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Glial effects of the lithium mineral water Maria from Malnas-Bai

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ABSTRACT

Objective: *To investigate the influence of lithium mineral waters and lithium salts upon the differentiation of glial cells.*

Material and methods*: Mixed glial cultures were prepared from neonatal Wistar rat cortex. Cultures derived from neonatal rat forebrain develop with a monolayer or large flat astrocytes attached to the culture dish, with many smaller cells of the oligodendrocytes lineage on their surface.*

Results: *Treatment of these cultures with lithium mineral waters from Maria spring compared to treatment with lithium chloride 2mM showed significant differences in cell morphology. Immunohistochemical studies for glycogen synthase kinase (GSK)-3β supported the protective effects of lithium mineral waters for glial cells, whereas lithium chloride 2mM determined cytotoxic effects and inhibited Wnt signalling pathway.*

Conclusions: *The results of this study indicate the fact that lithium chloride and lithium mineral waters induce changes in glial cells. The changes depend on the lithium level in the culture medium.*

Key words: lithium, glial cells, GSK-3β, GFAP, Laminin, Vimentin

INTRODUCTION

Lithium has been the simplest therapeutic agent available for the treatment of bipolar disorder for over 100 years and no definitive mechanism for its effect has been established. Many studies have been done on neurons and they pointed out that lithium has many influences on the neurotransmitter systems, second messenger systems and the level of membrane depolarization. Glial cells create a specific cellular environment for neurons. Lithium has also many influences on the morphology and physiology of glial cells, as many authors sustain.

Lithium ions enter into cells through the transporting proteins involved in Na^+ and K^+

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transportation. The main entranceways of lithium are sodium channels and Na^+/H^+ exchangers. Sodium channels are involved in Na^+ transport from the exterior to the cell interior, in the sense of concentration gradient. Because of equal permeability of these channels to sodium and lithium, lithium ions can use them to enter into the cell without energy consumption. Lithium concentration grows 5-10 fold at the level of dendrites after a stimuli salve, due to the opening of numerous sodium channels during the action potential (1).

Details regarding the molecular mechanism and target region that justify the stabilizing action of lithium salts on manic-depressive psychosis are not fully elucidated. It has been suggested that second messengers generating systems, which imply adenylate cyclase and phosphoinositols, are potential target sites. In human blood, lithium ions can interfere with almost any process that implies sodium, potassium, calcium, magnesium, calmoduline, other second messenger, hormones and many known neurotransmitters. Therapeutic action of lithium salts in bipolar disorders seems to be the result of many events that modify the neuronal activity at multiple levels.

The effect of lithium treatment on neural cytoskeleton has been investigated in detail (2). The GSK-3β target β-catenin has two functions within the cell: it enters the nucleus to affect changes in gene expression through its interaction with transcription factors, and it links the actin cytoskeleton to adherens junctions in the cell membrane. Current evidence points to microtubule-associated proteins (MAPs) as important cytoskeletal targets of GSK-3β. Together, these aspects demonstrate that GSK-3β phosphorylation, and hence lithium, has the potential to alter the neuronal architecture of the brain. The implication is that a possible mechanism for lithium therapy and, by extrapolation, a cause of depression, could be a result of structural change in the way neurons interact – in essence, it could be a problem of neural "hard-wiring" (3).

GSK-3β activity has been associated with a number of neuronal effects. GSK-3β overexpression correlates with neuronal degeneration and increased sensitivity to apoptotic stimuli in human neuroblastoma SH-SY5Y cells. β-Amiloid (Aβ) treatment of rat hippocampal neurons increases GSK-3β expression and induces apoptosis, which is blocked by antisense oligonucleotides directed at GSK-3β (4).

The effects of GSK-3 on transcription factors such as c-jun, heat shock factor-1 (HSF-1) and nuclear factor of activated T cells (NFAT) are particularly noteworthy, and have drawn considerable interest. Generally, GSK-3 activity results in suppression of the activity of transcription. Conversely, inhibition of GSK-3 appears to activate these transcription factors. Thus, GSK-3 is well positioned to integrate signals from multiple pathways, a function that is undoubtedly critical in the CNS (5).

In Romania exist sparkling water sources which have a high level of lithium salts, the so-called "lithium content sparkling waters", with lithium concentrations between 3 and 8 mg Li/l. One of these sources is Maria spring from Malnas-Bai, which has a lithium concentration of 8,03 mg lithium per litre. ❑

MATERIAL AND METHODS

The primary glial cells culture was initiated from the brain of Wistar rat pups aged 1-3 days, in keeping with the known techniques (6). After the meninx is removed, the brain is passed through a 60mm nytex and the cells thus obtained are directly plated on glass Petri dishes with a 60 mm diameter. The cells have been grown in a DMEM medium (Dulbecco's Modified Eagle's Medium, Sigma), with 4500 mg/l of glucose, 25 mM HEPES, 100-u/ml penicillin and 50 mg/ml neomycin (Sigma). The medium was supplemented with 15% calf fetal serum (Gibco). The medium was first replaced after 24 h and then every three days. The primary cell cultures were maintained in a humidified atmosphere, at 37°C, 5% CO_2.

Four culture groups have been used in the study:

1. Cultures maintained in medium with 2mM LiCl;
2. Cultures maintained in medium prepared with 25% Maria mineral water and 75% bidistilled water;
3. Cultures maintained in medium prepared with 100% Maria mineral water and no bidistilled water;
4. Control cultures maintained in medium prepared with 100% bidistilled water.

25% Maria mineral water dilution was used in order to simulate the physiological conditions for the ingestion of mineral water.

Morphological analyses were performed by standard procedures with hematoxylin-eosin staining. After the medium was removed, the cell cultures were washed with TFS and fixed in

formaldehyde (10%). Glial cells were stained with hematoxylin for 10 minutes, and with eosin for 5 minutes.

Immunohistochemical detection of GFAP, GSK-3β (Glial Fibrillary Acidic Protein), vimentin and laminin was made by an indirect immunoperoxidase method. Glial cells were fixed for 10 min., at room temperature in Bouin solution. After washing, the cultures were sequentially incubated in 3% H_2O_2 to remove endogenous peroxidase (10 min.) washed in PBS and incubated with 2% bovine serum albumin (BSA, fraction V) to remove non-specific background staining (30 min.). The cells were incubated overnight at 4^0C with primary antibodies. The following primary antibodies were used: anti GFAP goat polyclonal antibodies (Santa Cruz Biotechnology) – dilution 1:100; anti-GSK-3β goat polyclonal antibodies (Santa Cruz Biotechnology)– dilution 1:100; anti Vimentin mouse monoclonal antibodies (Sigma) – dilution 1:200; and anti Laminin goat polyclonal antibodies (Santa Cruz Biotechnology) – dilution 1:100. All antibodies were diluted in PBS with 2% bovine serum albumin. After washing with PBS, glial cells were incubated with horseradish peroxidase-conjugated secondary antibody for an hour, at room temperature. For goat antibodies detection were used rabbit anti goat secondary antibodies (Sigma), coupled with peroxidase and diluted 1:150 in PBS. For mouse antibodies detection were used rabbit anti mouse secondary antibodies (Sigma), coupled with peroxidase and diluted 1:350 in PBS. After washing with PBS, immune complex was detected in the presence of 0.05% 3,3 diaminobenzidine tetrachloride and 0.015% H_2O_2 in PBS. Glial cells nuclei were counter-stained with Harris hematoxylin.

The chemical content of Maria lithium mineral water was determined by standard chemical and physical procedures in the Chemistry Lab of National Institute of Rehabilitation, Physical Medicine and Balneoclimatology, Bucharest, Romania. The microbiological purity of Maria water was assured by micro-filtration. ❑

RESULTS

Lithium mineral water from Maria Source, Covasna, Romania has the chemical content presented in Table 1. The concentration of lithium is 8.03 mg/l, which correspond to 1.15 mM lithium, a value situated in the therapeutic range (0.2-1.5 mM).

Cell morphology depends on microenvironment and in control culture, glial cells are of various types: astrocytes, oligodendrocytes, mature or immature. Standard conditions for culture growing permit a monolayer distribution on Petri dishes as showed in Figure 1. Treatment of glial cell cultures with ¼ lithium mineral water Maria does not significantly change the normal aspect as shown in Figure 2. Glial cells have a normal aspect and their cytoplasmic projections are very clear.

Culture observations by means of light microscopy after hematoxylin-eosin staining pointed to the fact that lithium chloride; at a concentration of 2mM has a mitogenic effect on glial cells in the first 11-12 days. After that lapse of time, manifestations of toxicity appear – Figure 3. The cells become swollen, round, vacuoles appear in the cytoplasm. Cytoplasmic projections become narrower and their number decreases. With time, cell lysis occurs and the monolayer acquires the appearance of a loose network, as "gaps" appear in it. After 18-20 days of culture, glial cells treated with 2mM LiCl become detached from the substrate.

When the culture medium was prepared using only Maria mineral water the cell morpho-

	Content in 1 litre of mineral water				
	mg	mM	mEq	mg %	mEq %
ANIONS					
Cl^-	1.009,1	28,460	28,460	10,922	24,866
Br^-	2.,9	0,036	0,036	0,031	0,032
I^-	0,7	0,006	0,006	0,008	0,017
NO_3^-	9,9	0,160	0,160	0,107	0,139
SO_4^-	25,1	0,261	0,261	0,272	0,457
HCO_3^-	5202,0	85,256	85,256	56,304	74,489
			114,455		100,000
CATIONS					
Na^+	2263,8	98,441	98,441	24,503	86,009
K^+	70,5	1,803	1,803	0,763	1,575
Li^+	8,03	1,152	1,152	0,087	1,007
NH^+	0,70	0,039	0,039	0,008	0,034
Ca^{2+}	212,6	5,304	5,304	2,301	9,269
Mg^{2+}	28,3	1,164	1,164	0,306	2,033
Fe^{2+}	2,2	0,039	0,039	0,024	0,070
Mn^{2+}	0,1	0,002	0,002	0,001	0,003
			114,455		100,000
H_2SiO_3	21,5	0,275	–	0,233	–
HBO_2	372,2	8,492		4,029	
NH_2	7,0	0,437		0,076	
O_2	2,4	0,150		0026	
CO_2	748,0	17,000			
Mineralization	9239,0	231,477	228,909	100	

TABLE 1. Chemical content of Maria mineral water

logy is completely changed. After 10 days there is a very high pathological index as can be seen in Figure 4 and after 20 days, the survivor cells are smaller, and with bigger projections – Figure 5.

Immunodetection of GFAP shows a weak positive reaction for control glial cells culture – Figure 6. Treatment with Maria mineral water weakly intensifies this pattern – Figure 7. 2mM LiCl determine an intensification of GFAP expression – Figure 8.

Vimentin is very well expressed in glial cells culture and our results reflect this aspect very clear in Figure 9. Affected disposition and altered quantity of vimentin occur in glial cells culture after treatment with 2mM LiCl, fact shown in Figure 11. Maria water treated cultures manifest an intense reaction for vimentin too, but the distribution is uniform in the cytoplasm and the quantity of vimentin is less than for 2mM LiCl – Figure 10.

After 10 days in culture, immature astrocytes show an intense reaction to laminin, Figure 12. Besides, for some cells laminin localization was seen on the cytoplasmic projections. In glial cells cultures treated with ¼ Maria water, laminin was detected perinuclear, Figure 13 and after 20 days, the reaction was negative. For glial cells cultures treated with 2mM LiCl, immunohistochemical reaction for laminin presents an altered distribution of these filaments. Laminin has been identified in perinuclear aggregates in vacuolised cells – Figure 14.

Immunodetection of GSK-3β shows a moderate reaction for control glial cells culture – Figure 15. This protein is situated more often in a limited cytoplasmic area near the nucleus. Treatment with ¼ Maria mineral water intensifies this pattern, Figure 16 and figure 17. In contrast, 2mM LiCl determine the absence of GSK-3β expression (Figure 18). ❑

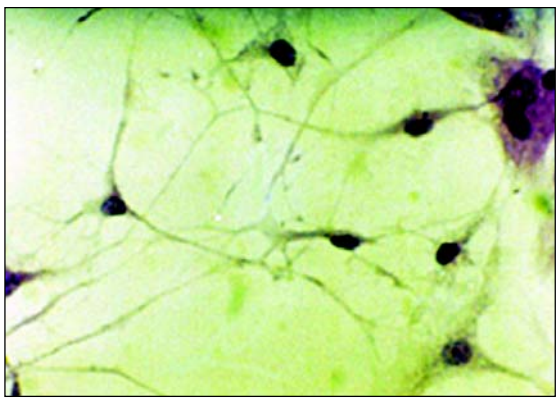

FIGURE 1. Glial cells culture after 20 days, hematoxylin-eosin, x250

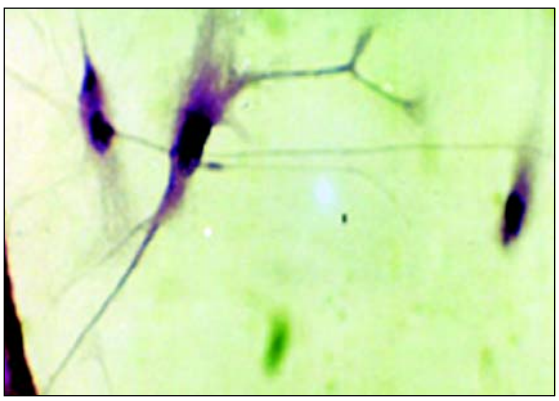

FIGURE 2. Glial cells after treatment with ¼ Maria mineral water for 20 days, hematoxylin-eosin, x400

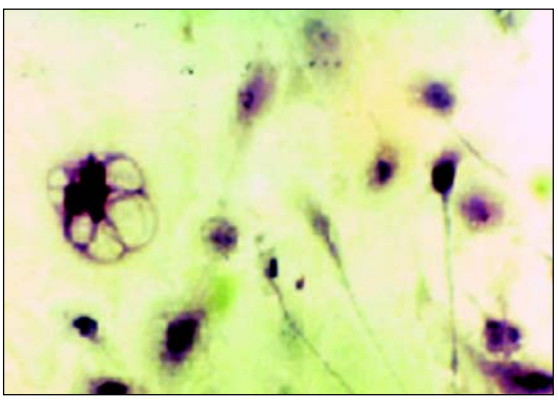

FIGURE 3. Vacuolised glial cell after treatment with 2 mM LiCl for 10 days, hematoxylin-eosin, x250

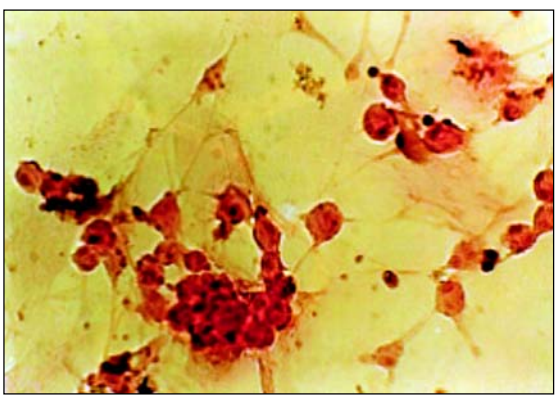

FIGURE 4. Glial cells after treatment with 100% Maria mineral water for 10 days, hematoxylin-eosin, x200

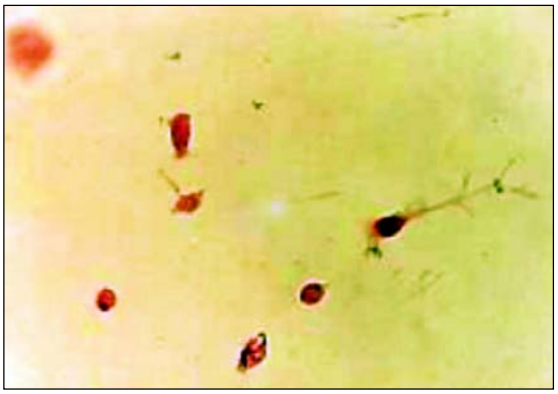

FIGURE 5. Glial cells after treatment with 100% Maria mineral water for 20 days, hematoxylin-eosin, x100

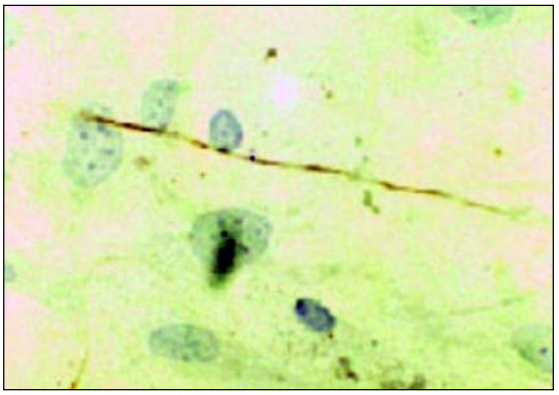

FIGURE 6. GFAP positive glial cells culture after 10 days, x400

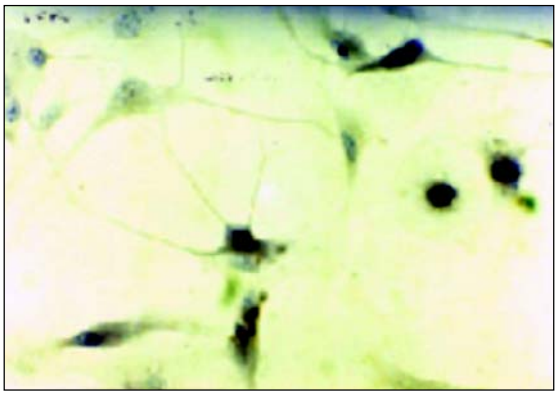

FIGURE 7. Low GFAP reaction for glial cells after treatment with ¼ Maria mineral water for 10 days, x400

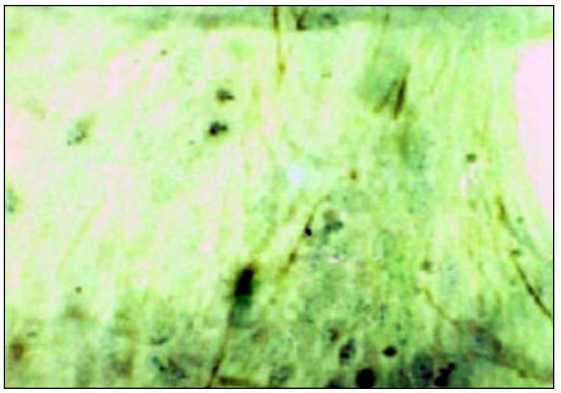

FIGURE 8. GFAP localization in glial cells treated with 2mM LiCl for 10 days, x400

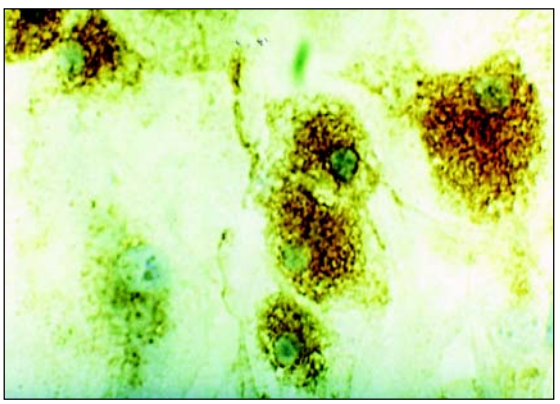

FIGURE 9. Vimentin localization in control glial cells after 10 days, x400

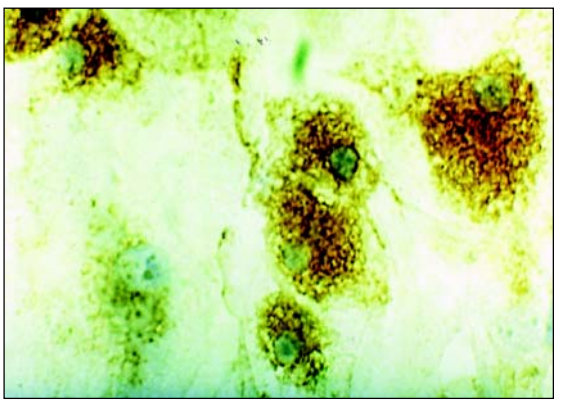

FIGURE 10. Vimentin localization in glial cells treated with Maria water for 10 days, x400

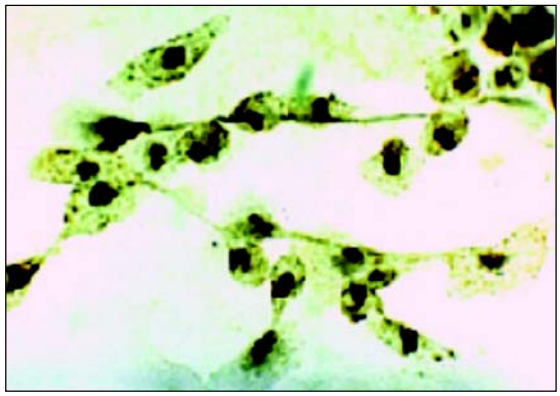

FIGURE 11. Vimentin cytoplasmic localization in glial cells treated with 2mM LiCl for 10 days, x400

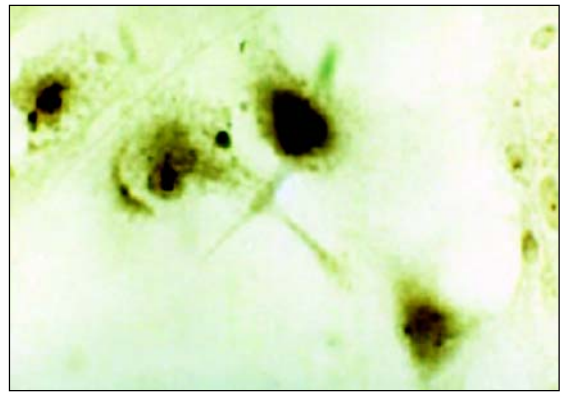

FIGURE 12. Laminin cytoplasmic localization in control glial cells after 10 days, x400

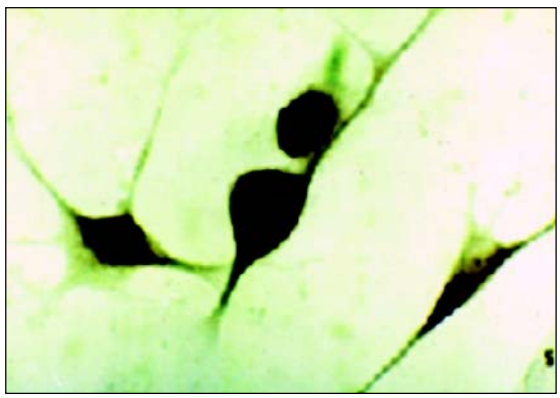

FIGURE 13. Laminin localization in glial cells treated with Maria water for 10 days, x400

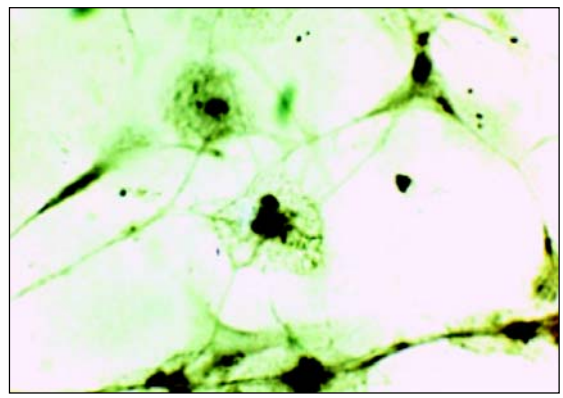

FIGURE 14. Altered laminin localization in glial cells treated with 2mM LiCl for 10 days, x400

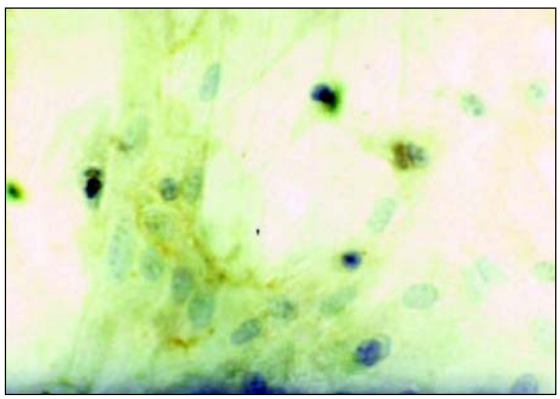

FIGURE 15. Low GSK-3β reaction for control glial cells after 10 days, x250

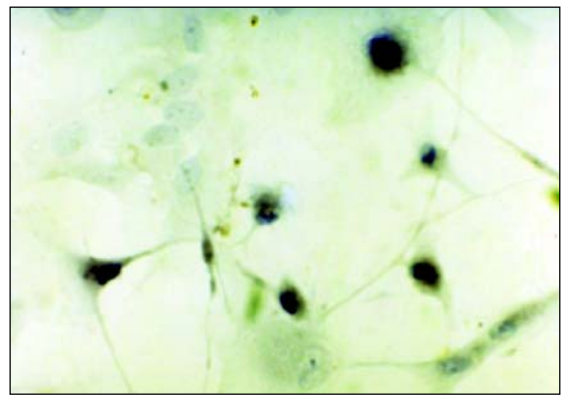

FIGURE 16. GSK-3β reaction in glial cells after treatment with Maria mineral water for 10 days, x250

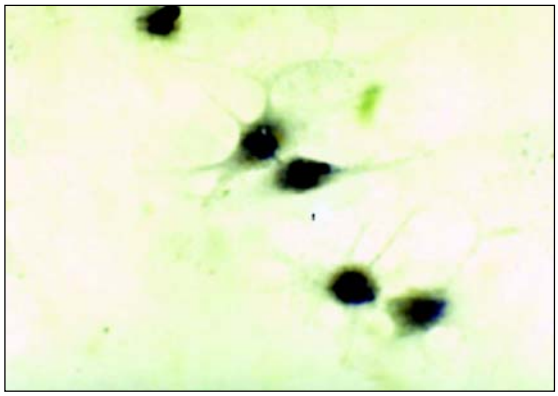

FIGURE 17. GSK-3β cytoplasmic localization in glial cells treated with Maria mineral water for 10 days, x 400

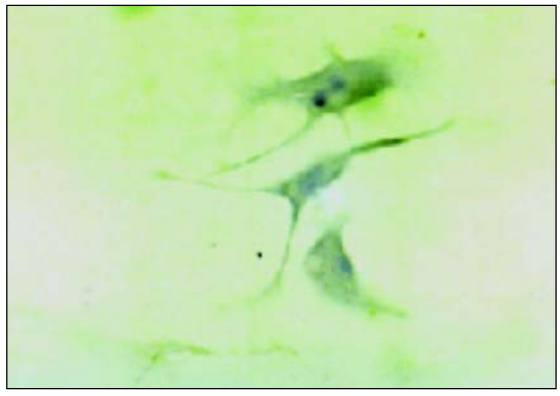

FIGURE 18. GSK-3β negative reaction in glial cell after treatment with 2mM LiCl for 10 days, x400

DISCUSSIONS

A major challenge in modern biology is to understand the basic mechanism controlling both neural activity and pattern formation during early development (5). The fact that both processes can be modulated by lithium is intriguing, especially as it seems it may be working through a common mechanism. Our hypothesis is that lithium is acting on the cell through influencing the cybernetic biochemical network (1) of the cell – Diagram 1. The fact that with mineral waters many factors are implied is evident for the cell physiology, but the

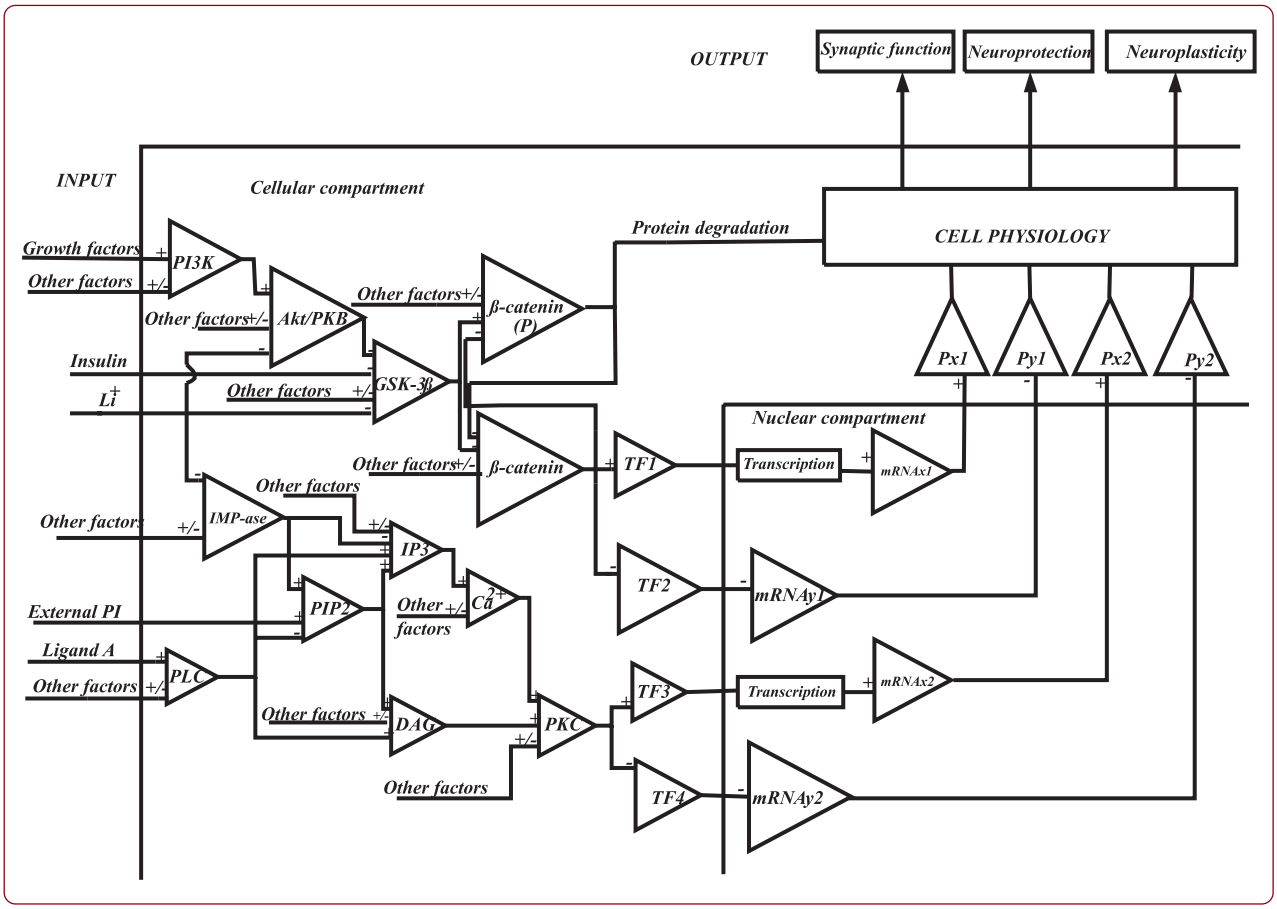

DIAGRAM 1. Cybernetic model for the lithium action on cell level

Abbreviations: PI, phosphatidylinositol; PI_3K, phosphatidylinositide 3-kinase; PKB, protein kinase B; PKC, protein kinase C; IMPase, inositol monophosphatase; PIP_2, phosphatidylinositol 3,4-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PLC, phospholipase C; TF, transcription factor; Px1, Px2, Py1, Py2 – proteins.

cellular responses are limited by the terms of adapting cell to the new environment created. If it is impossible for the cell to adapt to the disturbance factors, they present pathological changes like: swelling, vacuolisation, granulation, apoptosis or necrosis.

GFAP is a very sensitive and specific marker for rapid astrocytic response to injury and disease. Increase of GFAP in astrocytes occurs gradually throughout the adult lifespan of mice, rat and humans. Since GFAP normally increases with age and there is a wide variation in the collection and processing of human brain tissue, it is difficult to demonstrate mild gliosis by immunocytochemistry. It is now well established that GFAP is the principal 8-9 nm intermediate filament in mature astrocytes (2). In control glial cells cultures, immunodetection of GFAP shows a weak reaction. Treatment with Maria mineral water weakly intensifies this pattern. In contrast, 2mM LiCl determine an intensification of GFAP expression.

In the neuroectoderm, vimentin is a specific marker for astrocytes and ependymal cells. It is expressed in the mouse in astrocytes and glial precursors well before the onset of GFAP expression and might therefore serve as an early marker of glial differentiation (7). Our results reflect an affected disposition and altered quantity of vimentin in glial cells culture after treatment with 2mM LiCl. Maria water treated cultures manifest an intense reaction for vimentin too, but the distribution is uniform in the cytoplasm and the quantity of vimentin is less than for 2mM LiCl.

Laminin is a protein of early astrocytes and deposited by them in primary culture (8), thus suggesting a role for this glycoprotein in the development of the central nervous system. All the nonneuronal cells are strongly to moderately positive for laminin. Laminin has a cytoplasmic granular distribution. The cytoplasmic laminin immunoreactivity in astrocytes gradually disappears depending on the age of the animal and time in culture (2). These results are in accordance with our experiments, which indicate positive laminin cells after 10 days in culture and a negative reaction after 20 days. For glial cells cultures treated with 2mM LiCl, immunohistochemical reaction for laminin presents an altered distribution of these filaments. Laminin has been identified in perinuclear aggregates in vacuolised cells.

The potential role of glycogen synthase kinase-3β in modulating apoptosis was examined in human SH-SY5Y neuroblastoma cells. Although often considered a constitutively active enzyme, GSK-3β can be both activated and inhibited. Activation has been shown to occur subsequent to phosphorylation of Tyr-216 and recently by transient increases in intracellular calcium. Inhibition of GSK-3β can be induced by activation of the Wnt pathway or by agents, including lithium, that activate a signalling cascade that commences when growth factors or insulin bind to their respective receptors, resulting in the recruitment and activation of phosphatidylinositol-3 kinase (9).

The results presented here suggest that at 2 mM, LiCl the inhibition of GSK-3β expression is much accentuated and this correspond to the majority of papers which indicate GSK-3β as main target of lithium action.

Klein and Melton investigated in 1996 (10) the kinetic nature of the inhibition of GSK-3β by lithium, by measuring initial velocity as a function of substrate concentration at several concentrations of LiCl. The data suggest that lithium acts as an uncompetitive inhibitor of GSK-3β (Ki for LiCl = 2.1± 0.6 mM). Thus, inhibition of GSK-3β by lithium should not be overcome by increasing substrate concentration. The hypothesis that GSK-3β is the endogenous target of lithium action is supported by genetic data as well as in vivo biochemical data (11).

Since GSK-3β plays a critical role in the central nervous system by regulating various cytoskeletal processes as well as long-term nuclear events, its inhibition may underlie some of the long-term therapeutic effects of mood-stabilising agents (12).

There is a major interest in the development of novel, potent inhibitors of GSK-3, and most of the large pharmaceutical companies have a GSK-3 inhibitor development program. Unfortunately, the primary impetus for these inhibitors is not for the treatment of bipolar disorder, but rather for the treatment of others diseases, especially Alzheimer's disease and diabetes (2). ❑

CONCLUSION

The results of this study indicate the fact that lithium chloride and lithium mineral waters induce changes in cells, *in vitro*. Morphological and biochemical changes depend on the

concentration of lithium and on its time of action. Moreover, different types of cells tolerate lithium concentration levels in different ways.

Lithium has an intracellular target in GSK-3b. Lithium chloride and lithium mineral waters induce alterations in cells. The type of changes induced by lithium are similar to those determined by alteration of GSK-3b expression. Morphological and biochemical alterations depend on the concentration of lithium and on its time of action. Our study indicates the inhibition of GSK-3b expression at 2mM LiCl. Instead, Maria mineral water intensifies the expression of GSK-3b.

The use of lithium in medicine is a significant success in the field of inorganic pharmacology, and it is of particular interest since lithium is the lightest solid element whose chemistry is relatively simple. It may be assumed, therefore, that whatever lithium does, it acts on fundamental cell processes. ❑

ACKNOWLEDGEMENTS

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