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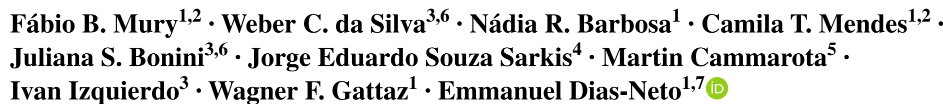
ORIGINAL PAPER

Lithium activates brain phospholipase A2 and improves memory in rats: implications for Alzheimer's disease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Abstract Phospholipase A2 (Pla2) is required for memory retrieval, and its inhibition in the hippocampus has been reported to impair memory acquisition in rats. Moreover, cognitive decline and memory deficits showed to be reduced in animal models after lithium treatment, prompting us to evaluate possible links between Pla2, lithium and memory. Here, we evaluated the possible modulation of Pla2 activity by a long-term treatment of rats with low doses of lithium and its impact in memory. Wistar rats were trained for the inhibitory avoidance task, treated with lithium for 100 days and tested for perdurability of long-term memory. Hippocampal samples were used for quantifying the expression of 19 brain-expressed *Pla2* genes and for evaluating the enzymatic activity of Pla2 using group-specific radio-enzymatic assays. Our data pointed to a significant perdurability of long-term memory, which correlated with increased transcriptional and enzymatic activities of certain members of the Pla2 family (iPla2 and sPla2) after the chronic lithium treatment. Our data suggest new possible targets of lithium, add more information on its pharmacological activity and reinforce the possible use of low doses of lithium for the treatment of neurodegenerative conditions such as the Alzheimer's disease.

Keywords Lithium · Memory · PLA2 · Step-down inhibitory avoidance task · Hippocampus · Gene expression

Introduction

Lithium salts have been used for decades as the drug of choice in the treatment of manic-depressive episodes and as a mood stabilizer in patients with bipolar disorder (BPD) [1–4]. Clinical benefits derived from lithium treatment comprise acute anti-manic, antidepressant and long-term

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prophylactic effects, including the reduction in suicide rates [5, 6].

Over the years, the mechanisms of action of lithium have been mainly evaluated from the perspective of its effects over the enzyme glycogen synthase kinase 3-beta (GSK3B) [7–10], as well as over neurotransmitters and G-protein-coupled second messenger systems [11, 12]. However, some aspects of the therapeutic basis of lithium remain to be revealed. It has been hypothesized that the effects of the chronic lithium treatment are due to its ability to alter the balance between neurotransmitter and neuropeptide signaling pathways [13]. Moreover, chronic lithium treatment seems to regulate gene expression in the brain [14], and an investigation of lithium effects over the transcriptome of a human neuronal cell culture indicated the up-regulation of neuroprotective genes together with the downregulation of genes encoding pro-apoptotic proteins [15]. The lithium treatment of neurons derived from bipolar disease patients altered the uptake and the intracellular levels of calcium and redirected neural stem cell fate [16].

The effects of lithium on cognitive functioning remain ambiguous [17]. Animal studies suggest that lithium improves memory deficits induced by neuropathological insults [18, 19] and indicate its potential use as a therapeutic agent in cognitive decline [20]. Nocjar et al. [21] found that a 4-week lithium treatment significantly improved memory recognition and learning in rats and promoted hippocampal synaptic plasticity. We recently reported a synergistic and additive effect of lithium and enriched environment on the generation of new cells in the mouse hippocampus [22]. These data, together with reports that lithium reduced the development of neurofibrillary tangles and the production of amyloid-beta [10, 18, 23], suggest that lithium treatment may be useful for patients with Alzheimer's disease (AD). The effects of lithium in different types of memory in humans have been the focus of several studies, involving BPD patients [24–27] and non-diseased controls [28, 29], with conflicting results regarding its effects over short- and long-term memory performance.

Our group has found that chronic lithium treatment reduced the prevalence of AD in elderly BPD patients when compared to matched, non-lithium-treated BPD patients [30]. Moreover, in individuals with mild cognitive impairment (MCI), we found that low-dose lithium intake over 4 years reduced the conversion to AD [31]. As MCI is considered a pre-Alzheimer condition [32], these findings suggest that lithium may have disease-modifying properties capable of reducing AD risk. Similarly, other in vivo and in vitro studies have also confirmed the neuroprotective properties of lithium in neurodegenerative diseases, particularly in AD [33], including its capability to stimulate neurogenesis in the hippocampus [14, 34] of adult rodents. Apart from this, chronic lithium treatment up-regulates synaptosomal uptake of glutamate and promotes the enhancing of long-term potentiation in the rat dentate gyrus [35, 36]. In addition, by means of a powerful inhibitory action of the enzyme glycogen synthase kinase 3 (Gsk3), lithium exerts a favorable function on the two main pathogenic pathways of the AD: the amyloid-beta cascade and the formation of the neurofibrillary tangles [10]. Finally, lithium promoted an improvement in learning, motor coordination and memory in a knock-in mouse model of spinocerebellar ataxia type 1 (SCA1), characterized by hippocampal neuropathology and multiple behavioral changes [37].

A major focus of neurodegenerative disease research is the identification of molecules and processes mediating memory formation and retrieval. Previous studies of our group evidenced a correlation between abnormally low PLA2 activity and more severe clinical aspects of AD [38–40], as well as with memory decline, more specifically in long-term memory retrieval in animal models [41]. A reduced PLA2 activity has been demonstrated in the cerebrospinal fluid of patients with AD and vascular dementias [42], whereas the cognitive training of elderly subjects induced an increased platelet PLA2 activity [43].

The links between lithium–memory and memory–Pla2 prompted us to investigate the effects of the chronic treatment of rats with low doses of lithium, examining its nephrotoxicity, its impact over memory retrieval and its effect on the expression of all brain-active *Pla2* genes as well as the enzyme activity of distinct Pla2 subgroups.

Materials and methods

Animal studies

Male Wistar rats (8–10 weeks), weighing 200–250 g, were obtained from the central animal house of Universidade Federal de São Paulo, São Paulo, Brazil. In conformity to the "Guide for the Care and Use of Laboratory Animals" (ISBN 0-309-05377-3) of the National Institute of Health (NIH Publication No. 86-23, revised 1985), all efforts were made to reduce the number of animals used and to minimize their suffering. This study was approved by the local Animal Care Committee at the Universidade de São Paulo, Brazil.

During the experimental phase, animals were housed in polypropylene cages (four animals/cage), at a temperature of 22 ± 2 °C with 12-h light/dark cycles, being fed a standard diet with free access to water. Treatment of the animals started after 7 days of acclimatization. Forty rats were divided in three groups: T1 fifteen animals receiving approximately 5 mg LiCl/day (0.125 mg LiCl/mL of water); T2 fifteen animals receiving approximately 50 mg

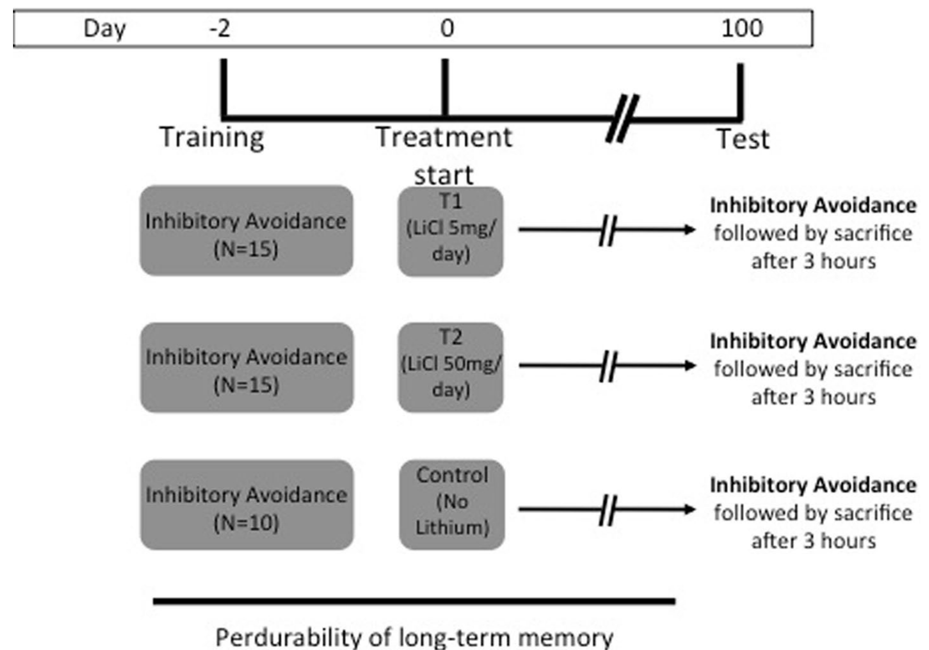

Fig. 1 Experimental design. The *figure* indicates the training, treatment and test points, as well as experimental groups and time intervals

LiCl/day (1.25 mg LiCl/mL of water); and control ten animals receiving regular water (with no added LiCl). Lithium concentrations used to achieve the desired daily doses were determined based on an average daily water consumption projected according to the expected weight of the animals in the midpoint of the treatment period (estimated water consumption: 10 mL/100 g) [44]. Animals were treated for 100 consecutive days, and during this time, periodic body weight measurements, as well as standard locomotion tests, were taken to appraise their overall health status and to estimate lithium intake. After 100 days of treatment with LiCl, behavioral procedures tasks (inhibitory avoidance testing, open-field habituation and elevated plus-maze; Fig. 1) were performed as described below.

Behavioral procedures

Inhibitory avoidance

Animals were trained in inhibitory avoidance [45–47] 2 days before the beginning of the LiCl treatment, aiming to evaluate the effect of chronic lithium use over the perdurability of already consolidated long-term memories. This learning task, previously used by Izquierdo et al. [48] who demonstrated its capability to generate a mnemonic trace that lasts for the time of this work, was used here in an attempt to emulate what would happen to humans in clinical reality. Briefly, animals were placed on an 8.0-cm-wide, 5.0-cm-high platform at the left of a 50-cm-wide, 25-cm-deep, 25-cm-high acrylic box with a grid made of a series of 1-mm-caliber bronze bars spaced 1 cm apart. Latency to step down onto the grid with all four paws was measured; upon stepping down, the animals received a 0.8 mA, 4-s scrambled footshock and were immediately withdrawn from the training apparatus. This longer (4 s instead of 2 s) and more intense footshock (0.8 mA instead of 0.5 mA) warrants persistence of the inhibitory avoidance memory for the length of the treatment period [48]. In the test session, conducted 100 days after the beginning of LiCl treatment, animals were placed again on the platform, but the footshock was omitted and step-down latency was used as a measure of memory retention (Fig. 1).

Open-field habituation

In order to control for possible effects of lithium on locomotion, animals were exposed for 5 min to a 40 cm × 50 cm × 60 cm open-field apparatus whose brown linoleum floor was divided by white lines into 12 equal squares. In the session, all animals of the three treatment groups were placed in the rear left square and left to explore it freely [47]. The number of line crossings and rearing events were compared among control and the lithium treatment groups.

Elevated plus-maze test

Effects of lithium on the mobility, locomotion and anxiety-related behaviors were also evaluated with the elevated plus-maze test [49]. Briefly, the elevated plus-maze consisted of a central platform (10 × 10 cm) with four 45 × 10 cm arms, of which two were open and two were

closed. Arms were arranged in a way that the two arms of each type were opposite to each other. The maze was kept 88 cm above floor level, and tests were carried out under dim red light. Animals were placed individually on the central platform of the elevated plus-maze facing an open arm. For 5 min, two observers recorded the number of rearings, the time spent in the open and in the enclosed arms and the number of entries in each arm. The percentage of time spent in the enclosed arms and the number of entries in these arms were used as a measure of anxiety [48, 50].

Plasma lithium determinations

Prior to the end of the treatment period (100 days), blood was collected by tail-cuff to determine lithium concentration in the sera of all animals. Prior to analysis, samples were diluted 1:20 with a mixture of 0.5 g of serum, 9 g of 2 % HNO3 and 0.5 g of a 100 ng/g solution of indium. All solutions were directly weighed in disposable trace metal clean polypropylene auto-sampler tubes. Stock standard solutions were prepared from 1 mg/g stock solutions of lithium and indium (Spex Industries Inc.—Edson, New Jersey, USA). All standard solutions were made up in 2 % HNO_3. Nitric acid was purified by sub-boiling distillation in quartz still. Lithium quantification was performed by external calibration with seven serial dilutions of a standard solution varying from 1 to 70 ng/g with 1 ml of In internal standard solution. Blood lithium concentration was determined for each animal by using sector-field inductively coupled plasma mass spectrometer (SF-ICPMS) ELEMENT 1—Finnigan MAT (Bremen, Germany), with a low-flow (1 mL/min) concentric nebulizer (Meinhard, Santa Ana, CA, USA), and a Scott-type spray chamber cooled to 5 °C. Isotopes were acquired in E-scan mode. Three replicate analyses were performed on each sample after a 60-s uptake and 60-s stabilization period. The SF-ICPMS operating conditions are summarized in Supplementary Table S1.

mRNA and protein analysis

After treatment, animals were killed by decapitation, and hippocampi were dissected in cooled phosphate-buffered saline solution (PBS). Tissue aliquots were mixed with a buffer (5 mM Tris–HCl pH 7.4, 4 °C), homogenized (B. Braun Biotech International, South Africa), divided in aliquots and immediately stored at −80 °C for subsequent enzymatic and mRNA analyses of *Pla2* genes.

Brain expression of Pla2 genes

The *Pla2* genes studied here were all those annotated or predicted in the rat genome and that were also expected to be expressed in the brain, based on our in silico searches against transcriptional data available in public databases, essentially composed of cDNA transcript fragments such as Expressed Sequence Tags (ESTs) and Serial Analysis of Gene Expression (SAGE) evaluated using the programs BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and BLAT (http://genome.ucsc.edu). The putative brain expression of a *Pla2* gene was considered when rat, mouse or human gene tags corresponding to that gene, derived from brain cDNA libraries, were available. For all brain-expressed *Pla2* genes suggested by in silico analyses, experimental validation was performed by PCR amplification of hippocampal cDNA. New rat *Pla2* genes were identified based on sequence conservation with mouse and human orthologs, followed by cDNA amplification using oligonucleotide primers based on the most conserved genomic regions among these three species, and confirmed by DNA sequencing. Special attention was given for selecting PCR primers, which were designed in distinct exons to avoid genomic DNA amplification, and targeted to amplify specific members of the Pla2 family. To preclude the amplification of paralogs, regions conserved at nucleotide level in different *Pla2* genes were avoided. Primers were designed with Primer3 (http://frodo.wi.mit.edu) or *PrimerExpress*® (Applied Biosystems, Foster City, CA, EUA), after masking the repetitive sequences using RepeatMasker (http://www.repeatmasker.org). Primer sequences and expected amplicon sizes of *Pla2* genes and reference genes are given in Supplementary Table S2.

RNA extraction and cDNA synthesis

Hippocampi were rapidly dissected and immediately stored at −80 °C. Tissues were later homogenized in ten volumes of 5 mM Tris–HCl buffer pH 7.4, and 30 % of each sample homogenate was used for RNA extraction with TRIzol®Reagent (Gibco BRL, Rockville, MD), according to [51]. Quality and quantity of RNA samples were determined using NanoDrop® (ND-1000—Thermo Fisher Scientific, Inc—USA) and the integrity of the total RNA was checked by electrophoresis in 1 % agarose gels containing 1 M of guanidine isothiocyanate. cDNA synthesis was performed at 42 °C for 90 min, using 1 µg of total RNA, 200U of ImProm-II™ Reverse Transcriptase (Promega, Madison, WI, USA), 0.5 µg oligo $(dT)_{12-18}$, 0.5 mM dNTPs, 3 mM $MgCl_2$ and 1 × reaction buffer in a 20 µl reaction volume.

Real-time PCR analysis of gene expression

Differential gene expression was evaluated by quantitative real-time PCR (qPCR), using SYBR Green PCR Master Mix in an ABI 7500 Sequence Detection System (Applied-Biosystems, Foster City, CA, USA). The qPCR experiment

workflow and analysis are in compliance with the MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines [52]. Reference genes were selected by geNorm [53] and used to normalize the amounts of cDNA used in each experiment. For each qPCR, we used 7.5 ng cDNA equivalents and 3.75 pmol of each primer in 15 µl reactions. For each target gene, we included a no-template control as well as two geNorm-selected reference genes. The thermal amplification profile consisted of an initial step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. qPCR data were captured using the software *Sequence Detector System* (SDS) 1.3.1 (Life Technologies). Relative gene expression levels were normalized by the geometric mean of the reference genes [53].

Radioenzymatic analysis of Pla2 activity

Aliquots of hippocampus protein homogenates were used to determine the activity of Pla2 subtypes by radioenzymatic assays as described [38, 54]. Protein concentration was determined with the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA, USA), modified from Lowry et al. [55]. Briefly, the substrate used was L-α-1-palmitoyl-2-arachidonyl-phosphatidyl-choline labeled with [l-^{14}C] in the arachidonyl tail at position sn-2 (^{14}C-PC) (48 mCi/mmol specific activity, PerkinElmer, Boston, MA) [56–60], with slight modifications. Prior to the enzymatic reaction, a mixture of arachidonyl-l-^{14}C-PC and toluol–ethanol–butylhydroxytoluol antioxidants (1:10, v/v) was evaporated under a nitrogen stream (0.075 µCi per sample), resuspended in a solution of 0.3 mg/mL BSA in ultrapure water and homogenized by sonication. Total brain tissue homogenates were diluted to a final protein concentration of 1.5 mg/mL with 50 mM Tris–HCl (pH 8.5 for sPla2 and cPla2 or pH 7.5 for iPla2). The assays contained 100 mM Tris–HCl buffer (pH 8.5 or pH 7.5), 1 µM (for cPLA2 and iPLA2) or 2 mM $CaCl_2$ (for sPla2), 100 µM BEL (Biomol, Plymouth Meeting, PA, USA), 300 µg of protein from diluted homogenates and 0.075 µCi arachidonyl-1-^{14}C-PC. After 30 min incubation at 37 °C, reactions were interrupted by adding a mixture of HCl–isopropanol (1:12, v/v). The released [l-^{14}C] AA was extracted, and the radioactivity of ^{14}C-AA was measured in a liquid scintillation counter (Tri-Carb 2100 TR; Packard, Meriden, CT, USA) for calculating Pla2 activities (pmol mg protein min^{-1}). All Pla2 activity determinations were performed in triplicates.

Statistical analysis

Most results are expressed as mean ± SEM and analyzed by regular parametric statistics (one-way ANOVA followed by post hoc Tukey test was performed with the significance level set at $p < 0.05$). The behavioral data (step-down latency, in seconds) are expressed as median ± interquartile range because a 600-s ceiling was applied to the tests, and therefore, the distribution is not normal and required nonparametric statistics (Duncan multiple range test with the significance level set at $p < 0.05$). The correlations between step-down latency and PLA2 enzymatic activity for each Pla2 group were calculated using R program (version 3.0.2) and measured by Pearson's coefficient. Nonlinear regression models were employed for those resembling nonlinear patterns.

Results

Our analyses of transcriptional databases provided in silico support for the brain expression of 20 out of the 27 *Pla2* genes (human, mouse or rat data). Experimental validation of hippocampal expression of 19 of these 20 genes was performed by reverse-transcriptase PCR. For one particular gene (*Pla2g6e or iPLA2 ζ*), we were not able to design PCR primers with the criteria required for this study (primers located in distinct exons, out of repetitive elements and with no significant matches to other *Pla2* genes), to avoid false amplification results such as the amplification of contaminant genomic DNA or paralogous genes. For the remaining 19 genes, expected amplicons were obtained and confirmed by DNA sequencing, allowing to confirm their expression in the hippocampus of male adult rats. One of the genes partially sequenced here represents the first evidence of the expression of *Pla2g4e* (*cPLA2ε*)in rat, and its partial cDNA sequence was deposited in the GenBank (Accession Code EF011108). All *Pla2* genes scrutinized here, as well as the in silico transcriptional evidences and experimental validations, are presented in the Supplementary Table S3.

After determining the set of *Pla2* genes active in the rat hippocampus, we evaluated the effects of chronic LiCl treatment over their expression. First, we confirmed the effectiveness of treatment by determining the lithium plasma levels of all 40 animals using Sector-Field Inductively Coupled Plasma Mass Spectrometer (SF-ICPMS). The resulting blood lithium levels observed for animals receiving 5 mg (T1) or 50 mg (T2) lithium/day were, respectively, 0.1 ± 0.019 (T1) and 0.35 ± 0.066 (T2) mEq/L. No traces of lithium were detected in the plasma of control animals (not treated with LiCl). In parallel, higher hippocampal mRNA levels were observed for 6 out of the 19 *Pla2* genes investigated: iPla2s: *Pla2g6b* (*Pla2g6γ)* and *Pla2g6d* (*Pla2g6ε*); Paf-acetylhydrolases: *Paf-ah* and *Pafah1b1*; and sPla2s: *Pla2g2d* and *Pla2g12a* (Fig. 2).

All these alterations, with a single exception of the *Pla2g12a* gene, were seen only for the groups that received

higher (i.e., 50 mg/day) lithium doses. mRNA levels of the remaining *Pla2* genes remained unaltered in both treatment groups.

Next, we investigated whether this transcriptional *Pla2* augmentation paralleled to higher Pla2 enzymatic activities of the corresponding protein groups, as measured by radioenzymatic assays. As shown in Fig. 3, iPla2 activity showed significant augmentation for the two therapeutic regimens used ($p < 0.001$), whereas for sPla2, a significant increase was seen in the higher blood lithium level group when compared to controls ($p < 0.001$) (Fig. 3a, b). In line with the mRNA findings, no differences in cPla2 activity were observed (Fig. 3c).

Moreover, we observed that lithium treatment resulted in memory enhancement, reflected in the statistically significant better performance observed in the step-down inhibitory avoidance task after 100 days of treatment for both treatment groups (Fig. 4). Memory performance showed a positive correlation with the iPLA2 group enzymatic activity: Higher step-down latency times corresponded to higher activity as measured by pmol/mg protein/min (p value 0.0133). No significant body weight alterations were seen for the animals in any of the two treatment groups (data not shown), and no kidney lesions could be observed by histology (see Supplementary Fig S1, available online). Open-field and elevated plus-maze tests showed no differences between treated and untreated groups ($p > 0.05$, Table 1), suggesting that the step-down results are not due to alterations in anxiety or in the spontaneous locomotion of animals which otherwise may have been caused by the chronic lithium treatment.

Discussion

Our in silico analysis allowed us to build a transcriptional map indicating the brain expression of most *Pla2* genes. This approach showed to be reliable as the hippocampal expression could be confirmed for all 19 *Pla2* selected genes. After defining and experimentally validating the brain-related *Pla2* set, we investigated their possible modulation by lithium. Therapeutic and even sub-therapeutic lithium concentrations have been shown to be capable of modulating *Gsk3b* mRNA [9] and protein [61, 62], and it also seems to be capable of rescuing or attenuating long-term potentiation deficits due to GSK-3 dysfunction [63], improving learning and memory in rats [21, 64] and reducing subsequent dementia rates in chronically treated elderly bipolar patients [30], which was further replicated in a large cohort of 4856 patients with a diagnosis of a manic or mixed episode or bipolar disorder [64]. Moreover, lithium treatment over 2 years reduced the conversion to AD in individuals with mild cognitive impairment [11, 65]. Importantly, whereas the long-term treatment of elderly patients with low doses of lithium showed to be clinically safe [66], lithium was able to regulate nitric oxide levels in bipolar disorder patients [67]. As the Pla2 activity is significantly reduced in brain tissues [38, 60] as well cerebrospinal fluid of patients with AD when compared to controls [42], we investigated whether LiCl would modulate *Pla2* mRNA levels and/or enzymatic activity.

Phospholipid metabolism alterations resulting from altered PLA2 levels have been reported in AD [38, 39, 69–71], and these changes are correlated with the density of neuritic plaques and neurofibrillary tangles in the brain cortex [40]. Moreover, individuals with lower baseline PLA2 activity had an increased risk of converting to AD during a 4-year follow-up [72]. Here, we show experimental evidence that lithium treatment correlated with an increase in the mRNA levels of 6/19 *Pla2* genes evaluated (Fig. 2), and this elevation paralleled to a raise in the enzymatic activities of the corresponding Pla2 subgroups in the rat hippocampus (Fig. 3).

Our findings also demonstrated the effectiveness of small doses of lithium, which are less likely to lead to harmful side effects. The low lithium doses used here resulted in peripheral blood lithium levels corresponding to 0.1 ± 0.019 and 0.35 ± 0.066 mEq/L for T1 and T2 groups, which are far below the usual 0.8–1.2 mEq/L therapeutic interval [73]. Whereas this experiment was designed to evaluate the effects of lithium in preserving memories formed before the beginning of the treatment, it does not allow us to verify possible effects over memories formed during or after this treatment. This treatment regimen enhanced the perdurability of a strong aversive memory as detected by step-down inhibitory avoidance task, in line with reports of lithium neuroprotective effects after long-term administration in therapeutic levels [71], which lead to neuroprotective effects capable of negatively impacting the cognitive decline [30, 74]. Importantly, this same test has been shown by others to also parallel positive memories [75], implying that the results shown here are probably not limited to negative memories. Whereas these low lithium concentrations caused no detectable nephrotoxicity, they were capable of positively impacting memory retrieval after 100 days of treatment (Fig. 4). To eliminate the possibility of locomotor or anxiolytic effects given by these doses of lithium on memory performance results, we have used open-field test and elevated plus-maze, which allows to measure these aspects [47]. Lithium in these doses had no effects on behavior in the open-field or in the elevated plus-maze, suggesting that they did not affect general activity, exploration or anxiety levels (Table 1). Due to the large time interval elapsed between learning training and memory test, the inhibitory avoidance task was the most appropriate learning task to evaluate the possible effect of lithium treatment on perdurability memory [48]. Other

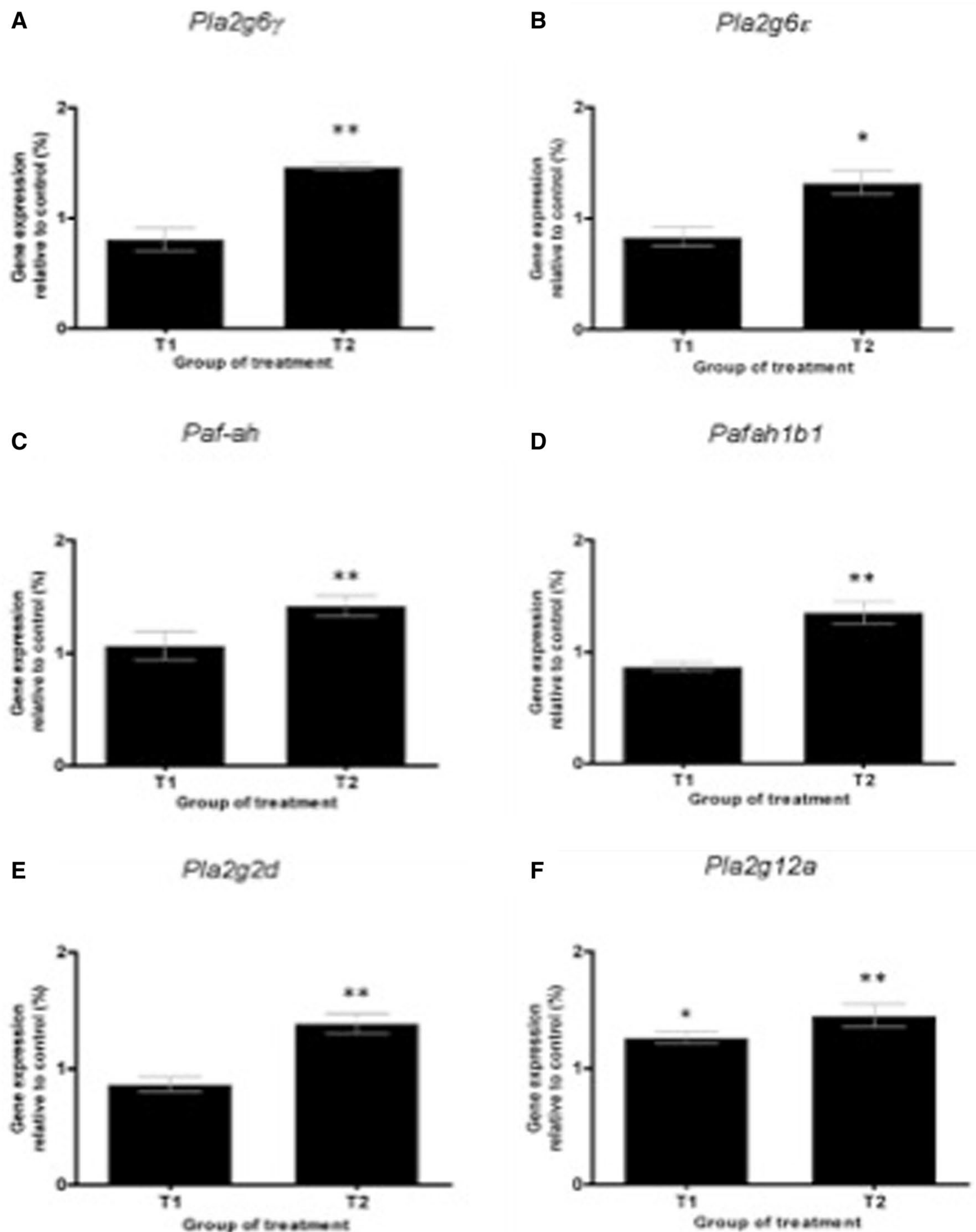

Fig. 2 *Pla2* gene expression analysis by quantitative real-time PCR in rat hippocampi. The expression of 19 *Pla2* genes was evaluated by real-time PCR. Relative expression was normalized by endogenous controls, compared to untreated controls and is presented for all genes that gave statistically significant *p* values ($p < 0.05$). Significant increments in expression were observed for (iPla2)—**a** *Pla2g6γ* (NM_001108020.1) and **b** *Pla2g6d* (XM_343302); (Paf-acetylhydrolase)—**c** *Paf-ah* (NM_001009353) and **d** *Pahah1b1* (NM_031763); (sPla2)—**e** *Pla2g2d* (NM_001013428), and **f** *Pla2g12a* (NM_001108565). Expression data were evaluated by analysis of variance (ANOVA) followed by Tukey test, $*p < 0.05$ and $**p < 0.01$. T1 = treatment group receiving 5 mg lithium/day (blood lithium concentration: 0.10 ± 0.019 mEq/L); T2 = treatment group receiving 50 mg lithium/day (blood lithium concentration: 0.35 ± 0.066 mEq/L)

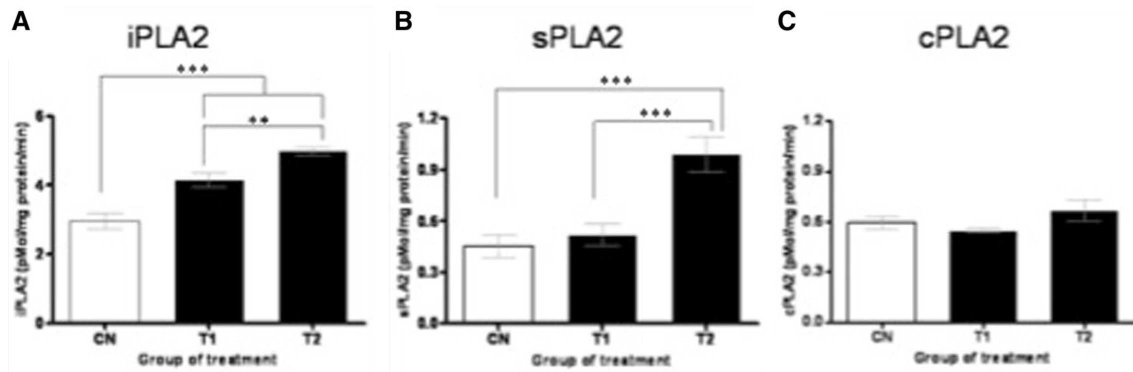

Fig. 3 Quantitative enzymatic activities of Pla2 subgroups in rat hippocampus. Radioenzymatic assays were used to determine the activity of Pla2 in controls ($n = 10$ animals) or LiCl-treated animals ($n = 10$–15), as described. Specific activities of calcium-independent phospholipase A2 (iPla2) (**a**), secretory phospholipase A2 (sPla2) (**b**) and cytosolic phospholipase A2 (cPla2) (**c**) are presented for both treatment groups. Significant differences were seen for iPla2 and sPla2 but not for cPla2. Values shown are mean Pla2 activity (pMol/mg protein/min) plus SEM. *Asterisks* indicate ** = $p < 0.01$ and *** = $p < 0.001$. Statistical analysis was conducted by one-way ANOVA test with post hoc Tukey test. CN = untreated control; T1 = treatment group receiving 5 mg lithium/day (blood lithium concentration 0.10 ± 0.019 mEq/L); T2 = treatment group receiving 50 mg lithium/day (blood lithium concentration 0.35 ± 0.066 mEq/L)

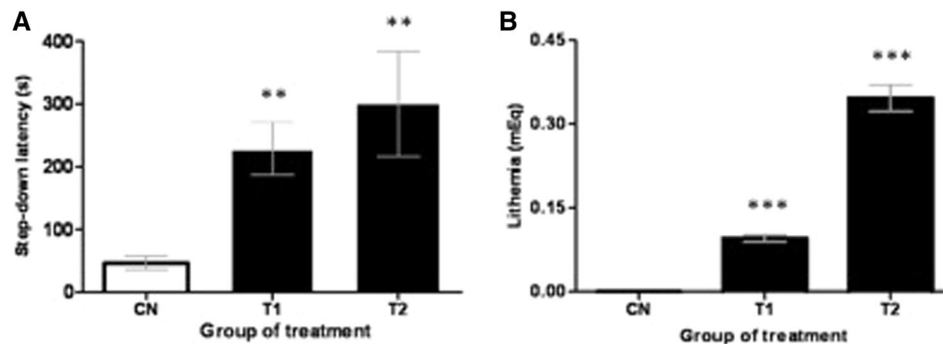

Fig. 4 Effects of lithium on the step-down inhibitory avoidance task (**a**) and corresponding plasmatic lithium levels (**b**). Animals were treated for 100 days with 5 or 50 mg lithium/day (T1 and T2, respectively). **a** An enhancement of retention test performance was seen with both doses; difference in median (interquartile range) retention scores (step-down latency, in secs) between T1 and T2 was not significant, but both were higher than those of the vehicle group at a $p < 0.01$ level (Duncan multiple range test). **b** Plasmatic levels of lithium in the control and in both treatment groups. As expected, the lithium doses in the treatment groups corresponded to the plasmatic levels of lithium, as shown in (**b**). In this case, lithium levels were bellow the detection limit for untreated animals and significantly different between T1 and T2 treatment groups ($p < 0.001$). Data points were evaluated by analysis of variance (ANOVA) followed by Tukey test. Differences in mean value were considered to be statistically significant at $p < 0.05$. *Double asterisks* indicate $p < 0.01$, and *triple asterisks* indicate $p < 0.001$. Data are presented as mean ±SEM of 10–15 animals in each group. *CN* control, *T1* (0.10 ± 0.019 mEq/L) and *T2* (0.35 ± 0.066 mEq/L) treatments

Table 1 Open-field and elevated plus-maze

	Controls	T1 (5 mg/LiCl/Day)	T2 (50 mg/LiCl/Day)
Rearings	21.9 ± 4.8	15.6 ± 5.7	17.6 ± 4.2
Crossings	34.1 ± 10.3	24.9 ± 14.6	24.9 ± 8.8
Percentage of time spent in the open arms (mins)	28.6 ± 27.5	36.4 ± 41.6	30.8 ± 31.0
Entries into open arms	1.5 ± 1.3	1.4 ± 1.7	1.8 ± 1.6
Total entries	4.3 ± 2.2	3.0 ± 2.5	3.3 ± 1.9

Data are expressed as mean ± SEM of the number of crossings and rearings (open field; $N = 10$–15 per group), as well as the total number of entries, the number of entries into the open arms and the percentage of time spent in the open arms (plus-maze; $N = 10$–15 animals per group). No differences among any of the groups reached statistical significance in one-way ANOVA or Duncan multiple range tests at $p > 0.05$

types of memories measured in other learning tasks are not so strong like aversive memories, as corroborated in a group of rats subjected to water-maze learning task, where both control and lithium-treated rats did not remember the spatial mnemonic trace learned 100 days before on a strong massive water-maze learning protocol (data not shown). It might be interesting to design an experiment that would evaluate the fall of the mnemonic performance over time in this task, in each group.

Previous studies conducted by us evidenced that the simultaneous inhibition of cPla2 and iPla2 as well as the selective inhibition of iPla2 in the CA1 region of the rat's hippocampus impaired the acquisition of short- and long-term memory [76], as well as the retrieval of long-term memory [41] in a contextual fear task (step-down inhibitory avoidance). This could in fact be attributed to a significant reduction in intracellular and plasmatic membrane fluidity, which was observed after the inhibition of the Pla2 in the rat's hippocampus [77].

Experiments with cPla2 knockout mice showed alterations in the arachidonic acid (AA) metabolism straightforwardly influenced the membrane's fluidity [78], thereby directly implicating Pla2 and long-term potentiation (LTP). According to Fujita et al. [79], cPla2 and iPla2 are directly involved in the creation of LTP, which is a form of activity-dependent synaptic plasticity and is considered the foundation of the construction of memory [80]. Also, the catalytic activity of Pla2 is responsible for the production of AA, which is implicated in the formation of LTP. Others have demonstrated that LTP Ca^{2+}-sensitive induction in rat hippocampus slices was facilitated by the addition of AA [81]. However, the selective inhibition of Pla2 in rat's hippocampus slices led to a cease of memory formation, but could then be re-initiated by the addition of AA [79, 82]. Therefore, it is plausible to suggest that higher Pla2 activity, or specifically the iPla2 subgroup, could play a key role in the genesis of a better memory retrieval, which in our study was observed in animals treated with lithium. Furthermore, our group recently demonstrated that rats fed with a high conjugated linoleic acid diet (which modulates PLA2 activity) showed increased memory and higher hippocampal PLA2 levels [83], in line with other groups that showed the positive impact of polyunsaturated fatty acids and memory in elderly subjects [84, 85].

Besides the positive results presented here, we should note some of its limitations. First, due to the explorative character of the study, results are presented without error probability correction. If a Bonferroni adjustment of the type I error probability was applied, no significant differences between lithium-treated groups and controls would remain. However, with an adjustment of the error probability, the power of detecting existing mean differences would be too low. Second, we should also point out that we have used low doses of lithium in this study, which could perhaps lead to results different from regular clinical doses. Furthermore, we should point that we have only used male animals, and a further limitation was the use of the total hippocampus without punching relevant sub-regions such as the dentate gyrus or CA1/subiculum. Finally, additional behavioral experiments before and after treatment should be performed to better detail the effects of lithium on different forms of memory and even further step-down latencies resulting from our higher lithium dose.

Even in face of these limitations, our results are consistent with the best cognitive performances achieved by lithium-treated animals, which could involve Pla2 signaling, reinforcing the results of Nunes et al. [30], who showed a reduction in the conversion rate of dementia, specifically AD in patients with bipolar disorder treated with lithium. We should note that imbalances of PLA2 have been shown to be opposite between schizophrenia and Alzheimer's disease patients [38, 39, 69, 72, 82, 86, 89], diseases that also present distinct cognitive deficits [90]. Our findings reinforce the positive impact of the chronic use of lithium in memory—even when given at low concentrations that should reduce its cytotoxicity—and suggest that Pla2 may have a role in neurochemical cascades that affect memory. Moreover, these results show new specific Pla2 targets for lithium, which may be relevant to explain the mechanism of action on both aspects: neuroprotection and in reverting deficits in learning/memory in AD. This suggests a close association between lithium administration and memory improvement and provides further support of its therapeutic utility, particularly in AD.

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Compliance with ethical standards

Conflict of interest None.

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