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Pharmacology, Biochemistry and Behavior 79 (2004) 607–621

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

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Chronic lithium decreases Nurr1 expression in the rat brain and impairs spatial discrimination☆

Mumna Al Banchaabouchi, Sandra Peña de Ortíz, Raïssa Menéndez, Keqin Ren, Carmen S. Maldonado-Vlaar*

Department of Biology, University of Puerto Rico, Gándara Avenue, Julio García Díaz Building Room 201, P.O. Box 23360, San Juan 00931-3360, Puerto Rico

Received 20 February 2004; received in revised form 7 July 2004; accepted 9 September 2004

Abstract

Lithium (Li^+) is a drug used for the treatment of neuropsychiatric disorders, whereas Nuclear receptor-related factor 1 (Nurr1) has been implicated in normal and aberrant cognitive processes. Li^+'s effects on cognition and Nurr1 expression were examined. Rats were exposed to Li^+ in their diet for 4 weeks and only those reaching Li^+ blood concentrations within the established clinically therapeutic range were used. Li^+ decreased rearing activity in rats, but did not affect horizontal locomotion nor object recognition memory. In contrast, Li^+ treated animals were significantly impaired in the initial, but not late, stages of acquisition of a hippocampal-dependent spatial discrimination task. In agreement with the behavioral results, chronic Li^+ caused a significant downregulation of basal Nurr1 expression in several brain regions. In particular, a significant negative correlation between Li^+ blood levels and Nurr1 expression was identified in the CA1 hippocampal subregion, but not in CA3, perirhinal cortex or the dorsal endopiriform nucleus. Upregulation of hippocampal Nurr1 levels to those of controls were observed in Li^+ treated rats following training in the spatial task. Overall, the results suggest that the effects of Li^+ on the brain may be particularly relevant to hippocampal-dependent cognitive processes involving Nurr1 expression.

Keywords: Lithium; Affective disorders; Nurr1; Hippocampus; Spatial learning; Transcription factor

1. Introduction

Li^+ is an important mood-stabilizing agent for the treatment of manic-depression and other affective disorders (Vestergaard and Licht, 2001). The therapeutically relevant effects of chronic Li^+ in mood disorders and its proposed mechanisms of action in the brain are still in debate. Overall, the effects of Li^+ are thought to involve a progressive to long-term neuroplastic changes involving intracellular signaling pathways, transcription factors, and regulation of gene expression. Specifically, earlier studies showed the influence of Li^+ on neurotransmitter systems (Ahluwalia and Singhal, 1984; Odagaki et al., 1990) and on cellular signal transduction mechanisms (Manji and Lenox, 1999; Manji et al., 1995). Chronic Li^+ causes downregulation of the expression of several transcription factors, including c-Fos, c-Jun and the cAMP responsive element binding protein (CREB) (Williams and Jope, 1994; Miller and Mathe, 1997; Swank, 1999; Chen et al., 1999; Yuan et al., 1999; Bosetti et al., 2002). However, little is known about its effects on the immediate-early gene Nurr1.

Nuclear receptor-related factor 1 (Nurr1, also known as HZF-3, RNR-1; NOT; NR4A2) is a member of the inducible ligand-independent nuclear receptor family of transcription factors (Law et al., 1992; Peña de Ortiz et al., 1994). Several reports have implicated Nurr1 in neuronal plasticity and its

☆ Financial support: This work was supported by NIH (NIDA-DA11665-04 grant to CSMV; COBRE: NCRR-NIH 5P20 RR15565-02 to CSMV and SPO; SOGGMO 8102-26S1 to SPO).

* Corresponding author. Tel.: +1 787 764 0000x2712; fax: +1 787 764 3875.

E-mail address: cmalvlar@caribe.net (C.S. Maldonado-Vlaar).

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doi:10.1016/j.pbb.2004.09.015

neuronal expression is induced by a variety of stimuli, including membrane depolarization, ischemia, seizures and kindling (Peña de Ortiz and Jamieson, 1996; Honkaniemi and Sharp, 1996; Crispino et al., 1998). More recent studies have also shown that the neural expression of the gene encoding Nurr1 is dynamically regulated during acquisition of spatial discrimination learning, fear conditioning, or conditioned taste aversion (Peña de Ortiz et al., 2000; Ressler et al., 2002; Ge et al., 2003). Thus, Nurr1 expression seems to be relevant to normal cognitive processes. In addition, several studies have demonstrated that the gene encoding Nurr1 plays a crucial role in the survival, differentiation, and induction of the dopaminergic cell phenotype during embryonic development (Zetterstrom et al., 1997; Le et al., 1999; Saucedo-Cardenas et al., 1997; 1998). In addition, Nurr1 has been implicated in the maturation and maintenance of midbrain dopaminergic neurons in adulthood (Ichinose et al., 1999; Chung et al., 2002). Furthermore, this gene has also been associated to cocaine addiction, schizophrenia and manic-depression (Bannon et al., 2002; Buervenich et al., 2000).

Interestingly, Li^+ was shown to act on tyrosine hydroxylase (TH) (Chen et al., 1998), on dopamine transporter (DAT) (Carli et al., 1997) and on the vesicular monoamine transporter 2 (VMAT2) (Cordeiro et al., 2002). Both TH and VMAT2 are known gene targets of Nurr1 transcriptional regulation (Sakurada et al., 1999; Kim et al., 2003; Sacchetti et al., 2001; Smits et al., 2003) and proposed substrates of neuropsychiatric diseases. Since both Nurr1 and Li^+ seem to act on dopaminergic transmission, and because of the proposed role of Nurr1 on cognition, we set out to investigate the effects of Li^+ on both behavior and Nurr1 expression. The present results demonstrate that chronic Li^+ has a suppressive effect on the basal expression of Nurr1 in the hippocampus, which may be associated with the impaired initial acquisition of spatial discrimination that was also caused by chronic Li^+. However, Li^+ treated rats were able to overcome their initial impairment in the task, a phenomenon which could be due in part to their ability to upregulate hippocampal Nurr1 levels during mid acquisition.

2. Materials and methods

2.1. Animals

Male Long Evans (Charles River, USA) weighing 250–275 g were used in these studies. All experiments were conducted according to the guidelines of the ethics committee of the National Institutes of Health for the use and ethical handling of the animals. Rats were placed in pairs in plastic cages with food and water ad libitum upon arrival and kept on a 12-h on–off light/dark cycle and acclimatized 2 to 3 days before biochemical and behavioral manipulations started.

2.2. Li^+ treatment

The animals were fed Purina Lab rat chow with or without Li^+ carbonate (Li_2CO_3, Harlan Teklad, Madison, WI). For the first week, rats were exposed to a dose of 0.1% Li_2CO_3. Next, the dose was increased to 0.2% Li_2CO_3 for 3 weeks. Saline (0.9% NaCl) was added to the drinking water of Li^+ treated rats to reduce toxicity as previously reported (Smith and Amdisen, 1983). Body weight was monitored weekly in both control and Li^+ treated rats. After 4 weeks of dietary Li^+, rats were randomly divided for behavioral and biochemical studies.

2.3. Procedure for plasma Li^+ determination

At the end of the experiments, all animals were sacrificed and blood samples were collected either from the trunk when decapitated or from the liver when perfused. The blood collected in a mictrotube were immediately centrifuged at 14,000 rpm at 4 °C for 10 min, plasma was collected then stored in −20 °C until analysis for the levels of Li^+. Concentration of plasma Li^+ was prepared according to the method of Pybus and Bowers (1970) and was analyzed using the Atomic Absorption Spectrophotometer (Model 1100A, Perkin-Elmer, USA).

3. Behavioral assessments

3.1. Motor activity

Animals subjected to the *behavioral tests* were used 2 days before the end of the treatment. To measure locomotor activity, animals (Controls, n=11; Li^+ animals, n=11) were placed individually in a Plexiglas activity test chamber, with no prior habituation. Spontaneous ambulation and rearing behaviors were measured at 10 min intervals during a 60 min time period by continuous automated counting of photobeam interruptions using Tru Scan Photobeam Activity System (Coulbourn Instruments, Allentown, USA).

3.2. Object recognition task

The apparatus consisted of a closed box (100×100×50 cm high) made of black plexiglass. The objects to be discriminated were three wooden objects that differed in shape. Two days after the motor activity test, rats (Controls, n=5; Li^+ animals, n=5) were subjected to a habituation trial for 3 days in which they were exposed, first per pair then individually, to the empty arena and allowed to explore for 15 min. The rats were then observed during acquisition and retention trials both directly and on videotape recorded by a camera placed above the test arena. On the fourth day, the animals were exposed to two unfamiliar objects, designated A or B positioned diagonally in each corner of the apparatus. The rats were allowed to explore the objects for

a total of 15 min, of which only the initial 5 min were recorded and analyzed. The retention test was given 24 h later and consisted in placing the animals in the same testing arena with one of the two objects explored during acquisition (familiar object) and a novel object, designated object C, in the same locations. Animals were allowed to explore the objects for 5 min. Two stopwatches were used to count the time of investigation for each individual object during the acquisition and retention trials. In addition to the total investigation time for each object, a preference index for each object was also calculated for both acquisition and retention using the following formula: (investigation time for object *X*)÷(investigation time for object *X*+investigation time for object *Y*).

3.3. Holeboard spatial discrimination task

The holeboard is a square arena (100×100×50 cm high) made of black plexiglass containing four rows of four equidistant holes in the floor plate. In the present experiments, small pieces of chocolate chips were used as bait in 4 out of the 16 holes of the maze as described by Douma et al. (1998). For these experiments, control rats (n=8) were pair-fed during the last 3 weeks of treatment in order they maintain body weights similar to the Li^+ treated rats (n=8). None of the subjects of these experiments were hungry or had their diet restricted. Pair feeding consisted of calculating the amount of food that was given to the control rats based on the food intake of the Li^+ treated rats of the previous 24 h. The day previous to start of habituation, both control and Li^+ treated rats were familiarized with chocolate chips in their home cage. Subsequently, rats were habituated to the maze by allowing them to explore and freely eat in a fully baited maze for 5 min in three separate sessions a day for 2 days. Acquisition training started the next day. Pair-fed controls and Li^+ treated rats were subjected to a morning and afternoon sessions. Each session consisted of 5 trials (Peña de Ortiz et al., 2000) after which animals were rewarded with a few chocolate chips in their home cage. Normal food was available only at the end of the afternoon session. A total of 6 training sessions were given over a 3-day period. Performance of the task was assessed by recording the latency to complete the task (searching time), and the number of reference and working errors as described by us previously (Robles et al., 2003). An additional set of control and Li^+ treated rats was sacrificed by decapitation immediately after the third session of training. Their hippocampi were obtained as described below and stored at −80 °C until used for protein extraction and Western blot analysis.

4. Biochemical analysis

Naïve animals used for the biochemical studies were sacrificed after the 4 weeks of treatment. For the immunohistochemistry studies, the rats were deeply anesthetized with pentobarbital (50 mg/kg body weight, i.p.), then transcardially perfused with phosphate buffer saline (PBS, 0.1 M, pH 7.4), followed by 4% paraformaldehyde in 0.1 M PB. The brains were removed (Controls, n=10; Li^+ animals, n=7), post-fixed in the same fixative for 30 min, cryoprotected in PB containing 10% sucrose until they sunk in the tube and then stored in −80 °C until used for cryosectioning. Perfused frozen coronal sections (25 μm thick), six per slide (Controls, n=10; Li^+ animals, n=7), of the entire brain were obtained in a cryostat at −20 °C and stored at −80 °C until used. For Nurr1 immunohistochemical detection (Ge et al., 2003), sections were allowed to air dry for 20 min and were then washed twice with PBS, 5 min each time. Permeabilization was done with 0.1% Triton X-100 in 0.1% of sodium citrate for 5 min and washed as described above. The sections were then incubated with 0.3% H_2O_2 and methanol for 15 min and washed with PBS, which was followed by incubation with 5% goat serum in PBS for 30 min. Primary anti-Nurr1 polyclonal antibody (Santa Cruz Biotech.) was diluted at 1:100 in 1% goat serum in PBS (blocking buffer) and added to the sections. Then sections were incubated overnight at 4 °C in a moist chamber. The primary anti-Nurr1 was a rabbit polyclonal antibody raised against the carboxy-terminal region of the rat Nurr1 peptide. The next day and following two PBS washes, sections were incubated for 2 h at room temperature with a goat anti-rabbit biotinylated secondary antibody diluted at 9:1000 in 1% goat serum in PBS and then washed with PBS again. The sections were next incubated with ABC peroxidase reagent (avidin–biotin peroxidase complex, Pierce) for 1 h at room temperature and washed with PBS. Diaminobenzidene (DAB) was added to the sections for 10 min, followed by PBS washing. Omission of the primary antibody, as a negative immunohistochemical control, resulted in no Nurr1 staining (data not shown). The slides were mounted using permanent mounting medium (Vector Laboratories). Counting in a particular brain structure was generally performed on at least four representative histological sections from every animal. Areas were selected from regions CA1 and CA3 of the hippocampus, from the perirhinal (Prh) cortex and the dorsal endopiriform nucleus (DEN). Anatomical localization and regional nomenclature were defined according to Paxinos and Watson (1998). The distribution of Nurr1-like immunoreactive neurons from the area of interest was plotted onto a drawing. The immunoreactive cell nuclei displaying brownish black staining (DAB oxidized brown precipitates) in the various brain regions were automatically counted separately with the aid of an Olympus BX40 microscope. The microscope was equipped with a Polaroid DMC digital camera (1600×1200 dpi) with 10× objective magnification, using unbiased stereology with the Image J program (http://rsb.info.nih.gov/ij/). Following background subtraction, the threshold was adjusted so the counting program could

equally recognize those pale- and deep-stained nuclei. The controls comprised one batch: one animal from each group. Counting was not carried out on the control brains, because of the lack of Nurr1 staining.

4.1. Western blot analysis

Rats were rapidly sedated in an etherized chamber and then decapitated. The brains were obtained (Controls, n=4; Li animals, n=4), rinsed in cooled PBS, chilled on dry ice, and dissected to obtain the hippocampi, which were frozen on dry ice and stored at −80 °C until used for protein extraction for further Western blot analysis (Ge et al., 2003). Tissue samples were homogenized with the Brinkmann Polytron Homogenizer (Brinkmann Instruments) in extraction buffer (30 mM Hepes/KOH, pH 7.9; 5.0 mM $MgCl_2$; 1.0 mM EDTA; 0.5 M KCl; 20% glycerol; 2.0 mM dithiothreitol (DTT); 0.5 mM phenylmethyl–sulfonyl fluoride (PMSF); 1.0 μg/ml each of leupeptin and aprotinin). The samples were vortexed and centrifuged at 4 °C with a speed of 52,000 rpm for 2 h in an Optima TLX Ultracentrifuge (Beckman Coulter). The supernatant was dialyzed against dialysis buffer (30 mM Hepes/KOH, pH 7.9; 5 mM $MgCl_2$, 0.2 mM EDTA, 50 mM KCl, 10% glycerol, 0.6 mM DTT, 0.5 PMSF, 1.0 μg/ml leupeptin, 1.0 μg/ml aprotinin) at overnight. The dialyzed samples were obtained and centrifuged in Eppendorf Microcentrifuge 5415 C (Brinkmann Instruments) at 14,000 rpm for 30 min at 4 °C. Protein concentration was determined using the Bradford method. Samples were kept at −80 °C until used. The lysates, 40 μg of protein were loaded onto an 8% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) for electrophoresis. Proteins were then transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech) and electroblotted at 10 V at 4 °C overnight using the TransBlot SD semi-dry Transfer Cell (Bio-Rad). The next day, the membranes were subjected to Western blot analysis following standard procedures. After blocking in 5% dry milk and washed three times with PBS-Tween 20 (PBS-T) washes, the membrane containing the hippocampi protein samples were incubated for 1 h at room temperature (RT) with the primary anti-rabbit Nurr1 polyclonal antibody (Santa Cruz, CA) diluted at 1:500 in blocking solution. Following three additional PBS-T washes, the membranes were incubated with the anti-rabbit horseradish peroxidase conjugated secondary antibody (Amersham Pharmacia Biotech), diluted at 1:5000 for 1 h at room temperature. After three PBS-T washes, the membranes were soaked briefly in detection reagent (ECL Western Blotting Analysis System, Amersham Pharmacia Biotech) and exposed to X-ray film. For the specificity tests, the Nurr1 antibody (Santa Cruz Biotech.) was incubated overnight at 4 °C with three times the amount of Nurr1 blocking peptide (Santa Cruz Biotech.). The pretreated antibody was then diluted in the desired amount of 5% milk and Western Blotting was continued as previously described. The membrane were stripped and probed for actin to exclude differences in protein loading between the samples. Films were scanned and analyzed in a densitometer (Personal Densitometer SI, Molecular Dynamics, Amersham Biosciences, NJ, USA). Normalization was done, first by dividing actin optical density (ODs) of each sample by the highest value of actin obtained in the corresponding membrane. Then, the mean of Nurr1 OD per animal and per condition was divided by the corresponding actin OD.

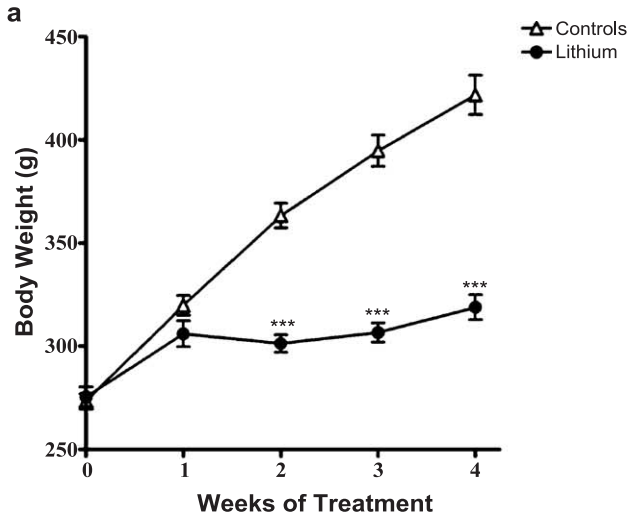
5. Statistical data analysis

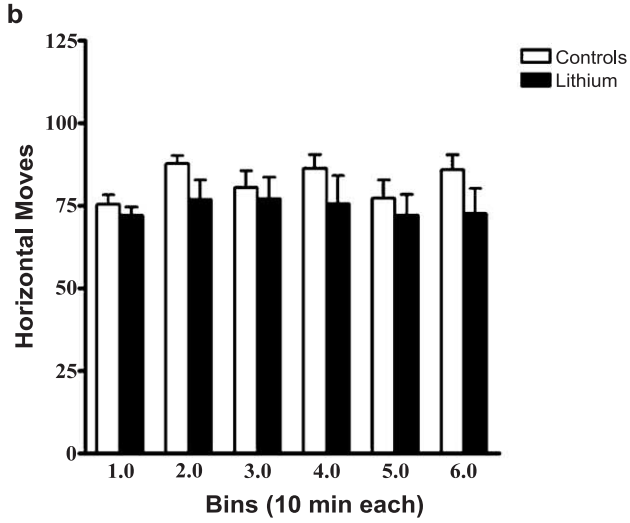
The body weight data was analyzed using two-way analysis of variance (ANOVA) coupled to Bonferroni post-tests for multiple comparisons. Horizontal locomotion and rearing between controls and Li^+ treated rats were assessed using a two-tailed Student's t-test or two-way analysis of variance (ANOVA). Object recognition and hole-board task was analyzed by two-way ANOVA and Bonferroni post-hoc analysis. A p value of <0.05 was accepted as statistically significant. Pearson correlation analysis was done between the Li^+ blood levels and the number of Nurr1 positive nuclei in the various brain regions analyzed. In addition, the Li^+ blood level and immunohistological findings were subjected to linear regression using the minimum sum of squares method. The standard error of the slopes was obtained by calculating the 95% confidence intervals and the linear relationship between Li^+ blood levels and Nurr1 expression was determined by an F-test to determine whether the slopes were significantly different from zero. The data from the CA1 region and of the Western blot were analyzed by the Student's t-test.

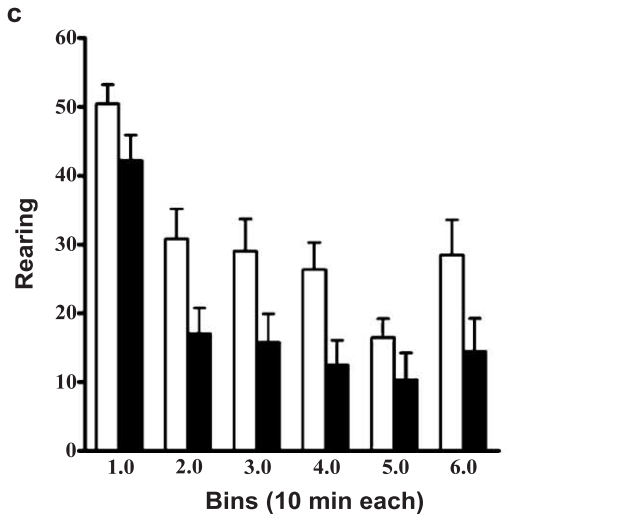
6. Results

Chronic Li^+ is known to cause decreased body weight in rats (Baptista et al., 1995). Hence, the changes in body weight of control and Li^+ treated animals were monitored (Fig. 1a). The data suggested that the Li^+ treatment halted increase in body weight. Two-way ANOVA confirmed significant differences between the groups caused by the treatment (F (1, 149)=194.4, ***p<0.0001) and duration (weeks) of treatment (F (4, 149)=75.5, ***p<0.0001) factors. A significant interaction occurred between the two factors (F (4, 149)=29.01, ***p<0.0001) because the differences in weight gain between the groups were only evident after the second week of treatment. Multiple comparisons post-testing showed that significant differences in body weight between the groups occurred after 2, 3, and 4 weeks of treatment (***p<0.001). Atomic Absorption Analysis revealed that only a subset of the Li^+ treated rats (31%) reached or surpassed the established clinically relevant therapeutic concentration in the blood of

0.63±0.05 mEq/l (data not shown). Hence, for the following studies, only the data from such animals was considered for the experiments.







7. Behavioral analysis

7.1. Motor activity

The effects of chronic Li^+ treatment on general locomotor activity of the animals were examined. Note that throughout the behavioral testing, the Li^+ treatment was not discontinued. As seen in Fig. 1b, Li^+ did not cause significant differences in horizontal locomotion (Student's *t*-test: $t(20)=1.4$, $p>0.1$). This finding suggests that Li^+ treatment did not produce ataxia or motoric impairments in these animals. However, Li^+ treated rats showed significantly less rearing activity (Fig. 1c) than controls (Student's *t*-test: $t(20)=2.76$, $p<0.05$). This result is consistent with previous behavioral evidence in rats (Kofman and Belmaker, 1990).

7.2. Object recognition task

Next, the effects of chronic Li^+ on object recognition memory (Fig. 2) were assessed. During acquisition training, both controls and Li^+ treated rats spent more time exploring object A than object B (Fig. 2a). In fact, two-way ANOVA identified a significant effect by the object factor ($F(1, 16)=6.533$, $*p<0.05$) indicating that the animals of both groups discriminated between the two objects. Interestingly, the analysis also identified a significant effect by the treatment factor ($F(1, 16)=5.447$, $*p<0.05$). No significant interaction occurred between the object and treatment factors ($F(1, 16)=0.1333$, $*p>0.05$). Overall, these data suggest that the Li^+ treated animals spent less time exploring either of the objects than controls, although post-hoc analysis identified no specific differences in exploration time between the groups for each of the two objects. Accordingly, when the preference of the animals to explore each of the objects was compared, a significant effect was identified for the object factor ($F(1, 16)=13.97$, $**p<0.005$), but no significant effect by treatment was observed (Fig. 2b). Importantly, the results from the retention test show that both groups were able to remember the familiar object presented 24 h earlier (Fig. 2c). Again, two way ANOVA identified a significant effect by the object factor ($F(1, 16)=6.418$, $*p<0.05$), but no significant effect by the treatment factor. Similar results were obtained when we analyzed the preference of the groups to

Fig. 1. Effects of chronic Li^+ treatment on body weight and locomotor activity. (a) Body weight across the 4 weeks of treatment period in rats. Data are mean±S.E.M. While the control group (open circles) gained normal weight, Li^+ treated group (closed circles) gained significantly less weight (two-way ANOVA: treatment factor, $***p<0.0001$; week of treatment factor, $***p<0.0001$). The asterisks depict the results of multiple comparisons post-testing showing significant differences in body weight between the groups at 2, 3, and 4 weeks of treatment ($***p<0.001$). (b) Effects of Li^+ on horizontal locomotor activity. Data are mean±S.E.M. A mild decrease in horizontal locomotion was observed for the Li^+ treated rats compared to controls. (c) Effects of Li^+ on vertical locomotor activity. Li^+ treated rats showed marked decrease in rearing activity when compared to the controls.

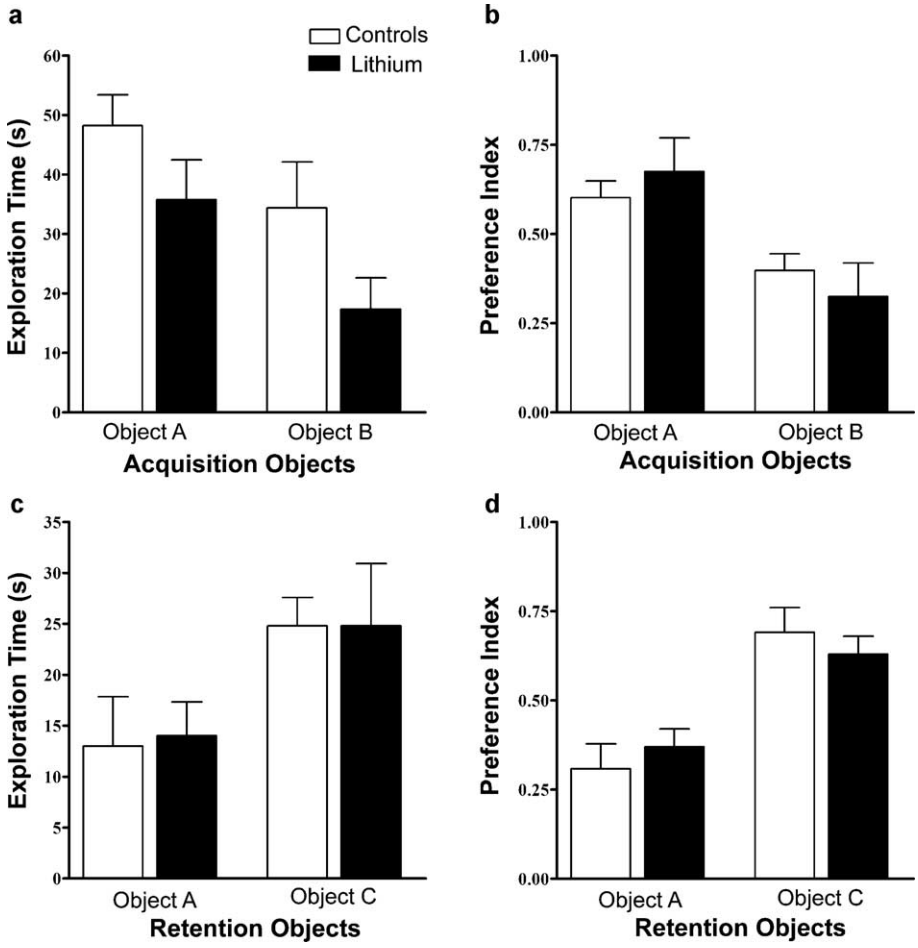


Fig. 2. Effects of chronic Li^+ treatment on object recognition. (a) Mean (±S.E.M.) time spent exploring the two objects for controls (open bars) and Li^+ treated rats (closed bars) during the acquisition phase. Both groups spent more time exploring object A than object B (two-way ANOVA: object factor (*p<0.05). The analysis also identified an effect by treatment factor (*p<0.05), although overall no significance was determined when using post-hoc testing. (b) Mean (±S.E.M.) Preference Index for each object calculated for the acquisition phase. Two-way ANOVA found a significant effect by the object factor (**p<0.005), but not by treatment. (c) Mean (±S.E.M.) time spent exploring the two objects for controls (open bars) and Li^+ treated rats (closed bars) during the retention phase. While two-way ANOVA identified a significant effect by the object factor (*p<0.05), no significant effect was observed by Li^+ treatment. (d) Mean (±S.E.M.) Preference Index for each object calculated for the retention phase. While two-way ANOVA identified a significant effect by the object factor (***p<0.0001), no significant effect was observed by Li^+ treatment.

explore either of the two objects (Fig. 2d). Both groups showed a similar and significant preference to investigate the novel object (object C) than the familiar object (two-way ANOVA, object factor (F (1, 16)=27.85, ***p<0.0001); treatment and interaction (p>0.05). The present results suggest that even though the Li^+ treated rats showed a lower interest compared to controls for investigating both novel objects during acquisition training, they were able to remember the familiar object on the next day, as did controls. Furthermore, the Li^+ treated rats preferred to explore the novel object instead during the retention test.

7.3. Spatial discrimination task

In order to further characterize the effects of Li^+ treatment on learning, we utilized a more complex task, the hole-board maze that measures spatial discrimination learning ability in animals. Rats were subjected to two training sessions a day with a total of six sessions in 3 days. Because of decreased body weight under Li^+ treatment, pair-fed controls were used and kept at similar body weights as the Li^+ rats. During these experiments, animals were not food deprived. When analyzing the trials factor, the results showed that rats in both groups displayed spatial learning as shown by a significant decrease in the number of reference errors (Fig. 3a, left) and searching time (Fig. 3b, left) throughout acquisition training (two-way ANOVA, trials factor: (F (29, 420)=8.234, ***p<0.0001 and (F (29, 420)=4.479, ***p<0.0001, respectively). However, the analysis of the trials data revealed that Li^+ treated rats committed significantly more reference errors and had significantly longer searching times than control rats (two-way ANOVA, treatment factor: (F (1, 420)=59.61, ***p<0.0001 and (F (1, 420)=47.87, ***p<0.0001, respectively). While the level of interaction between the trials and treatment factors did not reach statistical significance for either reference errors or

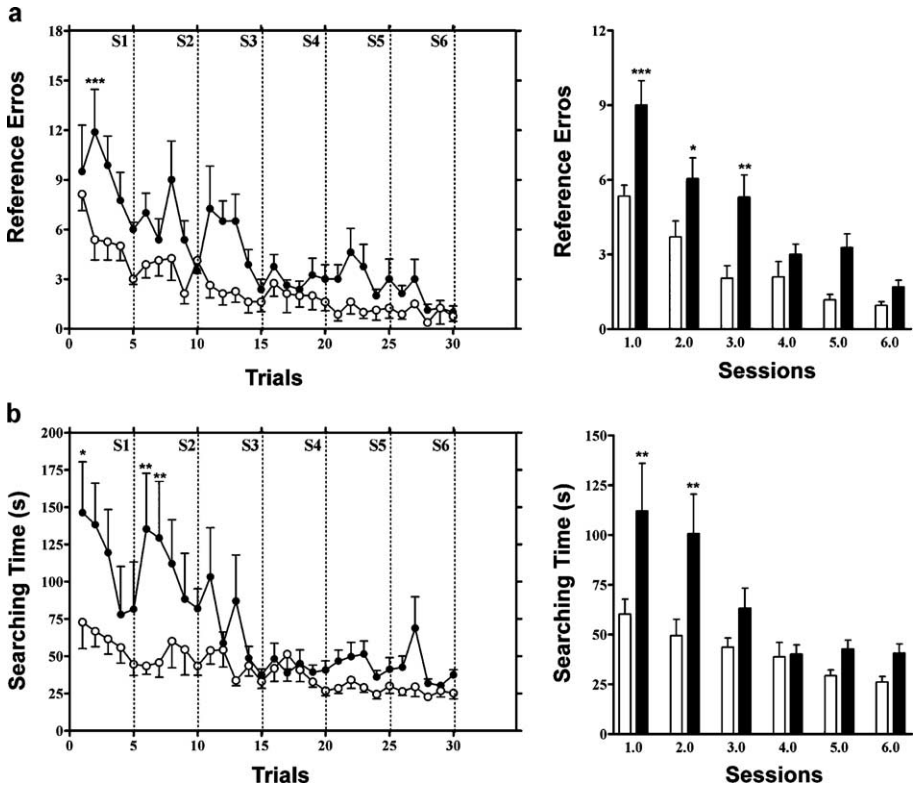


Fig. 3. Effects of chronic Li^+ treatment on spatial discrimination learning. (a) Mean (±S.E.M.) number of reference errors in training trials (left) and sessions (right) for control (open circles/bars) and Li^+ treated rats (closed circles/bars). For the trials data, Li^+ treated rats committed significantly more reference errors than control rats (two-way ANOVA, treatment factor: ***p<0.0001; post-hoc tests, trial 2: ***p<0.001). Similar results were obtained with the sessions data (two-way ANOVA, treatment factor: ***p<0.0001; post-hoc tests, sessions 1 (***p<0.001), 2 (*p<0.05) and 3 (**p<0.01)). (b) Mean (±S.E.M.) number of searching time in training trials (left) and sessions (right) for control (open circles/bars) and Li^+ treated rats (closed circles/bars). For the trials data, Li^+ treated rats spent significantly more time searching than control rats (two-way ANOVA, treatment factor: ***p<0.0001; post-hoc tests, trials 1 (*p<0.05), 6 (**p<0.01), and 7 (**p<0.01)). Similar results were obtained with the sessions data (two-way ANOVA, treatment factor: ***p<0.0005; post-hoc tests, sessions 1 (**p<0.01) and 2 (**p<0.01).

searching time, the latter behavioral measure showed a nearly significant level of interaction between the two factors (p=0.07). This finding suggests that the effects of Li^+ might be expressed differently during different stages of acquisition. This was supported by the results of our post-hoc analysis. Multiple comparisons testing identified specific significant differences between the groups on trial 2 (***p<0.001) for reference errors and on trials 1 (*p<0.05), 6 (**p<0.01), and 7 (**p<0.01) for searching time. These results suggest that the effects of Li^+ on acquisition were more evident during the initial two sessions of acquisition training. Analysis of the sessions data for reference errors and searching time confirmed these findings (Fig. 3a and b, respectively). As with the trials data, rats in both groups displayed a significant decrease in the number of reference errors (Fig. 3a, right) and searching time (Fig. 3b, right) throughout acquisition training (two-way ANOVA, sessions factor: (F (5, 84)=25.13, ***p<0.0001 and (F (5, 90)=7.944, ***p<0.0001, respectively). Also in agreement with the trials data, the sessions data showed that Li^+ treated rats committed significantly more reference errors and had significantly longer searching times than control rats (two-way ANOVA, treatment factor: (F (1, 84)=39.19, ***p<0.0001 and (F (1, 90)=15.65, ***p<0.0005, respectively). No significant interaction was observed between the two factors for either the reference errors or searching time data. Multiple comparisons testing identified specific significant differences between the groups on sessions 1 (***p<0.001), 2 (*p<0.05) and 3 (**p<0.01) for reference errors and on sessions 1 and 2 (**p<0.01 each comparison) for searching time. These results suggest that the effects of Li^+ on acquisition were more evident during the initial two sessions of acquisition training. Similar results were obtained for the data on the number of working errors except that post-hoc analysis identified no specific significant differences between the groups (data not shown). Finally, the retention test, 24 h later, showed no difference between the two groups in any of the behavioral parameters (data not shown).

8. Biochemical analysis

8.1. Inmunohistochemistry

So far, the results suggested that chronic Li^+ treatment resulted in a selective impairment in hippocampal dependent

Table 1
Results of Pearson correlation analysis of Li^+ plasma levels and the number of Nurr1 positive nuclei in different brain regions for both controls and Li^+ treated rats

Brain region	Pearson correlation			
	Control		Li	
	R^2	p value	R^2	p value
CA1	0.1902	>0.2	0.9593	<0.005**
CA3	0.0812	>0.4	0.1156	>0.5
Prh Cortex	0.3631	>0.05	0.0067	>0.8
DEN	0.0625	>0.5	0.0261	>0.7

Significant correlation between the levels of Li^+ in the blood and the number of Nurr1 positive nuclei was only identified in the CA1 hippocampal subregion of animals treated with Li^+ (R^2=0.9593, **p<0.005). Abbreviations: CA1, CA3: hippocampal subregions; Prh: perirhinal cortex; DEN: dorsal endopiriform nucleus.

learning. Next, an attempt to identify a molecular correlate to the behavioral findings was made. The next experiments were focused on the Nurr1 protein because of its implication in neuropsychiatric disorders and learning processes (Bannon et al., 2002; Buervenich et al., 2000). The levels of Nurr1 were examined in several brain regions after chronic treatment with Li^+. The number of Nurr1 positive nuclei was determined in the hippocampal CA1 and CA3 regions, as well as in the Prh cortex and the DEN. These are all regions shown previously to express the *Nurr1* gene (Xing et al., 1997). First, the relation between Li^+ blood levels and the number of Nurr1 positive nuclei in several brain regions were compared between controls and Li^+ treated rats. Table 1 depicts the results of Pearson correlation analysis of our data. Interestingly, a significant correlation between the levels of Li^+ in the blood and the number of Nurr1 positive nuclei was only identified in the CA1 hippocampal subregion of animals treated with Li^+ (R^2=0.9593, **p<0.005; Fig. 4a). Linear regression analysis of the data showed that in the control animals there was no significant relation between Li^+ blood concentrations and the number of Nurr1 positive nuclei in the hippocampal CA1 region

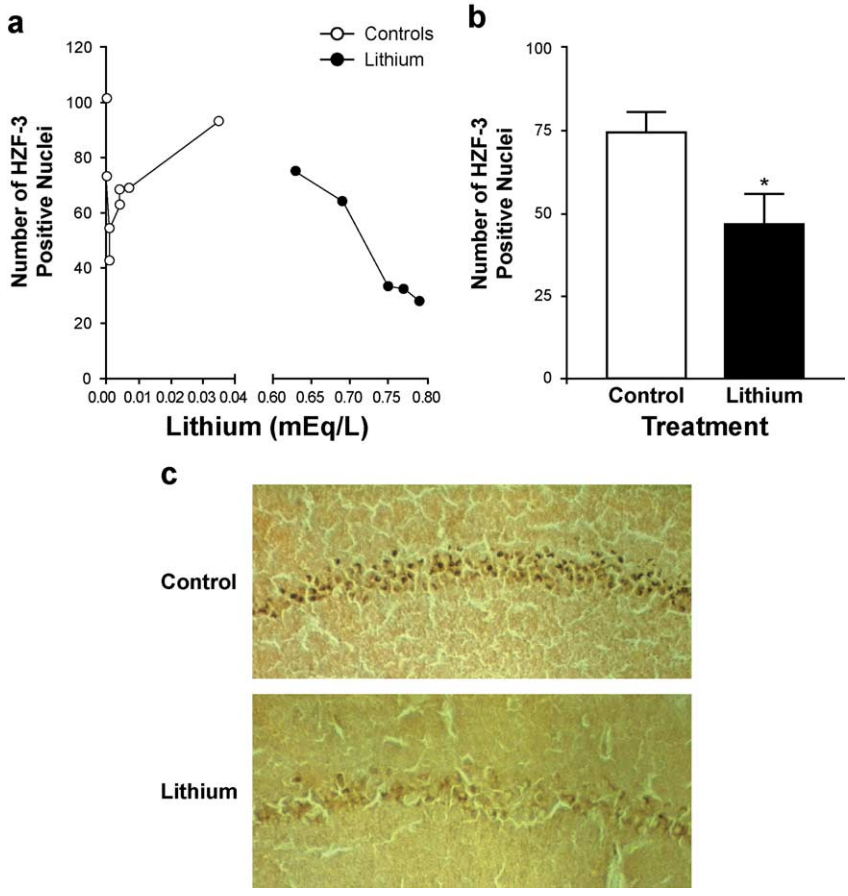


Fig. 4. Effects of chronic Li^+ treatment on hippocampal Nurr1 expression determined by immunohistochemistry. (a) Graph plotting the number of Nurr1 positive nuclei in CA1 (Y axis) in relation to the levels of Li^+ in the blood (X axis) for both controls (open circles) and Li^+ treated rats (closed circles). No significant relation between Li^+ blood concentrations and the number of Nurr1 positive nuclei in the hippocampal CA1 region was observed for controls, since the slope of the line was not significantly different from zero (F test: p>0.2). For the Li^+ treated animals, a significant negative correlation between Li^+ blood concentrations and the number of Nurr1 positive nuclei in CA1 was determined (slope: −320.6±38.14; F test: **p<0.005). (b) Bar graph showing the number of Nurr1 positive nuclei in CA1 of the control and Li^+ treated groups. The Li^+ group showed significantly less expression of Nurr1 in CA1 than controls (Student's t-test: *p<0.05). (c) Representative photomicrographs of the CA1 hippocampal subregion of controls (upper panel) and Li^+ treated rats (lower panel).

(slope=710.7±598.7). The slope was not significantly different from zero (F test: F (1, 6)=1.409, p>0.2). In contrast, for the Li^+ treated animals we found a significant negative correlation between Li^+ blood concentrations and Nurr1 expression in the CA1 region with a slope value of −320.6±38.14, which was significantly different from zero in an F test (F (1, 3)=70.68, **p<0.005). Fig. 4b shows a bar graph comparing the levels of Nurr1 in CA1 of the control and Li^+ treated groups as a whole. A Student's t-test determined that chronic Li^+ treatment caused a significant reduction in the levels of Nurr1 in the hippocampal CA1 region, compared to controls (t_{10}=2.55, *p<0.05). Similar results were also obtained in the CA3 region (t_{10}=2.289, *p<0.05), but not with the other regions examined (data not shown). Fig. 4c shows representative photomicrographs from the CA1 hippocampal subregions of controls and Li^+ treated rats depicting the decrease in Nurr1 expression within that region as a result of chronic Li^+ treatment.

8.2. Western blot

To confirm the immunohistochemistry results, Western blot analysis was also performed of hippocampal Nurr1 levels in separate control (n=4) and Li^+ treated rats (n=4). Western blot analysis of whole cell protein extracts prepared from the entire dorsal hippocampus revealed a clear band with an approximate molecular weight of 66 kDa, corresponding to the Nurr1 protein (Fig. 5a). Samples from Li^+ treated rats showed significantly lower levels of Nurr1 (normalized to actin) than non-treated controls (t_{10}=2.542, *p<0.05), replicating our immunohistochemical findings that Nurr1 in the hippocampus was decreased by chronic Li^+ treatment. One may speculate that the observed early impairment in hippocampal-dependent-spatial learning observed in the Li^+ treated rats (Fig. 3) could be associated to the low levels of hippocampal Nurr1 in these animals as observed in our biochemical studies (Fig. 4).

However, the behavioral data did show that even though the Li^+ treated rats were initially impaired in hippocampal dependent learning, these animals did eventually reach similar rate of learning with the control animals during the mid and final stages of acquisition. It had been previously shown that the levels of *Nurr1* mRNA increased in the CA1 and CA3 hippocampal subregion during mid acquisition of a hole-board task similar to the one used in these studies (Peña de Ortiz et al., 2000). Particularly, those studies found

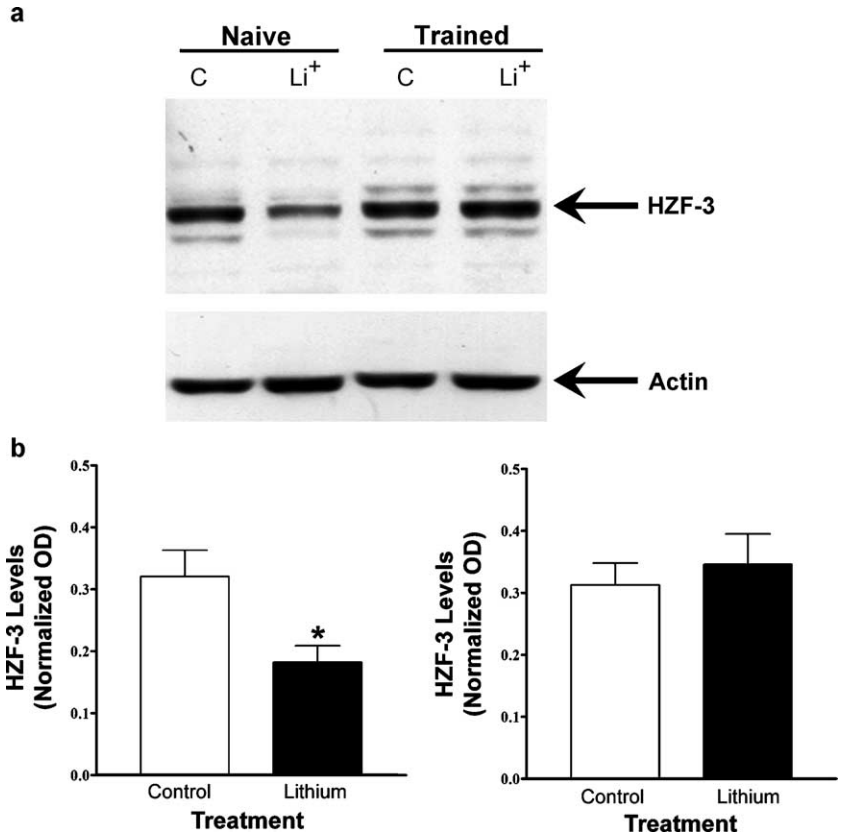


Fig. 5. Analysis of Nurr1 protein in hippocampal tissue. Left: Representative Western blot showing NURR1 (Top) and Actin (bottom) in the hippocampus of controls and chronically treated Li^+ rats after they were subjected to spatial discrimination training. The trained animals were sacrificed 3 h after session 3 of training, corresponding to the first session of day 2 of acquisition. (b) Left: Bar graph, mean±S.E.M., showing the densitometric analysis of Nurr1 for control (open bars) and Li^+ treated rats (closed bars). A Student's t-test identified a significant (*p<0.05) decrease in hippocampal Nurr1 in Li^+ animals compared to controls. Right: Bar graph, mean±S.E.M., showing the densitometric analysis of Nurr1 for Trained control (open bars) and Li^+ treated rats (closed bars). No difference was detected between the Trained groups using a Student's t-test.

significant increases in hippocampal *Nurr1* expression after the third session of acquisition training. Thus, the levels of hippocampal Nurr1 in control and Li^+ treated rats were compared after receiving three training sessions in the hole-board. Remarkably, there were no significant differences between the groups in rats sacrificed after the third session of training in the version of the spatial discrimination task used in these studies (Fig. 5b). This finding may suggest that the Li^+ treated animals were capable of upregulating the hippocampal expression of Nurr1 in response to the active learning experience.

9. Discussion

In the present study the effects of chronic Li^+ were investigated on rat's behavior and Nurr1 expression. In these experiments, rats showed physiological side effects of Li^+ treatment that had been previously reported. For example, rats decreased in body weight which has been previously attributed to gastrointestinal malaise (Smith and Amdisen, 1983; Launchbaugh and Provenza, 1994) and the ensuing decline in food intake (Baptista et al., 1995; Alessandri et al., 1994). In addition, Li^+ treatment also resulted in polydipsia and polyuria in the treated rats as observed in human patients (Smith and Amdisen, 1983; Mailman, 1983; Martines-Maldonado et al., 1975). In spite of these physiological effects, the Li^+ treated rats presented a healthy general appearance. When the animals were tested for spontaneous motor behavior, no difference was found in general motor activity between controls and Li^+ treated rats suggesting no toxic effects of chronic Li^+ treatment. However, the Li^+ treated animals showed significantly less rearing activity. As previously reported (Carroll and Sharp, 1971; Cox et al., 1971; Pascual and Gonzalez, 1995), Li^+ caused a decrease in rearing activity in the animals during a 60 min session, which could be associated to a depressive effect of Li^+ on exploratory behavior. In previous reports, a significant interaction was found between Li^+-induced inositol monophosphatase inhibition and decreased rearing (Kofman and Belmaker, 1990, 1993). Moreover, disruptions in brain DA transmission suppress locomotion (Pitts and Horvitz, 2000), therefore another explanation for our results could be an effect of Li^+ on central DA transmission that may have modified spontaneous exploration and vertical motor activity (Staunton et al., 1982; Carli et al., 1994).

Furthermore, the effect of chronic Li^+ treatment on cognitive function in rats was examined. Numerous controversial clinical findings are inconclusive with regard to whether Li^+ affects or not cognitive functions in humans (Kusumo and Vaughan, 1977; Kropf and Muller-Oerlinghausen, 1979; Squire et al., 1980; Stip et al., 2000). Separate groups of controls and Li^+ treated animals were used and tested in two specific behavioral tasks, the object recognition task and the spatial discrimination food search task. Since we were interested in determining the effects of Li^+ on spatial learning, testing this aspect of exploratory behavior allowed furthering characterizing the possible effects of Li^+ in eliciting a response to novelty. Object recognition memory, is agreed to be dependent on the perirhinal cortex (Murray and Richmond, 2001; Brown and Aggleton, 2001; Hannesson et al., 2004; Norman and Eacott, 2004). Whether it is a hippocampal or non-hippocampal dependent task is still in debate. While some studies reported that the task does not require the hippocampus (Mumby et al., 2002), several recent studies support the fact that the hippocampus contributes to learned object familiarity (Brown and Aggleton, 2001; Pittenger et al., 2002; Gaskin et al., 2003; Hammond et al., 2004). Object recognition memory is assessed by the preference that normal animals display for exploring novel, rather than familiar, complex objects (Ennaceur et al., 1997). In the present study, although the two groups discriminated between the two objects during acquisition training, Li^+ treated animals exhibited less explorative behavior for the objects when compared to controls. This data agrees with previous findings showing that Li^+ treatment reduces the approaches to novel objects (Crawley, 1983) and may be indicative of apprehension toward novel experiences or deficits in attention. This apparent initial apprehension to novelty could be related to the decrease found in Nurr1 expression in the hippocampus. Moreover, it is hypothesized that the other behavioral aspects of object recognition that stay intact in our findings may be perhaps the result of the lack of Nurr1 expression changes in the perirhinal cortex following Li^+ treatment. This assumption is supported by the data taken from the retention test, where the Li^+ treated rats were similar in object recognition memory as controls. They were able to remember the familiar object, as did controls, and were able to learn new discriminations. Thus, while chronic Li^+ affected exploratory behavior toward novel objects it did not affect object recognition memory. Previous biochemical experiments have shown that Nurr1 is prominently expressed in the deeper layers of perirhinal cortex (Xing et al., 1997). The perirhinal cortex is known to be involved in object recognition memory as studied in different species (Meunier et al., 1993; Mumby and Pinel, 1994; Buffalo et al., 1999; Abe et al., 2004). It is hypothesized that Nurr1 was not affected by chronic Li^+ treatment in the Prh cortex because higher concentrations of Li^+ and different route of administration (i.p. or s.c.) were needed to disrupt Nurr1 expression in this brain region. More experiments are needed to further characterize this finding.

The hole-board maze was first used to test deficits in attention and thought disorders (Oades, 1982). The task enables animals to discriminate relevant (baited) from irrelevant (unbaited) holes indicating a learning process where the choice accuracy is improved throughout the testing period. As opposed to the most commonly used laboratory tool in learning and memory, the Morris water

maze, in which animals are forced to swim in opaque water to find an escape platform (Morris et al., 1982), the hole-board task relies on a more ethological behavior for rodents: food searching. Like the Morris water maze, the hole-board task is sensitive to hippocampal lesions and has been used in various learning and memory studies (Oades, 1981, 1982; Oades and Isaacson, 1978; Peña de Ortiz et al., 2000; Vazquez et al., 2000; Robles et al., 2003; Van der Zee et al., 1992; Isaacson et al., 1994; Maldonado-Irizarry and Kelley, 1995; Van der Staay, 1999; Van der Staay et al., 1990).

Importantly, in the present experiment, despite the fact that Li^+ treated rats showed learning delays and significant poor performance in the first three sessions, they displayed spatial learning throughout the acquisition training and did not differ from the controls during the last three sessions. These results suggest that chronic Li^+ treatment delays but does not block spatial memory formation in the hole-board. The present findings further demonstrate that this latency in initial learning could be the result of spatial deficits and not other physiological, motoric or motivational factors. Our behavioral data support this notion. First, animals did not show overt Li^+ intestinal toxicity since they ate the chocolate chips that were presented to them as bait. Second, diminished motor activity was not reported since animals showed normal horizontal locomotion (Fig. 1b). However, the constant decreased level of rearing activity in these animals (Fig. 1c,d) may partly explain their initial learning impairment. It was observed that in order to establish proper spatial associations between food containing holes and extramaze cues rats normally used rearing during their learning trials. Interestingly, we also demonstrated that Li^+ impaired initially spatial reference memory formation but not working memory. Previous studies also support this finding. For example, rats chronically treated with Li^+ showed effects in reference memory errors but not in working memory errors in the Morris Water maze (Vasconcellos et al., 2003). Finally, decreased motivation was not observed in our animals since they showed a normal arousal level in the object recognition task, which is not influenced by response to any reward. Taken together, the evidence suggests that Li^+ may affect various spatial memory processes differentially. Furthermore, we may conclude from these behavioral data that the effects of Li^+ may be specific to particular cognitive processes, especially those involving the hippocampus, as has been previously suggested (Stip et al., 2000; Engelsmann et al., 1992).

To better understand the molecular substrates of this selective impairment in hippocampal-related learning following chronic Li^+ treatment, special interest was given to Nurr1. As previously mentioned, Nurr 1 has been implicated in cognitive processes (Peña de Ortiz et al., 2000; Ressler et al., 2002; Ge et al., 2003) and neuropsychiatric disorders (Bannon et al., 2002; Buervenich et al., 2000). First, brain samples from untrained control and Li^+ groups that were sacrificed immediately after the 4 weeks of treatment were analyzed. The present immunohistochemical results demonstrated that chronic Li^+ treatment reduced the basal levels of Nurr 1 like previously shown for other immediate early transcription factors (Williams and Jope, 1994; Miller and Mathe, 1997; Swank, 1999; Chen et al., 1999; Yuan et al., 1999; Bosetti et al., 2002). Nurr 1 is known to be present within the Prh cortex, the CA1–3 area of the hippocampus and the DEN (Peña de Ortiz et al., 1994; Näkki et al., 1996; Xing et al., 1997). In particular, a significant negative correlation between Li^+ levels in plasma and the number of Nurr1 positive nuclei was obtained for the CA1 hippocampal subregion, but not other brain regions of Li^+ treated animals.

The hippocampal decrease in Nurr1 expression caused by Li^+ treatment was confirmed by Western blotting. Previous studies showed that chronic Li^+ treatment decreased CREB phosphorylation in the hippocampus (Chen et al., 1999). CREB is a nuclear protein that modulates the transcription of genes with cAMP responsive elements in their promoters (Impey et al., 1998; Silva et al., 1998). Since the proximal promoter region of the *Nurr1* gene contains a cAMP responsive element (Saucedo-Cardenas et al., 1997), it is possible that the observed Li^+-induced decreases in Nurr1 expression in the brains of naïve rats are associated to its effects on CREB activation. Another plausible explanation for this Nurr1 decrease comes from molecular studies that have shown that chronic Li^+ treatment inhibited PKC function in the hippocampus and other cortical areas (Wang et al., 2001; Manji et al., 1993; Lenox et al., 1992). PKC is an intracellular enzyme crucial for synaptic plasticity in several species of animals and seems to be also an intracellular target of Li^+ actions (Manji and Lenox, 1999, review). Moreover, PKC has been reported to modulate Mitogen-Activated Protein Kinase (MAPK) actions on CREB in CA1 hippocampal regions (Roberson et al., 1999). Therefore, it is hypothesized that an inhibition of PKC signaling pathways could result in a decrease in CREB phosphorylation which in turn reduce Nurr1 expression during Li^+ treatment.

Interestingly, however, unlike our findings with naïve rats, when hippocampal Nurr1 levels in spatially trained Li^+ treated and untreated rats were compared, we observed no difference between the groups. Thus, the suppressive effect of chronic Li^+ on hippocampal Nurr1 expression at the basal level may be reversed by enhanced neuronal activity during active learning. Corroborating this possibility, we had previously shown that Nurr1 mRNA increases in the CA1 and CA3 subregions of the hippocampus after training in the hole-board spatial discrimination task (Peña de Ortiz et al., 2000). We suggest that while chronic Li^+ treatment caused a decrease in the basal levels of Nurr1 in the brain, neuronal activity related to learning processes may have enhanced Nurr 1 expression.

It is hypothesized that a rebound effect of particular upstream cellular mechanisms could have led to hippocampal induction of Nurr1 expression during mid acquisition of the hole-board task. It has been reported that Protein kinase A regulate mitogen-activated protein kinase (MAPK) activity and CREB phosphorylation within the CA1 region of the hippocampus (Roberson et al., 1999). Therefore, we postulate that during the late stages of the spatial learning acquisition, upstream mechanisms are being activated and triggering the phosphorylation of CREB and subsequently the activation of Nurr1. Moreover, unpublished data from our laboratory has also revealed that within the nucleus accumbens (a brain area also known to be involved in spatial learning) CREB phosphorylation reaches its peak around the same time point the animals in the present study seem to override their spatial learning deficits in the hole-board task. This finding is consistent with previous studies that reported a biphasic pattern of CREB phosphorylation during long-term potentiation in dentate gyrus of the rat (Schulz et al., 1999). Therefore, it is plausible that these cellular cascades are playing an important role in the regulation of gene expression within hippocampal neurons and inducing compensatory biochemical effects that result in the delayed acquisition of spatial learning in Li^+ treated animals.

Another explanation for the present findings could be related to actions of Nurr1 in the dopamine (DA) system. Previous studies have shown that DA plays a role in learning and memory processes (McNamara and Skelton, 1993; Myhrer, 2003) and reduced expression of Nurr1 affects DA neurotransmission in the mesolimbic and mesocortical system (Eells et al., 2002). In addition, forced Nurr1 activation in differentiated and undifferentiated adult hippocampus-derived progenitor cells elicits endogenous tyrosine hydroxylase (TH) expression suggesting a direct Nurr1 action on TH gene transcription (Sakurada et al., 1999; Pothos, 2002). More recently, lesion studies using striatal 6-hydroxydopamine microinjections produced an enhancement in the amount of Nurr1 positive cells in the substantia nigra, which also suggest that Nurr1 is related to dopamine neuronal function (Ojeda et al., 2003). Moreover, Li^+ also has direct actions in the DA. Previous experimental data have demonstrated that chronic Li^+ treatment induced a decrease in the number of D2-like receptors in the hippocampus as well as altered functioning of inositol phosphate metabolism in hippocampal slices (Marinho et al., 1998). Since our present data report a close interaction between Li^+ and Nurr1, it is possible to suggest that Li^+ treatment may be affecting DA function through its action in Nurr1. Thus, Li^+ could be an intracellular target for Nurr1 that act via DA neurotransmitter system.

Overall, our results suggest that the initial impairment in spatial discrimination learning caused by chronic Li^+ might be associated with the reduced basal levels of Nurr1 observed in the hippocampus of Li^+ treated animals. On the other hand, the capacity of the Li^+ treated animals to overcome their initial impairment and complete acquisition of the task at similar levels than controls could be due to the retained ability of these animals to upregulate hippocampal Nurr1 in response to active learning. In addition, these findings also suggest that hippocampal Nurr1 expression might be important for the initial as well as the late stages of acquisition of spatial discrimination. We conclude that expression of the *Nurr1* gene in the brain is associated with long-term spatial memory processes (Peña de Ortiz et al., 2000). Its anatomical specificity within the brain suggests that Nurr1 could be involved in functions related to memory or cognition.

In conclusion, these experiments provide evidence that the hippocampus is a possible site for Li^+'s effects by temporarily attenuating cognitive and memory abilities in complex learning tasks. Furthermore, that Nurr1 is involved in functions related to memory or cognition, not associated with object recognition memory but important for complex spatial learning tasks. Moreover, the chronic effect of Li^+ reduced hippocampal Nurr1 expression, a fact that may highlight the possibility of this transcription factor being a candidate risk-conferring factor for affective disorders and a target for pharmacological treatment in these conditions. Finally, it is interesting to speculate on the implications of the present findings and suggest that the therapeutic effects of chronic Li^+ on the brain may be particularly relevant to hippocampal-dependent cognitive processes involving Nurr1 expression.

Acknowledgements

We thank Hong Ge for her contribution with the immunohistochemistry studies described on this work and Pedro Valdes for his technical assistance with the Atomic Absorption Analysis (AAS).

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