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ORIGINAL PAPER

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Lithium reduces Gsk3b mRNA levels: implications for Alzheimer Disease

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Abstract *Background* There is evidence of increased systemic expression of active GSK3B in Alzheimer's disease patients, which apparently is associated with the formation of senile plaques and neurofibrillary tangles. Due to its central role in the pathogenesis of AD, GSK3B is currently a promising target of the pharmaceutical industry. Whilst trials with specific GSK inhibitors in AD are under way, major attention has been focused on the neuroprotective effects of lithium. Whereas the direct and indirect inhibitory effects of lithium over GSK3 activity have been documented by several groups, its effects over *Gsk3* transcription have not yet been addressed. *Methods* We used quantitative PCR to evaluate the transcriptional regulation of *Gsk3a* and *Gsk3b* in lithium-treated primary cultures of rat cortical and hippocampal neurons. *Results* We found a significant and dose-dependent reduction in the expression of *Gsk3b*, which was specific to hippocampal cells. This same effect was further confirmed *in vivo* by measuring Gsk3 expression in different brain regions and in peripheral leukocytes of adult rats treated with lithium. *Conclusion* Our studies show that LiCl can modulate *Gsk3b* transcription *in vitro* and *in vivo*. This observation suggest new regulatory effects of lithium over *Gsk3b*, contributing to the better understanding of its mechanisms of action, offering a new and complementary explanation for *Gsk3b* modulation and reinforcing its potential for the inhibition of key pathological pathways in Alzheimer's disease.

Key words lithium · *Gsk3a* · *Gsk3b* · Alzheimer disease · transcription · hippocampus

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Introduction

Glycogen synthase kinase-3 (GSK3) is a serine–threonine kinase that intermediates various intracellular signaling pathways. Although the two GSK3 isoforms A and B have subtle differences, including sub-cellular localization and certain specialized functions [13, 26], they also share a number of similar properties. GSK3 has been shown to phosphorylate several brain proteins, including amyloid precursor protein (APP) [1], neurofilaments [10] and the microtubule-associated protein Tau [23]. It has been suggested that a dysfunction of GSK3 A and B could play a role in diseases that are characterized by the deposition of amyloid plaques and/or the aggregation of Tau in hyper-phos-

phorylated state, such as Alzheimer's disease (AD), fronto-temporal dementia (FTD) and other tauopathies. In patients with AD, there is evidence of increased systemic expression of active GSK3B [14], and in animal and *in vitro* models of AD, GSK3B appears to be associated with biological mechanisms that lead to the formation of senile plaques and neurofibrillary tangles.

The over-expression of GSK3B in transgenic mice is associated with many neuropathologic features linked to neurodegeneration [7] and cognitive impairment [11] probably derived from axonal transport defects, that lead to abnormal behavioral phenotypes [19]. Interestingly, most of the structural and functional deficits associated with overactive Gsk3b can be rescued or attenuated by its inhibition [12].

In view of its central role in the pathogenesis of AD, GSK3B is currently one of the most promising targets in the development of pharmaceutical compounds with potential disease-modifying properties. Numerous intracellular signaling pathways converge on GSK3 and regulate its activity via inhibitory serine-phosphorylation [24]. Whilst trials with specific GSK3 inhibitors in AD are under way, major attention has been focused on the neuroprotective effects of lithium, one of the most used and studied GSK3B inhibitors. Lithium inhibits GSK3 activity *in vitro* at concentrations of 1.0–2.0 mM, within the plasmatic therapeutic window required for clinical use, i.e., 0.6 to 1.2 mEq/L [16, 23]. Lithium prevents the accumulation of amyloid-β in several cell culture models, an effect attributed to the inhibition of GSK3 [25]. Phiel et al. [22] showed that a three-week treatment with lithium significantly decreased the levels of amyloid-β peptide in the brains of the AD model APP-Swedish/Tg2576 mice and suggested that GSK3A is involved in the amyloidogenic cleavage of the APP. Thus the inhibition of GSK3 reinforces the potential use of lithium in the treatment and, possibly, in the prevention of AD. In fact, we have recently provided the first clinical evidence that chronic LiCl treatment may reduce the risk for AD, in a sample of elderly patients with bipolar disorder [21].

The inhibitory effects of lithium on GSK3 activity have been documented by several groups [2, 6, 15, 16, 23]. It is accepted that lithium inhibits GSK3 directly and indirectly. In the direct inhibition lithium competes with magnesium at the cationic binding site of GSK3, which is a requirement for enzymatic activation. Some authors suggest that the indirect inhibition of GSK3 by lithium could involve the activation of protein kinase B (AKT/PKB), usually in response to insulin or insulin growth factors, leading to the phosphorylation of single serine residues (serine-21 for GSK3A; serine-9 for GSK3B) of its regulatory aminoterminal domain [4, 8, 28].

To the best of our knowledge, the effects of lithium on the mRNA levels of Gsk3 have not yet been addressed. To investigate this question we used quantitative PCR (qPCR) to evaluate the transcriptional levels of *Gsk3a* and *Gsk3b* in lithium-treated primary cultures of rat cortical and hippocampal neurons. We found a significant, dose-dependent and localized reduction in the expression of *Gsk3b*, without significant alterations of mRNA levels of other genes of this same pathway. The findings of lithium effects over *Gsk3* were then confirmed *in vivo*, after measuring the expression of these genes in LiCl-treated adult rats, which reinforced the veracity of this regulation.

Methods

All procedures involving laboratory animals were approved by the local Animal Care Committee and conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" (ISBN 0-309-05377-3, NIH publication No. 86-23, revised 1985). All experiments were performed at the Laboratory of Neuroscience (LIM-27) at the Institute of Psychiatry, University of Sao Paulo, Brazil.

Treatment of primary cultures of rat cortical and hippocampal neurons with LiCl

Wistar rats were sacrificed at gestational day 18 (E18), and their embryos were collected by laparotomy. Whole embryonic brains were isolated and maintained immersed in Hank's balanced salt solution (HBSS—Gibco BRL, USA). Cortices and hippocampi were dissected, and fragments were dissociated by trypsinization, followed by mechanical dissociation in Hank's solution containing inactivated horse serum (10%), Hepes buffer (10 mM), DNAse (10 μg/ml) and $MgCl_2$ (8 mM). Cells were counted and re-suspended in neurobasal medium containing B-27 supplement (Gibco BRL), glutamine (2 mM), penicillin (100 I.U.), streptomycin (100 μg/ml), and 5% fetal calf serum (Gibco BRL) and plated onto poly-D-lysine coated dishes (1×10^7 cells/plate). Culture medium was replaced by serum-free medium after 24 h of incubation and half-changed every 48 h. Cells were incubated at 37°C and 5% CO_2. Neuronal viability was microscopically ascertained prior to experimentation. After 5 days in culture (DIC), cultures were incubated for another 5 days with different concentrations of LiCl (Merck, Germany) ranging from 0.02 to 2 mM. All results presented here were derived from three independent primary neuronal cultures, and all real-time qPCR were performed in triplicates.

Treatment of adult rats

The effects of LiCl treatment on the expression of *Gsk3* were also investigated *in vivo*. Twelve male Wistar rats, 2.5–3 months old were obtained from the Central Animal Laboratory House (UNIFESP, Brazil), housed in polypropylene cages maintained at 22 ± 2°C and 12-h light/dark cycles, and provided with a standard diet and free access to water. After 2 weeks of acclimatization, animals were divided in three groups of four animals, and each group was treated twice a day with 1 ml intra-peritoneal injections of 0.9% NaCl (saline control) or LiCl dissolved in saline, in the final concentrations of 5 mg (0.12 mMol) or 10 mg (0.24 mMol) of LiCl/Kg of body weight. No anesthetics were used for the injections and all animals were treated for 35 consecutive days. During this period, blood samples were collected once a week (tail cuff method), 12 h after the last administration and immediately before the next one, and used for determining serum lithium levels. After 35 days, blood was collected, animals were sacrificed by decapitation, following the immediate removal of the frontal cortex and both hippocampi, which were then stored at −80°C for further mRNA analysis.

Table 1

Gene	Forward (5′–3′)	Reverse (5′ – 3′)
Gsk3a	TCAAGGCTCTCCCCACTAGA	GTGAGGAGGGATGAGAATGG
Gsk3b	AGCCTATATCCATTCCTTGG	CCTCGGACCAGCTGCTTT
Actb	GTCTTCCCCTCCATCGTG	AGGATGCCTCTCTTGCTCTG
Npy	GAGGACATGGCCAGATACTACTC	GCATTTTCTGTGCTTTCTCTCA
Hprt	GCCCTTGACTATAATGAGCACTTCAG	GTAGATTCAACTTGCCGCTGTCTT
Bace1	CCCTACACCCAGGGCAAGTG	CCAATGATCATGCTCCCTCC
Psenen	CAGAGCCAAATCAAAGGCTA	GAAGGAGAGGTAGTCCCCAAG
Ctnnb1	CTCGCTCGGGATGTTCACA	ACAGAGGACCCCTGCAGCTA
Wnt7a	GGGTGCGAGCATCTGTA	ATTGCGGAACTGAAACTGACACTT
Tau	AAGACAGACCATGGAGCAGAAATC	CGGCTAACGTGGCAAGCT
Akt1	CGTGTGGCAAGATGTGTATGAGA	TGAGCTGTGAACTCCTCATCAAA
Inpp1	CAGTATATCAAAGGTTCTGCCAATGT	GGACGCAAACGGCTGATT

mRNA extraction and cDNA synthesis

For each treatment group, three distinct primary neuronal cultures or samples of four rat brains were homogenized in TRIzol® Reagent (Gibco BRL) and RNA was prepared according to Chomczynski and Sacchi [5]. For RNA extraction of leukocytes, white blood cells were disrupted in RLT buffer (Qiagen, Valencia, CA, USA), homogenized with a QIAshredder and RNA was purified using the QIAamp ® RNA Blood Kit (Qiagen). cDNA synthesis was performed in a 20 µl reactions containing 2 µg of total RNA, 110 pmol of oligo $dT_{12\text{-}18}$, 3 mM $MgCl_2$, 0.5 mM of each dNTP and 200 units of ImProm-II™ Reverse Transcriptase (Promega, Madison, WI-USA).

Gene expression analyses by quantitative PCR

Oligonucleotide primers were designed using the software Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3). To reduce genomic DNA amplifications, primers were designed to anneal to different exons of the studied genes. Repetitive regions were masked (http://www.repeatmasker.org) and duplicates of 10 ng total RNA equivalents of cDNA were used in each amplification, together with 0.25 pmol of each primer. qPCR was performed in an Applied Biosystems 7500 Real-Time PCR System using SYBR Green I (Applied Biosystems, USA) in a final volume of 15 µl. The thermal amplification profile consisted of 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Each assay included a no-template control as well as three endogenous control genes for normalization (*Actb*, *Npy* and *Hprt1*—selected by geNorm) [27]. The sequences of the oligonucleotide primers used in this study are presented in Table 1. Fluorescence was continually monitored during the melting process and no nonspecific amplification products or primer-dimer artifacts were detected.

Data analyses were performed using the Sequence Detection Software, version 1.3.1 (Applied Biosystems). Relative gene expression levels were normalized to the geometric mean of the endogenous controls [27].

Statistical analysis

The experimental results are expressed as means ± S.E.M. with the number of experiments indicated. One-way ANOVA followed by correction using post hoc Tukey was used to determine significant differences in the *in vitro* and *in vivo* studies. $P \leq 0.05$ were considered to be statistically significant.

Results

Effects of LiCl on GSK expression in cultured neurons

Baseline mRNA levels indicative of *Gsk3* expression were significantly different according to the brain areas from which cultures were started. Primary cortical neurons displayed a 1.6-fold higher expression of *Gsk3a* and a 2.3-fold higher expression of *Gsk3b* as compared to primary hippocampal neurons. The results of three independent cultures of cortical and hippocampal neurons showed that the treatment with LiCl for 5 days resulted in a statistically signifi-

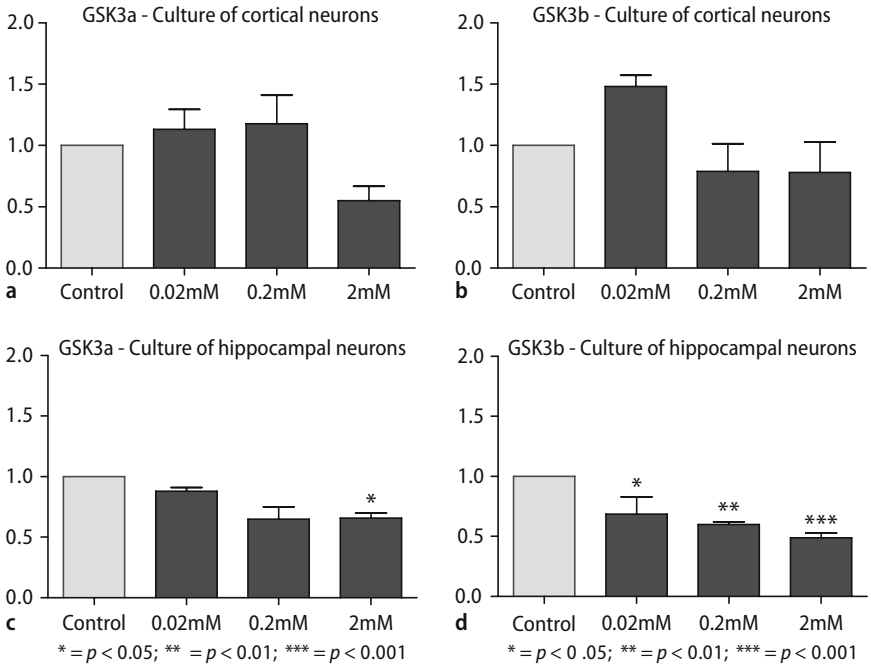

Fig. 1 Effects of LiCl treatment on *Gsk3a* and *Gsk3b* mRNA levels in primary cultures of hippocampal and cortical neurons, as compared to untreated controls. Three distinct primary cultures of neurons were incubated for five days, in triplicates, with different concentrations of LiCl, ranging from 20 nM to 2 mM. Total RNA was extracted and expression levels of *Gsk3a* and *Gsk3b* were measured in triplicates by qPCR. Data are presented as the means ± S.E.M. and *bars* represent the percent changes in mRNA expression for each experimental condition, after normalizing by the average expression of three endogenous controls, as compared to controls (100%). ANOVA analysis showed that no significant alterations could be observed for any of the genes in cortical neurons (**a**, **b**) or in hippocampal *Gsk3a* (**c**). However, LiCl treatment resulted in a significant decrease in *Gsk3b* mRNA levels hippocampal cultures compared to controls (**d**). *$P = 0.001$ for LiCl 2 mM; $P = 0.004$ for LiCl 0.2 mM and $P = 0.024$ for LiCl 0.02 mM

cant reduction in the *Gsk3b* mRNA levels in cultured hippocampal, but no inhibition was found in cortical neurons. The findings not only suggest a region-specific effect of lithium, but the results clearly demonstrate a dose-dependent effect, which reinforces the veracity of these findings. While no alterations could be seen in cortical cells, hippocampal neurons treated with LiCl showed significant reductions of mRNA levels of *Gsk3b*. LiCl concentrations of 2 mM, 0.2 mM and even 0.02 mM resulted in significant reductions of *Gsk3b*, respectively reducing the mRNA levels in 50 ± 7% ($P = 0.001$), 40 ± 4% ($P = 0.004$) and 32 ± 20% ($P = 0.024$). No significant alterations in *Gsk3b* mRNA could be seen when 0.002 mM of LiCl was used (data not shown). For *Gsk3a*, the only alteration observed was when the higher dose of LiCl (2 mM) was used to treat hippocampal neurons in culture. In this situation, a significant reduction (34 ± 5%, $P = 0.03$) was observed for this gene. Overall, the experiments in culture showed a consistent effect of LiCl leading to a reduction of *Gsk3b* expression (Fig. 1).

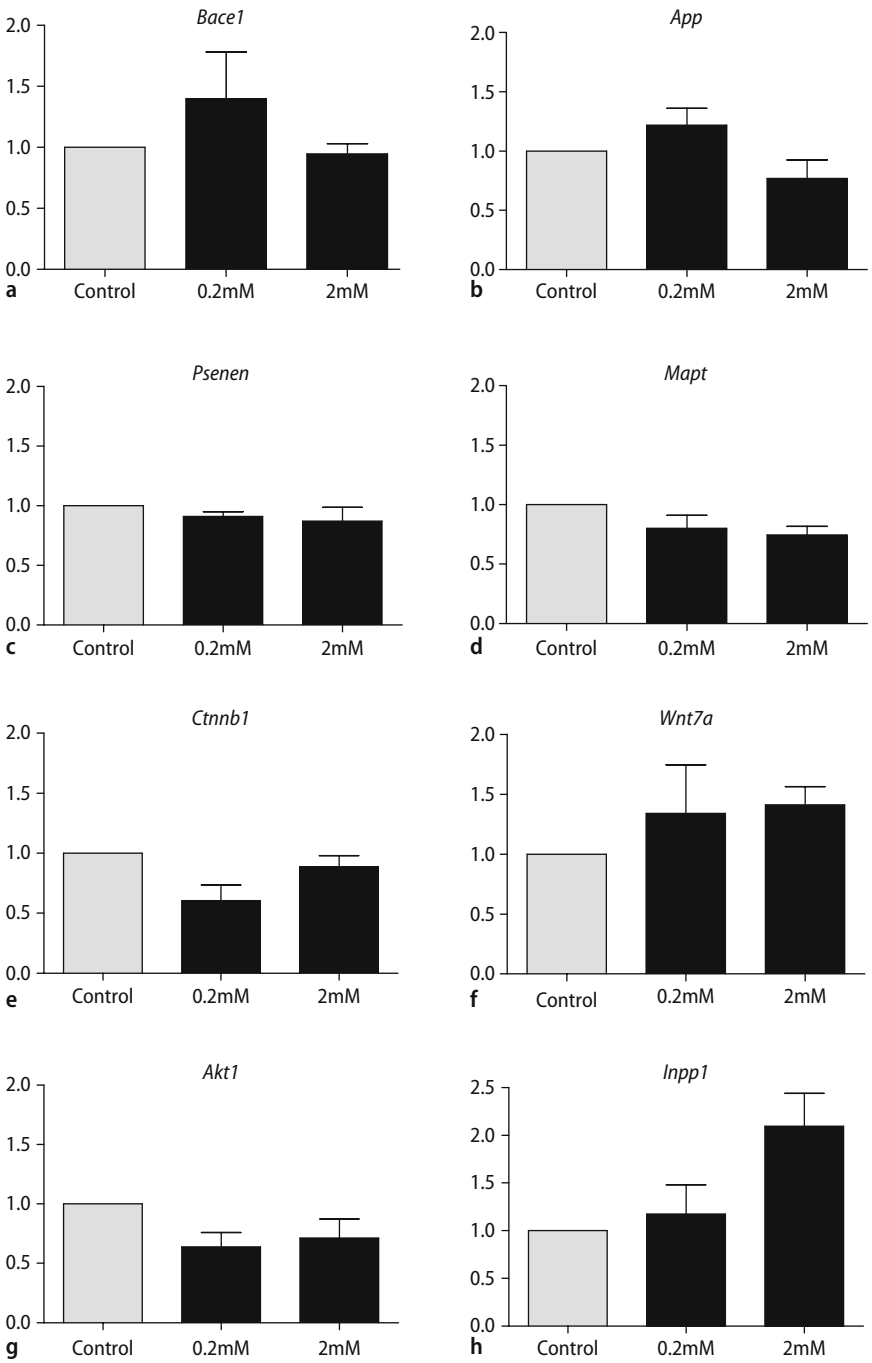

Fig. 2 Effects of LiCl treatment of primary cultures of hippocampal neurons, over the expression of genes related to Gsk3 pathways. Three distinct primary cultures of neurons were incubated for 5 days, in triplicates, with 0.2 or 2.0 mM of LiCl. Total RNA was extracted and expression levels of *Bace1, App, Psenen, Mapt, Ctnnb1, Wnt7a, Akt1, Inpp1* were determined in triplicates by qPCR. Data are presented as the means ± S.E.M. and bars represent the percent changes in the normalized mRNA expression for each experimental condition as compared to controls (100%). Statistical analysis showed no significant alterations for any of these genes ($P > 0.05$)

In vitro effects of LiCl on the expression of genes encoding Gsk3-related proteins

We also evaluated the effects of LiCl treatment over the expression of another eight genes of the GSK3 pathway, in order to evaluate if the results observed could be due to a broad depression of the neuronal transcriptional machinery. The genes evaluated for this purpose were *Bace1, App, Psenen, Mapt, Ctnnb1, Wnt7a, Akt1, Inpp1*. As can be seen in Fig. 2, no statistically significant alterations could be seen in the expression of any of these genes, suggesting the specific effects of LiCl over the expression of Gsk3b.

Effects of LiCl on *Gsk3* mRNA levels *in vivo*

The intracerebral modulation of *Gsk3b* expression by lithium was also observed *in vivo*. Adult rats treated with 5 mg/kg/day or 10 mg/kg/day of LiCl attained mean lithium serum levels of 0.1 ± 0.04 and 0.6 ± 0.2 mEq/l (respectively). No significant effects on body weight were observed as a consequence of LiCl treatment (data not shown). To evaluate a possible tissue-specificity of LiCl regulation, *Gsk3* levels were investigated in two brain areas and in leukocytes of these animals. No significant differences were observed in the mean mRNA levels of *Gsk3a* in the frontal cortex, hippocampus or leukocytes for both treatment groups as compared to controls (Fig. 2). However, we found a significant reduction of *Gsk3b* mRNA levels in the hippocampus, but not in the cortex nor in the peripheral leukocytes of LiCl treated rats. The mean percent reduction of hippocampal *Gsk3b* expression was 39 ± 6% ($P = 0.001$) and 63 ± 6% ($P < 0.001$), respectively in rats treated with 5 mg/kg/day ($N = 4$) or 10 mg/kg/day ($N = 4$) of LiCl (Fig. 3). LiCl-treatment resulted, once again, in a specific, dose-dependent and hippocampal-restricted reduction of *Gsk3b*.

Discussion

Lithium has been widely used for over five decades in the treatment of major psychiatric disorders [3]. The exact mechanisms that underlie its effects as a mood-stabilizing agent are still poorly understood, but probably include its capability of inhibiting GSK3B

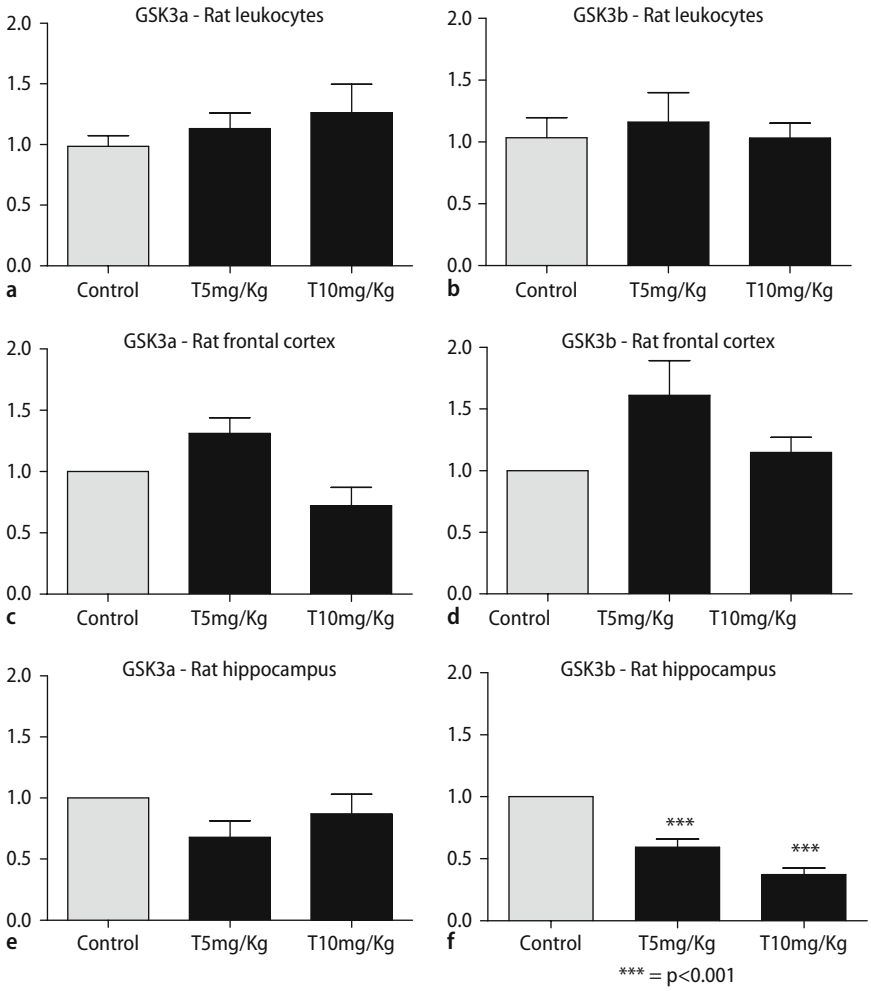

Fig. 3 *In vivo* effects of LiCl treatment over mRNA expression of *Gsk3a* and *Gsk3b* in leukocytes, brain cortex and hippocampus of rats. Twelve adult rats were treated for 35 consecutive days with two daily intra-peritoneal injections of saline ($N = 4$), or LiCl at two distinct doses (5 mg/kg/day ($N = 4$) or 10 mg/kg/day ($N = 4$). Total RNA was extracted from leukocytes or from homogenates of cortical and hippocampal tissues. *Gsk3a* and *Gsk3b* levels were quantified by qPCR as described. Data are presented as relative means ± S.E.M. and *bars* represent the percent changes in mRNA expression for each experimental condition as compared to controls (100%). ANOVA analysis showed no significant alterations for any of the investigated genes in leukocytes (**a** and **b**) or in the brain cortex (**c** and **d**) ($P > 0.05$). This same analysis showed that lithium treatment caused no alterations in the transcription levels of hippocampal *Gsk3a* (*e*), but resulted in a significant reduction of *Gsk3b* in hippocampus for both drug concentrations ($P = 0.001$), (f)

[16, 23]. The neurobiological effects of lithium on GSK3 have so far been attributed to the direct and indirect inhibition of enzymatic activity. In the current study, we provide experimental evidences that lithium may also affect the transcriptional regulation of *Gsk3b*.

Given the central role of *GSK3B* in the pathogenesis of AD, bipolar disorder and other conditions, the identification of its specific modulators will be of great importance. However, due to the structural and regulatory similarities between GSK3A and GSK3B, the development of drugs that can specifically inhibit one of these two isoforms may be a difficult task. At the aminoacid level, the kinase domains of GSK3A and GSK3B are almost identical and these proteins have apparently the same functions in the Wnt pathway. However, the sequence diversity observed at the amino-and carboxyl-terminus may be responsible for the distinct biological properties of each isoform. In the search for specific inhibitors, a way to overcome the close protein similarity between these proteins would be to identify agents that target *GSK3B* at its nucleotide level, by either blocking its transcription or inhibiting its translation. Experiments based on this rationale have been proposed by Mussmann et al. [20].

Our results, both in cultured neurons and *in vivo*, indicate that therapeutic (or even lower) LiCl concentrations, leads to a remarkable hippocampal specific, dose-dependent, reduciton of GSK3B gene expression (above 50%, $P < 0.001$).

Gould et al. [9], using cytosolic β-catenin as an indirect marker of *in vivo* GSK3 activity, reported a reduction of *Ctnnb1* (*β-catenin*) mRNA levels in rats treated with lithium in concentrations equivalent to therapeutic levels in humans. However, because in our experiments the effect of lithium on the expression of *Gsk3b* occurred at lower concentrations than those required to inhibit the transcription of *Gsk3a*, we speculate that the modulation of *Gsk3* isoform's expression may be even more complex. In an analysis of the papers of Lau et al. [17] and Lee et al. [18], it is noteworthy that the promoters of *Gsk3a* and *Gsk3b* have a remarkably different structure, in terms of regulatory elements and transcription initiator factors. This distinct promoter structure organization reinforces the diversity of the transcriptional regulation of these genes and may explain the differential responses to LiCl observed here.

In AD, overactive GSK3B favors the hyper-phosphorylation of Tau and the amyloidogenic cleavage of APP, which are critical events in the formation of neurofibrillary tangles and neuritic plaques. In mice [22] and in *Drosophila* models [19], lithium reversed the pathological effects of GSK3 over-expression. Accordingly, in elderly patients, the chronic exposure to lithium used to treat bipolar disorder, was associated with a smaller prevalence of dementia and AD, as compared to age-matched bipolar patients treated with other mood-stabilizing drugs [21]. Our findings suggest a new complementary mechanism for LiCl as a GSK3B inhibitor. The observation of a significant reduction in the *Gsk3b* transcription, even at very low concentrations, together with the data of Nunes et al. [21] may suggest the possible use of this agent in the treatment or even in the prevention of Alzheimer's disease.

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Conflict of interest The authors declare no conflict of interest

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