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Research Article

Verification of epigenetic inheritance in a unicellular model system: multigenerational effects of hormonal imprinting

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Abstract

The unicellular *Tetrahymena* has receptors for hormones of higher vertebrates, produces these hormones, and their signal pathways are similar. The first encounter with a hormone in higher dose provokes the phenomenon of hormonal imprinting, by which the reaction of the cell is quantitatively modified. This modification is transmitted to the progeny generations. The duration of the single imprinter effect of two representative signal molecules, insulin and 5-HT (5-hydroxytryptamine), in two concentrations (10^{-6} and 10^{-15} M) were studied. The effects of imprinting were followed in 5 physiological indices: (i) insulin binding, (ii) 5-HT synthesis, (iii) swimming behaviour, (iv) cell growth and (v) chemotaxis in progeny generations 500 and 1000. The result of each index was different from the non-imprinted control functions, growth rate, swimming behaviour and chemotactic activity to insulin being enhanced, while others, e.g. synthesis and chemotactic responsiveness of 5-HT and the binding of insulin were reduced. This means that a function-specific heritable epigenetic change during imprinting occurs, and generally a single encounter with a femtomolar hormone concentration is enough for provoking durable and heritable imprinting in *Tetrahymena*. The experiments demonstrate the possibility of epigenetic effects at a unicellular level and call attention to the possibility that the character of unicellular organisms has changed through to the present day due to an enormous amount of non-physiological imprinter substances in their environment. The results – together with results obtained earlier in mammals – point to the validity of epigenetic imprinting effects throughout the animal world.

Keywords: epigenetic inheritance; hormonal imprinting; 5-hydroxytryptamine; *Tetrahymena*; unicellular model system

1. Introduction

The unicellular ciliate, *Tetrahymena pyriformis* is a frequently used model in cell biological experiments, which was used also in two Nobel-prize winning experimental series: self-splicing character of RNA (Cech, 1990, 2002) and telomers and telomerase (Blackburn, 2005). In the early 1970s, it was demonstrated at first that the *Tetrahymena* can recognize the hormones of higher ranked animals. Related hormones were distinguished on a high level by the binding sites and responses were also more or less specific (Csaba and Lantos, 1973, 1975a). Histamine enhanced phagocytosis while 5-HT (5-hydroxytryptamine) and its relative, the plant hormone, 5-hydroxy-indole-acetic acid were ineffective. Insulin increased glucose metabolism (Csaba and Lantos, 1975b), triiodothyronine enhanced growth and the precursors of these hormones also had increasing effects (Csaba et al., 1982b). Later the structure of the *Tetrahymena* insulin receptor was studied and proved to be similar to that of the mammalian one (Zipser et al., 1988; Christopher and Sundermann, 1995; Leick et al., 2001; Christensen et al., 2003). Signal transduction pathways were also demonstrated to show high homology with mammalian ones (Kuno et al., 1979; Zipser et al., 1988; Kovács and Csaba, 1990; Kőhidai et al., 1992). At the same time, production of more mammalian hormones had been observed and verified in *Tetrahymena* (LeRoith et al., 1980, 1982, 1983). Extremely low hormone concentrations were also able to influence hormone production as well as the binding capacity of the identical receptors (Csaba et al., 2006, 2007).

The first encounter between the *Tetrahymena* and an exogenously given hormone provokes the phenomenon of hormonal imprinting. The cell and its progeny generations remember the first encounter and respond to the repeatedly given hormone with an altered reaction (Csaba, 1980, 1984, 1985). The effect of hormonal imprinting prevails by the help of changes in DNA methylation (Csaba and Kovács, 1990), which shows that it is an epigenetic process. Former experiments have proved that imprinting with diiodotyrosine, the precursor of the thyroid hormones, increases the growth rate/multiplication of the cells (Csaba et al., 1982a), which gradually diminished by the time, but was still observed in generation 500 (Csaba et al., 1982b).

Development of hormonal imprinting can be investigated from different aspects. The most common and classical way is when (i) the imprinter chemical is applied in a second encounter and in the same or lower concentrations as it the first time. However, radical effects of the first treatment are detectable not only at the second encounter, but also as (ii) altered – regularly more sensitive – cell physiological responsiveness of the cells or (iii) as changed – regularly increased – binding characteristics of the surface membrane or cytoplasmic components (Figure 1).

In the present work, our objectives were (i) to study the effect of hormonal imprinting over as many as 1000 offspring generations; (ii) to determine the permanent character of the hormonal imprinting by testing essential physiological indices and (iii) to show the wide diversity of aspects of evaluation of hormonal imprinting in long-lasting dimensions. Two reference signal

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Abbreviation: 5-HT, 5-hydroxytryptamine.

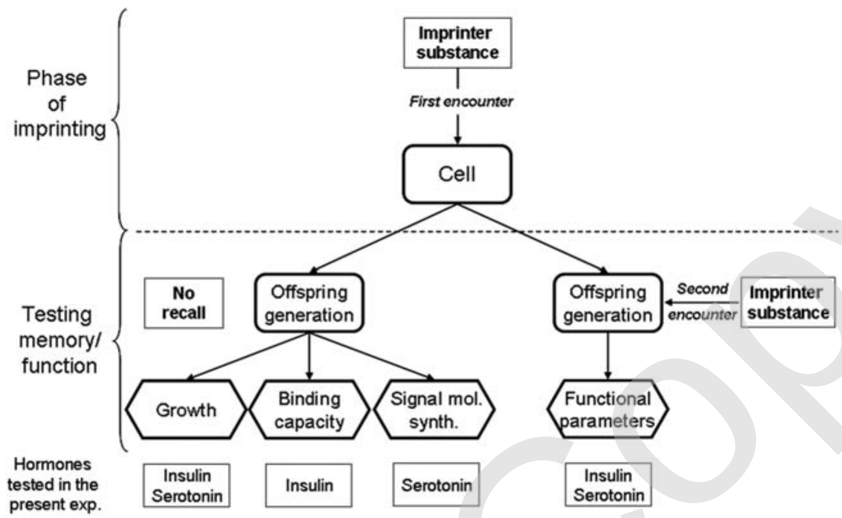

Figure 1 Review scheme of investigations of the long lasting permanent character of hormonal imprinting

molecules were used as imprinters: insulin, which has proven receptors in *Tetrahymena,* and 5-HT, which is present widely in the animal and plant kingdom.

2. Materials and methods

2.1. Cells and treatments

Tetrahymena pyriformis GL strain was used in the logarithmic phase of growth. The cells were cultured at 28°C in tryptone medium (Sigma–Aldrich) containing 0.1% yeast extract (Difco), for 24 h. The density of *Tetrahymena* cultures studied was 10^4 cell/ml. There were control samples given no treatment and samples treated with 10^{-15} M or 10^{-6} M concentrations of 5-HT (Sigma–Aldrich) or insulin (Actrapid, Novo) for 60 min. The control and pretreated cells were maintained in tryptone/yeast medium and transferred twice weekly for 120 days, the only difference between the two groups being the pretreatment of the imprinted cells, and other stimuli were excluded. At day 60 (~500th generation) and day 120 (~1000th generation) test-indices as (i) 5-HT content (in the case of 5-HT imprinting), (ii) FITC-insulin binding (in the case of insulin imprinting), (iii) cell growth (after 60 and 120 days) as well as (iv) swimming and (v) chemotactic behaviours (in the case of both imprinters at 60 and 120 days old cultures and also after pretreatments with 10^{-15} M or 10^{-6} M concentration of hormones) were assessed (Figure 1).

2.2. Flow cytometric analysis of the intracellular 5-HT content and insulin binding

After 60 or 120 days of imprinting, cells were fixed with 4% (w/v) PFA (paraformaldehyde) in pH 7.2 PBS for 5 min. For the analysis of the 5-HT content, cells were washed twice in buffer (0.1% BSA, 20 mM Tris/HCl, 0.9% NaCl and 0.05% Nonidet P40, pH 8.2), but the wash did not contain Nonidet P40 in the case of the insulin-binding assay.

To block non-specific binding of anti-5-HT antibodies, cells were treated with blocking buffer (1% BSA in PBS) for 30 min at room temperature. Aliquots of cell suspensions (50 μl) were transferred to tubes and 50 μl of primary antibody (anti-5-HT purchased from Sigma–Aldrich) or FITC-labelled insulin (FITC-insulin, Sigma–Aldrich) diluted 1:200 in antibody buffer (1% BSA in wash buffer) were added to the cells for 30 min at room temperature. Samples were washed 4 times with buffer (1% BSA solved in PBS for analysis of 5-HT content; PBS for insulin-binding assay) to remove excess primary antibody or FITC-insulin. The cells were imprinted with 5-HT and incubated with FITC-labelled secondary antibody (anti-rabbit IgG; Sigma–Aldrich; dilution 1:50 with antibody buffer – PBS) for 30 min at room temperature.

To control the specificity of immunocytochemical reactions, the autofluorescence of the cells and non-specificity of the secondary antibody were tested: (i) fluorescence of cells treated only with PBS was evaluated (insulin binding); (ii) fluorescence of cells treated only with the secondary antibody (without the specific first antibody) was measured (5-HT content). The measurement was done with a FACS Calibur flow cytometer (Becton Dickinson), using 5000 cells for each measurement. Hormone content in the cell populations was studied in this way. Dead and living cells were measured separately in the analysis. As dead cells lose their membrane integrity, FS/SS (forward scatter and side scatter values; in dot plot) were used to exclude debris and dead cells. CellQuest Pro software was used for the measurement and analysis of data. The numerical comparison of detected values (always one treated group to the control) was done by the comparison of changes in percentage of geometric mean channel values (Geo-mean) relative to the appropriate control groups.

2.3. Cell growth

Following the imprinting, *Tetrahymena* cultures were maintained in glass tubes. Samples of the groups in generations 500 and 1000 after imprinting were taken from the stock cultures (cell density 10^4 cells/ml) and grown in flasks (starter density 10^3 cell/ml). The growth characteristics of cultures were taken by sampling at 6, 18, 24 and 48 h under sterile conditions. To determine cell number, a CASY TT® (Innovatis-Roche) cell counter was used, which also gave the distribution of cell sizes and viability.

2.4. Chemotaxis assay

The chemotactic ability of *Tetrahymena pyriformis* cells was determined by a modified version of Leick's two chamber capillary chemotaxis assay (Leick and Helle, 1983; Kőhidai et al., 1995). Pipette tips of an 8-channel micropipette filled with the test substances were used as the upper chamber. Wells of a microtitration plate filled with cell cultures served as lower chambers. The incubation time was 15 min, which had proved to be optimal by previous experiments, as the concentration gradient is still present in the chamber (Sáfár et al., 2011). The chemotactic responses of the imprinted cell populations and the control (not pretreated) cells were investigated in generation 500 (after 60 days) and generation 1000 (after 120 days). The control (not pretreated) cells were assayed with cell culture medium without hormones and with insulin and 5-HT at 10^{-15} and 10^{-6} M. The imprinted cell populations were tested with control medium and the 10^{-15} or 10^{-6} M of the identical hormone. After incubation, the samples were fixed with 4% formaldehyde dissolved in PBS (pH 7.2). The number of cells was determined by CASY TT® cell counter and analyser (Innovatis-Roche). Pulse area analysis method was applied in the system to count the cells, and to characterize viability and morphometric properties of the samples. The main setting parameters allowed us to measure 400 μl samples (100 μl sample of cell culture diluted in 5 ml of CASY ton) in triplicates by using the 150 μm pore size capillary. All experiments were repeated 5 times. The resulting values were normalized to the control and given as the 'Chemotaxis index' (Chtx. ind.) in percent.

2.5. Swimming behaviour

Generation 1000 (120 days) cells were used after imprinting, without further treatment and after retreatment with 5-HT or insulin. The data were compared with the responsiveness of non-imprinted controls. The following groups were formed (the first symbol indicates the type of imprinting, while the second symbol shows the hormone applied on the 120 days old cultures; C, control; Ins-6, insulin 10^{-6} M; Ins-15, insulin 10^{-15} M; Ser^6, 5-HT 10^{-6} M; Ser^{15}, 5-HT 10^{-15} M): C_C; C_Ins-6; C_Ins-15; C_Ser-6; C_Ser-15; Ins-6_Ins-6; Ins-6_Ins-15; Ins-15_Ins-6; Ins-15_Ins-15, Ser-6_Ser-6; Ser-6_Ser-15; Ser-15_Ser-6; Ser-15_Ser-15.

The swimming behaviour of cells was observed in an AxioObserver invert microscope (Carl Zeiss MicroImaging GmbH) using AxioVision Rel 4.7.1 software. The swimming tracks of cells were registered with the time-lapse module (5 ms time duration, maximal picture speed). The movement analysis was done by the tracker module of the software. Characteristics of tracking are: 25 cells/visual field, four parallel fields and 2×25 frame long analysis time. For characterizing the swimming behaviour, the mean velocity of cells (normalized to the control) and the tortuosity of the swimming tracks were used. The latter is the ratio of the distance of starting and end points of the path and the actual length of the path taken by the cell.

2.6. Statistical analysis

The data generated with the CellQuest Pro, AxioVision Rel 4.7.1 or CASYexcell 2.3 software were exported to Excel, and the additional statistical analysis of data was done by Origin Pro8.0. Data shown in the figures represent means ± S.D. values. The level of significance was obtained by ANOVA and is shown as follows: *$P<0.05$; **$P<0.01$.

3. Results

3.1. Flow cytometry analysis

3.1.1. Insulin binding after insulin imprinting

The insulin binding significantly decreased after 10^{-15} M as well as 10^{-6} M insulin imprinting (10^{-15} M: generation 500 – 81.6%, generation 1000 – 76.4%; 10^{-6} M: generation 500 – 82.2%, generation 1000 – 79.0%), independently of the time passed (Table 1). The values elicited by the two concentrations were very similar.

3.2. 5-HT content after 5-HT imprinting

The 5-HT contents of cells imprinted with either 10^{-15} or 10^{-6} M 5-HT were significantly lower (10^{-15} M – 61.0%; 10^{-6} M – 70.7%) in generation 500 after imprinting (Table 2). However, in generation 1000 in cells imprinted with 10^{-6} M 5-HT, the difference was not detectable (103.2%), while in the group imprinted with 10^{-15} M 5-HT a clear elevation in 5-HT content (119.0%) was registered.

3.3. Cell growth

3.3.1. Insulin imprinting

In generation 500, the 10^{-15} M insulin imprinting had a significant negative effect on the cell density in the short term (6 h) while at

Table 1 Insulin binding of *Tetrahymena* imprinted by insulin in generations 500 and 1000
The geometric mean channel values are expressed in percentage of the non-imprinted control. Data represent the means of 5 parallels, the errors indicating S.D. Level of significance (*P*) is related to the control (n.s.=not significant).

Imprinting	Binding, generation 500 Geo means ± S.D. (%)	Significance to control	Binding, generation 1000 Geo means ± S.D. (%)	Significance to control
Non-imprinted control	100 ± 5.23	n.s.	100 ± 2.46	n.s.
Insulin 10^{-15}	81.55 ± 6.49	$P=0.01$	76.37 ± 1.51	$P<0.01$
Insulin 10^{-6}	82.2 ± 5.16	$P<0.01$	79.02 ± 1.28	$P<0.01$

Table 2 **5-HT content of *Tetrahymena* imprinted by serotonin in generations 500 and 1000**
The geometric mean channel values are expressed in percentage of the non-imprinted control. Data represent the means of five parallels, and the errors indicate S.D. Level of significance (*P*) is related to the control (n.s.=not significant).

Imprinting	Content, generation 500 Geo means ± S.D. (%)	Significance to control	Content, generation 1000 Geo means ± S.D. (%)	Significance to control
Non-imprinted control	100 ± 5.3	n.s.	100 ± 2.86	n.s.
5-HT 10^{-15}	61.04 ± 2.05	$P<0.01$	118.96 ± 1.61	$P<0.01$
5-HT 10^{-6}	70.68 ± 1.6	$P<0.01$	103.22 ± 2.59	n.s.

other time points the cultures imprinted with 10^{-15} M insulin showed no change in growth (Figure 2Aa). To the contrary, after imprinting with 10^{-6} M insulin a significant elevation of growth rate was detected at the later time points (18, 24 and 48 h). In parallel, the viability of the cells imprinted by 10^{-15} M insulin was slightly, but significantly, depressed after 48 h (Figure 2Ab). After 1000 generations in the group imprinted with 10^{-15} M insulin, an increased cell density was measured at 6 h, while a significant decrease was registered at 48 h (Figure 2Ba). Imprinting with 10^{-6} M insulin had no effect by generation 1000, the cell number of this population was similar to the control at each time point studied. Similarly the viability was significantly decreased in cells imprinted by 10^{-15} M insulin for 48 h of the proliferation assay (Figure 2Bb).

3.4. 5-HT imprinting

In generation 500, the proliferation rate of the cells imprinted with either concentration of 5-HT decreased by 6 h (Figure 3Aa). At later time points, 10^{-15} M imprinting failed to affect cell division, while the growth rate of cells imprinted with 10^{-6} M increased, being significant only at 24 h. According to the time course of our study, only after 1000 generations did 10^{-6} M imprinting elevate the cell number and solely at 6 h (Figure 3Ba). The growth rate of the cells imprinted with 10^{-15} M 5-HT remained at the control level. The viability of imprinted cultures showed similar or even better indicators than their relevant controls (Figure 3Ab and 3Bb).

3.5. Chemotaxis

3.5.1. Insulin imprinting

A slight negative effect of the 10^{-15} M imprinting on the chemotactic response of *Tetrahymena* was observed at generation 500 after imprinting (Ins-15_C: 81.4%), while this effect had subsided to the control level by generation 1000 (Figure 4). The migratory activity was in the control range in cells, which met the 10^{-6} M insulin only at pretreatment either 500 or 1000 generations before the test. No chemotactic effect of insulin was

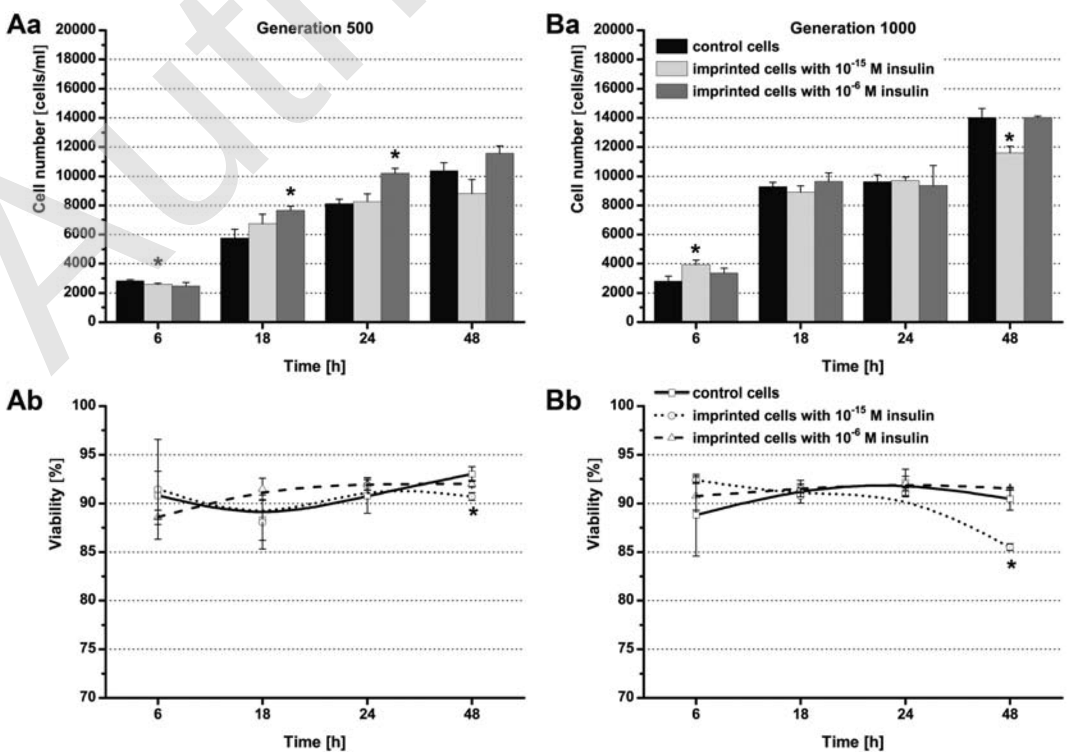

Figure 2 **Time and concentration course study of the growth and viability of *Tetrahymena pyriformis* imprinted by insulin in generations 500 (A) and 1000 (B)**
Data represent means ± S.D. of three parallel measurements. The significance is related to the control at given time point. *$P<0.05$.

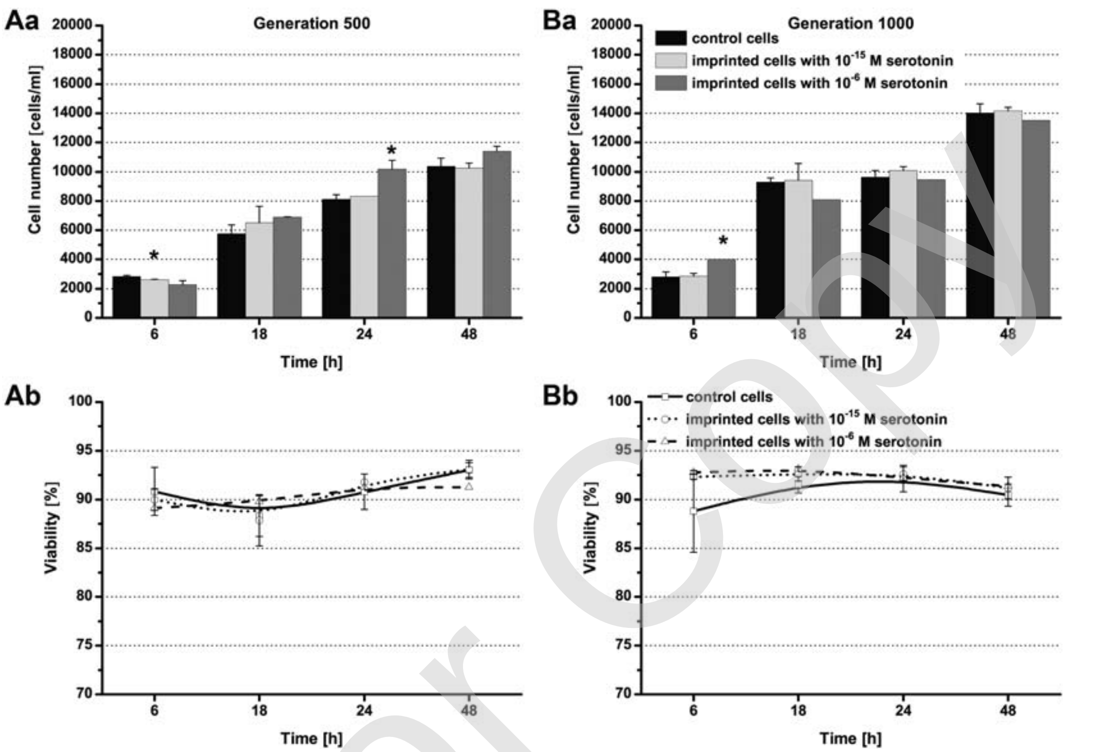

Figure 3 Time and concentration course study on the growth and viability of *Tetrahymena pyriformis* imprinted by 5-HT in generations 500 (A) and 1000 (B)
Data represent the means $\pm$ S.D. of three parallel measurements. The significance is related to the control at given time point. *$P<0.05$.

found between control cells and cells imprinted with different concentrations of insulin in generation 500.

The chemotactic character at 10^{-6} M insulin proved to be slightly attractive in those cells pretreated with 10^{-15} M insulin (Ins-15_Ins-6: 121.2%) in generation 1000 (Figure 4). However, the chemotactic effect of insulin was slightly repellent at both concentrations (Ins-6_Ins-15: 78.3%; Ins-6_Ins-6: 82.7%) in generation 1000 after imprinting with 10^{-6} M insulin. A similar avoidance of the control cells to the insulin was detected, however, but only in high concentration (C_Ins-6: 80.5%). Considering the results in generation 500, the trends in chemotactic responsiveness in the groups discussed above was opposite.

3.5.2. 5-HT imprinting

The single pretreatment with both 10^{-15} and 10^{-6} M 5-HT in generation 500 decreased the migratory response of *Tetrahymena* cells in a similar way (Ser-15_C: 87.4%; Ser-6_C: 87.1%) (Figure 5). The chemotactic effect of 5-HT was neutral at the first encounter (non-imprinted cells), as observed in generation 500 in cells imprinted with 10^{-15} M 5-HT. Nevertheless, the strength of the chemotactic response of these imprinted cells was less than the activity of the control cells. In cells imprinted by 10^{-6} M 5-HT, the chemotactic activity of the re-exposure to 5-HT was slightly repellent (Ser-6_Ser-15: 78%, Ser-6_Ser-6: 79%).

Interestingly with this kind of ligand, the negative result of the first encounter (imprinting) remained durable and increased in significance (Ser-15_C: 78.5%) only in imprinting with 10^{-15} M 5-HT in generation 1000 (Figure 5). The neutral effect of 10^{-6} M 5-HT in generation 1000 control cells turned into one of repellence in the case when 10^{-6} M 5-HT was applied on 5-HT imprinted cells at the second encounter (Ser-15_Ser-6: 67.5%; Ser-6_Ser-6: 81.2%). At this later time-point, the chemotactic reaction of the cells imprinted with 10^{-15} M 5-HT was lower than in the group imprinted with 10^{-6} M 5-HT.

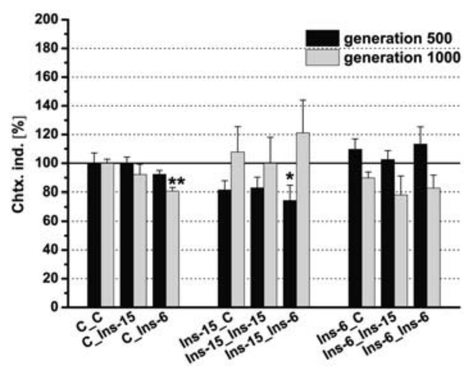

Figure 4 Concentration- and time-dependent chemotactic effect induced by retreatment with insulin in *Tetrahymena pyriformis* imprinted by insulin
The 'Chemotaxis index' (Chtx. ind.) is expressed as percentage of the non-imprinted, non-retreated control (C_C). Data represent the means $\pm$ S.D. of 4 parallel measurements. The level of significance is related to the C_C: *$P<0.05$; **$P<0.01$.

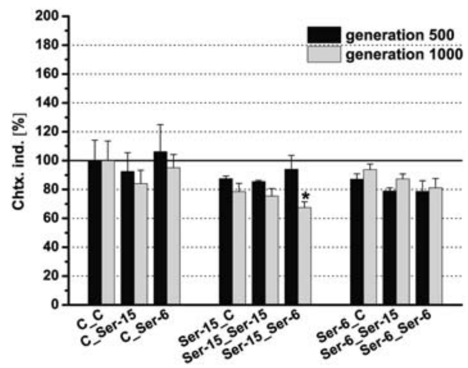

Figure 5 Concentration and time-dependent chemotactic effect induced by retreatment with 5-HT in *Tetrahymena pyriformis* imprinted by 5-HT
The 'Chemotaxis index' (Chtx. ind.) is expressed as percentage of the non-imprinted, non-retreated control (C_C). Data represent means ± S.D. of 4 parallel measurements. The level of significance is related to C_C: $^{*}P<0.05$.

3.6. Swimming behaviour (mean velocity and tortuosity)

3.6.1. Insulin imprinting

In generation 1000, the swimming velocity of the cells imprinted with 10^{-15} insulin (Ins-15_C: 97%) was similar to the control cells. The second encounter with 10^{-15} insulin had a slight negative effect (Ins-15_Ins-15: 88.5%). When the imprinting was done with 10^{-6} M insulin, the mean velocity of swimming cells was significantly higher (Ins-6_C: 126%) than the controls (Figure 6A). Repeated treatment with 10^{-15} or 10^{-6} M insulin decreased the swimming speed both in non-imprinted *Tetrahymena* (C_Ins-15/Ins-6: 86.9–87%) and in cells imprinted with 10^{-6} M insulin (Ins-6_Ins-15: 83.6%, Ins-6_Ins-6: 87.2%).

In generation 1000, there was no difference in the level of tortuosity of cell paths imprinted with 10^{-15} M insulin (Ins-15_C: 1.44) compared with the control (C_C: 1.53), while after imprinting with 10^{-6} M insulin it was significantly lower (Ins-6_C: 1.25) than the controls (Figure 6B). The application of insulin enhanced the serpentine-like movement in the non-imprinted cells, and the retreatment with the lower concentration (10^{-15} M) of insulin proved to be more effective (C_Ins-15: 1.80; C_Ins-6: 1.63). The same tendency was detected in cells after insulin imprinting (10^{-6} M); however, the values of tortuosity could not reach the level of the identical control (Ins-6_Ins-15: 1.57; Ins-6_Ins-6: 1.37). Retreatment with 10^{-6} M insulin reduced the tortuosity of swimming of cells imprinted with 10^{-15} M insulin (Ins-15_Ins-6: 1.15).

3.6.2. 5-HT imprinting

The velocity of swimming was significantly increased after imprinting independently of the imprinter concentration (Ser-15_C: 135.1%; Ser-6_C: 126.7%). However, retreatment with either concentration decreased the velocity in all the tested groups, but was more pronounced after 10^{-6} M pretreatment (Ser-6_Ser-15: 55.4%; Ser-6_Ser-6: 80.8%) (Figure 7A). In both groups of imprinting, 10^{-15} M 5-HT was more effective.

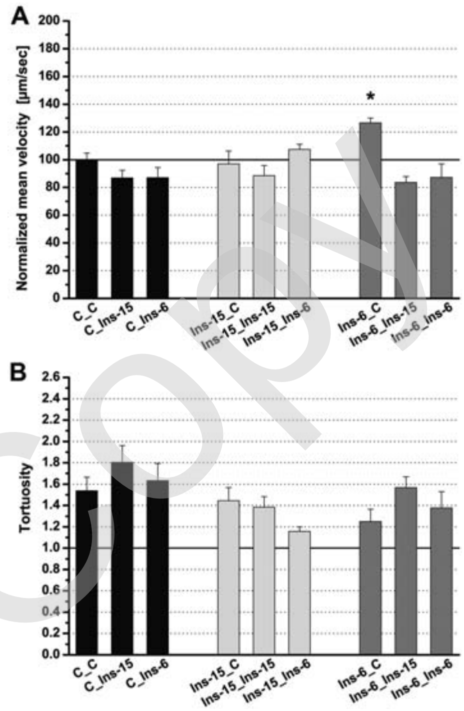

Figure 6 Effects of insulin re-exposure on the swimming behaviour of insulin imprinted *Tetrahymena pyriformis* in generation 1000
Values of ± S.D. are in the range 7.33–9.89% (**A**) and 0.19–0.30 (**B**). The mean velocity is expressed as percentage of the non-imprinted, non-retreated control (C_C). Data represent the mean of four parallel measurements. The level of significance is related to the C_C: $^{*}P<0.05$.

The movement of the cells became more rectilinear in parallel with the increasing velocity of the cells following the imprinting (C_C: 1.53; Ser-15_C: 1.15; Ser-6_C: 1.34) (Figure 7B). In the 10^{-15} M 5-HT imprinted cultures, retreatment with 5-HT (10^{-6} M) resulted in a more winding path (Ser-15_Ser-6: 1.47) compared with 10^{-15} M retreatment (Ser-15_Ser-15: 1.27). Nevertheless, the addition of 10^{-15} M 5-HT to the non-imprinted cells caused the most notable tortuosity of *Tetrahymena* (C_Ser-15: 2.18) and this effect in imprinted groups was still observed, but at a lower level (Ser-15_Ser-15: 1.27; Ser-6_Ser-15: 1.76).

4. Discussion

The unicellular ciliate, *Tetrahymena,* has a hormonal system, which regulates different functions (Csaba, 1985, 2000). The individual members of a population have very sensitive receptors to extremely low concentrations of hormones produced by the cells themselves in their watery milieu (Csaba et al., 2007), as well as signal pathways by which the reaction of the cell is stimulated (Christensen et al., 1998). In addition, the first encounter with the exogenously given hormone induces the phenomenon of hormonal imprinting, which means that the hormone could be 'memorized' by the cell and is transmitted to the progeny (Csaba, 1980, 1985, 1994, 2008). Usually, hormonal imprinting is represented by the enhanced reaction of the cell to the hormone

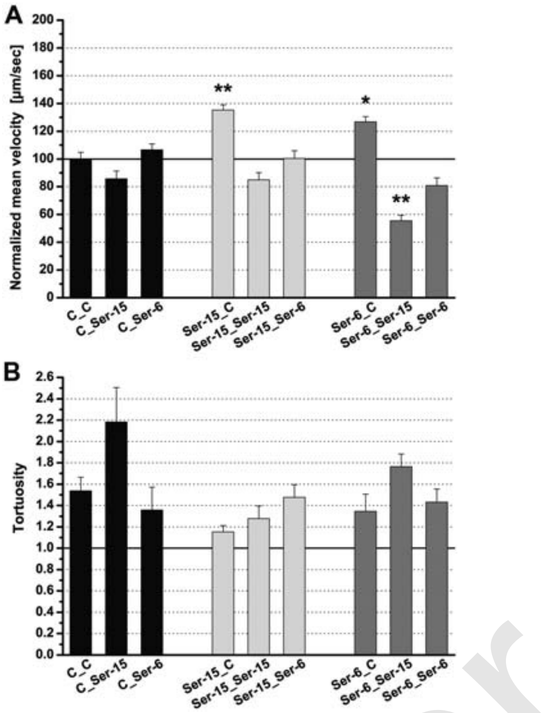

Figure 7 Effects of 5-HT re-exposure on the swimming behaviour of 5-HT imprinted *Tetrahymena pyriformis at* generation 1000
The mean velocity is expressed as percentage of the non-imprinted, non-retreated control (C_C). Data represent the mean of 4 parallel measurements. Values of $\pm$S.D. are in the range 8.23–10.5% (**A**) and 0.24–0.35% (**B**). The level of significance is related to the C_C: **P*<0.05; ***P*<0.01.

(or a material recognizable by the same receptors) on the next occasion and at lower concentrations. Theoretically this could help the cell to approach more readily a substance that has a hormonal character or can be receptorially recognized, or it can help the cell to escape from harmful substances when present at much lower concentrations.

Development of 'memory' (hormonal imprinting) seems to be an epigenetic process, as the imprinters are non-mutagenic and their effect is heritable for hundreds of generations (Kőhidai et al., 1990). However, there is also direct evidence of an epigenetic effect. 5-Azacytidine, which can replace cytidine during methylation of DNA, can deeply influence the outcome of hormonal imprinting (Csaba and Kovács, 1990).

In numerous earlier experiments, the effect of imprinting have been studied 24 h (~8 generations) after treatment. In this case, the effect of imprinting was complete, with the test indices being quantitatively changed (Fülöp and Csaba, 1994, 1997; Csaba and Kovács, 2000; Csaba, 2000). The main aim of the present experiments was to measure the durability of the imprinting up to generations 500 and 1000 i.e. culture for 60–120 days, and allows us to suppose that an epigenetic change provoked by a hormone is definitive. Two hormones were chosen for testing because insulin has a thoroughly studied receptor in *Tetrahymena* (Christopher and Sundermann, 1995; Leick et al., 2001; Christensen et al., 2003). Similarly 5-HT, has also been studied in many experiments (Essmann, 1987; Quinones-Maldonado and Renaud, 1987; Castrodad et al., 1988). The two hormone concentrations used in the experiments were selected by experiences gained in previous experiments (Csaba et al., 2007). The indices tested are considered those essential in physiology of cells in general; nevertheless, they are also characteristic of *Tetrahymena*. The individual life of a *Tetrahymena* cell is short; it divides 8–10 times per day (Scherbaum, 1957; Prescott, 1959).

In all of the indices tested, the results of imprinted cells were different from those of the control cells, which shows the permanency of the effect. The alterations are quantitative: offspring generations of the cells imprinted about 1000 generations before, express significantly increased or decreased reactions, without any further stimulus with the hormone as well as – in the case of swimming behaviour and chemotaxis – after repeated treatments. In addition, the strength of the imprinting is dependent on the type of hormone given and the test-reaction studied.

As mentioned, *Tetrahymena* has a complete endocrine system, producing hormones, possessing receptors and signal transduction pathways, all of which are similar to those of mammalian ones. The system was affected very deeply by imprinting. Insulin binding was reduced by a high (10^{-6} M) and also by a very low (10^{-15} M) insulin imprinting by ~20% in generations 500 and 1000, which means that it is a permanent reduction. 5-HT imprinting with both concentrations caused a similar effect (decrease) on 5-HT production up to generation 500; however, the negative character of 10^{-6} M had disappeared by generation 1000, while in the case of 10^{-15} M imprinting, the negative effect was significantly reversed into an elevation of hormone content.

Insulin has a very important role in the life of *Tetrahymena*, (Christensen, 1993; Christensen et al., 1996), while 5-HT also has regulatory functions. However, it is not known as a 'life-saving factor'. This might explain the difference in durability of the two imprinters. However, insulin is a polypeptide hormone and 5-HT is an amino acid type one, which might also explain the difference. Imprinting by an amino acid-type hormone precursor, diiodotyrosine, which had been studied for decades also permanently changed the responsiveness for 500 generations with a gradual decline (Csaba et al., 1982b).

After 10^{-6} M imprinting, cell proliferation as well as the swimming velocity were higher to generation 500 and 1000. This shows that not only the hormonal system is affected durably by imprinting but also fundamental cellular functions. In addition, the effect of imprinting is complex: while the swimming velocity is faster after 10^{-6} M imprinting, insulin re-exposure decreases it (in generation 1000), which demonstrates the difference between the first (imprinting) and the second encounter (message). In our previous experiments, the second encounter with insulin 24 h after insulin (10^{-6} M) imprinting also reduced the percentage ratio of the relatively fast spiral movement; however, the effect of the imprinting itself in that short period was insignificant (Kovács et al., 1994).

The results of swimming behaviour unambiguously show that the sensitivity of model cells to the ligands is different. The second encounter with the hormones, independently of the type and concentrations of the signal molecules applied, had negative effects on the swimming velocity in the groups imprinted with 10^{-6} M of imprinter. At lower concentrations, 10^{-15} M, of the imprinter substances, the phenomenon was not general. The 10^{-15} M insulin imprinting did not develop the phenomenon mentioned above.

The parameters – mean velocity and tortuosity – describing the swimming behaviour of *Tetrahymena* are well correlated with one another. Due to the ciliary way of migration, it is likely that the rapid movements can be carried out in a linear path. As a consequence, the slower elements of movement are possibly performed on a more winding track (Muto et al., 2010). This seems to be confirmed by the two data sets obtained with insulin and 5-HT, while in the case of 5-HT two exceptions could be also detected (Ser-15_Ser-6 and Ser-15_Ser-15).

The chemotactic responsiveness of insulin imprinted *Tetrahymena* proved to be concentration-dependent in respect of itself in the imprinting and at the responses elicited at the second encounters. Insulin imprinting itself in the short term (24–48 h) results in an increased chemotactic response; however, this is highly dependent on the type (porcine or bovine) and condition (crystalline or amorphous) of the insulin molecules used to develop imprinting (Csaba et al., 1994). Evaluation of long-term characteristics of the responsiveness shows that relatively high (10^{-6} M) and low (10^{-15} M) concentrations of insulin as an imprinter shifts the profile: enhanced responsiveness is detectable to 10^{-6} M insulin imprinting in generation 500, compared with the identical groups of generation 1000; while in the case of 10^{-15} M insulin-imprinted cells an increased chemotaxis to insulin was elicited in generation 1000 compared with generation 500.

The molecular dependency of memory developed by imprinting is well demonstrated in the chemotaxis experiments with 5-HT. In contrast with insulin, 5-HT was previously reported as a chemorepellent substance in the short term (Kőhidai et al., 1994). Our present results show that this negative moiety of 5-HT is well conserved in the long-term (generations 500 and 1000) relations following imprinting. The robustness of the chemorepellent character is embodied in that, in this case, no concentration dependency was observed; 5-HT worked in general as negative imprinter for chemotaxis.

Overall, we can conclude that certain functions are transgenerationally enhanced by imprinting (growth rate, swimming velocity for both hormones and chemotactic activity in case of insulin), while others are diminished (hormone binding to insulin; chemotactic responsiveness to 5-HT). It seems likely that the hormone concentration itself, produced by the cells into the watery milieu, does not provoke imprinting as its concentration is supposed to be far less than our use of 10^{-15} M.

The phenomenon of hormonal imprinting (in unicellular organisms and mammals) has been observed and described by us over 30 years ago. Earlier, its epigenetic character was not known as epigenetics itself was in its infancy. Today the epigenetic effect of it (also supported by the present results) is justified across the animal world, including humans, and its effect on human evolution needs also to be considered (Csaba, 2008). Our present results could establish that the effect of hormonal imprinting on *Tetrahymena* is transmitted from generation to generation, and represents a permanent change in gene expression. This means that hormonal imprinting is an epigenetic phenomenon not only in mammals but also at the unicellular level. This was also previously supposed when azacytidine treatment had been shown to significantly influence insulin imprinting (Csaba and Kovács, 1990), and has been justified now, when the duration of imprinting effect has been taken much further. The imprinters of *Tetrahymena* are not mutagenic substances, but physiological ones. They have an imprinter effect in higher than normal concentrations; however, sometimes this concentration can be as low as 10^{-15} M (femtomolar concentration), as in the present experiments. Nevertheless, non-physiological materials that can effect through receptor-triggered pathways (e.g. hormone analogues, pesticides, insecticides, aromatic hydrocarbons and scent materials) can also imprint unicellular organisms (Csaba, 2008). It can also be supposed that the present natural *Tetrahymena* population is not identical with its archaic progenitors, not only because of the eventual mutations but also because of epigenetic changes caused by the increasing mass of (hormone-like) chemical contaminants (Csaba, 2011) of the waters of today. There is a theoretical possibility that specific subtypes of cells are growing under the pressure of epigenetic imprinting. In earlier experiments, a significant difference was found between cell clones of imprinted sister (*Tetrahymena*) cells. However, all of the progeny cells were functionally altered by the imprinting (Csaba et al., 1989).

Author contribution

László Kőhidai performed and evaluated experiments, and was in control of the paper. Eszter Lajkó performed and evaluated experiments. Éva Pállinger performed and evaluated experiments. György Csaba had the idea of the experiments and wrote the paper.

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