Effects of lithium and valproate on amphetamine-induced oxidative stress generation in an animal model of mania

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Objective: Previous studies have suggested that oxidative stress may play a role in the pathophysiology of bipolar disorder (BD). Moreover, recent studies indicate that lithium and valproate exert neuroprotective effects against oxidative stress. We studied the effects of the mood stabilizers lithium and valproate on amphetamine-induced oxidative stress in an animal model of mania. **Methods:** In the first model (reversal treatment), adult male Wistar rats received d-amphetamine or saline for 14 days, and between the 8th and 14th days, they were treated with lithium, valproate or saline. In the second model (prevention treatment), rats were pretreated with lithium, valproate or saline, and between the 8th and 14th days, they received d-amphetamine or saline. We assessed locomotor activity with the open-field task. We measured thiobarbituric acid reactive substances (TBARS) and protein carbonyl formation, as parameters of oxidative stress, and superoxide dismutase (SOD) and catalase (CAT), the major antioxidant enzymes, in the prefrontal cortex and hippocampus. **Results:** Lithium and valproate reversed (reversal treatment model) and prevented (prevention treatment model) amphetamine-induced hyperactivity and reversed and prevented amphetamine-induced TBARS formation in both experiments. However, the co-administration of lithium or valproate with amphetamine increased lipid peroxidation, depending on the brain region and treatment regimen. No changes in protein carbonyl formation were observed. SOD activity varied with different treatment regimens, and CAT activity increased when the index of lipid peroxidation was more robust. **Conclusion:** Our findings suggest that lithium and valproate exert protective effects against amphetamine-induced oxidative stress in vivo, further supporting the hypothesis that oxidative stress may be associated with the pathophysiology of BD.

Objectif : Des études antérieures ont indiqué que le stress oxydant peut jouer un rôle dans la pathophysiologie du trouble bipolaire. Des études récentes indiquent de plus que le lithium et le valproate ont un effet neuroprotecteur contre le stress oxydant. Nous avons étudié les effets du lithium et du valproate, agents thymorégulateurs, sur le stress oxydant provoqué par des amphétamines dans un modèle animal de la manie. **Méthodes :** Dans le premier modèle (traitement d'inversion), des rats Wistar mâles adultes ont reçu de la d-amphétamine ou une solution physiologique pendant 14 jours et, entre le 8e et le 14e jour, on leur a administré du lithium, du valproate ou une solution physiologique. Dans le deuxième modèle (traitement de prévention), on a traité au préalable les rats en leur administrant du lithium, du valproate ou une solution physiologique, et ils ont reçu, entre le 8e et le 14e jour, de la d-amphétamine ou une solution physi-

ologique. Nous avons évalué l'activité locomotrice au moyen de la tâche en champ ouvert. Nous avons mesuré les substances réactives à l'acide thiobarbiturique (SRATB) et la formation du complexe protéine carbonyle comme paramètres du stress oxydant, ainsi que la superoxyde-dismutase (SOD) et la catalase (CAT), les principales enzymes anti-oxydantes, dans le cortex préfrontal et l'hippocampe. **Résultats :** Le lithium et le valproate ont inversé (modèle du traitement d'inversion) et évité (modèle du traitement de prévention) l'hyperactivité causée par l'amphétamine et inversé et évité la formation de SRATB provoquée par l'amphétamine au cours des deux expériences. L'administration simultanée de lithium et de valproate avec l'amphétamine a toutefois accru la peroxydation des lipides, selon la région du cerveau et le traitement. On n'a observé aucun changement de la formation du complexe protéine carbonyle. L'activité de la SOD a varié en fonction des différents traitements et celle de la CAT a augmenté lorsque l'indice de peroxydation des lipides était plus élevé. **Conclusion :** Nos constatations indiquent que le lithium et le valproate ont un effet protecteur contre le stress oxydant provoqué par les amphétamines in vivo, ce qui appuie encore l'hypothèse selon laquelle il peut y avoir un lien entre le stress oxydant et la pathophysiologie du trouble bipolaire.

Introduction

Recent studies have consistently reported increased products of lipid peroxidation and alterations of the major antioxidant enzymes in people with bipolar disorder (BD).[1–3] It has been widely demonstrated that the generation of reactive oxygen species (ROS) plays a critical role in the pathophysiology of several neuropsychiatric disorders.[4,5] The brain is particularly vulnerable to ROS production because it metabolizes 20% of total body oxygen and has a limited amount of antioxidant capacity.[6] In situations where the generation of free radicals exceeds the capacity of antioxidant defence, oxidative stress may lead to membrane degradation, cellular dysfunction and apoptosis. This might be relevant for the pathogenesis of BD, because in-vivo magnetic resonance spectroscopy studies have demonstrated changes in brain compounds related to oxidative phosphorylation, energy production and phospholipid metabolism.[7] In addition, it has been hypothesized that BD is associated with mitochodrial dysfunction,[8,9] and abnormalities in respiratory complex activity and energy production may lead to cellular degeneration.[5]

Additional associations between oxidative and antioxidative systems in BD have been demonstrated in pharmacological studies. In primary cultured neuronal cells, lithium and valproate (first-line mood stabilizers) prevent glutamate-induced oxidative stress[10] and increase mRNA and protein levels of glutathione S-transferase M1 isoenzyme;[11] and valproate inhibits $FeCl_3$-induced lipid peroxidation and protein oxidation.[12] More recently, King and Jope[13] showed that lithium protects against caspase activation that is induced by intrinsic and extrinsic sources of oxidative stress in human neuroblastoma SH-SY5Y cells. Together, these findings strongly suggest that the modulation of ROS generation may be relevant in the mood-stabilizing effects of lithium and valproate; however, the effects of lithium and valproate on oxidative stress have not been studied in vivo. We recently found that repeated amphetamine exposure increases thiobarbituric acid-reactive species and protein carbonyl formation in the rat hippocampus and cerebral cortex, 2 brain regions related to mood regulation.[14] In addition, recent genetic and postmortem studies suggest that BD may be associated with altered dopaminergic transmission.[15–17] Because of these findings, we designed the present study to investigate the effects of lithium and valproate on lipid and protein oxidation levels (markers of oxidative stress) and on superoxide dismutase (SOD) and catalase (CAT) activities (the major antioxidant enzymes) in a dopaminergic model of mania.

Methods

We conducted the study, using adult male Wistar rats obtained from our breeding colony. The animals were housed 5 to a cage, on a 12-hour light/dark cycle (lights on at 7:00 am), with free access to food and water. All experimental procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behaviour (SBNeC) recommendations for animal care. This study was approved by the local ethics committee (Comitê de Ética em Pesquisa da Universidade do Extremo Sul Catarinense).

Reversal treatment

In this model, we reproduced the treatment of an acute manic episode. Rats received either a daily injection of d-amphetamine, 2 mg/kg (Sigma, St. Louis, Mo.), or saline for 14 days. Between the 8th and the 14th days, the animals were divided into 3 experimental groups (12–15 animals per group): 1 group received lithium, 47.5 mg/kg intraperitoneal (IP), twice a day; the second group received valproate, 200 mg/kg IP, twice a day; and the third group received saline IP twice a day. Locomotor activity was assessed 2 hours after the last injection, and rats were sacrificed by decapitation immediately following the open-field task. The prefrontal cortex and hippocampus were dissected, rapidly frozen and stored at –80°C until assayed.

Prevention treatment

In this model, we reproduced the maintenance treatment of BD. Rats received either lithium, 47.5 mg/kg; valproate, 200 mg/kg; or saline IP twice a day for 14 days. The animals were then divided into 2 groups (12–15 animals per group). Between the 8th and the 14th days, each group received one daily IP injection of d-amphetamine, 2 mg/kg, or saline. Locomotor activity was assessed 2 hours after the last injection,

and rats were sacrificed by decapitation immediately following the open-field task. The prefrontal cortex and hippocampus were dissected, rapidly frozen and stored at –80°C until assayed. All lithium-treated animals presented lithium levels between 0.6–1.2 mEq/L in plasma, as recommended in the treatment of patients with BD.

Locomotor activity

We used the open-field task to assess locomotor activity. The task was performed in a 40 ∞ 60 cm open field surrounded by 50 cm-high walls made of brown plywood with a frontal glass wall. The floor of the open field was divided into 12 equal rectangles by black lines. The animals were gently placed on the left rear rectangle and were allowed to explore the arena. Crossings of the black lines and rearings were counted for 5 minutes.

Oxidative stress parameters

To determine oxidative damage, we measured the formation of thiobarbituric acid reactive species (TBARS) during an acid-heating reaction, as previously described.[18] The samples were mixed with 1 mL of trichloroacetic acid (TCA) 10% and 1 mL of thiobarbituric acid (TBA) 0.67% and were then heated in a boiling water bath for 15 minutes. TBARS were determined by the absorbance at 535 nm. Oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with dinitrophenylhidrazine (DNPH), as previously described.[19] Proteins were precipitated by the addition of 20% trichloroacetic acid and were re-dissolved in DNPH; the absorbance was read at 370 nm.

To determine CAT activity, the brain tissue was sonicated in 50 mmoL/L phosphate buffer (pH 7.0), and the resulting suspension was centrifuged at 3000 g for 10 minutes. The supernatant was used for enzyme assay. CAT activity was measured by the rate of decrease in hydrogen peroxide absorbance at 240 nm.[20] SOD activity was assayed by measuring the inhibition of adrenaline auto-oxidation, as previously described[21] All biochemical measures were normalized to the protein content, with bovine albumin as standard.[22]

All data are presented as mean and standard error of the mean. Differences among the experimental groups evaluating exploratory behaviour were determined by 1-way analysis of variance (ANOVA), followed by the Tukey post hoc test. Biochemical data were analyzed by 1-way ANOVA, and multiple comparisons were performed with the Newman-Keuls test. In all comparisons, statistical significance was set at $p < 0.05$.

Results

Behaviour

In the reversal and prevention models, amphetamine increased locomotor and rearing behaviours, and the administration of lithium and valproate reversed and prevented amphetamine-induced hyperactivity (Fig. 1, Fig. 2).

Lithium and valproate did not affect behavioural measures in animals treated with saline, suggesting that the effects of mood stabilizers on animals treated with amphetamine
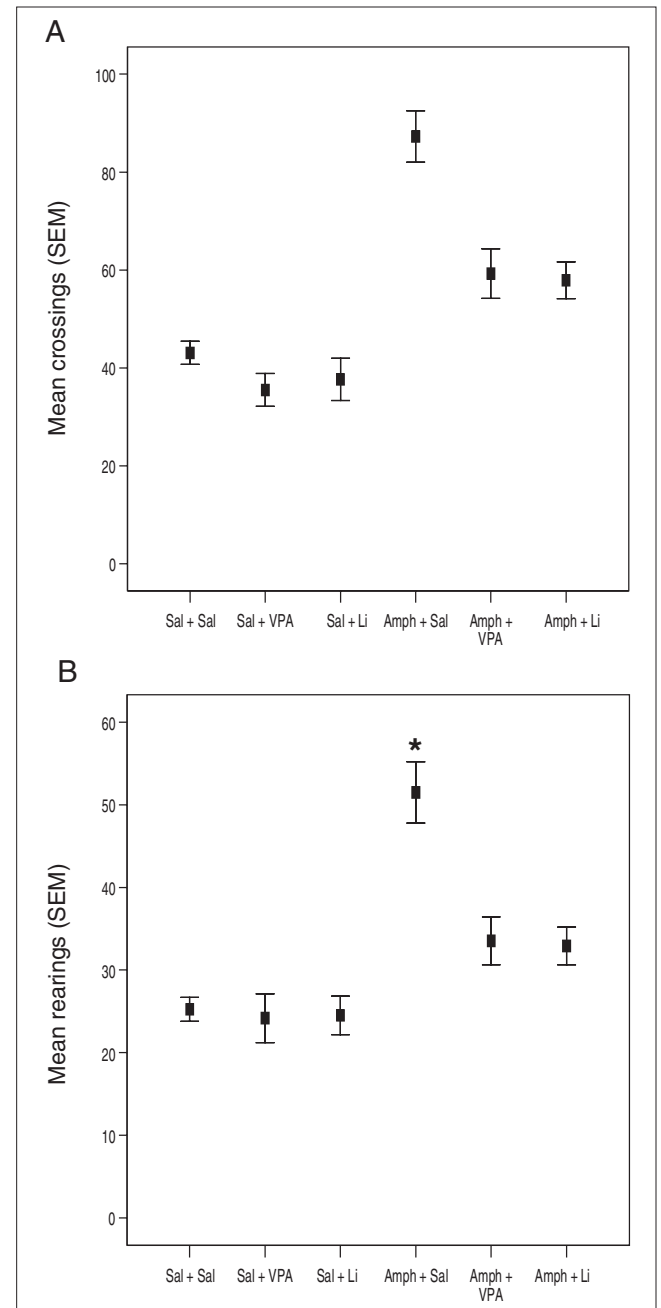

Fig. 1: Numbers of crossings (A) and rearings (B) in the reversal model (n = 12 for each group). Bars represent means; error bars represent standard error of the means (SEM). Crossings = 1-way analysis of variance (ANOVA); $F_{5,66}$ = 22.68; $p < 0.001$; *different from all groups (Tukey's post hoc; $p < 0.001$). Rearings = 1-way ANOVA; $F_{5,66}$ = 15.28; $p < 0.001$; *different from all groups (Tukey's post hoc; $p < 0.001$). Rats were pretreated with amphetamine (Amph) for 7 days and then treated with Amph plus mood stabilizers between the 8th and the 14th days. Li = lithium, Sal = saline, VPA = valproate.

were not related to sedation. These results replicate our recent findings[23] and confirm the reproducibility of this behavioural model.
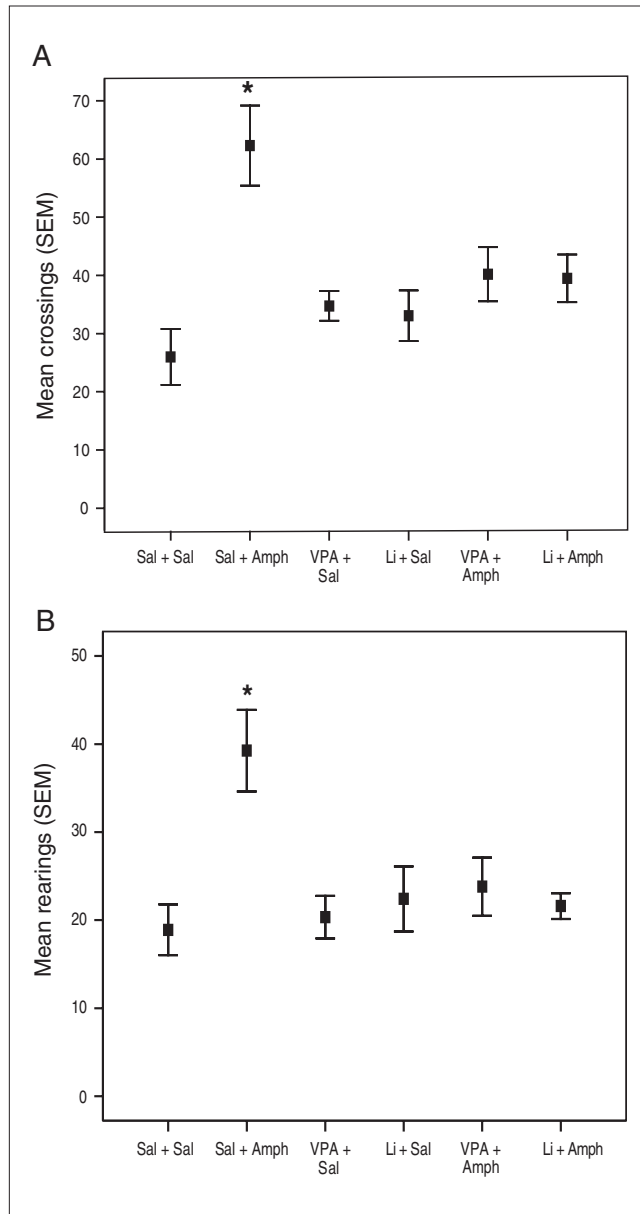

Fig. 2: Numbers of crossings (A) and rearings (B) in the prevention model (*n* = 12 for each group). Bars represent means; error bars represent standard error of the means (SEM). Crossings = 1-way analysis of variance (ANOVA); $F_{5,66}$ = 7.04; *p* < 0.001; *different from all groups (Tukey's post hoc; *p* < 0.001 from Sal + Sal and Li + Sal; *p* < 0.001 from VPA + Sal; *p* = 0.018 from Li + Amph; *p* = 0.023 from VPA + Amph). Rearings = 1-way ANOVA; $F_{5,66}$ = 4.80; *p* < 0.001; *different from all groups (Tukey's post hoc; *p* < 0.001 from Sal + Sal and VPA + Sal; *p* = 0.006 from Li + Sal and VPA + Amph; *p* = 0.022 from Li + Amph). Rats were pretreated with mood stabilizers for 7 days and then treated with mood stabilizers plus amphetamine (Amph) between the 8th and the 14th days. Sal = saline, VPA = valproate.

Oxidative stress parameters

Reversal treatment

Amphetamine increased TBARS levels in the prefrontal cortex, and this effect was reversed by both mood stabilizers (Fig. 3a). The administration of lithium and valproate
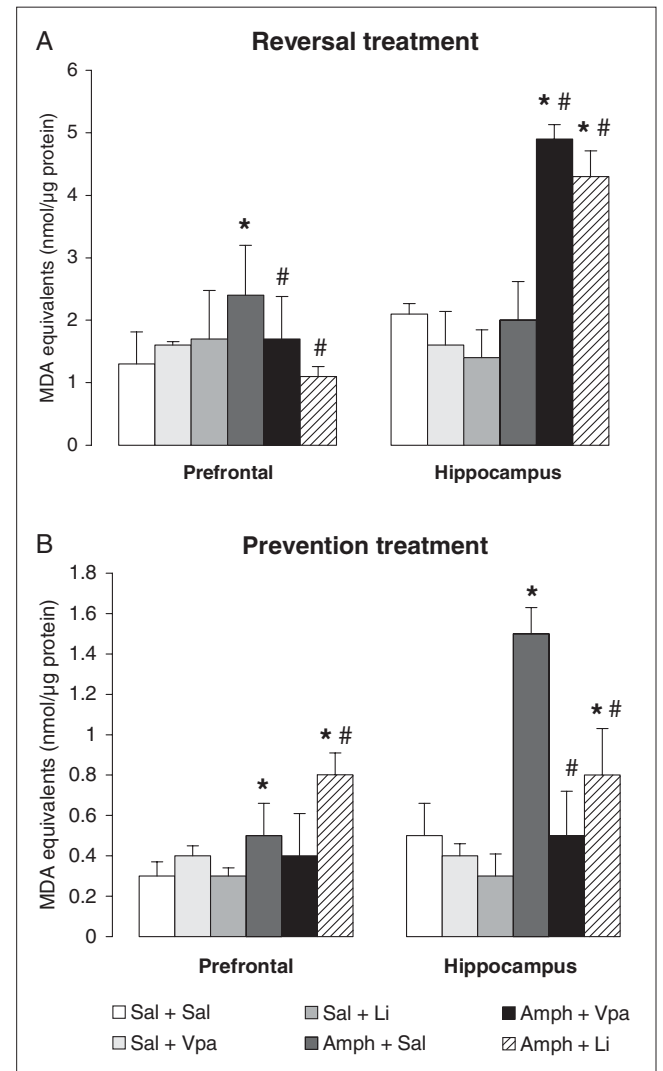

Fig. 3: TBARS levels in the prefrontal cortex and hippocampus after reversal (A) and prevention (B) treatments (*n* = 5 for each group). Prefrontal cortex in the reversal treatment group = 1-way analysis of variance (ANOVA); $F_{5,24}$ = 3.14; *p* = 0.02. Hippocampus in the reversal treatment group = 1-way ANOVA; $F_{5,24}$ = 22.44; *p* < 0.001. Prefrontal cortex in the prevention treatment group = 1-way ANOVA; $F_{5,24}$ = 7.78; *p* = 0.001. Hippocampus in the prevention treatment group = 1-way ANOVA; $F_{5,24}$ = 15.66; *p* < 0.001. Bars represent means; error bars represent standard error of the means (SEM); *different from the Sal + Sal group (Newman-Keuls post hoc; *p* < 0.05); # different from the Amph + Sal group (Newman-Keuls post hoc; *p* < 0.05). Amph = amphetamine, Li = lithium, MDA = malondialdehyde, Sal = saline, TBARS = thiobarbituric acid reactive species.

increased TBARS formation in the hippocampus of rats pretreated with amphetamine. No changes were observed in protein carbonyl formation (data not shown).

Prevention treatment

Amphetamine increased TBARS levels in the prefrontal cortex and hippocampus, and these effects were prevented by valproate pretreatment (Fig. 3b). Lithium pretreatment partially prevented amphetamine-induced lipid peroxidation in the rat hippocampus but augmented amphetamine-induced lipid peroxidation in the prefrontal cortex. As in the reversal treatment, no changes were observed in protein carbonyl formation (data not shown). Taken together, our findings suggest that the neuroprotective effects of lithium and valproate on amphetamine-induced oxidative stress vary with brain region and treatment regimen. When interpreting the results, it
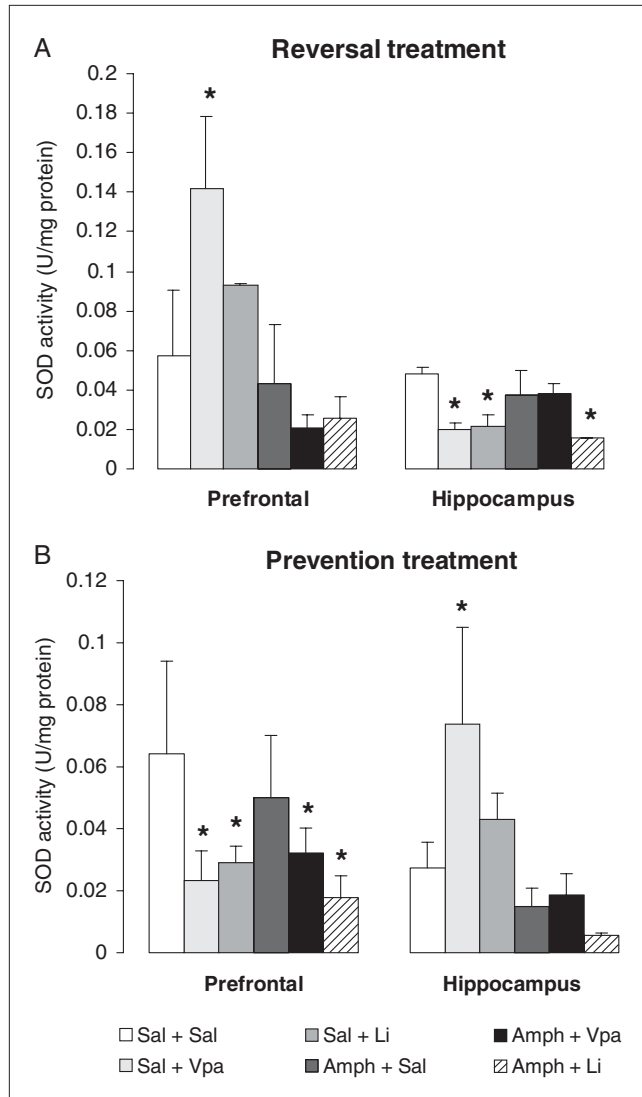

Fig. 4: Superoxide dismutase (SOD) levels in the prefrontal cortex and hippocampus after reversal (A) and prevention (B) treatments ($n = 5$ for each group). Prefrontal cortex in the reversal treatment group = 1-way analysis of variance (ANOVA); $F_{5,24} = 8.58$; $p = 0.02$. Hippocampus in the reversal treatment group = 1-way ANOVA; $F_{5,24} = 8.94$; $p = 0.01$. Prefrontal cortex in the prevention treatment group = 1-way ANOVA; $F_{5,24} = 4.48$; $p = 0.011$. Hippocampus in the prevention treatment group = 1-way ANOVA; $F_{5,24} = 8.36$; $p = 0.001$. Bars represent means; error bars represent standard error of the means (SEM); *different from the Sal + Sal group (Newman-Keuls post hoc; $p < 0.05$); Amph = amphetamine, Li = lithium, Sal = saline, Val = valproate.
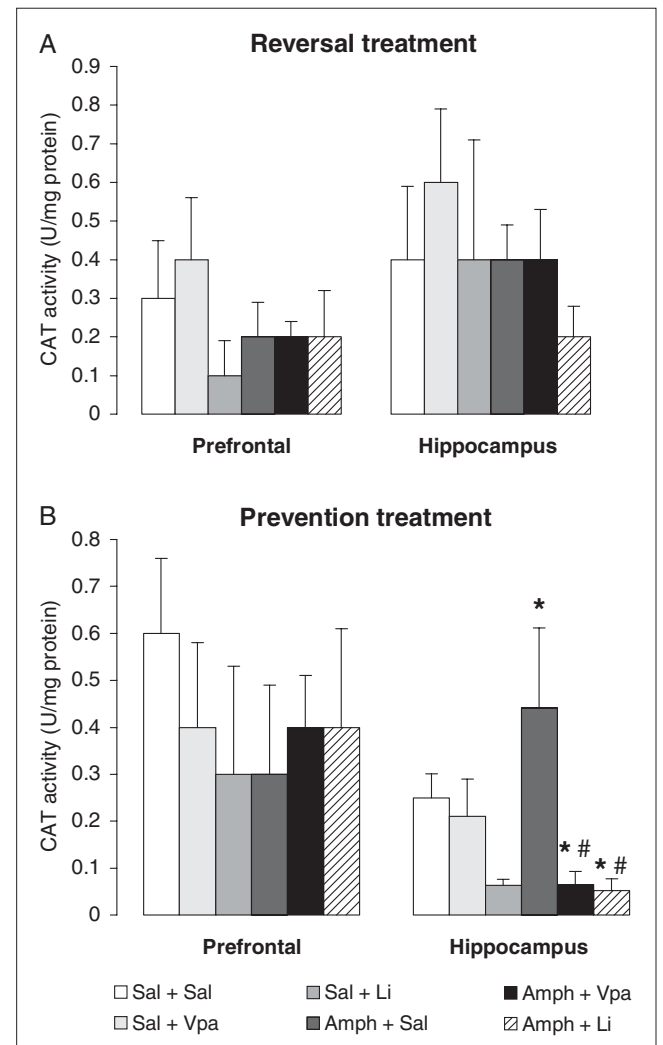

Fig. 5: Catalase (CAT) levels in the prefrontal cortex and hippocampus after reversal (A) and prevention (B) treatments ($n = 5$ for each group). Prefrontal cortex in the reversal treatment group = 1-way analysis of variance (ANOVA); $F_{5,24} = 2.57$; $p = 0.069$. Hippocampus in the reversal treatment group = 1-way ANOVA; $F_{5,24} = 2.35$; $p = 0.088$. Prefrontal cortex in the prevention treatment group = 1-way ANOVA; $F_{5,24} = 0.63$; $p = 0.679$. Hippocampus in the prevention treatment group = 1-way ANOVA; $F_{5,24} = 8.91$; $p = 0.001$. Bars represent means; error bars represent standard error of the means (SEM); *different from the Sal + Sal group (Newman-Keuls post hoc; $p < 0.05$); #different from the Amph + Sal group (Newman-Keuls post hoc; $p < 0.05$). Amph = amphetamine, Li = lithium, Sal = saline, Vpa = valproate.

is worth noting that the administration of lithium and valproate alone had no effect on oxidative stress.

Antioxidant enzyme activity

Reversal treatment

In this model, valproate increased SOD levels in the prefrontal cortex, whereas lithium, valproate and lithium plus amphetamine decreased SOD in the rat hippocampus (Fig. 4a). No changes were observed on CAT activity (Fig. 5a).

Prevention treatment

Lithium, valproate, lithium plus amphetamine and valproate plus amphetamine decreased SOD levels in the prefrontal cortex, whereas valproate increased SOD in the hippocampus (Fig. 4b). Amphetamine increased CAT levels, whereas lithium plus amphetamine, and valproate plus amphetamine decreased CAT levels in the rat hippocampus (Fig. 5b).

Discussion

In this study, we demonstrated that lithium and valproate reversed amphetamine-induced lipid peroxidation in the prefrontal cortex and prevented amphetamine-induced lipid peroxidation in the hippocampus. Using the present model, we were able to reproduce previous findings of the neuroprotective effects of mood stabilizers in response to oxidative stress.[10–13] Our results are consistent with existing evidence that mood stabilizers share significant neuroprotective properties.[24] However, we also found that the co-administration of lithium or valproate with amphetamine can increase TBARS formation, suggesting that their effects on oxidative stress vary depending on the brain region and treatment regimen. Because the administration of lithium or valproate alone did not induce oxidative damage, it is conceivable that they might augment amphetamine-induced oxidative stress in some situations. Amphetamine can enhance ROS formation through several pathways, for example, through autooxidation of dopamine with the formation of highly reactive quinones,[25] direct inhibition of mitochondrial electron transport chain complexes[26] and increased glutamate release.[27] Although the mechanisms by which mood stabilizers decrease ROS generation are poorly understood, it has been suggested that they might involve the induction of the molecular chaperone GRP78,[28] buffering $[Ca^{2+}]i$ levels,[10] and by stabilizing mitochondrial function.[10] Interestingly, Carli and colleagues[29] demonstrated that chronic lithium administration modifies the affinity of dopamine transporters, thereby decreasing overactive dopamine transmission. In addition, divalproex sodium has been shown to decrease $[^{18}F]$-dopa uptake constants in the striatum of patients with mania, suggesting that divalproex sodium treatment decreases aromatic amino acid decarboxylase activity, which should decrease the rate of dopamine synthesis.[30] Thus, lithium and valproate could decrease amphetamine-induced ROS generation, at least in

part, by decreasing the amount of dopamine available for release from presynaptic neurons.

We did not find any change in protein carbonyl formation in the present model. This is contrary to our previous finding that repeated d-amphetamine administration increases protein carbonyl levels in the rat hippocampus.[14] Differences in experimental design may account for this discrepancy. The effects of amphetamine, lithium and valproate on antioxidant enzymes were highly variable in our experiments. In fact, there is a paucity of data assessing the effects of these drugs on antioxidant systems. D'Almeida and others[31] administered methamphetamine, 2.5 mg/kg daily, for 5 months and found no differences in SOD or CAT activity in the rat brain. More recently, Carvalho and colleagues[32] administered d-amphetamine, 20 mg/kg daily, for 14 days and reported increased CAT activity in medial prefrontal cortex but no effect on SOD or CAT activity in the rat hippocampus. During physiological states, SOD metabolizes superoxide anion (O_2^-), producing hydrogen peroxide (H_2O_2), which can react with iron to generate highly reactant hydroxyl radicals via the Fenton reaction. CAT is the most important peroxidase in detoxifying excess hydrogen peroxide to prevent hydroxyl production. Thus an increase in SOD or CAT levels per se does not necessarily indicate increased oxidative stress, whereas an imbalance between SOD and CAT activities could lead to an excessive generation of free radicals.[33,34] In the context of the present model, we specifically found increased CAT activity when the degree of lipid peroxidation was more robust (Fig. 3b, Fig. 5b). It is likely that CAT activity increased in response to increased H_2O_2 production induced by amphetamine metabolism. We also found changes in SOD activity without evidence of lipid or protein oxidation when lithium and valproate were administered alone. This is in line with a previous finding that electroconvulsive shocks increased SOD and CAT activity in the rat hippocampus but did not induce oxidative stress.[35]

In conclusion, we have demonstrated that lithium and valproate can reverse and prevent amphetamine-induced oxidative stress in vivo. Our findings further support the notion that neuroprotection may be one of the mechanisms of action of mood stabilizers and that oxidative stress may play a role in the pathophysiology of BD.

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