Developmental/Plasticity/Repair

Lithium Administration to Preadolescent Rats Causes Long-Lasting Increases in Anxiety-Like Behavior and Has Molecular Consequences

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Lithium (Li) is frequently used in the treatment of bipolar disorder (BPD), a debilitating condition that is increasingly diagnosed in children and adolescents. Because the symptoms of BPD in children are different from the typical symptoms in adulthood and have significant overlap with other childhood psychiatric disorders, this disorder is notoriously difficult to diagnose. This raises the possibility that some children not affected by BPD are treated with Li during key periods of brain development. The objective of this investigation was to examine the long-term effects of Li on the developing brain via a series of behavioral and molecular studies in rats. Rat pups were reared on Li chow for 3 weeks. Parallel groups were tested while on Li chow or 2 and 6 weeks after discontinuation of treatment. We found increased measures of anxiety-like behavior at all times tested. Gene microarray studies of the amygdala revealed that Li affected the expression of gene transcripts of the synapse and the cytoskeleton, suggesting that the treatment induced synaptic adjustments. Our study indicates that Li can alter the trajectory of brain development. Although the effects of Li on the normal brain seems unfavorable, effects on the abnormal brain cannot be determined from these studies alone and may well be therapeutic. Our results indicate that Li administration to the normal brain has the potential for lasting adverse effects.

Key words: **amygdala; anxiety; behavior; development; elevated plus maze; gene expression microarrays; lithium**

Introduction

Lithium (Li) is frequently used to treat bipolar disorder (BPD) in adults (American Psychiatric Association, 1994). BPD patients have often symptoms before adulthood (Carlson et al., 1977; Joyce, 1984), and 1% of adolescents ages 14–18 years meet criteria of BPD or cyclothymia, considered a milder form of BPD (Lewinsohn et al., 1995). In recent years, diagnosis in juveniles has increased markedly, but controversy surrounds the diagnostic criteria in childhood and adolescence (Carlson, 1990; Geller and Luby, 1997; Wozniak et al., 2001; Harpaz-Rotem et al., 2005). BPD symptoms in childhood and adolescence deviate from the symptom criteria established for adults (Steele and Fisman, 1997; Wozniak et al., 2001), and diagnosis at an early age is complicated by the overlap with other childhood psychiatric disorders such as attention-deficit-hyperactivity disorder (ADHD) and conduct disorder (Geller and Luby, 1997; Hechtman and Greenfield, 1997; Geller et al., 1998; Davanzo and McCracken, 2000). Despite these problems, treatment is often initiated at an early age and, in some cases, even preschool age (Geller et al., 1995; Wozniak et al., 2001; Biederman et al., 2005).

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Because Li has therapeutic effects in adults with BPD, it seems a reasonable choice for younger patients. Li can be effective in children and adolescents for the treatment of mania (Biederman et al., 1998; Kowatch et al., 2000; Kafantaris et al., 2003), conduct disorder (Silva et al., 1993), and aggression (Sheard, 1975). However, drug trials tailored to children and adolescents are needed before adult medications can be deemed safe for younger patients. The immature CNS is vulnerable to the latent cognitive and neurological impact of drugs (Tueth et al., 1998), and drug-induced modifications have the potential to alter the developmental trajectory of the brain (Carlezon and Konradi, 2004).

In the present study, we examined the effects of chronic Li exposure on preadolescent rats to address the question of whether Li has any long-lasting effects on brain development and behavior. We used normal rats as a model to examine how Li would affect brain development in children who receive the drug but are later rediagnosed as not having BPD. We focused on fear- and anxiety-like behaviors, which are prevalent in pediatric BPD patients diagnosed with BPD (46.0–78%) (Masi et al., 2001; Engstrom et al., 2003; Dickstein et al., 2005). Behavioral measures used included the open-field paradigm, elevated plus maze (EPM), fear-potentiated startle (FPS), locomotor activity, and the Morris water maze. These studies were combined with a gene expression microarray analysis in the amygdala, an area involved in fear and anxiety (Davis, 1997; LeDoux, 2003) that has been shown to be reduced in size in pediatric BPD patients (Chang et al., 2005).

Rat preadolescence/early adolescence was defined by the 3

week period after weaning (Andersen, 2003; Spear, 2004), during which Li was added to the rat chow. Experiments were designed to determine behavioral adaptations after 3 weeks of Li treatment and 2 and 6 weeks after the termination of Li treatment. Molecular adaptations were examined during and 2 weeks after Li treatment.

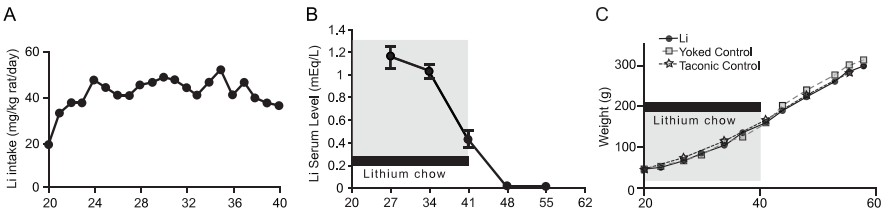

Figure 1. Li intake, Li serum levels, and weight gain. ***A***, Average Li intake over 3 weeks is shown in a representative group ($n = 12$). ***B***, At the end of each week, a group of rats was killed ($n = 4$) and serum was analyzed for Li levels. ***C***, Representative growth curve for Li rats ($n = 12$) and Co rats ($n = 12$). The growth curve was in line with the growth curve for male Sprague Dawley rats provided by Taconic Farms. ***B***, ***C***, Data are average ± SEM (in some charts, smaller than the symbol).

Materials and Methods

Li treatment and health maintenance

Male Sprague Dawley rats (Taconic Farms, Germantown, NY), postnatal day 16 (P16), were shipped to our facility with a lactating female. A total of 340 rats was used for all experiments. Pups were housed in family units and allowed to acclimate for 4 d. On P20, the pups were weaned and each litter was split between lithium and control chow, with four rats per cage. Care was taken that all experiments performed subsequently had equal numbers of rats of all litters. Pups were provided with preweighed aliquots of 0.15% Li carbonate chow or control chow (Co) balanced for nutrient content (Harlan Teklad, Madison, WI). The colony room was maintained on a 12 h light/dark cycle.

Pups were reared on Li/Co chow for 3 weeks (P20–P41). Food weight was recorded daily and once on the weekend, and additional chow was added as needed. Control rats were yoked to Li food intake on a cage-by-cage basis. All rats were weighed twice a week, and a representative weight curve is shown (Fig. 1*C*). Although overall health in rats on Li diet is normal (Cappeliez, 1986; Laursen et al., 2004), rats do display polydypsia/polyurea, accompanied by sodium wasting (Lee et al., 1971; Singer et al., 1972). Therefore, all cages were equipped with a bottle of 450 mM NaCl solution, in addition to ample drinking water. Cages and bedding were changed daily for Li and Co rats. One rat in a control group died of unknown causes; no deaths attributable to Li treatment occurred.

Calculation of Li intake

For each 24 h period, the weight of Li chow consumed by one cage of rats was divided by the number of rats (four) and multiplied by the percentage of Li in the chow (0.15% Li_2CO_3 = 0.03% Li corrected for carbonate). This value was then divided by the average weight of the rats in that cage. The resulting value, approximate Li intake, was expressed as milligrams of Li consumed per kilogram rat per 24 h (Fig. 1*A*). Li serum levels (LabCorp, Burlington, NC) were determined in independent groups of rats ($n = 4$ per group) that were killed at the end of each week (Fig. 1*B*).

Timing of behavioral testing

Rats on Li/Co chow were subjected to behavioral testing in the middle of their third week (P36). To prevent withdrawal effects during behavioral testing, Li/Co chow was extended until P44 when all testing was complete. Rats tested for prior Li/Co [2w] exposure were kept on Li or Co chow for 3 weeks and then switched to normal laboratory chow *ad libitum* for 2 weeks before behavioral testing began. One group of rats and respective controls (prior Li/Co [6w]) were tested in the open-field paradigm 6 weeks after discontinuation of Li/Co chow. All testing was performed in parallel in Li and weight-matched Co groups.

The data for each behavioral test were collected from two to three independent experiments performed over the course of several months. Final values of *n* are reported individually for each test. Generally, all tests were performed in independent groups of rats. However, open field and EPM were considered non-invasive, and, in some groups of rats, one of these tests was performed before FPS, Morris water maze, or molecular analysis. Rats had at least 3 d of rest between both tests. Control groups were always treated exactly the same as Li groups. Open field and EPM were never performed in the same group of rats.

Open field

At P37, P51, and P83, respectively, Li/Co, prior Li/Co [2w], and prior Li/Co [6w] rats were placed for 1 h in automated, 43 × 43 × 30.5 cm (length × width - height) locomotor activity chambers (Med Associates, St. Albans, VT) to record activity levels and open-field behavior. Each unit had a white enamel floor and Plexiglas walls lined with photo beams enclosed within a sound- and light-attenuating outer chamber. A 7.5 cm perimeter was designated as the "exterior" zone, and the remaining square was the "interior" zone. "Box size," an internal parameter that accounts for the size of the test subject and represents the number of photobeams to be broken before a movement is considered ambulatory, was set at 2, 3, and 4 for ages P37 (Li/Co), P51 (prior Li/Co [2w]), and P83 (prior Li/Co [6w]), respectively. Data were analyzed for time spent in the interior zone/time spent in the exterior zone, distance traveled, and number of zone crossings.

Elevated plus maze

The EPM (Hamilton Kinder, Poway, CA) consisted of four black Plexiglas arms at right angles elevated 85 cm above ground. Two of the arms were enclosed by panels 40 cm high (closed arms), whereas the other two were open to the room (open arms). Each arm was 10.8 cm wide and 50 cm long.

Rats were allowed to acclimate to the procedure room in their cages for 1–2 h before testing. Every effort was made to prevent acoustic and visual disturbances during this time. The room was lit by a single light bulb in a corner, providing the maze with a small amount of indirect light. Sessions were filmed from overhead with a Sony (Tokyo, Japan) Handycam camcorder with "night shot" setting.

After acclimation, each rat was placed in the center facing an open arm, and behavior was recorded for exactly 5 min. The maze was cleaned thoroughly between rats. Groups were alternated to prevent any time-of-day effects. Video of the trials was scored by a blinded observer for time spent in closed versus open arms, as well as number of open and closed arm entries. A rat was considered in an arm when its hindlegs crossed the threshold. Data were expressed as time spent in open arms over time spent in all arms.

Fear-potentiated startle

Training and testing of animals was conducted in startle cages similar to those described previously (Carlezon et al., 2005). The visual conditioned stimulus (CS) was a light produced by an 8 W fluorescent bulb (15 ms rise time) located 10 cm behind, and at a 45° angle above, the startle cage. The unconditioned stimulus (US) was a shock delivered to the floor bars of each cage by a shocker/scrambler module. The calibration, presentation, and sequencing of all stimuli were under the control of the personal computer using specially designed software.

During training, rats received a 5 min acclimation period followed by 10 light–shock pairings consisting of a 3.7 s light (CS) coterminating with a 0.5 s, 0.6 mA footshock (US). Shock reactivity (displacement of the cage in response to the shock) was recorded after each light–shock presentation. The mean intertrial interval (for training, defined as the onset interval between successive light–shock pairings) was 3 min (range of 2–4 min). Rats received two training sessions spaced 48 h apart. Twenty-four hours after the second training session, rats were placed in the startle cages, and, after a 5 min acclimation period, rats were presented with nine startle stimuli, three at each of three intensities (95, 100, and 105 dB) in a semi-random order with a 30 s interstimulus interval (ISI). These initial stimuli were presented to allow the startle response to become habituated and, therefore, more stable before the collection of the test

data. Rats were then presented with nine noise-alone and nine light–noise trials (95, 100, and 105 dB noise for both trial types). For the light–noise trials, the startle-eliciting stimulus occurred 3.2 s after the onset of the light (i.e., the time when the shock would have occurred). All trial types were presented in a pseudorandom order (30 s ISI) with the constraint that each trial type occurred only once in each consecutive six-trial block.

Morris water maze

The Morris water maze tank was 175 cm in diameter and 63.5 cm high, filled with 35.5 cm of water at 22°C made opaque by the addition of powdered milk. A clear Plexiglas platform, 10 cm square, was placed ~2.5 cm below the surface of the water least 36 cm away from any wall. The internal perimeter of the tank was divided into six equally spaced drop spots, each labeled above the water level by a number and a high-contrast visual cue. Training took place on 4 consecutive days (P41–P44 for rat on Li/Co, P56–P59 for prior Li/Co [2w]). On days 1–3, each rat was subjected to six swimming trials a day with no more than 10 min between trials. During each trial, the rat was placed in the tank facing the wall at one of the six drop spots and allowed to swim freely for 1 min. If the rat located the platform within the time limit, it was removed from the tank. Escape latency (1–60 s) was recorded. Rats that failed to find the platform were guided to the proper location and left there for 10 s before being removed. In such cases, escape latency was recorded as 90 s. Drop spots were randomized across animals, and each animal was placed at each location once each day. On day 4, the platform was in the original position for the first four trials but was moved to a new location in a different quadrant of the tank for the next four trials (reversal). All sessions were filmed and analyzed for crossings into quadrants and total time spent in the quadrant where the platform was previously located.

Statistical analyses

The JMP program (release 5) and StatView (version 4.5) (both from SAS, Cary, NC) were used for the statistical analyses. Factorial ANOVAs and Fisher's *post hoc* protected *t* tests were used to find significant changes. Values that deviated >2 SDs from the average were removed from the analysis, which affected between 0 and 8% of all measures within an experiment.

Gene expression microarray analysis

Treatment groups and tissue dissection. Gene array analysis was performed in the amygdala of four groups of rats ($n = 7$ per group), Li/Co (Li chow from P20 to P41, killed at P41), and prior Li/Co [2w] (Li chow from P20 to P41, killed at P55). Rats were decapitated, and brains were removed and immediately frozen in isopentane/dry ice. Amygdala was dissected in 2 mm round tissue punches at −1.7 mm bregma and −2.8 mm bregma, yielding four punches (two slices, left and right side of brain). Each punch was 0.8 mm thick (Paxinos and Watson, 1998). The punches contained the central amygdaloid nucleus, basolateral amygdaloid nucleus, and basomedial amygdaloid nucleus with all their subdivisions.

RNA extraction. RNA was extracted from the tissue punches using the RNAgent kit (Promega, Madison, WI). RNA quality was assessed in an analytical gel, and 5 μg of total RNA was used for cDNA synthesis with the SuperScript double-stranded cDNA synthesis kit (Invitrogen, Carlsbad, CA). *In vitro* transcription was performed with the Enzo-IVT kit (Enzo Biochem, Farmingdale, NY). Biotinylated RNA was hybridized to the rat RAE230A array (Affymetrix, Santa Clara, CA), and washing and staining was performed according to company protocol. Samples from individual rats were hybridized to individual arrays. The Affymetrix RAE230A array contains ~15,000 genes; each gene is represented by 11 perfectly matched 25-mer oligonucleotides and the same number of one-mismatch oligonucleotides to provide values for nonspecific binding.

Quality control criteria. Tissue preparation and RNA extractions were performed in a single batch by the same investigators to limit experimental variability. All quality control criteria defined by Affymetrix and DNA-Chip Analyzer (dChip) (Li and Wong, 2001) were met by the samples, and no differences between the experimental groups were observed. The average percentage "present" call across all arrays was 60.2 ± 2.9%, and the 3′/5′ glyceraldehyde-3-phosphate dehydrogenase and β-actin ratios were 1.7 ± 1.0 and 2.2 ± 0.5 (average ± SD). Background (58.5 ± 5.7) and noise (raw Q, 2.2 ± 0.2) were comparable between all treatment groups.

Data analysis. The Gene Chip Operating Software (Affymetrix) was used to scan the chips, determine cell intensities, and examine sample quality (see above). RMAExpress (Bolstad et al., 2003; Irizarry et al., 2003) was used to calculate gene expression levels, and dChip was used for group comparisons, ANOVA, hierarchical clustering, gene classification, and linear discriminant analysis clustering. Gene Microarray Pathway Profiler (GenMAPP) (Dahlquist et al., 2002; Doniger et al., 2003) and NIH David (Dennis et al., 2003) were used to examine the biological context of the findings with help of public databases, and >400 local MAPPs were assembled by the investigator or obtained from www.genmapp.org. In local MAPPs, genes were organized according to their function, such as second-messenger pathways, neurotransmitter systems, kinases, phosphatases, enzymes involved in glycolysis, subunits of the proteasome, presynaptic and postsynaptic proteins, proteins of the mitochondrial respiratory chain, G-protein-coupled receptors, etc. All MAPPs were established before data analysis and were not influenced by the results.

Real-time quantitative PCR

Complementary DNA was synthesized from 500 ng of total RNA with the SuperScript First-Strand Synthesis System for real-time quantitative PCR (Q-PCR) (Invitrogen) and oligonucleotide deoxythymidine primer. A primer set for each gene was designed with the Primer3 software (www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi), for amplicons of 100–200 bp. Melt curve analysis and PAGE were used to confirm the specificity of each primer pair. A Q-PCR kit (iQ SYBR Green supermix; Bio-Rad, Hercules, CA) was used for the experiment that was performed on a DNA engine Opticon 2 (MJ Research, Waltham, MA) in a volume of 20 μl, with 4 μl of 1:10 diluted cDNA samples and 0.3 μM primers. The PCR cycling conditions were initially 50°C for 2 min, followed by 95°C for 7 min, followed by 39 cycles of 94°C for 10 s, 55°C for 15 s, and 72°C for 30 s. Data were collected between 72°C and 82°C depending on amplicon melt temperature. A melt curve analysis was performed at the end of each Q-PCR experiment. Dilution curves were generated for each primer pair in every experiment by diluting complementary DNA from a vehicle sample to a final concentration of 1.00, 0.2, 0.04, 0.008, and 0.0016. The logarithm of the dilution values was plotted against the cycle values for the standard curve. Opticon Monitor Data Analysis Software version 1.4 (MJ Research) was used to analyze the data. Blanks were run with each dilution curve to control for cross-contamination. Dilution curves, blanks, and samples were run in duplicate. Reported values were normalized to general transcription factor IIB (GTFIIB UniGene ID; Rn.6109), which was not regulated in the gene arrays. β-Actin was not used because it was induced in the gene arrays.

Results

Li Treatment, Li serum levels, and weight gain

During the 21 d on Li chow, Li intake remained relatively stable ~40 $mg \cdot kg^{-1} \cdot d^{-1}$, a dose range identical to that used in children and adolescents (Tueth et al., 1998) (Fig. 1*A*). Serum Li levels ranged from 0.4 to 1.2 milliequivalents per liter (mEq/L), with a sharp decline in week 3, while rats were still on Li chow (Fig. 1*B*). These levels are comparable with serum Li levels in bipolar disorder patients, which range from 0.4 to 1.0 mEq/L (Perlis et al., 2002). Interestingly, Li serum levels in adult rats 1 week on Li chow were similar to the levels observed in adolescent rats 3 weeks on Li chow (0.4 mEq/L; $n = 8$ rats; 275 g), suggesting that the higher levels of Li observed in the serum of younger rats are probably a reflection of a different metabolism in preadolescent rats rather than the decrease in Li serum levels reflecting a metabolic adaptation. Three weeks after the start of Li treatment, at P41, the rats seemed to have reached adult Li metabolism.

Li was undetectable 1 and 2 weeks after discontinuation of Li chow and in Co rats. Thus, prior Li rats ([2w] and [6w]) had no detectable Li serum levels at the time of testing or in gene expression microarray analysis. Rats appeared to be well groomed and

showed no unusual lethargy or aggression. Unlike adult rats on Li chow, which, in a control experiment, had a 50% lower weight gain than their matched controls (control rats, from 250 to 369 g; Li chow rats, from 250 to 312 g over 3 weeks; $n = 8$ per group), adolescent Li rats had normal weight gain while on Li chow and after being taken off (Fig. 1*C*). Thus, although controls were yoked, they were not subjected to food deprivation.

Li-treated rats show increased anxiety-like behavior in the open-field test

Rats on Li and prior Li rats ([2w] and [6w]) spent significantly less time than their respective controls in the interior part of the locomotor chamber (Fig. 2*A*). A significant effect of treatment ($F_{(5,159)} = 30.3$; $p < 0.0001$) and age ($F_{(5,159)} = 126.6$; $p < 0.0001$) was observed, whereby Li treatment decreased, and age increased, time spent in the interior part of the chamber. The effect increased with age (age × treatment $F_{(5,159)} = 12.6$; $p < 0.0001$), demonstrating that rats that were not on Li chow had retained, and even increased, their avoidance of the open field. *Post hoc* Fisher's PLSD tests showed a significant difference between Li and control rats at all time points tested. Minor motor effects were observed in the open field, which affected rats that were on Li chow but not rats that were previously on Li chow. Total distance traveled was affected by age but not by treatment (Fig. 2*C*) (treatment, $F_{(5,159)} = 1.6$, $p = 0.17$; age, $F_{(5,159)} = 32.9$, $p < 0.0001$), whereas zone crossings were reduced in rats on Li chow (Fig. 2*D*) (treatment, $F_{(5,159)} = 12.4$, $p < 0.0005$; age, $F_{(5,159)} = 155.4$, $p < 0.0001$; *post hoc* Fisher's PLSD, $p \leq 0.001$ in Li/Co; not significant in either prior Li/Co[2w] or prior Li/Co[6w]).

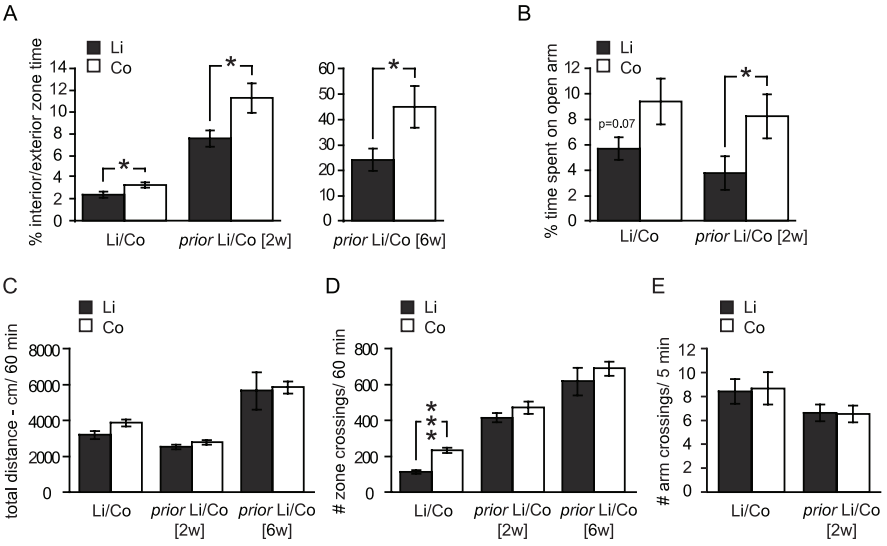

Figure 2. Open-field behavior and EPM after chronic exposure to Li. ***A***, Rats on Li chow spent significantly less time in the inner part of the locomotor chamber than their matched controls, during Li exposure, 2 weeks later (prior Li/Co [2w]), and 6 weeks later (prior Li/Co [6w]). $n = 51$ for Li, 45 for Co, 24 for prior Li [2w], 24 for prior Co [2w], 10 for prior Li [6w], and 10 for prior Co [6w]. ***B***, Rats exposed to Li during preadolescence spent less time on the open arm of the EPM than their matched controls. $n = 23$ for Li, 24 for Co, 19 for prior Li [2w], and 21 for prior Co [2w]. ***C***, No significant differences in total distance traveled in the locomotor chambers was observed between treatment groups; for *n* values, see ***A***. ***D***, Zone crossings in the locomotor chambers were significantly reduced in Li-treated rats on Li but not in prior Li [2w] or in prior Li [6w] rats; for *n* values, see ***A***. ***E***, No differences in arm crossing were seen on the EPM; for *n* values, see ***B***. All data are average ± SEM. $*p \leq 0.05$; $***p \leq 0.001$.

Li-treated rats show increased anxiety-like behavior in the elevated plus maze test

In the EPM, Li and prior Li [2w] rats spent less time on the open arm than their matched controls. Treatment had a significant effect, whereas age had no effect (Fig. 2*B*) (treatment, $F_{(3,83)} = 8.5$, $p < 0.005$; age, $F_{(3,83)} = 0.9$, $p < 0.4$). *Post hoc* Fisher's PLSD tests showed a significant difference between Li and control rats 2 weeks after Li chow was discontinued but showed only a trend for rats on Li chow ($p \leq 0.07$). No differences were observed in arm crossings on the EPM (Fig. 2*E*).

Li-treated rats show normal conditioned fear response in the fear-potentiated startle paradigm

FPS was used to examine whether rats treated with Li had an altered response to conditioned fear (Fig. 3*A*, *B*). Neither juvenile rats on Li nor adult rats exposed to Li during preadolescence had an altered response in the FPS paradigm (Fig. 3*A*) ($F_{(3,41)} = 1.5$; $P \leq 0.22$). A trend toward reduced shock reactivity was observed in rats exposed to Li, but this trend did not reach significance (Fig. 3*B*) and did not seem to affect FPS performance (Fig. 3*B*, inset) ($r = 0.215$; $p = 0.142$). Indeed, FPS was similar (Fig. 3*C*) in a subset of animals that were matched to have equivalent levels of shock reactivity (Fig. 3*D*). Because FPS is influenced by learning and memory processes, we examined potential learning deficits that could interfere with FPS in an independent group of rats in the Morris water maze. No difference was observed between Li and prior Li [2w] rats and their respective control groups in learning and remembering the location of the platform (Fig. 3*E*, days 1–4). There was also no difference in relearning a new position of the platform (Fig. 3*E*, All Reversals). Thus, juvenile Li exposure seems to affect innate anxiety but not learned fear.

Gene expression microarray analysis

Gene expression microarray analysis was performed in the amygdala. All genes that reached a p value of ≤ 0.05 and were above detection threshold ("present") in at least 50% of all samples were subjected to MAPPfinder analysis. In rats on Li, 6.9% of all genes present in at least 50% of all samples were differently regulated, with almost equal percentages upregulated and downregulated. In prior Li [2w] rats, 5.7% of all genes present in at least 50% of all samples were differently regulated, most of them upregulated (Fig. 4*A*). Transcripts with altered expression levels during Li treatment were generally different from transcripts expressed in prior Li [2w] rats (Fig. 4*B–D*, see numbers of overlapping genes between "on Li" and "prior Li" rats in Venn diagrams; Table 1). However, genes differently regulated in rats on Li had similar trends in prior Li [2w] rats. This is demonstrated by the many genes that reached significance when both groups were combined (Fig. 4*B–D*, "all Li versus all controls"). Only five genes were regulated in opposite directions (Fig. 4*E*,*F*). Many of the transcripts affected by Li treatment indicated structural adjustments. They included synaptic vesicle genes, cytoskeletal genes, and genes involved in cell adhesion (Table 1). Genes involved in the inositol phosphate pathway were also affected by Li treatment, in line with the notion that Li may exert its therapeutic action by interfering with the metabolism of phosphoinositides (Baraban et al., 1989), although this theory is controversial (Berry et al., 2004). Many genes affected by Li treatment clustered into functional groups such as GTP metabolism, potassium channels, and

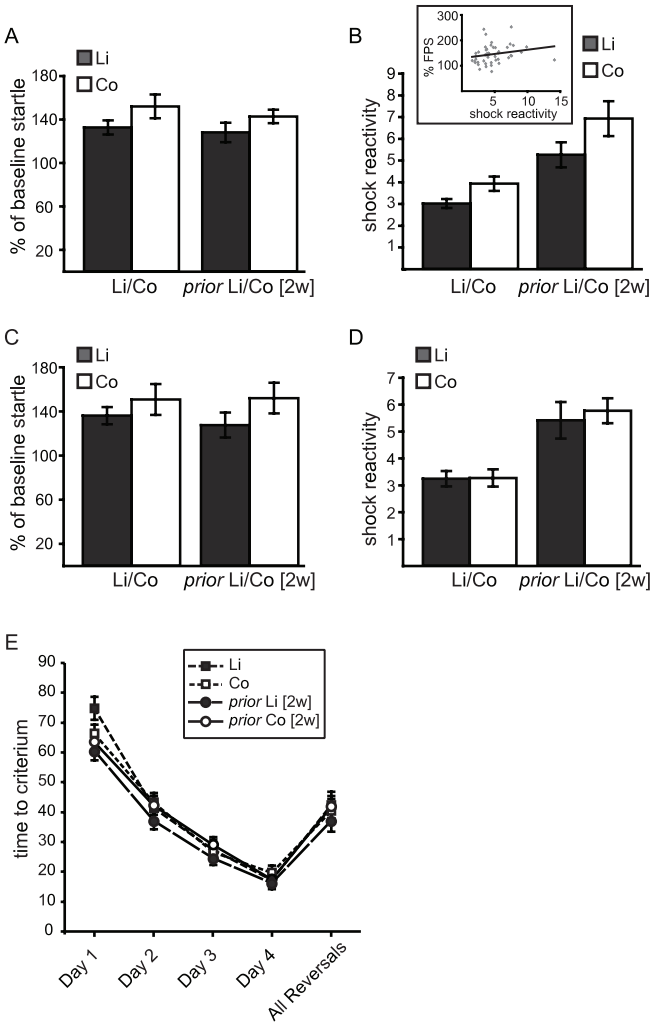

Figure 3. Fear-potentiated startle and Morris water maze. ***A***, FPS as percentage of baseline startle (ratio of response on light + startle trials − startle alone trials/startle alone) in juvenile rats on Li chow (left) and rats 2 weeks after discontinuation of Li chow (right). No difference in FPS was observed in either group; $n = 11$ per group. ***B***, Rats on Li or prior Li [2w] had a trend toward lower shock reactivity, but differences were not significant; $n = 12$ per group. A nonsignificant correlation was observed between shock reactivity and percentage of FPS (inset; $r = 0.215$; $p = 0.142$). ***C***, Subgroups of rats with comparable shock reactivity (***D***) did not show differences in percentage of FPS either; $n = 9$ per group for ***C*** and ***D***. ***E***, No differences were observed in the ability to learn, remember, or relearn the position of the platform of the Morris water maze ($n = 10$ in Li and control; $n = 18$ in prior Li [2w] and prior Co [2w]).

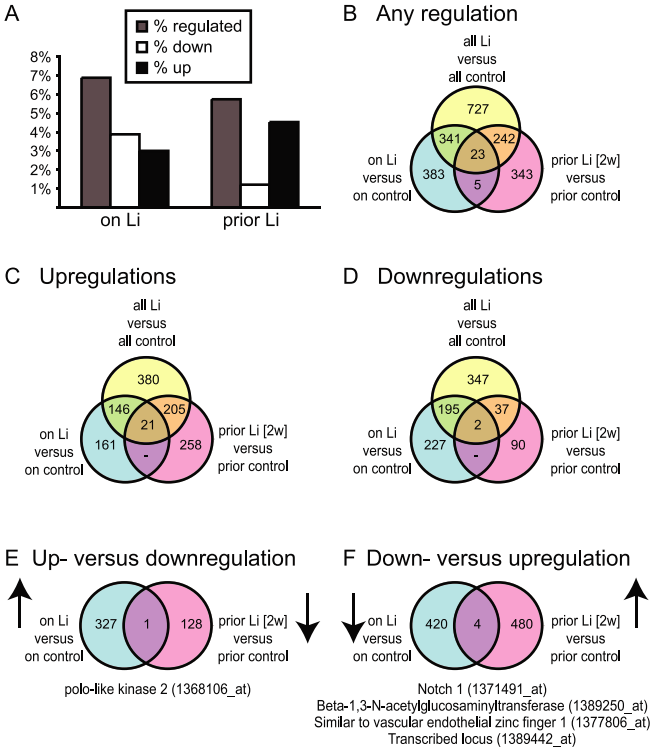

Figure 4. Gene expression patterns in the amygdala. Percentage of all genes with altered regulation in rats on Li and prior Li [2w] rats. Only genes that were expressed in at least 50% of all samples ($n = 10{,}933$ in rats on Li and 10,685 in prior Li [2w]) were included in the calculation. ***B–F***, Venn diagrams of genes with overlapping regulations. $n = 6$ per group.

ATPase activity. Four transcripts were subjected to Q-PCR analysis (Fig. 5), which reflected the general pattern of regulation observed in gene arrays. The transcripts were chosen based on fold difference and level of significance in the gene expression microarray analysis, combined with their representation of individual groups of altered transcripts.

Discussion

Rats treated with Li during preadolescence showed increased measures of innate anxiety in the open field and on the EPM. The open-field test relies on a rodent's innate exploratory behavior counteracted by its natural aversion to open space. The time spent in the interior of the box is related to the exploratory behavior of a rat and inversely related to the anxiety level (Crawley, 1985). Avoidance of the open field was observed during Li administration and 2 weeks after Li administration was concluded. A group of rats was tested in the locomotor chambers 6 weeks after discontinuation of Li chow. At this point, in adulthood, they still spent less time in the open/inner area of the locomotor box than their age- and weight-matched controls. The anxiety-like behavior was more pronounced after Li administration had ended, suggesting that preadolescent Li administration alters brain function beyond the time of exposure. Li and Co rats had similar locomotor measures in the open-field chamber 2 and 6 weeks after discontinuation of Li/Co exposure. Therefore, the altered behavior in the locomotor chamber was unlikely to be attributable to locomotor differences. Increased anxiety in Li rats was further supported by results with the EPM, which is one of the most widely used behavioral tests to examine innate anxiety. Normal rats placed in the maze for the first time tend to spent much of their time in the closed arms. Treatment with anxiolytic drugs increases the time spent in the open arms and the number of open arm entries, whereas anxiogenic drugs have the opposite effect (Pellow et al., 1985; Brett and Pratt, 1990; Treit et al., 1993; Dawson and Tricklebank, 1995). Rats exposed to Li in our study spent less time on the open arm than their matched controls, an anxiogenic-like effect that was significant in rats on prior Li [2w] chow. Arm crossings were the same in all groups, indicating no differences in motor activity.

Results from the FPS paradigm suggest that conditioned fear is not significantly affected by Li exposure. FPS is a measure of fear-like behaviors induced by pairing an electric shock with a conditioned stimulus (light) and using the acoustic startle response as a measurable response to the conditioned stimulus (Brown et al., 1951; Davis and Astrachan, 1978; Grillon and Davis, 1997). In the present study, we found that this conditioned response (the potentiation of startle in the presence of the light) was similar between control and Li-treated rats. Shock reactivity

Table 1.

				on Li		prior Li/Co [2w]	
synaptic vesicles	probe set	gene	LocusLink ID	fold change	P value	fold change	P value
upregulated by lithium	1368865_at	synaptoporin	66030	1.26	0.009	1.01	0.791
	1368276_at	synaptophysin	24804	1.19	0.037	-1.22	0.078
	1370718_at	synaptotagmin X	60567	1.10	0.050	1.05	0.257
downregulated by lithium	1368246_at	adaptor-related protein complex 3, mu 2 subunit	140667	-2.02	0.043	-1.30	0.258
	1369058_at	synaptotagmin 3	25731	-1.43	0.014	-1.23	0.070
	1388193_at	huntingtin interacting protein 1	192154	-1.38	0.017	-1.24	0.164
	1372950_at	blocked early in transport 1-like	54400	-1.36	0.027	-1.13	0.299
	1373510_at	vesicle-associated membrane protein 1	25624	-1.24	0.018	1.09	0.386
	1370840_at	syntaxin binding protein 1	25558	-1.22	0.023	-1.13	0.205
	1398330_at	syntaxin binding protein 1	25558	-1.13	0.018	-1.08	0.103
upregulated in prior lithium	1372474_at	pantophysin	366595	1.13	0.302	1.46	0.005
	1373024_at	adaptor-related protein complex 3, sigma 1 subunit	302290	1.13	0.399	1.37	0.027
	1398843_at	vesicle-associated membrane protein, associated protein a	58857	1.09	0.399	1.23	0.030
	1372628_at	adaptor-related protein complex AP-4, sigma 1	366618	1.09	0.573	1.32	0.034
downregulated in prior lithium	1369627_at	synaptic vesicle glycoprotein 2b	117556	1.02	0.914	-2.41	0.047
	1369332_a_at	regulating synaptic membrane exocytosis 1	84556	-1.07	0.649	-1.35	0.044

				on Li		prior Li/Co [2w]	
cell adhesion	probe set	gene	LocusLink ID	fold change	P value	fold change	P value
upregulated by lithium	1367888_at	MT-protocadherin	93662	1.33	0.018	-1.05	0.690
	1369351_at	contactin 3	54279	1.12	0.019	1.15	0.017
downregulated by lithium	1372447_at	fibroblast growth factor receptor 1	79114	-1.50	0.001	-1.14	0.355
	1373636_at	sparc/osteonectin, cwcv and kazal-like domains proteoglycan 1	306759	-1.25	0.005	1.05	0.450
	1373470_at	beta-catenin	84353	-1.13	0.006	-1.00	0.963
	1367807_at	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1	116552	-1.28	0.006	1.05	0.690
	1371921_at	catenin, alpha 1, 102kDa	307505	-1.16	0.013	1.04	0.195
	1387498_a_at	fibroblast growth factor receptor 1	79114	-1.85	0.013	-1.24	0.312
	1368699_at	Down syndrome cell adhesion molecule	171119	-1.23	0.017	-1.10	0.405
	1387320_a_at	densin-180	117284	-1.17	0.018	-1.14	0.210
	1368685_at	chondroitin sulfate proteoglycan 4	81651	-1.18	0.019	1.01	0.939
	1370954_at	procollagen-proline, 2-oxoglutarate 4-dioxygenase, alpha 1	64475	-1.33	0.021	1.09	0.335
	1368577_at	gap junction membrane channel protein beta 6	84403	-1.36	0.025	-1.07	0.616
	1373124_at	tyrosine protein kinase pp60-c-src	83805	-1.49	0.028	-1.29	0.136
	1389030_a_at	tyrosine protein kinase pp60-c-src	83805	-1.37	0.028	-1.15	0.220
	1388703_at	endothelial cell adhesion molecule	300519	-1.23	0.029	-1.04	0.606
	1367880_at	laminin, beta 2	25473	-1.27	0.033	-1.23	0.156
	1398922_at	collagen alpha1 type VI		-1.69	0.040	-1.43	0.166
	1374239_at	FERM, RhoGEF and pleckstrin domain protein 2	316639	-1.14	0.044	-1.03	0.501
	1369103_at	fyn proto-oncogene	25150	-1.51	0.045	-1.07	0.543
	1388388_at	bonecartilage proteclycan 1		-1.26	0.047	1.08	0.615
	1373101_at	phosphatidylinositol glycan, class K	295543	-1.24	0.049	-1.08	0.552
upregulated in prior lithium	1374870_at	collagen, type XXVII, alpha 1	298101	1.03	0.568	1.13	0.031
	1388045_a_at	cadherin 22	29182	1.06	0.619	1.20	0.042
downregulated in prior lithium	1369793_a_at	melanoma cell adhesion molecule	78967	-1.28	0.136	-1.31	0.034
	1368895_at	neuroligin 2	117096	1.07	0.342	-1.19	0.014
	1388038_at	attractin	83526	-1.02	0.806	-1.18	0.031

				on Li		prior Li/Co [2w]	
cytoskeleton	probe set	gene	LocusLink ID	fold change	P value	fold change	P value
upregulated by lithium	1372195_at	troponin C2, fast	296369	1.32	0.005	-1.10	0.117
	1370697_a_at	nexilin	246172	1.20	0.046	1.11	0.127
	1370513_at	tropomyosin 1, alpha	24851	1.14	0.001	1.06	0.412
	1370158_at	myosin heavy chain 10	79433	1.15	0.008	1.01	0.690
	1371511_at	actin related protein 2/3 complex, subunit 2	301511	1.14	0.044	1.11	0.174
	1388128_at	actin-related protein 3	81732	1.05	0.041	-1.02	0.724
	1367457_at	beclin 1	114558	1.07	0.025	1.05	0.242
downregulated by lithium	1373478_at	myosin binding protein H		-1.67	0.036	-1.37	0.115
	1371866_at	myosin XVIIIa	287905	-1.52	0.041	-1.16	0.521
	1373706_at	actin dependent regulator of chromatin, a-like 1	316477	-1.46	0.009	-1.05	0.654
	1368250_at	tektin 1	85270	-1.43	0.019	-1.22	0.392
	1377692_at	smooth muscle myosin heavy chain 11 isoform SM1-like	362697	-1.26	0.032	-1.02	0.757
	1387866_at	myosin IXb	25486	-1.22	0.021	-1.02	0.827
upregulated in prior lithium	1371087_a_at	microtubule-associated protein 6	29457	1.17	0.323	1.32	0.046
	1376785_at	synaptonemal complex protein 3		1.18	0.274	1.31	0.036
	1371716_at	actin dependent regulator of chromatin, c2	362815	-1.17	0.181	1.30	0.035
	1373849_at	actin-related protein BAF53a	361925	1.09	0.204	1.22	0.010
	1375863_a_at	gamma-tubulin complex component 4	368168	1.05	0.505	1.21	0.008
	1376005_at	kinesin family 1B	117548	1.05	0.543	1.21	0.013
	1372444_at	kinesin heavy	117550	1.22	0.055	1.21	0.035
	1374573_at	dynein 2 light intermediate chain	298767	1.07	0.271	1.13	0.017
downregulated in prior lithium	1393418_at	tropomodulin 2	58814	-1.05	0.933	-1.46	0.024
	1369541_at	tropomodulin 2	58814	-1.10	0.490	-1.32	0.010
	1373268_at	microtubule-associated protein 4	367171	1.04	0.718	-1.33	0.043
	1368137_at	microtubule-associated protein tau	29477	-1.10	0.369	-1.10	0.049
	1369720_at	myosin Ib	117057	1.23	0.364	-1.31	0.010
	1370933_at	myosin IE	25484	-1.06	0.532	-1.14	0.045
	1368450_at	myosin Va	25017	1.14	0.169	-1.14	0.020
	1371053_at	myosin, heavy 8	252942	1.01	0.825	-1.07	0.024
	1374403_at	ephrin B1	25186	-1.12	0.119	-1.11	0.013
	1387552_at	discs, large homolog-associated protein 2	116681	-1.05	0.328	-1.23	0.009

(*Table continues*)

Table 1. *Continued*

				on Li		prior Li/Co [2w]	
inositol phosphate pathway	probe set	gene	LocusLink ID	fold change	P value	fold change	P value
upregulated by lithium	1370585_a_at	protein kinase C, beta 1	25023	1.23	0.004	-1.02	0.819
	1370025_at	phosphatidylinositol-4-phosphate 5-kinase, type II, gamma	140607	1.23	0.032	-1.08	0.172
	1371014_at	phospholipase C, beta 1	24654	1.23	0.042	1.14	0.159
	1399125_at	inositol polyphosphate-1-phosphatase	316376	1.18	0.010	1.01	0.945
	1388502_at	inositol-1,3,4,5-trisphosphate 5-phosphatase, type II	362590	1.11	0.014	1.06	0.374
downregulated by lithium	1370114_a_at	phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1	25513	-2.19	0.040	-1.33	0.055
	1387904_at	inositol hexaphosphate kinase 1	50560	-1.62	0.013	-1.24	0.260
	1372074_at	diphosphoinositol polyphosphate phosphohydrolase; DIPP	294292	-1.34	0.043	-1.15	0.201
	1388933_at	inositol 1,3,4-triphosphate 5/6 kinase	314398	-1.28	0.017	-1.08	0.354
	1373101_at	phosphatidylinositol glycan, class K	295543	-1.24	0.049	-1.08	0.552
	1375272_at	phosphatidylinositol 4-kinase type II	114554	-1.20	0.036	-1.05	0.543
	1389723_at	phosphoinositide-3-kinase, regulatory subunit 4, p150	363131	-1.17	0.025	-1.05	0.453
upregulated in prior lithium	1371776_at	phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1	25513	1.39	0.188	1.58	0.018
	1370948_a_at	myristoylated alanine-rich C-kinase substrate (MARCKS)	294446	1.11	0.566	1.46	0.037
	1373432_at	myristoylated alanine-rich C-kinase substrate (MARCKS)	294446	1.10	0.619	1.35	0.027
	1369944_at	MARCKS-like protein	81520	-1.02	0.858	1.18	0.016
	1389143_at	phosphatidylinositol 3-kinase, catalytic, alpha polypeptide	170911	1.13	0.550	1.44	0.022
	1377735_at	phosphoinositol 4-phosphate adaptor Protein-1	295674	1.16	0.282	1.34	0.019
	1389176_at	inositol polyphosphate-5-phosphatase F	309008	1.10	0.269	1.28	0.013
	1375600_at	Similar to phosphatidylinositol-glycan biosynthesis, class O	313341	1.09	0.233	1.15	0.034
	1386883_at	glycogen synthase kinase 3 alpha		1.04	0.579	1.12	0.028
downregulated in prior lithium	1369039_at	phosphatidylinositol 4-kinase, catalytic, beta polypeptide	81747	-1.12	0.557	-1.61	0.033
	1387847_at	phosphatidylinositol 3-kinase, catalytic, beta polypeptide	85243	-1.06	0.538	-1.31	0.033

				on Li		prior Li/Co [2w]	
GTP/GDP synthesis	probe set	gene	LocusLink ID	fold change	P value	fold change	P value
upregulated by lithium	1368154_at	guanylate cyclase 1, soluble, alpha 3	25201	1.39	0.029	1.24	0.137
	1369097_s_at	guanylate cyclase 1, soluble, beta 3	25202	1.28	0.001	1.19	0.254
	1374389_at	guanylate cyclase 1, soluble, beta 3	25202	1.26	0.019	1.22	0.066
	1374872_at	RAS guanyl releasing protein 2	361714	1.52	0.039	1.01	0.985
	1383322_at	RAS-like family 11 member B	305302	1.42	0.006	1.05	0.607
	1368505_at	regulator of G-protein signaling 4	29480	1.38	0.005	-1.11	0.307
	1368500_a_at	regulator of G-protein signaling 9	29481	1.48	0.036	-1.02	0.765
	1372065_at	ADP-ribosyltransferase 3	305235	1.30	0.009	1.04	0.456
	1382105_at	guanine nucleotide binding protein, beta 5	83579	1.28	0.027	-1.01	0.917
	1370122_at	RAB27B	363410	1.11	0.021	1.11	0.074
	1376573_at	RAB34	360571	1.17	0.023	1.06	0.421
downregulated by lithium	1373572_at	GTP binding protein 5	296462	-1.27	0.037	1.06	0.553
	1370202_at	HRAS like suppressor	24913	-1.41	0.009	-1.03	0.891
	1371836_at	RAB5C	287709	-1.49	0.020	-1.28	0.119
upregulated in prior lithium	1388800_at	RAB5A	64633	1.13	0.320	1.32	0.044
	1368847_at	RAB10	50993	1.19	0.292	1.42	0.046
	1372404_at	RAS-related C3 botulinum substrate 2	366957	1.00	0.908	1.14	0.022
	1369614_at	RAP2B	170923	-1.01	0.863	1.30	0.028
	1373658_at	GTPase-activating protein	315298	-1.03	0.537	1.10	0.009
downregulated in prior lithium	1371723_at	Ras-related GTP binding C	298514	1.05	0.546	-1.18	0.039
	1373894_at	RAB31	246324	1.03	0.807	-1.27	0.048
	1371659_at	ras homolog gene family, member C	295342	1.02	0.901	-1.18	0.033

				on Li		prior Li/Co [2w]	
potassium channel	probe set	gene	LocusLink ID	fold change	P value	fold change	P value
upregulated by lithium	1369487_a_at	potassium inwardly-rectifying channel, subfamily J, member 4	116649	1.51	0.007	-1.09	0.823
	1371114_at	potassium inwardly-rectifying channel, subfamily J, member 4	116649	1.28	0.028	1.03	0.719
	1387881_at	potassium channel, subfamily V, member 1	60326	1.36	0.009	1.02	0.741
	1370439_a_at	potassium voltage gated channel, Shaw-related subfamily, member 2	246153	1.35	0.016	1.18	0.252
	1368524_at	potassium voltage gated channel, Shaw-related, member 1	25327	1.24	0.010	-1.06	0.521
	1388225_at	potassium voltage gated channel, Shaw-related, member 2	246153	1.16	0.026	-1.01	0.906

				on Li		prior Li/Co [2w]	
ATPases	probe set	gene	LocusLink ID	fold change	P value	fold change	P value
upregulated by lithium	1368698_at	ATPase, Ca++ transporting, plasma membrane 2	24215	1.34	0.041	-1.37	0.065
	1386937_at	ATPase, Na+/K+ transporting, beta 1	25650	1.10	0.050	1.01	0.831
downregulated by lithium	1398862_at	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	29693	-1.52	0.020	-1.15	0.438
	1371925_at	ATPase type 13A	290673	-1.33	0.029	-1.13	0.274
	1374431_at	ATPase, H+ transporting, V1 subunit A, isoform 1	360716	-1.09	0.036	-1.03	0.575
downregulated in prior lithium	1371402_at	ATPase, H+ transporting, lysosomal, beta 56/58 kDa, isoform 2	117596	-1.13	0.180	-1.27	0.011

levels (a measure of sensorimotor processes) were also similar between control and Li-treated rats, suggesting that exposure to lithium does not interfere with the ability to detect or respond to painful stimuli. Because FPS is also a measure of learning and memory processes, we tested Li-treated rats in the Morris water maze to examine whether a potential impairment of memory processes might have obstructed fear potentiation (D'Hooge and De Deyn, 2001). However, no differences were evident in the acquisition or retention phase, or in relearning the new location of the platform. Together, these results suggest that behavioral measures of innate anxiety (open field and EPM), but not conditioned fear (FPS) or spatial memory (Morris water maze), reveal the enduring consequences of Li exposure early in life.

Because of the lasting behavioral effects of adolescent Li administration, we examined whether Li alters gene expression and may thereby convey synaptic reorganization and "neuronal memory" of Li exposure (Bailey et al., 1996; Silva, 2003; Klann and Dever, 2004). Because the strength of gene array experiments is their emphasis on broad, biological themes rather than on specific genes (Konradi, 2005), we analyzed gene expression pat-

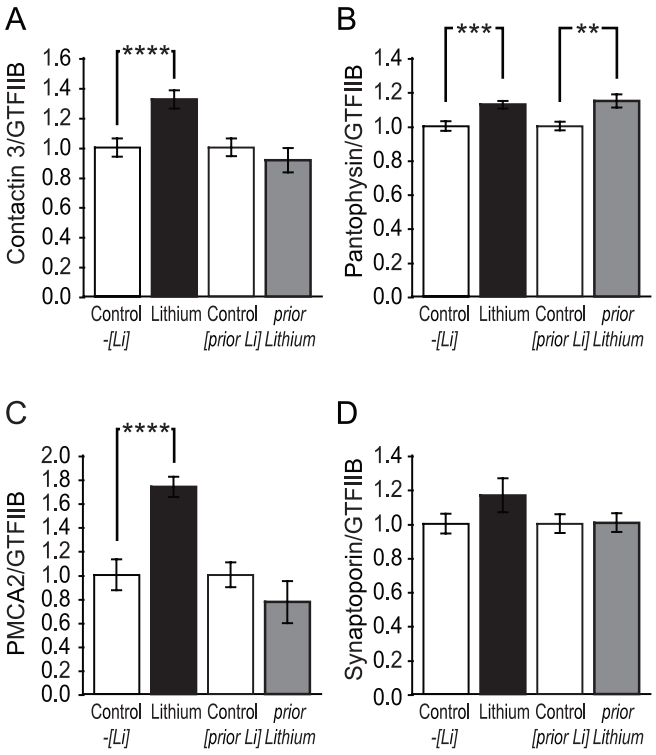

Figure 5. Q-PCR verification of gene array data. The expression of four genes was tested with Q-PCR. ***A***, Contactin 3; ***B***, pantophysin; ***C***, plasma membrane Ca^{2+}-transporting ATPase (PMCA2); and ***D***, synaptoporin. All genes were normalized to general transcription factor IIB (*GTFIIB*), which was not regulated in the gene arrays. Data are mean ± SEM of $n = 6–8$ samples. $^{**}p \leq 0.01$; $^{***}p \leq 0.005$; $^{****}p \leq 0.001$.

terns during and 2 weeks after Li administration in a gene expression microarray approach. We chose the amygdala as the brain area of interest, because it is known to be involved in mechanisms of fear and anxiety (Davis, 1997; LeDoux, 2003). Gene expression in the amygdala was altered by Li treatment and affected transcripts that are involved in cell adhesion and in the structure of synapses and cytoskeleton. These changes, if translated into protein levels, indicate structural rearrangements of neurons. Although most of the altered gene expression was transient and not observed 2 weeks after discontinuation of Li administration, it cannot be concluded that the rearrangements themselves were reversible. Even if gene expression patterns went back to control levels, synaptic connections might have changed during the time of altered gene expression. Neuronal or dendritic rearrangements could have far-reaching consequences particularly in an immature brain, and they could explain the long-lasting effects on anxiety observed in our study. Two weeks after discontinuation of Li exposure, gene expression changes were observed in the same gene families affected during Li treatment but in a different set of transcripts and to a lesser degree. However, these modifications did not normalize the anxiety-like behavior.

Other gene families affected by Li treatment included the inositol phosphate pathway and GTPases, two pathways that can interact with each other (Alonso et al., 1988; Huang et al., 1988). Depletion of brain inositol levels has been hypothesized to be important for the therapeutic action of Li (Berridge et al., 1982; Williams et al., 2002; Harwood, 2005), although this theory is controversial (Berry et al., 2004). Interestingly, peptidylglycine α-amidating monooxygenase, an enzyme that was shown previously to be upregulated by inositol depletion in adult rat cortical slices (Brandish et al., 2005), was downregulated in the amygdala in our study after chronic Li treatment. This finding could be explained as differences between chronic and acute treatment paradigms, by the different experimental approaches applied, or as differences in gene expression patterns in different brain areas, as has been shown previously (MacDonald et al., 2004).

Two more gene families affected by Li were potassium channel transcripts and ATPases. These data suggest altered ion flux/ion homeostasis after Li exposure. Potassium channels are chiefly responsible for repolarizing cell membranes after action potentials. Six potassium channel subunits were upregulated during Li exposure, indicating an increased demand for repolarization, possibly caused by increased depolarization or by hypopolarization of nerve cells. ATPases are enzymes involved in ion transport, vesicle transport, and lysosomal acidification. We found changes predominantly in Ca^{2+}, Na^{+}, and K^{+} transporting ATPase transcripts. These data suggest that Li affects membrane potentials and electrophysiological properties of cells in the amygdala.

It is important to emphasize that we tested normal rats in the present study; no genetic models of BPD exist. Although the data show that normal rats might be unfavorably affected by Li treatment (and, by inference, the results might caution against treating juveniles with Li in cases in which the clinical diagnosis is equivocal), they should be considered in light of the positive impact Li can have in the management of BPD. In children and adolescents, BPD is not only characterized by anxiety but also by irritability and disruptive behavior with features of ADHD and conduct disorder (http://www.nimh.nih.gov/publicat/bipolarupdate.cfm). These other behaviors were not addressed in our study. Genetic manipulations that can mimic some of these symptoms are needed to further address the effect of Li on BPD-like behaviors.

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