the C-terminal sequence Leu-Trp-NH_2 (the Hydra-LWamides, also called Hym-53, -54, -248, -249, -331; Fig. 1D); (3) one gene that codes for a preprohormone that contains a neuropeptide with the C-terminal sequence Lys-Val-NH_2 (Hydra-KVamide, also called Hym-176; Fig. 1E); and (4) one preprohormone gene that codes for a neuropeptide with the C-terminal sequence Arg-Gly-NH_2 (Hydra-RGamide, also called Hym-355; Fig. 1F).

In two previous papers (Mitgutsch et al. 1999; Hansen et al. 2000) we have investigated the expression of the first five preprohormone genes (represented in Fig. 1A–E) in both intact, "steady state" *Hydra* and in regenerating animals and found that the preprohormone-A gene is expressed in neurons of the hypostome (a region around the mouth), upper gastric region (just under the tentacles), and peduncle (a region just above the foot, or pedal disk); the preprohormone-B gene expression is confined to neurons of the hypostome and upper gastric region; the preprohormone-C gene is only expressed in tentacle neurons; although the LWamide gene is expressed in neurons throughout *Hydra*, it is mainly expressed in neurons of the head and pedal disk; the KVamide gene is expressed in a band of neurons in the penduncle and in very few neurons of the upper gastric region. All genes are expressed by different populations of neurons, except for the band of neurons in the peduncle that expresses both the preprohormone-A and the KVamide genes (Mitgutsch et al. 1999; Hansen et al. 2000).

In the present paper, we studied the expression of the Hydra-RGamide gene, especially with respect to the possibility of coexpression with one of the other *Hydra* neuropeptide genes. This work is important, because it helps us to determine and understand the complexity of the *Hydra* nervous system.

Materials and methods

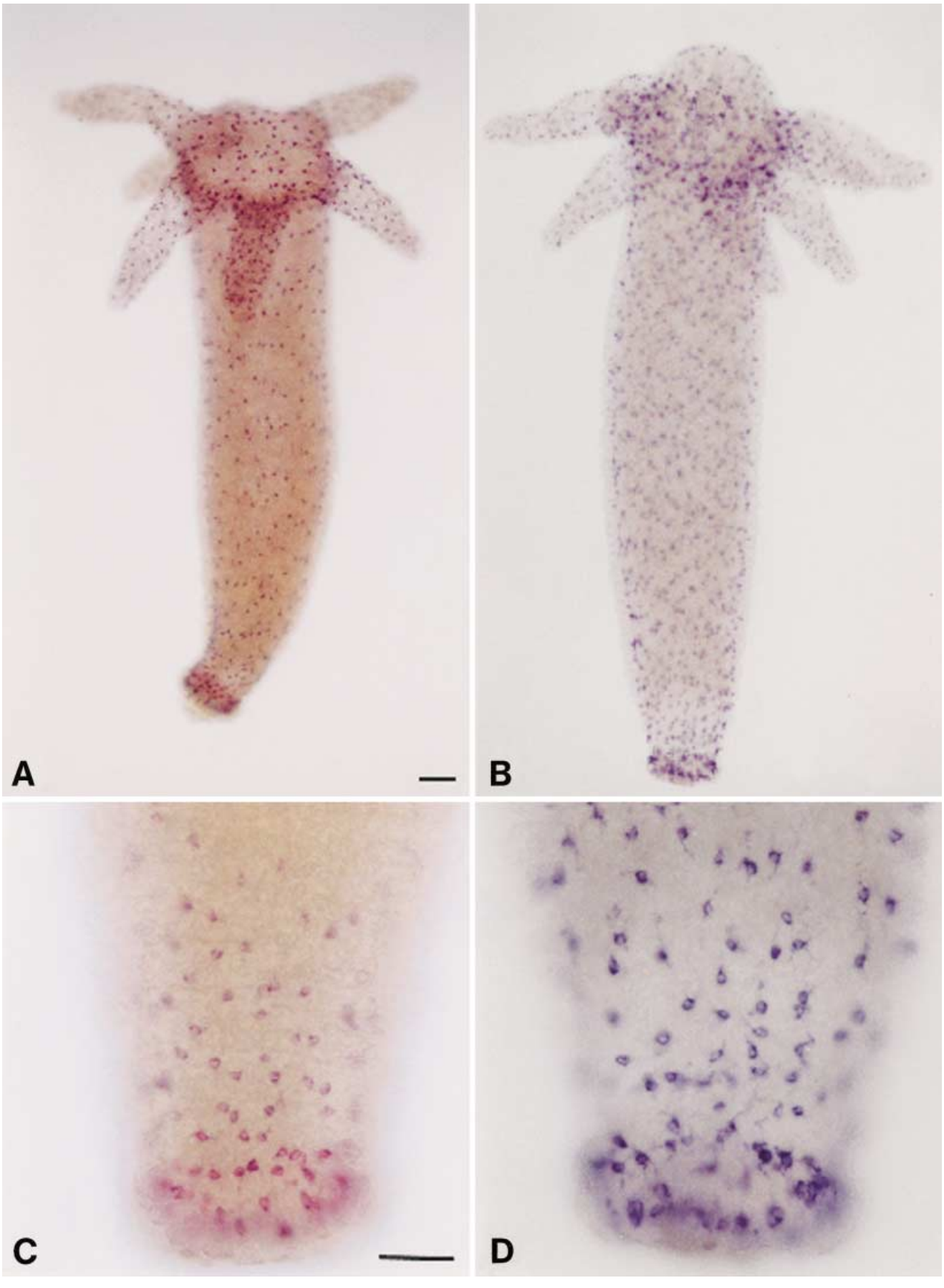
The same *H. magnipapillata* strain and methods were used as described by Hansen et al. (2000), with the following small changes or additions: (1) the digoxigenin (DIG) RNA and fluorescein RNA labeling kits, as well as transfer RNA (tRNA), were obtained from Roche Molecular Biochemicals (Mannheim, Germany); (2) the labeled RNA probe was used in a concentration of 0.125–0.250 µg/ml; (3) either BM purple or nitrobluetetrazolium/bromochloroindolyl phosphate (NBT/BICP; both from Roche) was used to obtain a blue/purple staining; the NBT/BICP staining was stronger and, therefore, stained more neurons than BM purple; (4) the Hydra-RGamide probe was made in the following way: the cDNA coding for prepro-Hydra-Hym-355 was obtained by PCR, using Hydra cDNA as a template and the sense/antisense primers CTAACCGTGATGCTACTGACT/CATCTCTTTTGCCTCCTCT-TG. This PCR generated a product corresponding to nucleotide positions 122–316 of Fig. 2 by Takahashi et al. (2000). This was cloned into vector pCR4-TOPO for preparation of the cRNA probe.

Results

We used whole-mount *Hydra* and a two-color double-labeling in situ hybridization technique (Hauptmann and Gerster 1994; Grens et al. 1996; Mitgutsch et al. 1999; Hansen et al. 2000) to study the possible coexpression of the Hydra-RGamide gene with one of the other neuropeptide genes from *Hydra*. For the two colors, we used Fast Red (to visualize cells, containing one set of mRNA) and either BM purple or NBT/BICP (to visualize cells containing the other set of mRNA). NBT/BICP is a stronger stain that BM purple, thereby revealing more neurons that express one set of mRNA (because it also reveals cells expressing low concentrations of mRNA), but it has the disadvantage that its color is not a stable blue, i.e., it tends to be more violet than BM purple, especially after a double-staining procedure with Fast Red, which often results in a yellowish background. This makes NBT/BICP less suitable than BM purple for two-color double-labeling experiments, but we have used this stain on several occasions to show as many cells as possible.

We found that neurons from many regions in *Hydra* coexpress the Hydra-RGamide and -LWamide genes. For the tentacles, this phenomenon is shown in Fig. 2C–E. Figure 2E shows neurons expressing Hydra-LWamide mRNA (Fast Red, a clear red color); Fig. 2C displays neurons expressing Hydra-RGamide mRNA (BM purple, a clear blue color); and Fig. 2D shows neurons coexpressing the two genes (violet, a color in between the ones shown in Fig. 2C, E). Figure 2A, B exhibits overall pictures of *Hydra*. Figure 2B shows staining for Hydra-LWamide mRNA alone (Fast Red, a clear red color), and Fig. 2A shows coexpression of the Hydra-LWamide and -RGamide genes in various neurons distributed over all body parts of *Hydra*, but mainly localized in the basal disk and head (violet color). The neurons in the body column express the Hydra-LWamide gene more weakly

Fig. 3A–D In situ hybridization of whole-mount *Hydra* with cRNA probes, coding for either the Hydra-RGamide or Hydra-LWamide preprohormone. **A** Two-color double-labeling in situ hybridization of a young *Hydra*, using a probe coding for Hydra-LWamide (stained with Fast Red) and another coding for Hydra-RGamide (stained with nitrobluetetrazolium/bromochloroindolyl phosphate, NBT/BICP). Note that the neurons have a violet staining that is clearly different from the one in **B** and Fig. 2B, E, showing that they coexpress the two preprohormone genes. **B** In situ hybridization, using a probe coding for the Hydra-RGamide preprohormone (staining with NBT/BICP). Note the large number of neurons stained. **C** Double-labeling in situ hybridization of the foot region of *Hydra* using probes, coding for either the Hydra-LWamide preprohormone (stained by Fast Red), or the Hydra-RGamide preprohormone (stained with NBT/BICP). The neurons exhibit various nuances of violet, which are clearly different from the color in **D** and Fig. 2B, E, showing that they coexpress the two preprohormone genes. Note that the neurons in the basal disk have a more redish nuance, and the neurons in the peduncle/lower gastric region a more bluish nuance, suggesting that they contain the two mRNAs in different concentrations. **D** In situ hybridization with a probe coding for the Hydra-RGamide preprohormone (stained with NBT/BICP). Note that the RGamide neurons form a ring at the edge of the basal disk. *Bars* **A**, **B** 100 µm; **C**, **D** 50 µm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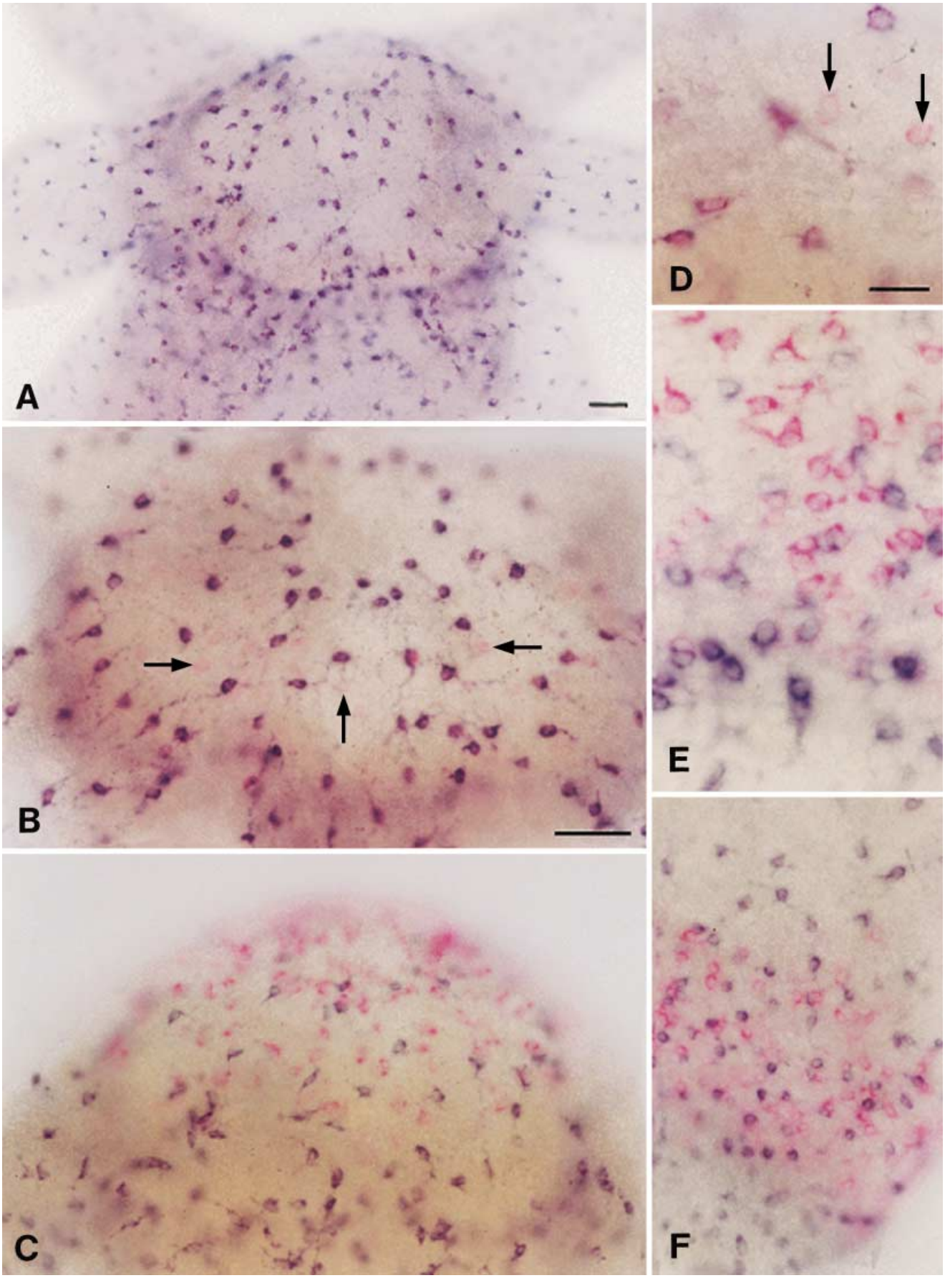
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than the -RGamide gene, sometimes giving the impression that these neurons only express Hydra-RGamide mRNA (cf. Fig. 2A, B). In our opinion, however, there is always coexpression in the body column (the color is never pure blue).

Figure 3B shows the maximal number of neurons in a whole-mount *Hydra* that we are able to stain for Hydra-RGamide mRNA (the staining is with NBT/BICP). Figure 3A shows that, in a young *Hydra*, these neurons coexpress the Hydra-LWamide gene. Figure 3D shows a detail of the foot (basal disk and peduncle), with a ring-like structure of neurons in the basal disk, expressing the Hydra-RGamide gene. The same neurons express the Hydra-LWamide gene (Fig. 3C; violet staining). Note that the neurons in the body column appear to express more Hydra-RGamide than -LWamide mRNA (Fig. 3C; the color tends to be more bluish than in the basal disk).

Figure 4A shows neurons in the head (hypostome and tentacles) that express the Hydra-RGamide gene. These neurons coexpress the Hydra-LWamide gene (Fig. 4B), but, in addition to these neurons, another population of neurons in the hypostome exists that only (weakly) expresses the Hydra-LWamide gene (Fig. 4B, D; red neurons, indicated by arrows). There are, therefore, two populations of Hydra-LWamide neurons in the hypostome.

The *Hydra* preprohormone-B gene is only expressed in neurons of the hypostome and upper gastric region (Mitgutsch et al. 1999; Hansen et al. 2000). These neurons do not coexpress the Hydra-RGamide gene (Fig. 4C). Similarly, the preprohormone-A, -C, and Hydra-KVamide genes are not expressed together with the Hydra-RGamide gene. Examples of these separate expression patterns in neurons of the peduncle are shown in Fig. 4E, F.

◀ **Fig. 4A–F** Whole-mount in situ hybridization of *Hydra*, using cRNA probes, coding for various *Hydra* preprohormones. **A** In situ hybridization of the head (hypostome and tentacle) region, using a probe coding for the Hydra-RGamide preprohormone (stained with NBT/BICP). **B** Double-labeling in situ hybridization, using probes specific for either the Hydra-LWamide preprohormone (stained with Fast Red), or the Hydra-RGamide preprohormone (stained with NBT/BICP). This experiment is similar to that shown in Fig. 3A, but with the magnification used now two populations of neurons can be discerned: one coexpressing the two preprohormone genes (*violet*), and one expressing the Hydra-LWamide preprohormone gene only (*weakly red*, *arrows*). **C** Two-color double-labeling in situ hybridization of the head region of *Hydra*, using probes specific for either the *Hydra* preprohormone-B mRNA (stained with Fast Red), or the Hydra-RGamide preprohormone (stained with NBT/BICP). The two preprohormone genes are expressed by different populations of neurons. **D** The same experiment as **B**, but now at higher magnification, showing more clearly the hypostomal neurons only expressing the Hydra-LWamide preprohormone (*red*, *arrows*). **E** Two-color double-labeling in situ hybridization of the peduncle region of *Hydra*, using probes specific for either the Hydra-KVamide preprohormone mRNA (stained with Fast Red), or the Hydra-RGamide preprohormone mRNA (stained with NBT/BICP). The two preprohormone genes are expressed by different populations of neurons. **F** Double-labeling in situ hybridization of the peduncle region of *Hydra*, using probes for either *Hydra* preprohormone-A (stained with Fast Red) or the Hydra-RGamide preprohormone (stained with NBT/BICP). The two preprohormone genes are expressed by different neurons. *Bars* **A** 50 µm; **B**, **C**, **F** 50 µm; **D**, **E** 20 µm

Discussion

In a previous paper, we have found that a population of neurons in the peduncle of *Hydra* coexpresses the genes for preprohormone-A and the Hydra-KVamide preprohormone, but that, in another region (the upper gastric region), these genes are separately expressed (Hansen et al. 2000). In the present paper, we found a second example of preprohormone gene coexpression, i.e., that of the recently published Hydra-RGamide preprohormone (Takahashi et al. 2000) and of the Hydra-LWamide preprohormone (Leviev et al. 1997), which occurs in a large number of neurons spread all over *Hydra* (Figs. 2, 3). These results show that simple, evolutionarily old nervous systems such as those of cnidarians, already have invented the principle of neuropeptide coexpression, a phenomenon that has been conserved throughout evolution and that is also widely found in mammals (Lundberg et al. 1980; Hökfelt 1991; Lundberg 1996; De Lange et al. 1997; Hökfelt et al. 2000). Simple nervous systems, therefore, generally use neuropeptide cocktails for signal transmission, which can either be neuropeptide cocktails derived from a single preprohormone such as the preprohormones-A, -B, and -C, which each contain various different neuropeptide copies (Fig. 1A–C), or cocktails derived from two different preprohormones such as those giving rise to Hydra-RGamide and -LWamide (Fig. 1D–F).

From both higher invertebrates and mammals, we know that neurons that coexpress two neurotransmitters use these transmitters for a different but related function (sometimes complementary) to steer in an organized and controlled way a certain overall process in an organism (Hökfelt 1991; Lundberg 1996; Hökfelt et al. 2000). A classic example of this phenomenon is that of vasoactive intestinal polypeptide (VIP) and acetylcholine, which are colocalized in parasympathetic neurons, innervating the salivary gland of mammals (Lundberg et al. 1980). Acetylcholine mainly produces salivary secretion via its muscarinic acetylcholine receptors, whereas VIP mainly produces vasodilatation. The two peptides added together, however, give a prominent potentiation of both vasodilatation and secretion in the salivary gland, which is remarkably long-lasting, showing that the two substances cooperate (Lundberg et al. 1980). Another classic example in higher invertebrates is that of the egg-laying hormone (ELH) and bag-cell peptides (BCPs) produced by the bag cells in the abdominal ganglion of the marine snail *Aplysia californica*. The ELH preprohormone gives rise to ELH, which acts on the sexual organs of the snail, but also to the BCPs, which exert a positive autocrine feedback on the bag cells, thereby causing a massive release of ELH from these cells in a period where this is needed, i.e., shortly before egg laying (Scheller et al. 1983; Brown and Mayeri 1989).

An obvious question to be raised, therefore, is: In how far do Hydra-RGamide and Hydra-LWamide potentiate or supplement each other in their actions? It has been reported that Hydra-RGamide stimulates neuron differentiation by inducing its stem cells, the I-cells, to enter into the neuronal pathway, although these effects are not very large (Takahashi et al. 2000). The Hydra-LWamides and other cnidarian LWamide peptides have dual actions. First, they act excitatory on epitheliomuscular tissues of *Hydra*, other hydrozoans, and sea anemones; and second, they induce metamorphosis in planula larvae of the marine hydrozoan *Hydractinia echinata*, i.e., they have an effect on neuronal development (Leitz et al. 1994; Takahashi et al. 1997; Leitz 2001). It would be interesting, therefore, to investigate whether the Hydra-LWamides also have an effect on neuronal development in *Hydra* and whether they potentiate, inhibit, or supplement the effects of Hydra-RGamide. This would give a meaning for their colocalization.

An alternative reason for coexpression would be that Hydra-RGamide is involved in muscle contraction. Hydra-RGamide added alone to *Hydra* did not induce muscle contraction (Takahshi et al. 2000). But, in the light of our present findings, the obvious experiment to do is to add Hydra-RGamide together with the Hydra-LWamides and to see whether it potentiates (or inhibits) the effects of the latter.

We have already found, in 1983, clear evidence for neuropeptide coexistence in *Hydra* (Grimmelikhuijzen 1983). However, this work was done with vertebrate neuropeptide antisera, cross-reacting with, at that time, unknown intrinsic *Hydra* neuropeptides. Thus, using a fluorescence technique for simultaneous visualization of two antigens in one section, we found the coexistence of oxytocin/vasopressin-like material, with bombesin-like material in neurons of the basal disk, gastric region, and tentacles of *Hydra* (Grimmelikhuijzen 1983). Based on their location (basal disk) and anatomy, these neurons are very likely to be identical to the neurons that we now find, coexpressing the Hydra-RGamide and Hydra-LWamide preprohormones. This conclusion is supported by the fact that the oxytocin/vasopressin antisera used in 1983 cross-reacted with oxytocin, vasopressin, mesotocin, isotocin, and vasotocin, showing that those IgG subpopulations stained, which recognized common portions of these neuropeptides (Grimmelikhuijzen 1983). Hydra-RGamide has the C-terminal sequence PXGamide (where X is R, K, I, L) in common with all the above-mentioned oxytocin/vasopressin-like peptides, and could, thus, be recognized by these IgG subpopulations. The *Hydra* neurons that, so far, have been named oxytocin/vasopressin neurons, therefore, are likely to be Hydra-RGamide neurons (Grimmelikhuijzen et al. 1982; Grimmelikhuijzen 1983; Koizumi and Bode 1991; Sakaguchi et al. 1996). Similarly, the Hydra-LWamides have the C-terminal sequence Leu-X-amide (where X is W, or its conserved amino acid residue M) in common with bombesin, and IgG fractions of the bombesin antisera used (Grimmelikhuijzen 1983) might have recognized the Hydra-LWamides. Thus, our present finding of coexpression of the Hydra-RGamide and -LWamide genes could be regarded as a rediscovery of our old finding of neuropeptide coexistence in *Hydra* neurons, with the important difference, however, that we now know the structures of the preprohormones and neuropeptides that are coexpressed.

So far, six neuropeptide genes have been cloned from *Hydra* (Fig. 1). Five populations of neurons express only one type of neuropeptide gene. The Hydra-RGamide gene is always coexpressed with the Hydra-LWamide gene and, in addition, a population of neurons exists in the *Hydra* peduncle that coexpresses the Hydra-KVamide gene together with the preprohormone-A gene (Hansen et al. 2000). This brings the total number of neurochemically different populations of neurons in *Hydra* to at least seven. This number is, of course, much lower than in mammals (Hökfelt et al. 2000). But the interesting conclusion for *Hydra* is that, although its nervous system is primitive, it has already acquired several of the sophisticated principles that we know from higher animals.

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