Mood stabilizer lithium inhibits amphetamine-increased 4-hydroxynonenal-protein adducts in rat frontal cortex

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Abstract

Recent studies indicate that bipolar disorder is associated with mitochondrial dysfunction and oxidative stress. Previous studies in our laboratory have shown that the mood stabilizer lithium inhibits oxidative stress. The α,β-unsaturated aldehyde 4-hydroxy-2-nonenal (4-HNE), a major product of lipid peroxidation, is able to exert cytotoxicity and disturb cellular function by forming protein adducts. The purpose of this study is to determine whether chronic lithium treatment prevents 4-HNE-protein adduction in an amphetamine-induced hyperactive mania-like model. We found that repeated amphetamine stimulation significantly induced hyperactive behaviour, decreased activities of mitochondrial complexes I and III, and increased 4-HNE-protein adducts in rat frontal cortex, and that chronic lithium treatment inhibited both amphetamine-induced hyperactivity and 4-HNE-protein adduction. Monoamine neurotransmitters are involved in the aetiology and pathology of bipolar disorder and other psychiatric diseases, and also contribute significantly to amphetamine-induced behavioural effects. Vesicular monoamine transporter 2 (VMAT2) is critical in packaging monoamine neurotransmitters. We found that 4-HNE can form protein adducts with VMAT2. Repeated amphetamine stimulation significantly increased 4-HNE-VMAT2 adducts, while chronic lithium treatment reduced amphetamine-increased 4-HNE-VMAT2 adducts in rat frontal cortex. Our findings suggest that chronic lithium treatment may inhibit amphetamine-induced hyperactive mania-like behaviour by preventing 4-HNE-VMAT2 adduction. This finding also indicates that prevention of 4-HNE-VMAT2 adduction may contribute in part to the pharmacological action of lithium for the treatment of bipolar disorder.

Introduction

Bipolar disorder (BD) is a chronic illness with recurring episodes of mania and depression affecting approximately 1.5% of the population. Lithium is the most commonly used mood-stabilizing agent for the treatment of BD, and is useful both for treatment of acute manic episodes and for prophylaxis against future episodes. It is widely acknowledged that chronic treatment with lithium is required to generate

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therapeutic effects, to improve the course of illness and prevent relapses of mania and depression. Although many studies have shown that lithium targets various cellular components including glycogen synthase kinase-3 (GSK3), Akt, inositol phosphatases and others, the relationship between these targets and lithium's therapeutic effect are not yet fully clear. A growing body of evidence shows that chronic lithium treatment is neuroprotective both *in vitro* and *in vivo* (Rowe & Chuang, 2004; Schloesser *et al.* 2008) and this neuroprotective mechanism is currently undergoing extensive study. Previously we found that chronic treatment with lithium inhibited oxidative damage to lipid and protein in primary cultured rat cerebral cortical cells (Shao *et al.* 2005; Wang *et al.*

2003). A number of independent laboratories also found that chronic lithium treatment reduced hydrogen peroxide radical-induced cytochrome c release, caspase-3 activation and cell death in human neuroblastoma cells and murine hippocampal cells (King & Jope, 2005; Lai *et al.* 2006; Schafer *et al.* 2004). These studies suggest that chronic lithium treatment produces a neuroprotective effect against oxidative stress.

The α,β-unsaturated aldehyde 4-hydroxy-2-nonenal (4-HNE) is a major product of lipid peroxidation. 4-HNE not only is toxic to cells, but also reacts with many proteins to form 4-HNE-protein adducts, further affecting the function of these proteins (Petersen & Doorn, 2004). Recently, we found that 4-HNE-protein adducts are increased in post-mortem brain of subjects with BD and schizophrenia compared to controls (Wang *et al.* 2009), indicating that 4-HNE-induced protein modification has an important role in the pathological progress of these psychiatric diseases.

Amphetamine, as a psychostimulant, can induce hyperactivity, enhanced mood, racing thoughts, high energy and restlessness that are similar to symptoms of psychotic mania (Gerner *et al.* 1976; Jacobs & Silverstone, 1986). Considering the difficulty of modelling the highly complex mood-swinging nature of BD, amphetamine-induced hyperactivity has been used to analyse the efficacy of anti-manic treatments, even though hyperactivity occurs not only in BD but also in other disorders (Gould & Einat, 2007; Machado-Vieira *et al.* 2004). It has been reported that lithium treatment reduces both single and repeated administration of low-dose amphetamine-induced hyperactivity in rats (Correa *et al.* 2007; Cox *et al.* 1971; Frey *et al.* 2006; Gould *et al.* 2007; McNamara *et al.* 2008; Valvassori *et al.* 2010). However, repeated administration of amphetamine has consistently been reported to induce oxidative stress (Andreazza *et al.* 2008; Frey *et al.* 2006).

Monoamine neurotransmitters are involved in the aetiology and pathology of BD and other psychiatric diseases (Carlborg *et al.* 2011; Cousins *et al.* 2009; Hirschfeld, 2000; Wiste *et al.* 2008), and also contribute significantly to amphetamine-induced behavioural effects (Robertson *et al.* 2009; Rothman & Baumann, 2006). These neurotransmitters are synthesized by cytosolic enzymes, and then packed into synaptic vesicles. Vesicular monoamine transporter 2 (VMAT2) is responsible for the packaging process of monoamine neurotransmitters (Guillot & Miller, 2009). In the present study we determine whether repeated amphetamine stimulation increases adduction of 4-HNE with VMAT2 in rat frontal cortex and whether chronic lithium treatment prevents formation of 4-HNE-protein adducts in the amphetamine-induced hyperactive mania-like animal model.

Materials and methods

Animal treatment

Male Sprague–Dawley rats (Charles River, Canada) weighing approximately 230–270 g were fed normal chow or chow containing 0.24% Li_2CO_3 by weight (BioServ, USA) for 3 wk. Beginning at day 8, rats were given an intraperitoneal (i.p.) injection of vehicle or amphetamine at 2 mg/kg daily for 2 wk. Two weeks' treatment with amphetamine at this dose has been reported to increase levels of malondialdehyde, another lipid peroxidation product (Andreazza *et al.* 2008; Frey *et al.* 2006). Chow was available to animals *ad libitum*, along with 0.9% saline and regular drinking water to prevent dehydration. Rats were weighed every other day (before treatment: control group 246 ± 6 g, lithium group 248 ± 7 g, amphetamine group 248 ± 9 g, and lithium + amphetamine group 251 ± 8 g; after treatment: control group 333 ± 21 g, lithium group 305 ± 17 g, amphetamine group 325 ± 16 g, and lithium + amphetamine group 312 ± 19 g). Blood was collected to measure serum lithium levels. Serum lithium level testing was performed by Vancouver Coastal Health laboratory. There were no detectable lithium levels in the control and amphetamine treatment groups. Serum lithium concentrations were 0.68 ± 0.17 mM in the lithium treatment group and 0.55 ± 0.08 mM in the lithium + amphetamine treatment group, which are within the therapeutic range of the drug. All experiments followed the guidelines of the University of British Columbia Animal Care Committee and the Canadian Council on Animal Care.

Open-field test

Locomotor activity was monitored in an open field consisting of a black Plexiglas square box ($50 \times 50 \times 50$ cm). Open-field test was performed in the morning. Before any behavioural tests, all animals were first handled in the colony room for 5 min daily for 3 d, and then placed individually in the box for 30 min daily for another 2 d before the drug injection procedure began in order to become habituated to testing boxes. Locomotor activity was analysed 2 h after the last injection with normal saline or amphetamine. Rat locomotor activity was recorded with a video camera connected to Ethovision Video Tracking System software Noldus XT5.1 (Noldus Information Technology Inc., USA). This software is able to record and track the

distance travelled of each rat during the behavioural test.

Immunohistochemistry

Rats were anaesthetized with urethane and perfused transcardially with 250 ml of 100 mM phosphate buffered saline (PBS) (pH 7.4) at room temperature, followed by 4% paraformaldehyde in PBS. The brain was then removed, post-fixed in the same paraformaldehyde solution overnight at 4 °C, and transferred into 30% sucrose at 4 °C until the brain sank to the bottom of the container. The brains were then sectioned coronally at −18 °C using a cryostat (Leica Microsystems, Germany), and 30-μm thick coronal sections were collected from the levels of anterior cingulate cortex, prelimbic cortex and infralimbic cortex according to a rat brain atlas (Paxinos & Watson, 1998). The tissue sections were kept in a cryoprotectant solution containing 50 mM PBS, 25% ethylene glycol and 25% glycerol. The sections were mounted on glass slides (Fisher Scientific, Canada). Each slide contained corresponding sections from one animal in each treatment group (control, lithium, amphetamine, amphetamine + lithium groups) to allow comparisons across groups and minimize differences in background. Slides were then air-dried, washed with PBS three times, and immediately used in immunohistochemistry.

Tissue sections were blocked with 5% normal goat serum with 0.3% Triton X-100 for 1 h at room temperature. The sections were labelled with a polyclonal antibody for 4-HNE-protein adducts (QED Bioscience Inc., USA), or double-labelled with an antibody for 4-HNE-protein adducts and a monoclonal antibody for neuronal marker NeuN [or a monoclonal antibody for astrocyte marker glial fibrillary acidic protein (GFAP)] at 4 °C overnight (Chemicon International, USA). After washing with PBS, sections were further incubated with Alexa-568 conjugated anti-goat IgG (Invitrogen Canada Inc., Canada) or Alexa-488 conjugated anti-mouse IgG (Invitrogen) for 2 h at room temperature. Then the sections were washed with PBS and coverslipped using fluorescent antifade mounting media (Invitrogen).

Images were captured in anterior cingulate cortex, prelimbic cortex or infralimbic cortex at 40× magnification using an Eclipse E600 microscope with NIS-Elements Advanced Research v. 3.0 software (Nikon Canada, Canada). The intensity value of each positively labelled cell for each brain region was collected. Regions without fluorescence staining served as background. All images were captured with uniform threshold and intensity settings. Fluorescence intensities of each brain region for every slide were calculated as follows:

Fluorescence intensity =

(intensity of cell$_1$ − intensity of background)
+ (intensity of cell$_2$ − intensity of background)
+ ... + (intensity of cell$_n$ − intensity of background)

Three adjacent sections were processed and evaluated for each rat.

Preparation of P2 fraction

Rat frontal cortex was homogenized in 10:1 (v/w) ice-cold isolation buffer containing 0.32 M sucrose, 5 mM Hepes, 1% (v/v) protease inhibitor cocktail (Cayman Chemical Company, USA) and centrifuged at 1000 g for 10 min at 4 °C. The supernatant was collected and centrifuged again at 30 000 g for 30 min at 4 °C. The supernatant was discarded, while the precipitate (P2) enriched with synaptosomes and mitochondria was resuspended either in a buffer containing 0.21 M mannitol, 0.07 M sucrose, 10 mM Tris–HCl (pH 7.4) (used for enzyme assay) or lysed on ice in lysis buffer containing 1% Triton X-100, 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail (used for immunoprecipitation and immunoblotting analysis).

Analysis of mitochondrial complexes I and III activities

Complex I activity was analysed by measuring NADH oxidation. Sixyty-five μl of P2 extraction, 15 μl of 0.5 mM NADH, 10 μl of 0.5 mM CoQ1 and 10 μl of assay buffer [50 mM KCl, 10 mM Tris–HCl, 1 mM EDTA, 2 mM KCN (pH 7.4)] were mixed, incubated at 30 °C and recorded at a 340 nm *vs.* time for 5 min with a spectrophotometer. Rotenone is an inhibitor of complex I. The rotenone insensitive activity was determined simultaneously with 10 μl of 10 mM rotenone in the reaction mixture. Complex I activity was calculated as total enzyme activity minus rotenone insensitive enzyme activity. Complex III activity was determined by measuring the reduction of cytochrome c. Forty μl of P2 extraction, 40 μl of 0.25 mM cytochrome c, 40 μl of the reaction mixture [containing 0.25 mM decylubiquinol, 20 mM NaN$_3$ and 250 mM Tris–HCl (pH 7.4)] were mixed, incubated at 30 °C and recorded at a 550 nm *vs.* time for 5 min with a spectrophotometer. Antimycin A is an inhibitor of complex III. The Antimycin A insensitive activity was determined simultaneously with 10 mM antimycin A present in the reaction mixture. Complex III activity was
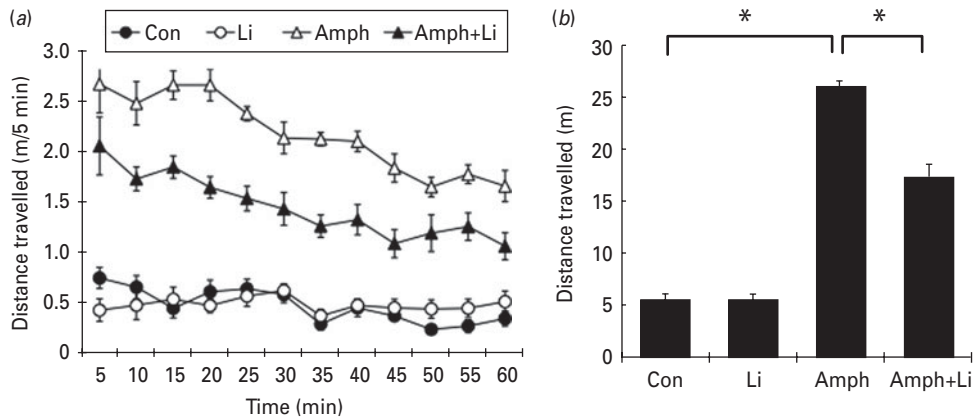
Fig. 1. The effect of lithium (Li) on hyperactivity induced by repeated amphetamine (Amph) stimulation. Rats were fed with normal chow (Con) or Li chow for 3 wk. At day 8, rats were also given Amph at 2 mg/kg (i.p.) daily for 14 d. The distance travelled was recorded 2 h after final Amph injection. (*a*) Distance travelled for each rat in every 5 min within 60 min; (*b*) total distance travelled. Data are displayed as mean ± S.E.M. (*n* = 8). * Indicates $p < 0.05$, ANOVA followed by *post-hoc* analysis.

calculated as total enzyme activity minus antimycin A insensitive enzyme activity.

Immunoprecipitation and immunoblotting analysis

Immunoprecipitation was performed using crude synaptosomal P2 fractions obtained from rat frontal cortex. P2 fractions were incubated with an antibody for VMAT2 (Millipore Corporation, USA). Immunoprecipitated proteins were further incubated with Protein A/G beads (Santa Cruz Biotechnology, USA) overnight at 4 °C. Protein A/G beads were then washed in cold lysis buffer. Immunoprecipitated proteins were eluted with loading buffer containing 100 mM Tris–HCl (pH 6.8), 4% SDS, 0.2% Bromophenol Blue and 20% glycerol.

Immunoprecipitated proteins or lysated P2 fractions were run on a SDS–PAGE with 10% acrylamide gel for 1.5 h at 100 V and transferred to polyvinylidene fluoride (PVDF) membranes at 110 V for 1.5 h. The membranes were then blocked with 5% milk in PBS for 1 h at room temperature, incubated with an antibody for 4-HNE-protein adducts or VMAT2 overnight at 4 °C, and further incubated for 2 h at room temperature with a secondary antibody conjugated to horseradish peroxidase. Immunoreactive bands were detected with the enhanced chemiluminescence reagent (PerkinElmer, Canada). The membranes were imaged with a Fujifilm LAS-3000 system (Fujifilm Medical Systems USA Inc., USA) and analysed by Gene Tool software (PerkinElmer). When 4-HNE-VMAT2 adductions were quantified, the membranes probed with an antibody for 4-HNE-protein adducts were stripped and re-probed with an antibody for VMAT2 for checking loading control.

Statistical analysis

Statistical analysis was performed using SPSS 16 software (SPSS Inc., USA). All results were expressed as mean ± standard error of the mean. Locomotor activity was quantified as distance travelled. The distance travelled by each rat in 5-min blocks of time over 1 h was calculated and analysed by two-way repeated-measures analysis of variance (ANOVA) using treatment group as the between-subjects factor and time as the repeated measure. Significant differences between treatment groups were determined by a *post-hoc* LSD test. Total distance travelled among groups within 1 h was analysed by one-way ANOVA followed by a *post-hoc* LSD test. The results from immunohistochemistry and immunoblotting analysis were also analysed by one-way ANOVA followed by a *post-hoc* LSD test. Statistical significance was set as $p < 0.05$.

Results

Chronic treatment with lithium reduced hyperactivity induced by repeated amphetamine stimulation

Rats were chronically treated with lithium for 21 d. Beginning at day 8, rats also received vehicle or amphetamine at 2 mg/kg (i.p.) once daily for 14 d. Two-way repeated-measures ANOVA indicated a significant effect of group [$F_{(3, 28)} = 165.4$, $p < 0.05$], a significant effect of time [$F_{(11, 308)} = 14.2$, $p < 0.05$] and a significant interaction between group and time [$F_{(33, 308)} = 2.7$, $p < 0.05$] (Fig. 1*a*). *Post-hoc* analysis indicated that chronic treatment with lithium reduced hyperactivity induced by repeated amphetamine stimulation ($p < 0.05$). Further, the total distance
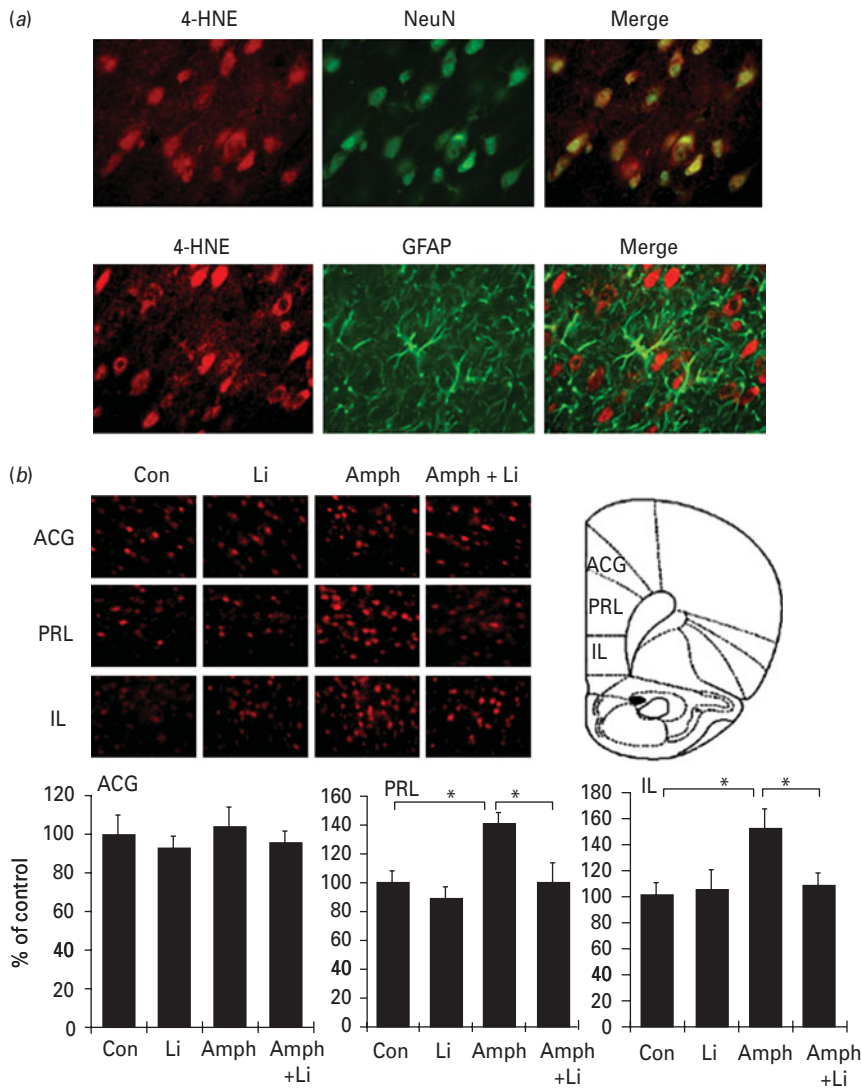
Fig. 2. The effect of lithium (Li) on amphetamine (Amph)-increased 4-HNE-protein adducts in rat medial frontal cortex. (*a*) Immunostaining of 4-HNE-protein adducts in neurons and glial cells of rat frontal cortex. Immunohistochemical staining of 4-HNE, NeuN and GFAP was performed using immunohistochemistry with a polyclonal antibody recognizing 4-HNE-protein adducts, a monoclonal antibody recognizing neuronal marker NeuN and a monoclonal antibody recognizing glial marker GFAP. (*b*) The effect of Li on Amph-increased 4-HNE-protein adducts in anterior cingulate (ACG), prelimbic (PRL) and infralimbic (IL) regions of rat medial frontal cortex. Rats were fed with normal chow (Con) or Li chow for 3 wk. At day 8, rats were also given Amph at 2 mg/kg (i.p.) daily for 14 d. Results are the mean±S.E.M. (n=8). * Indicates $p < 0.05$, ANOVA followed by *post-hoc* analysis.

travelled within 60 min was also calculated. We found a significant difference in total distance travelled among control, lithium, amphetamine and lithium + amphetamine treatment groups [$F(3, 28) = 165.3$, $p < 0.05$] (Fig. 1*b*). *Post-hoc* analysis showed that repeated amphetamine stimulation significantly increased the total distance travelled compared to controls ($p < 0.05$), while lithium treatment significantly reduced the amphetamine-increased total distance travelled ($p < 0.05$).

Chronic treatment with lithium reduced 4-HNE-protein adducts increased by repeated amphetamine stimulation

Using double staining with 4-HNE-protein adducts antibody and NeuN antibody or GFAP antibody, we found that 4-HNE-protein adducts were predominately in neurons in the rat frontal cortex (Fig. 2*a*), therefore we solely analysed neuronal 4-HNE-protein adducts in further experiments. As shown in Fig. 2*b*,
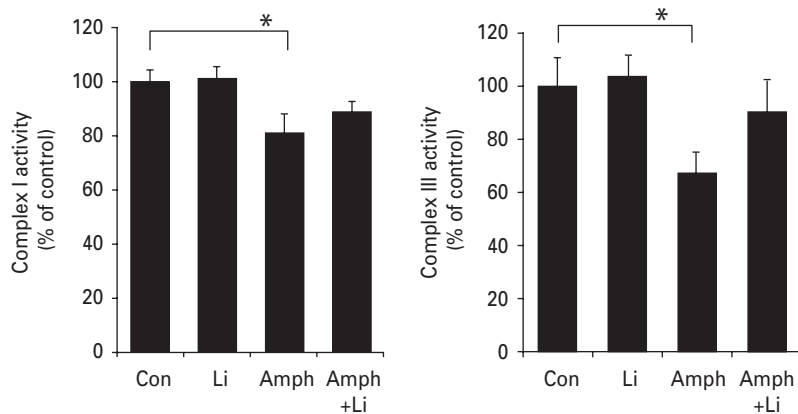
Fig. 3. The effects of lithium (Li) and amphetamine (Amph) on activities of mitochondrial complexes I and III in rat frontal cortex. Rats were fed with normal chow (Con) or Li chow for 3 wk. At day 8, rats were also given Amph at 2 mg/kg (i.p.) daily for 14 d. P2 extracts from rat frontal cortex were used to analyse activities of complexes I and III. Results are the mean ± s.e.m. ($n = 8$). * Indicates $p < 0.05$, ANOVA followed by *post-hoc* analysis.

an overall significant difference in levels of 4-HNE-protein adducts among control, lithium, amphetamine and lithium + amphetamine treatment groups was detected in prelimbic [$F(3, 28) = 7.8$, $p < 0.05$] and infralimbic [$F(3, 28) = 3.2$, $p < 0.05$] regions of rat frontal cortex, but not in the anterior cingulate region. *Post-hoc* analysis showed that repeated amphetamine stimulation significantly increased levels of 4-HNE-protein adducts in prelimbic ($p < 0.05$) and infralimbic ($p < 0.05$) regions. Although chronic lithium treatment has no effect on 4-HNE-protein adducts, this treatment significantly inhibited amphetamine-increased 4-HNE-protein adducts ($p < 0.05$ for prelimbic region, $p < 0.05$ for infralimbic region).

The effects of amphetamine and lithium on activities of mitochondrial complexes I and III

Dysfunctional mitochondrial complexes I and III are major sites for production of reactive oxygen species (ROS) (Nohl *et al.* 2005). We found a significant difference in activities of complex I [$F(3, 28) = 3.2$, $p < 0.05$] and complex III [$F(3, 28) = 3.0$, $p = 0.05$] among control, lithium, amphetamine and lithium + amphetamine treatment groups in rat frontal cortex. *Post-hoc* analysis showed that repeated amphetamine stimulation significantly decreased activities of complex I activity ($p < 0.05$) and complex III activity ($p < 0.05$) compared to controls. However, chronic treatment with lithium did not produce any effect on either basal activities of complexes I and III or amphetamine-decreased activities of complexes I and III (Fig. 3).

Chronic treatment with lithium reduced 4-HNE-VMAT2 adducts increased by repeated amphetamine stimulation

It has been shown that amphetamine regulates monoamine neurotransmitters (Robertson *et al.* 2009; Rothman & Baumann, 2006). Because VMAT2 plays a critical role in monoamine neurotransmitter packing, we used immunoprecipitation to examine whether amphetamine-increased 4-HNE-protein adduction with VMAT2 in rat frontal cortex. First, we determined if 4-HNE directly forms protein adducts with VMAT2. P2 fraction from rat frontal cortex was immuno-precipitated using an antibody for VMAT2. Immuno-precipitated proteins were further treated with 100 μM 4-HNE for 1 h and 4-HNE-protein adducts in immunoprecipitated proteins were examined by immunoblotting analysis using an antibody for 4-HNE adducts. As shown in Fig. 4*a*, 4-HNE can form protein adducts with VMAT2 in rat frontal cortex. Second, we examined if treatments with amphetamine and lithium affect 4-HNE-VMAT2 adducts. P2 fraction from rat frontal cortex was immunoprecipitated using an antibody for VMAT2 and immunoprecipitated proteins were further examined by immunoblotting analysis using an antibody for 4-HNE adducts. We found a significant difference in levels of 4-HNE-VMAT2 adducts [$F(3, 28) = 3.2$, $p < 0.05$] among control, lithium, amphetamine and lithium + amphetamine treatment groups in rat frontal cortex. *Post-hoc* analysis showed that repeated amphetamine stimulation significantly increased levels of 4-HNE-VMAT2 adducts ($p < 0.05$) (Fig. 4*b*, *c*). Although chronic lithium
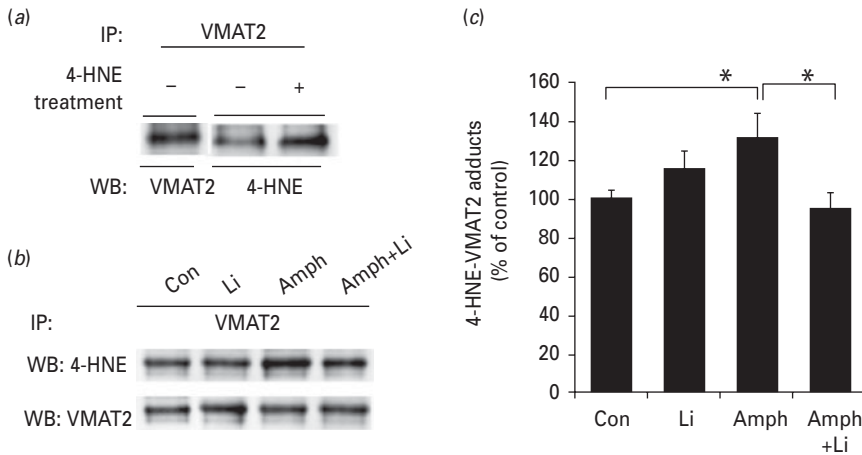
Fig. 4. The effects of lithium (Li) on amphetamine (Amph)-increased 4-HNE-VMAT2 adduction in rat frontal cortex. (*a*) Determining if 4-HNE forms protein adducts with VMAT2. P2 extracts from rat frontal cortex were immunoprecipitated (IP) using an antibody for VMAT2. IP proteins were then incubated with vehicle (−) or 100 μM 4-HNE (+) for 1 h. 4-HNE-treated IP proteins were analysed using Western blot (WB) analysis with an antibody for 4-HNE-protein adducts or VMAT2. (*b, c*) Determining the effect of Li on Amph-increased 4-HNE-VMAT2 adducts. Rats were fed with normal chow (Con) or Li chow for 3 wk. At day 8, rats were also given Amph at 2 mg/kg (i.p.) daily for 14 d. P2 extracts from rat frontal cortex were immunoprecipitated using an antibody for VMAT2, followed by WB analysis to detect 4-HNE-VMAT2 adducts using an antibody for 4-HNE-protein adducts. Membranes were then stripped and re-probed with an antibody for VMAT2 for checking loading control. Results are the mean ± S.E.M. ($n=8$). * Indicates $p<0.05$, ANOVA followed by *post-hoc* analysis.
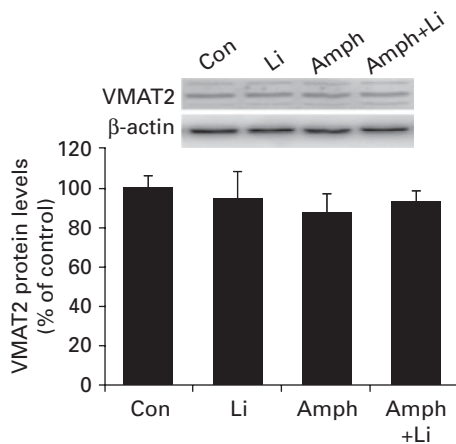

Fig. 5. The effects of lithium (Li) and amphetamine (Amph) on protein levels of VMAT2 in rat frontal cortex. Rats were fed with normal chow (Con) or Li chow for 3 wk. At day 8, rats were also given Amph at 2 mg/kg (i.p.) daily for 14 d. Protein levels of VMAT2 were determined in P2 extracts from rat frontal cortex using immunoblotting analysis with an antibody for VMAT2. Housekeeping protein β-actin was used as loading control. Results are the mean ± S.E.M. ($n=8$). * Indicates $p<0.05$, ANOVA followed by *post-hoc* analysis.

treatment did not affect basal levels of 4-HNE-VMAT2 adducts, this treatment significantly decreased levels of 4-HNE-VMAT2 adducts ($p<0.05$) induced by amphetamine stimulation (Fig. 4*b, c*). Third, we also found that treatment with either amphetamine or lithium did not affect proteins levels of VMAT2 (Fig. 5).

Discussion

In the present study, we found that chronic treatment with the mood stabilizer lithium at a therapeutically relevant concentration decreased repeated amphetamine stimulation-increased locomotor activity. This result is consistent with previous findings (Correa *et al.* 2007; Frey *et al.* 2006; Valvassori *et al.* 2010) and also supports the view that the amphetamine-induced hyperactive mania-like model has a good predictive validity.

4-HNE, as a major product of the lipid peroxidation process, can modify various proteins by reacting with cysteine, histidine or lysine residues to form 4-HNE-protein adducts (Petersen & Doorn, 2004). We found that 4-HNE-protein adducts are predominantly located in neurons, not glia, which may be due to the fact that glial cells contain high levels of endogenous antioxidants (Bolanos *et al.* 1995; Dringen & Hirrlinger, 2003; Oshiro *et al.* 2000). Therefore, glial cells are more resistant to oxidative stress than neuronal cells. We found in rat medial frontal cortex that repeated amphetamine stimulation increased 4-HNE-protein adducts, while chronic lithium treatment decreased amphetamine-induced 4-HNE-protein adducts.

We found previously that chronic lithium treatment protected cultured rat cerebral cortex cells from lipid peroxidation (Shao *et al.* 2005; Wang *et al.* 2003). These findings suggest that chronic lithium treatment produces a neuroprotective effect against oxidative stress, further preventing oxidative stress-induced protein modification. The decrease by chronic lithium treatment of both hyperactive mania-like behaviour and oxidative stress-induced protein modification suggests that a property of neuroprotection against oxidative stress may contribute in part to lithium's mood-stabilizing pharmacological action.

Mitochondria, enriched in presynaptic terminals, produce most cellular ATP and buffer excesses of cytoplasmic calcium. Mitochondrial complexes I and III are the main sites where electrons are leaked to oxygen, producing superoxide radicals (Nohl *et al.* 2005). Superoxide radicals are further dismutated by superoxide dismutase to hydrogen peroxide radicals that produce highly reactive hydroxyl radicals under the Fenton reaction (Maher & Schubert, 2000). We found here that repeated amphetamine stimulation decreased activities of complexes I and III in P2 fractions enriched with mitochondria and synaptosomes from rat frontal cortex. Previously, amphetamine treatment was reported to decrease the mitochondrial membrane potential in cultured rat cortical cells (Cunha-Oliveira *et al.* 2006) and decrease expression of 39 kDa complex I subunit in SK-N-SH human neuroblastoma cells (Klongpanichapak *et al.* 2008). These studies together suggest that amphetamine stimulation may induce mitochondrial dysfunction, resulting in accumulation of ROS. Oxidation of monoamine neurotransmitters catalysed by monoamine oxidases has been known to increase ROS production (Yamamoto & Raudensky, 2008). In addition, dopamine auto-oxidation can result in ROS production and reactive quinones (Miyazaki & Asanuma, 2008). Mitochondrial dysfunction, enzymatic oxidation of monoamine neurotransmitters and dopamine auto-oxidation may contribute to amphetamine-induced oxidative damage to lipids, resulting in increased 4-HNE levels. In the current study, we also found that chronic treatment with lithium at a therapeutically relevant concentration neither altered basal activities of complexes I and III nor prevented amphetamine-decreased activities of complexes I and III. This result suggests that lithium may not directly target mitochondrial complexes but instead may regulate the downstream targets of dysfunctional complexes I and III in order to prevent oxidative stress. Previously, we found that chronic lithium treatment increased expression and activity of glutathione *S*-transferase type 4 (GSTA4) (Shao *et al.* 2008). Because GSTA4 specifically catalyses conjugation of 4-HNE with glutathione to scavenge 4-HNE (Awasthi *et al.* 2004), our studies suggest that chronic lithium treatment may prevent amphetamine-induced 4-HNE-protein adduction by targeting GSTA4. Lithium is also known to inhibit GSK3β (Klein & Melton, 1996). GSK3β has been reported to inhibit antioxidant responsive element (ARE)-driven gene expression, and further promote oxidative stress (Rojo *et al.* 2008; Salazar *et al.* 2006). It is interesting that GSTA4 expression can be transcriptionally induced through ARE (Tjalkens *et al.* 1998). It is possible that inhibition of GSK3β by lithium may further increase expression of GSTA4 expression via ARE and subsequently prevent 4-HNE-VMAT2 adduction. Arachidonic acid is a polyunsaturated fatty acid contained in membrane phospholipids that is abundant in the brain. Oxidation of arachidonic acid and its metabolites can generate 4-HNE and other cytotoxic aldehydes. Recently, amphetamine stimulation has been reported to increase D_2 dopamine receptor-mediated arachidonic acid release in rat brain, while chronic treatment with lithium can inhibit this action (Basselin *et al.* 2005; Bhattacharjee *et al.* 2006). Therefore inhibition of arachidonic acid release by lithium may also prevent 4-HNE-protein adduction. A clear understanding of the protective mechanism of lithium against 4-HNE-protein adduction requires further investigation.

Evidence indicates that free radicals are able to react with many amino acids and induce oxidative post-translational protein modification. Hydroxyl radicals can cause protein carbonylation by introducing carbonyl groups to lysine, proline, arginine, and threonine residues of proteins, while 4-HNE can also introduce carbonyl groups into proteins by forming 4-HNE-protein adducts. Peroxynitrite radicals can induce tyrosine nitration, and nitrogen oxide radicals induce cysteine nitrosylation (Grimsrud *et al.* 2008; Stadtman, 2006). VMAT2 is critical for the packaging of monoamine neurotransmitters, and contributes to amphetamine-induced pharmacological action. Previously, free radicals have been found to oxidatively modify VMAT2. Rotenone, a mitochondrial complex I inhibitor, has been shown to increase tyrosine nitration of VMAT2 in human dopaminergic SH-SY5Y cells, subsequently inducing dopamine redistribution from vesicles to the cytosol (Watabe & Nakaki, 2008). Cysteine residues of VMAT2 in rat striatal vesicles have also been found to be nitrosylated by methamphetamine treatment (Eyerman & Yamamoto, 2007). We found in the current study that 4-HNE can form adducts with VMAT2. Although amphetamine

treatment has no effect on VMAT2 protein levels, this treatment significantly increased 4-HNE-VMAT2 adduction. It has been indicated that amphetamine may increase release of monoamine neurotransmitters partially by interrupting neurotransmitter uptake by the monoamine vesicles, subsequently elevating cytosolic neurotransmitter levels, resulting in neurotransmitter reverse transport out of the nerve terminal (Yamamoto & Raudensky, 2008). It is possible that oxidative modifications of VMAT2 may mediate amphetamine-interrupted vesicular sequestration of neurotransmitters and subsequently cause increase of neurotransmitters in cytosol. We also found that chronic treatment with lithium inhibited amphetamine-induced 4-HNE-VMAT2 adduction in rat frontal cortex, which suggests that lithium may inhibit amphetamine-induced hyperactive behaviour by preventing oxidative modification of VMAT2.

Increasing evidence suggests that brain mitochondrial dysfunction is associated with BD. This evidence includes decreased ATP, phosphocreatine and pH value, and increased anaerobic glycolysis in BD (Dager *et al.* 2004; Hamakawa *et al.* 2004; Kato *et al.* 1994, 1995). Studies in post-mortem brain tissue have revealed that expression of many mRNAs coding for subunits of mitochondrial complexes I–V is decreased in BD subjects (Konradi *et al.* 2004; Sun *et al.* 2006). Recently it has been found that oxidative stress is increased in prefrontal cortex, cingulate cortex and hippocampus of BD and schizophrenia subjects (Andreazza *et al.* 2010; Che *et al.* 2010; Wang *et al.* 2009). Clinical studies also report that treatment with *N*-acetylcysteine, an orally bioavailable precursor of antioxidant glutathione, as an add-on to the mood stabilizer medication, results in significant pharmacological effects (Berk *et al.* 2008; Magalhaes *et al.* 2011). These findings suggest that BD may be associated with mitochondrial dysfunction-induced oxidative stress. Together with our current finding, this also raises the possibility that neuroprotection against oxidative stress is one of the mechanisms for mood-stabilizing action.

Given that we have used repeated amphetamine stimulation-induced hyperactivity as a model for mania, some caution should be considered in the interpretation of our results. BD is a heterogeneous and complex disorder with multiple symptoms, while amphetamine-induced hyperactive behaviour only mimics one aspect of it. In addition, the neurobiology of BD still remains largely unknown, and dysfunctional monoamine neurotransmitter systems may only contribute to a part of its pathological development. Therefore, the neuroprotective mechanism of lithium needs to be further explored in various other manic-like animal models in order to understand the role of neuroprotection against oxidative stress in the pharmacological treatment with lithium for BD.

In conclusion: repeated amphetamine stimulation significantly induced hyperactive mania-like behaviour, decreased activities of complexes I and III, and increased 4-HNE-protein adducts in rat frontal cortex, while chronic lithium treatment inhibited amphetamine-induced hyperactivity and prevented formation of 4-HNE-protein adducts. 4-HNE can also form protein adducts with VMAT2. Repeated amphetamine stimulation significantly increased 4-HNE-VMAT2 adducts, while chronic lithium treatment inhibited amphetamine-induced 4-HNE-VMAT2 adducts in rat frontal cortex. These findings suggest that chronic lithium treatment may inhibit amphetamine-induced hyperactive mania-like behaviour by preventing 4-HNE-VMAT2 adduction. This finding also indicates that prevention of 4-HNE-VMAT2 adduction may contribute in part to the pharmacological action of lithium for the treatment of BD.

Statement of Interest

In addition to their primary employers, Dr Trevor Young is an occasional speaker for Eli Lilly and AstraZeneca. Dr William Honer reports receiving consulting fees or sitting on paid advisory boards for: the Alberta Heritage Medical Research Foundation, the Canadian Agency for Drugs and Technologies in Health, Janssen, Novartis In-silico (unpaid) and AstraZeneca; receiving lecture fees from: the Canadian Academy of Psychiatry and the Law, Partners in Psychiatry, Hotel Dieu Hospital (Kingston), Rush University, University of Calgary, the Capital Mental Health Association (Victoria), Université de Montréal, Janssen and AstraZeneca, and educational grant support from Janssen, Eli Lilly and AstraZeneca.

References

Andreazza AC, Kauer-Sant'Anna M, Frey BN, Stertz L, et al. (2008). Effects of mood stabilizers on DNA damage in an animal model of mania. *Journal of Psychiatry and Neuroscience* **33**, 516–524.

Andreazza AC, Shao L, Wang JF, Young LT (2010). Decreased mitochondrial complex i activity and increased

oxidative damage to mitochondrial proteinsin the prefrontal cortex of patients with bipolar disorder. *Archives of General Psychiatry* **67**, 360–368.

Awasthi YC, Yang Y, Tiwari NK, Patrick B, et al. (2004). Regulation of 4-hydroxynonenal-mediated signaling by glutathione S-transferases. *Free Radical Biology and Medicine* **37**, 607–619.

Basselin M, Chang L, Bell JM, Rapoport SI (2005). Chronic lithium chloride administration to unanesthetized rats attenuates brain dopamine D(2)-like receptor-initiated signaling via arachidonic acid. *Neuropsychopharmacology* **30**, 1064–1075.

Berk M, Copolov DL, Dean O, Lu K, et al. (2008). *N*-acetyl cysteine for depressive symptoms in bipolar disorder – a double-blind randomized placebo-controlled trial. *Biological Psychiatry* **64**, 468–475.

Bhattacharjee AK, Chang L, White L, Bazinet RP, et al. (2006). D-Amphetamine stimulates D(2) dopamine receptor-mediated brain signaling involving arachidonic acid in unanesthetized rats. *Journal of Cerebral Blood Flow and Metabolism* **26**, 1378–1388.

Bolanos JP, Heales SJ, Land JM, Clark JB (1995). Effect of peroxynitrite on the mitochondrial respiratory chain : differential susceptibility of neurones and astrocytes in primary culture. *Journal of Neurochemistry* **64**, 1965–1972.

Carlborg A, Jokinen J, Nordström AL, Jönsson EG, et al. (2011). Early death and CSF monoamine metabolites in schizophrenia spectrum psychosis. *Nordic Journal of Psychiatry* **65**, 101–105.

Che Y, Wang JF, Shao L, Young LT (2010). Oxidative damage to RNA but not DNA occurs in hippocampus of subjects with major mental illness. *Journal of Psychiatry and Neuroscience* **35**, 296–302.

Correa C, Amboni G, Assis LC, Martins MR, et al. (2007). Effects of lithium and valproate on hippocampus citrate synthase activity in an animal model of mania. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* **31**, 887–891.

Cousins DA, Butts K, Young AH (2009). The role of dopamine in bipolar disorder. *Bipolar Disorders* **11**, 787–806.

Cox C, Harrison-Read PE, Steinberg H, Tomkiewicz M (1971). Lithium attenuates drug-induced hyperactivity in rats. *Nature* **232**, 336–338.

Cunha-Oliveira T, Rego AC, Cardoso SM, Borges F, et al. (2006). Mitochondrial dysfunction and caspase activation in rat cortical neurons treated with cocaine or amphetamine. *Brain Research* **1089**, 44–54.

Dager SR, Friedman SD, Parow A, Demopulos C, et al. (2004). Brain metabolic alterations in medication-free patients with bipolar disorder. *Archives of General Psychiatry* **61**, 450–458.

Dringen R, Hirrlinger J (2003). Glutathione pathways in the brain. *Biological Chemistry* **384**, 505–516.

Eyerman DJ, Yamamoto BK (2007). A rapid oxidation and persistent decrease in the vesicular monoamine transporter 2 after methamphetamine. *Journal of Neurochemistry* **103**, 1219–1227.

Frey BN, Valvassori SS, Reus GZ, Martins MR, et al. (2006). Effects of lithium and valproate on amphetamine-induced oxidative stress generation in an animal model of mania. *Journal of Psychiatry and Neuroscience* **31**, 326–332.

Gerner RH, Post RM, Bunney Jr. WE (1976). A dopaminergic mechanism in mania. *American Journal of Psychiatry* **133**, 1177–1180.

Gould TD, Einat H (2007). Animal models of bipolar disorder and mood stabilizer efficacy : a critical need for improvement. *Neuroscience and Biobehavioral Reviews* **31**, 825–831.

Gould TD, Einat H, O'Donnell KC, Picchini AM, et al. (2007). Beta-catenin overexpression in the mouse brain phenocopies lithium-sensitive behaviors. *Neuropsychopharmacology* **32**, 2173–2183.

Grimsrud PA, Xie H, Griffin TJ, Bernlohr DA (2008). Oxidative stress and covalent modification of protein with bioactive aldehydes. *Journal of Biological Chemistry* **283**, 21837–21841.

Guillot TS, Miller GW (2009). Protective actions of the vesicular monoamine transporter 2 (VMAT2) in monoaminergic neurons. *Molecular Neurobiology* **9**, 149–170.

Hamakawa H, Murashita J, Yamada N, Inubushi T, et al. (2004). Reduced intracellular pH in the basal ganglia and whole brain measured by ^{31}P-MRS in bipolar disorder. *Psychiatry and Clinical Neurosciences* **58**, 82–88.

Hirschfeld RM (2000). History and evolution of the monoamine hypothesis of depression. *Journal of Clinical Psychiatry* **61**, 4–6.

Jacobs D, Silverstone T (1986). Dextroamphetamine-induced arousal in human subjects as a model for mania. *Psychological Medicine* **16**, 323–329.

Kato T, Shioiri T, Murashita J, Hamakawa H, et al. (1995). Lateralized abnormality of high energy phosphate metabolism in the frontal lobes of patients with bipolar disorder detected by phase-encoded 31P-MRS. *Psychological Medicine* **25**, 557–566.

Kato T, Takahashi S, Shioiri T, Murashita J, et al. (1994). Reduction of brain phosphocreatine in bipolar II disorder detected by phosphorus-31 magnetic resonance spectroscopy. *Journal of Affective Disorders* **31**, 125–133.

King TD, Jope RS (2005). Inhibition of glycogen synthase kinase-3 protects cells from intrinsic but not extrinsic oxidative stress. *Neuroreport* **16**, 597–601.

Klein PS, Melton DA (1996). A molecular mechanism for the effect of lithium on development. *Proceedings of the National Academy of Sciences USA* **93**, 8455–8459.

Klongpanichapak S, Phansuwan-Pujito P, Ebadi M, Govitrapong P (2008). Melatonin inhibits amphetamine-induced increase in alpha-synuclein and decrease in phosphorylated tyrosine hydroxylase in SK-N-SH cells. *Neuroscience Letters* **436**, 309–313.

Konradi C, Eaton M, MacDonald ML, Walsh J, et al. (2004). Molecular evidence for mitochondrial dysfunction in bipolar disorder. *Archives of General Psychiatry* **61**, 300–308.

Lai JS, Zhao C, Warsh JJ, Li PP (2006). Cytoprotection by lithium and valproate varies between cell types and

cellular stresses. *European Journal of Pharmacology* **539**, 18–26.

Machado-Vieira R, Kapczinski F, Soares JC (2004). Perspectives for the development of animal models of bipolar disorder. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* **28**, 209–224.

Magalhaes PV, Dean OM, Bush AI, Copolov DL, *et al.* (2011). *N*-acetyl cysteine add-on treatment for bipolar II disorder: a subgroup analysis of a randomized placebo-controlled trial. *Journal of Affective Disorders* **129**, 317–320.

Maher P, Schubert D (2000). Signaling by reactive oxygen species in the nervous system. *Cellular and Molecular Life Sciences* **57**, 1287–1305.

McNamara RK, Sullivan J, Richtand NM (2008). Omega-3 fatty acid deficiency augments amphetamine-induced behavioral sensitization in adult mice: prevention by chronic lithium treatment. *Journal of Psychiatric Research* **42**, 458–468.

Miyazaki I, Asanuma M (2008). Dopaminergic neuron-specific oxidative stress caused by dopamine itself. *Acta Medica Okayama* **62**, 141–150.

Nohl H, Gille L, Staniek K (2005). Intracellular generation of reactive oxygen species by mitochondria. *Biochemical Pharmacology* **69**, 719–723.

Oshiro S, Kawahara M, Kuroda Y, Zhang C, *et al.* (2000). Glial cells contribute more to iron and aluminum accumulation but are more resistant to oxidative stress than neuronal cells. *Biochimica et Biophysica Acta* **1502**, 405–414.

Paxinos G, Watson C (1998). *The Rat Brain in Stereotaxic Coordinates*, 4th edn. Academic Press: San Diego.

Petersen DR, Doorn JA (2004). Reactions of 4-hydroxynonenal with proteins and cellular targets. *Free Radical Biology and Medicine* **37**, 937–945.

Robertson SD, Matthies HJ, Galli A (2009). A closer look at amphetamine-induced reverse transport and trafficking of the dopamine and norepinephrine transporters. *Molecular Neurobiology* **39**, 73–80.

Rojo AI, Sagarra MR, Cuadrado A (2008). GSK-3beta down-regulates the transcription factor Nrf2 after oxidant damage: relevance to exposure of neuronal cells to oxidative stress. *Journal of Neurochemistry* **105**, 192–202.

Rothman RB, Baumann MH (2006). Balance between dopamine and serotonin release modulates behavioral effects of amphetamine-type drugs. *Annals of the New York Academy of Sciences* **1074**, 245–260.

Rowe MK, Chuang DM (2004). Lithium neuroprotection: molecular mechanisms and clinical implications. *Expert Reviews in Molecular Medicine* **18**, 1–18.

Salazar M, Rojo AI, Velasco D, de Sagarra RM, *et al.* (2006). Glycogen synthase kinase-3beta inhibits the xenobiotic and antioxidant cell response by direct phosphorylation and

nuclear exclusion of the transcription factor Nrf2. *Journal of Biological Chemistry* **281**, 14841–14851.

Schafer M, Goodenough S, Moosmann B, Behl C (2004). Inhibition of glycogen synthase kinase 3 beta is involved in the resistance to oxidative stress in neuronal HT22 cells. *Brain Research* **1005**, 84–89.

Schloesser RJ, Song J, Klein PS, Manji HK (2008). Cellular plasticity cascades in the pathophysiology and treatment of bipolar disorder. *Neuropsychopharmacology Reviews* **33**, 110–133.

Shao L, Cui J, Young LT, Wang JF (2008). The effect of mood stabilizer lithium on expression and activity of glutathione S-transferase isoenzymes. *Neuroscience* **151**, 518–524.

Shao L, Young LT, Wang JF (2005). Chronic treatment with mood stabilizers lithium and valproate prevents excitotoxicity by inhibiting oxidative stress in rat cerebral cortical cells. *Biological Psychiatry* **58**, 879–884.

Stadtman ER (2006). Protein oxidation and aging. *Free Radical Research* **40**, 1250–1258.

Sun X, Wang JF, Tseng M, Young LT (2006). Down regulation in components of mitochondrial electron transport chain in post-mortem frontal cortex from subjects with bipolar disorder. *Journal of Psychiatry and Neuroscience* **31**, 189–196.

Tjalkens RB, Luckey SW, Kroll DJ, Petersen DR (1998). Alpha,beta-unsaturated aldehydes increase glutathione s-transferase mRNA and protein: correlation with activation of the antioxidant response element. *Archives of Biochemistry and Biophysics* **359**, 42–50.

Valvassori SS, Rezin GT, Ferreira CL, Moretti M, *et al.* (2010). Effects of mood stabilizers on mitochondrial respiratory chain activity in brain of rats treated with d-amphetamine. *Journal of Psychiatric Research* **44**, 903–909.

Wang JF, Azzam JE, Young LT (2003). Valproate inhibits oxidative damage to lipid and protein in primary cultured rat cerebrocortical cells. *Neuroscience* **116**, 485–489.

Wang JF, Shao L, Sun X, Young LT (2009). Increased oxidative stress in anterior cingulate cortex of subjects with bipolar disorder and schizophrenia. *Bipolar Disorders* **11**, 523–529.

Watabe M, Nakaki T (2008). Mitochondrial complex I inhibitor rotenone inhibits and redistributes vesicular monoamine transporter 2 via nitration in human dopaminergic SH-SY5Y cells. *Molecular Pharmacology* **74**, 933–940.

Wiste AK, Arango V, Ellis SP, Mann JJ, *et al.* (2008). Norepinephrine and serotonin imbalance in the locus coeruleus in bipolar disorder. *Bipolar Disorders* **10**, 349–359.

Yamamoto BK, Raudensky J (2008). The role of oxidative stress, metabolic compromise, and inflammation in neuronal injury produced by amphetamine-related drugs of abuse. *Journal of Neuroimmune Pharmacology* **3**, 203–217.