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Neuronal control of development in hydra

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Abstract **Hydra is an excellent model system for developmental biology, because pattern formation processes can be easily studied in regeneration, transplantation, and reaggregation experiments. At the cellular level hydra has the advantage that it contains only a few basic cell types and that differentiation pathways are short. Two types of signals, produced and released by nerve cells, control the spatial and temporal patterns of differentiation. Positive signals induce specific local differentiation events, and negative signals inhibit the spread of such inductions to larger areas. Head-specific growth and differentiation are controlled by head activator and head inhibitor, foot-specific processes are regulated by foot activator and foot inhibitor. The activators are peptides, the inhibitors are low-molecular-weight substances. The sequence of the head activator is known, and it is conserved throughout the animal kingdom. At the cellular level head activator exerts three types of effects in hydra. It stimulates cells to divide, and it is responsible for the determination and the final differentiation of nerve cells and head-specific epithelial cells. For nerve-cell differentiation the cAMP pathway is used as second messenger system. Components of this pathway were identified in hydra. In mammals head activator is produced by nerve and neuro-endocrine cells, and it acts as mitogen on cells of neural origin. It is present early in neural development and in abnormal neural development, such as brain and neuroendocrine tumours.**

KEY WORDS: *hydra, head activator, foot activator, nerve-cell differentiation, signal transduction*

The nervous system of hydra

Within the animal kingdom hydra, or coelenterates in general, are the first organisms with true nerve cells. Hydra is a simple animal, containing a head with hypostome and tentacles at one end, and a foot with peduncle and basal disc at the other end of a gastric column. The body wall of hydra consists of two cell layers, ectoderm and endoderm, separated by a collagenous extracellular structure, the mesoglea (Fig. 1). As main structural components, ectoderm and endoderm contain epithelio-muscular cells. In tentacles and foot they are in a terminally differentiated state, whereas in the gastric region they are uncommitted and able to differentiate in a head- or foot-specific direction. Between the epithelial cells all the other cell types including nerve cells and their precursor cells, the interstitial cells, are located (Fig. 1). Interstitial stem cells occur in the gastric region only. They can give rise to nerve cells, nematocytes, gland cells, mucous cells, and, in the sexual cycle, to oocytes and sperm cells (David and Gierer, 1974; Bode *et al.*, 1987; Bosch and David, 1987).

The nervous system of hydra is primitive in so far, as it has no specialized nervous organs such as brain or ganglia, and no glial cells. The nerve cells are organized in a nerve net with condensations to ganglion-like structures in the head and in the foot region (Fig. 2). Hydra nerve cells are able to make synapses with other nerve cells or with target cells. They can initiate and propagate action potentials, and their morphology corresponds in all criteria to nerve cells found in higher organisms. From their location and shape two types of neurons can be distinguished in hydra, sensory nerve cells and ganglion cells. Sensory nerve cells are located between epithelio-muscular cells close to the surface of ecto- and endoderm, extending one process into the extracellular space. They are able to respond to tactile, chemical, and light stimuli. Ganglion cells lie at the base of epithelio-muscular cells and they have at least two or more processes. The local environment of a neuron changes during tissue movement and during regeneration. As a consequence of this a certain plasticity is observed with regard to expression of neuropeptides and other neuronal markers (Bode, 1992).

The nervous system of hydra serves two functions. It is responsible for the coordination of movement and behaviour, and it controls morphogenesis. For the coordination of movement this evolutionary oldest nervous system uses peptides for neurotransmission. Classical transmitters like acetylcholine, serotonin and catecholamines and their processing enzymes do not seem to be present in nerve cells of hydra, but neuropeptides are abundant both in hydra and in other coelenterates (Grimmelikhuijzen *et al.*, 1992). Using immunochemical methods neuropeptides were isolated from different coelenterates. Most of these belong to the RFamide family. The respective genes were cloned and shown to contain multiple copies of RFamide-like and additional putative neuropeptide sequences (Grimmelikhuijzen *et al.*, 1992). Some of the isolated peptides

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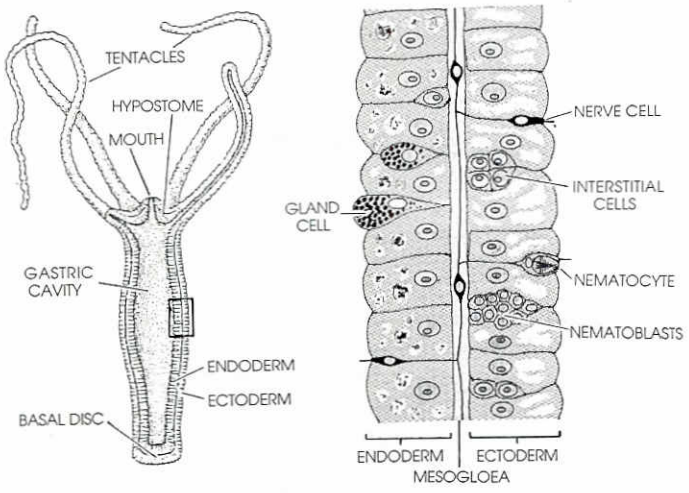


Fig. 1. Schematic presentation of a hydra longitudinal cross section (left), enlarged view of the indicated rectangle from the gastric region (right).

were assayed for transmitter function and found to have both excitatory and inhibitory actions on isolated muscle preparations (McFarlane *et al.,* 1991). The location of RFamide-like material in dense-cored neurosecretory granules in nerve processes in contact with epithelio-muscular cells supports a neuromuscular function in hydra (Koizumi *et al.,* 1989).

For the control of morphogenesis both peptides and non-peptides serve as transmitters. We found that two sets of substances, secreted by nerve cells, regulate head- and foot-specific differentiation events and thus pattern formation in hydra, an activator and an inhibitor of head formation, and a second set for foot formation (reviewed in Schaller *et al.,* 1989b). The two activators are small peptides, with molecular masses above 1000 Da. The two inhibitors have no peptide bonds, are hydrophilic, transmitter-like molecules of relative molecular masses lower than 500 Da. The sequence of the head activator (HA) was determined to be pGlu-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe (Schaller and Bodenmüller, 1981). All four factors are released from nerve cells into the intercellular space, where they act on target cells. The distribution of sources for both HA and head inhibitor have their maximal concentration in the head area, and the foot factors in the foot area (Fig. 3). The head inhibitor controls where a head structure is induced, primarily by regulating, presynaptically, the release of the head activator and also its own release. The head inhibitor, as a small, very hydrophilic molecule, is able to diffuse over long distances. It is bound to a large molecular-weight carrier complex of 600-800 kDa. It, therefore, cannot diffuse freely. Binding of HA to the carrier-complex is non-covalent (Schaller *et al.,* 1986). The HA-carrier complex binds to heparin suggesting an additional barrier to diffusion by interaction with extracellular matrix components. The carrier-bound HA is biologically active and is the most suitable ligand for interaction with the HA receptor (Neubauer *et al.,* 1991). The free HA peptide, once released from the carrier, or offered as synthetic HA, inactivates itself rapidly by dimerization or is degraded by enzymes (Bodenmüller *et al.,* 1986). Dimeric HA has no biological activity, and it is an inadequate ligand for the HA receptor (Neubauer *et al.,* 1991). As a consequence of the different diffusion and degradation parameters for inhibitor and activator, at the release site, there will be predominance of activator over inhibitor and at a greater distance predominance of inhibitor over activator, ensuring induction of head or foot structures in a locally restricted area.

Action of the foot activator

The foot factors like the head factors occur as a gradient along the longitudinal axis of hydra, but with their maximal concentration in the foot (Fig. 3). For the determination of their relative concentration a biological assay is used. Foot-specific epithelial cells, the foot mucous cells, contain a peroxidase activity which can be easily quantified photometrically by the use of a soluble substrate for peroxidases (Hoffmeister and Schaller, 1985). Measurement of the enzyme activity, therefore, provides a rapid and reliable test for monitoring the differentiation of foot-specific cells. To quantify the amount of foot factors in a given extract, feet are removed from intact animals. The foot-regenerating parts are incubated in medium containing the extracts to be assayed. Foot peroxidase activity is measured 22 h and 24 h after cutting, respectively. In the presence of foot inhibitor, the reappearance of the peroxidase activity and, thus, of foot-specific epithelial cells is retarded, while it is accelerated in the presence of foot activator. The respective effects are dose-dependent and specific. At the cellular level foot activator acts as a mitogen by pushing epithelial and interstitial cells, which are arrested in G2, into mitosis. Due to the presence of foot activator, epithelial cells are stimulated to differentiate into foot mucous cells, and interstitial nerve precursor cells differentiate into mature nerve cells (Hoffmeister, 1989). So far we could not show any effect of foot activator on the determination of interstitial stem cells to undergo foot-specific neurogenesis. This finding may provide a reason why the head system dominates over the foot system as was suggested by transplantation experiments (Hicklin *et al.,* 1973; Hicklin and Wolpert, 1973; Bode and Bode, 1980).

The analysis of a foot-regeneration deficient strain of *Hydra oligactis* (Hoffmeister, 1991) demonstrates the autonomy and importance of the foot factors for pattern formation in hydra. *Hydra oligactis* possess a very low content of foot activator, being reduced to 42% compared to *Hydra vulgaris,* whereas the content of foot inhibitor is as high as in *Hydra vulgaris.* Moreover, the foot activator is present in *Hydra oligactis* as a much steeper gradient than in *Hydra vulgaris,* the gradient of the foot inhibitor is very flat. In *Hydra oligactis* this unfavorable ratio of foot activator over foot inhibitor in foot regenerating upper halves leads to a deficiency in foot regeneration. This shows that the foot activator plays an essential role during foot-specific differentiation processes.

Action of HA at the cellular level in hydra

HA has three effects at the cellular level in hydra: (1) It acts as mitogen in the G2/mitosis transition by inducing cells to divide. This mitogenic effect of HA is not cell-type specific. All dividing cell types in hydra, arrested in the G2-phase of the cell cycle respond in this way, and low concentrations of HA ($>10^{-13}$

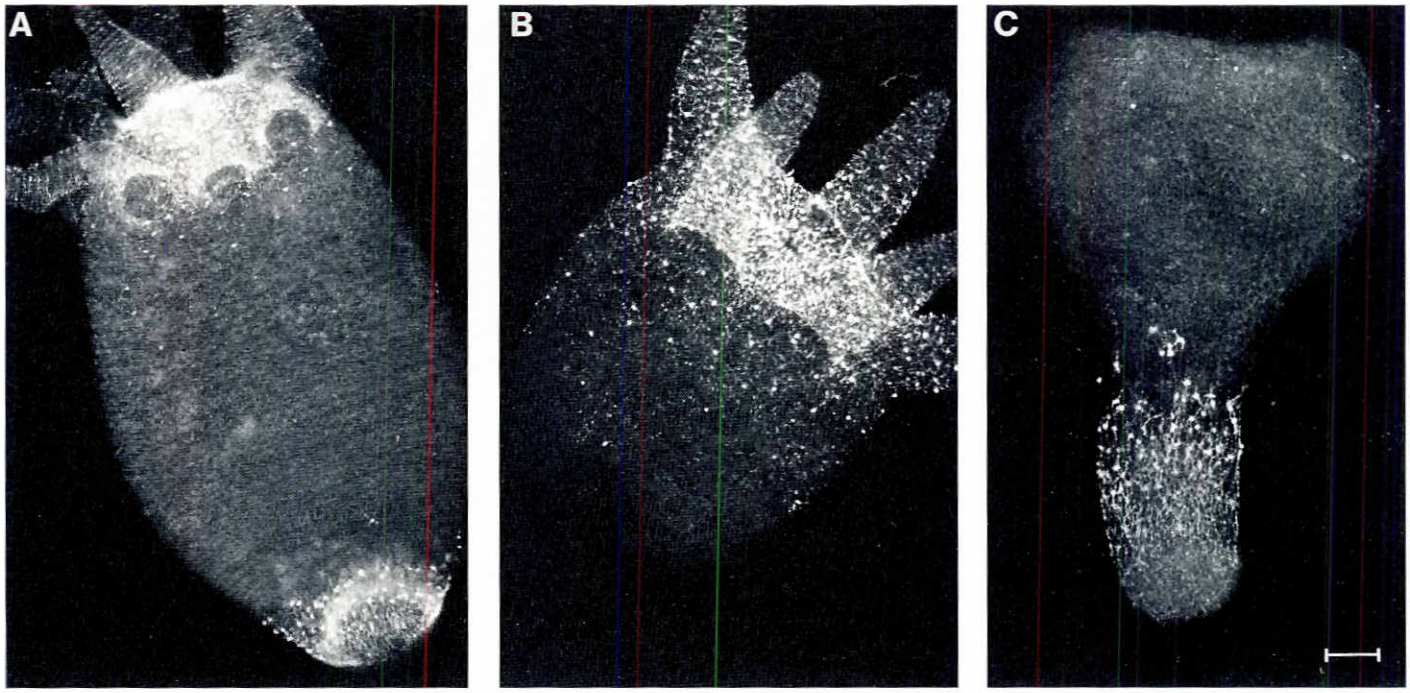


Fig. 2. Nerve net in hydra. *Nerve cells were made visible by staining with an FMRFamide antiserum (kind gift of C.J.P. Grimmelikhuijzen), which reacts with a subset of nerve cells in the head and peduncle regions of hydra.* **(A)** *Intact hydra,* **(B)** *upper, and* **(C)** *lower half of a hydra of the species Hydra vulgaris. Bar, 500 μm.*

M) achieve this effect. (2) The fate of a cell, in hydra like in other organisms, is decided in the S-phase, and requires DNA synthesis. In the presence of high concentrations of HA ($>10^{-11}$M) during S-phase interstitial stem cells become determined for the nerve-cell pathway, and uncommitted epithelial cells of the gastric region for head-specific differentiation. (3) HA, or predominance of activators over inhibitors, are required for final differentiation. Thus epithelial cells are arrested in G2 and differentiate to their predetermined fate only, if HA or foot activator are present. Similarly, interstitial cells determined for the nerve-cell pathway, undergo a final mitosis, before they terminally differentiate to nerve cells. Low concentrations of HA ($<10^{-12}$ M), foot activator or disinhibition by wounding stimulate this last step of nerve-cell differentiation (Hoffmeister 1989; Schaller *et al.,* 1990; Neubauer *et al.,* 1991).

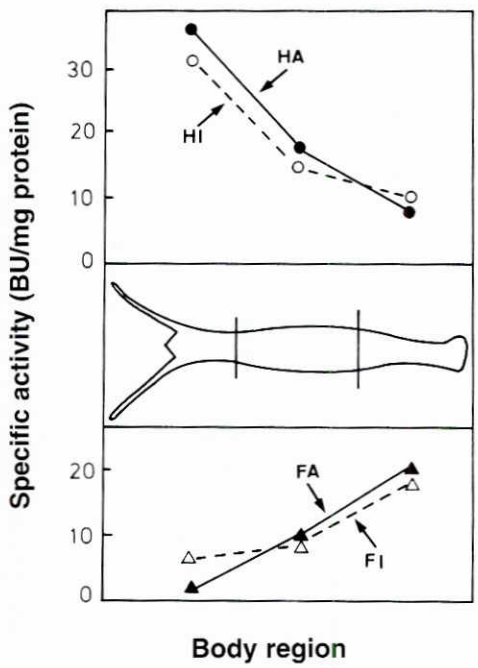
The effect of HA on nerve-cell differentiation and determination is mediated by the cAMP pathway

In hydra the nervous system is continuously renewed by differentiation from interstitial cells (Fig. 4). On average, an individual nerve cell has a half life of seven days. Nerve cells can differentiate from uncommitted interstitial stem cells in a single cell cycle, which, under favorable conditions, requires at least 24 h (Fig. 5). In this pathway from cycling interstitial stem cell to mature nerve cell, HA can act at three restriction points in the cell cycle: At the first restriction point (R1), HA stimulates cycling stem cells to divide and enter the S-phase. In the early S-phase, at restriction point 2 (R2), HA is required to induce nerve-cell determination. In the subsequent G2-phase, cells become arrested at restriction point 3 (R3). HA is able to function as signal at R3 to trigger final mitosis, before differentiation to nerve cells (reviewed in Schaller *et al.,* 1989).

To study what happens at R3, HA analogues and molecules involved in signal transduction, were assayed for their effect on nerve-cell differentiation and determination. Circumstantial evidence had suggested that cAMP may be active as second messenger at R3 (Holstein *et al.,* 1986). Using a water-soluble membrane permeable cAMP agonist and antagonist, we indeed found (Fenger *et al.,* 1994), that the effect of HA on nerve-cell differentiation can be mimicked by the cAMP agonist, Sp-cAMPS (Fig. 6A), and is counteracted by the cAMP antagonist, Rp-cAMPS (Fig. 6B).

Likewise in a different experimental set-up which allowed analysis of the effect of HA on nerve-cell determination, HA could be replaced by the cAMP agonist, and its action inhibited by the cAMP antagonist (Fig. 7). From this is concluded that in nerve-cell determination, and differentiation, cAMP is acting as second messenger in the HA signal transduction cascade.

For signal transduction from the transmembrane HA receptor (Neubauer *et al.,* 1991; Christians *et al.,* 1993) to adenylyl cyclase a helper protein is involved, named CAP, adenylyl cyclase associated protein. CAP was originally isolated from yeast where it mediates signal transduction over RAS or other G proteins to adenylyl cyclase (Field *et al.,* 1990; Gerst *et al.,* 1991). We isolated a gene coding for CAP from hydra and found that treatment of hydra with CAP antisense oligonucleotides blocked the effect of HA, but not of the cAMP agonist, SP-cAMPS on nerve-cell determination and differentiation (Fenger *et al.,* 1994). The cAMP analogues directly interact with protein kinase A (Wang *et al.,* 1991). The fact that CAP antisense oligonucleotides inhibited HA, but not cAMP action, places the



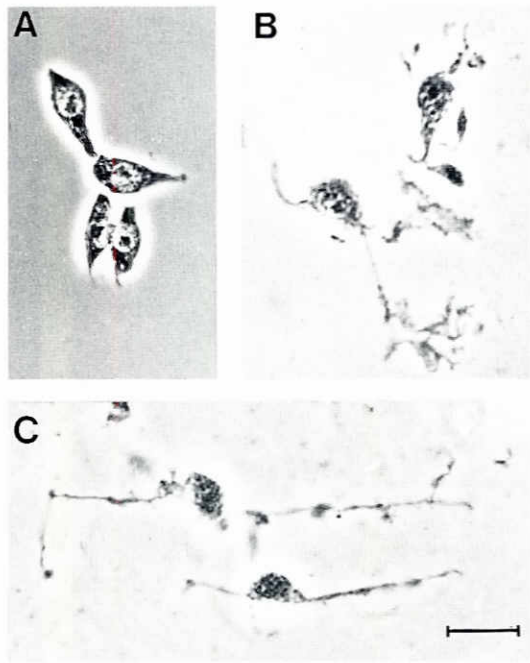


Fig. 3 (left). Concentration of head activator (HA), head inhibitor (HI), foot activator (FA), and foot inhibitor (FI) in different body regions of hydra. *The concentration is expressed in biological units (BU) per mg of protein as defined by respective bioassays (Schaller et al., 1979; Hoffmeister and Schaller, 1985).*

Fig. 4 (right). Morphology of interstitial cells differentiating to nerve cells. (A) *Interstitial cells,* **(B)** *developing nerve cells with nuclear morphology, resembling that of interstitial cells, and* **(C)** *mature nerve cells. Bar, 20 μm.*

function of CAP between HA-receptor and adenylyl cyclase (Fig. 8).

Protein kinase A consists of two catalytic and two regulatory subunits. Upon binding of cAMP to the regulatory subunits, the catalytic subunits are released. One of their functions is the activation of transcription factors by phosphorylation. Especially the transcription factor CREB, which interacts with promoter regions containing a cAMP response element, CRE, is activated by protein kinase A (Fig. 8). We found that in hydra CREB activity is increased after HA-treatment and during regeneration (Galliot *et al.*, 1995).

Hydra is one of the oldest species with a well defined axial polarity. In other organisms anterior-posterior development is under the control of homeobox containing genes and their encoded transcription factors. Using the guessmer strategy for screening (Galliot and Schummer, 1993) nine different homeobox containing genes were isolated from hydra of which two (*cnox* 1 and *cnox* 2) are related to the HOX/HOM gene family. By using quantitative PCR a differential expression pattern of these genes was found during head regeneration in hydra. This indicates that homeobox-containing genes have a similar function in anterior-posterior development in hydra as in other organisms (Schummer *et al.*, 1992).

During regeneration and as a consequence of HA treatment, an early activation of CREB activity was observed preceding changes in expression of the homeobox containing transcription factors. This may indicate that CREB triggers their expression cascade.

Occurrence and function of HA in mammals

HA was originally isolated from hydra, but later also from two mammalian sources, hypothalamus and intestine. Its amino acid sequence was found to be identical (Bodenmüller and Schaller, 1981). Using immunological assays in combination with reverse-phase high-pressure liquid chromatography, it was found that HA is present in other animals (insects, amphibia, birds, crustaceans). In human tissues, high amounts of HA were found in tumors of neural and endocrine origins, in cell lines derived from such tumors, and in blood of patients with such tumors (Schaller *et al.*, 1988, Winnikes *et al.*, 1992). The role of HA as growth factor for neural development was assayed on the neural cell line NH15-CA2. It was found that presence of HA leads to an increase in cell number which is due to the mitogenic action of HA, stimulating these cells to pass through G2 and enter mitosis 1-2 h later (Schaller *et al.*, 1989a). This effect seems to be specific for neural and neural crest derived cells, since no other cell type showed any responses.

Conclusion

The differentiation of a stem cell to a head- or foot-specific cell in hydra is a multistep process with several restriction points in the cell cycle at which the four morphogenetic signals act. The fate of a cell, in hydra like in other organisms, is decided in the S-phase and requires DNA-synthesis. In the presence of high concentrations of HA during S-phase interstitial stem cells become determined for the nerve cell pathway, and epithelial stem cells for head-specific differentiation (Schaller, 1976;

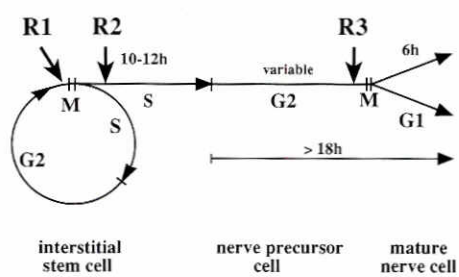


Fig. 5. Restriction points (R) in the pathway from uncommitted interstitial stem cell to mature nerve cell, at which HA acts as positive signal.

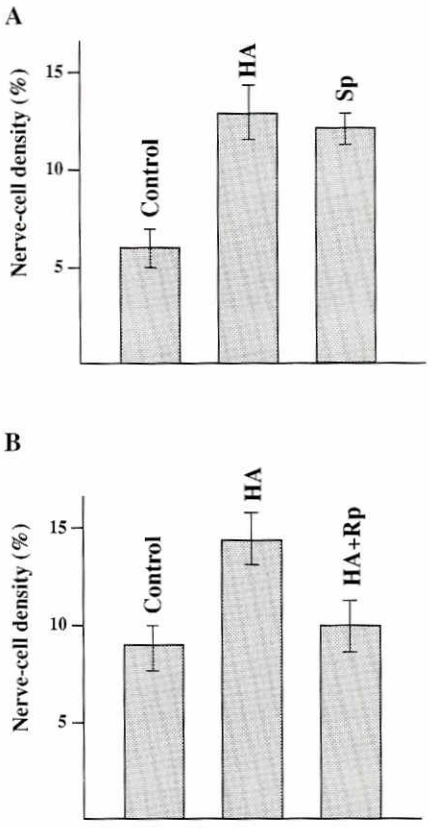


Fig. 6. The effect of HA on nerve-cell differentiation is (A) mimicked by the cAMP agonist, Sp-cAMPS (Sp) and (B) blocked by a cAMP antagonist Rp-cAMPS (Rp). *Hydra without buds of the species Hydra vulgaris were incubated for 8 h in the HA analogue, C_8-HA^2 (10^{-10} M; Neubauer et al., 1991), in Sp-cAMPS (10^{-5} M), and in Rp-cAMPS ($2x10^{-5}$ M). At 8 h gastric regions were cut out, dissociated into cells, and the nerve-cell density was determined as ratio of nerve cells to epithelial cells. Due to their slow turn-over rates of three days, epithelial cells are suited as standard.*

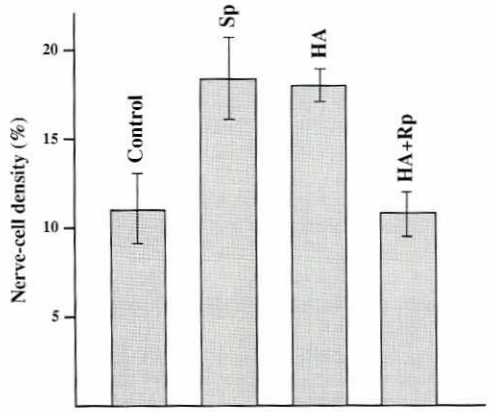


Fig. 7. The effect of HA on nerve-cell determination is mimicked by the Sp-cAMPS (Sp) and blocked by Rp-cAMPS (Rp). *Hydra were incubated for 8 h in C_8-HA^2 (10^{-10} M), Sp-cAMPS (10^{-5} M), and in Rp-cAMPS (10^{-4} M). Isolates were prepared by cutting off head and foot, and by incubating the isolated gastric regions in hydra medium for another 18-20 h. During these 18-20 h nerve cells differentiate due to the wounding stimulus. An increase in nerve-cell density over controls reflects nerve-cell determination having occurred in the treated animals before wounding.*

Holstein *et al.*, 1986; Schaller *et al.*, 1990). Such determined cells can differentiate only, if HA or foot activator are present. For this latter effect HA and foot activator seem to be interchangeable and low concentrations suffice. Thus, specificity is achieved at the determination level and requires high concentrations of the specific morphogen. Progression to final differentiation is a less selective step, where HA and foot activator act as positive, head inhibitor (and possibly also foot inhibitor) as negative signals.

From these data, the following model is derived: Close to the head region where head activator concentration is highest many stem cells become determined for differentiation, interstitial cells for the nerve-cell pathway and epithelial cells for head-specific differentiation. They differentiate fast to head-specific nerve cells and to hypostomal or tentacle-specific epithelial cells. In the gastric region, fewer stem cells become determined and they become arrested in G2 due to high concentrations of head inhibitor. During the gradual tissue displacement from the subhypostomal to the basal region, interstitial cells committed to the nerve-cell pathway reach the foot where, under the influence of foot activator, they differentiate to nerve cells, but now in a foot-specific manner. Head activator and head inhibitor are produced by nerve cells of the head, foot activator and foot inhibitor by nerve cells of the foot. This nerve cell differentiation scheme represents a complicated autocrine control loop which in the end ensures that head-specific growth and differentiation is maintained in the head region, that foot-specific processes occur in the foot, and that the head system dominates over the foot system as postulated from other biological experiments (Bode and Bode, 1980; Hoffmeister, 1991).

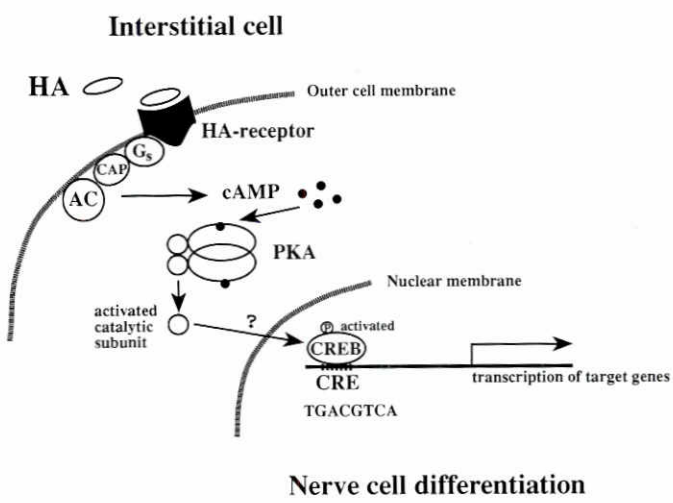


Fig. 8. HA signal transduction cascade. *HA interacts with a transmembrane receptor (R) which is most likely G protein linked. Activation of the adenylyl cyclase (AC) is mediated by CAP. The production of cAMP activates protein kinase A (PKA) which by phosphorylation of CREB, the cAMP response element binding protein, stimulates transcription of genes containing a CRE element in their promoter region.*

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