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LITHIUM MINERAL WATERS EFFECT ON WNT PATHWAY SIGNALLING OF GLIAL CELLS

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ABSTRACT. Lithium adjusts signalling activities regulating second messengers, transcription factors, and gene expression. The neurotrophic intermediary molecule GSK-3 was identified in 1996 as the target of lithium responsible for the developmental effects in *Xenopus* embryos. GSK-3 plays a broad role in cellular metabolism, including contributions to signalling activities, growth, and differentiation. GSK-3β has been shown to phosphorylate numerous substrates, including several transcription factors such as c-jun, c-myc, heat shock factor 1, cytoskeletal proteins such as the microtubule-associated protein tau, and the multifunctional protein β-catenin. The fact that inhibiting GSK-3 results in antiapoptotic effects strongly implicates this enzyme as mediator of lithium's neuroprotective effects. 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. Treatment of these cultures with lithium mineral waters from Mary Source compared to treatment with lithium chloride 2mM showed significant differences in cell morphology. Immunohistochemical studies for GSK-3β supported the protective effects of lithium mineral waters for glial cells, instead lithium chloride 2mM determined cytotoxic effects and inhibited Wnt signalling pathway. The immunohistochemical reaction for GSK-3β was absent in this case.

KEY WORDS. glial cells, lithium mineral waters, Wnt signalling

INTRODUCTION

Lithium occurs naturally in biological tissues and hence in foodstuffs, drinking water. Natural waters containing high concentrations of this and other metals are sold as "mineral waters" with supposed medicinal properties (2).

The effect of lithium treatment on neural cytoskeleton has been investigated in detail (9). 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" (10).

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, diverse, signalling pathways, a function that is undoubtedly critical in the CNS (3).

This serine / threonine protein kinase family is highly conserved throughout the eukaryotes, and is found as two isoformes, GSK-3α and GSK-3β, in vertebrates. Enzyme kinetic experiments suggest that again Li^+ inhibits through competition for Mg^{2+}. GSK-3 enzymes in vitro require a high Mg^{2+} concentration for maximal activity, the free Mg^{2+} concentration in the cell is much lower than this (0.5-1.5mM) and at these lower concentrations Li^+ gives half-maximal inhibition at 0.8mM – a good fit to the therapeutic concentration range (4).

More long-term cytoskeletal changes are seen in response to Wnt ligand mediated regulation of GSK-3. This does not involve changes in GSK-3 phosphorylation, but instead controls formation of a complex between GSK-3 and the scaffold protein Axin. When Wnt binds a receptor complex between Frizzled and LRP 5 or 6, it mediates dissociation of GSK-3 from Axin in a process that requires the protein Dvl. Wnt stimulation, or Li^+ treatment, alters microtubule dynamics in neuronal growth cones and increases branching along the axon. This requires both Dvl and Axin proteins and via a mechanism not yet understood regulates GSK-3 phosphorylation of the microtubule binding protein, MAP1B. Wnt proteins also interact with an atypical tyrosine kinase receptor, Ryk, to control neuronal out growth

and axon guidance. The relationship of this Wnt pathway to regulation of the cytoskeleton is not known, but Wnt stimulation Ryk appears to activate the Wnt-Frizzled pathway (6) – **figure10.**

Altered GSK-3 activity could therefore regulate both cell number and morphology in the brain. Potentially, this could have profound effects on the complexity of neuronal interactions, although this has yet to be demonstrated. Recent results however have shown that Li^+ and specific GSK-3 inhibitors cause similar changes in behaviour of mice and rats to that seen with antidepressant treatment (4).

MATERIAL AND METHODS

The primary glial cells culture was initiated from the brain of rat pups aged 1-3 days, in keeping with the known techniques (8). After the meninx is removed, the brain is passed through a 60µm nytex and the cells thus obtained are directly plated on Petri plates 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 hours 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: control cultures, cultures treated with 1mM LiCl, cultures treated with 2mM LiCl, cultures treated with ¼ Mary mineral water, cultures treated with 1mM LiCl and with ¼ Mary mineral water.

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. LDH activity will be assessed in the culture medium and in the cell homogenate by means of a DiaSys kit. Results were expressed as percentage of released LDH.

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.

Immunohistochemical detections of GSK-3β 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 over night at 4^0C with primary antibodies: anti-GSK-3β goat polyclonal antibodies (Santa Cruz Biotechnology)– dilution 1:100. The antibodies were diluted in PBS with 2% bovine serum albumin. After washing with PBS, glial cells were reincubated 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. 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.

When the medium was prepared with Mary mineral water and was added 1mM LiCl, glial cells showed an intense apoptotic process, fact identified in **figure 3**.

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 4**. 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 GSK-3β shows a moderate reaction for control glial cells culture - **figure 5**. This protein is situated more often in a limited cytoplasmic area near nucleus.

Treatment with Mary mineral water does not change this pattern, **figure 6**. In addition, 1mM LiCl and Mary mineral water ¼ maintain the same distribution for GSK-3β in glial cells, **figure 7**. Instead, 2mM LiCl determine the absence of GSK-3β expression, **figure 8**.

Table 2 presents the cell released LDH. In the glial cells cultures medium appears an increase in LDH released after the culture treatment with LiCl 2mM. The cells treated with Mary mineral water present the same values for released LDH as the control, **figure 9**. These data are in agreement with the morphological aspects of cultures. It is supposed that the increase in LDH concentration in cells reflects an intensification of glycolysis in order to compensate the deficiency in oxidative phosphorylation.

DISCUSSION

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 (1).

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.

The kinetic nature of the inhibition of GSK-3β by lithium was investigated by Klein and Melton, 1996, 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 (5).

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 (7).

There exists 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 (9).

Treatment of glial cells cultures with Mary mineral water does not determine an intense inhibition of GSK-3β. The total amount of lithium is below the toxic dose, and the Wnt signalling pathway is not profoundly altered in this case.

CONCLUSIONS

The results of this study indicate the fact that lithium has an intracellular target represented by GSK-3β. 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-3β expression. Morphological and biochemical alterations depend on the concentration of lithium and on its time of action.

Our study clearly indicates the inhibition of GSK-3β at 2mM LiCl. Instead, Mary mineral water does not affect the expression of GSK-3β.

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**	

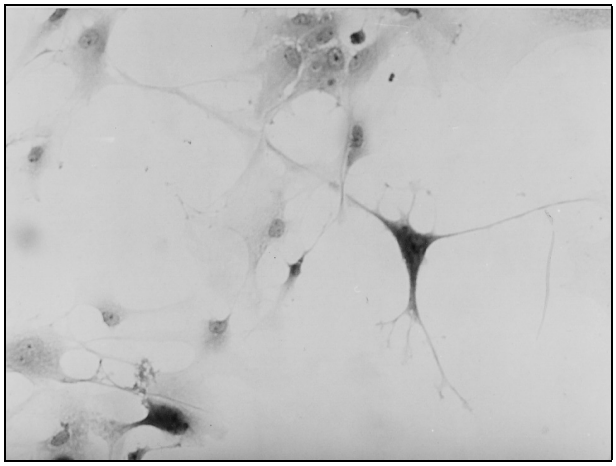


Fig. 1. *Glial cells culture after 10 days, hematoxylin-eosin, x250*

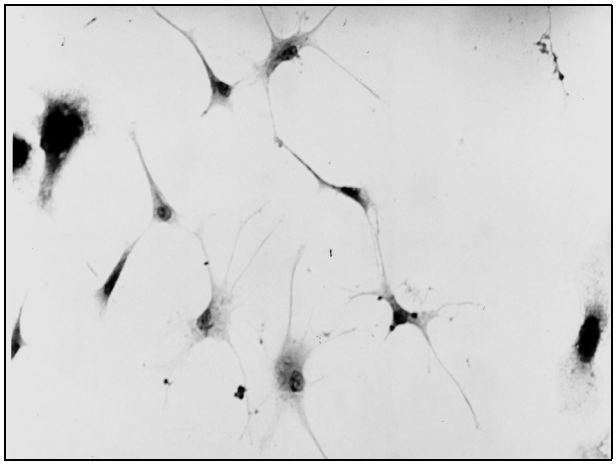


Fig. 2. *Glial cells treated with Mary mineral water for 10 days, hematoxylin-eosin, x250*

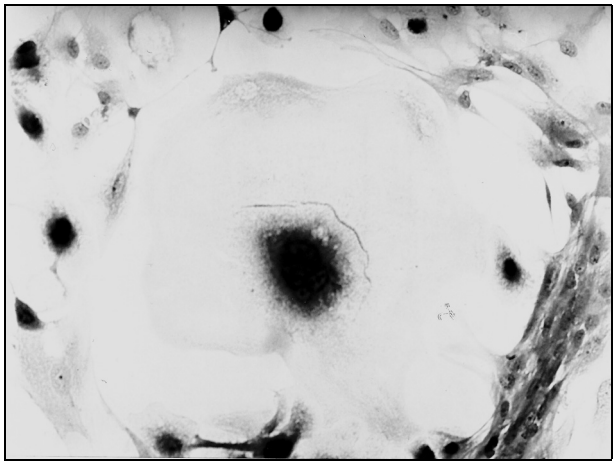


Fig. 3. *Alterated glial cell treated with Mary mineral water and 1mM LiCl for 10 days, hematoxylin-eosin, x250*

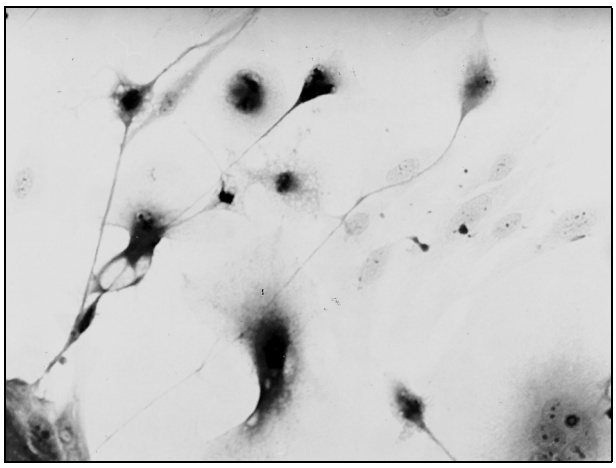


Fig. 4. *Glial cell after treatment with 2mM LiCl for 10 days, hematoxylin-eosin, x250*

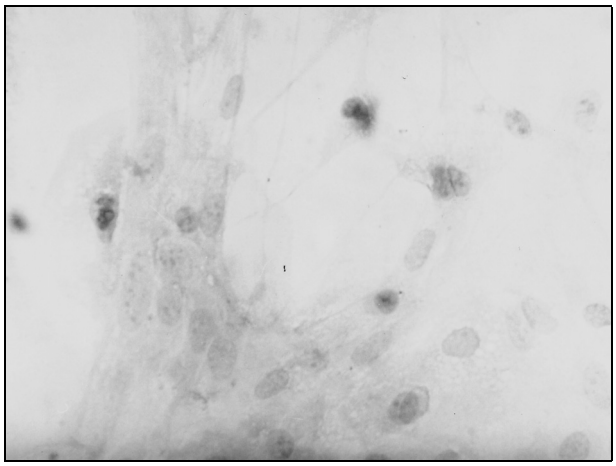


Fig. 5. *Low GSK-3β reaction for control glial cells after 10 days, x400*

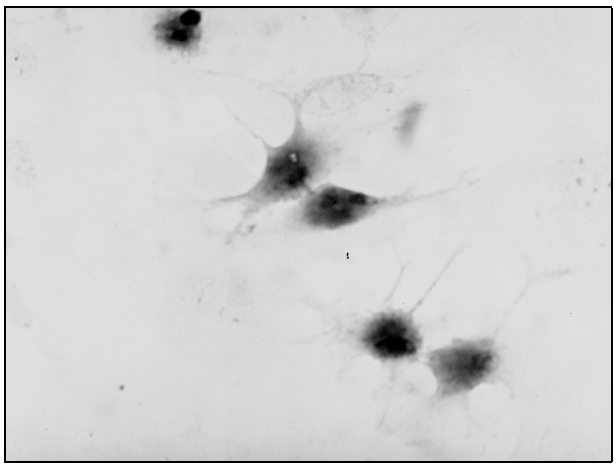


Fig. 6. *GSK-3β citoplasmatic localization in glial cells treated with Mary mineral water for 10 days, x400*

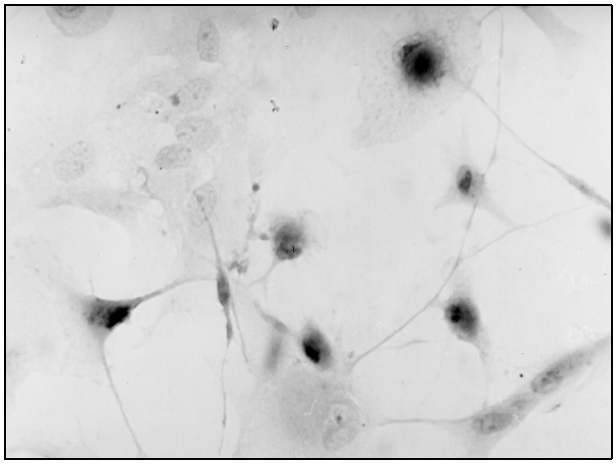


Fig. 7. *Low GSK-3β reaction in glial cells after treatment with Mary mineral water and 1mM LiCl for 10 days, x400*

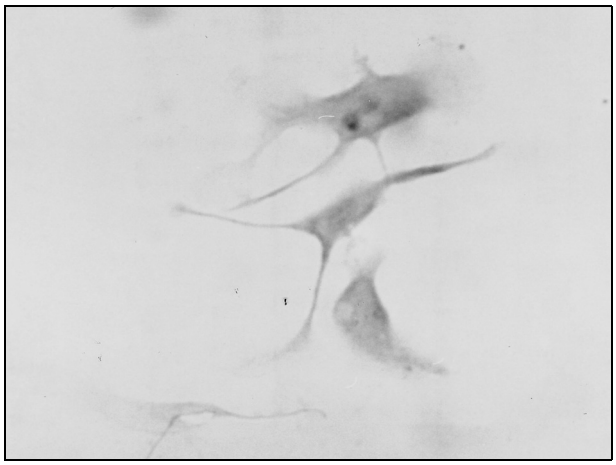


Fig. 8. *GSK-3β negative reaction in glial cell after treatment with 2mM LiCl for 10 days, x400*

Table 2. Released LDH in glial cells culture medium after treatment with lithium mineral water or lithium chloride

Released LDH	Control	Mary mineral water	Mary mineral water + LiCl 1mM	LiCl 1 mM	LiCl 2 mM
Experiment 1	105	110	128	162	170
Experiment 2	123	130	121	165	188
Experiment 3	120	133	124	163	179
Average	116	124,3333	124,3333	163,3333	179

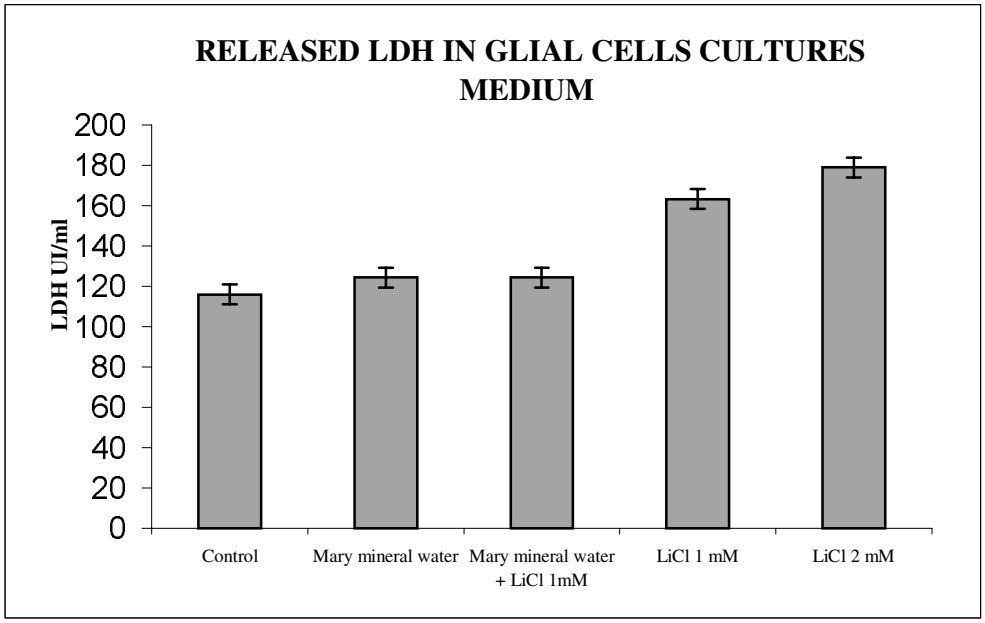
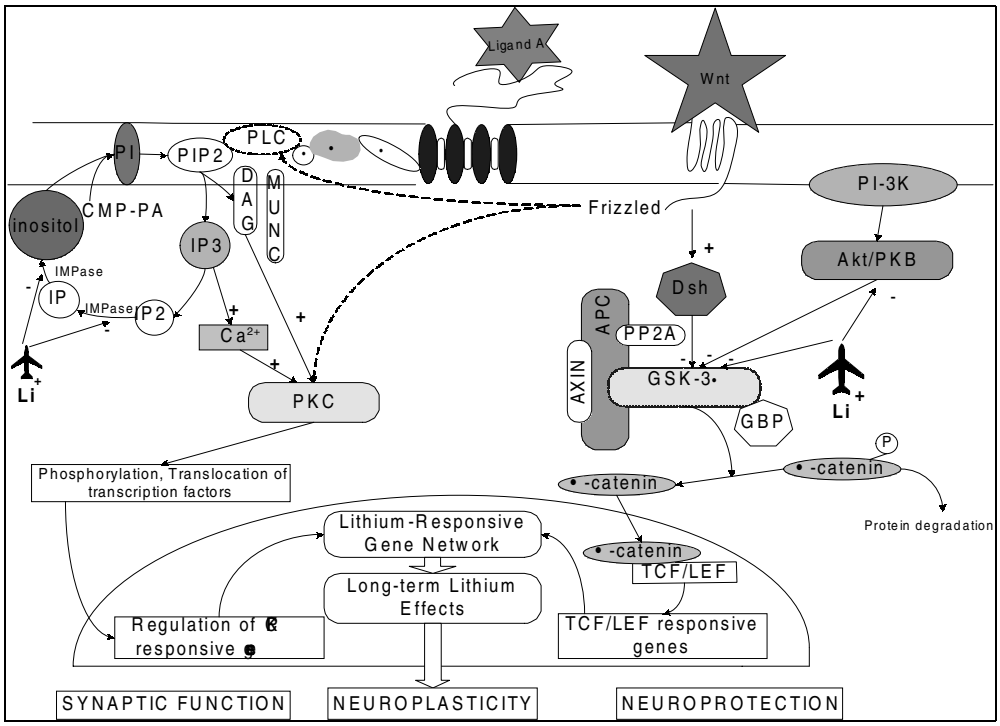


Fig. 9. *Densitogram of released LDH in glial cells culture medium after treatment with lithium mineral water or lithium chloride*



Adapted after Lenox and all, 2003

Fig. 10. Bio*logical model of lithium action at the cellular level*.