Role of the neuropeptide head activator for growth and development in hydra and mammals

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Summary

In hydra, HA is produced by nerve cells and released into the intercellular space bound to large-molecular-weight carrier(s). By additional interaction with extracellular matrix components and selfinactivation by dimerisation, a local action is ensured. HA acts as a mitogen on all dividing cell types in hydra forcing them to pass through G₂, divide, and either start a new round of cell division or terminally differentiate. In addition, HA is required for head-specific determination and differentiation processes. To become a head-specific nerve cell, for example, an interstitial stem cell requires HA in early S-phase to become determined to the nerve cell pathway, in late G₂ to progress through mitosis, and/or in G₁ to differentiate to a head- and not to a foot-specific nerve cell.

HA (with identical amino acid sequence) occurs in other animals including mammals. In mammals, it is produced by nerve or endocrine cells and it probably acts, as in hydra, on nerve-precursor cells. On the neural cell line NH15-CA2 and on the pituitary cell line AtT20, HA acts as mitogen by stimulating cells arrested in G₂ to enter mitosis. The presence of HA early in neural development and in abnormal neural development, such as in brain and neuroendocrine tumors, are consistent with a function in growth control for HA in mammals.

Key words: head activator, morphogen, mitogen, cell differentiation, extracellular matrix.

Introduction

Intercellular communication is important for the ordered spatial and temporal pattern of cellular differentiations that results in morphogenesis. Two types of signals are required for communication, positive signals that induce specific, local differentiation events, and negative signals that inhibit the spread of inductive events to larger areas. By definition, substances responsible for induction should have restricted diffusion properties to ensure local action, whereas inhibitors should be easily diffusible to be able to communicate with cells at greater distances (Gierer and Meinhardt, 1972; Kemnner, 1984). To study such substances, we originally used hydra as a model system. When we found that one of our inducing substances, the head activator, is present also in other animals including mammals, we extended our investigations to them. The role of the hydra head activator (HA) in the development and cellular differentiation of both hydra and mammals is described. The special molecular properties of the HA, namely binding to large molecular weight carriers and selfinactivation by dimerisation, suit it for its function as a locally acting growth and differentiation signal.

Hydra, a small freshwater coelenterate about 1 cm in length (Fig. 1), is suited as a model system because it is simple, contains few cell types with short differentiation pathways, and is very amenable to experimental manipulations such as tissue grafting, regeneration and reaggregation of cells. For developmental studies, the central part of hydra, the gastric column, is the most interesting, since it contains undetermined, undifferentiated tissue. The structures at the ends, the head with hypostome and tentacles, and the foot with peduncle and basal disk contain predominantly committed cells in the process of or in terminal differentiation. With daily feeding, hydra reproduces asexually by budding, having a 3-day doubling time. Under starvation conditions and in the cold, the animal enters a sexual cycle in which gonads are formed instead of buds (reviewed in Lenhoff, 1982).

Hydra consists of only two cell layers, ectoderm and endoderm, separated by a collagenous extracellular structure, the mesoglea (Fig. 2). Ectoderm and endoderm are made up of epithelio-muscular cells, which in the gastric region contain cells able to differentiate to head- or foot-specific epithelial cells. Thus, if a hydra is cut horizontally into two parts, gastric cells will differentiate into the specific cells of the missing structure, head or foot, and this occurs within a single cell cycle. Thus, foot-specific ectodermal epithelial cells, characterised by the production of a peroxidase-like enzyme as a marker (Hoffmeister and Schaller, 1985), become
visible 20–24 h after cutting. Similarly, head-specific ectodermal epithelial cells characterised by a head-specific monoclonal antibody (Javois et al. 1986), appear at about the same time, each marker restricted to the appropriate half (Dübel et al. 1987). Ectodermal and endodermal epithelial cells do not interconvert and must therefore derive from different gastric stem cells (Smid and Tardent, 1982).

The most interesting stem-cell population in hydra are interstitial cells which, as their name implies, are located between epithelial cells (Fig. 2). Interstitial cells are stem cells for nerve cells, nematocytes, endodermal gland and mucous cells, and, in the sexual cycle, also for oocytes and sperm cells (David and Gierer, 1974; David and Murphy, 1977; Bode and David, 1978; Bode et al. 1987; Bosch and David, 1987). Nerve cells differentiate from stem cells in a single cell cycle, nematocytes require two to four cell cycles giving rise from one stem cell to (4), 8, 16, (32) clonally derived nematocytes. The differentiation of interstitial stem cells is, like that of epithelial cells, dependent on their position within the animal. Close to the end structures, interstitial stem cells differentiate preferentially to nerve cells whereas commitment to the nematocyte cell lineage occurs in a graded fashion along the gastric column. Under steady-state conditions, a constant ratio between stem cells and differentiating cells is strictly maintained (Bode and Flick, 1976; Bode et al. 1976).

Purification and chemical analysis of inducers and inhibitors of differentiation and morphogenesis in hydra

We found that two sets of substances regulate head- and foot-specific differentiation events in hydra, an activator and an inhibitor of head formation and a second set for foot formation. The effect of substances influencing head- and foot-specific differentiation events can most easily be measured as acceleration or inhibition of head or foot regeneration (for a review see Schaller et al. 1979). For that purpose, heads or feet are removed from intact animals, and the head- or footless parts incubated in medium with and without the factors to be assayed. In the presence of inhibitors, regeneration is retarded or at higher concentrations completely inhibited. In the presence of the activators, regeneration is accelerated. The effects are specific such that, for example, the head activator accelerates the rate of head regeneration, but not that of foot regeneration (Schaller et al. 1979). Underlying these gross morphogenetic effects are changes at the cellular level which can also be used to assay the respective substance. The
appearance of foot-specific ectodermal epithelial cells is thus accompanied by the production of a peroxidase activity which can easily be utilised to quantify foot-specific cellular differentiation (Hoffmeister and Schaller, 1985).

By means of such quantifiable assays, we separated the four substances and purified the two activators extensively, the two inhibitors to a lesser extent (Table 1). The two activators are small peptides with relative molecular masses of around 1000. 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). The analysis of the foot activator (FA) is close to completion. The two inhibitors, both nonpeptide, hydrophilic molecules of relative molecular masses lower than 500 are difficult to purify, since so far no special property has distinguished them from other small molecules. For purification, in general, a crude extract is subjected to an organic solvent extraction with 90 % methanol to remove large molecules and to separate the active components from possible carriers or substances to which they adhere. Lipids are removed by ether and chloroform extraction, and the active components separated by ion-exchange, molecular-sieve and other conventional chromatographic procedures. As a last step, HPLC methods allow final purification. Both activators can be purified by using reverse-phase columns from which FA elutes under neutral conditions at 25 % methanol, whereas 60 % methanol is required for HA elution indicating that FA is a more polar molecule than HA. The inhibitors do not adsorb to such reverse-phase columns and require separation on nonreverse-phase material such as silica. From such silica HPLC columns, the head inhibitor (HI) elutes faster than the foot inhibitor (FI) when solvent gradients with decreasing hydrophobicity are applied indicating that FI is more polar than HI. This purification of the inhibitors is sufficient for biological experiments but not yet for chemical analysis.

The crux of the problem as shown in Table 1 is the low molar activity of all four substances. The activators are active at picomolar and the inhibitors at nanomolar concentrations. This means that a hydra needs, and contains, low amounts of the respective substance. A hydra contains, for example, less than one femtomole (10^-15 moles) of HA. For analysis at least one nanomole of purified peptide is required. With an optimistic 10 % yield this implies that at least 10^7 hydra have to be extracted. For the purification of HA, we made use of the fact that other coelenterates also contain HA. We thus processed 200 kg of the sea anemone, *Anthopleura elegantissima*, to obtain 20 nanomoles (20 μg) of pure HA (Schaller and Bodenmüller, 1981).

### Biological and morphogenetic properties of activators and inhibitors in hydra

In hydra, all four substances occur in gradients with maximal concentrations for the head factors in the hypostomal region and the foot factors in the basal disk (Fig. 3). Copurification with nerve cells was taken as evidence that in normal hydra all four factors are products of nerve cells (Schaller and Gierer, 1973; Berking, 1977; Grimmelikhuijzen, 1979; Schmidt and Schaller, 1980). For HA this was confirmed by copurification with neurosecretory granules (Schaller and Gierer, 1973) and, as shown in Fig. 4, by immunocytochemical localisation of HA in developing nerve cells (Schawaller et al. 1988).

All four factors are released from nerve cells into the intercellular space where they act on target cells. The inhibitors control where a certain structure is induced.

### Table 1. Properties of head and foot factors from hydra

<table>
<thead>
<tr>
<th>Morphogen</th>
<th>Molecular mass (D)</th>
<th>Purification (x-fold)</th>
<th>Active concentration</th>
<th>Gradient</th>
<th>T,</th>
<th>Range of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head activator</td>
<td>1124</td>
<td>10^9</td>
<td>10^-13 M</td>
<td>head→foot</td>
<td>4 h</td>
<td>local</td>
</tr>
<tr>
<td>Head inhibitor</td>
<td>&lt;500</td>
<td>10^5</td>
<td>&lt;10^-13 M</td>
<td>head→foot</td>
<td>4 h</td>
<td>global</td>
</tr>
<tr>
<td>Foot activator</td>
<td>~1000</td>
<td>10^4</td>
<td>&lt;10^-12 M</td>
<td>foot→head</td>
<td>Long</td>
<td>local</td>
</tr>
<tr>
<td>Foot inhibitor</td>
<td>&lt;500</td>
<td>10^4</td>
<td>&lt;10^-8 M</td>
<td>foot→head</td>
<td>Short</td>
<td>global</td>
</tr>
</tbody>
</table>

*Fig. 3. 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) as defined by respective bioassays per mg of protein (Schaller et al. 1979; Hoffmeister and Schaller, 1985).*
Fig. 4. Immunocytochemical localisation of head activator in interstitial cells developing to nerve cells in hydra. Cryosections of hydra were fixed with 1 % carbodiimide and 1 % formaldehyde and reacted with the antibody 12/4 (1:103) (Schawaller et al. 1988). Enlargement x250.

primarily by regulating the release of the activator and also their own release. Thus we found that HI at very low concentrations inhibits the release of HA and at 20-fold higher concentrations it also inhibits its own release (Schaller, 1976; Kemmner and Schaller, 1984). The morphological action of HI is best illustrated by mutants of hydra which differ in HI content and consequently in size. Under normal feeding conditions, hydra asexually reproduce by budding, maintaining a constant growth rate and size. A young animal after detachment from the parent animal grows to a certain size, then growth stops, and newly arising cells form buds. The location where a bud is induced is regulated predominantly by the head and to a lesser extent by the foot through the mediation of the respective inhibitors. In mutants with high HI content, the distance between head and bud becomes very large resulting in a maxi phenotype; in mutants with low HI content, the distance shrinks to produce mini animals (Fig. 5). This indicates that the larger HI concentration in maxi over mini results in a greater inhibited area (Schaller et al. 1977). It also shows that HI is able to diffuse over long distances.

In the absence of inhibitors induction occurs. If a hydra is cut horizontally into two pieces of equal size, in the upper half the head inhibits the release of HA at the cut surface, whereas, in the lower half release of HA is allowed. Vice versa, in the lower half the foot inhibits FA release, whereas, in the upper half FA release is allowed. This results in a local induction of head or foot regeneration leading to complete restoration of the missing structure within 2 days.

Corelease of activators with carrier molecules

HA and HI are both small molecules with very similar molecular masses. It was unclear why they should have different diffusion properties. As an explanation we found that HI is released in its naked low molecular mass form, whereas HA is bound to a large molecular mass carrier (Schaller et al. 1986). If HA is extracted from hydra tissue with aqueous solvents or if medium is collected from animals regenerating a head, all HA activity elutes from S-300 columns with an apparent relative molecular mass of 600–800×10³ (Fig. 6). Binding is noncovalent as demonstrated by quantitative extractability with organic solvents such as methanol and/or high salt (2 M-NaCl). The complex binds to heparin (Scheerer, unpublished results) suggesting an additional barrier to diffusion by a possible interaction with extracellular matrix components. This carrier-bound HA is active at 10⁻¹³ M indicating that binding to the carrier does not inhibit the biological effect. HA either binds to its receptor as HA-carrier complex and/or the affinity of the free HA for its receptor is higher than that for the carrier. The free HA peptide, once released from the carrier or offered as synthetic HA, has little chance of long persistence, since it either inactivates itself rapidly by dimerisation (Fig. 7) or is degraded by enzymes. We found that dimeric HA has no biological action on hydra (Bodenmüller et al. 1986). The rapid inactivation fulfills an additional prerequisite for local action, namely that a molecule once released cannot diffuse far. The head inhibitor is not associated
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Fig. 6. Gel filtration of medium collected from hydra during the first 3 h of head regeneration on Sephacryl S-300 columns equilibrated with 25 mM-ammonium bicarbonate, pH 7.5, at 4°C. Total volume: 40 ml, fraction size: 0-95 ml. 125I-Tyr1-HA was added to monitor the position of free HA.

Fig. 7. Diagram depicting release of HA bound to a carrier molecule from producer cell and subsequent interaction with HA receptors on target cells or selfinactivation by dimerisation.

with carrier molecules (Fig. 6) and is therefore not hindered in its free diffusion over long distances. Likewise the foot inhibitor is also released in a low molecular mass form, whereas the foot activator is bound to a large molecular mass carrier. We therefore assume that this represents a general principle. Binding to carrier fulfils two functions: it inhibits diffusion and it prevents degradation thus ensuring the longer half-life (Table 1) of activators over inhibitors. It also explains why substances of low molecular mass, by definition diffusible, can nevertheless be morphogens with local action.

Action of HA at the cellular level in hydra

At the cellular level, HA has two effects in hydra. It stimulates cells to divide, and it is responsible for head-specific differentiations (Schaller, 1976a,b; Holstein et al. 1986; Hoffmeister and Schaller, 1987). The effects as a growth factor can most easily be demonstrated by adding HA to 24 h starved animals and counting mitotic cells 1–2 h later. In hydra this can be monitored as an effect on mitotic index shown in Fig. 8 for interstitial cells. The effect is dose dependent (Hoffmeister and Schaller, 1987), and it is used in our laboratory as a convenient, fast biological assay for determining HA concentrations. The 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 (Schaller, 1976a). HA thus acts as a true mitogen in contrast to growth factors in higher animals, which usually act in the transition from Go to G1.

Differentiation pathways in hydra are short. From stem cell to fully differentiated cells only one or a few cell cycles are required. Hydra contains two main stem cell populations, epithelial cells, which are the main structural components of the two cell layers ectoderm and endoderm, and interstitial cells from which, as main differentiation products, nerve cells and nematocytes derive.

Epithelial cell differentiation occurs in one cell cycle, as evidenced by the appearance of head- or foot-specific epithelial cells in head or foot regenerating tissue one day after cutting (Dübel et al. 1987). After treatment of gastric tissue with foot activator, foot-specific epithelial cells appear (Hoffmeister and Schaller, 1987). In analogy, HA is required for head-specific epithelial cell differentiation.

The decision which pathway is chosen may be a multistep process. Interstitial cells require HA at least twice, first for the determination to enter the nerve-cell

Fig. 8. Effect of HA stimulating mitosis of interstitial cells in hydra. O--O cells treated with HA (10^{-12}M), ▲--▲ non-treated controls. For each time point 8000 cells were counted.
pathway, and second for the decision to differentiate to a head-, as opposed to a foot-, specific nerve cell. Using pulse labeling with $^{3}$H]thymidine applied before or after treatment with head activator and assaying for labeled nerve cells, we could show that head activator is required in early S-phase to allow the determination of stem cells to nerve cells (Schaller, 1976). In C. N. David's laboratory a convenient method to assay determination was developed. After treatment with head activator, a piece of tissue from the gastric region is removed from the inhibitory influence of head and foot by isolation. In such isolates up to 50% of all the stem cells differentiated to nerve cells compared to only 10% in an intact animal (Holstein et al. 1986). Head inhibitor at high concentrations could antagonise the effect on nerve cell determination (Bering, 1977). After determination in S-phase, such a cell will traverse through the G2-phase and its final mitosis only if head activator or foot activator is present (Fig. 9). The differentiation is inhibited in the presence of relatively low concentrations of head inhibitor. Thus, for the determination to the nerve cell pathway, head activator is absolutely necessary, head inhibitor is able to antagonise this effect and foot activator has no influence. For the final differentiation, both head activator and foot activator may be used as signals, but both can be antagonised by head and maybe also by foot inhibitor.

From these data, the following model is derived: close to the head region where head activator concentration is highest many stem cells become determined to nerve cells and differentiate to nerve cells fast and in a head-specific manner. In the gastric region, fewer stem cells become determined to nerve cells, and most of them are prevented from differentiation to nerve cells by the head inhibitor. During the gradual tissue displacement from the subhypostomal to the basal region, these cells reach the foot where, under the influence of foot activator, they differentiate to nerve cells, 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 very 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 et al. 1980).

**Occurrence of HA in mammals**

HA was originally isolated and sequenced from two main mammalian sources, hypothalamus and intestine. Its amino sequence was found to be identical to the hydra peptide (Bodenmüller and Schaller, 1981). Using immunological assays in combination with reverse-phase high-pressure liquid chromatography (Bodenmüller and Roberge, 1985), we discovered that HA is present in other animals (insects, amphibia, birds, crustaceans) and, in addition to brain (Table 2), in other tissue of neural or endocrine origin. High amounts of HA were found in tumors of such origins (Fig. 10), in cell lines derived from such tumors, and in blood of patients with such tumors (Schaller et al. 1988).

HA is not only present in tumor tissue and in tumor cell lines, but also in developing tissue. Thus we found that in human and rat embryos HA is present in the developing brain and in the developing intestine (Schaller et al. 1977). In addition, elevated levels were produced by human placental cells at 3–4 months of gestation and found in the milk of lactating mothers. This suggests that HA also acts as a growth factor in normal development.

To study which cell types in mammals produce HA, we made use of the teratoma cell line P19 which by treatment with retinoic acid and cell clumping can differentiate after several cell divisions and a 8- to 14-day lag phase into different cell types including nerve cells (McBurney et al. 1982). Such early nerve cells produce high amounts of HA as shown by direct extraction from the cells and by immunocytochemistry (Fig. 11).

**Table 2. Occurrence of head activator in human tissue**

<table>
<thead>
<tr>
<th>Source of tissue</th>
<th>Concentration of HA (fm mg$^{-1}$ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>12</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>22</td>
</tr>
<tr>
<td>Pons</td>
<td>18</td>
</tr>
<tr>
<td>Medulla</td>
<td>65</td>
</tr>
<tr>
<td>Thalamus</td>
<td>65</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>1670</td>
</tr>
<tr>
<td>Retina</td>
<td>800</td>
</tr>
</tbody>
</table>
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Fig. 10. Occurrence of HA in human brain tumors. Broken line indicates upper limit of HA concentration found in nonhypothalamic nontumor brain tissue.

Fig. 11. Immunocytochemical localisation of HA in the teratoma cell line P19 induced to nerve cell differentiation by treatment with retinoic acid (5×10^{-7} M). Reaction with the monoclonal HA antibody E 21 (1:200) (Schawaller et al. 1988).

Action of HA as a growth factor in mammals

To investigate HA's action on mammalian cells, we used the cell lines NH15-CA2 and AtT20. We found that, in both cases, the presence of HA leads to an increase in cell number. This is due to the mitogenic action of HA, stimulating cells to pass through G_2 and enter mitosis. This is shown in Fig. 12, where the action of HA was monitored as an increase in mitotic index on NH15-CA2 cells. Fig. 12 also demonstrates that the effect of HA can be mimicked by anti-idiotypic antibodies produced against monoclonal HA antibodies. Localisation of HA receptors on cells in mitosis confirms HA's action as a growth factor acting in the transition from G_2 to mitosis (Schawaller et al. 1988).

Other possible functions of HA in mammals

In mammals, the head activator is not only found in growing tissue like embryos and tumors but also later in differentiated tissue, such as in the hypothalamus of an 85-year-old man, and in blood samples of all ages. We therefore assume that the head activator not only acts as a growth factor but, like other mammalian neuropeptides, also has additional functions in intercellular communication.

Neuropeptides in general may act as neurotransmitters or modulators directly at synapses, at target sites nearby or as neurohormones over long distances following release into the blood. We found that the head activator is present in the blood of all mammals including humans. When trying to analyse its action in the blood, we discovered that injected head activator was degraded within minutes (Roberge et al. 1984). Most of this degradation was due to the angiotensin-converting enzyme. An astonishing finding was that endogenous head activator was stable and unavailable for degradation by angiotensin-converting enzyme. As M. Roberge in our laboratory could show this was due to the fact that HA, in the blood, like in hydra, is bound to a high molecular weight carrier from which it can be released by salt treatment or methanol extraction indicating that HA is not covalently bound, but only attached to the carrier. Angiotensin-converting enzyme is a dipeptidyl carboxypeptidase which cuts off dipeptides from the carboxy end. The fact that carrier-bound head activator is not degraded by angiotensin-converting enzyme shows that the carboxy end of the head activator, which is very hydrophobic, is hidden in or protected by the carrier. Likewise antibodies directed against the carboxy terminus of HA also do not recognize the carrier-bound HA suggesting that the amino end is also hidden by, or buried in, the carrier.

In human blood, the HA level increased 30- to 200-fold within 15–30 min after a meal (Bodenmüller and Roberge, 1985). Since the gastrointestinal tract is the
highest source of HA besides the brain, we assume that HA is released from there.

As a possible action, we found that HA, like many other neuropeptides, stimulates exocrine pancreatic secretion as measured by amylase release from isolated pancreatic lobules. This may suggest a role for HA in digestion control processes (Bodnemüller and Roberge, 1985).


References


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