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Presence of benzodiazepine binding sites (receptors) and amplification thereof by imprinting in *Tetrahymena*

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Summary. Live *Tetrahymena* cells bound ^{3}H-diazepam specifically, as demonstrated by autoradiographic evidence of displacement of about 25% of labeled diazepam in the presence of a 1000-fold amount of cold diazepam. The ^{3}H-diazepam bound to membrane preparations isolated from untreated (control) cells was not displaced by cold diazepam, whereas cells involved in primary interaction (imprinting) with diazepam showed amplification and specificity of diazepam binding in both in vivo (cell suspension) and in vitro (pellicle) systems, as well as displacement of bound label in the presence of 1000-fold cold diazepam. It appears that diazepam induced imprinting and, consequently, also the formation of specific receptors in *Tetrahymena*.
Key words. Hormonal imprinting; receptors; benzodiazepine; *Tetrahymena*.

The unicellular organism *Tetrahymena* does contain certain vertebrate hormones [1–4], and is able to respond to these, in many cases even specifically [5–7]. For example histamine, which stimulates phagocytosis in higher organisms [8, 9], has a similar effect on *Tetrahymena* [10], and the binding sites presented by the membrane of the unicellular organism can differentiate histamine from its antagonists [11]. Phagocytosis stimulant action and selectivity have also been observed with serotonin [10, 11]. Insulin [12] and adrenalin [13] enhance the glucose uptake of the protozoan, and the effect of polypeptide hormones is measurable by stimulation of RNA synthesis [14]. *Tetrahymena* also presents receptors for opioids, detectable by displacement of bound label [15].

Primary interaction with a hormone amplifies the potential binding sites or, if such structures are lacking, induces the formation of these [5–7]. This phenomenon, termed hormonal imprinting, endows the unicellular organism with a 'memory' of the primary interaction, which persists over as many as 500 generations [16]. The increased responsiveness to the hormone shown by imprinted cells can be explained by a greater binding capacity, which has been studied mainly in connection with polypeptide hormones.

In the present study we investigated whether or not *Tetrahymena* also possessed, or could present as a result of imprinting, receptors for a non-hormone (drug) molecule acting at receptor level.

The experiments were performed partly by autoradiography, partly on membrane preparations.

1. *Autoradiography. Tetrahymena pyriformis* GL cells, maintained in 0.1% yeast extract containing 1% Bacto tryptone medium (Difco, Michigan, U.S.A.) under continuous shaking at 28 °C, were used. One-day mass cultures were treated or not treated with 1 mM diazepam solution (in ethanol) for 24 h (the ethanol content of the medium was 0.1%). After treatment the cells were washed twice by centrifugation, and were returned to plain medium for one day or one week (with a single passaging in the latter case). Finally the pretreated and not pretreated mass cultures were incubated in the presence of ^{3}H-diazepam (N-methyl-^{3}H, Amersham, England; sp. act. 3.15 TBq/mmol), added at 0.1 ng/ml, 1 ng/ml or 10 ng/ml concentration for 30 min. At the 1 ng/ml level of treatment, part of the cultures were additionally treated with a 10-, 100- or 1000-fold amount of non-labeled (cold) diazepam.

The cells were incubated for 30 min at 4 °C, fixed in 2% glutaraldehyde (in Losina solution), embedded in araldite, and cut into semi-thin sections. These were coated with Ilford G5 emulsion and exposed for 6 months, then developed in an ORWO R9 developing solution. Quantitative autoradiography was based on grain counting exclusively above those cells whose nucleus was visible in the longitudinal section. Inter-group differences were analyzed for significance with Student's two-sample t-test.

2. *Examination of cytoplasmic membrane preparations. Tetrahymena* cells treated with 10^{-6}M diazepam for 48 h were washed in three changes of sterile Losina solution, and were returned to fresh plain medium for culturing for a further period of 48 h at 28 °C, under mild shaking. A control series of untreated cells was set up in parallel in diazepam-free medium. The cells were washed and transferred to fresh medium after 48 h, exactly like the treated series. The cell density of the 48-h cultures was 10^5/ml.

For binding studies the cells were separated from the nutrient medium by centrifugation at 400 × g for 10 min and pellicle preparations were made as described by Nozawa and Thompson [17]. The homogenate was centrifuged at 100 × g for 10 min at 4 °C, the supernatant and the layer above the whole-cell pellet was withdrawn, the sedimented cells were washed in two changes of 10 mM TRIS-HCl buffer (at 10,000 × g for 10 min at 4 °C) and were resuspended in the same buffer solution. The protein content of the suspension, determined according to Lowry et al. [18], was 1.84 mg/ml.

For determination of ^{3}H-diazepam binding we incubated 160 µl pellicle preparation in the presence of 20 µl 20 nM ^{3}H-diazepam and 20 µl 20 µM non-labeled diazepam or 20 µl buffer for 2 h at 4 °C. After incubation the suspension

was centrifuged at 10,000 × g for 5 min at 4 °C, the supernatant was discarded, and the pellet was washed in two changes of ice-cold buffer, resuspended in 1 ml buffer, pipetted into 10 ml Aquasol containing scintillation cuvettes, and assayed for radioactivity in a Beckmann LS 900 scintillation counter.

The natural environment of *Tetrahymena* presents a great variety of challenges for the protozoan, for it contains many materials which are either useful or deleterious, and the recognition and 'memory' of these properties is of vital importance for the survival of *Tetrahymena* and its progeny. Recognition presupposes an extraordinarily dynamic membrane, whose protein configurations are continuously reassembling and disassembling and are, if required, capable of behaving as receptors. It appears that these non-specific, dynamically changing structures present the binding sites for environmental molecules, although they always appear by chance coincidence.

However, after primary interaction with an active molecule, which is able to bind to the binding site produced by chance, the latter becomes amplified and reappears regularly in the membrane. This can explain the experimental observation that primary interaction with a hormone (hormonal imprinting) results in a greater binding capacity for the hormone, which persists over many subsequent generations. At the same time, cellular response to the hormone is also increased.

In the light of the foregoing considerations there is reason to postulate that the dynamic membrane of *Tetrahymena* may also present binding structures for diazepam. In toto autoradiographic studies and examinations on plasma membrane preparations equally support this hypothetical conclusion.

Autoradiography demonstrated a parallelism of binding increase with the concentration increase (table 1; fig.). In the presence of 1 ng/ml labeled diazepam even 100 ng/ml cold diazepam displaced a considerable amount of bound label from the receptors (table 2), and 1000 ng/ml cold diazepam accounted for a significant (about 25%) decrease in the amount of bound label. The fact that the greater part of bound label persisted on the receptors, also in the presence of a one-thousandfold amount of cold diazepam, suggested that the proportion of non-specific binding was high. Since, however, live cells were exposed to ^{3}H-diazepam, part of it may have been incorporated and may have been, therefore, no longer available for displacement. Taking this circumstance into consideration, we may state that the approximately 25% proportion of displacement indicated the specificity of diazepam receptors.

Table 1. Saturation values of diazepam [grain count ±SE (n)/cells in longitudinal section]

Diazepam (ng/ml)	Control	Pretreatment + 1 day	Pretreatment + 1 week
0.1	11.0 ± 1.0 (25)	10.4 ± 0.7 (25)	12.2 ± 0.9 (25)
1	24.7 ± 1.7 (25)	25.5 ± 1.0 (25)	27.1 ± 1.2 (25)
10	94.8 ± 3.5 (20)	143.7 ± 4.8 (20)*	134.6 ± 5.2 (20)*

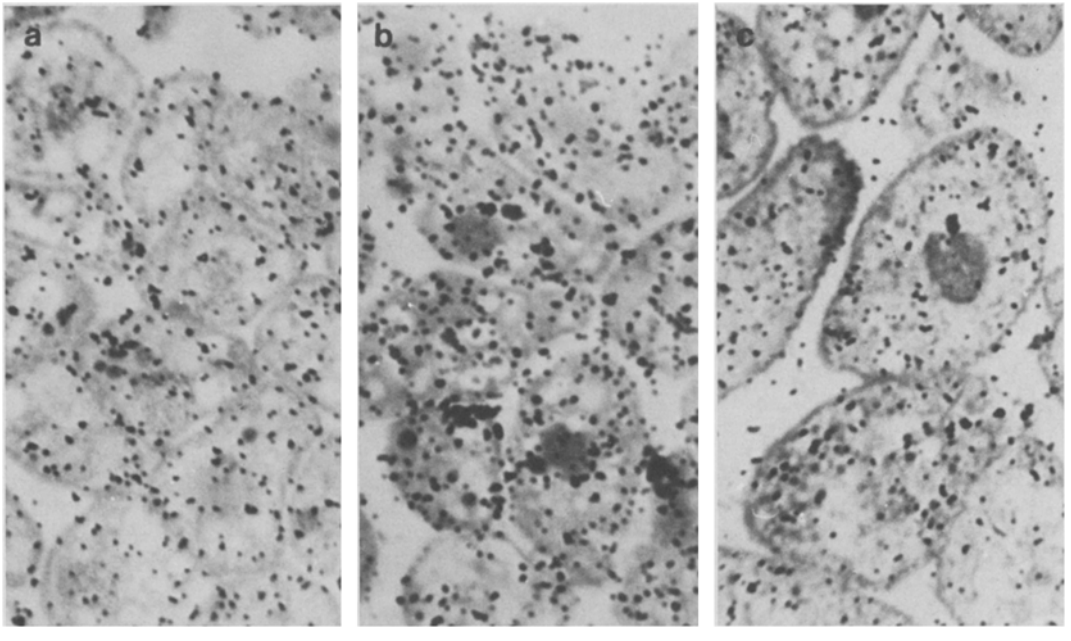
* Significance related to the control $p < 0.01$.

Table 2. Displacement of ^{3}H-diazepam (1 ng/ml) by different concentrations of cold drug

Cold (ng/ml)	Control	Pretreatment + 1 day	Pretreatment + 1 week
0	24.7 ± 1.7 (25)	25.5 ± 1.0 (25)	27.1 ± 1.2 (25)
10	–	23.6 ± 1.3 (19)	24.7 ± 2.1 (25)
100	21.6 ± 0.9 (25)	20.7 ± 1.4 (25)	23.0 ± 1.3 (25)
1000	16.9 ± 0.9 (31)*	17.2 ± 1.2 (25)*	17.5 ± 1.2 (25)*

* Significance, related to the control $p < 0.01$.

The behavior of the cells pretreated (imprinted) with 0.1 or 1.0 ng/ml diazepam did not differ from that of the control cells either after one day, or after a week (table 1), whereas those pretreated with 10 ng/ml showed a considerable increase in diazepam binding capacity both after one day and one week. It follows that at a low level of labeled diazepam the spontaneously present binding sites were sufficient for a maximal uptake, which was not further increased by the enhanced binding capacity of the receptors. However, at a higher level of diazepam, the uptake increased considerably over the control, indicating that diazepam imprinting had in



Grains over control (a) and diazepam pretreated (b: one day, c: one week, before) *Tetrahymena* (concentration of ^{3}H-diazepam: 10 ng/ml). Magnification × 750.

Table 3. ^{3}H diazepam binding of imprinted and not imprinted *Tetrahymena* pellicle related to the control as 100

Binding %		Binding %	
Control	100	100	Imprinted
Control + 1000 × cold diazepam	100	59.0*	Imprinted + 1000 × cold diazepam

* = $p < 0.05$

fact taken place in *Tetrahymena*. It deserves special mention that the difference between the cells imprinted and not imprinted with diazepam was still demonstrable after one week, in which about 50 generation changes occurred. Thus imprinting maintained a 'memory' of primary interaction with diazepam over many progeny generations, to judge from the greater binding and uptake of the drug by these relative to the control.

The plasma membrane preparations represented an entirely different experimental system, in which incorporation of ^{3}H-diazepam into the cells was a priori impossible. Displacement of the ^{3}H-diazepam molecules bound to the membrane of the not-pretreated cells failed to take place in that system, whereas more than 40% ($p < 0.05$) of bound ^{3}H-diazepam was displaced on the imprinted cells in presence of an 1000-fold amount of unlabeled (cold) diazepam (table 3). Although these observations fail to substantiate the specificity of the spontaneous binding sites, such as are presented by the control cells, they substantiate unequivocally the establishment of diazepam imprinting in *Tetrahymena*, and the specificity of the receptors induced by imprinting.

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Inhibition of collagen synthesis by interleukin-1 in three-dimensional collagen lattice cultures of fibroblasts

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Summary. Interleukin-1 (Il-1) was added to collagen lattice cultures of human skin fibroblasts. No cell division was induced, the ability of fibroblasts to contract the lattices was decreased and a dose-related inhibition of collagen synthesis without effect on non-collagen proteins was found. Indomethacin had no influence on these effects.
Key words. Collagen lattice culture; collagen synthesis; interleukin-1; indomethacin; fibroblast culture.

Cultures of fibroblasts in collagen lattices, which have been described by several authors [1–3], constitute a valuable tool for the study of the behavior of cells embedded in an extracellular matrix, in a physiological situation comparable to the one which they occupy in vivo. In this complex medium, it has been demonstrated that fibroblasts remain more or less quiescent. They do not divide, and their rate of collagen synthesis is reduced to a negligible level compared to their activity in monolayer cultures [4]. A very noticeable property of these lattice cultures is the contraction which operates during the first days of culture and results in the structuring of a resistant, organized, coherent lattice [5, 6]. The mechanism of this lattice contraction is not well understood at the present time. On the other hand, many efforts have been devoted to establish the reasons why cells are in apparent dormancy in these collagen lattices. For instance, several growth factors have been tried, more or less successfully, in order to restart cell division and protein synthesis in lattice cultures [7, 8]. Up to now, interleukin-1 (Il-1), a cytokine capable of modulating a number of biological activities of fibroblasts, has not been used in that system. Il-1 is shown to be a multifunctional factor, acting as a lymphocyte activator, an inducer of acute phase protein production and an endogenous pyrogen [9]. In connective tissue, Il-1 induces the secretion of proteolytic enzymes including collagenase [10] plasminogen activator [11] and stromelysin [12], a process that promotes matrix breakdown. Il-1 also stimulates the production of prostaglandin E_2 by mesenchymal cells [10]. On the other hand, Il-1 is a well-known mitogenic factor for fibroblastic cells [13]. Furthermore, Il-1 has been reported to increase the steady-state level of collagen mRNAs in fibroblasts [14, 15], synovial cells [14, 16] and chondrocytes [14]. However, contradictory results have been obtained in studies on the effect of Il-1 on collagen protein production [14–21]. This discrepancy can be explained by variations in the experimental procedures and the cell lines used [22]. In particular, it has been suggested that the Il-1 induced prostaglandin PGE_2 release could be responsible for an inhibition of collagen synthesis,