Antimanic-like activity of candesartan in mice: Possible involvement of antioxidant, anti-inflammatory and neurotrophic mechanisms

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Abstract
Activation of the brain angiotensin II type 1 receptor (AT1R) triggers pro-oxidant and pro-inflammatory mechanisms which are involved in the neurobiology of bipolar disorder (BD). Candesartan (CDS) is an AT1 receptor antagonist with potential neuroprotective properties. Herein we investigated CDS effects against oxidative, neurotrophic inflammatory and cognitive effects of amphetamine (AMPH)-induced mania. In the reversal protocol adult mice were given AMPH 2 mg/kg i.p. or saline and between days 8 and 14 received CDS 0.1, 0.3 or 1 mg/kg orally, lithium (Li) 47.5 mg/kg i.p., or saline. In the prevention treatment, mice were pretreated with...
1. Introduction

Although advances have been made, understanding of the pathophysiology of BD remains elusive. Animal models have been important tools for investigating BD neurobiology and novel treatment approaches (Young et al., 2011). The pharmacological animal model of mania induced by d-amphetamine (AMPH) is associated with behavioral and biochemical manifestations including oxidative imbalance, mitochondrial damage and alterations in neuroplasticity (Frey et al., 2006b) which resemble BD pathophysiology (Berk et al., 2011).

Postmortem brain and clinical studies have demonstrated peripheral as well as central nervous system alterations in diverse parameters of oxidative stress and antioxidant enzymes (Andreazza et al., 2008), including decreased levels of glutathione (GSH) and increased thiobarbituric acid reactive substance (TBARS), a marker of lipid peroxidation. Deficits in GSH result in oxidative imbalance and subsequent oxidation of phospholipids, proteins, and DNA (Kulak et al., 2013).

Involvement of inflammatory mechanisms and neuroimmune alterations is seen in the pathophysiology of BD (Berk et al., 2011). Immune disturbances have been related to the severity and recurrence of mood episodes, illness progression, cognitive dysfunction and higher rates of comorbidities (Berk et al., 2011). Accordingly, increased peripheral levels of pro-inflammatory cytokines have been reported at differing stages of the illness, such as IL-6 and TNF-α, during depressive episodes as well as IL-2, IL-4, IL-6 and TNF-α in mania when compared with euthymic patients and healthy subjects (Kim et al., 2007). To date, as far as we know no studies have evaluated alterations in TNF-α levels in animals submitted to the AMPH-induced model of mania.

Neurotrophic signaling pathways are also compromised in BD and, in this regard, brain-derived neurotrophic factor (BDNF) and glycogen synthase kinase 3-beta (GSK-3beta) are two important targets related to this pathway (Picchini et al., 2004). Serum BDNF is reduced in BD during manic and depressive episodes when compared and euthymic patients and healthy controls, even in drug-naive patients (de Oliveira et al., 2009). This neurotrophin is also decreased in discrete brain areas of AMPH induced animal models of mania (Frey et al., 2006a; Macêdo et al., 2012). Regarding GSK-3beta, a growing body of evidence indicates a link between this kinase and the therapeutic effects of mood stabilizers (Gould and Manji, 2005). Lithium inhibits GSK-3beta both directly and indirectly, and these mechanisms may be related to the neuroprotective effects of this mood stabilizer (Picchini et al., 2004).

There are substantial unmet needs in the pharmacotherapy of BD. For example, the chronic use of the classical mood stabilizer lithium is related to insufficient response, unwanted side effects, i.e. renal and thyroid dysfunction, subtle emotional and cognitive impairment and weight gain (Licht, 2012). There are few available treatments for the management of cognitive dysfunction in BD (Dean et al., 2012). This reinforces the need for genuinely novel pharmacotherapies for BD.

The last decade had witnessed an increased interest in the study of the brain renin-angiotensin system (RAS) (McKinley et al., 2003). This system is related to pro-inflammatory and pro-oxidant effects in brain areas related to mood regulation, such as the hippocampus (Bild et al., 2013). Angiotensin II (AngII) receptor blockers (ARBs) are pleiotropic agents with anxiolytic (Saavedra et al., 2006) and neuroprotective activities (Yagi et al., 2013). Additionally these substances present antioxidant action (Bild et al., 2013) as well as up-regulate BDNF levels (Kishi et al., 2012).

Based on the reported psychopharmacological effects of ARBs, we recently hypothesized that these agents may provide a novel treatment opportunity for the prevention and/or treatment of BD (de Gois Queiroz et al., 2013). Noteworthy, there is an excess burden of cardiovascular risk factors in BD patients compared to the general population (Kilbourne et al., 2004). Furthermore, in many forms of experimental hypertension, the neuropeptide AngII as well as components of the intrinsic brain RAS presents with an increased expression and activity (Veerasingham and Raizada, 2003). There is limited data to suggest a higher rate of hypertension in BD than the general population (Kilbourne et al., 2004). In keeping with this view the RAS system may play a role in the shared biology linking BD to cardiovascular morbidity.

Thus, based on the preclinical and neuroprotective effects of ARBs (Lu et al., 2005), in the present study we aimed to investigate the effects of the AngII type 1 receptor antagonist candesartan (CDS) against behavioral (hyperlomotion and cognitive deficits) and neurochemical (oxidative imbalance, increase in TNF-α levels and decrement in...
BDNF and phospho-GSK-3beta) alterations induced by AMPH in mice, an acknowledged animal model of mania. The neurochemical evaluations were conducted in brain areas related to BD pathophysiology namely hippocampus (Frey et al., 2006b) and cerebellar vermis (Singh et al., 2011).

2. Experimental procedures

2.1. Animals

Two hundred seventy four treatment-naive adult male mice, weighing 20-25 g, were obtained from the animal house facility of Federal University of Ceará and were group-housed, 6-8 per cage, in standard polycarbonate cages (42 x 20.5 x 20 cm) with standard environmental conditions (22 ± 1 °C, humidity 60 ± 5% and 12 h light-dark cycle) with ad libitum access to food and water. The experimental procedures were performed from 8:00 a.m. to 2:00 p.m. in accordance to the Guide for the Care and Use of Laboratory Animals, from the US Department of Health and Human Services. The experimental protocol was approved by the Federal University of Ceará Animal Care and Use Committee (Protocol number 065/2013). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Drugs

D-amphetamine (AMPH) and lithium carbonate were obtained from Sigma (St. Louis, MO, USA) and were directly dissolved in saline solution (NaCl 0.9%, w/v). Candesartan (CDS) was purchased from AstraZeneca Laboratories (Cotia, SP, Brazil) and suspended in 0.5% carboxyl methyl cellulose (CMC). The drugs were made up freshly within 1-2 h of dosing and were administered in a volume of 0.1 ml/10 g body weight. The doses of CDS and lithium used here were based on previous reports (Lu et al., 2005; Macêdo et al., 2012).

2.3. Study design

Two treatment protocols (prevention and reversal) were used and are schematically shown in Fig. 1. Each group consisted of 6-10 randomly allocated animals. In the reversal model, we mimicked the treatment of acute mania in accordance to a previously proposed protocol (Frey et al., 2006b). For this, mice received one daily intraperitoneal injection (i.p.) of AMPH 2 mg/kg or saline (SAL), for 14 days. Between the 8th and 14th day, AMPH and saline-treated animals additionally received CDS [0.1, 0.3 or 1 mg/kg orally (p.o.)], lithium (47.5 mg/kg i.p.) or SAL daily. In the prevention model (Fig. 1), we simulated the maintenance phase of BD treatment (Frey et al., 2006b). Animals were treated daily with lithium (47.5 mg/kg i.p.), CDS [0.1, 0.3 or 1 mg/kg p.o.] or SAL (p.o.) for 14 days. On the 8th treatment day, animals were additionally treated with either AMPH (2 mg/kg; i.p.) or SAL once a day. The time interval between drugs administration in all situations was 30 min. Since the administration of lithium or CDS alone caused no behavioral or oxidative alterations, we decided to remove these groups from the enzyme immunoassay (ELISA) determinations. Again, due to the absence of a dose-response effect by CDS we decided to exclude the group administered CDS 0.1 mg/kg from these measurements. This decision was based on the rational use of laboratory animals (and rules from the ethical committee of our institution).

Behavioral determinations of locomotor activity were performed by means of the open field test and spatial working memory was evaluated through the Y-maze task, which were registered at the 14th day of treatment, 2 h after the last drug administration. A minimum of six animals was kept per group. Distinct animals were used for each behavioral determination, i.e., 6-10 animals per group were allocated to open field test while 7 animals per group were allocated to the Y maze task. Following behavioral determinations, mice were sacrificed by decapitation and the hippocampus and cerebellar vermis were dissected, rapidly frozen, and stored at −80 °C until neurochemical determinations were performed. For neurochemical assays, i.e. ELISA or oxidative stress determinations, the groups were formed by random samples from animals submitted to each one of the behavioral tests in order to avoid bias in the neurochemical results. Furthermore, ELISA and oxidative stress determinations were performed in different brain samples, since these methods require different preparation of sample homogenate as described in Section 2.6.

2.4. Serum lithium measurement

The serum of lithium-treated animals was separated by centrifugation. Assays were performed with a digital flame photometer (Labnova AP-1500 series, São Paulo, Brazil). The serum levels of lithium in each animal ranged from 0.8 to 1.1 mEq/L, as recommended for the routine treatment of BD (Hopkins and Gelenberg, 2000).

2.5. Behavioral determinations

2.5.1. Open-field test (Archer, 1973)

The open-field area was made of acrylic (with transparent walls and a black floor, 30 cm x 30 cm x 15 cm) divided into nine squares of...
equal areas. The apparatus was placed in a red light room. The animals were gently placed on the left corner and allowed to freely explore the arena for 5 min. The number of squares crossed was used to determine horizontal activity (crossing) and rearing behavior was used to determine vertical activity. This observational time has been shown to discriminate putative antimanic agents in the AMPH-induced animal model of mania (Frey et al., 2006b; Macêdo et al., 2013).

2.5.2. Spontaneous alternation in the Y-maze (Conrad et al., 1996)
Each mouse was placed at the end of one arm of the maze and allowed to freely move through the apparatus during 8 min. The apparatus consisted of a Y-shaped maze with three white, opaque plastic arms (length 35, width 5, wall height 10) at a 120° angle from each other arms. A correct alternation was defined as entries in all three arms on consecutive occasions. The percentage of correct alternations was calculated as follows: total of alternations/(total arm entries \(C_3^2\)), as described in detail elsewhere (Dall'Igna et al., 2007).

2.6. Biochemical determinations

2.6.1. Tissue preparation
For oxidative stress analysis, the hippocampus and cerebellar vermis were homogenized ten times [weight/volume (w/v)] with ice-cold 0.1 M phosphate buffer (pH 7.4). The homogenates were centrifuged at 10,000 rpm for 15 min, for collection of the supernatant.

For ELISA determinations, tissue samples were homogenized eight times (w/v) in the case of TNF-α determination, and 20 times (w/v) in the case of phospho-Ser9-GSK-3beta and BDNF with cold phosphate-buffered saline (PBS, pH 7.4). A protease inhibitor cocktail (Sigma-Aldrich) was added to the buffer and the homogenate was centrifuged at 14,000 rpm for 30 min.

2.6.2. Neurochemical assays
GSH, TBARS and TNF-α were determined in the hippocampus and cerebellar vermis and BDNF and phospho-Ser9-GSK-3beta only in the hippocampus.

2.6.3. Assay for GSH (Sedlak and Lindsay, 1968)
The method was based on Ellman’s reagent (DTNB) reaction with free thiol groups. Briefly, the supernatants were mixed with 0.4 M Tris-HCl buffer, pH 8.9 and 0.01 M DTNB. Reduced glutathione levels were determined by the absorbance at 412 nm and were expressed as ng of GSH/g wet tissue.

2.6.4. Measurements of lipid peroxidation
Lipid peroxides formation was analyzed by measuring thiobarbituric acid reacting substances (TBARS) levels according to a method described in detail elsewhere (Ohkawa et al., 1979). The lipid peroxidation was determined by the absorbance at 535 nm and was expressed as \(\mu\)mol of malondialdehyde (MDA)/mg of protein.

2.6.5. Determination of TNF-α, BDNF and phospho-Ser9-GSK-3beta levels
The levels of TNF-α and BDNF (R&D systems, Minneapolis, MN, USA) as well as phospho-Ser9-GSK-3beta [Human/Mouse/Rat Phospho-GSK-3beta (S9) ELISA; Millipore, USA] were determined in each sample by enzyme immunoassays according to each specific manufacturers’ directions. Results are expressed as pg/g tissue.

2.7. Statistical analysis
Behavioral and biochemical data (GSH and lipid peroxidation levels) were statistically analyzed using a 2 \(\times\) 2 factorial ANOVA. Two between-subjects factors were used: lithium (0 and 47.5 mg/kg) or CDS (0, 0.1, 0.3 and 1 mg/kg) and AMPH treatment (0 and 2 mg/kg) for oxidative stress analysis and the hippocampus and cerebellar vermis and BDNF and phospho-Ser9-GSK-3beta only in the hippocampus.}

Fig. 2 Number of crossings in the prevention (A) and reversal (B) protocols. Number of rearings in the prevention (C) and reversal (D) protocols. Prevention protocol \(n=6-10\) animals per group and reversal protocol \(n=6-8\) animals per group. Bars represent mean \(\pm\) SEM of the number of crossings or rearings. CDS=candesartan; AMPH=amphetamine; Li=lithium; SAL=saline. *\(P<0.05\) versus SAL; \(P<0.05\) versus SAL+AMPH according to two-way ANOVA followed by Tukey’s post-hoc test.
was the within-subjects factor. In the case of a significant interaction between factors Tukey’s post-hoc tests were used.

In the analysis of BDNF and phospho-GSK-3β levels a one-way ANOVA was used. When a main effect of treatment was detected (P<0.05), Tukey’s post-hoc test was used to determine the level of significance for each drug treatment (lithium and 0.1, 0.3 or 1 mg/kg CDS). Nonparametric analyses with Kruskal-Wallis test followed by Dunn’s multiple comparisons test were performed for TNF-α results. Before ANOVA, D’Agostino-Pearson omnibus test was conducted to verify the normal distribution of the data. All results are expressed as mean±standard error of the mean (SEM). For all analyses, the significance level was set at P≤0.05. Data analyses were performed using GraphPad Prism software, version 6.0f for Mac (San Diego California, USA).

3. Results

3.1. Candesartan prevented and reversed hyperlocomotion induced by AMPH

As illustrated in Fig. 2A, the analysis of the number of crossings in the prevention protocol revealed significant interaction between lithium [F (1, 31)=10.75, P=0.0026] and CDS [F (3, 56)=10.61, P<0.0001] × AMPH. Amphetamine administration significantly increased the number of crossings when compared to SAL-treated mice (P<0.0001). When lithium [F (1, 31)=12.66, P<0.0012] or CDS [F (3, 56)=9.964, P<0.0001] were used as factors significant main effects of these treatments were observed. The administration of lithium significantly prevented the hyperlocomotion induced by SAL+AMPH (P<0.001). Candesartan 0.1, 0.3 or 1 mg/kg also significantly prevented the alterations induced by SAL+AMPH (P<0.0001). In the reversal protocol (Fig. 2B) a significant interaction between lithium [F (1, 23)=16.59, P=0.0005] and CDS [F (3, 45)=10.71, P<0.0001] × AMPH was observed, with significant main effects of both lithium and CDS (lithium: [F (1, 23)=9.416, 0.0054]; CDS: [F (3, 45)=4.765, P=0.0057]). In this protocol SAL+AMPH significantly increased the number of crossings when compared to SAL-treated animals (P<0.0001), while lithium (P<0.0001) or CDS 0.1 (P<0.0001), 0.3 (P<0.0001) or 1 (P<0.0001) mg/kg significantly reversed this increase.

The evaluation of rearing in the prevention (Fig. 2C) and reversal (Fig. 2D) protocols revealed a significant interaction between lithium × AMPH and CDS × AMPH (prevention protocol: lithium × AMPH [F (1, 31)=24.28, P<0.0001], CDS × AMPH [F (3, 56)=16.27, P<0.0001]; reversal protocol: lithium × AMPH [F (1, 23)=8.998, P=0.0066], CDS × AMPH [F (3, 45)=5.934, P=0.0019]), with significant main effects of lithium (prevention protocol: [F (1, 31)=14.33, P=0.0009], reversal protocol [F (1, 23)=8.528, P=0.0079]) and CDS treatments (prevention protocol: [F (3, 56)=9.881, P<0.0001], reversal protocol [F (3, 45)=9.742, P<0.0001]). In both prevention and reversal protocols SAL+AMPH significantly increased the number of rearings when compared to SAL-treated mice (P<0.0001) while lithium (P<0.0001) or CDS 0.1 (P<0.0001), 0.3 (P<0.0001) or 1 (P<0.0001) mg/kg significantly reversed this increase.

3.2. Candesartan, but not lithium, prevented and reversed AMPH-induced deficits in working memory

Bipolar disorder patients during mania present substantial deficits in working memory compared to healthy controls (Martinez-Arán et al., 2004), based on this evidence, we tested the occurrence of working memory deficits in mice submitted to the AMPH-induced model of mania as well as the effects of CDS or lithium in this behavioral paradigm using the Y-maze task (Fig. 3). The two-way ANOVA analysis of Y maze results in the prevention protocol (Fig. 3A) revealed no significant interaction between lithium × AMPH [F (1, 24)=1.959, P=0.1744], although a main effect of AMPH treatment was observed [F (1, 24)=28.29, P<0.0001]. When CDS was used as factor a significant CDS × AMPH interaction was observed [F (3, 48)=8.614, P=0.0001] with the significant main effect of CDS treatment [F (3, 48)=3.365, P=0.0261]. Tukey’s post-hoc test revealed that the administration of SAL+AMPH increased working memory deficits when compared to SAL-treated animals (P<0.0001). The administration of lithium before AMPH also significantly increased working memory deficits when compared to SAL or SAL+lithium-treated mice. On the contrary, CDS 0.1 (P<0.001), 0.3 (P<0.001) or 1 (P<0.001) mg/kg significantly prevented this alteration. In the reversal protocol (Fig. 3B) there was a significant lithium × AMPH interaction [F (1, 24)=6.492, P=0.0177]. In this protocol the administration of SAL+AMPH or lithium+AMPH increased working memory deficits when compared to SAL-treated animals. When CDS was used as factor a significant interaction between CDS × AMPH was observed [F (3, 48)=6.423, P=0.0010] with significant main effect of CDS [F (3, 48)=4.095, P=0.0115]. Post-hoc test showed that CDS 0.1

![Fig. 3](image-url) Percentage of correct alternations in the Y maze of animals submitted to the prevention and reversal treatments (n=7 animals per group). Bars represent mean±SEM of the % of correct alternations. CDS=candesartan; AMPH=amphetamine; Li=lithium; SAL=saline. *P<0.05 versus SAL; †P<0.05 versus SAL+lithium; ‡P<0.05 versus SAL+AMPH according to two-way ANOVA followed by Tukey’s post-hoc test.
CDS detected with the signification protocol (Fig. 4B) no signification between lithium × AMPH was observed. When CDS was used as factor there was a significant CDS × AMPH interaction \[ F(3, 52) = 4.108, P = 0.0115 \] without the main effect of CDS. In this brain area we observed significant decreased levels of GSH in animals administered SAL + AMPH \( P < 0.05 \), lithium + AMPH \( P < 0.05 \) or CDS 0.3 + AMPH \( P < 0.05 \) when compared to SAL-treated. In the reversal protocol (Fig. 4D) no significant interaction between lithium × AMPH or CDS × AMPH was detected.

The analysis of hippocampal lipid peroxidation of animals submitted to the prevention protocol (Fig. 4A) revealed a significant decrease in GSH levels in animals administered SAL + AMPH \( P < 0.01 \) or CDS 1 + AMPH \( P < 0.01 \) when compared to SAL-treated mice. Lithium + AMPH-treated animals also presented decreased levels of GSH when compared to SAL \( P < 0.05 \) or lithium + SAL-treated \( P < 0.05 \) ones. A significant increase in GSH levels was observed only in mice treated with CDS 0.3 + AMPH when compared to SAL + AMPH \( P < 0.05 \). The administration of CDS 1 + SAL increased GSH levels when compared to SAL \( P < 0.05 \).

In the cerebellar vermis, prevention protocol (Fig. 4C), no significant interaction between lithium × AMPH was observed. When CDS was used as factor there was a significant CDS × AMPH interaction \[ F(3, 46) = 13.15, P = 0.0013 \] and CDS × AMPH \[ F(3, 45) = 13.81, P = 0.0001 \] with significant main effects of lithium \[ F(1, 24) = 6.508, P = 0.0175 \] and CDS \[ F(3, 45) = 8.395, P = 0.0002 \]. Post-hoc test showed an increase in lipid peroxidation in SAL + AMPH-treated mice when compared to SAL-treated \( P < 0.001 \) ones that was prevented by lithium \( P < 0.001 \),

![Fig. 4](image)

**Fig. 4** Reduced glutathione (GSH) levels in the hippocampus (top panel) and cerebellar vermis (bottom panel) after prevention and reversal treatments \( n = 6-8 \) animals per group. Bars represent mean ± SEM. CDS = candesartan; AMPH = amphetamine; Li = lithium; SAL = saline. *\( P < 0.05 \) versus SAL; **\( P < 0.05 \) versus SAL + lithium; \( P < 0.05 \) versus SAL + AMPH according to two-way ANOVA followed by Tukey’s post-hoc test.

3.3. Candesartan and lithium prevent and reverse AMPH-induced alterations in oxidative stress parameters

Since pro-oxidative alterations are observed in BD patients (Andreazza et al., 2008) and also in animal models of mania (Macêdo et al., 2013), we evaluated the levels of the main endogenous antioxidant, GSH and lipid peroxidation in the hippocampus and cerebellar vermis. In the evaluation of hippocampal levels of GSH in the prevention protocol (Fig. 4A) we observed a significant interaction between lithium × AMPH \[ F(1, 25) = 5.954, P = 0.0221 \] and CDS × AMPH \[ F(3, 52) = 8.739, P < 0.001 \], in this latter case with the significant main effect of CDS treatment \[ F(3, 52) = 8.599, P = 0.0001 \]. Tukey’s test showed a decrease in GSH levels in SAL + AMPH-treated mice when compared to SAL-treated ones \( P < 0.001 \). Candesartan 0.3 \( P < 0.001 \) or 1 \( P < 0.0001 \) mg/kg significantly prevented this decrease. In the reversal protocol (Fig. 4B) no significant interaction between lithium × AMPH was observed. On the other hand, a significant CDS × AMPH interaction \[ F(3, 50) = 15.22, P < 0.001 \] was detected with the significant main effect of CDS treatment \[ F(3, 50) = 4.153, P = 0.0105 \]. Post-hoc analysis revealed a significant decrease in GSH levels in animals administered SAL + AMPH \( P < 0.01 \) or CDS 1 + AMPH \( P < 0.01 \) when compared to SAL-treated mice. Lithium + AMPH-treated animals also presented decreased levels of GSH when compared to SAL \( P < 0.05 \) or lithium + SAL-treated \( P < 0.05 \) ones. A significant increase in GSH levels was observed only in mice treated with CDS 0.3 + AMPH when compared to SAL + AMPH \( P < 0.05 \). The administration of CDS 1 + SAL increased GSH levels when compared to SAL \( P < 0.05 \).
or CDS in the three doses studied (P<0.01). The animals administered CDS 0.3+AMPH presented decreased levels of lipid peroxidation also when compared to SAL-treated mice (P<0.05). In the reversal protocol (Fig. 5B) there was no significant lithium × AMPH interaction. When CDS was used as factor a significant CDS × AMPH interaction was observed [F (3, 47)=5.252, P=0.0033] with the main effect of CDS treatment [F (3, 47)=4.824, P=0.0052]. According to post-hoc analysis CDS 0.1 (P<0.01), 0.3 (P<0.01) or 1 (P<0.01) mg/kg significantly decreased the levels of lipid peroxidation when compared to SAL+AMPH-treated animals, although these levels remained increased when compared to saline-treated mice and drug-matched controls (P<0.01).

Considering lipid peroxidation levels in the cerebellar vermis, in the prevention protocol (Fig. 5C) significant lithium × AMPH [F (1, 23)=10.30, P=0.0039] and CDS × AMPH...
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3.5. The administration of candesartan and lithium alters BDNF and phospho-GSK-3beta levels in the brain areas of mice submitted to AMPH-induced mania

Because the mechanism of action of lithium involves alterations in neurotrophins and GSK-3beta (Gould and Manji, 2005), we decided to evaluate the effect of CDS in these parameters in the hippocampus. As illustrated in Fig. 6, in the prevention protocol, the administration of SAL+AMPH significantly decreased the levels of both parameters when compared to control animals (BDNF: [F(4,32)=6.426, P<0.01]; phospho-Ser9-GSK-3beta: [F(4,37)=30.10, P<0.001]). The administration of lithium (P<0.001), CDS 0.3 (P<0.001) or 1 (P<0.001) mg/kg significantly prevented the alterations in BDNF levels induced by AMPH (Fig. 6A). In relation to phospho-Ser9-GSK-3beta, only lithium (P<0.01) significantly prevented AMPH-induced alterations in this parameter (Fig. 6B). In the reversal protocol, the administration of SAL+AMPH decreased BDNF levels, which was significantly reversed by lithium or CDS at both doses [F(4,29)=4.882, P<0.001] (Fig. 6A). Similarly to the observed in the prevention protocol decreased levels of phospho-Ser9-GSK-3beta were observed in SAL+AMPH-treated animals (P<0.01) when compared to SAL-treated ones whereas only lithium (P<0.001) significantly reversed this alteration.

4. Discussion

To our knowledge this is the first study to demonstrate that CDS, an ARB, prevented and reversed AMPH-induced hyperlocomotion in mice, acknowledged as a pharmacological animal model of mania (Young et al., 2011). Furthermore, CDS prevented and reversed working memory deficits and the majority of AMPH-induced neurochemical abnormalities akin to the effects of lithium, with the exception of working memory deficits that were not ameliorated by lithium administration. Additionally, as far as we know, this is the first study to demonstrate, in an animal model of mania induced by AMPH: i) the presence of important alterations in the cerebellar vermis, a brain area altered in youth at risk for BD (Singh et al., 2011) and ii) increments in TNF-α levels in the hippocampus and cerebellar vermis, a cytokine suggested as a possible biological target for BD (Brietzke and Kapczinski, 2008).

Regarding behavioral alterations during manic episodes, psychomotor agitation is a hallmark manifestation (Young et al., 2011) and is assessed in animal models of mania by hyperlocomotion in the open field test (Frey et al., 2006b). This is concordant with the hypothesis of dopamine dysregulation as a core component of the pathophysiology of BD.

3.4. Candesartan and lithium administration decreases TNF-α levels in mice submitted to the prevention and reversal protocols

Increased levels of the pro-inflammatory cytokine TNF-α have been reported during manic and depressive episodes of BD (Kim et al., 2007). Accordingly, in the prevention protocol significant increased levels of TNF-α were detected in the animals administered SAL+AMPH when compared to controls in both hippocampus (P<0.001) and cerebellar vermis (P<0.01) as depicted in Table 1. In the hippocampus pre-treatment with CDS 0.3 (P<0.0001) or 1 mg/kg (P<0.0001) completely abolished the increase in TNF-α levels induced by SAL+AMPH. In the cerebellar vermis in the same way as hippocampus CDS 0.3 (P<0.01) or 1 mg/kg (P<0.01) normalized the levels of TNF-α increased by SAL+AMPH. In the reversal procedure, SAL+AMPH caused no significant alteration in TNF-α levels either in the hippocampus or in the cerebellar vermis. Post-treatment with lithium significantly decreased the levels of TNF-α in both brain areas when compared to SAL+AMPH-treated animals (P<0.05). The same was observed with the post-administration of CDS 1 mg/kg (P<0.01).

![Fig. 6](https://example.com/figure6.png)

**Fig. 6** Levels of BDNF (A) and phospho-Ser9-GSK-3beta (B) in the hippocampus of mice submitted to the prevention and reversal protocols (n=6-8 animals per group). Bars represent mean±SEM. CDS=candesartan; AMPH=amphetamine; Li=lithium; SAL=saline. *P<0.05 versus SAL; P<0.05 versus SAL+AMPH according to one-way ANOVA followed by Tukey’s post-hoc test.

\[ F(3, 46)=8.099, P=0.0002 \] interactions were observed with the significant main effect of CDS \[ F(3, 46)=2.826, P=0.0499 \]. Tukey’s test revealed increased levels of lipid peroxidation in SAL+AMPH-treated mice when compared to SAL (P<0.0001). The levels of lipid peroxidation were significantly decreased in mice administered CDS 1+AMPH when compared to SAL+AMPH (P<0.001). The AMPH-treated animals pretreated with lithium, CDS 0.1, 0.3 or 1 mg/kg presented higher levels of lipid peroxidation when compared to SAL and each respective drug-matched control. In the reversal protocol (Fig. 5D) we also observed significant interactions between lithium × AMPH \[ F(1, 22)=1.11, P=0.0030 \] and CDS × AMPH \[ F(3, 42)=10.24, P<0.0001 \] with the significant main effect of CDS \[ F(3, 42)=10.18, P<0.0001 \]. In this brain area the animals treated with SAL+AMPH (P<0.001) or lithium+AMPH (P<0.001) presented higher levels of lipid peroxidation when compared to SAL. On the other hand, the administration of CDS 0.1, 0.3 or 1+AMPH increased the levels of lipid peroxidation when compared to SAL or each respective drug-matched control (P<0.0001).
and the role of AMPH as a dopamine agonist in this paradigm (Berk et al., 2007). In keeping with this view, in the present study, AMPH administration substantially increased locomotion in the open field test, which was prevented and reversed by lithium, thereby reproducing the predictive validity of the model (Macêdo et al., 2013). Likewise, CDS at the three doses studied prevented and reversed AMPH-induced hyperlocomotion, which suggests it may possess antimanic-like activity.

In recent years the validity of animal models of mania has been queried (Young et al., 2011). In this regard, some researchers have emphasized the need in these models to address more behavioral parameters than just levels of activity (Einat, 2006). A clinically relevant phenotype would be to measure cognition (Young et al., 2011) based on the well documented cognitive impairments observed in BD patients (Martínez-Arán et al., 2004). In this context, impairments in executive function and verbal memory in BD are frequently observed alterations (Martínez-Arán et al., 2004). In our experimental conditions, AMPH was able to induce working memory deficits which were prevented and reversed by CDS, but not lithium. This result obtained with CDS seems to be of interest, because lithium, a well-known mood stabilizer used to treat BD for over 50 years (Licht, 2012), has been associated with some reports of cognitive blurring and specific memory deficits in BD patients (Senturk et al., 2007). However, there are some reports that individuals who are excellent lithium responders may have preserved cognition (Rybakowski et al., 2009). In hypertensive rats (Kishi et al., 2012) ARBs prevented cognitive deficits, suggesting that this system may have an important pathophysiological role in cognitive impairment (Yagi et al., 2013).

Overall, the dysregulation of brain RAS leads to an excessive activation of AT1 receptors by AngII which is associated with an increase in sympathetic drive along with hormonal responses to stress, activation of pro-inflammatory pathways as well as changes in glutamate release and ROS formation (Jung et al., 2007). The mechanisms underlying AngII neurotoxic effects include: i) induction of ROS production by activation of the reduced nicotinamide adenine dinucleotide phosphate NADPH-oxidase complex (NOX) (Seshiah et al., 2002), ii) regulation of the activity of several transcription factors, leading to an increase in the synthesis of pro-inflammatory mediators, e.g. EPAC1 (exchange protein directly activated by cAMP) activation (Xie et al., 2014) and iii) activation of GSK-3beta (Agarwal et al., 2013). In keeping with this view, the AT1 receptor blockade has been recently proposed by our research group as a potential target for the pharmacotherapy for BD (de Gois Queiroz et al., 2013).

The repeated administration of AMPH has been associated with oxidative imbalance, inflammatory activation, mitochondrial damage and alterations in neuroplasticity (Macêdo et al., 2013; Valvassori et al., 2010) which mirror relevant pathways involved in BD pathophysiology (Berk et al., 2011). Therefore, we decided to determine the possible involvement of these pathways in the antimanic effects of CDS, based on the previously demonstrated neuroprotective and neurotrophic properties of this drug (Lu et al., 2005).

Importantly, our study sought abnormalities in the cerebellar vermis in an animal model of mania. This brain area is referred to as “limbic cerebellum” based on its involvement in the integration of affective and cognitive behaviors (Schmahmann et al., 2007). The involvement of the cerebellar vermis in the pathophysiology of BD has been suggested by previous clinical studies (Singh et al., 2011), although it has not been examined in preclinical models of BD.

One of the novelties of the present study is the demonstration of oxidative imbalance and increased TNF-α levels in the cerebellar vermis of mice submitted to AMPH-induced mania, which directs attention to a closer evaluation of this brain area in future studies addressing mania.

Candesartan prevented and reversed the oxidative imbalance caused by AMPH, although, regarding the evaluation of lipid peroxidation in the cerebellar vermis only CDS 1 mg/kg prevented AMPH-induced increase in this parameter. In our experimental protocol, lithium was not able to significantly prevent or reverse the alteration in GSH and in some cases lipid peroxidation levels, being capable to prevent the increase in lipid peroxidation induced by AMPH in the hippocampus. A previous study in which lithium was administered twice daily had shown that depending on the brain area and protocol (i.e., prevention or reversal) studied, co-administration of lithium and AMPH increased lipid peroxidation when compared to control animals (Frey et al., 2006b). A tentative explanation for the discrepancy between these previous findings (Frey et al., 2006b) and ours is that we used a single-dose schedule for lithium administration. On the other hand, in the present study CDS prevented and reversed the majority of AMPH-induced oxidative changes. Hence, ARBs present antioxidant properties in brain tissues (Lu et al., 2005) and especially CDS was able to restore GSH levels and decrease lipid peroxidation in the hippocampus of rats submitted to chronic cerebral hypoperfusion (Ozacmak et al., 2007). It is worth to mention that in the present study, alterations caused by CDS did not display a dose-response relationship. Hence previous preclinical studies using similar doses of CDS (i.e., 0.1, 0.3 and 1 mg/kg) in other experimental designs have not dose-dependent effects for CDS (Kozak et al., 2008).

While clinical studies have shown immunological abnormalities in the pathophysiology of BD (e.g., increased peripheral levels of pro-inflammatory cytokines) (Brietzke and Kapczinski, 2008; Kim et al., 2007), our preclinical approach is the first to show, alterations in TNF-α levels in the hippocampus and cerebellar vermis of mice submitted to AMPH-induced mania.

TNF-α has an important role in neuroplasticity, cell resilience and neuronal survival (Brietzke and Kapczinski, 2008). Previous report observed increased levels of this cytokine during manic and depressive episodes of BD (Kim et al., 2007) calling, thus, the attention for TNF-α as an interesting novel target for BD treatment (Brietzke and Kapczinski, 2008).

In our study, the administration of AMPH in the prevention protocol increased TNF-α in both areas studied, indicating that inflammation may contribute to AMPH-induced behavioral changes. Candesartan, similarly to lithium, presented immunomodulatory activity, decreasing TNF-α levels. Some previous studies highlight the putative anti-inflammatory effects of lithium (Wang et al., 2013). Lithium inhibits the GSK-3beta/NF-κB signaling pathways, leading to a down-regulation of the inflammatory process (Wang et al., 2013), in addition to the down-regulation of the expression of several genes related to inflammation (Padmos et al., 2008). Accordingly, in BD patients an inverse correlation between TNF-α level and...
lithium response was observed (Guloksuz et al., 2012). Regarding CDS, this ARB reduces the expression of pro-inflammatory cytokines and their receptors in brain areas involved in the regulation of emotion, cognition, and memory in animals submitted to lipopolysaccharide-induced neuroinflammation (Benicky et al., 2011).

Herein, the increase in TNF-α levels induced by AMPH was accompanied by a decrease in BDNF and phospho-GSK-3beta (Ser-9) in the hippocampus. Interestingly, both lithium and CDS reduced TNF-α and restored BDNF levels, but only lithium increased phospho-GSK-3beta (Ser9) levels in this brain area.

BDNF binds to the tyrosine receptor kinase B (TrkB) receptor resulting in PI3K activation and consequently the regulation of GSK-3beta (Bondy and Cheng, 2004). GSK-3 is a pluripotent kinase which is at the convergence of a wide array of signaling pathways regulated by lithium and diverse mood stabilizer drugs (Gould and Manji, 2005). Hence, the neuroprotective effect of lithium involves GSK-3 inhibition (Li et al., 2010). GSK-3 is constitutively activated and its phosphorylation at Ser-9 is a step whereby multiple protective signaling pathways converge (Gould and Manji, 2005).

Levels of phospho-Ser9-GSK-3beta in BD patients treated with lithium are higher than in healthy controls (Li et al., 2010). In the present study, lithium prevented AMPH-induced alterations in phospho-GSK-3beta, whereas CDS lacked such effects. Conversely, CDS decreased phospho-GSK-3beta (Ser-9) levels. As mentioned above, GSK-3beta is inhibited by N-terminal phosphorylation at Ser-9 and is activated by Tyrosine phosphorylation at Tyr-216. Agarwal et al. (2013) reported a slight but significant increase in the phosphorylation of GSK-3beta (Ser-9) and an up-regulation in the phosphorylation of GSK-3beta (Tyr-216) in neuronal cells exposed to AngII, suggesting that this peptide is related to GSK-3beta activation (Bijur and Jope, 2003). Therefore, in the present study CDS may have caused a reduced activation, and not an increase in inactivation of GSK-3beta. Thus, future studies addressing the ratio of p-GSK-3beta (Tyr-216)/p-GSK-3beta (Ser-9) expression are necessary to indicate the level of activation of GSK-3beta in animals submitted to CDS administration.

The present study has some limitations that need to be addressed. First, the AMPH-induced animal model of mania does not precisely mimic all the symptoms of the related human disorder and also does not address the developmental component of BD. Furthermore, animal models of mania do not have pathological validity outside the dopamine dysregulation hypothesis. Second, the ratio of protein expression of p-GSK-3beta (Tyr-216)/p-GSK-3beta (Ser-9) should be determined in future studies to better account for CDS effects in this neurochemical parameter.

In conclusion, the present study suggests that CDS may have antimanic effects. The possible mechanisms involved in this action may include antioxidant, anti-inflammatory and neurotrophic effect in brain areas related to BD pathophysiology, namely the cerebellar vermis and hippocampus. Our findings strengthens the hypothesis that blockage of AT1 receptors may be a novel target for the management of BD. Our study opens perspectives for the design of proof-of-concept trials of AT1 antagonists either as an adjunctive treatment for bipolar disorder. Furthermore, this novel target may act as a cognitive enhancer for BD.

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Contributors

Authors JASG, GCS, DFL and LMC contributed to the design of the study and performed the behavioral experiments. Authors DM and JQ designed the study and wrote the paper while MB and AFC analyzed the data and wrote the paper. Authors FCFS and JB performed the biochemical experiments.

Conflict of interests

The authors declare no conflict of interests.

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