The mechanism of receptor development as implied by hormonal imprinting studies on unicellular organisms

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In higher organisms, the development of hormone receptors is genetically encoded and forms an integral part of cell membrane differentiation[6]. Full maturation of the receptor nevertheless requires the presence of the hormone for amplification (hormonal imprinting)[4,7]. Hormonal imprinting takes place at the primary interaction between target cell and hormone, and amplifies the cellular interacting structure (receptor) for specific binding of the hormone. It has been shown experimentally that hormonal imprinting can also occur in unicellular organisms[4,6] if the hormone is present in their environment[3]. Thus the phenomenon of hormonal imprinting can be reproduced experimentally in unicellular model systems[7]. The unicellular organism *Tetrahymena* has proved to be an ideal model organism for hormone receptor studies from several points of view. First and foremost, the *Tetrahymena* cells do not require separation for culturing, which ensures the integrity of their membrane, and they can be easily maintained and propagated in culture. A still greater advantage is that preformed specific receptors are relatively numerous in the *Tetrahymena,* and the inducibility and formation of binding sites (receptors) for hormones can therefore be relatively easily studied by adequate methods. Evidence has also been obtained that the membrane receptors of the *Tetrahymena* are in many respects similar to those of higher organisms, e.g. of mammals. A cautious extrapolation of observation on the receptors of the *Tetrahymena* to those of higher organisms is therefore possible.

The *Tetrahymena* is a free-living ciliated unicellular organism for which adaptation to environmental changes is vitally important. Adaptation presupposes the presence in the cell membrane of receiver structures capable of recognizing environmental changes, and of mediating these to an effector system which brings about an appropriate cellular response(s)[6]. Such receiver structures may play a role in the uptake of nutrients, recognition of poisonous materials (molecules), and of individuals of the opposite sex in the case of sexually reproducing species, etc. Since the environmental changes are very varied, and in some cases also abrupt, the cellular responses are necessarily similarly prompt and manifold[5]. The environmental changes presumably alter the membrane itself, in a manner that enables it to respond to the next similar change more intensively and promptly than it originally responded. We believe that such events are involved in hormone receptor formation, too. The environmental changes act not only on the cell membrane, but also on the entire system of cellular functions, activating thereby an effector mechanism which controls, at transcription level, the intracellular response to the stimulus received. For example, the presence of metals in the environment of *Tetrahymena* causes it to synthesize metal-binding proteins[44].

The above implications have been substantiated by experimental observations[2]. We have been able to induce in the membrane of the *Tetrahymena* specific-appearing receptors to certain materials (hormones), which neither are, nor have ever been present in its natural environment. The experimental induction of structures acting as receptors permits an insight into the mechanism of receptor formation.

Although several hormones and hormone-like materials have been demonstrated in the body of *Tetrahymena*[41,42], it seems unlikely that this protozoon possesses preformed receptors for vertebrate hormones. According to present knowledge, few receptor structures can be regarded as preformed at the unicellular level. A preformed structure is, for example, the nutrient receptor[40], which accounts primarily for peptide binding; the polypeptide-type hormone receptors presumably developed from the primitive nutrient receptor structures. However, certain experimental observations suggest that receptors for hormones other than those of a polypeptide type can also occur in the membrane of *Tetrahymena*[24,25,28,31].

The substantiation of hormonal imprinting, and detection of the specific hormone binding sites, requires extremely sensitive procedures, and the study of adequate parameters which can show conclusively an increase (or a decrease) in the number of receptors. Such procedures are on the one hand methods for the detection of hormone binding, and on the other, those assessing certain physiological responses which portray the quantitative relations of hormone reception (phagocytic activity, growth rate, metabolic rate, ect.). Much valuable information has also been derived from investigations into changes in the quantitative relations of hormone receptor components, e.g. lectin binding studies can precisely reveal quantitative alterations of the saccharide components of membrane receptors[10].

The supposition that the hormone(s) added to the maintenance medium of the *Tetrahymena* cells act by mediation of the cell membrane leads to the presumption that the hormone-induced changes depend on the following factors: 1) physiological state, composition, and fluidity of the membrane; 2) integrity of the membrane-associated adenyl-cyclase-cAMP-cPDE and guanylate cyclase systems; quantitiy and undisturbed operation of other second messengers, such as Ca^{2+}, inositol trisphosphate, etc.; 4) appropriate functioning of the calmodulin system; 5) whether the hormonal imprinting persists over many subsequent generations in which case the collaboration of certain nuclear regions is highly possible. Thus the hormone-induced changes presumably also depend on nuclear functions and on the undisturbed course of protein synthesis, which are indispensable for receptor formation.

The constancy of the culture conditions is an essential

prerequisite of investigations into the factors influencing hormone action, because changes in the environmental conditions (ambient temperature, photoperiod, nutrient supply, osmotic conditions) can greatly influence the composition of the cell membrane, and thereby the inducibilitiy of imprinting as well[30,35].

The role of the cell membrane in hormonal imprinting

Imprinting by polypeptide hormones acting on membrane receptors can take place only in physiological conditions of membrane composition and fluidity. Changes in membrane fluidity and culturing temperature (which alter the relative proportion of unsaturated fatty acids)[53], or in the membrane steroids (e.g. replacement of membrane tetrahymenol by ergosterol, which accounts for rigidity of the membrane) interfere with the normal course of imprinting[37]. Membrane fluidity and, consequently, the mechanism of imprinting, is also affected by local anesthetics and phenothiazine derivatives[45]. Those membrane components which are integral parts of the receiver structures, for example certain saccharides, play a major role in hormonal imprinting. The membrane of the *Tetrahymena* binds concanavalin-A (Con-A), and since the insulin receptor contains saccharide components which are also able to Con-A, pretreatment with Con-A inhibits insulin binding to fixed cells. However, if Con-A and insulin treatment are applied either simultaneously, or on an overlapping scheme, the progeny generations show a significantly greater binding affinity for both ligands than on treatment with either ligand in itself[33]. Similar observations were made in hepatoma cell cultures, in which exposure to Con-A increased not only the binding of insulin to the membrane, but also the internalization of insulin[51]. Con-A presumably enhances the formation of additional binding sites for peptides (insulin), and accounts thereby for a greater intensity of imprinting. It appears that Con-A and insulin act on cPDE-activity, which is one active factor of the Ca^{2+}-calmodulin system, not only in vertebrate target cells, but also in the unicellular *Tetrahymena*[52].

Hormone-membrane interaction seems to be an indispensable prerequisite of imprinting for those hormones which act on membrane receptors. For example, imprinting failed to take place if insulin was administered into the cytoplasm of the *Tetrahymena* in a liposome-coated form, which excluded a membrane-hormone interaction[47].

Agents causing cell membrane perturbation, for example endotoxins, also prevent hormonal imprinting. However, the same agents do not appreciably interfere with hormonal imprinting if they are applied 4 h after hormone exposure. It appears that the establishment of imprinting requires a certain time, and no membrane perturbation can extinguish it once it has become established[46].

Agents known to inhibit clustering during receptor mediated endocytosis, such as methylamine, etc., do not inhibit imprinting[39].

The above experimental observations indicate that the main prerequisite of imprinting is the physiological integrity of the cell membrane, because changes in its normal physical-chemical state inhibit the development of imprinting.

The role of second messengers in hormonal imprinting

The second messengers, such as Ca^{2+}, cAMP, cGMP, etc., play a key role in mediation of the hormonal signal. Since hormonal imprinting presumably involves mediation to the nucleus of the information carried by the signal (hormone) molecule, dysfunction of the intracellular effector system hampers the normal course of hormonal imprinting.

Binding of the hormone to the membrane receptor alters the Ca^{2+}-binding capacity of the membrane[49]. It is known that insulin depresses, whereas glucagon enhances the binding of Ca^{2+}. Inhibition of Ca-binding with La^{3+} does not interfere with the insulin-imprinting of *Tetrahymena* cells (insulin depresses itself the binding of Ca) but prevents the binding of TSH[34]. Conversely, EDTA and EGTA, which form chelates with Ca^{2+} and Mg^{2+}, and alter the influence of the ciliary-membrane-associated guanylate-cyclase-calmodulin complex on the intracellular Ca-level of the *Tetrahymena*[50], inhibit imprinting by insulin, but enhance rather than depress imprinting by TSH, to judge from a significant increase in TSH-binding after imprinting in the presence of EDTA or EGTA[34]. EDTA also accounted for a roughly 100% increased in the hepatocellular response to glucagon[1].

TMB-8, which prevents the establishment of a normal intracellular Ca^{2+}-level, and the Ca^{2+}-antagonizing Ni^{2+} ions as well, have been recognized as further inhibitors of hormonal imprinting[45].

Endogenous cAMP acts as second messenger also in the *Tetrahymena*. Exogenous cAMP modifies the mechanism of imprinting. Treatment of the *Tetrahymena* with dibutyryl-cAMP, or with the cPDE-inhibitor theophylline, equally inhibits imprinting by insulin and TSH[12].

Lithium ions inhibit specifically the action of TSH on the thyroid cell membrane[1], and also inhibit imprinting of the *Tetrahymena* by TSH[12].

The experimental observations outlined above unequivocally suggest that imprinting is inhibited by any gross interference with the function of the intracellular system responsible for mediation of the signals received by the cell membrane. The normal course of imprinting is severely disturbed by any dysfunction of the second messengers (Ca^{2+}; cAMP).

Impact of the inhibition of endocytosis, transcription and translation on hormonal imprinting

The influence of transcription and translation inhibitors on hormonal imprinting has been little studied. Actinomycin was found to inhibit imprinting by insulin[39], but did not inhibit imprinting by diiodotyrosine (T_2). Cycloheximide also inhibited imprinting by insulin, but puromycin had no influence on it[39].

Of the endocytosis inhibitors, colchicine and cytochalasin generally inhibit hormonal imprinting[39], except that colchicine does not interfere with imprinting by T_2[17].

The mechanism of hormonal imprinting seems to be highly sensitive to a great variety of external factors and internal changes. Hormonal imprinting of the target cell takes place only in a state of physiological integrity, which makes possible the mediation to the effector system of the signal which has arisen from receptor-hor-

mone interaction, and subsequent recycling of it to the membrane as well. Dysfunction of any element of the effector system can account for the failure of hormonal imprinting.

Impact of the hormone concentration and time factor on hormonal imprinting

The applied hormone concentration and the duration of hormone exposure play a decisive role in hormonal imprinting.

A single treatment with T_2 increases the growth rate of the *Tetrahymena,* and this effect is concentration-dependent. A practically linear relationship has been demonstrated between the quantity of hormone and the growth rate of the cells in the concentration range 10^{-9}–10^{-15} M[18]. A single exposure to 10^{-18} M T_2 had in itself no influence on the growth rate, but a measurable increase occurred on reexposure at the same concentration, from which it follows that although the first exposure had not altered the growth rate, it did give rise to hormonal imprinting[22]. The growth rate showed a linear increase with the length of exposure from 10 min to 24 h, in cultures examined immediately after treatment, and a similar, although less distinct linearity was still demonstrable one week later. Reexposure to the hormone after one week did not significantly increase the growth rate of the cells preexposed for a short time (from 10 min to 1 h), but increased it significantly, if preexposure lasted 4 h or longer. Similar observations were made in cultures reexposed after two weeks[18].

It appears that although low hormone concentrations have no direct stimulatory effect on cell growth, they do induce imprinting, whereas high concentrations (10^{-9} M) have a direct stimulatory effect at short (1-h) exposure, but fail to induce imprinting. Thus the concentration of the hormone seems to play, at least within certain limits, a less important role in imprinting than the length of hormone exposure. If Koch's[29] dynamic receptor pattern generation theory (the continuous formation of membrane patterns from subpatterns, inquiring the milieu) is true, there is reason to postulate that the membrane-associated information carrier molecules require a certain time for assembling to a (receptor) pattern complementary to the signal molecule; hence, short-term exposures obviously cannot give rise to a durable imprinting.

In the case of insulin the optimal length of imprinting time (primary exposure) proved to be 24 h, when assessed 1 day after treatment, and 4 h, when assessed 28 days after it, although the cells preexposed for 4 h had bound less hormone than the control cells at 1 day after treatment. A 10-min primary exposure proved to be ineffective at both sampling intervals[36].

Higher than effective hormone concentrations (e.g. 10^{-4} M T_2), do not provoke hormonal imprinting[23].

Which materials can induce imprinting?

If the existence of imprinting is regarded as an established fact, the question may be justly posed, which types of molecules are capable of inducing the formation of binding sites in the membrane of the *Tetrahymena*?

From the point of view of hormone phylogenesis it should be taken into consideration that in many cases the *Tetrahymena* is more responsive to the phylogenetically lower hormone precursor(s) than to the hormone proper which represents the most active (signal) molecule in higher (e.g. mammalian) organisms. The classical example in this context is the thyroxine series (tyrosine, T_1, T_2, T_3 and T_4), in which T_2 (diiodotyrosine) represents the most active signal molecule for the *Tetrahymena*[15]. In contrast, in the tryptophane series (tryptophane, tryptamine, 5-HTP and 5 HT) the most 'developed' molecule, 5-HT (serotonin) has the strongest action at the unicellular level. In other words, while the omnipresent hormone serotonin, which also occurs in the *Tetrahymena,* is universally most active in its proper form, the non-omnipresent vertebrate hormone thyroxine acts on the unicellular organism most strongly in its precursor (T_2) form[3,15]. This phenomenon supports the implication that at lower levels of phylogenesis, the cell receptors also are phylogenetically lower structures than in higher organisms, although they appear to be less variable in their evolutionary course than the hormones[43].

The binding sites of insulin and Con-A overlap not only in mammalian cells, but also in the *Tetrahymena,* for they have common saccharide components at both phylogenetic levels. Several cellular responses elicited by insulin can also be provoked with Con-A. This prompted investigations into the imprinting capacity of Con-A for itself and for insulin. However, while insulin-imprinting accounted for a quantitative increase in membrane binding sites for Con-A (owing to common sugar components), Con-A-treatment failed to imprint the *Tetrahymena* for insulin, and imprinted it only temporarily for itself as well[36].

Non-hormone polypeptides, such as bovine serum albumin, protamine, etc. also failed to induce imprinting of similar duration to that induced by polypeptide hormones, such as insulin, glucagon, TSH and ACTH[16].

These observations strongly suggest that durable imprinting can only be induced by those signal molecules whose steric configuration enables them to act as a hormone; presumably molecules of this type have been transformed into hormones in the course of evolution[6].

Study of induced receptors in the Tetrahymena

It is important to clarify whether the receiver structures induced in the *Tetrahymena* by hormonal imprinting can be regarded as true receptors, and if they can, the following aspects remain to be investigated: 1) mode of receptor fixing and length of receptor persistence (demonstrability); 2) structural similarity to hormone receptors of higher organisms; 3) degree of receptor specificity, and hormone overlap phenomena on the receptor structure, of the kind observed in mammalian cells.

Investigations along these lines may throw light on similarities and dissimilarities between the hormone receptors of the *Tetrahymena* and those of higher organisms.

Receptor 'memory' in unicellular model systems

Diiodotyrosine (T_2) increases considerably the growth rate of the *Tetrahymena,* and the increase, although it tends to become less as time progresses, persists as long as 12 weeks, during which about 500 generation changes occur. Reexposure to T_2 elicits a still greater growth response, from which existence of a receptor 'memory'[20] and gene-level transmission of the information received can be deduced. Remarkably, repeated reexposures do not further increase the effect of the primary exposure (imprinting), but do not extinguish the 'memory' thereof. Receptor 'memory' also operates in those cases in which the primary exposure (imprinting) does not in itself increase the growth rate over the control (e.g. in the case of epinephrine treatment), but the repeated treatment gives rise to a significant elevation of growth rate[19]. Induction of receptor 'memory' is not the privilege only of those hormones which are present in the body of the *Tetrahymena* (e.g. epinephrine), for 'foreign' hormones, such as the plant hormone gibberellin, can also evoke it[19].

Imprinting of the *Tetrahymena* with histamine gave rise to a measurable increase in the number of histamine receptors in as many as 50 subsequent generations, but reexposure to histamine 6 days after imprinting was followed by a significant decrease in histamine binding. In this particular case, receptor 'memory' operated for 'down-regulation'[11].

Certain similarities have been detected between receptor 'memory' and neuronal 'memory'. Four 1-h exposures to T_2 increased the growth rate of the *Tetrahymena* to a significantly greater degree than did a single uninterrupted exposure for 4 h[22]. This is still more remarkable, if it is taken into consideration that a single 1-h exposure could not induce hormonal imprinting in itself[18], but repeated short treatments evoked a long-lasting memory. So-called retroactive interference was observed in those cases in which treatment with other hormones (serotonin, gramine, epinephrine, dopamine, or combinations of these) followed immediately after a 4-h primary exposure to T_2. In these conditions reexposure to a 'foreign' hormone depressed imprinting by T_2 or, more precisely, development of a receptor 'memory', to a considerable degree[22]. The only exception was serotonin, which depressed the growth stimulant action of T_2 only to a negligible degree, as assessed one week later. However, the 'foreign' hormones do not extinguish receptor 'memory' after it has become established, i.e. at a later time after preexposure. Membrane perturbations induced after the establishment of receptor 'memory' did not abolish imprinting either[46].

The theory of gene-level fixing of receptor 'memory', i.e. of hormonal imprinting (supported by the existence of 'memory' after 500 generations), is in good agreement with Robertson's[48] hypothesis that memory is a gene-level rather than membrane-level phenomenon.

In *Tetrahymena* cells once imprinted, 'memory' survives maintenance in anaerobic conditions, without medium exchange, for as long as a year. In these conditions the cells fail to divide, but their life span is extremely prolonged[8]. Investigations along this line may throw light on the true cause of decline of the memory with progressing time, i.e. whether mutation ('rejuvenation') of the cells in the course of serial divisions, or an inadequate coding of the new information, is responsible for the gradual loss of memory. At all events, the results of these experiments are inexplicable unless a gene-level fixing of the information is postulated, for the effect of imprinting by T_2 was still demonstrable after 9 to 12 months of growth arrest.

Important information has emerged from those experiments, in which the *Tetrahymena* cells were treated immediately after imprinting with materials structurally similar to, but functionally different from, the hormone used or, conversely, with others which were similar to the hormone in function, but differed from it in structure. Structural similarities had a more adverse influence on imprinting than functional ones[21], from which it follows that the conformation of the interacting molecule plays the leading role in hormonal imprinting.

Structural studies on membrane receptors of Tetrahymena

All receptor detection studies described in the foregoing sections were based on indirect approaches (assessment of growth rate, etc.), which do not supply adequate information on receptor structure. For structural studies, immunological tests are the method of choice.

In experimental conditions, rat hepatocellular insulin receptors provoke the formation of specific antibodies, which bind to insulin-imprinted *Tetrahymena* cells to a significantly greater degree than to intact control cells, and induce the formation of additional insulin-binding sites in the membrane of the *Tetrahymena.* It appears that the induced insulin binding sites of the *Tetrahymena* are immunologically similar to the insulin receptors of the rat hepatocellular membrane[13].

Rabbits immunized with live *Tetrahymena* cells develop antibodies presumably to the surface components of the cells, which also include insulin receptors of the *Tetrahymena* cells used for immunization had previously been exposed to insulin. Treatment of rat hepatocytes with antibodies to *Tetrahymena* was followed by a decrease in their insulin binding capacity. Antisera prepared with insulin-treated *Tetrahymena* cells accounted for a significantly greater decrease than those prepared with intact cells. FITC-labeled antiserum to insulin-treated *Tetrahymena* increased hepatocellular insulin binding capacity to a significantly greater degreee in insulin-treated than in untreated control cells[35]. These phenomena, too, suggest a structural similarity between the induced insulin binding sites of the *Tetrahymena* and the insulin receptors of rat hepatocytes. It also follows from the above experimental observations that the insulin receptors of the *Tetrahymena* are not preformed structures, but arise under the influence of the hormone, since hormonal presence is an indispensable prerequisite of receptor formation.

Specificity of the hormone receptors of Tetrahymena

Experimental studies have unequivocally shown that the receptors of *Tetrahymena* are highly specific. The H_1- and H_2-type histamine receptors of the unicellular organism have dissimilar saccharide components[38]. The chemically histamine-like histamine antagonists bind to the same

sites as histamine itself, while the structurally dissimilar ones bind either to other sites, or to those regions of the histamine receptor which do not interfere with histamine binding but prevent histamine action. Obviously, a marked specialization of binding sites exists also at the lowest levels of phylogenesis.

In mammalian cells, structurally related hormones (e.g. TSH and FSH) overlap on each other's binding sites, so that imprinting with TSH amplifies the receptor not only for itself, but also for FSH, and vice versa[26]. Such overlaps are also expressed in physiological responses[9]. TSH-FSH and FSH-TSH overlaps have also been demonstrated in *Tetrahymena* cells[32], although in these only TSH amplified for both itself and FSH, whereas FSH amplified to a lesser degree for TSH than for itself. Similar phenomena were observed in mammalian cell cultures, too[26]. The differences between the imprinting actions of the two hormones indicate that hormone overlaps are not greater at the unicellular than at the mammalian level, and the *Tetrahymena* cell, too, is capable of differentiating structurally related hormones from one another.

Experiments with serotonin and other structurally related hormones (5-HTP, 5-methoxytroptophane, tryptamine, gramine, indoleacetic acid) have also presented evidence in support of receptor specificity. There are indications that the receptor recognizes the basic structure of the signal molecules. Presence or absence of a hydroxyl group had little influence on hormone-receptor interaction, but the presence of two methyl groups practically excluded any cellular response[14]. Presence of a methoxy- or hydroxyl group in position 5 of serotonin and related molecules increases binding affinity for the receptor, as has been observed earlier in mammalian cells[27].

Summarizing, we may state that *Tetrahymena* is an ideal model for investigations into the mechanism of hormonal imprinting. Experiments on that model can provide important information on the course of imprinting, and the properties, structure, 'memory' and specificity of the induced receptors can be relatively easily studied by an appropriate experimental approach.

It appears that, although there are certain essential differences between the response of unicellular *(Tetrahymena)* and higher (e.g. mammalian) organisms to hormones, hormonal imprinting is in many respects similar at the low and high levels of phylogenesis. Similarities between *Tetrahymena* and mammalian cells include the immunologically-studied nature of the hormone receptors; the overlaps of lectin and hormone binding sites; and the critical stages of the imprinting mechanism. In view of this, the information emerging from receptor studies on the *Tetrahymena* can be – with great caution – extrapolated to the cells of higher organisms. Experimental observations on receptor formation in the *Tetrahymena* are in good agreement with current conceptions on the phylogeny and ontogeny of receptors[2-4, 6, 7], and unequivocally support the hypothesis that phylogenetically, receptor development had its origin in the imprinting mechanism, which is reproduced during ontogenesis.

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The phylogeny of the endocrine system

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Key words. Endocrine phylogeny; thyroid; steroids; peptide hormones; endocrine diversification; multiple sites; invertebrate hormones.

The scope of phylogeny

Phylogeny, which is a representation of the evolutionary history of taxa, requires critical interpretation of biological diversity and of the relationship of this with the unity of organisation which underlies living systems. Some degree of personal judgement may be involved in particular cases, and so, because of this subjective element, phylogenetic propositions are always open to discussion in the light of new information. On the larger issues, however, and on many smaller ones as well, there is substantial agreement, founded on the rich classical resources of descriptive anatomy, which take account of large numbers of species, both living and extinct, and with embryo-