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CYTOLOGICAL ASPECTS OF GLIAL CELLS AFTER TREATMENT WITH LITHIUM MINERAL WATERS

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ABSTRACT. Glial cells are no longer considered to be passive structural and trophic supports for neurons, but rather play important and integral roles in the physiological functioning of the central nervous system in both health and disease. Lithium effects on *in vitro* models have been the subjects of many studies since lithium was recognized as an effective drug in the treatment of bipolar disorder. The primary glial cells culture was initiated from the brain of Wistar rats aged 3-4 days. In Romania, there are some sources of mineral waters, which contain between 3-8 mg Li^+/l. One of these waters is Mary Source from Malnaş-Băi, with 8.03 mg Li^+/l. The purpose of this study is to observe how is changed the morphological aspect of cultured glial cells after treatment with lithium chloride or lithium mineral waters. After 12 days, morphological changes caused by lithium cytotoxicity occur in culture such as cell swelling, cytoplasm granulation, and vacuolisation. Using lithium mineral waters to a dilution of ¼, we obtained a positive effect for the development of glial cells culture. When we added 1mM LiCl and lithium mineral waters to a dilution of ¼, the effect was still positive for the culture. The efficacy of lithium mineral waters was tested by compeering control glial cells, cultivated in standard medium, with those cultivated in medium with lithium mineral waters or in the presence of lithium chloride in various concentrations.

KEY WORDS. lithium, glial cells, lithium mineral waters, bipolar disorder

INTRODUCTION

Lithium is the most commonly used drug for the treatment of manic-depressive illness. The precise mechanisms underlying its clinical efficacy remain unknown. At present, lithium is the standard prophylactic agent for bipolar disorder and is taken by 1% of the population. It is also effective against recurrent unipolar depression and is used in conjunction with antidepressants or as a temporary mood stabilizer between treatments. The molecular basis of these affective disorders and their relationship to the effects of lithium remain unknown. Lithium modulates signals impacting on the cytoskeleton, a dynamic system contributing to neural plasticity, at multiple levels, including glycogen synthase kinase -3β, cyclic AMP-dependent kinase, and protein kinase C, which may be critical for the neural plasticity involved in mood recovery and stabilization (7). Recent studies in the fields of cell and developmental biology provide new insights into the action of lithium and the aim of this article is to examine the results of lithium and lithium mineral waters treatment on glial cells, in the context of central nervous system.

In 1817, August Arfvedson discovered lithium while working in a Swedish laboratory. Berzelius named it lithion (Greek: lithos; stone). When people realized lithium could be found in mineral waters that have been used for healing, they started to connect lithium with the power to heal (Fieve, 1984). Garrod (1859) first described its medical use for the treatment of rheumatic conditions and gout and particularly mentions lithium use in “brain gout”, a depressive disorder.

Major depressive disorders have traditionally been considered to have a neurochemical basis, but recent studies have associated these complex disorders with regional reductions in central nervous system volume, as well as in the numbers and/or size of glial and neurons in discrete brain areas (10).

Since its discovery, lithium has been shown to act upon various neurotransmitter systems at multiple levels of signalling in the brain. Lithium, affecting each neurotransmitter systems within complex interactivity neuronal networks, is suggested to restore the balance among aberrant signalling pathway in critical regions of the brain. Lithium action has been tested in a number of different systems such as fibroblasts, kidney and mammary gland epithelial cells, leukemic cells, carcinomas, pheochromocytomas, adipocytes, neural cells, thyroid cells and parathyroid cells (2).

Many of the proposed mechanisms for lithium cellular action have suggested an inhibitory effect on components of various signalling pathways, such as cyclic AMP formation, cyclic GMP formation, G proteins, or inositol phosphate metabolism (3).

Lithium has been shown to be an inhibitor of a number of structurally similar magnesium – dependent phosphomonoesterases at concentrations values within the therapeutically relevant range of concentrations (0,2 – 1,5 mM). Biochemical and genetic studies subsequently identified the upstream inositol polyphosphatase as an additional target for lithium (8).

Critical to the attribution of any observed biochemical effect to therapeutic relevance is the observation that the action of lithium to stabilize mood cycling of

bipolar illness requires a lag period for the efficacy to occur and is not immediately reversed upon discontinuation of treatment. Thus, the mechanisms of action of lithium appears to involve its long term effects in stabilizing mood in manic depressive patients, perhaps by resetting ion homeostasis or neurotransmitter receptor balance (13).

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 (11). After the meninx is removed, the brain is passed through a 60μm 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, 100 μg/ml streptomycin and 50 μg/ml neomycin (Sigma). The medium was supplemented with 15% calf fetal serum (Gibco). The medium was first replaced after 24 hrs and then every three days. The primary cells culture will be maintained in a humidified atmosphere, at 37^0C, with 5% CO_2.

Five culture lots have been used in the study: cultures treated with 1mM LiCl, cultures treated with 2mM LiCl, cultures treated with ¼ Mary mineral water, cultures treated with ¼ Mary mineral water supplemented with 1mM LiCl, and control cultures.

The efficacy of lithium mineral waters was tested by compeering control glial cells, cultivated in standard medium, with those cultivated in medium with lithium mineral waters or in the presence of lithium chloride in various concentrations.

Behaviour characterization of glial cells in the presence of lithium mineral waters and lithium chloride has been done both morphologically and immunohistochemically. The morphological aspects of cells, in control, lithium-treated and mineral water-treated, have been studied by means of light microscopy after staining with haematoxylin-eosin. For the immunohistochemical evaluation were used some markers for glial cells such as GFAP, vimentin and laminin.

Immunohistochemical detections of GFAP, vimentin and laminin were 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 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 counterstained with Harris hematoxylin.

The chemical content of Mary lithium mineral water were made 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 Mary water was assured by micro-filtration.

RESULTS

Lithium mineral water from Mary 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 Mary has resulted in protection of cells by the effects of lithium as shown in **figure 2.** Glial cells have a normal aspect and their cytoplasmic projections are very clear.

Culture observations by means of phase contrast 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. In the lot treated with 1mM LiCl and in the control lot, the evolution is similar, although no manifestations attributed to lithium toxicity.

Immunodetection of GFAP shows a weak reaction for control glial cells culture – **figure 4**. Treatment with Mary mineral water does not change this pattern – **figure 5**. Instead, 2mM LiCl determine an intensification of GFAP expression – **figure 6**.

Vimentin is very well expressed in glial cells culture and our results reflect this aspect very clear in **figure 7.** Affected disposition and altered quantity of vimentin occur in glial cells culture after treatment with 2mM LiCl, fact shown in **figure 9.** Mary 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 8.**

After 10 days in culture, immature astrocytes show an intense reaction to laminin, **figure 10**. Besides, for some cells the laminin localization was seen on the cytoplasmic projections.

In glial cells cultures treated with Mary water, laminin was detected perinuclear, **figure 11** 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 12**.

DISCUSSION

A major challenge in modern biology is to understand the basic mechanism controlling both neural activity and pattern formation during early development (3). 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 (12) of the cell –**diagram 1**. The fact that with mineral waters many factors are implied is evident for the cell physiology, but the 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.

In the case of other types of neural cells (PC12), lithium (5-10 mM) determines cell survival in the short term (2 days), in the absence of serum and NGF (2). The same authors show that treatment of immature granular cells with 10mM lithium results in cellular apoptosis. After maturation *in vitro*, certain lithium concentrations are likely to extend cell survival. These alterations of cell morphology may be because lithium interferes with the phosphoinositols cycle, altering the amount of free inositol and thus modifying membrane architecture (1,5)

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 (6). In control glial cells cultures, immunodetection of GFAP shows a weak reaction. Treatment with Mary mineral water does not change this pattern. Instead, 2mM LiCl determine an intensification of GFAP expression.

In the neuroectoderm, vimentin is a specific marker for astrocytes and ependymal cells. It is expresed 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 (14). Our results reflect an affected disposition and altered quantity of vimentin in glial cells culture after treatment with 2mM LiCl**.** Mary 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, 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 (9). These results are in accordance with our experiments, which indicate positive laminin cells after 10 days in culture 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.

CONCLUSIONS

The results of this study indicate the fact that lithium chloride and lithium mineral waters induce alterations in cells, *in vitro*. Morphological and biochemical alterations depend on the concentration of lithium and on its time of action. Moreover, different types of cells tolerate lithium concentration levels in different ways.

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, its action is on fundamental processes. For this reason, it may be important as a probe to investigate the molecular interactions of more complex drugs with their receptors. If we can discover whatever it is that lithium does at molecular level, which makes it so effective in psychiatry, we may gain insights into the most basic features of the cellular response to drugs. Lithium does not have a large and convoluted structure, which can make multiple contacts with receptors, which may lead to modification of receptor activation. Whatever lithium does, it achieves because it is a highly charged cation with a large hydrated radius and chemical properties, which are similar to magnesium (4).

ACKNOWLEDGEMENTS

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Table 1. Chemical content of Mary mineral water

		Content in 1Kg of water				
		mg	**mM**	**m.eq.**	**mg %**	**m eq. %**
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**	

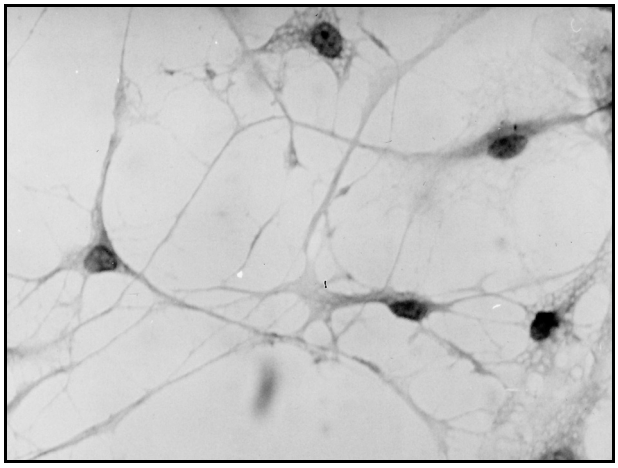

Fig 1. *Glial cells culture after 20 days, hematoxylin-eosin, x400*

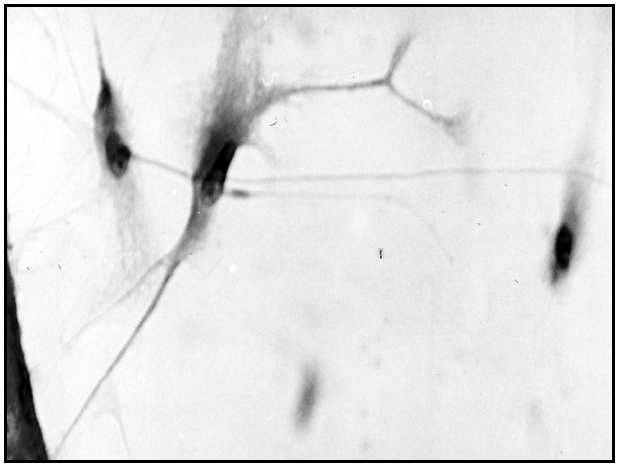

Fig 2. *Glial cells after treatment with Mary mineral water for 20 days, hematoxylin-eosin, x400*

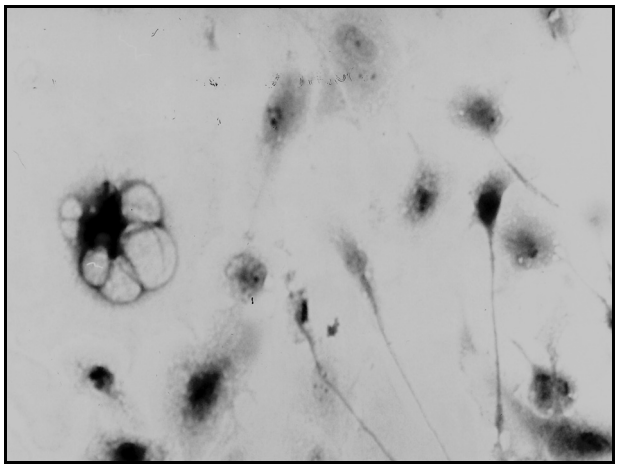

Fig 3. *Vacuolised glial cell after treatment with 2mM LiCl for 10 days, hematoxylin-eosin, x250*

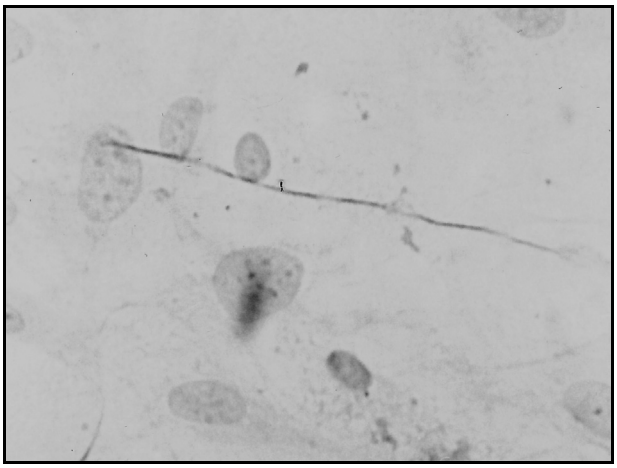

Fig 4. *GFAP positive glial cells culture after 10 days, x400*

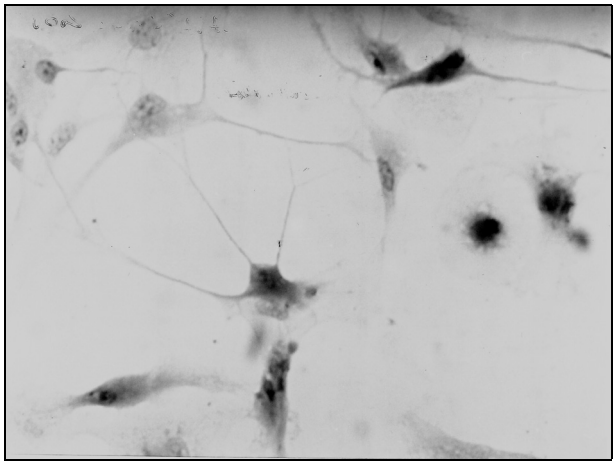

Fig 5. *Low GFAP reaction for glial cells after treatment with Mary mineral water for 10 days, x400*

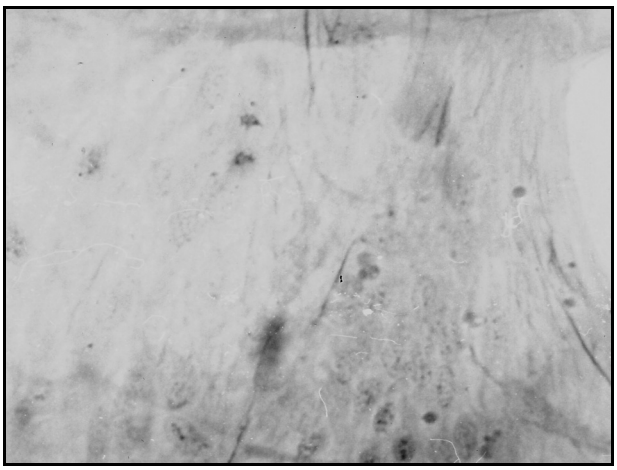

Fig 6. *GFAP localization in glial cells treated with 2mM LiCl for 10 days, x400*

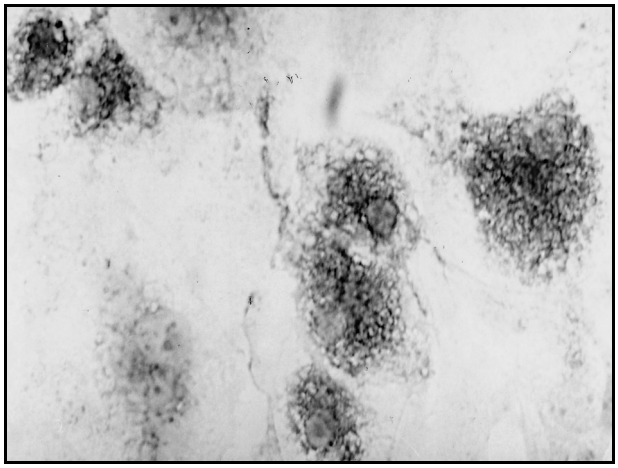

Fig 7. *Vimentin localization in control glial cells after 10 days, x400*

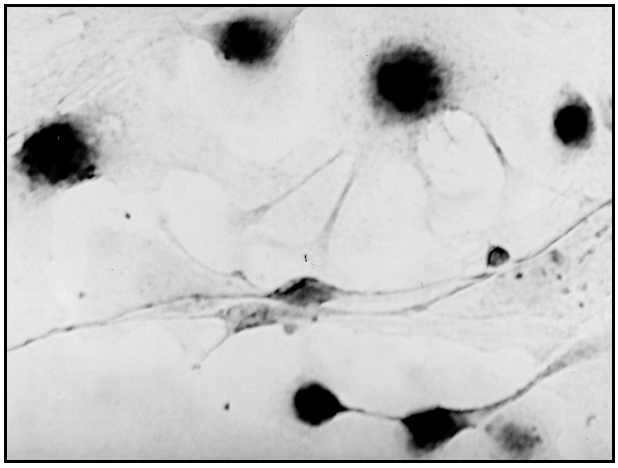

Fig 8. *Vimentin localization in glial cells treated with Mary water for 10 days, x400*

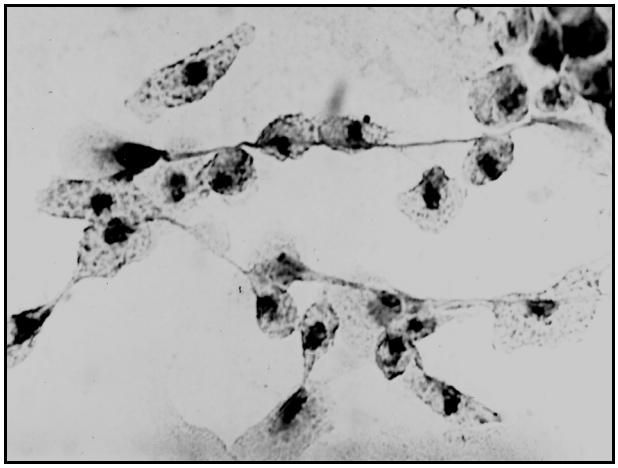

Fig 9. *Vimentin citoplasmatic localization in glial cells treated with 2mM LiCl for 10 days, x400*

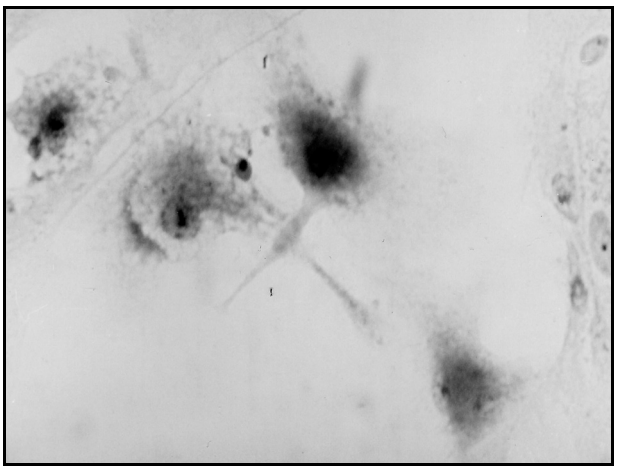

Fig 10. *Laminin citoplasmatic localization in control glial cells after 10 days, x400*

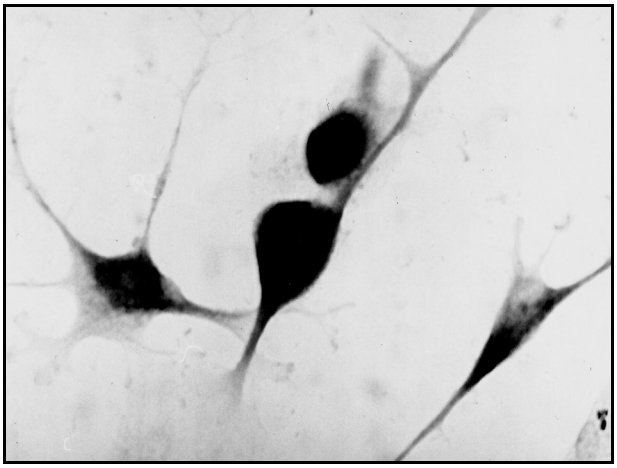

Fig 11. *Laminin localization in glial cells treated with Mary water for 10 days, x400*

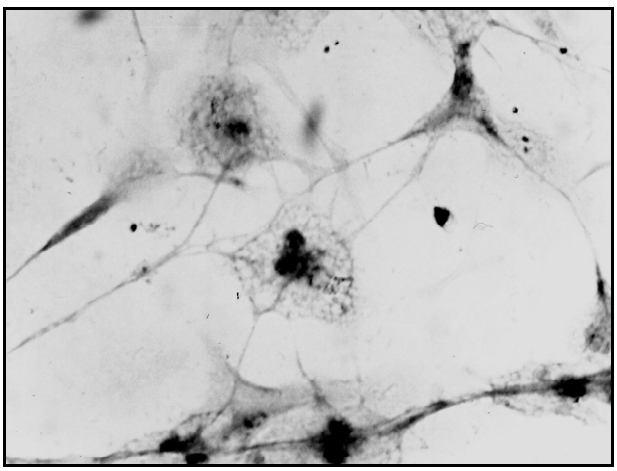

Fig 12. *Altered laminin localization in glial cells treated with 2mM LiCl for 10 days, x400*

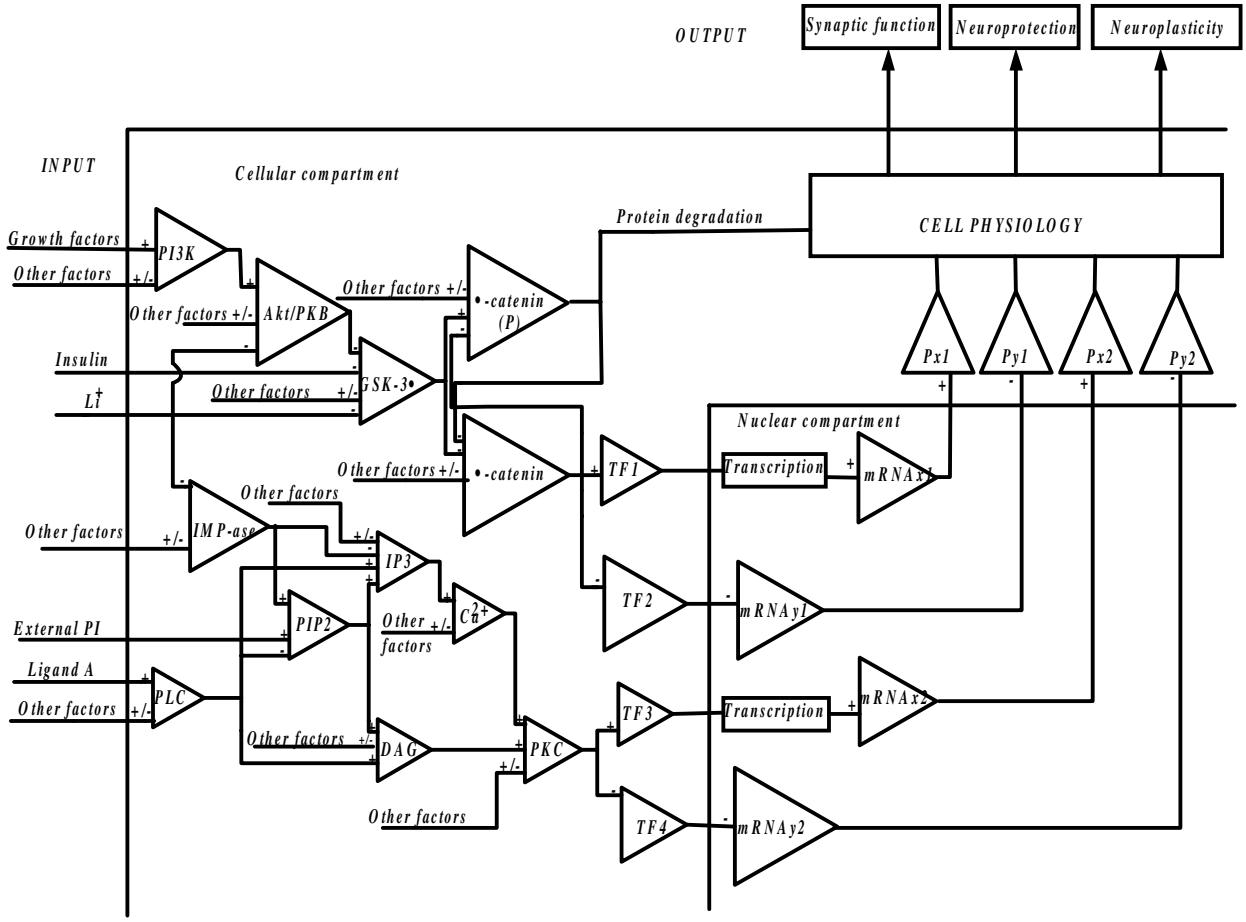

Diagram 1. *Cybernetic model for the lithium action on cell level*