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# Effect of LIF-withdrawal on acetylcholine synthesis in the embryonic stem cell line CGR8 is not mediated by STAT3, PI3Ks or cAMP/PKA pathways

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ABSTRACT

Acetylcholine (ACh) acts as a local cellular signaling molecule and is widely expressed in nature, including mammalian cells and embryonic stem cells. The murine embryonic stem cell line CGR8 synthesizes and releases substantial amounts of ACh. Particularly during early differentiation – a period associated with multiple alterations in geno-/phenotype functions – synthesis and release of ACh are increased by 10-fold. In murine stem cells second messengers of the STAT-3, PI3K and cAMP/PKA pathways are involved in maintaining self-renewal and pluripotency. The present experiments were designed to test whether blockers of these signaling pathways enhance ACh cell content in the presence of LIF, i.e. when CGR8 is pluripotent. NSC74859, an inhibitor of STAT-3, affected neither the proliferation rate nor ACh cell content, whereas the more sensitive STAT-3 inhibitor FLLL31 reduced the proliferation rate and increased ACh cell content by about 3-fold. The PI3K inhibitor LY294002 reduced the proliferation rate but did not modify the ACh cell content, whereas the PKA inhibitor H89 produced effects comparable to FLLL31. Interestingly, in control experiments a strong inverse correlation was found between cell density and ACh cell content, which could explain the 3-fold increase in the ACh cell content observed in the presence of FLLL31 and H89. Forskolin, a PKA activator, had no effect. In conclusion, it appears unlikely that the 10-fold increase in ACh cell content induced by LIF removal, i.e. during early differentiation, is mediated by second messengers of the STAT-3, PI3K and cAMP/PKA pathways. However, the PI3K pathway appears to be involved in control of the inverse relation between cell density and ACh cell content, because this correlation was significantly attenuated in the presence of LY294002.



## 1. Introduction

Embryonic stem cells were first established from mouse blastocysts in co-culture with inactivated fibroblast cells [1]. Later on, the factor originating from fibroblasts to maintain self-renewal and pluripotency was identified as leukemia inhibitory factor (LIF), a member of the Il-6 cytokine family [2]. Thus, murine embryonic stem cells remain pluripotent when they are cultured in the presence of LIF, but show early differentiation upon the removal of LIF [3,4]. Recently, the synthesis of non-neuronal acetylcholine was demonstrated in the murine embryonic stem cell line CGR8; ACh synthesis and release were substantially (10-fold) increased during early differentiation, i.e. upon the removal of LIF [4].

The property of LIF to maintain pluripotency of murine embryonic stem cells depends on the activation of signal transducer and activation of transcription (STAT3), but also phosphoinositide 3-kinase (PI3K) activation is important for self-renewal and pluripotency [5–7]. Moreover, the cAMP/PKA pathway modifies pluripotency and self-renewal; inhibitors of PKA reduced the expression of the pluripotency marker Oct-4 in the presence of LIF, therewith facilitating differentiation. However, in the absence of LIF or in the presence of the differentiation factor retinoic acid, forskolin, an activator of PKA, significantly promotes self renewal and increases Oct-4 expression, thus promoting pluripotency [8]. It is evident that inhibitors of the three described pathways promote early differentiation of CGR8 cells, which is comparable to the removal of LIF and may result in an enhanced synthesis of ACh, when one of these pathways is coupled to the regulation of ACh synthesis during this transient period. Therefore, in the present experiments the effect of inhibitors of the three described signaling pathways was tested with respect to the proliferation rate (as monitored by cell number) and ACh content in the presence of LIF.

*Abbreviations:* ACh, acetylcholine; HPLC, high pressure liquid chromatography; DMSO, dimethylsulfoxide; FCS, fetal calf serum; FLLL31, (E,E)-1,7-Bis(3,4-dimethoxyphenyl)-4,4-dimethyl-1,6-heptadiene-3,5-dione; H89, N-[2-(p-Bromocin-amino) ethyl]-5-isoquinolinesulfonamide dihydrochloride; IL-6, interleukin-6; LIF, leukemia inhibitory factor; LY294002, 2-(4-morpholino)-8-phenyl-4H-1-benzo-pyran-4-one; NSC74859, 2-hydroxy-4-(((4-methylphenyl)sulfonyloxy)acetyl) amino-benzoic acid; STAT3, signal transducer and activator of transcription 3; PI3Ks, phosphatidylinositol-3-kinases; PKA, proteinkinase A; cAMP, cyclic adenosine monophosphate.

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## 2. Methods

### 2.1. Culture of CGR8 cells

The murine embryonic stem cell line CGR8 was a generous gift from Dr. J. Hescheler, University of Cologne, Germany. Undifferentiated cells were cultured under standard conditions in gelatinized flasks with Glasgow's buffered minimal essential medium (Gibco, Germany), supplemented with 100 U/ml leukemia inhibitory factor (LIF; Sigma Aldrich, Germany) to maintain pluripotency. In addition, the medium contained 0.05 mM beta-mercaptoethanol, 2 mM L-glutamine, 100 units/ml penicillin, 0.01 mg/ml streptomycin and 10 vol.% fetal calf serum (Sigma Aldrich, Germany). Cells grown in 25 $cm^2$ flasks reached confluence after 4 days. Thereafter CGR8 cells were passaged and seeded at a density of $1 \times 10^6$ cells on 75 $cm^2$ flasks. After an additional 2 day period under regular cell culture conditions the following inhibitors were added to the medium and the cells were incubated for the next 72 h, when CGR8 cells substantially proliferate after the preceding passage. The following test substances were used and the medium replaced once a day: NSC74859 (STAT3 Inhibitor VI, S3I-201, MerckMillipore, Darmstadt, Germany) and FLLL31, both inhibitors of the STAT3 pathway; LY294002 for PI3K inhibition; and H89, an inhibitor of PKA, which was present for 24 or 72 h (all Sigma Aldrich, Germany). Additionally, the effect of forskolin, a PKA activator was also tested after an exposure time of 24 h. The inhibitors of the STAT3 and PI3Ks pathways as well as forskolin required dissolution in DMSO (final maximal concentration 0.3 vol.%). Therefore, DMSO control experiments were performed in parallel, i.e. 0.3 vol.% DMSO was present in the culture medium during the last 3 days. After the incubation times indicated adherent cells were collected (0.05% trypsin/0.02% EDTA) for ACh measurement. Cells were pelleted by centrifugation (500 ×*g* for 10 min). The supernatant was removed and the extraction of ACh was performed as described previously [4,9]. Cell pellets were stored at −20 °C. Cell number was measured using a CASY® Cell Counter (Schärfe Systems GmbH, Reutlingen, Germany).

When indicated, seeding was also performed at varying cell counts of 0.2 and $1.5 \times 10^6$ cells on 75 $cm^2$ flasks, to investigate the dependence between ACh cell content and cell density. After an additional 3 to 5 days of culture with medium change intervals of 24 h cells were used for the determination of ACh cell content.

### 2.2. Measurement of ACh

Cell pellets were suspended in a mixture of acetone and formic acid (85/15; vol%/vol%) and placed on ice for 1 h. After sedimentation on ice the supernatant was evaporated to dryness by nitrogen. The dried sample was resuspended in 600–1000 μl of the mobile phase (70 mM phosphate buffer with 0.3 mM EDTA; pH 8.5 adjusted) of the HPLC system, filtered and 20 μl was injected. A cationic exchange high-pressure liquid chromatography (HPLC) unit combined with bioreactor and electrochemical detection was used [9].

### 2.3. Calculations and statistics

Results were expressed as mean value ± sem of n experiments. Statistical analysis of the results was performed by Student's *t*-test; *p* values of $<0.05$ indicate a significant difference.

## 3. Results

### 3.1. Effects of inhibitors of STAT3 pathway on ACh content in the presence of LIF

Cells ($1 \times 10^6$) were grown in 75 $cm^2$ flasks and after a culture period of 5–6 days a monolayer was obtained ($63 \pm 6.6 \times 10^6$ cells; $n = 7$; see also Fig. 1a). The ACh content was found to be $0.67 \pm 0.07$ pmol/$10^6$ cells ($n = 7$). As the inhibitors have to be dissolved in DMSO (final volume 0.3 vol.%), respective DMSO controls were performed. DMSO neither affected the proliferation rate ($68.8 \pm 14.1 \times 10^6$ cells; $n = 3$) nor the acetylcholine content ($0.78 \pm 0.13$ pmol/$10^6$ cells) (see also Table 1 and Fig. 2). NSC74859, a STAT3 inhibitor, did not affect the proliferation rate or ACh cell content (see Table 1 and Fig. 2). FLLL31 represents a more sensitive STAT inhibitor and was tested at a concentration of 1.25 μM. This inhibitor reduced proliferation rate substantially and increased ACh content about 3-fold (Table 1 and Fig. 2). Higher concentrations of the STAT3 inhibitor FLLL31 (2.5 μM) reduced cell viability, i.e. CGR8 cells lost their property to adhere and detached into the supernatant. Therefore, only the results with the lower concentration were used for data analysis.

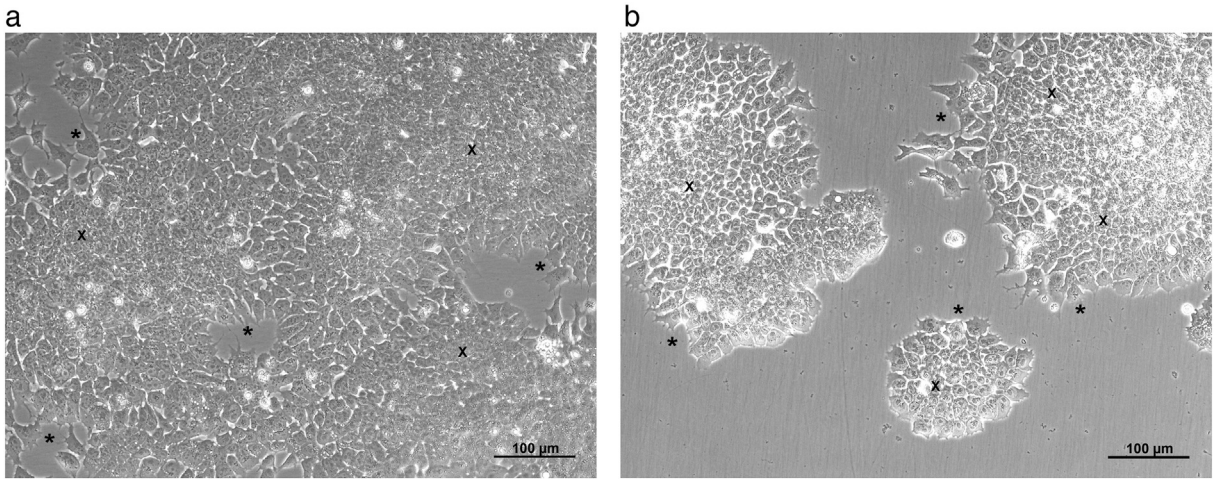


**Fig. 1.** Phase-contrast microscopy of CGR8 cells after seeding at a cell density of $1 \times 10^6$ cells (a) or $0.1 \times 10^6$ cells (b). CGR8 cells were seeded as indicated and cultured in the presence of LIF. a: After 5 days a monolayer was found after seeding at high cell density, but the initial cell clusters are still visible. In the center of these clusters the individual cells show a smaller size (x), whereas the border cells (*) are elongated. b: Shown are 3 cell clusters after seeding at low cell density after 5 culture days. A large area of the 75 $cm^2$ flask is not covered with cells at all. Within the cell clusters the border cells are enlarged (*) and the central cells (x) show a smaller size. Thus, a similar pattern is found as in panel a.

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