Time course effects of lithium administration on spatial memory acquisition and cholinergic marker expression in rats

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ABSTRACT

Background: The effects of chronic lithium exposure on spatial memory in rats remain controversial. In this study a time course of the effects of lithium, administered systemically, on spatial memory acquisition in Morris water maze was investigated.

Material and Methods: Lithium (600 mg/L) was administered to four groups of rats in their drinking water; the first group of animals received lithium for one week, the second group for two weeks, the third group for three weeks, and the fourth group for four weeks. As controls, four groups of animals received only normal drinking water for the same period of time. Toward the end of their lithium or water treatment, all animals were trained for four days; each day included one block and each block contained four trials. Test trials were conducted 48 hrs after completion of the lithium treatment. Escape latency, traveled distance and swimming speed were evaluated during testing trials. Brain tissues from animals were processed according to the standard protocols for immunohistochemical analysis.

Results: Lithium treatment decreased escape latency and traveled distance, but not swimming speed, compared with controls, suggesting significant spatial memory acquisition enhancement by lithium. Quantitative analysis showed that lithium, particularly after four weeks of exposure, significantly increased the number and density of immunostained ChAT-containing (choline acetyltransferase) neurons in the medial septal area in comparison with control groups. There was also a significant correlation between the number of immunostained ChAT neurons and behavioral measures.

Conclusion: These results suggest that chronic oral administration of lithium causes spatial memory acquisition improvement in rats and an increase in ChAT immunostaining levels in medial septal nuclei.

Keywords: Lithium; Morris water maze; Spatial Memory; Cholinacetyltransferase; Medial septal nucleus

INTRODUCTION

Lithium (Li$^+$) is an effective drug for treatment and prophylaxis of bipolar disorder (1,2). This cation is distributed in all body fluids with volume of distribution equal to total body water (3). Although the effect of lithium in treatment of mania and bipolar affective disorder is generally recognized, its proposed mechanisms and modes of action in the brain are not fully understood. Overall, lithium effects are believed progressive to long-term neuroplastic alterations involving phosphoinositide intracellular pathways, transcription factors, and regulation of gene expression (4-6). Moreover, previous studies have shown that lithium affects several neurotransmitter systems and cellular transcription mechanisms (5,7,8). Although the effects of lithium on learning and memory have been evaluated in several studies, the results are somewhat inconsistent. While some studies have shown that lithium has

neuroprotective effects in neurodegenerative disorders other studies have failed, to demonstrate lithium-induced memory impairment (5,9). In addition, while several clinical investigations have shown cognitive impairment in short-term and long-term memory in patients taking lithium (10-14) which often leads to non-compliance with treatment (11), some other clinical studies have demonstrated that lithium did not affect memory function (15,16).

A considerable body of evidence indicates that lithium affects memory by interaction with acetylcholine (Ach), serotonin (5HT), dopamine (DA), N-methyl-D-aspartic acid (NMDA), prostaglandins, kinases and neurotrophic factors in different areas of the brain (3,17-25). These observations suggest an important role for lithium in synaptic plasticity and regulation of cognition.

ACh-containing neurons of the basal forebrain are known to be affected significantly in Alzheimer's disease (AD) (26-32) and the resulting ACh deficit has been shown to correlate with memory and cognitive impairments in this disorder (26,33-36).

Choline acetyl transferase (ChAT) is the enzyme that synthesizes ACh and vesicular acetylcholine transporter (VAChT) is responsible for transport of ACh into synaptic vesicles for regulated release. ChAT and VAChT proteins are strongly expressed in cholinergic neurons and are required for vesicular release of ACh and cholinergic transmission (37,38).

Moreover, it has been reported that the septohippocampal and the basal forebrain-neocortical pathways of the cholinergic system are primarily affected in AD (39). Thus, a decrease in cholinergic neurotransmission is thought to be one of the important causes of dementia and cognitive deficit in AD (40). In addition, our previous work showed that inhibition of protein kinase A type II (PKA II) caused memory retention deficits as well as a reduction in expression of ChAT in the medial septal area and dorsal hippocampus (41).

The interaction between lithium and cholinergic system has been reported previously (3,21,42-44). For instance, it was shown that hippocampal cholinergic neurotransmission was enhanced through 5-HT$_{1A}$ receptor-mediated pathways by a repeated lithium treatment (21). The aim of this study was to examine the time-course of the effects of systemic Li$^+$-treatment on spatial memory acquisition in rats and on ChAT immunoreactivity in their medial septal areas as well as to evaluate correlations between its immunoreactivity with spatial memory parameters and time-course of lithium administration.

MATERIAL AND METHODS

Animals
Male Wistar rats (200-240 g) were purchased from Pasteur Institute of Iran and maintained on 12hrs light/dark cycle with free access to food and water. All the procedures involving the use of animals were performed in accordance with the guidelines of the Helsinki on animal care. The animals were randomly assigned to eight groups with 8 animals in each group. Four groups of animals were subjected to receiving lithium (600 mg/L) in their drinking water; First group received lithium for one week (7 days), second group for two weeks, third group for three weeks and fourth group for four weeks. Similarly, as controls, four additional groups of animals received only normal drinking water (tap water) for the same periods of time.

Drugs
All drugs used in this study were purchased from Sigma (St Louis, MO, USA).

Behavioral training and testing
In this study, animals were subjected to training trials during the last four days of the administration of lithium which was terminated at the end of the training trials. Spatial memory acquisition was then tested 48 hrs after termination of the lithium administration.
Training of all groups of rats was conducted in the Morris Water Maze task. The water maze was a black circular tank (136 cm in diameter and 60 cm in height), which was filled with water (20±2 ºC) and placed in a room containing several extra maze cues. The Plexiglas escape platform that was used for the spatial task was submerged at a depth of 1 cm from water surface. Rats received one training session consisting of four trials in a day. The complete method of training and testing was explained in the previous work (41). The interval between the last training and the first testing trials was 48 hours. The testing included 1 block of 4 trials.

Determination of serum Li$^+$ levels
Lithium measurement was made after four weeks of lithium treatment. The blood samples were obtained directly from the heart of animals and collected into siliconized tubes. Serum from each animal was immediately separated by centrifugation in a microcentrifuge and diluted for estimation of Li$^+$ in distilled and de-ionized water. Li$^+$ levels in serum samples were then determined using a Shimadzu AA-670 Atomic absorption spectrophotometer. Readings were made in triplicate at the wavelength of 670.8 nm. Peak

height measurements were compared with values for standards of known concentration prepared similarly in diluted serum.

Immunohistochemical staining procedure
Brain tissue from five to six animals in each group were obtained and processed according to standard protocols (27,41,45). Animals were deeply anesthetized and then transcardially perfused with 100 ml of PBS (phosphate-buffered solution) followed by 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer containing 0.15% picric acid at 4°C. The brains were then postfixed in the paraformaldehyde/ picric acid solution overnight followed by incubation in a PBS solution containing 30% sucrose. After being embedded in Optimal Cutting Temperature (O.C.T.), the brains were sectioned at 40-micron intervals through the basal forebrain in the frontal plane, according to the atlas of Paxinos and Watson (1997). These tissue sections were immunostained to determine the presence and the extent of ChAT immunoreactivity. In this study, all of the immunohistochemical sections from all animals were processed at the same time. The details of immunohistochemical staining procedure for evaluation of ChAT immu-noreactivity were explained in the previous studies (38, 41). After finishing different steps of staining, the sections were then mounted on gelatin-coated Super Frost Plus glass slides (Fisher Scientific, USA), dried in air overnight, washed briefly with distilled water, incubated in xylene for 5 min, and coverslipped with a Cytoseal® mounting medium containing xylene (Baxter Diagnostics, Inc., McGrow Park, IL, USA). These tissue sections were analyzed with a BX51 Olympus microscope. On the basis of anatomical landmarks, the medial septal area, located anteroposterior (AP), 0–0.7; lateral (L), 0; ventral (V), 5 (Paxinos and Watson 1997), was selected with the microscope set at 10X. All photomicrographs were taken at 10X. The number of neurons was determined by counting the number of neurons in all of the brain slices collected for any given brain region of interest.

Quantification of ChAT- immunostaining
Following immunohistochemical staining, ChAT immunoreactivity was evaluated by light microscopy and the captured images were digitized using Olysia software (Olympus, Japan). In the medial septal area of each animal, the number of ChAT-containing neurons was directly counted. The collected values were then averaged and compared statistically between control and lithium-treated animals in each group. The average number of th**e** immunostained ChAT-

containing neurons in various groups was used for preparation of the bar graphs in Fig.7A and 7B .

Statistical Analysis
One-way ANOVA used in this study. A Newman-Keulz multiple comparison test was performed to assess differences in behavioral scores. The ChAT immunoreactivity measurements in control and lithium-treated animals were analyzed using one-way ANOVA and Newman-Keulz multiple comparison post-test. A *P*-value of 0.05 or less was considered statistically significant.

RESULTS

Effects of four days of training on escape latency, traveled distance and swimming speed in the Morris water maze
Four days training of animals in this study caused a significant decrease in escape latency and traveled distance for finding a hidden platform in the Morris water maze. As is shown in figure 1 (a and b), there was a significant difference between the first and the fourth day of training in terms of escape latency (***, $P<0.001$,) and traveled distance (***, $P<0.001$). The data in figure 1 shows the mean of all groups of animals that were used in this study including control group and lithium-treated animals (for one, two, three and four weeks). The swimming speed did not change significantly during training days in any of the animal groups (Figure 1c).

Time-course of the effects of systemic administration of lithium on escape latency, traveled distance and swimming speed
Exposure of animals to lithium chloride (600 mg/L) through their drinking water for one, two, three and four weeks caused alternations in escape latency and traveled distance during testing trials. The animals that were treated with lithium (600 mg/L) for either one, two or three weeks did not show any significant alternations in escape latency, traveled distance and swimming speed (Fig. 2a, 2b, and 2c). Although, a significant reduction in escape latency (*, $P<0.05$) and traveled distance (*, $P<0.05$) was observed after four weeks of lithium (600 mg/L) administration compared to the respective control group (Fig 2a and 2b), the swimming speed did not changed significantly by the lithium administration in different periods (Fig. 2c). The data suggests that exposure of animals to lithium (600 mg/L) for 4 weeks slightly but significantly improved spatial memory acquisition in comparison with respective control group (Fig. 2a, and 2b).

Figure 3A-D shows a visible reduction in traveled path following lithium administration compared to the control group (Fig. 3E). Administration of lithium (600 mg/L) for 4 weeks caused a significant reduction in the traveled path (Fig. 3D), when animals were tested 48 hrs after completion of training trials.

Time course of the effects of systemic administration of lithium on the cholinergic marker ChAT in the medial septal nucleus
Immediately after completion of testing trials, sections of the brains of rats from five lithium-treated animals in each period (i.e., one, two, three and four weeks of exposure) and control groups were immunostained with anti-ChAT antibodies.
As it is shown in Figure 4, representative rats receiving lithium (600 mg/L) for different periods showed an apparent increase in the number and density of cell bodies of the immunostained ChAT-containing (Fig. 4a-e) neurons in the medial septal area. These increases were particularly more evident and dramatic for animals which received lithium for four weeks.
Subsequent quantitative analysis of ChAT-immunostaining in the medial septal nucleus of tissue sections appears to confirm that there was a significant increase in ChAT (Fig 5) (**, $P<0.01$) immmunoreactivity in the four-weeks lithium-treated animals.

Assessment of the correlation between lithium exposure, cholinergic marker immunoreactivity and spatial memory parameters
A significant correlation between the time-course of lithium administration and the number of immunostained ChAT-containing neurons was observed in the medial septal area ($P<0.05$, $R^2=0.91$) (Fig 6).
In addition, a significant correlation was found between the number of immunostained ChAT-containing neurons in the medial septal nucleus and escape latency (**, $P<0.01$, $R^2=0.87$) (Fig. 7A) and traveled distance (**, $P<0.01$, $R^2=0.77$) (Fig. 7B) in rats treated with lithium for four weeks.

Results of determination of lithium concentration in blood of animals
The maximum concentration was observed up to three weeks after lithium administration (Table 1).

DISCUSSION
In the present study, spatial memory acquisition and expression of ChAT protein in the medial septal nucleus of rats which were exposed to lithium for either one, two, three or four weeks was evaluated. A significant increase in ChAT immunoreactivity was found in the medial septal nuclei of animals that were exposed to lithium, particularly for four weeks. This particular group also showed a significant improvement in the Morris Water Maze performance task that demonstrates spatial memory acquisition enhancement. Significant correlations were also found between ChAT immunostaining in the medial septal area with time-course of lithium administration and behavioral measures.
In this study, measurement of the lithium level in the blood of the lithium-treated animals showed hat its concentration increased significantly during that lithium exposure.

Table 1. Whole blood lithium concentration in rats after chronic administration of lithium chloride (600 mg/L) in drinking water.

Time (day)	Whole blood lithium concentration(mmol/L)
Control	0.018±0.002
7	0.057±0.008***
14	0.280±0.020***
21	0.300±0.010***
28	0.260±0.030***

Animals received lithium chloride (600 mg/l) for 7, 14, 21 and 28 days in their drinking water. Control groups received tap water. Each point is the mean±SEM for all animals. (***, $P<0.001$, One-way ANOVA) different from control group.
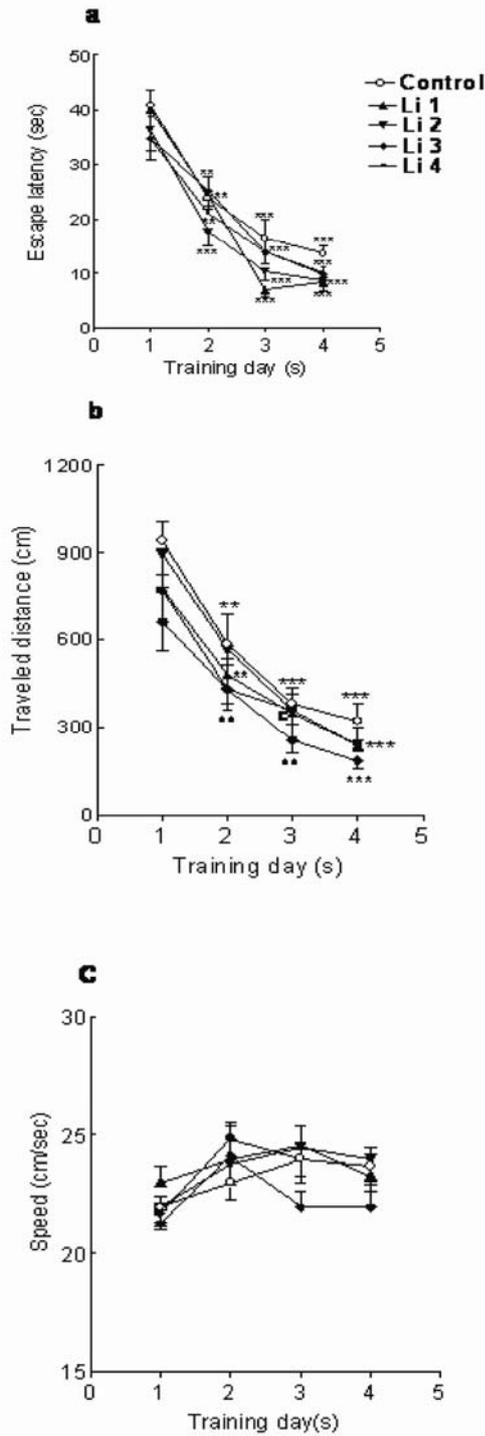
Figure 1. Effect of training on finding a hidden platform during training days in the Morris Water Maze task. that all groups of animals, including control and lithium-treated (Li1 for one, Li2 for two, Li3 for three and Li4 for four weeks) learned how to find the platform during training trials. There was a significant difference (***, $P<0.001$, **, $P<0.01$), One-way ANOVA) in escape latency (a) and traveled distance (b) between the first and fourth days of training. No significant difference was observed for swimming speed between first, second, third or fourth days (c). The results are presented as mean ±SEM.
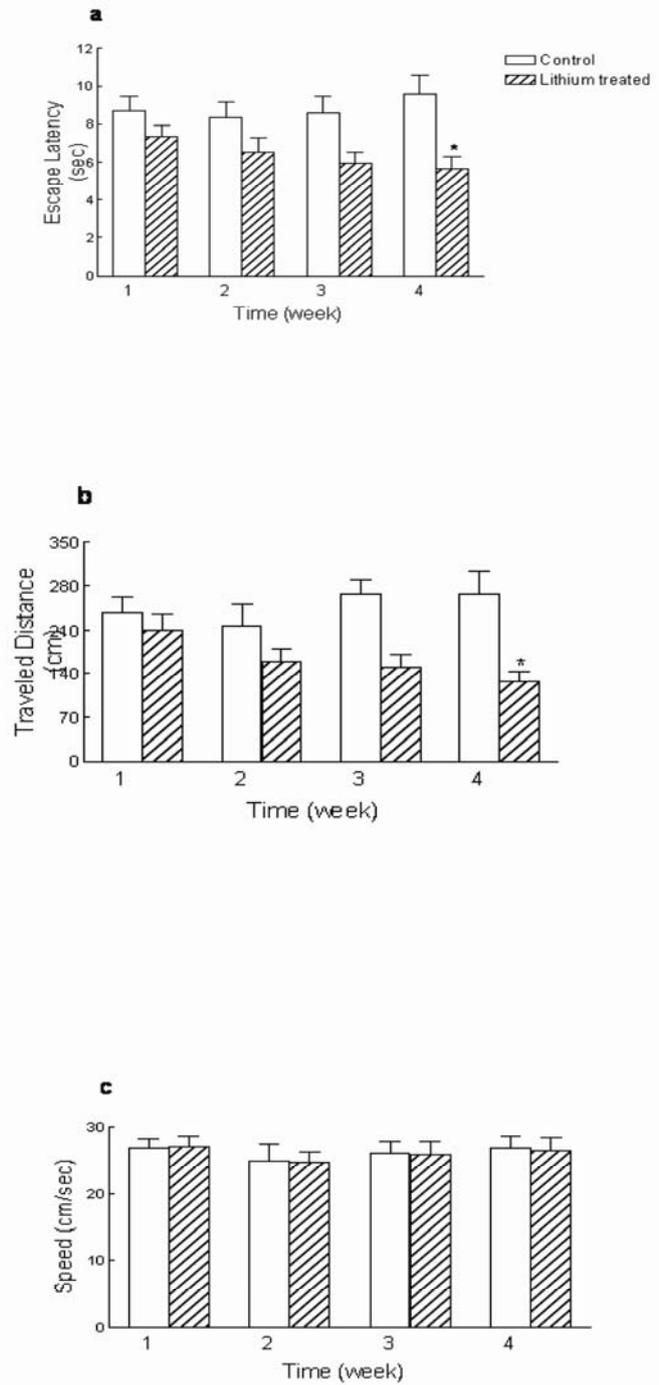
Figure 2. Effect of lithium administration on spatial memory acquisition. Administration of lithium (600 mg/L) for four weeks caused spatial memory improvement during the testing trials. The escape latency (a) and traveled distance (b) were significantly reduced after four weeks of lithium exposure compared to controls (*, $P<0.05$, One-way ANOVA and Newman-Keulz multiple comparison post test). The swimming speed did not show any significant alteration (c). The data show the mean±SEM of 8 animals.

No significant differences were found in swimming speed among the first, second, third, and fourth day of training. In additionally, animals which received lithium did not show any significant differences in swimming speed compared with those which received normal drinking water (respective control groups). These observations demonstrate that systemic lithium administration did not cause any apparent undesirable motor disturbances. Such observations provide additional evidence in support of our conclusion that systemic lithium administration enhances spatial memory acquisition in rats.

Several studies have shown the effects of lithium on spatial memory but the results are inconsistent and controversial. There are some evidence indicating that lithium treatment attenuates the effects of chronic stress on spatial memory (46) and has showing neuroprotective effects in neurodegenerative diseases (19,47-50). Some other studies have suggested that chronic lithium impairs spatial discrimination, which may be relevant to hippocampal-dependent cognitive processes (5,9).

The discrepancies between results of this and other studies and those of others can be related to the type of task employed, duration of lithium exposure, time of spatial memory acquisition evaluation, various effects of lithium on different areas of the brain and time and concentration-dependent effects of lithium on a variety of neurotransmitters, and neuronal pathways.

The interactions of lithium with cholinergic system have been reported previously in various studies (3,21,43,51). It has been reported that lithium increases ACh synthesis and release in the brain (44). Presynaptic facilitation of the release of ACh has also been proposed to be one of the important factors in the CNS (3).

It has also been shown that lithium induces a concentration- and time-dependent increase in the mAChR number (51). The agonist-induced down-regulation of the mAChR number has also been inhibited by lithium (51). Therefore, the improvement in spatial memory acquisition by lithium could be related to its interactions with the cholinergic system in the brain.

Lithium is known to increase cholinergic activity and to modify the expression of cholinergic markers (52). Thus, it is reasonable to assume that the spatial memory enhancement observed in this study was caused, in part, through the effects of lithium on cholinergic markers in the medial septal area. Because of the above findings, part of this study was focused on measurement and quantification of ChAT immunoreactivity in the medial septal area.

One of the major findings of this study is an increase in ChAT immunoreactivity. Our quantitative analysis showed a significant increase in the number of immunostained ChAT-containing neurons in the medial septal area in four week lithium-treated animals. Improvement in spatial memory may be partly due to an increase in ChAT protein expression levels caused by lithium-induced stimulation of the cholinergic activity.

In AD, elevated levels of tyrosine phosphoproteins and sustained activation of intracellular signaling pathways in the brain such as the P38MAPK (p38 mitogen-activated protein kinase) have been detected (52-55). Moreover, the P38MAPK pathway, a major proinflammatory signal transduction pathway, is hyperactive in the AD brain (56). Also, β-amyloid-induced P38MAPK activation is apparently associated with loss of cholinergic neurons (52). In addition, it has been demonstrated that lithium treatment antagonizes activation of the P38MAPK induced by neurotransmitters such as glutamate (19). Thus, it is reasonable to deduce that effects of lithium on spatial memory acquisition and expression of ChAT of this study may also be due to an interaction with the P38MAPK pathway.

Neurotrophin a family member of the brain-derived growth factor (BDNF) is thought to play a critical role in the neural plasticity and memory processes (47, 57-60). Lithium may also exert its effects on spatial memory and cholinergic activity through activation of certain growth factors. For instance, studies have showed that chronic treatment with lithium increases the levels of BDNF mRNA in the rat brain time-dependently (48,61,62).Thus, it is possible that a lithium-induced up regulation of certain growth factors proteins also contributes to the spatial memory enhancing effects of lithium which was found in this study.

In summary, in the present work, it was shown that systemic exposure to chronic lithium at 600 mg/L causes spatial memory acquisition enhancement and an increase in ChAT immunoreactivity in the medial septal area, particularly in animals receiving lithium for four weeks. Furthermore, a significant correlation was found between the increase in the number of immunostained ChAT-containing neurons and behavioral measures.
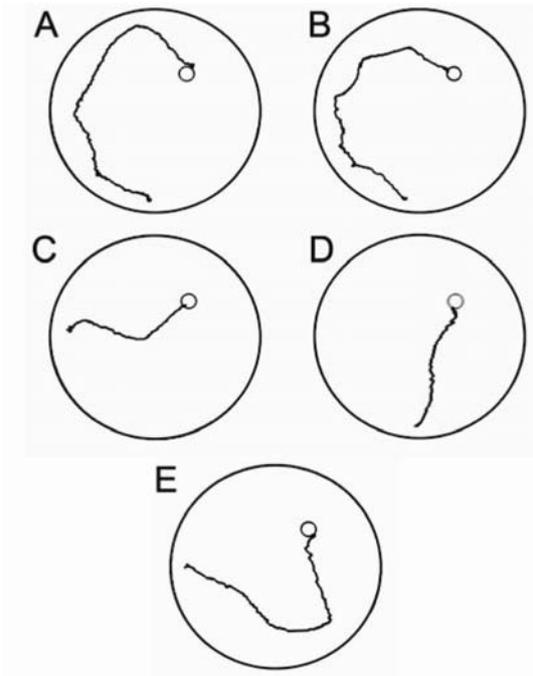
Figure 3. Diagrams A-E show representative traces of travel path in the lithium-exposed animals after one (A), two (B), three (C) and four (D) weeks of exposure as well as control (E).   Lithium reduced travel path in animals exposed to four weeks of lithium compared to control.   The open circles designate the position of the hidden platform.
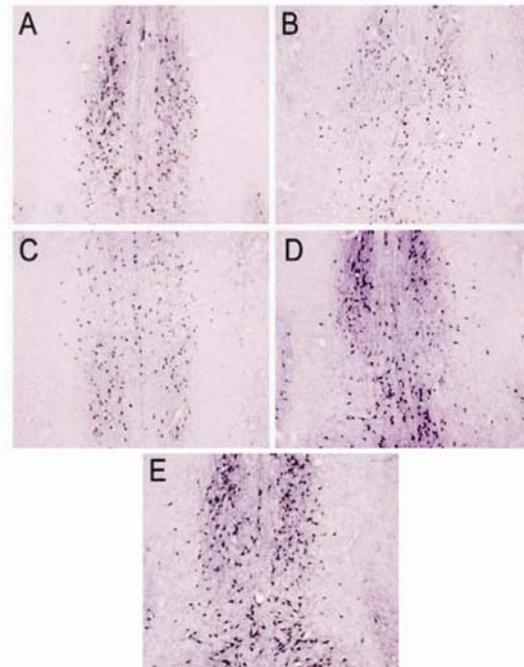
Figure 4. Alteration in ChAT immunostaining in the medial septum of rats exposed to lithium.   Brain tissue sections from control (a) and those treated with lithium for one (b), two (c) three (d) and four (e) weeks were immunostained with anti-ChAT antibody. There was an apparent increase in the number of immunostained ChAT-containing neurons after four weeks of lithium exposure (e).
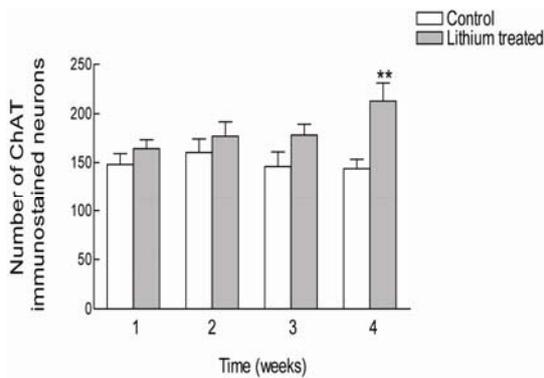

Figure 5. Quantification analysis of ChAT immunostaining in the rat medial septal area. The average number of immunostained ChAT-containing neurons in the medial septal area in four control groups and four groups exposed to lithium (600 mg/L). A significant increase in the numbers of immunostained ChAT-containing (**$P<0.01$, Newman-Keulz multiple comparison post test) neurons was observed after four weeks
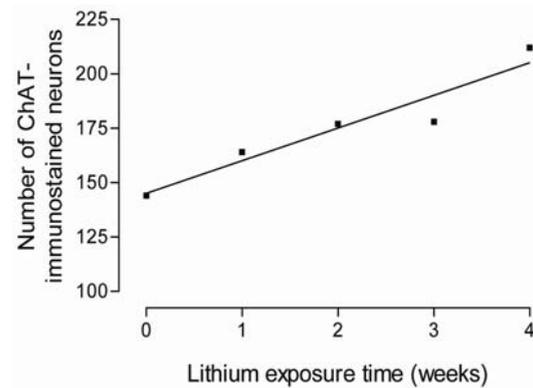
Figure 6. Correlation between the number of immunostained ChAT-neurons in the medial septum and the time-course of lithium administration. There was a significant correlation between the number of ChAT-containing neurons in the medial septum and a time-course of water exposure in the control groups (0) and lithium administration in groups exposed to lithium for one (1), two (2), three (3) and four (4) weeks ($P<0.05$, $R^2=0.91$)
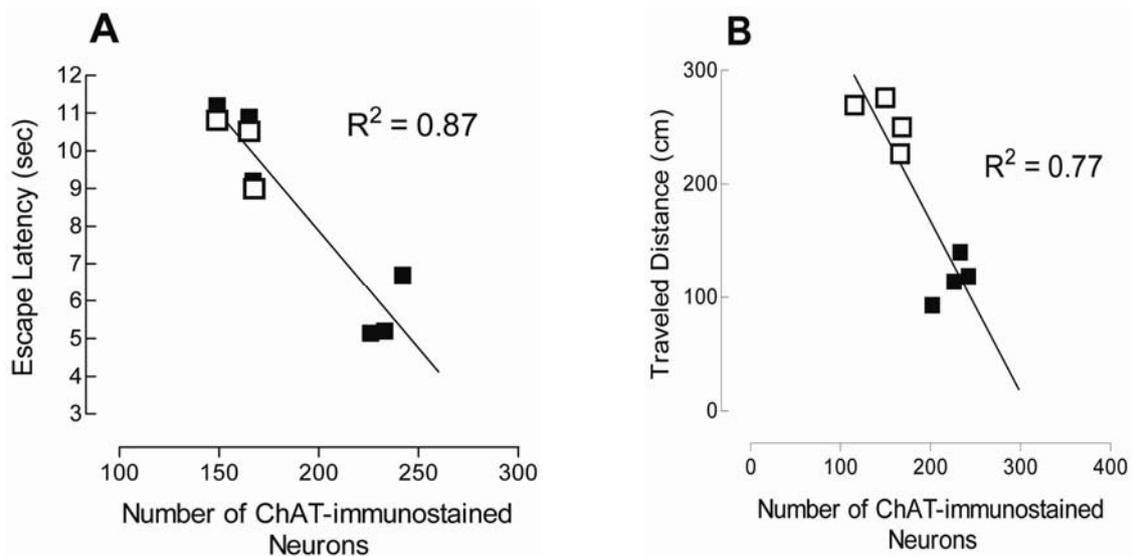
Figure 7. Correlation between the number of immunostained ChAT-containing neurons versus some behavioral parameters in the Morris Water Maze. An attempt was made to find correlations between ChAT, immunostaining levels in the medial septum versus escape latency and traveled distance for animals exposed to lithium (■) for four weeks (600 mg/L) and animals in the control group (□). There is a significant correlation between the average number of immunostained ChAT-containing neurons and escape latency ($P<0.01$, $R^2=0.86$) (A). There is also a significant correlation between the average number of immunostained ChAT-containing neurons and traveled distance ($P<0.01$, $R^2=0.78$) (B).

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