The antimanic-like effects of andrographolide and quercetin

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LUIZ KAE SALES KANAZAWA

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Luiz Kae Sales Kanazawa

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By

Luiz Kae Sales Kanazawa

Supervisor:

Prof. Dr. J. Prickaerts

Co-supervisor:

Dr. Roberto Andreatini, Paraná, Brazil

Evaluation committee:

Prof. Dr. D. van den Hove (Chair)

Prof. Dr. A. Blokland

Prof. Dr. G. Wegener, Aarhus, Denmark

Dr. J. Olivier, RU Groningen

Dr. N. Leibold

Prof. Dr. Maria Aparecida Barbato Frazão Vital, Federal University of Paraná

ABBREVIATIONS

AchE	Acetylcholinesterase
AD	Alzheimer's disease
Akt	Protein kinase B
ANDRO	Andrographolide
BD	Bipolar disorder
BDNF	Brain derived neurotrophic factor
CAT	Catalase
CNS	Central nervous system
CREB	cAMP response element binding-factor
DAG	Diacylglycerol
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSK3β	Glycogen synthase kinase 3 beta
GST	Glutathione-S-transferase
HD	Huntington’s disease
IP_3	Inositol triphosphate
LDX	Lisdexamfetamine
LPO	Lipid peroxidation
LPS	Lipopolysaccharide
MS	Multiple sclerosis
PD	Parkinson's disease
PFC	Prefrontal cortex
PI3K	Phosphoinositide-3-kinase
PIP_2	Phosphatidylinositol 4,5-biphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C

PNS	Peripheral nervous system
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SD	Sleep deprivation
SOD	Superoxide dismutase
USV	Ultrassonic vocalization

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CHAPTER 1

General introduction

1. Bipolar disorder

Bipolar disorder (BD) is a chronic mental illness that affects 1%-3% of the global population and is one of the major causes of disability worldwide (Goodwin and Jamison, 2007). It is characterized by recurrent episodes of depression and mania or hypomania (Abrial et al., 2015). BD can be mainly subdivided into BD-I and BD-II, in which BD-I is characterized by episodes of mania and depression and BD-II is characterized by episodes of hypomania and depression. The manic episodes in BD-I are periods of abnormally and persistently elevated, expansive, or irritable mood and increased goal-directed activity or energy. The same applies for hypomania in BD-II. However, the manic episode lasts at least one week and is present most of the day, nearly every day, while the hypomanic episode lasts at least four consecutive days and is present most of the day, nearly every day. Three (or more) of the following symptoms are present to a significant degree and denote a noticeable change from usual behavior in both mania and hypomania: inflated self-esteem or grandiosity; decreased need for sleep; talkativeness or pressure to keep talking; flight of ideas; distractibility; increase in goal-directed activity or psychomotor agitation; excessive involvement in activities that have a high potential for painful consequences. However, in hypomania, the episode is not severe enough to cause significant impairment in social or occupational functioning or to necessitate hospitalization (American Psychiatry Association, 2013).

The major depressive episodes present in BD-I or BD-II consist of five (or more) of the following symptoms present for a two-week period with changes in normal functioning (and at least one of the symptoms must be depressed mood or loss of interest/pleasure (anhedonia)): depressed mood most of the day, nearly every day; markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day; significant weight loss or weight gain; insomnia or hypersomnia nearly every day; psychomotor agitation or retardation nearly every day; fatigue or loss of energy; feelings of worthlessness or excessive or inappropriate guilt; diminished

ability to think or concentrate, or indecisiveness; recurrent thoughts of death or recurrent suicidal ideation (American Psychiatry Association, 2013).

Other forms of BD include: cyclothymic disorder, which is a chronic, fluctuating mood disturbance with many periods of hypomanic and depressive symptoms, for at least two years; mixed episodes, in which mania or hypomanic episodes occur with depressive features; substance/medication-induced BD, when induced by substances such as alcohol or psychostimulants, such as amphetamine; other specified BD, when full criteria for other subtypes of BD are not met; rapid cycling, in which there are, at least, four mood episodes in the previous twelve months; and BD with psychotic features, consisting of delusions or hallucinations that are present at any time in the episodes (American Psychiatry Association, 2013).

According to Nivoli (2011) and Judd et al. (2008), bipolar patients spend approximately 2/3 of their lives in depressed mood and 1/3 in other types of mood (manic, hypomanic or euthymic). Thus, greater attention is directed towards the depressive stages of the disorder and not so much towards the manic phase. However, just as the depressive symptoms, the manic symptoms also affect significantly the well-being of patients and are associated to several social and personal losses, significant distress or impairment in social and occupational functioning (American Psychiatry Association, 2013; Müller-Oerlinghausen et al., 2002). Suicidal behavior is frequent in patients with BD, as between 20 and 60% of them attempt suicide at least once in their lifetime, and between 4 and 19% of them end up committing suicide (Dome et al., 2019). In BD, the risk of death by suicide can be 30% higher than of the general population (Bauer et al., 2018). In addition, although there are several drugs for the treatment of the depressive stages, there are fewer pharmacological options for the management of the manic phases, which show many limitations regarding their clinical use, such as intolerance to several side effects or refractoriness to treatment (Cipriani et al., 2011; Keck, 2003). Also, even when the treatment strategies are adequate, the course of BD involves high rates of recurrent manic and depressive episodes, relapses and hospitalizations (Souza, 2011) and even with the remission of mood swings, subsyndromal symptoms can still persist in a large number of patients (Knapp and Isolan, 2005). The great number of side effects results in low medication adherence (Castro-Costa e Silva, 2011; Miklowitz and Johnson, 2006; Sajatovic et al., 2004), which contributes to medication nonresponse (Osterberg and Blaschke, 2005), increases in relapses (Gutiérrez-Rojas et al., 2010),

suicide and suicide attempts (Pompili et al., 2009), hospitalizations (Hong et al., 2011) and involvement with criminal justice (Robertson et al., 2014).

Among the main pharmacological treatments available for the treatment of BD, mood stabilizers such as lithium and sodium valproate are the main ones (Chiu et al., 2013; Miklowitz and Johnson, 2006). Antipsychotics such as risperidone, olanzapine, quetiapine, lurasidone, ziprasidone, paliperidone, asenapine, clozapine and aripiprazole, and anticonvulsants such as lamotrigine, carbamazepine and oxcarbazepine, have also been approved for the treatment of BD, also in combination with antidepressants (Bai et al., 2019; Bauer et al., 2019). Antipsychotics act on dopamine and serotonin receptors. Second-generation (atypical) antipsychotics have less affinity to D_2 receptors than first-generation antipsychotics, and more affinity to 5-HT_{2A} receptors (Jauhar and Young, 2019). An advantage of second-generation antipsychotics is the decreased propensity of causing movement disorders in comparison to first-generation antipsychotics (Carbon et al., 2017). The main adverse effects involved in the use of antipsychotics are extrapyramidal symptoms, weight gain, effects on glucose metabolism, sedation and sexual dysfunction (Young et al., 2015). Antidepressant monotherapy for acute bipolar depression is not recommended due to the possibility of manic switch or induction of rapid cycling (Bauer et al., 2012). Thus, antidepressants (such as tricyclics or serotonin/norepineprhine reuptake inhibitors) are combined with mood stabilizers (lithium or anticonvulsants) or antipsychotics for the management of BD (Bauer et al., 2012; Malhi et al., 2012).

The biochemical abnormalities underlying the pathophysiology of this disorder remain to be fully elucidated (Szabo et al., 2009). However, as will be discussed below, studies indicate in particular the involvement of increased brain oxidative stress, as well as increased activity of the enzymes glycogen synthase kinase 3β (GSK3β) and protein kinase C (PKC) in the pathophysiology of BD.

2. Oxidative stress

Studies show that among many other factors, increased oxidative stress is involved in the pathophysiology of BD, as there is increased production of free radicals and depletion of antioxidant enzymes and molecules in BD (Berk et al., 2011). One of the main processes involved in the generation of free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS) is oxidative phosphorylation. Among the

ROS, the most relevant are the singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), ozone (O_3), hypochlorous acid (HOCl) and the free radicals superoxide anion (O^{2-}) and the hydroxyl radical (OH^-). Some examples of RNS are nitric oxide (NO) and the free radical peroxynitrite ($ONOO^-$) (Kohen and Nyska, 2002). ROS and RNS are capable of reacting with practically every biomolecule, including DNA, RNA, proteins, carbohydrates and lipids, causing damage to these molecules (Diplock et al., 1998). The endogenous antioxidant defense system protects the cells from damage caused by free radicals, ROS and RNS. One of the most important antioxidant molecules in the prevention of oxidative damage is reduced glutathione (GSH), which is able to reduce proteins with oxidated sulphydryl groups and to also reduce the levels of O^{2-} and H_2O_2, with the co-activity of other enzymes, such as glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST) and catalase (CAT) (Rosa et al., 2014). Studies show that there are alterations in the levels of antioxidant enzymes in mania and BD. The GR and GST activities are increased in BD patients in late stages, when compared to BD patients in early stages (Andreazza et al., 2009). Studies demonstrate the consistent increase in lipid peroxidation (LPO) by free radicals and ROS and alterations in the levels of antioxidant enzymes in BD patients, independently of their stage (Berk et al., 2011; Andreazza et al., 2007; Machado-Vieira et al., 2007; Ozcan et al., 2004).

Brüning et al. (2012), in an animal model of mania, demonstrated the positive correlation between hyperlocomotion and increased LPO levels in rats. Valvassori et al. (2020) showed that intracerebroventricular (i.v) injection of lithium, tamoxifen or other drugs which can inhibit PKC prevented manic-like behaviors, such as crossings, rearings and groomings in the open-field test, induced by intraperitoneal (i.p.) administration of methamphetamine, a model of mania, in rats. In addition, these drugs also prevented methamphetamine-induced oxidative stress, by regulating oxidative parameters such as GPx, GR, 4-hydroxy-2-nonenal (4-HNE), 8-isoprostane (8-ISO), carbonyl groups and 3-nitrotyrosine (3-NT) levels in the frontal cortex, hippocampus and striatum of rats.

3. GSK3β, p-GSK3β and PKC

Pathways in which the enzyme GSK3β acts as a key regulator have been implicated in the development of many psychiatric diseases, including BD and that an

increase in GSK3β activity is linked to the occurrence of manic-like behaviors in animals (Prickaerts et al., 2006; Gould et al., 2004a).

The enzyme GSK3 is a serine/threonine kinase that regulates various signaling pathways (Prickaerts et al., 2006), including metabolic, signaling and structural proteins related to the modulation of G-coupled-, hormone- and ionotropic receptors and receptor trafficking (Beurel et al., 2016; Enman and Unterwald, 2012). GSK3 has two isoforms: GSK3α and GSK3β, which are serine/threonine kinases associated with regulation of glycogen synthesis in response to insulin (Dandekar et al., 2018; Frame and Cohen, 2001). It is well documented that GSK3β is generally pro-apoptotic (Rowe et al., 2007). GSK3β phosphorylates over 100 substrates and regulates multiple signaling pathways, involved in several cellular processes, such as gene expression, neurogenesis, neuronal death or survival and circadian rhythms (Jope and Roh, 2006). Impaired neurogenesis and neuronal plasticity are suggested to be crucial mechanisms in mood disorders and GSK3β is known to regulate both processes in the brain (Li and Jope, 2010).

GSK3β is a component of the Wnt signaling pathway, which is essential for embryonic development and also plays important roles in protein synthesis, cell proliferation and differentiation, microtubule dynamics and cell adhesion, for example (Frame and Cohen, 2001). GSK3β forms part of a β-catenin destruction complex that phosphorylates and thus facilitates the degradation of β-catenin, which is a protein related to the transcription of anti-apoptotic proteins (Serrano et al., 2014). In the absence of Wnt ligands, GSK3β, the transcriptional co-activator β-catenin and the tumor suppressor adenomatous polyposis coli (APC) bind directly to the scaffolding protein Axin in a complex that facilitates phosphorylation of β-catenin by GSK3β, which targets β-catenin for proteasome-dependent degradation (Valvezan and Klein, 2012). The pathway is activated by the binding of Wnt to a Frizzled family receptor, which passes the biological signal to the Dishevelled protein inside the cell (Zhang et al., 2010), inducing phosphorylation of the essential co-receptors low density lipoprotein receptor-related protein 5 (LRP5) and LRP6, which results in GSK3β inhibition and β-catenin stabilization, which is prevented from degradation and accumulates in the cytoplasm and nucleus. Stabilized β-catenin enters the nucleus and interacts with the lymphocyte enhancer factor/T-cell factor (LEF/TCF) family of transcription factors to

activate gene transcription (Valvezan and Klein, 2012; MacDonald et al., 2009; Clevers, 2006).

Evidence from pharmacological interventions and genetic models suggest the involvement of GSK3β in BD. For example, GSK3β knockout or GSK3β inhibitor-treated mice exhibited antidepressive- and antimanic-like behaviors, which suggests that GSK3β inhibition may be involved in the mechanism of mood-improving therapies (Gould et al., 2004b; O'Brien et al., 2004). Prickaerts et al. (2006) suggested that the use of transgenic mice with overexpression of GSK3β may be an animal model of mania, as these animals show manic-like behaviors, such as hyperlocomotion.

Lithium, the prototype mood stabilizer is both a direct and indirect inhibitor of GSK3β (Cole, 2013). In rat models of mania, specific GSK3β inhibitors reproduce behaviors mimicking the effects of lithium. Overexpression of GSK3β annuls the ameliorative behavioral effects caused by lithium treatment in mice. Polymorphisms in the GSK3β promoter region, in humans, are linked to earlier age of onset of BD, and therapeutic sensitivity to lithium (Polter et al., 2010). GSK3β is linked both to manic-like and depressive-like behaviors, and serine-9 (Ser^9) phosphorylation of GSK3β is the major regulatory mechanism inhibiting GSK3β (Polter et al., 2010).

The phosphorylation of GSK3 at an N-terminal serine residue (Ser^{21} at the α-isoform and Ser^9 at the β-isoform) creates a pseudosubstrate motif that inhibits GSK-3, allowing activation of downstream effectors such as glycogen synthase and mTOR (Proud, 2006). Of note, Ser^9-inhibition is on a pool of free GSK-3 that is not related to the Dishevelled/β-catenin complex. Proteins capable of phosphorylating GSK3β are widespread and include, for the Ser^9 site, protein kinase A (PKA) and protein kinase B (Akt) (Rowe et al., 2007). Disabling the inhibitory serine-phosphorylation of GSK3β can promote manic-like behavioral disturbances in animals, and serine-phosphorylation of GSK3β is reduced during manic states in animals and bipolar patients (Polter et al., 2010).

One of the actions of the mood stabilizer lithium is to inhibit GSK3β activity by either directly competitively inhibiting Mg^{2+} binding to the active site of the enzyme (Ryves and Harwood, 2001) or indirectly by stimulating the phosphoinositide 3-kinase (PI3K)/Akt pathway and enhancing phosphorylation of GSK3β at Ser^9 residues (Chiu et al., 2013; Kitagishi, 2012), which is associated with inhibition of GSK3β activity, and

this action is shared by agents such as valproate and antipsychotic drugs (Kozlovsky et al., 2006; DeSarno et al., 2002; Chalecka-Franaszek and Chuang, 1999).

Manic bipolar patients show higher levels of GSK3β compared to healthy controls (Li and Jope, 2010). Inhibitory serine-phosphorylation of GSK3β is lower in peripheral blood mononuclear cells of symptomatic bipolar cases as compared to healthy controls (Polter et al., 2010). Transgenic mice overexpressing GSK3β by substituting alanine by serine at position 9 show increased locomotor activity, modeling mania-like behavior (Polter et al., 2010). The phosphorylated/unphosphorylated GSK3β ratio (p-Ser^{9}-GSK3β/GSK3β ratio) can be an index of GSK3β activity and can correlate with behavioral aspects (Grabinski and Kanaan, 2016; De Sousa et al., 2015).

Another enzyme involved in the pathophysiology of BD is PKC. PKC is a family of, at least, 10 serine/threonine kinases, subdivided in three subfamilies, namely conventional (or classical) PKC (cPKC: α, βI, βII, γ; regulated by phospholipids, calcium ions and diacylglycerol (DAG)), novel PKC (nPKC: ε, δ, η, θ; regulated by phospholipids and DAG), and atypical PKC (aPKC: ζ, ι, λ; regulated by phospholipids but not by calcium ions or DAG), that are involved in intracellular signaling pathways (Saxena et al., 2017; Way et al., 2000). The activation of a variety of subtypes of G_q-coupled receptors stimulates phospholipase C (PLC), which, when activated, catalyzes the conversion of phosphatidylinositol 4,5-biphosphate (PIP_2) in two second messengers: triphosphate inositol (IP_3) and DAG. IP_3 stimulates the mobilization of intracellular calcium ions, whereas DAG activates PKC (Manji et al., 2001). Once activated, PKC migrates from the cytosol to the cellular membrane, as the enzyme bound to the membrane represents the active form of the enzyme, which is capable of phosphorylating its substrates properly (Parker and Murray-Rust, 2004).

PKC can be found in different cell types in different organs and its activity is related to the type of receptor activated and the cell type involved (Parker and Murray-Rust, 2004). PKC is highly expressed in the central nervous system (CNS) and most isoforms of PKC can be found in different brain areas, such as the hippocampus and the prefrontal cortex (PFC), and are involved in mood regulation (Abrial et al., 2011; Wetsel et al., 1992). PKC signaling is involved in processes that are altered in BD, such as neuroinflammation (Suganthy et al., 2016), oxidative stress (Hadley et al.,

2014), neuronal excitability (Pahl et al., 2014) and apoptotic pathway activation (Nam et al., 2015).

Studies show that manic patients have a higher membrane:cytosol PKC ratio in platelets, which is normalized after treatment with lithium (Friedman et al., 1993). *Post-mortem* studies show that there is increased expression, activity and translocation of PKCγ and PKCζ in the brains of bipolar patients, when compared to control individuals, with no psychiatric disorders (Wang and Friedman, 1996). Drugs used in the therapeutic management of BD, such as lithium and valproate, are capable of regulating PKC activity. Manji and Lenox (1999) showed that chronic (but not acute) treatment with lithium leads to an isoform-selective reduction of PKCα and PKCε in the PFC and hippocampus, with no significant alterations in the β, γ, δ or ζ isoforms. Both acute and chronic administration of amphetamine induces increased PKC activity and increased membrane:cytosol ratio, and increased growth associated protein-43 (GAP-43) phosphorylation, a substrate of PKC, leading to long term alterations in neurotransmission, which are linked to manic-like behavior (Einat et al., 2007). Chronic lithium administration was shown to decrease the levels of both cytosolic and membrane-bound PKC in platelets of BD patients (Soares et al., 2000).

4. Lithium

Lithium has been the cornerstone of maintenance treatment in BD since the 1960s (Jauhar and Young, 2019). Lithium regulates cell membrane transport, ion distribution and neurotransmitter regulation, as it inhibits excitatory neurotransmission by decreasing dopaminergic neurotransmission; and through its modulation of glutamatergic neurotransmission by downregulating NMDA receptors. Lithium regulates intracellular signaling and enzymes such as GSK3β, with inhibition of Akt phosphorylation and activation of neuroprotective pathways (Alda et al., 2015). Lithium inhibits GSK3β by regulating its inhibitory phosphorylation at Ser^9 residues (Malhi et al., 2013). GSK3β is activated under conditions of chronic stress, such as excessive dopaminergic neurotransmission during mania and has been shown to cause hyperactivity in animals (Prickaerts et al., 2006; Beaulieu et al., 2004). Lithium treatment antagonizes the development of dopamine-dependent locomotor behaviors in rodents by interfering with the regulation of Akt-GSK3β signaling pathway by D_2 receptors (Beaulieu et al., 2009). These results might indicate that the inactivation of

GSK3β could be one mechanism for the therapeutic response of lithium in BD patients (Dandekar et al., 2018).

Lithium also inhibits PKC by interfering with the phosphoinositol cycle (Malhi et al., 2013). After G_q-coupled receptor stimulation, PLC mediates the hydrolysis of PIP_2 to the secondary messengers DAG and IP_3. Inositol monophosphatase (IMPase) and inositol polyphosphate phosphatase (IPPase) facilitate the recycling of IP_3 back into myoinositol, which allows the phosphoinositol cycle to continue. Lithium inhibits the phosphoinositol cycle, as it inhibits the reuptake of inositol, and by direct inhibition of IMPase and IPPase. Therefore, with lithium-induced depletion of IP_3, there is less activation of PKC (Malhi et al., 2013). Lithium also directly inhibits MARCKS, a downstream target of PKC, that is responsible for neurotransmitter release (Machado-Vieira et al., 2009).

However, treatment with lithium involves the occurrence of many important side effects, such as nausea, vomiting, weight gain, xerostomia, polydipsia, polyuria, renal failure, thyroidal dysfunctions, somnolence, insulin resistance, and so on (Bai et al., 2019; Kemp et al., 2014; Chiu et al., 2013; Price and Marzani-Nissen, 2012; Souza, 2011; Müller-Oerlinghausen et al., 2002). Öhlund et al., 2018 showed that 44% of the patients with BD taking lithium discontinued the treatment due to side effects or perceived lack of effectiveness.

The inhibitory activity of lithium over GSK3β is believed to be one the main responsible factors for its antimanic action (Chiu et al., 2013). Many studies indicate that GSK3β is an interesting target for BD and that GSK3β inhibitors might possess antimanic-like properties. Kozikowski et al. (2007) affirm that the research regarding the therapeutic efficacy of GSK3β inhibitors as possible antimanic agents is of great importance. Valvassori et al. (2020) affirm that PKC and oxidative stress are also involved in BD and that PKC inhibition is strongly implied in the antioxidant and antimanic effects of several PKC inhibitors, such as lithium or tamoxifen. Thus, the search for other GSK3β- or PKC inhibitors in the context of mania in BD is of great relevance.

5. GSK3β or PKC inhibitors

Inhibition of GSK3β has beneficial effects and intensive efforts have been made in the search for and design of selective GSK3β inhibitors. These include inhibitors

isolated from natural sources, cations or small synthetic molecules, such as ATP-competitive inhibitors, non-ATP-competitive inhibitors or substrate-competitive inhibitors (Eldar-Finkelman and Martinez, 2011). Kalinichev and Dawson (2011) evaluated the antimanic-like properties of the GSK3β inhibitors indirubin, alsterpaullone, SB-627772, TDZD-8, AR-A014418, among others. The study showed that these drugs were capable of inhibiting the amphetamine-induced hyperlocomotion in rats, just like lithium, sodium valproate and other drugs that are employed in the pharmacotherapy of BD. GSK3β inhibitors such as CHIR99021, 6-BIO, SB216763 and alsterpaullone decreased amphetamine-induced hyperlocomotion (Muneer, 2017). Enman and Unterwald (2012) showed that valproic acid and the GSK3β inhibitor SB216763 markedly reduced amphetamine-induced hyperlocomotion and stereotypy. Valvassori et al. (2017) showed that lithium and valproate act on GSK3β to reverse the manic-like behavior in an ouabain-administration model of mania.

PKC inhibitors have also been shown to possess antimanic-like effects in preclinical studies. Chelerythrine, an alkaloid that inhibits PKC, was shown to prevent manic phenotypes induced by amphetamine administration (Abrial et al., 2013). Armani et al. (2012) showed that treatment with lithium, tamoxifen, which is actually an estrogen receptor modulator with inhibitory activity over PKC, or their combination prevented manic-like behavior after sleep deprivation (SD), an animal model of mania. Zarate et al. (2007) showed that tamoxifen administration for three weeks in BD patients led to an improvement of their symptoms. Myricitrin, another PKC inhibitor, prevented the increase in ultrasonic vocalizations (USVs) after amphetamine administration (Pereira et al., 2014). Monotherapy with endoxifen, a metabolite of tamoxifen, which also inhibits PKC, was shown to be as effective as valproate in reducing manic symptoms (Ahmad et al., 2016).

In addition to its inhibitory activity over PKC, it is believed that the antimanic effect of lithium is also related to its inhibitory activity over GSK3β, as well as to its antioxidant properties (Chiu et al., 2013; Malhi et al., 2013; Valvassori et al., 2020). Thus, research on drugs with inhibitory activity over the enzymes GSK3β and/or PKC with antioxidant properties is relevant in the search for new alternatives for BD therapy.

6. Andrographolide

The diterpene andrographolide (ANDRO) is known to be an inhibitor of GSK3β (Serrano et al., 2014). This compound is the major bioactive constituent of the plant *Andrographis paniculata*, which has been used for centuries in the Traditional Chinese Medicine, as well as in the Traditional Thai Medicine, Japan, Scandinavia and Indonesia over the centuries (Lu et al., 2019; Jayakumar et al., 2013). The plant expands to a height of 30-110 cm, mostly in moist shady places with glabrous leaves, and white flowers with rose purple spots on the petals (Kandanur et al., 2019). The whole plant has medicinal value, but the leaves contain the highest levels of ANDRO (Sareer et al., 2014).

ANDRO is a labdane diterpenoid lactone, which was first reported in the Indian Gazette in 1951, as an active constituent of *A. paniculata*. But it was not until 1984 that ANDRO was first described as a potential therapeutic agent for liver injury (Choudhury and Poddar, 1984). Many therapeutic effects for several ailments are reported for ANDRO: anti-diarrheal, anti-viral, anti-malarial, antioxidant, anti-inflammatory, hepatoprotective, for sexual dysfunctions, for cardiovascular disorders, to treat carbuncles, ulcers, colitis, herpes and venomous snake bites (Lim et al., 2012; Bharati et al., 2011). The plant is used in more than twenty-five Ayurvedic formulas for the management of diarrhea, sore throat, tonsilitis, jaundice, liver disorders, dermatological diseases, among others. It is very relevant in the Indian Pharmacopeia (Pandey et al., 2019). The herb *A. paniculata* is present in the United States Pharmacopeia as a dietary supplement (Aromdee et al., 2014).

The main pharmacological properties of ANDRO are mostly related to its anti-inflammatory and antioxidant properties. Mittal et al. (2016) showed that ANDRO exhibited protective effects against H_2O_2-induced cell death, ROS and LPO in HepG2 cells. The authors indicate that ANDRO administration led to inhibition of GSK3β, which led to retention of Nrf2 in the nucleus as well as sustained expression of HO-1 by binding to its antioxidant response elements (Are). Several data have reported the antioxidant properties of ANDRO in *in vitro* and *in vivo* models. They involve ROS scavenging, protective effects of ANDRO on mitochondria, inhibition of free radical-producing enzymes, such as NADPH oxidase and xanthine oxidase, as well as activation of antioxidant systems involving superoxide dismutase (SOD), CAT and GPx (Mussard et al., 2019).

Besides its antioxidant property, another pivotal mechanism of action of ANDRO involves its anti-inflammatory properties. For example, Pan et al. (2017) demonstrated that the hepatoprotective effects of ANDRO are linked to its anti-inflammatory and antioxidant properties, as it was capable of improving liver histology, decreasing the levels of aspartate transaminase (AST), alanine transaminase (ALT), myeloperoxidase (MPO), MDA, IL-1β, TNF-α and ROS levels. Li et al. (2017) showed that ANDRO possesses a beneficial effect in models of rheumatoid arthritis, due to its anti-inflammatory properties. ANDRO decreased the severity of arthritis and joint destruction in collagen-induced arthritis mouse model by inhibiting the MAPK signaling pathway, by decreasing IL-6, IL-1β, TNF-α, expression in the serum and reducing the phosphorylation of p38 MAPK and ERK1/2 expression. Tan et al. (2016) showed that ANDRO reversed pulmonary immune cell infiltration and pro-inflammatory cytokine production (TNF-α, IL-1β, and CXCL1, for example) in an animal model of lung inflammation in mice.

Also, treatment with ANDRO showed promising results in neurodegenerative disorders, such as Parkinson's disease (PD) and Alzheimer's disease (AD) (Rivera et al., 2016; Zhang et al., 2014). Rivera et al. (2016) showed that ANDRO treatment resulted in recovery of synaptic basal transmission and partial or complete protection of certain synaptic proteins in animal models of cognitive impairment. Serrano et al. (2014) showed that ANDRO has the capacity to induce a protection of long-term potentiation (LTP) and synaptic proteins against Aβ oligomers in an Alzheimer's animal model and that ANDRO has the property to inhibit the long-term depression (LTD) in a concentration-dependent manner, showing an accumulation of β-catenin and a reduction in the active state of GSK3β.

ANDRO was shown to inhibit the enzyme GSK3β possibly by directly interacting with the GSK3β binding site. ANDRO activates the Wnt/β-catenin and induces the transcription of Wnt genes and the phosphorylation of GSK3β at Ser^{9}, leading to its inactivation thus stimulating Akt-dependent GSK3β signaling (Tapia-Rojas et al., 2015). Serrano et al. (2014) showed that ANDRO was capable of increasing the levels of the inactive form of GSK3β and also increasing the levels of anti-apoptotic β-catenin.

Currently, ANDRO and *A. paniculata* extracts are taken orally by the general population (Tan et al., 2017). ANDRO is being tested clinically for several disorders.

For example, *A. paniculata* was tested in a double-blind, placebo-controlled study for the treatment of acute upper respiratory tract infection (Gabrielian et al., 2002). Also, *A. Paniculata* composition Paractin(R) showed promising beneficial effects in a phase 2 clinical study by reducing the levels of rheumatoid factor, IgA and C4 in a randomized placebo-controlled clinical trial of rheumatoid arthritis (Clinical Trial Identifier: NCT00749645),

Natural products have been the primary source of a diversity of biologically active molecules, driving pharmaceutical discoveries for centuries (Kandanur et al., 2019). Taking into consideration that ANDRO has an inhibitory activity over the enzyme GSK3β, as the antimanic drug lithium does, it is therefore relevant to evaluate if ANDRO may also have an antimanic-like activity by testing its effects in animal models of mania/BD.

7. Quercetin

Quercetin is the most widely distributed flavonoid in nature. This flavonoid can be found in plants as aglicones or in their glycosylated form (Lakhanpal and Rai, 2007). Quercetin-type flavonols can be found in apples, berries, grapes, onions, tomatoes, potatoes, orange, lettuce, eggplant, black and green teas, as well as in many seeds, nuts, flowers and leaves of, for example, *Ginkgo biloba* and *Hypericum perforatum* (Li et al., 2016). Quercetin is a brilliant yellow needle crystal, insoluble in cold water and poorly soluble in hot water (Li et al., 2016). The solubility can be changed by alterations in the attached glycosyl group (quercetin glycoside) (Li et al., 2016). Although its antioxidant properties are its most studied and elucidated pharmacological effects, quercetin possesses several other therapeutic properties still in investigation. It is hypothesized that its therapeutic properties are linked to its interaction with proteins from intracellular signaling cascades, such as Akt, MAPK, PI-3K and PKC (Dajas, 2012; Williams et al., 2004; Agullo et al., 1997).

Quercetin appears to be one of the most powerful flavonoids to protect against ROS produced by the normal metabolism or induced by damage (De Groot, 1994). The antioxidant properties of quercetin are related to the presence of several OH groups and conjugating pi bond orbital, which act as electron or proton donors and H_2O_2 scavengers (Alrawaiq and Abdullah, 2014). Several studies demonstrate other biological effects of quercetin, rather than its antioxidant properties, such as

antinociceptive (Filho et al., 2008), anti-inflammatory (Comalada et al., 2005), anxiolytic, antidepressant (Bhutada et al., 2010), neuroprotective (Dajas et al., 2015), antiproliferative (Russo et al., 2012), antibacterial (Rigano et al., 2007), and anti-ulcerative (De La Lastra et al., 1994), among other properties.

In an *in vitro* study, for example, quercetin inhibited neuroinflammation in astrocytes and pure neuronal cultures by inhibiting the release of IL-6, IL-8 and ROS production (Sharma et al., 2007), displaying a neuroprotective effect. Maurya and Vinayak (2015) show that PKC activation partially depends on ROS signaling. Thus, quercetin treatment reduced the total ROS level and downregulated PKC activity in ascite cells of Dalton's lymphoma-bearing mice. In an animal model of AD, quercetin administration reduced Aβ production in the hippocampus of APP/PS1 mice, reversing spatial learning deficit by stimulating the CREB/BDNF signaling pathway (Hou et al., 2010). In an animal model of PD induced by intracisternal 6-OHDA administration, quercetin treatment diminished striatal dopamine depletion, inhibiting GSH depletion through its antioxidant properties and improving neuronal survival (Haleagrahara et al., 2013). Sriraksa et al. (2012) showed that quercetin treatment improved spatial memory in rats by inhibiting acetylcholinesterase (AChE) activity and diminishing neuronal damage due to oxidative stress after 6-OHDA administration in rats. Quercetin administration was shown to attenuate myeloperoxidase activity, diminishing neutrophil infiltration and restoring GSH levels in a model of traumatic brain injury in rats (Graham et al., 2000). The neuroprotective properties of quercetin were demonstrated by Jiang et al. (2016), who showed that pre-treatment with 5 or 10 μmol/L quercetin in HT22 hippocampal neurons protected the cells against okadaic acid-induced neuronal injury, decreasing the levels of MDA and increasing the levels of SOD and GPx, and increasing the levels of p-Ser^{9}-GSK3β, the inactive phosphorylated form of the enzyme, and stimulating Akt.

Taking into account that quercetin exerts an inhibitory activity over the enzyme PKC and that it possesses antioxidant effects, as the antimanic drug lithium does, it is worthy to evaluate if quercetin may also have an antimanic-like activity by testing its effects in animal models of mania in BD.

8. Animal models of BD

Animal models of BD must be employed in order to evaluate the possible antimanic-like effects of drugs. Animal models of human diseases should meet three sets of criteria: construct validity, face validity, and predictive validity. Construct validity refers to commonalities between the mechanism of the model and the human disorder. Face validity refers to commonalities between the features of the model and of the symptoms of human disorder, whereas predictive validity refers to the efficacy of treatment drugs used for human disease for the phenotype of the animal model (Kato et al., 2007). To date, several putative animal models of mania or depression have been reported. They are classified into different categories: pharmacological models, nutritional models, environmental models, and genetic models (Kato et al., 2007). One of the reasons for the lack of successful drug development in BD is the absence of an established animal model and difficulty in assessing the prophylactic effect of mood stabilizers (Kato et al., 2007).

The administration of ouabain, a Na^+/K^+ ATPase inhibitor, is a model of mania, as rats display hyperactivity after ouabain administration (Decker et al., 2000). Nutritional models of mania/BD can also be employed, such as homocysteine administration or n-3 polyunsaturated fatty acids deprivation, yet also leading to behaviors such as aggressiveness (DeMar et al., 2006; Levine et al., 2005). Environmental models are also applied, such as SD (Gessa et al., 1995), as aberrations of the sleep-wake cycle and circadian rhythms belong to primary symptoms of patients suffering from BD and are used as diagnostic criteria (Gonzalez et al., 2014). Genetic models, such as usage of specific animal strains can be employed (Einat, 2007). CLOCK knockout mice or transgenic mice overexpressing GSK3β showed hyperactivity (Roybal et al., 2007; Prickaerts et al., 2006) and mutPOLG transgenic mice display manic-like behavior (Kubota et al., 2006). However, the most commonly used model of mania is the single administration of psychostimulants such as amphetamine (Frey et al., 2006), metamphetamine (Gould et al., 2001) or lisdexamfetamine (LDX) dimesylate (Macêdo et al., 2013). They cause hyperactivity and these models have been used to test the efficacy of antimanic treatment such as lithium or valproate.

9. Methylphenidate-induced manic-like behavior

The administration of psychostimulants, such as methylphenidate, affects several neurotransmitter systems, which is in line with the fact that many neurotransmitters are affected in patients with BD (Beyer and Freund, 2017). The catecholaminergic system is mainly involved in mania-like symptoms, as elevated levels of dopamine or norepinephrine are observed in patients with BD (Berk et al., 2007). Psychostimulants increase synaptic dopamine and norepinephrine through inhibition or reversing the corresponding reuptake mechanisms (Berk et al., 2007). Psychostimulants not only induces mania-like behavior in animals but also cause manic symptoms in healthy humans and BD patients, such as decreased need for sleep, elevated mood, hypersexuality, affecting sensorimotor function, learning and memory, for example (Corp et al., 2014; Cousins et al., 2009; Berk et al., 2007; Asghar et al., 2003; Jacobs and Silverstone, 1986). Psychostimulants can also precipitate manic episodes in patients with BD (Young et al., 2011). Lithium and valproate can attenuate the amphetamine mania-relevant behavior (Frey et al., 2006; Flemenbaum et al., 1974)

Psychostimulant-induced hyperlocomotion is the most frequently used animal model of mania (Einat, 2006). This pharmacological induction of manic-like behavior is reliable and shows face, construct and predictive validity (Einat, 2006; Machado-Vieira et al., 2004). To measure hyperlocomotion, the number of crossings in the open-field can be analyzed as an index of locomotor activity. The blocking or attenuation of hyperlocomotion after methylphenidate administration is indicative of an antimanic-like effect, at doses that do not impair locomotor activity *per se* (Gould et al., 2001; Sabioni et al., 2008).

10. Sleep deprivation (SD)-induced manic-like behavior

The SD model of mania is also widely used, as SD often precedes manic episodes (Kato et al., 2007). After SD, animals show insomnia, hyperactivity, irritability, aggressiveness, hypersexuality, and stereotypy (Gessa et al., 1995). These behavioral changes were improved by haloperidol and prevented by lithium (Gessa et al., 1995). The objective of the protocol is that, when animals standing in platforms surrounded by water for 24h, 72h or 96h, reach REM sleep, they display muscle

relaxation, falling into the water and, thus, having their sleep interrupted (Machado-Vieira et al., 2004). After this period of SD, animals show some behaviors such as hyperlocomotion and an increase in appetitive USVs, which can be correlated with manic-like behaviors (Wendler et al., 2019; Gessa et al., 1995).

The SD model involves several neurochemical alterations, such as down-regulation of the expression of the enzyme tyrosine hydroxylase in the substantia nigra pars compacta, as well as the decrease of dopaminergic neurotransmission in substantia nigra pars compacta and striatum (Lima et al., 2012), an increase of the expression of D_2 receptors in the striatum (Lima et al., 2007), as well as supersensibility of dopaminergic receptors (Tufik et al., 1978).

This non-pharmacological induction of manic-like behavior is reliable and shows face, construct and predictive validity (Einat, 2006; Gessa et al., 1995).

11. Lisdexamfetamine (LDX)-induced increases in 50-kHz USVs

The administration of the psychostimulant LDX dimesylate is another model of mania. LDX dimesylate is a long-acting *d*-amphetamine pro-drug employed in the therapeutic management of attention deficit/hyperactivity disorder (ADHD) (Ermer et al., 2016). It was developed to improve the therapeutic effects of stimulants such as *d*-amphetamine or methylphenidate, which are long-established treatments for ADHD, but of which the effects are not long lasting (Swanson et al., 2011). In the LDX molecule, a peptide links the amino group of *d*-amphetamine to the carboxyl group of L-lysine. After absorption in the small intestine, enzymatic hydrolysis by an unknown erythrocyte peptidase of this peptide bond releases the active *d*-amphetamine and the byproduct L-lysine (Ermer et al., 2016). *D*-amphetamine acts primarily by increasing the synaptic levels of dopamine and norepinephrine (Ward and Citrome, 2018). In adults with ADHD, the half-life of LDX is 0.5 hours, and the half-life of the *d*-amphetamine released is 17 hours, which allows a single daily dose (Adler et al., 2017).

As psychostimulant administration is a model of mania, LDX administration has also been used to mimic manic-like behavior in animals. Bristot et al. (2019) showed that 10 mg/kg once daily for 14 days LDX administration caused hyperlocomotion, which was reversed by a 7-day pre-treatment with 47.5 mg/kg lithium. Eger et al. (2016) used LDX administration as a model of mania to induce hyperlocomotion and

oxidative imbalance in rats, which were reversed by chronic pre-treatment with simvastatin. In addition to hyperlocomotion, Souza et al. (2015) showed that LDX administration led to oxidative stress, by causing depletion of GSH and induction of LPO in the PFC and striatum of rats, which were prevented or reversed by valproate.

Another parameter that can be analyzed in order to evaluate changes in behavior is alterations in the emission of USVs by the animal. Wendler et al. (2016) used LDX administration as model of mania to induce increases in 50-kHz USVs in rats. Studies show that psychostimulants and SD induce increases in 50-kHz USVs in rats, which can be inhibited by drugs used in the management of BD, such as antipsychotics and lithium (Wendler et al., 2019; Wendler et al., 2016; Barker et al., 2015; Rippberger et al., 2015; Pereira et al., 2014). Therefore, the evaluation of the effects of drugs on psychostimulant (e.g., LDX)-induced increases in 50-kHz USVs is of great relevance in the research for antimanic drugs.

Rodents can spontaneously emit USVs that occur above the range of human hearing (> 20 kHz) in positive and negative contexts to express emotional states or for communication with other rodents (Brudzynski, 2015; Burgdorf et al., 2011; Knutson et al., 2002). Adult rats emit high-frequency 50-kHz USVs in appetitive situations, such as playing with other rats, mating or due to psychostimulant administration (Burgdorf et al., 2011) Under aversive situations, such as painful or stressful stimuli, rats emit low-frequency 22-kHz USVs (Wöhr and Schwarting, 2013). Therefore, both appetitive 50-kHz and aversive 22-kHz USVs represent different affective and behavioral aspects of rats (Wöhr and Schwarting, 2013). Different types of USVs occur at different ages, with 40-kHz USVs predominating during infancy, and 22-kHz and 50-kHz USVs occurring during adolescence and into adulthood and then waning in frequency with senescence (Knutson et al., 2002).

40-kHz USVs have been most extensively documented in rat pups that have been separated from their mothers, and they range from 80 to 140 ms (Knutson et al., 2002). Although they have an average of 40-kHz, they can range from 30 to 65-kHz in frequency. Longer 22-kHz USV which are emitted by juvenile and adult rats during exposure to predators, aversive conditions, exposure to pain or threat cues are longer (ranging from 300 to 3000 ms) and lower in sound frequency (ranging from 18 to 32-kHz, with a typically narrow bandwidth of 1 to 6-kHz) (Brudzynski et al., 1993). The emitted shorter and higher-pitched 50-kHz USVs of juvenile and adult rats during playful activities, “rough-and-tumble" play, male and female social exploration

(Brudzynski and Pniak, 2002), locomotor activity, and rearing and exploration (Fu and Brudzynski, 1994), are short in length, range from 20 to 80 ms, and have a higher in sound frequency (ranging from 35 to 70-kHz with a bandwidth of 1 to 6-kHz) (Blanchard et al., 1993).

Based in frequency profile and call duration, three subtypes of appetitive calls (50-kHz USVs) have been described so far: flat calls, frequency modulated (which can be subdivided into step and mixed calls) and trill calls (Brudzynski, 2015), which can be seen in Figures 1 and 2. Both flat and frequency modulated remain within the same peak frequency range but differ in the sonographic profile and duration (Brudzynski, 2015). Flat 50-kHz calls appear to be a contact call, occurring at higher rates during non-positive affective social interactions (Burgdorf et al., 2008), however, frequency-modulated 50-kHz calls seem to be selective for positive affective social interactions (Burgdorf et al., 2011). Step calls are two adjoining frequencies with a jump (or step) between them (Grant et al., 2018). Trills, representing fast sin wave-like oscillations of the call frequency, were regarded as expressions of the highest state of arousal and motivation (Burgdorf et al., 2008). Repeated application of amphetamine causes sensitization with increased emission of trill-type 50-kHz USVs but not for flat 50-kHz calls (Ahrens et al., 2009).

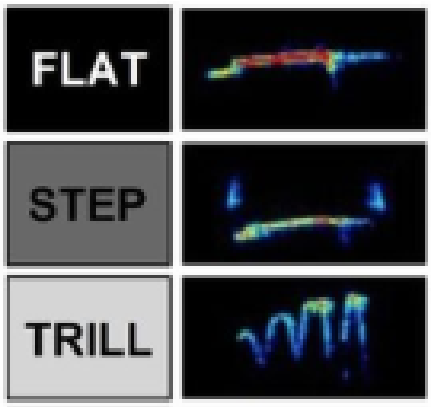

Fig. 1: Examples of flat, step and trill 50-kHz USVs. Adapted from: Kisko et al., 2018

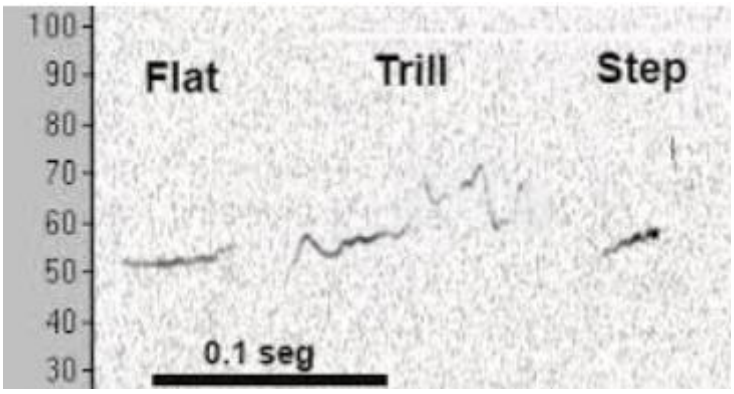

Fig. 2: Spectrograms of flat, trill and step 50-kHz USVs. Adapted from: Trein, 2017

Highly arousing aversive stimuli such as predatory odor, foot shock and bright light, decrease rates of 50-kHz USV emission, whereas rewarding stimuli increases rates of 50-kHz calls (Knutson et al., 2002, Burgdorf et al., 2011). The discovery that 50-kHz USVs reflect a positive state in rats allows these measures to be used effectively to monitor hedonic states in animal models of various psychiatric disorders (Burgdorf et al., 2011). The increased emission of 50-kHz induced by amphetamine administration is usually parallel to hyperlocomotion (Ahrens et al., 2013). Other psychostimulants, which strongly affect catecholamine release, such as cocaine, also increase 50-kHz USV emission rates (Wright et al., 2012). Peripheral or central administration of amphetamine unconditionally increases emission of 50-kHz USVs in a dose-dependent manner (Wintink and Brudzynski, 2001; Burgdorf et al., 2001). This effect can be reversed by peripheral injection of the dopamine antagonist haloperidol (Wintink and Brudzynski, 2001).

Rippberger et al. (2015) showed that *d*-amphetamine administration led to an increase in all subtypes of 50-kHz USVs. Drugs used in the management of BD, such as antipsychotics (e.g., risperidone and haloperidol), lithium and tamoxifen were shown to inhibit the increases in 50-kHz USVs induced by *d*-amphetamine or LDX (Wendler et al., 2016; Barker et al., 2015; Rippberger et al., 2015; Pereira et al., 2014). Pereira et al. (2014) studied the effects of the antimanic drugs lithium, the PKC-inhibitor tamoxifen and the nitric oxide inhibitor myricitrin on amphetamine-induced increases in 50-kHz USVs and hyperlocomotion in adult male rats. Lithium, tamoxifen and myricitrin abolished amphetamine-induced increases in 50-kHz USVs and reversed amphetamine-induced hyperlocomotion.

Aim and Outline of the Thesis

The aim of the thesis was to investigate the possible antimanic-like effect of chronic treatment with ANDRO and quercetin in animals submitted to different models of mania.

Chapter 2 is a review regarding the effects of ANDRO in disorders of the Central Nervous System, focusing on the effects of ANDRO in psychiatric disorders, in particular anxiety and mood. It was noted that ANDRO had not been tested for possible antimania effects yet, though it is evident that ANDRO has therapeutic potential for the treatment of BD. Therefore, ANDRO was tested in three models of BD (**chapter 3 and 4**). Besides ANDRO, quercetin is an interesting compound, which already attracted attention as a possible antimania drug. Therefore, we further investigated this compound in two models of BD (**chapters 5 and 6**). To briefly summarize the experiments:

In **chapter 3**, the results are shown of the experiments involving the chronic treatment with ANDRO in mice subjected to the SD- and methylphenidate-administration models of mania. The read-out effects of ANDRO were on SD- and methylphenidate-induced hyperlocomotion as well as on the levels of GSK3β and p-Ser^{9}-GSK3β in samples of PFC and striatum.

In **chapter 4**, the effects are evaluated of chronic treatment with ANDRO on the increase of 50-kHz USVs and hyperlocomotion elicited by administration of LDX as a rat model of mania.

In **chapter 5**, findings are described regarding the effects of quercetin on SD-induced hyperlocomotion and oxidative stress parameters (GSH and LPO levels) in the PFC, hippocampus and striatum of mice.

In **chapter 6**, results are shown regarding the effects of quercetin on methylphenidate-induced hyperlocomotion and oxidative stress parameters (GSH and LPO levels) in the PFC, hippocampus and striatum of mice.

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CHAPTER 2

Overview of the effects of andrographolide on disorders of the Central Nervous System

Kanazawa, L. K. S.[a]; Nelissen, E. [b], Prickaerts, J.[b] Andreatini, R.[a]

[a] *Laboratory of Physiology and Pharmacology of the Central Nervous System, Department of Pharmacology, Federal University of Paraná, Centro Politécnico, 81540-990 Curitiba, PR, Brazil*

[b] *Department of Psychiatry and Neuropsychiatry, School for Mental Health and Neuroscience (MHeNS), Maastricht University, Universiteitssingel 50, 6229 ER Maastricht, The Netherlands*

ABSTRACT

Andrographolide (ANDRO) is the major bioactive constituent of the plant *Andrographis paniculata*. This plant has been widely used for centuries for its several therapeutic properties. Preclinical studies show that ANDRO possesses anti-inflammatory and antioxidant properties, and is capable of inducing neurogenesis. In addition, ANDRO exhibits neuroprotective and antiapoptotic effects. These effects are mechanistically linked to increased expression of several neuroprotective proteins such as BDNF and Akt, as well as the inhibition of several anti-neuroplasticity proteins, such as GSK3β. These effects and mechanisms of actions have been linked to the attenuation of symptoms of several diseases and disorders. As for disorders of the central nervous system, many pre-clinical studies indeed demonstrate therapeutic potential of ANDRO for Alzheimer's disease, Parkinson's disease, multiple sclerosis, and depression. ANDRO is also already being tested in first clinical trials which makes it an even more interesting drug for further studies on its exact mechanism of action and to determine its potential efficacy for the management of neurodegenerative and neuropsychiatric disorders.

Key words: *Andrographis paniculata,* andrographolide, anxiety, central nervous system, depression, mood, neurodegenerative diseases, psychiatric disorders.

1. INTRODUCTION

The plant *Andrographis paniculata* (Burm.f.) Wall. ex Nees (*Acanthaceae*) has been used for centuries mainly in Asian traditional medicine. It is native to India, China and Sri Lanka, but can also be found in other Asian countries such as Taiwan, Malasia, Thailand, Pakistan and Indonesia, and in tropical areas of America and some countries in Africa (Hossain et al., 2014; Okhuarobo et al., 2014; Jayakumar et al., 2013). The genus *Andrographis* comprises more than 40 species of plants and *A. paniculata* is the most used in popular medicine. *A. paniculata* is an annual herbaceous plant that is branched, with an erect dark green stem, growing about 30 – 110 cm tall. It has white flowers with purple spots on the petals and it is known as "King of Bitters", due

to its bitter taste (Gupta et al., 2017; Tan et al., 2017; Lim et al., 2012). The whole plant has been used in Ayurvedic medicine for the treatment of several ailments such as dyspepsia, dysentery, influenza, and for snakebites and stings from insects (Okhuarobo et al., 2014). More specifically, the roots have been used as antihelmintic, antipyretic and stomachic, while the aerial parts have been used for the treatment of common cold, hypertension and urinary tract infection, among other ailments (Okhuarobo et al., 2014; Saxena et al., 1998). The leaves of the plant have been used to treat common cold, fever, gastrointestinal disorders, respiratory infections, and sores (Okhuarobo et al., 2014; Saxena et al., 1998).

The leaves of *A. paniculata* comprise the highest concentration of the major bioactive constituent of the plant: andrographolide (ANDRO), although it is also found in lesser concentrations in other parts of the plant (Jayakumar et al., 2013). ANDRO is a labdane diterpenoid lactone from the isoprenoid family that has the chemical formula $C_{20}H_{30}O_5$ (Islam et al., 2018). ANDRO was first isolated in a pure crystalline state, as the active constituent of *A. paniculata* by Gorter in 1911. In 1951, as reported in the Indian Medical Gazette, Chakravarti and Chakravarti described the usage of the roots and leaves of *A. paniculata* in cases of loss of appetite, bowel disorders and as febrifuge and antihelmintic, and they also elucidated the chemical structure of ANDRO ((3-[decahydro-6-hydroxy-5-hydroxymethyl)-5,8a-dimethyl-2-methylene-1-napthalenyl]ethylidene-4-hydroxy-2(3H)-furanone) (Chakravarti and Chakravarti, 1951). In 1984, laboratory tests were performed for the first time to evaluate the biological effects of ANDRO. In these tests, ANDRO was capable of reducing hepatic microsomal lipid peroxidation (LPO) induced by carbontetrachloride administration in male rats and also *in vitro* (Choudhury and Poddar, 1984). Ever since, several *in vitro*, *in vivo* and clinical studies have confirmed various pharmacological activities of *A. paniculata* extracts as well as ANDRO. In recent times, commercial preparations of this plant extract are also available as herbal supplements for health promotion (Hossain et al., 2014; Jarukamjorn and Nemoto, 2008).

2. EFFECTS OF ANDRO ON DISORDERS OF THE NERVOUS SYSTEM

Neurodegenerative disorders involve progressive dysfunction of neurons, oligodendrocytes or Schwann’s cells among others, which lead to disease-specific

symptoms as a consequence of impairments in the central nervous system (CNS) or peripheral nervous system (PNS) (Radi et al., 2014). ANDRO is known to possess anti-inflammatory and antiapoptotic properties, as well as offering protection against oxidative stress, which can all be very beneficial in the context of neurodegenerative disorders (Graverini et al., 2018; Yang et al., 2017).

2.1 Effects of ANDRO on brain oxidative stress

Due to various chemical reactions from diverse signaling pathways constantly occurring, the brain is susceptible to oxidative stress (Cobley et al., 2018). Glucose metabolism, glutamatergic neurotransmission, neurotransmitter auto-oxidation, microglial activation, and imbalanced mitochondrial activity increase the accumulation of reactive oxygen and nitrogen species (ROS and RNS, respectively) and oxidative stress (Cobley et al., 2018). The excessive production of ROS and RNS is involved in several acute and chronic pathological processes, including neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD) (Liguori et al., 2018). The vulnerability of the brain to oxidative stress can be due to its deficient endogenous antioxidant defense, high levels of polyunsaturated fatty acids, and high consumption of O_2 (Das et al., 2009). Therefore, antioxidant therapy may be helpful in the attenuation of oxidative damage (Liguori et al., 2018).

Lindsay et al. (2020) demonstrated that ANDRO significantly reduced the total amyloid-β (Aβ) levels, as well as astrogliosis and IL-6 levels in the brains of aged *Octodon degus*, used as models of AD as this is the only rodent that naturally develops AD-like pathology with age. ANDRO also reduced the levels of the oxidative stress markers 4-hydroxynonenal and N-tyrosine adducts in the hippocampus.

ANDRO (2.5 mg/kg p.o.) decreased blood glucose levels and also reduced the catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities in the hypothalamus, hippocampus, cerebellum and cerebral cortex of diabetic Sprague Dawley rats (Naik et al., 2017). ANDRO (250 mg/kg/day for 7 days i.p.) was capable of reversing nicotine-induced inhibition of mitochondrial electron transport chain complexes (I, II, III), overproduction of nitric oxide (NO) and suppression of mitochondrial oxidative stress scavenger system (CAT, glutathione reductase (GR), glutathione-S-transferase (GST) and glutathione (GSH), for example)

in different rat brain regions, such as the cerebral hemisphere, cerebellum and diencephalon (Das et al., 2009). The incubation of primary astrocyte cultures with 50μM ANDRO also upregulated the antioxidant molecule heme oxygenase-1 (HO-1) expression and potently activated its transcription factor, the nuclear factor erythroid 2 (Nrf2). This is related to ANDRO's effect on astrocyte-mediated antioxidant and anti-inflammatory responses and supports the potential of ANDRO as a therapeutic agent for neurological conditions that involve oxidative stress and neuroinflammation (Wong et al., 2016a).

2.2 Effects of ANDRO on neuroinflammation and apoptosis

Neuroinflammation has been implicated as a pathological factor in many neurodegenerative diseases. Overactivation of glial cells' pro-inflammatory activity may lead to increased cytokine release and ROS, which may result in CNS injury (Schain and Kreisl, 2017). Therefore, the anti-inflammatory effects of ANDRO could be beneficial for inflammation-related neurodegenerative disorders (Yang et al., 2017).

Pre- and post-treatment with ANDRO exhibited a significant protective effect against lipopolysaccharide (LPS)-induced neurotoxicity in mixed neuron-glia cultures, by attenuating LPS-induced microglial activation and production of ROS, TNF-α, NO, and prostaglandin E2 (PGE_2). Furthermore, ANDRO dose-dependently attenuated LPS-induced inducible nitric oxide synthase (iNOS) and ciclooxigenase-2 (COX2) expression in BV-2 microglia (Wang et al., 2004). Wong et al. (2014) demonstrated that ANDRO pretreatment (30 and 50 μM) of cultured rat primary astrocytes followed by incubation with IL-1β, attenuated the upregulation of the leukocyte chemoattractant chemokine C-C motif ligand 5 (CCL5). This effect could be related to ANDRO's capacity of reducing the phosphorylation of NF-κB, p65, and IκBα after IL-1β stimulation. Chemokines are divided into four main families (C-C, C-X-C, C-X3-C and C) and they are upregulated and secreted by primary microglia and astrocytes after IL-1β or TNF-α release during an inflammatory response (Ransohoff and Brown, 2012; de Haas et al., 2007). Despite the essential function of chemokines, dysregulation in their levels is implicated in neurological diseases and disorders such as AD, multiple sclerosis (MS) and traumatic brain injury (TBI) (Gyoneva and Ransohoff, 2015; Liu et al., 2014; Kerstetter et al., 2009). Thus, the anti-inflammatory effect of ANDRO in the context of inhibiting the upregulation and release of chemokines in the brain can be

beneficial for neurological disorders. Wong et al. (2016b) demonstrated that oral administration of ANDRO (25 or 50 mg/kg) 1h after intraperitoneal injection of LPS was also capable of attenuating mouse cortical C-C (CCL-2 and CCL-5) and C-X-C (CXCL-1, CXCL-2 and CXCL-9) chemokine levels. Also, 48h-incubation with ANDRO 4h before (10, 30 or 50 μM) or 4h after (50 μM) LPS incubation, caused abrogation of LPS-induced chemokines mRNA upregulation, as well as TNF-α in rat astrocytes (Wong et al., 2016b). 1h pre-treatment with ANDRO (5-10 μM) significantly protected primary microglia and BV-2 microglial cells against $A\beta_{(1-42)}$ toxicity and attenuated the release of TNF-α, IL-1β, NO, and PGE_2 by inhibiting the nuclear translocation of NF-kB by affecting IkB phosphorylation. It also downregulated the protein levels of iNOS and COX2 in microglial cells (Yang et al., 2017). In addition, cerebral endothelial cells of C57/BL6 mice were stimulated with LPS which subsequently increased the expression of pro-inflammatory enzymes, such as COX2 and iNOS. However, co-treatment with ANDRO markedly decreased the levels of both enzymes (Chang et al., 2014).

Apoptosis is a selective cell deletion process that is necessary for homeostasis. However, dysfunctions in the cell death program play a role in several human diseases, including neurodegenerative disorders such as AD, PD and HD, as a consequence of tissue damaged caused by increased apoptosis (Radi et al., 2014). Caspases play a pivotal role in apoptosis, as cytochrome c induces cleavage and activation of pro-caspase-9, which stimulates caspase-3 to induce degradation of several proteins and programmed cell death (Riedl and Shi, 2004). One of the main families of proteins involved in apoptosis is the Bcl-2 family, which comprises anti-apoptotic (Bcl-2, Bcl-XL, Mcl-1) or pro-apoptotic (Bid, Bax, and Bad) proteins (Cory and Adams, 2002).

Tzeng et al. (2012) showed that ANDRO and its derivative 14-deoxy-11,12-didehydroandrographolide reduced pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) levels, oxidative stress and chondroitin sulfate proteoglycans (CSPG) in H_2O_2-treated pheochromocytoma cell line 12 (PC12) cells in a TNF-α stimulated astrocyte conditioned medium. ANDRO (20 μM) administration is also capable of protecting and rescuing PC12 cells from $A\beta_{(1-42)}$-induced cell death and restoring alterations in nuclear morphology (Gu et al., 2018). In this study, ANDRO reduced the level of lactate dehydrogenase, malondialdehyde (MDA), intracellular ROS, NO, Bax and p-tau in

PC12 cells after $A\beta_{(1-42)}$ incubation. In addition, ANDRO administration increased the levels of the autophagy-related proteins beclin-1, p62, Atg (autophagy-related gene) 5 and 7 and AMBRA1 (a beclin-1 regulator). Finally, ANDRO increased the levels of Nrf2, which plays an important role in cell protection against oxidants, and also of p21, an anti-apoptotic protein.

The anti-inflammatory and antiapoptotic effects of ANDRO are corroborated by the results of Das et al. (2017). They showed that *in vitro* administration of ANDRO (1 μg/mL in Dulbecco's modified Eagle medium) in microglial cell culture, or in vivo, in BALB/c mice (1 mg/kg 3 times/week for one week i.p.), inhibited LPS-induced overexpression of high-mobility group box-1 protein (HMGB1), toll-like receptor 4 (TLR4), NFκB, COX2, iNOS and nitrite expression. Also, it decreased LPS-induced overexpression of protein markers like protein kinase C (PKC), p-CREB, Aβ, amyloid precursor protein (APP), p-tau, Bax, caspase-1 and -3, NLRP3. It also decreased the levels of TFN-α, IL-6, TGF-β and IL-1β, while increasing the levels of IL-10 and Bcl-2. In addition, ANDRO increased the levels of synapsin and post synaptic density-95 (PSD-95). Seo et al. (2017) showed that ANDRO (5 or 10 μM) increased the levels of Nrf2, nuclear Nrf2 and HO-1 in immortalized mouse hippocampal HT22 cell lines transfected with $A\beta_{42}$ plasmids. In addition, in murine microglial BV-2 cells, ANDRO (1, 5 or 10 μM) decreased intracellular levels of $A\beta_{42}$, IL-6, IL-1β, PGE_2, NO, iNOS, COX-2 and p-NF-kB. This can be due to the ability of ANDRO of inducing the expression and translocation of Nrf2 from the cytoplasm to the nucleus, enhancing HO-1 expression, as previously mentioned. Taken together, all these effects of ANDRO demonstrate its beneficial potential in the management of neurodegenerative diseases and psychiatric disorders with neuroinflammatory background, such as AD.

2.3 ANDRO and Alzheimer's disease

AD is the most prevalent neurodegenerative disorder and it is characterized by progressive cognitive impairment and its pathophysiology involves increased levels of amyloid-β and tau proteins (Kumar and Singh, 2015). An important model of AD is the use of APPswe/PS1ΔE9 transgenic mice, which display amyloidosis and, consequently, a progressive inflammatory response (Ruan et al., 2009). Serrano et al. (2014) showed that *in vivo*, ANDRO (2 mg/kg i.p.) prevents changes in neuropathology by reducing Aβ aggregates, tau phosphorylation and p-Tyr^{216}-GSK3β (the active form

of the enzyme) in the brain of 7-month-old AβPPswe/PS1 mice. ANDRO also increased the levels of PSD-95, GluA2, Shank and GluN2B. In the hippocampus and cortex, ANDRO increased the levels of p-Ser^{9}-GSK3β, as well as the levels of total and activated β-catenin. ANDRO recovered cognitive function of AβPPswe/PS-1 mice, in behavioral tests such as the memory flexibility test and the Morris water maze test. *In vitro* experiments showed that 10μM ANDRO increased synaptic transmission and protected LTP against Aβ oligomers (1 μM) in hippocampal slices. Also, ANDRO was capable of recovering the capacity of AβPPswe/PS1 mice hippocampal slices to induce LTP in comparison to AβPPswe/PS1 controls. This was seen as ANDRO increases slope of field excitatory postsynaptic potentials (fEPSP) in the CA1 region of hippocampal slices. The *in vitro* experiments also showed that in the hippocampal slices, ANDRO decreased the levels of p- Tyr^{216}-GSK3β and increased the levels of p-Ser^{9}-GSK3β and β-catenin, as well as inhibiting LTD induction.

In regards to the latter mechanism of action, several studies show that multiple beneficial effects of ANDRO may be related to the inhibitory activity of ANDRO on GSK3β. Tapia-Rojas et al. (2015), for example, showed that ANDRO treatment in both primary rat hippocampal neurons or HEK293 cells causes specific inhibition of GSK3β, as indicated by the increase in the inhibited form of the enzyme (p-Ser^{9}) and the decrease in the active form (p-Tyr^{216}). This correlates with an accumulation of β-catenin in a concentration-dependent manner. *In vivo* tests show that chronic ANDRO administration (2 mg/kg) in rats significantly decreased the level of phosphorylated β-catenin in Ser^{33}, Ser^{37} and Thr^{41} (phosphorylation in these epitopes leads β-catenin for ubiquitination and proteasomal degradation), at the same time increasing the levels of the inactive form of GSK3β (p-Ser^{9}) (Tapia-Rojas et al., 2015). The inhibition of GSK3β may be correlated to several pathways involved in neuronal survival and neuroprotection, such as protection against the damages caused by Aβ oligomers (Silva-Alvarez et al., 2013). The presence of Aβ oligomers induces an increase in GSK3β, and this facilitates LTD and the internalization of AMPA receptors from the postsynaptic region, consequently reducing the synaptic strength. However, ANDRO induces GSK3β inhibition, which, in turn, reduces PSD-95 phosphorylation, preventing the internalization of AMPA receptors and hindering the effect of Aβ oligomers on synaptic depression (Serrano et al., 2014). ANDRO also induces the translocation of β-catenin to the nucleus and the transcription of Wnt target genes (Tapia-Rojas et al.,

2015). In addition, studies demonstrate that the Wnt signaling, which can be activated after GSK3β by lithium, for example, reduces spatial memory impairment and neurodegeneration (Toledo and Inestrosa, 2010). In this context, Cisternas et al. (2018) showed that *in vitro* (50 μM) and *in vivo* (2 mg/kg i.p.) administration of ANDRO activates Wnt signaling and improves glucose utilization by increasing the expression and/or activity of hexokinase, phosphofructokinase and also the ATP and glycolytic rate levels in the brains of APPswe/PS1ΔE9 transgenic mice. In addition, in behavioral tests, ANDRO improved locomotor activity in the large open-field test, and improved spatial and recognition memory in the novel object recognition, novel object localization and memory flexibility tests with APPswe/PS1ΔE9 transgenic mice.

Varela-Nallar et al. (2015), also evaluated the effects of ANDRO (2 mg/kg i.p.) in APPswe/PS1ΔE9 transgenic mice. ANDRO increased neural progenitor cell proliferation (Nestin+/Ki67+ cells) and neurogenesis (BrdU+ and Ki67+ cells) in the subgranular zone of mice. Moreover, ANDRO increased cell proliferation and the density of immature neurons in the dentate gyrus. In addition, ANDRO induced the activation of the Wnt signaling pathway in the hippocampus of wild-type and APPswe/PS1ΔE9 mice, increasing the levels of β-catenin and of NeuroD1, a Wnt target gene involved in neurogenesis. Tapia-Rojas et al. (2016) showed that ANDRO, activating Wnt signaling reduces the levels of $A\beta_{(1-42)}$ increases Aβ40 levels, and reduces the Aβ42/Aβ40 ratio. These protective effects of ANDRO are related to activation of the Wnt pathway, from which ANDRO increased the levels of β-catenin and calcium/calmodulin-dependent protein kinase type IV (CAMKIV).

ANDRO sulfonate (2.5 or 5 mg/kg/day), given to the APP/PS1 transgenic mice in their drinking water for 7 months before the onset of Aβ plaque, prevented cognitive decline in spatial memory test. ANDRO increased the time in the target quadrant and decreased escape latency in the Morris water maze. ANDRO also increased the time in the new arm in the Y maze, as well as the time in the central area in the open-field test. In addition, ANDRO increased BDNF, synaptopodin, GDNF and NGF levels. Regarding oxidative stress factors, ANDRO increased SOD, CAT and decreased MDA levels. Finally, ANDRO increased ATP production and attenuated mitochondria swelling (Geng et al., 2017).

Without using transgenic mice, Rivera et al. (2016) showed that the beneficial effects of ANDRO were reproducible in *Octodon degus*. ANDRO (2 or 4 mg/kg i.p.)

was administered in aged degus (56-month-old) for 3 months and it led to recovery of spatial memory and learning performance in the novel local/object recognition and Barnes maze test. In addition, ANDRO led to recovery of synaptic basal transmission by reversing impaired hippocampal synaptic plasticity. Moreover, ANDRO increased the levels of GluN2A, VGluT1, PSD95 and decreased the levels of phosphorylated tau, Aβ40, Aβ42 and amyloid plaques in aged degus.

Another important aspect of AD is that cholinergic neurotransmission is severely decreased (Waite, 2015). Acethylcholinesterase (AchE) inhibitors, such as tacrine, donepezil, rivastigmine and galantamine are employed for the management of AD, as they increase acetylcholine levels. However, these drugs do not stop neuronal loss and the deterioration of cognitive function in AD patients (Godyn et al., 2016). Therefore, the research for new therapeutic strategies for the management of AD is necessary. In this context, Thakur et al. (2016) demonstrated that *A. paniculata* leaf extract (50, 100 or 200 mg/kg) or ANDRO (15, 30 and 60 mg/kg) oral administration in STZ-induced diabetic rats were capable of attenuating cognitive deficits, as seen by reductions in the escape latency in the Morris water maze test. Treatment with *A. paniculata* extract or ANDRO also reduced AchE activity in both pre-frontal cortex (PFC) and hippocampus, as seen by a photometric method using acetyltiocholine as a substrate. In addition, the treatments attenuated oxidative stress in the PFC and hippocampus of diabetic rats, by decreasing LPO levels and increasing CAT and SOD activity.

Taken together, these results indicate that ANDRO may have a beneficial effect for AD, as its mechanism of action attenuated important detrimental factors in AD pathophysiology.

2.4 ANDRO and Parkinson's disease

PD is a progressive neurodegenerative disorder characterized by the degeneration of dopaminergic neurons from the nigroestriatal pathway and the presence of Lewy bodies (Connolly and Lang, 2014; Deumens et al., 2002). The available pharmacological therapies, such as levodopa, monoamine oxidase inhibitors or dopamine agonists act mainly by counteracting the consequences of the loss of dopamine (Connolly and Lang, 2014). These drugs can improve quality of life for

patients, but they do not alter the underlying neurodegenerative process (Connolly and Lang, 2014). Thus, an alternative therapy for PD would be of great relevance.

In this context, Zhang et al. (2014) showed that the ANDRO-lipoic acid conjugate AL-1 significantly prevented 1-methyl-4-phenylpyridinium (MPP)-induced neurotoxicity in SH-SY5Y cells and primary cerebellar granule neurons. In a mouse model of PD, AL-1 rescued 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced loss of tyrosine hydroxylase-positive neurons. It also counteracted the MPTP-induced impairments of locomotor activity and gait and postural patterns, as seen in the open-field and Catwalk tests. AL-1 administration also elevated the striatal levels of dopamine and its metabolite 3,4-dihydroxyphenylacetic acid. Furthermore, AL-1 remarkably lowered the NO and MDA levels and increased the SOD level in the substantial nigra of MPTP-treated mice. AL-1 also scavenged free radicals such as $^{*}OH$, O_2^{*-}, $ONOO^-$ and $DPPH^-$, suggesting an attenuating effect of AL-1 on oxidative stress. The immunoblotting data showed that AL-1 significantly ameliorated the decreased expression of tyrosine hydroxylase (TH) protein in the substantia nigra and inhibited the up-regulation of phosphorylated NF-κB p65 *in vitro* and *in vivo*. Thus, ANDRO or its conjugates could be interesting to protect the nigrastiatal system from oxidative stress and attenuate its dopaminergic cell loss.

2.5 ANDRO and stroke

Ischemic brain injury inflicted by stroke is among the leading causes of death and disability worldwide (Soares et al., 2016). Cerebral ischemia is characterized by an interruption of the brain blood flow and decreased oxygen and glucose supply to the neurons, leading to neuronal death and the pathogenesis involves complex oxidative stress-related pathways, such as the Nrf2 and HO-1 pathways (Soares et al., 2016; Yen et al., 2013).

Du et al. (2018) demonstrated that 10μM ANDRO reduced cell apoptosis in hypoxia-injured mouse cortical astrocytes (C8-D1A). ANDRO was capable of reducing the levels of Bax as well as the ratio of pro/cleaved caspases 3 and 9, while increasing the levels of Bcl-2, which demonstrates an attenuation of apoptosis of hypoxic astrocytes. In addition, ANDRO increased the levels of pro-autophagic Beclin-1 and LC3-II, and lowered the levels of p62, showing that ANDRO promoted autophagy in hypoxic astrocytes. Autophagy (or type II programmed cellular death) involves

degradation of cellular components when homeostasis is disturbed (Mizushima and Komatsu, 2011). Thus, by promoting autophagy, ANDRO plays a protective role (Du et al., 2018). The neurotrophic factor S100B expression was upregulated after ANDRO administration as well as proteins from the JNK pathway, which regulates autophagy (Du et al., 2018).

Within this framework, Chan et al. (2010) demonstrated by histological analysis that i.p. administration of ANDRO in rats, 1 h after permanent middle cerebral artery occlusion (pMCAO), reduced infarct volume. In correlation, neurological deficits were also reduced by ANDRO. Treatment with ANDRO also significantly reduced pMCAO-induced activation of microglia and elevation of TNF-α, IL-1β and PGE_2 levels in the ischaemic brain areas. In addition, ANDRO suppressed the translocation of p65 from cytosol to nucleus, indicating by reduced NF-κB activation. These neuroprotective effects exhibited by ANDRO may have therapeutic value in the treatment of stroke.

Chern et al. (2011) showed that treatment of mice that have undergone cerebral ischemic/reperfusion (CI/R) injury with ANDRO (10 or 100 μg/kg i.v.) ameliorated CI/R-induced oxidative/nitrosative stress (increased ROS production and increased protein nitrosylation), brain infarction, and neurological deficits in the mice, and enhanced their survival rate. ANDRO reversed the enhanced expression of NOX2, iNOS, and the infiltration of CD11b cells due to activation of NF-κB and hypoxia-inducible factor 1-alpha (HIF-1 α). *In vitro*, ANDRO was capable of reversing oxygen-glucose deprivation-induced ROS and NO overproduction caused by upregulation of NOX2 and iNOS via the PI3K/Akt-dependent NF-κB and HIF-1α pathways in BV-2 cells. Wang et al. (2020) showed that rats submitted to the cerebral hypoperfusion-induced hippocampal neuronal damage model and treated with ANDRO showed decreased levels of caspase-3, p-PTEN and increased levels of p-Akt, when compared to controls, suggesting a neuroprotective property of ANDRO.

10 μM ANDRO modulated the MAPK-Nrf2-HO-1 signaling cascade in primary cerebral endothelial cells (CECs) from C57/BL6 mice. Moreover, it provided protection against middle cerebral artery occlusion (MCAO)-induced ischemic stroke in Wistar rats. Furthermore, ANDRO increased HO-1 protein and mRNA expressions, Nrf2 phosphorylation, and nuclear translocation in CECs. In addition, HO-1 knockdown attenuated the protective effect of ANDRO against oxygen-glucose deprivation-induced CEC death. *In vivo*, ANDRO (0.1 mg/kg) significantly suppressed free radical

formation, blood-brain barrier disruption, and brain infarction in MCAO-insulted rats. The mechanism is attributable to HO-1 activation, as directly evidenced by ANDRO-induced pronounced HO-1 expression in brain tissues. These findings provide strong evidence that ANDRO could be a therapeutic agent for treating ischemic stroke (Yen et al., 2013).

2.6 ANDRO and traumatic brain injury

One of the main factors involving brain ischemia after TBI is neuronal cell death and inflammation, which relates to excessive NO and/or cytokines release (Mauler et al., 2003). ANDRO treatment (0.5, 1 or 2 mg/kg i.p.) effectively reduced neuronal cell death and alleviated neurobehavioral disorders and brain edema in rats after intracerebral hemorrhage (ICH) induced by secondary brain injury. It also reduced the levels of IL-1β, LDH, NLRP3, p20, GSDMD-N and CASP-1. *In vitro*, ANDRO (1, 3, 10 or 30 μM) ameliorated microglia activation-induced neuronal cell death by oxyhemoglobin and also decreased cytokine levels, TNF-α and IL-6 due to the inhibition of NF-κB signaling pathway activation. Meanwhile, ANDRO decreased the levels of IL-1β and LDH as well as microglia pyroptosis induced by ICH by suppressing the assembly of the NLRP3 inflammasome (Li et al., 2018).

After ANDRO (1 mg/kg i.p.) administration, neurological effects were attenuated, and both cerebral edema and apoptosis in brain tissues were decreased following TBI induced by a weight drop model in Sprague-Dawley rats. ANDRO decreased brain water content, albumin levels in the peri-contusive cortex and the modified neurological severity score (mNSS) score after 24h of TBI. ANDRO inhibited both microglial activation and the expression of pro-inflammatory cytokines induced by TBI (TNF-α, IL-1β and IL-6). It also suppressed NF-κB p65 subunit translocation to the nucleus and expression levels of phosphorylated ERK and p38 MAPK after TBI (Tao et al., 2018).

Overall, these results demonstrate the relevance of ANDRO in the context of TBI, mainly with regards to its anti-inflammatory and antiapoptotic properties.

2.7 ANDRO and multiple sclerosis

Xu et al. (2016) demonstrated that ANDRO treatment (1.5625, 3.125 or 6.25 μM) of RSC96 Schwann cells enhanced DNA content, which marks enhanced cell proliferation. ANDRO also promoted gene expression of BDNF and S100β, a specific Schwann cell marker. By hematoxylin-eosin staining, it was shown that ANDRO maintained the Schwann cell phenotype. Therefore, ANDRO accelerated proliferation of RSC96 cells *in vitro*, while maintaining the Schwann cell phenotype; which could for example be beneficial for peripheral nerve injury (Xu et al., 2016). ANDRO also possesses immunomodulatory properties, as tested in a preclinical model of autoimmune encephalomyelitis induced by myelin oligodendrocyte glycoprotein (MOG) injection, which leads to chronic spinal cord demyelination and paralysis. ANDRO (4 mg/kg i.p.) suppressed T-cell function and IL-2 release in neural tissue stimulated by a mix lymphocyte reaction between C57BL/6 and BALB/c mice splenocytes. ANDRO also suppressed maturation process of dendritic cells and direct response of antibody to myelin antigens, by reducing IFN-γ, IL-2 and anti-MOG IgG (Iruretagoyena et al., 2005).

In addition, clinical studies with *A. paniculata* extracts or ANDRO tablets are being performed or have been completed in order to evaluate their effects on different diseases, such as MS (Phase I, NCT02280876). A pilot clinical trial, by Ciampi et al. (2020) showed that 140 mg ANDRO (p.o.) given twice daily for 24 months in patients with not active primary or secondary progressive MS led to reduction in brain atrophy and disability progression, compared to placebo.

2.8 ANDRO and brain cancer

Khan et al. (2018) showed that ANDRO inhibited colon cancer cell proliferation and migration, arrested cell cycle at G2/M phase and induced apoptosis through caspase independent pathway in HT-29 cells. Kumar et al. (2012) demonstrated that ANDRO suppresses breast tumor growth in an orthotopic NOD/SCID mice model and this was correlated with down-regulation of the PI3K/Akt signaling pathway and inhibition of expression of pro-angiogenic molecules such as osteopontin (OPN) and vascular endothelial growth factor (VEGF). Among all types of malignant brain primary tumors, glioblastomas are the most common. Li et al. (2012) showed that glioblastoma

U251 and U87 cells incubated with ANDRO (10 – 100 μM) had decreased proliferation levels in the MTT essay. ANDRO (50 and 70 μM) induced G2/M phase arrest in both glioblastoma cell lines, as determined by flow cytometry. Some studies suggest that the inactivation of PI3K/Akt signaling has been implicated as an important target for the treatment of glioblastoma, as Akt is overactivated in 70% of the cases (Gharbi et al., 2007; Koul et al., 2006). ANDRO downregulated the levels of Cdk1 and Cdc25C proteins, as well as the activity of PI3K/Akt signaling, in both cell lines, as the levels of p-PI3K, p-Akt, p-mTOR and p-p70s6k decreased. All these findings underline that ANDRO possesses an antitumor activity.

2.9 Effects of ANDRO on anxiety and mood

The antidepressant-like effects of ANDRO were shown in the study of Geng et al. (2019), where depressive-like behavior was induced by chronic unpredictable mild stress (CUMS) in C57BL/6 mice. The administration of ANDRO (5 mg/kg) markedly decreased immobility time in the forced swim test and reversed the anhedonic behavior induced by CUMS on the sucrose preference test. Additionally, ANDRO reversed the increase in immobility time in the tail suspension test induced by CUMS. Treatment with ANDRO was also able to reverse the reduction in the levels of BDNF, glial cell-derived neurotrophic factor (GDNF) and nerve growth factor (NGF) induced by CUMS. These neurotrophins are important for the maintenance and regeneration of neurons in the brain. All these antidepressant-like effects of ANDRO were similar to the effects of the selective serotonin reuptake inhibitor fluoxetine on these same tests. In this study, the possible mechanism of action of ANDRO seems to be related to its anti-inflammatory properties (Geng et al., 2019).

Zhang et al. (2019) also demonstrated the antidepressant-like effect of ANDRO (10, 20 and 50 mg/kg i.p.) as it reversed the increased immobility time in both the forced swim and tail suspension tests in naïve mice and mice submitted to the CUMS protocol. All these effects were similar to the effects of fluoxetine. Additionally, ANDRO was able to increase the levels of BDNF, pTrkB, pERK1 and 2, p-Akt and pCREB in the hippocampus of naive mice and mice submitted to CUMS. Zhang et al. (2019) also showed that CUMS induced a reduction in the number of DCX^+ cells and $NeuN^+/BrdU^+$ co-labelled cells in the dentate gyrus, and both ANDRO and fluoxetine reversed this reduction. Taken together, these results indicate that the antidepressant-like effects of

ANDRO possibly involve the BDNF pathway in the hippocampus, and also the promotion of hippocampal neurogenesis. It is known that the pathophysiology of depression involves a reduction in BDNF synthesis and in hippocampal neurogenesis (Dean and Keshavan, 2017). Indeed, the mechanism of action of several antidepressants involve stimulation of neurogenesis (Kraus et al., 2017). Some studies have attested the capacity of ANDRO of enhancing BDNF expression and inducing neurogenesis in the hippocampus (Xu et al., 2016; Varela-Nallar et al., 2015; Adlam and Zaman, 2013), which makes ANDRO an interesting object of study due to its antidepressant-like effects.

Another aspect regarding the pathophysiology of depression is neuroinflammation. The anti-inflammatory properties of pure ANDRO and an *A. paniculata* extract were also related to its antidepressant-like and anti-stress effects in the study of Thakur et al. (2014a), in chronic foot shock stressed rats. A 21-day pretreatment with ANDRO (30 and 60 mg/kg p.o.) and the *A. paniculata* extract decreased the number of escape failures and the immobility time of stressed rats in the learned helplessness test and behavioral despair test, respectively. ANDRO and the *A. paniculata* extract also reversed the increase expression of TNF-α, IL-10 and IL-1β in the blood and brain tissue of stressed rats. Thus, this is another indication that ANDRO can modulate behavioral alterations by regulating cytokine homeostasis (Thakur et al., 2014a).

As previously mentioned, the antioxidant properties of ANDRO have beneficial effects on neurological disorders (Naik et al., 2017; Wong et al., 2016a; Das et al., 2009). Thakur et al. (2014b) showed that the administration of an *A. paniculata* extract reversed the decreased immobility time and escape failures in the behavioral despair and learned helplessness tests, respectively, in type-2 diabetic rats. These effects were similar to the effects of antidepressant imipramine. The *A. paniculata* extract increased SOD and CAT levels, while lowering LPO in the frontal cortex of diabetic rats. The extract also normalized the levels of monoamines (norepinephrine, dopamine and serotonin) in the hippocampus of diabetic rats. The modulation of neurotransmitter levels by ANDRO or the *A. paniculata* extract was also depicted in Thakur and Kumar (2018). In this study, ANDRO and the *A. paniculata* extract reversed the decrease in head twitches after 5-hydroxytryptophan administration and the decrease in behavior score after L-dopa injection in diabetic Swiss mice. These

effects were similar to the effects of imipramine. The study implies that both ANDRO and the *A. paniculata* extract are involved in the modulation of dopaminergic and serotonergic neurotransmission and that this activity is related to their antidepressant-like effect in diabetic mice.

ANDRO also seems to possess an anxiolytic-like effect, as demonstrated by Thakur et al. (2014c), in which ANDRO was able to reverse stress (repeated foot shocks)-induced hyperthermia in a manner similar to anxiolytic drug diazepam. Additionally, ANDRO was able to potentiate pentobarbital hypnosis, as it prolonged the duration of sleep induced by pentobarbital in stressed animals. The study suggests that ANDRO exhibits an adaptogenic potential of normalizing stress-triggered thermal alterations and other physiological responses.

Overall, ANDRO is shown to stimulate hippocampal neurogenesis, enhance BDNF expression, inhibit neuroinflammation and brain oxidative stress, which are all aspects of the pathophysiology of several affective disorders.

3. DISCUSSION

The wide variety of biological effects of ANDRO have been shown in several papers describing both pre-clinical and clinical tests. These biological effects of ANDRO include its anti-inflammatory, antioxidant, antimicrobial, antihyperglycemic, hepatoprotective and anticancer properties, for example. ANDRO was shown to counteract neuroinflammation and apoptosis in models of AD, PD, stroke and TBI, among other aspects of the pathophysiology of these disorders. The lack of effective therapies without a large side-effect profile for the management of disorders such as AD and PD makes it important to search for new therapeutic agents. This review demonstrates the potentially beneficial effects of ANDRO treatment on the pathophysiology and symptomatology of disorders of the CNS. Interestingly, ANDRO could possibly be beneficial not only for neurodegenerative diseases, but also for psychiatric disorders. Throughout the review, several studies showing the effects and possible mechanisms of action of ANDRO in disorders of the CNS were described. Yet, more studies are necessary to elucidate its therapeutic potential and to state its efficacy for the management of these disorders.

However, it is noteworthy that ANDRO has a potential for becoming a pharmacological intervention. This is corroborated by the fact that ANDRO is already

sold and consumed worldwide as dietary supplement as *A. paniculata* pills (powdered plant) or Kalmegh pills, mainly for its anti-inflammatory, antioxidant and antimicrobial properties (Kataky and Handique, 2010). In addition, scientific research involving ANDRO does not rely solely upon pre-clinical tests, but several clinical studies have already been performed and are still being developed. In a randomized, double-blind, placebo-controlled trial, the therapeutic efficacy of tablets of *A. paniculata* extract (170 mg of *A. paniculata* containing 85 mg of ANDRO) was evaluated in subjects with relapsing-remitting MS receiving interferon therapy significantly improved MS-associated fatigue (following the Fatigue Severity Scores), in 44% compared to the placebo group (Bertoglio et al., 2016). A phase II randomized double-blind placebo-controlled clinical study for the evaluation of *A. paniculata* oral tablets in patients with MS was completed in 2015 (NCT02280876), but the results are yet to be published. Other clinical studies with *A. paniculata* or ANDRO are starting to develop. In 2017, a clinical study for the evaluation of the effects of a mixture of magnesium, partenium, *Andrographis*, co-enzyme Q10 and riboflavin on migraine disorders, started recruiting subjects (NCT03190044). At the end of 2018, the evaluation of a dietary supplement with *A. paniculata* (with 40 mg of ANDRO) and *Withania somnifera* for cognitive impairment in elderly subjects began (NCT03780621). Thus, there are great perspectives for the research on ANDRO and its therapeutic effects on the CNS.

4. CONCLUSIONS

Overall, several pre-clinical studies show therapeutic effects of ANDRO in neurodegenerative and neuropsychiatric diseases, such as AD, PD, MS, and depression. Its mechanisms of action appear to involve the induction of hippocampal neurogenesis, the attenuation of neuroinflammation and brain oxidative stress, the increased expression of several neuroprotective proteins such as BDNF and Akt, as well as the inhibition of several anti-neuroplasticity proteins, such as GSK3β. The fact that ANDRO is also already being tested in first clinical trials makes it an even more interesting drug to be further studied for its exact mechanism of action and to determine its potential efficacy for the management of CNS disorders.

Conflict of interest

The authors declare no conflict of interest.

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CHAPTER 3

Andrographolide prevents sleep deprivation- and methylphenidate-induced manic-like behavior mediated via GSK3β inhibition

Kanazawa, Luiz Kae Sales[a]; Nelissen, Ellis[b]; Aguiar, Rafael Pazinatto[c]; Prickaerts, Jos[b]; Andreatini, Roberto[a].

[a] *Laboratory of Physiology and Pharmacology of the Central Nervous System, Department of Pharmacology, Federal University of Paraná, Centro Politécnico, 81540-990 Curitiba, PR, Brazil*

[b] *Department of Psychiatry and Neuropsychiatry, School for Mental Health and Neuroscience (MHeNS), Maastricht University, Universiteitssingel 50, 6229 ER Maastricht, The Netherlands*

c Department of Pharmacology and Therapeutics, State University of Maringá, 87020-900, Maringá, PR, Brazil

ABSTRACT

The pathophysiological aspects of bipolar disorder (BD) are not yet completely elucidated. However, studies indicate that it involves increased activity of the enzyme glycogen synthase kinase 3 beta (GSK3β) in the brain. Inhibition of GSK3β activity has been shown to reduce manic symptoms, with the antimanic effects of lithium, a GSK3β inhibitor, as a clear example. Andrographolide (ANDRO), the major bioactive compound of the plant *Andrographis paniculata*, is also an inhibitor of GSK3β and, therefore, might possess antimanic-like properties. Thus, we aimed to investigate the effect of a 21 days chronic treatment with 0.5 mg/kg or 2 mg/kg ANDRO or 100 mg/kg lithium on mice submitted to different models of mania: 24-h paradoxical sleep deprivation (SD) and acute administration of 5 mg/kg methylphenidate (s.c.). Both models are known to induce hyperlocomotion and increased activity of GSK3β. The results showed that SD induced hyperlocomotion in mice, which was reversed by chronic treatment with lithium and both doses of ANDRO. We also found that SD decreased Ser^9 phosphorylation of GSK3β in the prefrontal cortex (PFC) of these mice, indicative of increased GSK3β activity. Chronic treatment with lithium or 2.0 mg/kg ANDRO was able to reverse this SD-induced decrease in p-Ser^9-GSK3β. In addition, chronic administration of lithium as well as 0.5 mg/kg and 2.0 mg/kg ANDRO reduced methylphenidate-induced hyperlocomotion. Methylphenidate induced decreased Ser^9 phosphorylation of GSK3β in the striatum, which was prevented by lithium and 2.0 mg/kg ANDRO. These results indicate that ANDRO has antimanic-like properties that may be mediated by inhibiting GSK3β activity in the frontostriatal system.

Key words: andrographolide, bipolar disorder, glycogen synthase kinase 3 beta, mania, methylphenidate, sleep deprivation.

Abbreviations: ANDRO, andrographolide; BD, bipolar disorder; GSK3β, glycogen synthase kinase-3 beta; OF, open-field; PFC, pre-frontal cortex; SD, sleep deprivation.

1. INTRODUCTION

Bipolar disorder (BD) is a disease defined by episodes of mania and depression with euthymic states in between (Logan and McClung, 2016). There is a high prevalence of psychiatric and medical comorbidities among BD patients, as well as high rates of suicide attempts, disability and mortality (Grande et al., 2016). The pathophysiological factors underlying BD are not completely elucidated. However, several studies show the involvement of molecular targets in intracellular signaling pathways, such as glycogen synthase kinase 3 beta (GSK3β), protein kinase C (PKC) and inositol monophosphates (Grande et al., 2016). The enzyme GSK3β is a constitutively active serine/threonine kinase, inactivated by phosphorylation of serine residues at serine 9 of the regulatory amino-terminal domains (Frame and Cohen, 2001). The enzyme acts as a downstream regulatory switch that determines the output of several signaling pathways (Prickaerts et al., 2006). This enzyme is involved in many complex biological alterations of BD, such as neuroinflammation, oxidative stress and alterations in membrane ion channels and in the circadian system (Luca et al., 2016). An increase in GSK3β expression is linked to the occurrence of manic-like behaviors in animals (Prickaerts et al., 2006; Gould et al., 2004). Manic bipolar patients showed higher levels of GSK3β compared to healthy controls (Li et al., 2010), and mood stabilizers such as lithium and valproate have been associated with selective inhibition of GSK3β in preclinical studies (Cechinel-Recco et al., 2012; Jope, 2011). In fact, the inhibitory activity of the mood stabilizer lithium over GSK3β is believed to be essential for its antimanic action (Chiu et al., 2013). Furthermore, it is known that GSK3β inhibitors reduced locomotor hyperactivity both in DAT knockout and amphetamine-treated wild-type mice (Beaulieu et al., 2004; Gould et al., 2004), which are models for manic-like behavior. Additionally, the inhibition of GSK3β seems to have a neuroprotective effect (Serrano et al., 2014).

The pharmacological management of BD usually consists of a mood stabilizer alone or in combination with antipsychotics or antidepressants (Vieta et al., 2013). However, many patients take years to achieve stabilization and the life-long treatment may lead to the occurrence of several adverse effects (Alda and Manchia, 2018). Therefore, research on alternative therapeutic options is needed and molecular targets in intracellular signaling pathways might act as a starting point to develop future treatments (Geddes and Miklowitz, 2013).

Andrographolide (ANDRO), which is the major bioactive compound isolated from *Andrographis paniculata*, a medicinal plant with anti-inflammatory and antioxidant properties (Yang et al., 2017; Jayakumar et al., 2013; Das et al., 2009), is known to induce GSK3β inhibition (Tapia-Rojas et al., 2015). This diterpenoid possesses anti-inflammatory, antiapoptotic and antioxidant properties which can be beneficial in many disorders (Graverini et al., 2018; Das et al., 2009). Serrano et al. (2014) showed that, similarly to the antimanic drug lithium, ANDRO was capable of increasing the levels of the inactive, serine 9 phosphorylated form of GSK3β in an Alzheimer's disease mouse model. Therefore, the possible antimanic-like effect of ANDRO, a GSK3β inhibitory drug, was investigated in sleep deprivation (SD)- and methylphenidate-induced animal models for mania. The administration of psychostimulants is considered to be a valid model of mania, as it induces behavioral and molecular changes seen in mania (Logan and McClung, 2016). SD also produces neurochemical alterations seen in bipolar patients (Arent et al., 2015). Both models also induce increased expression of GSK3β in different brain regions (Andrabi et al., 2020; Mines and Jope, 2012). Thus, the evaluation of the effects of ANDRO on SD- and methylphenidate-induced manic-like behavior can provide a better understanding of the neurobiology of mania and potentially offer perspectives on ANDRO as a treatment for the management of BD.

2. MATERIALS AND METHODS

2.1 Animals

Male Swiss mice (30 – 40 g) were housed socially (6-8/cage) with controlled temperature (22±2 ºC) in a 12h:12h light/dark cycle (with lights on between 7 a.m. and 7 p.m.) with free access to water and food. The animals were allowed to acclimate for one week before testing commenced. All experiments were performed in accordance with the Brazilian Law for Animal Experimental Ethics and Care (11.794/8 October 2008) and the Local Committee on the Care and Use of Laboratory Animals. The experimental procedures were approved by the Institutional Ethics Board (CEUA/BIO – protocol #1109). All efforts were made to minimize animal suffering and the number of animals used.

2.2 Drugs

Andrographolide (Sigma, São Paulo, Brazil) was administered at doses of 0.5 and 2 mg/kg, intraperitoneally (i.p.) (Chan et al., 2010; Niranjan et al., 2010). ANDRO was dissolved in saline with dimethyl sulfoxide (DMSO). The repeated treatment was performed throughout 21 days, 3 times a week.

Lithium carbonate (Eurofarma, Itapevi, Brazil) was used as positive control at a dose of 100 mg/kg. Lithium was dissolved in saline and the pH was adjusted to 7.4 by adding 2N HCl. Repeated treatment was performed throughout 21 days, once a day, i.p.

Methylphenidate (Novartis, São Paulo, Brazil) was used for the induction of manic-like behavior at a dose of 5 mg/kg, subcutaneously (s.c.), 30 minutes before the experiments, in a single administration.

All drugs were administered in a constant volume of 10 ml/kg body weight.

2.3 Sleep deprivation protocol

For the non-pharmacological induction of manic-like behavior, mice were submitted to the 24h SD protocol (Silva et al., 2004). Groups of six animals were placed in polypropylene cages (41 x 34 x 16 cm), each cage containing 12 platforms (3 cm in diameter x 5 cm in height), surrounded by water up to 1 cm below the surface of the platforms. The animals could move freely, jumping from one platform to the other. Food and water were available the whole time.

The objective of the protocol is that, when animals reach REM sleep, they display muscle relaxation, falling into the water and, thus, having their sleep interrupted (Machado-Vieira et al., 2004). After this period of SD, animals show some behaviors such as hyperlocomotion and an increase in appetitive ultrassonic vocalizations (USVs), which can be correlated with manic-like behaviors (Wendler et al., 2019; Armani et al., 2012; Gessa et al., 1995). SD also reproduces aspects of the manic episode such as hyperactivity, hypersexuality and aggressiveness of manic patients (Valvassori et al., 2017).

The animals were chronically treated for 21 days with either vehicle (saline + DMSO), 100 mg/kg lithium or 0.5 or 2.0 mg/kg ANDRO. On the last day of treatment, the animals were submitted to the 24h SD protocol as previously described. After this

period, the animals were placed in the open-field, where the number of crossings was measured for 5 minutes. The open-field apparatus is a round arena (42 cm in diameter x 28 cm in height) divided in three circles subdivided into 25 equal regions (Barbosa et al., 2011). Following the test, the animals were euthanized for the removal of their pre-frontal cortex (PFC) and striatum for further analysis.

2.4 Methylphenidate-induced hyperlocomotion

Psychostimulant-induced hyperlocomotion is the most frequently used animal model of mania (Einat, 2006). This pharmacological induction of manic-like behavior is reliable and shows face, construct and predictive validity (Einat, 2006; Machado-Vieira et al., 2004). Psychostimulants that are capable of increasing the levels of dopamine cause behavioral effects that resemble mania, such as hyperlocomotion (Hasler et al., 2006).

To measure hyperlocomotion, the number of crossings in the open field was analyzed as an index of locomotor activity. The blocking or attenuation of hyperlocomotion after methylphenidate administration is indicative of an antimanic-like effect, at doses that do not impair locomotor activity *per se* (Sabioni et al., 2008; Gould et al., 2001).

The animals were chronically treated for 21 days with either vehicle (saline + DMSO), 100 mg/kg lithium or 0.5 or 2.0 mg/kg ANDRO. On the test day, either vehicle or 5 mg/kg methylphenidate (s.c.) was administered to the animals. After 30 minutes, they were placed in the center of the open-field apparatus for the evaluation of the locomotor activity within 5 minutes (Barbosa et al., 2011). Following the test, the animals were euthanized for the removal of their PFC and striatum for further analysis.

2.5 Western Blot

Mice were euthanized by decapitation and the PFC and striatum were removed, immediately frozen in liquid nitrogen and kept at -80ºC. The samples were homogenized in 200 µl ice-cold lysis buffer (1 mM EDTA, 1 mM EGTA, 1% glycerol, 0.1% triton, and 1% IGEPAL CA-630 in phosphate-buffered saline (PBS)) containing protease and phosphatase inhibitors (Roche, Mannheim, Germany) by the use of a SpeedMill PLUS tissue homogenizer (Analytik Jena AG, Jena, Germany). The

homogenates were subsequently centrifuged at 14000 rpm for 20 minutes at 4ºC. The supernatant was used for protein determination (DC™ Protein Assay, Bio-Rad laboratories, Veenendaal, the Netherlands). 30µg of each sample was incubated at 100ºC for 7 min and separated on a 10% SDS-PAGE gel. After electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories) and subsequently blocked with Odyssey blocking buffer in PBS (Li-Cor, Lincoln, NE, USA) for 1h at room temperature. Afterwards, the membranes were incubated overnight at 4ºC with the following primary antibodies in blocking buffer and PBS: rabbit anti-GSK3β (#9315S, Cell Signaling Technologies, Beverly, MA, USA, 1:1000); rabbit anti-p-GSK3β (p-Ser^{9}) (#9336S, Cell Signaling Technologies, 1:1000) or mouse anti-β-actin (#A5441, Sigma-Aldrich, Darmstadt, Germany, 1:20000), for normalization. Membranes were washed with PBS or PBS-0.1% Tween 20 (PBS-T), incubated with goat anti-rabbit IRDye 800 and donkey anti-mouse IRDye 680 secondary antibodies (Li-Cor, 1:10000) for 1h at room temperature, and washed again. The membrane was dried and bands were visualized using an Odyssey CLx Infrared Imaging System (Li-Cor) and quantification was performed using the software Image Studio Lite Ver 5.2.

2.6 Statistical analysis

Data were analyzed by one-way ANOVA. The statistical analysis for the groups treated with lithium was performed separately from the groups treated with ANDRO. The differences between the groups were analyzed by the least significant difference (LSD) *post hoc* test. Differences were considered to be statistically significant when $p < 0.05$. Data was expressed as mean ± SEM. SPSS 16.0 (SPSS Inc., Chicago, IL) was used for the statistical analysis.

3. RESULTS

3.1 The effects of lithium and ANDRO on sleep deprivation-induced hyperlocomotion

Hyperlocomotion was measured in mice by recording the number of crossings in an open-field test. A one-way ANOVA ($F_{3,36} = 4.98$, $p < 0.01$) revealed that chronic treatment with lithium successfully reversed SD-induced hyperlocomotion (*post-hoc* LSD, $p < 0.01$; Figure 1A). Similar results were found for chronic ANDRO treatment

($F_{5,53}$ = 2.52, p < 0.05) at both 0.5 mg/kg (*post-hoc* LSD, p < 0.05) and 2.0 mg/kg (p < 0.01; Figure 1B). This confirms that ANDRO exerts effects similar to lithium on locomotor behavior of mice in a SD-induced model for mania.

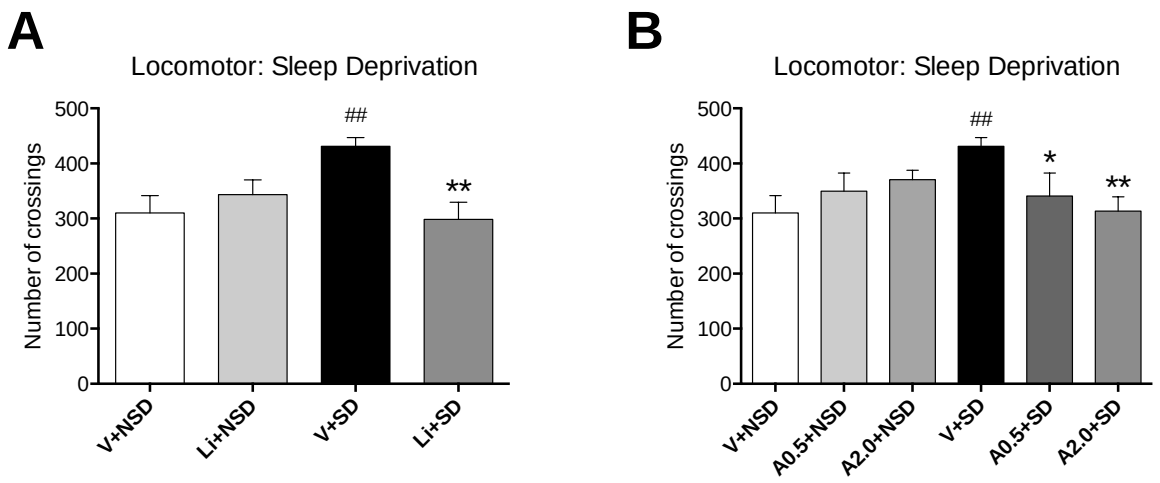

Fig 1. Effects of lithium and ANDRO on SD-induced hyperlocomotion. SD induced hyperlocomotion in mice, as was measured by an increased number of crossings in the open-field test (one-way ANOVA with *post-hoc* LSD, p < 0.01). Both A) lithium (i.p.) and B) 0.5mg/kg and 2.0 mg/kg ANDRO (i.p.), successfully reversed this SD-induced hyperlocomotion (p < 0.01). Data are represented as mean ± SEM; n=8-10. Hashes represent a significant difference from the V+NSD group, asterisks represent a significant difference from the V+SD group. $^{\#\#}$ p < 0.01; * p < 0.05, ** p < 0.01. V+NSD: vehicle + non-sleep deprivation; Li+NSD: lithium + non-sleep deprivation; V+SD: vehicle + sleep deprivation; Li+SD: lithium + sleep deprivation; A0.5+NSD: 0.5 mg/kg ANDRO + non-sleep deprivation; A2.0+NSD: 2.0 mg/kg ANDRO + non-sleep deprivation; A0.5+SD: 0.5 mg/kg ANDRO + sleep deprivation; A2.0+SD: 2.0 mg/kg ANDRO + sleep deprivation.

3.2 The effects of lithium and ANDRO on GSK3β phosphorylation in a SD model

Ser^9 phosphorylation levels of GSK3β as an indicator of its inactivity were measured in the PFC and striatum of mice chronically treated with lithium or ANDRO, alone or in combination with SD. A one-way ANOVA ($F_{3,35}$ = 10.08, p < 0.001) revealed that SD reduced Ser^9 phosphorylation of GSK3β (*post-hoc* LSD, p < 0.001) and that GSK3β inhibitor lithium prevented this decrease (p < 0.001, Figure 2A) in the PFC. Similar results ($F_{5,53}$ = 6.22, p < 0.001) were found for 2.0 mg/kg ANDRO, which successfully prevented a decrease in GSK3β Ser^9-phosphorylation (p < 0.001, Figure 2B) in the PFC. This effect was dose-dependent, since 0.5 mg/kg ANDRO did not

affect phosphorylation of Ser^{9}. This shows that ANDRO is able to dose-dependently inhibit GSK3β similar to lithium in a SD model for mania in the PFC of mice.

In the striatum, a one-way ANOVA revealed both treatment effects for the lithium groups ($F_{3,36}$ = 5.30, p < 0.01), as well as for the ANDRO groups ($F_{5,52}$ = 2.93, p < 0.05). However, contrary to the findings in the PFC, SD did not affect Ser^{9} phosphorylation of GSK3β in the striatum. Interestingly, both lithium (Figure 2D) and 2.0 mg/kg ANDRO (Figure 2E) enhanced GSK3β Ser^{9}-phosphorylation with and without SD. This shows that lithium and ANDRO both exert unspecific drug effects in the absence of SD model effect, or a disease model altogether. Additionally, the absence of SD induced effects on GSK3β Ser^{9}-phosphorylation in the striatum suggests that such effects are more specific to the PFC in comparison to the striatum.

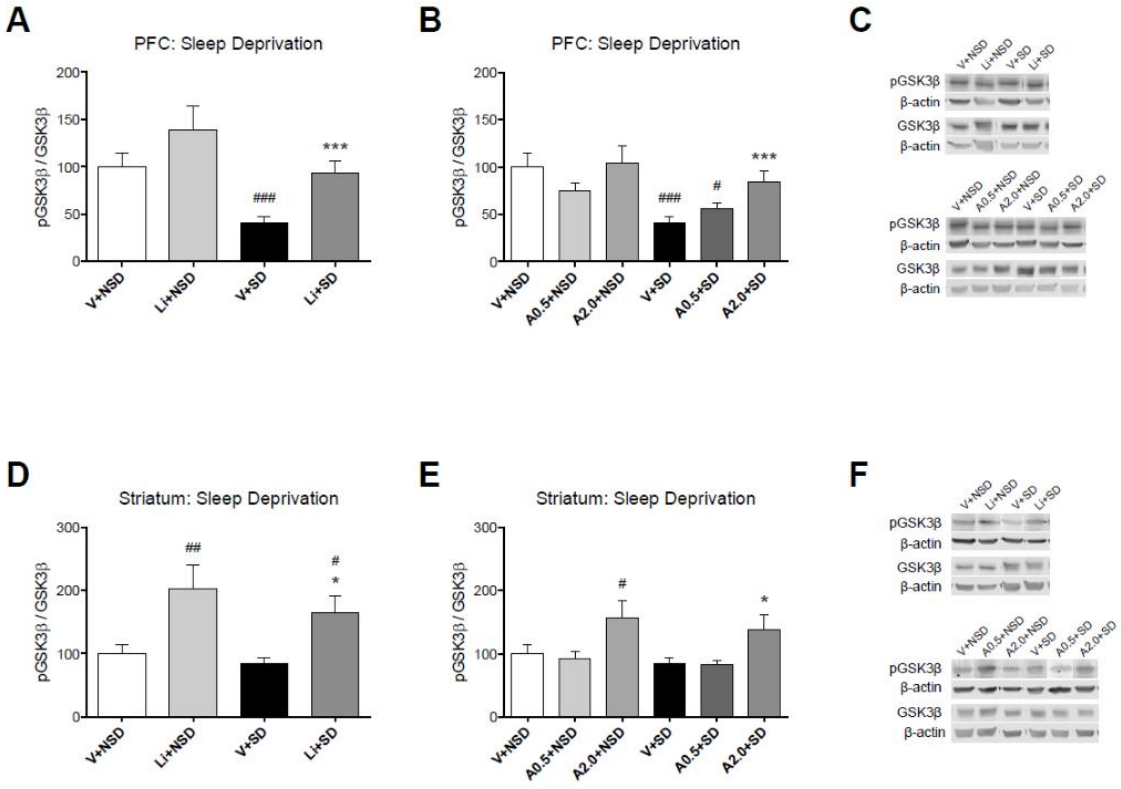

Fig 2. Effects of lithium and ANDRO on Ser^{9} phosphorylation of GSK3β in a SD model for mania. A) SD induced a reduction in Ser^{9} phosphorylation of GSK3β (one-way ANOVA with *post-hoc* LSD, p < 0.001) in the PFC, suggesting increased GSK3β activity. Chronic treatment with lithium (i.p.) or B) 2.0 mg/kg ANDRO (i.p.) was able to prevent this decrease in phosphorylation (p < 0.001). 0.5 mg/kg ANDRO did not affect GSK3β Ser^{9}-phosphorylation levels, suggesting a dose-dependent effect of ANDRO. C) Representative western blot bands for the PFC. D) Contrary to the PFC, SD did not affect serine-9 phosphorylation of GSK3β in the striatum. Yet, chronic treatment with either lithium (i.p.) or E) 2.0 mg/kg ANDRO (i.p.) enhanced GSK3β Ser^{9}-phosphorylation independent of SD model effects, or a disease model altogether, suggesting disease-unspecific effects of both drugs. F) Representative western blot bands for the striatum. Data are represented as mean ± SEM; n=8-10. Hashes represent a significant difference from the V+NSD group, asterisks represent a significant difference from the

V+SD group. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$; * $p < 0.05$, *** $p < 0.001$. V+NSD: vehicle + non-sleep deprivation; Li+NSD: lithium + non-sleep deprivation; V+SD: vehicle + sleep deprivation; Li+SD: lithium + sleep deprivation; A0.5+NSD: 0.5 mg/kg ANDRO + non-sleep deprivation; A2.0+NSD: 2.0 mg/kg ANDRO + non-sleep deprivation; A0.5+SD: 0.5 mg/kg ANDRO + sleep deprivation; A2.0+SD: 2.0 mg/kg ANDRO + sleep deprivation.

3.3 The effects of lithium and ANDRO on methylphenidate-induced hyperlocomotion

Similar to the SD model, hyperlocomotion was measured in mice by recording the number of crossings in an open-field test. A one-way ANOVA ($F_{3,36} = 20.38$, $p < 0.001$) revealed that chronic lithium treatment successfully reversed methylphenidate-induced hyperlocomotion (*post-hoc* LSD, $p < 0.001$; Figure 3A). Similar results were found for chronic treatment with ANDRO ($F_{5,54} = 13.71$, $p < 0.001$) at both 0.5 mg/kg and 2.0 mg/kg (*post-hoc* LSD, $p < 0.001$; Figure 1B). Similar to the SD findings, this confirms that ANDRO exerts effects on locomotor behavior of mice in a methylphenidate-induced model for mania that are similar to conventional treatment with lithium.

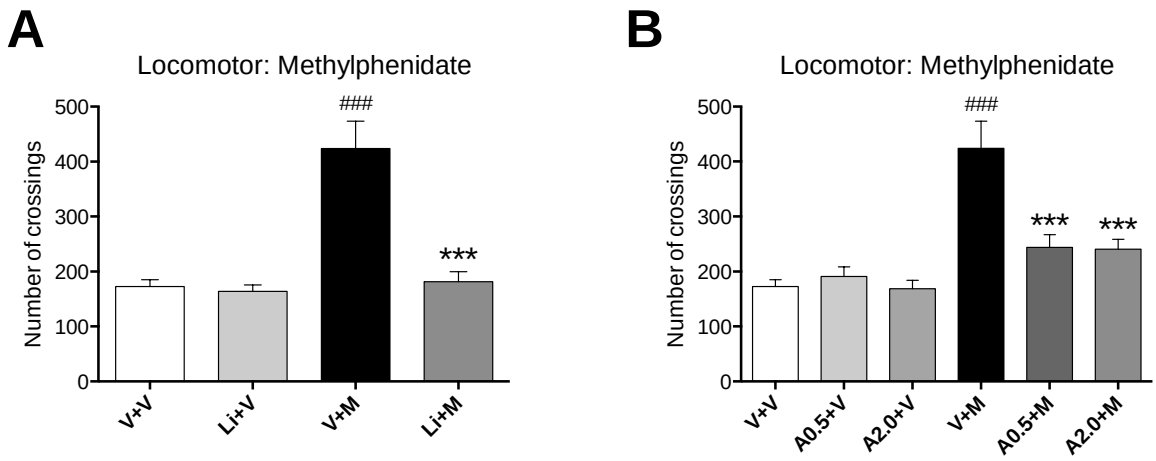

Fig 3. Effects of lithium and ANDRO on methylphenidate-induced hyperlocomotion. Methylphenidate treatment (s.c.) induced hyperlocomotion in mice, as was measured by an increased number of crossings in the open-field test (one-way ANOVA with *post-hoc* LSD, $p < 0.001$). Both A) lithium (i.p.) and B) 0.5mg/kg and 2.0 mg/kg ANDRO (i.p.), successfully reversed this methylphenidate-induced hyperlocomotion ($p < 0.001$). Data are represented as mean ± SEM; n=8-10. Hashes represent a significant difference from the V+V group, asterisks represent a significant difference from the V+M group. ### $p < 0.001$; *** $p < 0.001$. V+V: vehicle + vehicle; Li+V: lithium + vehicle; V+M: vehicle + methylphenidate; Li+M: lithium + methylphenidate; A0.5+V: 0.5 mg/kg ANDRO + vehicle; A2.0+V: 2.0 mg/kg ANDRO + vehicle; A0.5+M: 0.5 mg/kg ANDRO + methylphenidate; A2.0+M: 2.0 mg/kg ANDRO + methylphenidate.

3.4 The effects of lithium and ANDRO on GSK3β phosphorylation in a methylphenidate model

Again, phosphorylation levels of GSK3β on Ser^9 were measured in the PFC and striatum of mice as an indication of GSK3β protein inactivity levels, but now in the methylphenidate model for mania. A one-way ANOVA revealed chronic treatment effects for both the lithium groups ($F_{3,30} = 16.78$, $p < 0.001$) and the ANDRO groups ($F_{5,49} = 2.49$, $p < 0.05$) in the PFC of mice. However, methylphenidate treatment did not affect Ser^9 phosphorylation of GSK3β in the PFC. Still, both GSK3β inhibitor lithium ($p < 0.01$, Figure 4A), as well as 2.0 mg/kg ANDRO ($p < 0.05$, Figure 4B) showed unspecific treatment effects by enhancing GSK3β Ser^9 phosphorylation in the absence of a model effect of methylphenidate. Moreover, lithium even enhanced Ser^9 phosphorylation in the absence of methylphenidate treatment altogether, suggesting a drug-effect completely unspecific to the presence of GSK3β activity deficits. Interestingly, 0.5 mg/kg ANDRO in combination with methylphenidate reduced Ser^9 phosphorylation ($p < 0.05$), suggesting a differential dose-effect of ANDRO.

A one-way ANOVA ($F_{3,36} = 7.24$, $p < 0.001$) revealed that methylphenidate treatment did reduce Ser^9 phosphorylation (*post-hoc* LSD, $p < 0.05$) in the striatum, contrary to the PFC. Chronic treatment with GSK3β inhibitor lithium successfully prevented this decrease ($p < 0.001$; Figure 4D) and similar results ($F_{5,54} = 5.88$, $p < 0.001$) were found for 2.0 mg/kg ANDRO ($p < 0.001$), but not 0.5 mg/kg (Figure 4E). This suggests that, similar to lithium, 2.0 mg/kg ANDRO inhibited GSK3β activity through enhanced Ser^9 phosphorylation. Additionally, methylphenidate-induced enhancement of GSK3β activity through reduced Ser^9 phosphorylation seems to be specific for the striatum in comparison to the PFC. This is in contrast to sleep deprivation, which seems to specifically affect the PFC rather than the striatum.

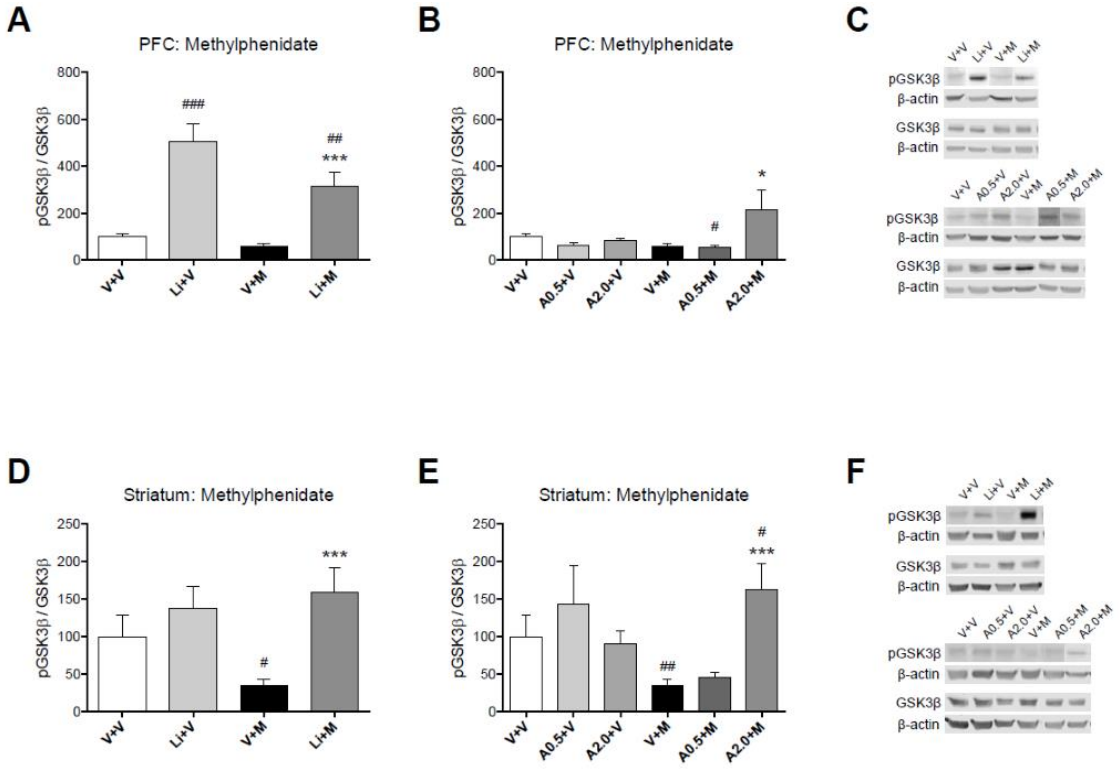

Fig 4. Effects of lithium and ANDRO on Ser^{9} phosphorylation of GSK3β in a methylphenidate model for mania. A) Methylphenidate treatment (s.c.) did not affect Ser^{9} phosphorylation of GSK3β (one-way ANOVA with *post-hoc* LSD, $p < 0.05$) in the PFC of mice. Still, both chronic treatment with lithium (i.p.) and B) 2.0 mg/kg ANDRO (i.p.) increased Ser^{9} phosphorylation of GSK3β ($p < 0.001$), suggesting an unspecific drug effect regardless of a methylphenidate model effect. Surprisingly, 0.5 mg/kg ANDRO reduced GSK3β Ser^{9} phosphorylation levels in the presence of the methylphenidate model, suggesting differential dose-effects of ANDRO. C) Representative western blot bands. D) Contrary to the PFC, methylphenidate treatment (s.c.) reduced GSK3β Ser^{9} phosphorylation in the striatum of mice. Both lithium (i.p.) and E) 2.0 mg/kg ANDRO (i.p.) chronic treatment prevented this decrease in Ser^{9} phosphorylation. 0.5 mg/kg ANDRO did not affect GSK3β Ser^{9} phosphorylation, suggesting dose-dependent effects of ANDRO. F) Representative western blot bands. Data are represented as mean ± SEM; n=8-10. Hashes represent a significant difference from the V+V group, asterisks represent a significant difference from the V+M group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; $^{*}p < 0.05$, $^{***}p < 0.001$. V+V: vehicle + vehicle; Li+V: lithium + vehicle; V+M: vehicle + methylphenidate; Li+M: lithium + methylphenidate; A0.5+V: 0.5 mg/kg ANDRO + vehicle; A2.0+V: 2.0 mg/kg ANDRO + vehicle; A0.5+M: 0.5 mg/kg ANDRO + methylphenidate; A2.0+M: 2.0 mg/kg ANDRO + methylphenidate.

4. DISCUSSION

4.1 Chronic ANDRO treatment reverses SD-induced changes in locomotor behavior and p-Ser^{9}-GSK3β phosphorylation in the PFC of mice

Our results showed that 24-h SD induced hyperlocomotion in mice. Chronic treatment with lithium was able to reverse this SD-induced hyperlocomotion. Importantly, this effect was seen at doses that did not affect spontaneous locomotor activity. These results are similar to reports in literature showing that SD induced hyperactivity in mice, which was reversed by lithium administration (Valvassori et al.,

2017; Armani et al., 2012). Additionally, Wendler et al. (2019) showed that rats submitted to the SD model, displayed increased locomotor activity, increased emission of 50-kHz USVs, as well as a change in the call profile characterized by an increase in the percentage of frequency modulated 50-kHz USVs, which is related to the SD-induced manic-like behaviors. All these behaviors were reversed by treatment with lithium. Abrial et al. (2015) showed also that SD triggers manic-like behaviors such as hyperlocomotion and increased sleep latency, which were reversed by lithium and aripiprazole. Interestingly, we found that chronic treatment with 0.5 mg/kg as well as 2.0 mg/kg ANDRO, which can inhibit GSK3β activity, displayed an antimanic-like effect by reversing SD-induced hyperlocomotion similar to lithium.

The enzyme GSK3β has numerous cellular and molecular functions, including cell development, gene transcription, metabolic homeostasis, neurogenesis, and apoptosis (Doble and Woodgett, 2003). However, animals that overexpress GSK3β show behaviors which can be correlated with mania in humans (Chen et al., 2009; Prickaerts et al., 2006). It was shown that phosphorylation of GSK3β at Ser^9 inhibits GSK3β activity, and previous studies have used the increase or decrease in p-Ser^9 levels as changes of cellular GSK3β activity (Jope and Johnson, 2004). Lithium, the prototype mood stabilizer, is both a direct and indirect inhibitor of GSK3β, which is proposed to be involved in the anti-manic action of the drug (Phiel and Klein, 2001). At therapeutic concentrations, lithium treatment can lead to inhibition of GSK3β by increasing phosphorylation at Ser^9 (Costemale-Lacoste et al., 2016; Sani et al., 2012). Li et al. (2007) showed an 8-fold increase in p-Ser^9-GSK3β levels in peripheral blood mononuclear cells of BD patients treated with lithium, compared to healthy controls. Therefore, we investigated the effects of SD on the Ser^9 phosphorylation status of GSK3β as an indicator of reduced GSK3β activity.

Our results showed that SD decreased Ser^9 phosphorylation of GSK3β in the PFC of mice, indicative of increased GSK3β activity. Chronic treatment with lithium or 2.0 mg/kg ANDRO were able to reverse this SD-induced decrease in p-Ser^9-GSK3β. Li et al. (2010) showed that the levels of total GSK3β were higher whereas Ser^9 phosphorylation of GSK3β was reduced in blood cells from manic patients than in those cells of healthy controls, and that chronic antimanic treatment increased the Ser^9 phosphorylation of GSK3β. Likewise, Andrabi et al. (2020) showed that SD induced an upregulation in GSK3β levels in the rat hippocampus, which was prevented by lithium treatment. Therefore, the increase in the levels of inactive Ser^9-phosphorylated

GSK3β by lithium and 2.0 mg/kg ANDRO can have a beneficial effect on manic-like behaviors. One of the antimanic actions of lithium involves indirect inhibitory effects on GSK3β activity, by activating Akt directly, which increases the levels of inactive p-Ser^{9}-GSK3β (Freland and Beaulieu, 2012).

Interestingly, in the striatum, SD did not affect the Ser^{9} phosphorylation status of GSK3β. However, both lithium and 2.0 mg/kg ANDRO increased phosphorylation of Ser^{9}-GSK3β, indicative of GSK3β inhibition, despite this absence of SD-induced effects on GSK3β in the striatum. This is indicative of a non-specific drug effect independent of the presence of a disease model, both for lithium and ANDRO. Our lack of an effect on Ser^{9}-GSK3β phosphorylation in the striatum is in contrast to SD-induced alterations in the PFC, so it might be argued that the rodent SD-induced model for mania is mainly affecting GSK3β activity in the PFC.

4.2 Chronic ANDRO treatment reverses methylphenidate-induced changes in locomotor behavior and Ser^{9}-GSK3β phosphorylation in the striatum of mice

The mechanism of action of methylphenidate is mediated by its ability to block the DAT and thus increase levels of extracellular dopamine in brain regions such as the striatum and the PFC (Bymaster et al., 2002; Volkow et al., 2002. Volkow et al., 2001). The administration of methylphenidate also induces hyperactivity which is reversed by lithium, sodium valproate and carbamazepine treatment (Logan and McClung, 2016; Tonelli et al., 2013; Barbosa et al., 2011). Methylphenidate monotherapy in bipolar patients is associated with increases in manic episodes, which are not observed when bipolar patients are using mood stabilizers (Viktorin et al., 2017). Moreover, in healthy volunteers, methylphenidate induced euphoric mood (Smith and Davies, 1977). These results indicated the validity of the methylphenidate administration model of mania. In our study, chronic administration of lithium as well as 0.5 mg/kg and 2.0 mg/kg ANDRO reduced methylphenidate-induced hyperlocomotion, which is an indicative of antimanic-like effect of ANDRO at doses that did not affect spontaneous locomotor activity.

Studies have shown that psychostimulant administration leads to an increased activity of GSK3β (Mines and Jope, 2012). For instance, GSK3α/β knock-in mice with

serine-to-alanine mutations to block serine phosphorylation, show increased sensibility to manic-like amphetamine-induced locomotor hyperactivity (Polter et al., 2010). Additionally, mice lacking one allele of the GSK3β gene show a significant reduction in locomotor responses to amphetamine (Beaulieu et al., 2004). In humans, the activity of GSK3β in the blood of BD patients is increased, when compared to healthy control subjects (Jacoby et al., 2016). Li et al. (2010) observed that antimanic treatment increases p-Ser^{9}-GSK3β in peripheral blood mononuclear cells of bipolar patients with a manic episode. Our study indicates that methylphenidate administration led to a reduction in the phosphorylation of Ser^{9}-GSK3β in the striatum of mice, as seen by a reduction in the p-GSK3β/GSK3β ratio. Additionally, chronic treatment with 2.0 mg/kg ANDRO and lithium reversed the methylphenidate-induced reduction of phosphorylation of Ser^{9}-GSK3β in the striatum of mice. In several rodent models of mania, synthetic inhibitors of GSK3β mimic the therapeutic effects of lithium (Kozikowski et al., 2011; Kozikowski et al., 2007). Mines and Jope (2012) showed that 8-day i.p. administration of 2 mg/kg amphetamine or 20 mg/kg methylphenidate led to decreased levels of p-Ser^{9}-GSK3β in the striatum of mice. Therefore, the attenuation of a methylphenidate-induced decrease in Ser^{9} phosphorylation of GSK3β in the striatum after treatment with ANDRO may be linked to ANDRO's behavioral antimanic-like effect.

Interestingly, methylphenidate did not affect Ser^{9} phosphorylation of GSK3β in the PFC of mice, while both lithium and 2.0 mg/kg ANDRO showed a non-specific drug effect independent by increasing phosphorylation of Ser^{9}-GSK3β in this absence of a methylphenidate-induced effects in the PFC. Additionally, 0.5 mg/kg ANDRO actually reduced phosphorylation of Ser^{9}-GSK3β in the presence of methylphenidate treatment, suggesting a dose-dependent differential effect of ANDRO, yet independent of any methylphenidate treatment. Due to the absence of methylphenidate-induced effects on p-Ser^{9}-GSK3β in the PFC, contrary to a reduction in phosphorylation of Ser^{9}-GSK3β in the striatum, it could be concluded that the rodent methylphenidate model for mania might mainly exerts its effects through striatal mechanisms over the PFC.

4.3 Differential roles of the striatum and PFC in methylphenidate and SD models for mania in mice

As already briefly pointed out above, we observed that SD-induced enhancement of GSK3β activity through reduced Ser^9-phosphorylation seems to be specific for the PFC in comparison to the striatum. This is in contrast to the methylphenidate effect, which seems to affect Ser^9-phosphorylation specifically in the striatum rather than the PFC.

Most human studies involving the effects of SD, measure parameters such as verbal fluency, logical reasoning, working memory, planning, inhibitory capabilities and decision-making, which are functions related to the PFC (Muzur et al., 2002; Harrison and Horne, 2000; Harrison et al., 2000). Indeed, defective frontal functioning was detected in these parameters after SD (Muzur et al., 2002). Neuroimaging studies showed detrimental effects of 24h SD on the blood flow in frontal brain areas, which were related to poor prefrontal task performance afterwards (Thomas et al., 2000; Drummond et al., 1999). Finally, electroencephalogram studies show that the PFC is particularly sensitive to SD, as the PFC shows the greatest changes in brain wave pattern from sleep to waking, as it is relatively inactive all through sleep (Maski and Kothare, 2013; Muzur et al., 2002). Therefore, it is not surprising that the PFC is mainly affected by SD in our study, contrary to the striatum which appears more resilient to the SD model.

In our study, methylphenidate administration did not significantly affect the levels of p-Ser^9-GSK3β in the PFC, as it did in the striatum, suggesting the striatum is more sensitive to the methylphenidate model for mania compared to the PFC. Indeed, Quansah and Zetterström (2019) showed that chronic methylphenidate administration in young rats increased the levels of dopamine-related genes and D_1 receptors mainly in the ventral striatum, in comparison to other brain areas such as the PFC. This indicates that methylphenidate affects primarily the striatum as opposed to the PFC, thereby supporting the findings in our current study. Additionally, a study in monkeys by Kodama et al. (2017), demonstrated that in the striatum, both high and low doses of methylphenidate induced consistent increases in DA release approximately 30 minutes after the administration. In the PFC on the other hand, a consistent increase in DA release was observed 1 hour after the administration of a high dose of methylphenidate, but not low doses. Finally, Gray et al. (2007) showed that methylphenidate decreased tyrosine hydroxylase-immunoreactivity in the striatum but increased in the mPFC. These results demonstrate that methylphenidate administration in different doses affects the striatum and/or the PFC in different

manners, supporting our findings that the striatum is more sensitive to methylphenidate opposed to the PFC.

5. CONCLUSION

Overall, our results show that SD and methylphenidate administration induced hyperlocomotion in mice, which was reversed by chronic treatment with lithium and 0.5 and 2.0 mg/kg ANDRO. Additionally, SD as well as methylphenidate administration led to a reduction in the p-Ser^{9}-GSK3β/GSK3β ratio in the PFC and striatum, respectively, indicating an increased activity of the enzyme. Chronic treatment with 2.0 mg/kg ANDRO and lithium increased the p-Ser^{9}-GSK3β/GSK3β ratio in the PFC and striatum. Overall, these results indicate that ANDRO has antimanic-like properties that may be mediated by the GSK3β pathway, though via divergent effects in the PFC and striatum dependent on the animal model of mania.

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CHAPTER 4

Andrographolide prevents increases in 50-kHz ultrassonic vocalizations, hyperlocomotion and oxidative stress induced by lisdexamfetamine in rats, an animal model of mania

Kanazawa, L. K. S.[a]; Radulski, D. R.[b]; Pereira, G. S.[b]; Prickaerts, J.[c]; Schwarting R.K.W.[d]; Acco, A.[b]; Andreatini, R[a].

[a] *Laboratory of Physiology and Pharmacology of the Central Nervous System, Department of Pharmacology, Federal University of Paraná, Centro Politécnico, 81540-990 Curitiba, PR, Brazil*

[b] *Laboratory of Pharmacology and Metabolism, Department of Pharmacology, Federal University of Paraná, Centro Politécnico, 81540-990, Curitiba, PR, Brazil*

[c] *Department of Psychiatry and Neuropsychiatry, School for Mental Health and Neuroscience (MHeNS), Maastricht University, Universiteitssingel 50, 6229 ER Maastricht, The Netherlands*

[d] *Behavioural Neuroscience, Experimental and Biological Psychology, and Center for Mind, Brain and Behavior (CMBB), Philipps-Universität Marburg, 35032 Marburg, Germany*

ABSTRACT

In rats, lisdexamfetamine (LDX) induces manic-like behaviors such as hyperlocomotion and increase in appetitive 50-kHz ultrasonic vocalizations (USVs), which are prevented by antimanic drugs, such as lithium. Inhibition of glycogen synthase kinase 3 beta (GSK3β) has been associated with antimanic effect. Thus, the aim of the present study was to evaluate the possible antimanic-like effect of andrographolide (ANDRO), a GSK3β inhibitor, on LDX-induced hyperlocomotion and 50-kHz USVs increase. In addition, the effect of ANDRO was studied on LDX-induced oxidative stress. Lithium was used as positive control. Adult Wistar rats were treated with vehicle, lithium (100 mg/kg i.p.) or ANDRO (2.0 mg/kg i.p.) 3 times a week for 21 days. On the test day, either 10 mg/kg LDX or saline was administered i.p. and USV calls and locomotor activity were recorded. LDX administration increased the number of 50-kHz calls as well as locomotor activity. Repeated treatment with lithium or ANDRO prevented these effects of LDX on 50-kHz USVs and locomotor activity. LDX increased lipid peroxidation (LPO) levels in rat striatum and both lithium and ANDRO prevented this effect. LPO levels in rat striatum were positively correlated with increases in 50-kHz USV emission as well as hyperlocomotion. In conclusion, the present results indicate that ANDRO has antimanic-like and antioxidant effects in an animal model of mania.

Key words: andrographolide, bipolar disorder, glutathione, GSK3β, lisdexamfetamine, lipid peroxidation, mania, oxidative stress, ultrassonic vocalizations

Abbreviations: ANDRO: andrographolide; GSH: reduced glutathione; GSK3β : glycogen synthase kinase-3β; LDX: lisdexamfetamine; LPO: lipid peroxidation; PFC: pre-frontal cortex; USVs: ultrassonic vocalizations.

1. INTRODUCTION

Manic episodes of bipolar disorder (BD) consist of elevated or irritable mood with enhanced energy, psychomotor agitation, risk behavior, pressured speech and tachylalia, for example (American Psychiatry Association, 2013). The pharmacological treatments for the management of manic phases of BD include mood stabilizers, such as lithium or sodium valproate, as well as antipsychotics and tamoxifen (Geddes and Miklowitz, 2013). However, these treatments are associated with the occurrence of several adverse effects, which negatively affect treatment adhesion (Baldessarini et al., 2018).

Oxidative stress has been associated with mania and antimanic drugs (Saxena et al., 2017; Malhi et al., 2013). Machado-Vieira et al. (2007a) found an increase in thiobarbituric acid reactive substances (TBARS) in manic non-medicated patients, which was reduced after lithium treatment. Lv et al. (2020) observed that malondialdehyde is higher in manic patients, decreasing after 6 weeks of effective electroconvulsive therapy. Moreover, increased oxidative stress was found in different animal models of mania, which could be reduced by treatment with lithium, valproate or antipsychotics (Menegas et al., 2020; Dal-Pont et al., 2019; Valvassori et al., 2019; Hodes et al., 2018; Valvassori et al., 2017; Souza et al., 2015; Arunagiri et al., 2014; Gazal et al., 2014; Brocardo et al., 2010; Frey et al., 2006).

Oxidative stress has been linked to the enzyme glycogen synthase kinase 3β (GSK3β), which is proposed as the target of the antimanic effects of lithium (Dal-Pont et al., 2019; Dandekar et al., 2018; Valvassori et al., 2017; Malhi et al., 2013). Furthermore, GSK3β activity is enhanced in peripheral blood mononuclear cells of bipolar patients in manic states (Li et al., 2010) and mice that overexpressed GSK3β showed manic-like behaviors (Prickaerts et al., 2006). In this line, it had been suggested that lithium and valproic acid prevented ouabain-induced manic-like behavior in rats through GSK3β inhibition (Valvassori et al., 2017). Moreover, the antimanic-like effect of GSK3β inhibition was associated to an antioxidant effect (Dal-Pont et al., 2019; Machado-Vieira et al., 2007a). Andrographolide (ANDRO), the main bioactive constitutive of the plant *Andrographis paniculata*, possesses anti-inflammatory, antioxidant and neuroprotective effects (Mittal et al., 2016; Tan et al., 2016; Serrano et al., 2014; Lim et al., 2012). Importantly, ANDRO also inhibits

GSK3β (Varela-Nallar et al., 2015; Serrano et al., 2014). Thus, it can be hypothesized that ANDRO might display antimanic-like effects.

Psychostimulant administration is the pharmacologically-induced animal model of mania most frequently used (Young et al., 2011). This model is based on the drug-induced increase in locomotor activity mainly (Hernandez-Miranda et al., 2017; Young et al., 2011). More recently, we proposed that 50-kHz ultrasonic vocalizations (USVs), which are related to positive affect, can serve as additional readouts for manic-like states (Wendler et al., 2019; Engelhardt et al., 2017; Hernandez-Miranda et al., 2017; Wendler et al., 2016; Pereira et al., 2014). In this line, lisdexamfetamine (LDX), a pro-drug of amphetamine, induces an increase of locomotor activity and 50-kHz USVs that are prevented by lithium or valproate administration (Bristot et al., 2019; Wendler, et al., 2016; Souza et al., 2015; Macêdo et al., 2013).

The aim of the present study was to evaluate the possible antimanic-like effect of repeated treatment with ANDRO on LDX-induced increases in 50-kHz USVs and locomotor activity of rats. In addition, the effect of ANDRO was studied on LDX-induced decreases in glutathione (GSH) levels and increases in lipid peroxidation (LPO) levels in rat prefrontal cortex (PFC) and striatum. Lithium was used as a positive control.

2. METHODS

2.1 Animals

Adults Wistar male rats (280-300 g) were socially housed (3-4 rats per cage) in polycarbonate cages (41 x 34 x 16 cm) and maintained in a room with controlled temperature (22±2 ºC) and constant 12h:12h light/dark cycle (with lights on between 7 a.m. and 7 p.m.), with water and standard laboratory chow access *ad libitum*. The experiments started one week after the rats arrived in our facility. All experiments were performed in accordance with the Brazilian Law for Animal Experimental Ethics and Care (11.794/8 October 2008) and the Local Committee on the Care and Use of Laboratory Animals. The experimental procedures were approved by the Institutional Ethics Board (CEUA/BIO – protocol # 1109). All efforts were made in order to minimize the number of animals used and their suffering.

2.2 Drugs and treatment protocol

ANDRO (Sigma, São Paulo, Brazil) was administered at a dose of 2 mg/kg, intraperitoneally (i.p.) (Chan et al., 2010; Niranjan et al., 2010). ANDRO was dissolved in saline with dimethyl sulfoxide (DMSO 2% v/v). The repeated treatment was performed throughout 21 days, 3 times/week (Monday, Wednesday and Friday). Lithium carbonate (Eurofarma, Itapevi, Brazil) was used as positive control at a dose of 100 mg/kg. Lithium was dissolved in saline and the pH was adjusted to 7.4 by adding 2N HCl. Repeated treatment was performed throughout 21 days, once a day. LDX (Venvanse®, Shire, São Paulo, Brazil) was dissolved in saline at a dose of 10 mg/kg. All drugs were administered i.p in a constant volume of 1 ml/kg body weight

The animals were treated with vehicle (saline + DMSO), 100 mg/kg lithium or 2.0 mg/kg ANDRO for 21 days. On the test day, 10 mg/kg LDX or saline was administered (i.p.) 1 h before the test. One hour after LDX administration, the rats were placed individually in an acrylic box (40 x 40 x 40 cm) for the recording of USV calls and locomotor activity, as shown in Figure 1.

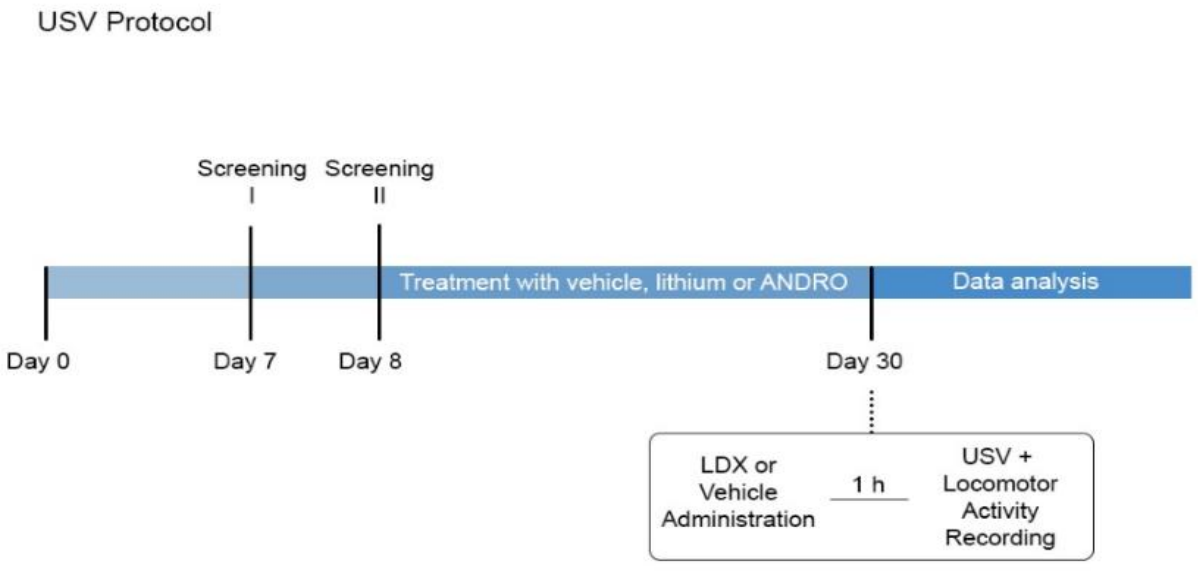

Fig 1. Experimental protocol. ANDRO: andrographolide; LDX: lisdexamfetamine; USV: ultrasonic vocalization recording. Screening test: individual USV recording in a clean homecage.

2.3 Screening test

In order to control for inter-individual variability that could affect USV, the rats were tested for their levels of spontaneous USV in a polycarbonate cage with clean bedding as a screening test. This test was performed on two consecutive days (5 minutes each) and the number of spontaneous 50-kHz USV calls were recorded. The rats were divided into the experimental groups (vehicle + saline, lithium + saline, ANDRO + saline, vehicle + LDX, lithium + LDX, ANDRO + LDX) according to the average number of 50-kHz USVs emitted on the two test days. Lights were dimmed to 4 lux for all tests (Natusch and Schwarting, 2010).

2.4 Locomotor activity test

USV calls and locomotor activity were recorded simultaneously. On the test day (day 30), 1h after LDX (or saline) injection, the rats were placed individually in an acrylic box (40 x 40 x 40 cm), with fresh bedding, and observed for 20 minutes. Recordings of the locomotor activity were made by a camera placed on top of the acrylic box. On the monitor screen the box image was divided virtually into 9 equally sized squares and a blind observer counted the number of squares crossed by the rats.

2.5 Ultrasonic vocalizations and analysis

USV emission were recorded by an UltraSound Gate Condenser Microphone (CM16; Avisoft Bioacustics, Berlin, Germany), sensible to frequencies between 15 and 180-kHz, which was placed 45 cm above the acrylic box and connected to a computer with the Avisoft Recorder 2.7 software (Wendler et al, 2019; Wendler et al., 2016; Pereira et al., 2014). Spectrograms from the USV recordings were generated at a frequency resolution of 488-Hz and a time resolution of 0.512 ms and were manually quantified, according to previous studies (Wendler et al., 2019; Pereira et al., 2014; Natusch and Schwarting, 2010). All USV emitted over 33-kHz were considered as 50-kHz USV (Wendler et al., 2016; Wöhr et al., 2015; Pereira et al., 2014).

2.6 Evaluation of oxidative stress parameters in the mouse brain

2.6.1 Brain samples

The animals were euthanized by decapitation immediately after the recording of USV emission and locomotor activity. The PFC and striatum were dissected, frozen in liquid nitrogen, and stored at -80ºC until further analysis. The brain samples were homogenized in potassium phosphate buffer (0.1 M, pH 6.5) in a 1:10 dilution. One part of the homogenate was used to determine the GSH levels, and the other was centrifuged at 9000 × *g* in a micro-high-speed refrigerated centrifuge (VS-15000 CFNII, Vision Scientific, Daejeon, South Korea) for 20 min. The supernatant was used to evaluate LPO.

2.6.2 Evaluation of GSH levels

To measure GSH levels, 100 μl of the homogenate was mixed with 80 μl of 12.5% trichloroacetic acid and centrifuged at 7600 x *g* for 15 min at 4ºC. Next, 20 μl of the supernatant was mixed with 280 μl of Tris buffer (0.4 M, pH 8.9) and 5 μl of DTNB (5,5'-dithiobis-[2-nitrobenzoic acid] in methanol, following the protocol originally described by Sedlak and Lindsay (1968), with minor modifications. Absorbance was read at 415 nm using a multi-mode microplate reader (BioTek Synergy HT, BioTek Instruments, Highland Park, VT, USA). The individual values were interpolated in a standard curve of GSH (0.375-3.0 μg) to corroborate the linearity of the reaction (r^2 must be > 0.99), and the values were divided by a correction factor. The results are expressed as μg/g of tissue.

2.6.3 Evaluation of LPO levels

Lipid peroxidation was determined according to the protocol described by Jiang et al. (1992), with minor modifications. First, 100 μl of the supernatant was suspended in 100 μl of methanol, vortexed, and centrifuged at 5400 x *g* for 5 min at 4ºC. Next, 100 μl of the supernatant was added to 900 μl of FOX2 reagent (Wolff's reagent; 4 mM BHT, 250 μM $FeSO_4$, 250 mM H_2SO_4, and 100 mM xylenol orange). The samples were then vortexed and incubated for 30 min in the dark at room temperature. Absorbance was read at 560 nm using a multi-mode microplate reader (BioTek

Synergy HT, BioTek Instruments, Highland Park, VT, USA). The results are expressed as nmol.mg.protein^{-1}.

2.6.4 Quantification of proteins

The quantification of proteins (mg.ml^{-1}) in the PFC and striatum samples was performed according to the method designed by Bradford (1976) and used to express the LPO data. The reaction was examined at 595 nm in a microplate reader (BioTek Synergy HT, BioTek Instruments, Highland Park, VT, USA) using bovine serum albumin (BSA) as protein standard.

2.7 Statistical analysis

Data were analyzed by two-way ANOVA (factor LDX treatment: saline or LDX; factor repeated treatment: vehicle, lithium or ANDRO) followed by the Newman-Keuls. Since some variables (total 50-kHz USVs, total time, and number of trill and flat subtypes) did not show homoscedasticity, the raw data were transformed in square root before statistical analysis. Differences were considered statistically significant when $p < 0.05$. Pearson's correlation index was used to evaluate the degree of association between variables. Data was expressed as mean ± SEM of raw data. Statistica 7.0, StatSoft (Tulsa, USA) was used for the statistical analysis.

3. RESULTS

3.1 Repeated lithium and ANDRO treatment reversed LDX-induced increases in 50-kHz USV

Two-way ANOVA of the number of USVs showed effects of LDX administration ($F_{1,37} = 8.570$, $p < 0.001$), of repeated treatment ($F_{2,37} = 13.87$, $p < 0.001$) and LDX administration x repeated treatment interaction ($F_{2,37} = 4.42$, $p < 0.05$; Figure 3A). The *post hoc* test indicated that LDX administration significantly increased the number of calls ($p < 0.001$). Treatment with lithium and ANDRO prevented the increases in the number of 50-kHz calls induced by LDX ($p < 0.001$ and $p < 0.01$, respectively). Lithium or ANDRO alone did not reduce the number of calls ($p > 0.05$).

Call subtypes: regarding *flat calls*, there was an effect of LDX administration ($F_{1,37}$ = 14.23, p < 0.001), of repeated treatment ($F_{2,37}$ = 9.17, p < 0.001) and a significant LDX administration x repeated treatment interaction ($F_{2,37}$ = 3.92, p < 0.05; Table 1). LDX increased flat calls (p < 0.001) and repeated lithium and ANDRO prevented this effect (both p < 0.01). On *trill calls*, there was an effect of LDX administration ($F_{1,37}$ = 6.36, p < 0.001), of repeated treatment ($F_{2,37}$ = 11.01, p < 0.01) and an LDX administration x repeated treatment interaction ($F_{2,37}$ = 5.06, p < 0.05). LDX increased trill calls (p < 0.001) and repeated lithium and ANDRO prevented this effect (p < 0.001 and < 0.05, respectively). On *step calls* there was an effect of LDX treatment ($F_{1,37}$ = 9.28, p < 0.01), of repeated treatment ($F_{2,37}$ = 3.27, p < 0.05) but not for LDX administration x repeated treatment interaction ($F_{2,37}$ = 2.89, NS). LDX increased step calls (p < 0.01) independently from repeated treatment (Table 1).

On temporal parameters of 50-kHz USV (Table 1), there were no effects of LDX administration or repeated treatment with lithium or ANDRO on the duration of calls (LDX administration: $F_{1,37}$ = 1.09, NS; repeated treatment: $F_{2,37}$ = 1.00, NS; factors interaction: $F_{2,37}$ = 1.28, NS) and on the latency for the first call (LDX administration: $F_{1,37}$ = 0.04, NS; repeated treatment: $F_{2,37}$ = 0.40, NS; factors interaction: $F_{2,37}$ = 0.33, NS). On the other hand, on total calling time there was an effect of LDX administration ($F_{1,37}$ = 10.75, p < 0.01), of the repeated treatment ($F_{2,37}$ = 6.16, p < 0.01) and a significant LDX administration x repeated treatment interaction ($F_{2,37}$ = 4.74, p < 0.05). LDX increased total calling time (p < 0.001), an effect that was prevented by lithium and ANDRO treatment (both p < 0.01; Table 1).

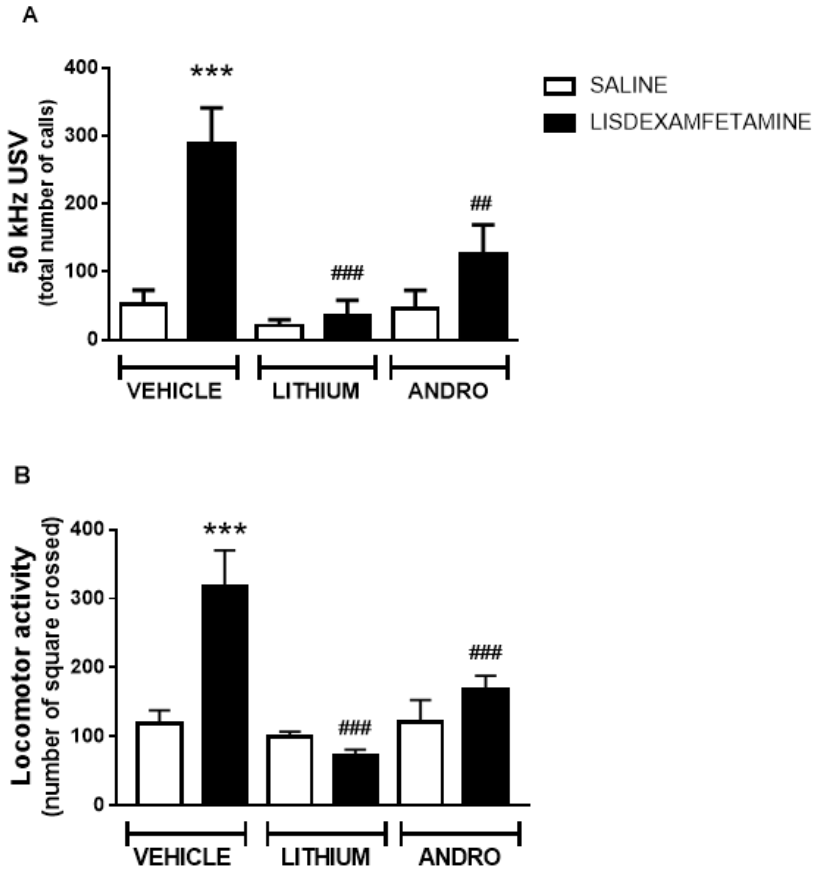

Fig 2. Effects of 21 days treatment with lithium (100 mg/kg i.p.), ANDRO (2.0 mg/kg i.p.) or vehicle on LDX (10 mg/kg i.p.)-induced increase in the number 50-kHz USV calls (A) and locomotor activity (B). Vehicle: saline + DMSO. Data are expressed by mean ± SEM. *n* = 5-8 rats/group. ***p < 0.001, compared with rats treated with vehicle + saline; $^{\#\#}p$ < 0.01 and $^{\#\#\#}p$ < 0.001, compared to the vehicle + LDX (two-way ANOVA followed by the Newman-Keuls *post hoc* test).

3.2 Repeated lithium and ANDRO treatment prevented LDX-induced hyperlocomotion

There were effects of LDX administration ($F_{2,37}$= 19.80, p < 0.001), of repeated treatment ($F_{1,37}$= 9.35, p < 0.001) and LDX administration x repeated treatment interaction ($F_{2,37}$= 4.07, p < 0.05). LDX administration increased locomotor activity (p < 0.001; Figure 3B), and treatment with lithium and ANDRO prevented such hyperlocomotion (p < 0.001 and p < 0.01, respectively). Lithium or ANDRO alone did not reduce locomotor activity (both p > 0.05).

Table 1 – Effects of repeated lithium and ANDRO on acute effects of LDX on call subtypes and temporal parameters of 50-kHz USVs.

	Saline			Lisdexamfetamine		
	Vehicle	Lithium	ANDRO	Vehicle	Lithium	ANDRO
Call subtypes						
Flat	40 ± 16	13 ± 5	28 ± 14	204 ± 39*	26 ±17	84 ± 32
Trill	6 ± 4	4 ± 1	10 ± 5	49 ± 11*	3 ± 2	22 ± 8
Step	4 ± 1	3 ± 2	4 ± 3	20 ± 31$	5 ± 3$	10 ± 4$
Temporal Parameters						
Call duration	5.2± 2.8	11.8±8.2	3.7±2.2	8.2±4.6	7.8±6.1	7.1±3.5
Latency 1st call	0.03±0.01	0.08±0.05	0.03±0.02	0.04±0.01	0.03±0.01	0.03±0.01
Total Time	1.69±0.67	1.14±0.40	1.73±1.19	11.46±2.56*	1.26±0.71	4.54±1.75

Data represent mean ± SEM.

Call duration, latency to first call, and total time in seconds; call subtypes: number of calls.

*$p < 0.05$ compared to all other groups

$^{\$}p < 0.05$ compared to all saline treated rats (LDX administration factor)

3.3 Repeated ANDRO administration prevented LDX-induced lipid peroxidation in rat striatum

LPO levels

In the striatum, two-way ANOVA showed effects of repeated treatment ($F_{2,37}$ = 6.33, $p < 0.01$), LDX administration ($F_{1,37}$ = 71.52, $p < 0.001$) and LDX administration x repeated treatment interaction ($F_{2,37}$ = 13.94, $p < 0.001$) in LPO levels. The Newman-Keuls test indicated that LDX administration increased LPO levels ($p < 0.001$) and lithium and ANDRO repeated treatment prevented LDX-induced increases in LPO levels ($p < 0.001$ and $p < 0.05$, respectively; Figure 3A).

In PFC, two-way ANOVA indicated that there were no effects of repeated treatment ($F_{2,37}$ = 2.38, NS), LDX administration ($F_{1,37}$ = 0.22, NS) or LDX administration x repeated treatment interaction ($F_{2,37}$ = 1.08, NS) in the levels of LPO (Figure 3B).

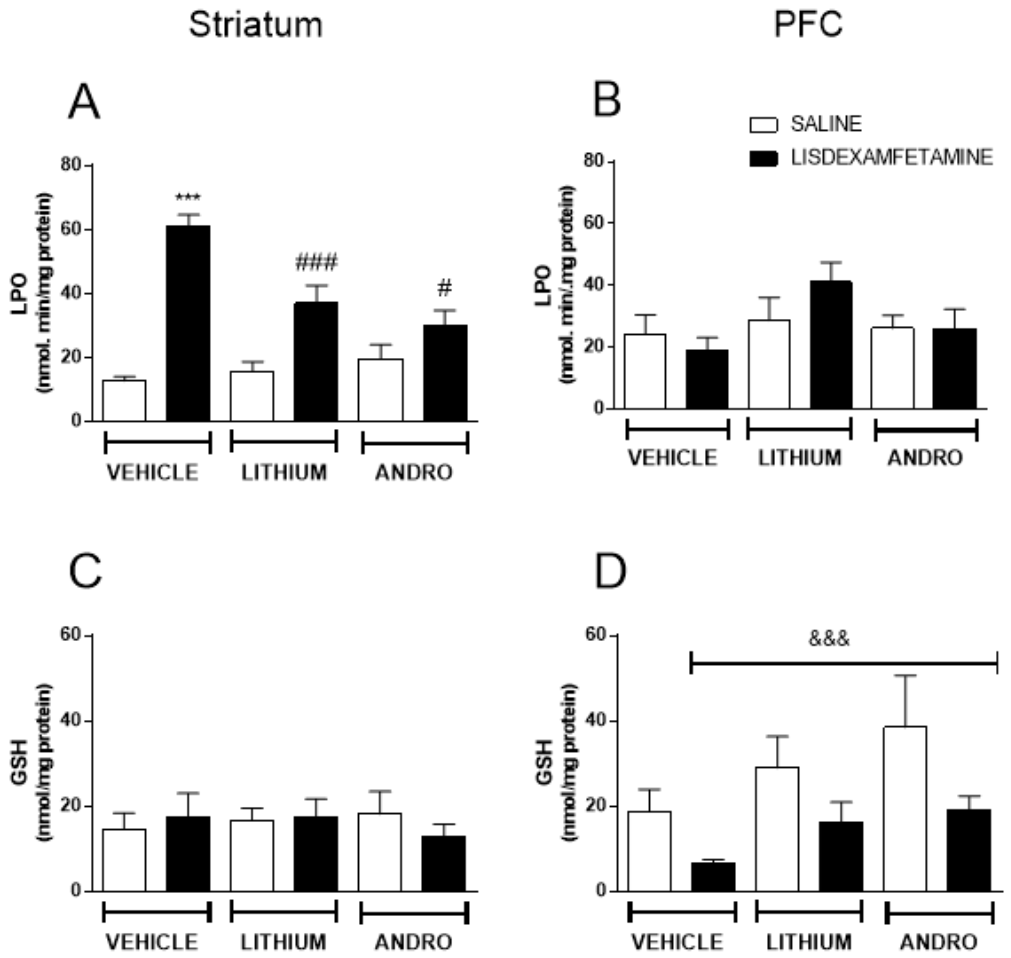

Fig 3. Effects of 21 days treatment with lithium (100 mg/kg i.p.), ANDRO (2.0 mg/kg i.p.) or vehicle on LDX (10 mg/kg i.p.)-decreases in GSH and increases in LPO levels in the PFC and striatum. A) LPO levels in the striatum; (B) LPO levels in the PFC; (C) GSH levels in the striatum; (D) GSH levels in the PFC. Vehicle: saline + DMSO. Data are expressed by mean ± SEM. *n* = 5-8 rats/group. ***$p < 0.001$, compared with rats treated with vehicle + vehicle; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$, compared with rats treated with vehicle + LDX; $^{\&\&\&}$p<0.05 compared to vehicle treated rats (LDX administration factor). Two-way ANOVA followed by the Newman-Keuls test).

GSH levels

In striatum, two-way ANOVA did not indicate effects of repeated treatment ($F_{1,37}$ = 0.055, NS), LDX administration ($F_{1,37}$ = 0.02, NS) or LDX administration x repeated treatment interaction ($F_{1,37}$ = 0.02, NS) in the levels of GSH (Figure 3C).

In PFC, two-way ANOVA showed effects of repeated treatment ($F_{2,37}$ = 3.35, $p < 0.05$) and LDX administration ($F_{1,37}$ = 7.59, $p < 0.01$) but not repeated treatment-LDX administration interaction $F_{1,37}$ = 0.20, NS). The Newman-Keuls test indicated that LDX administration decreased GSH levels in rat PFC ($p < 0.01$; Figure 3D).

3.4 Correlations

The Pearson's correlation test showed positive correlations between LPO levels in rat striatum with 50-kHz USVs calls (r= 0.62, p < 0.001) and with locomotor activity (r= 0.61, p < 0.001), as seen in Figure 4.

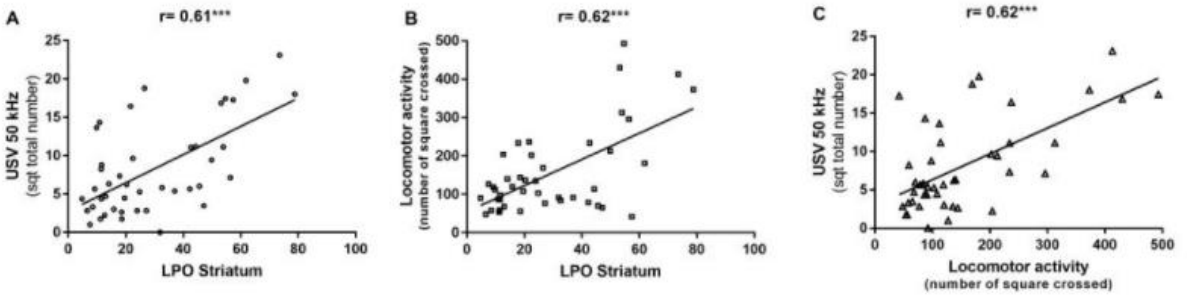

Fig 4. Pearson's correlation between the LPO levels in the striatum with 50-kHz USV calls (A), LPO levels with locomotor activity (B) and between locomotor activity with 50-kHz USV calls (C).

4. DISCUSSION

The present study showed that repeated treatment with ANDRO prevented LDX-induced manic-like behaviors (hyperlocomotion and increases in 50-kHz USVs) and striatal LPO levels. Similar results were obtained with repeated treatment with lithium, a clinically effective antimanic drug, which was used as a positive control. These results suggest that ANDRO possesses an antimanic-like behavioral effect and exerts antioxidant activity in the LDX model of mania.

The administration of psychostimulants (e.g., *d*-amphetamine) is a common method for inducing manic-like behavior in animal models, such as hyperlocomotion. LDX is a long-acting *d*-amphetamine pro-drug employed in the therapeutic management of attention deficit/hyperactivity disorder (Ermer et al., 2016). LDX administration has also been used to mimic manic-like behavior in animals (Bristot et al., 2019; Ascoli et al., 2017; Eger et al., 2016; Wendler et al., 2016; Souza et al., 2015; Macêdo et al., 2013). These manic-like behaviors were reversed or prevented by treatment with lithium or valproate (Lv et al., 2020; Bristot et al., 2019; Ascoli et al., 2017; Souza et al., 2015; Macêdo et al., 2013; Malhi et al., 2013; Machado-Vieira et al., 2007a; Cunha et al., 2006). LDX administration also increases oxidative stress and reduced BDNF levels, as observed in plasma as well as serum of bipolar patients in manic state (Caldiroli et al., 2020; Machado-Vieira et al., 2007b). Thus, LDX is a valid model for the study of mania and antimanic-like drugs. In the present study, repeated

ANDRO and lithium administration prevented LDX-induced hyperlocomotion at a dose that did not affect spontaneous locomotor activity, a profile suggestive of an antimanic-like effect (Young et al., 2011).

In addition to hyperlocomotion, LDX administration also showed an increase in 50-kHz USVs. Adult rats emit high-frequency 50-kHz USVs in appetitive situations, such as playing with other rats, mating or after psychostimulant administration (Rippberger et al., 2015; Burgdorf et al., 2011) Therefore, it is proposed that 50-kHz can represent a positive affective state in rats (Brudzynski et al., 2018; Wöhr and Schwarting, 2013). Considering that 50-kHz USVs may reflect a positive state in rats, this behavior can be used to monitor hedonic states in animal models of various psychiatric disorders (Burgdorf et al., 2011). In this line, 50-kHz USVs were found to be increased in different animal models of mania such as amphetamine or LDX administration and sleep deprivation (Wendler et al., 2019; Engelhardt et al., 2017; Wendler et al., 2016; Pereira et al., 2014). This increase in 50-kHz USVs was blocked by the antimanic drugs lithium, tamoxifen and antipsychotics (Wendler et al., 2019; Wendler et al., 2016; Barker et al., 2015; Pereira et al., 2014; Wintink and Brudzynski, 2001). Thus, 50-kHz USVs have been proposed as a new marker in animal models of mania, representing the increase in the positive affect (Wendler et al., 2019; Engelhardt et al., 2017; Hernandez-Miranda et al., 2017; Wendler et al., 2016). In the present study, repeated treatment with ANDRO and lithium prevented LDX effects in the total number of 50-kHz calls, in the number of trill and flat calls subtypes and in total calling time. ANDRO and lithium alone did not affect 50-kHz USVs. These results indicated an antimanic-like effect of ANDRO on 50-kHz USVs.

The present study also shows that LDX administration can lead to increased LPO in striatum and decreased GSH levels in PFC. Repeated lithium and ANDRO treatment prevented LDX-induced increases in LPO in rat striatum. Increased generation of reactive oxygen species and free radicals are involved in the pathophysiology of BD, as LPO markers are present in different phases of BD in the serum/plasma of BD patients and are associated to illness severity and/or the number of manic episodes (Akarsu et al., 2018; Sowa-Kucma et al., 2017; Brown et al., 2014; Andreazza et al., 2007; Machado-Vieira et al., 2007a). In this line, high concentrations of phospholipids in brain tissues make the brain more vulnerable to oxidative stress induced by LPO (Banerjee et al., 2012). Lipid hydroperoxide chain reactions eventually cause the formation of reactive aldehydes and this can damage lipid membranes

(Maes et al., 2018). Increased oxidative stress leads to derangement of signal transduction, structural plasticity and cellular resilience in brain tissue (Schäfer et al., 2004). Lv et al. (2020) showed that LPO levels were higher in the plasma of treatment-resistant BD patients and that decreased after 6 weeks of electroconvulsive therapy.

Oxidative stress in the brain was observed in different models of mania such as psychostimulants (Chaves Filho et al., 2020; Valvassori et al., 2019; Hodes et al., 2018; Sharma et al., 2016; Frey et al., 2006), ouabain (Dal-Pont et al., 2019; Valvassori et al., 2017), sleep deprivation (Kanazawa et al., 2016) and ketamine (Gazal et al., 2014). The observation that mood stabilizing agents such as lithium and sodium valproate also exert antioxidant effects, reinforces the idea that oxidative stress is involved in the pathophysiology and treatment of mania (de Queiroz et al., 2018; Valvassori et al., 2017; Kanazawa et al., 2016; Brown et al., 2014; Banerjee et al., 2012; Andreazza et al., 2007). However, the antioxidant effects of lithium may be specific to certain brain regions and they can vary depending on experimental variables including type of drug and age of animals. For example, in mice, repeated administration of amphetamine increased LPO in PFC, hippocampus and amygdala and doxycycline, but not lithium, reversed LPO increasing in hippocampus (Chaves Filho et al., 2020). On the other hand, Hodes et al. (2018) found that acute administration of amphetamine increased LPO in the hippocampus of mice but not in the PFC. Repeated methylphenidate administration led to LPO and protein damage in the PFC, but not in the striatum, cerebellum or hippocampus of juvenile rats (Schmitz et al., 2012). Particularly to LDX, it increased LPO in rat PFC, hippocampus and striatum, and lithium and valproate were able to prevent and reverse the effects of repeated LDX administration on LPO in these brain areas (Macêdo et al., 2013; de Souza et al., 2015). In the present study, acute LDX administration increased LPO levels in the striatum, but not in the PFC. Treatment with ANDRO and lithium reduced LDX-induced increase in LPO levels in rat striatum. In addition, there was a positive correlation between LPO levels in rat striatum and increases in 50-kHz USVs, as well as LDX-induced hyperlocomotion. Menegas et al. (2020), using the m-amphetamine administration model of mania, also observed correlations between hyperlocomotion and lipid damage parameters in the striatum of rats. Clinically, a positive correlation between mania severity (Young Mania Rating Scale) and oxidative stress index was also (Akarsu et al., 2018) observed which supports the relevance of our pre-clinical approach.

GSH is a non-enzymatic antioxidant molecule and its depletion can lead to neuronal dysfunctions and various disorders (Gaucher et al., 2018). In the present study, LDX administration reduced GSH levels in rat PFC. ANDRO and lithium treatment, however, had no effects on LDX-induced decrease on GSH level. Studies also showed divergent results regarding GSH levels in the brain. Macêdo et al. (2013), for example, showed that LDX administration induced hyperlocomotion and decreased GSH content in rat PFC and striatum, which was prevented by lithium in both brain areas. However, only lithium reversed the reduction of GSH in the PFC. These heterogeneous results can be dependent of methodological differences including subjects, experimental protocol or acute/repeated drug administration.

The antimanic effect of lithium has been partly related to its inhibitory activity on the enzyme GSK3β, which is upregulated in the brain of BD patients (Li et al., 2010). Moreover, GSK3β has been linked to oxidative stress in mania models. AR-A014418, a GSK3β inhibitor, reverted ouabain-induced hyperlocomotion and oxidative stress in mice brain (Dal-Pont et al., 2019). In this line, ANDRO also inhibits GSK3β (Varela-Nallar et al., 2015; Serrano et al., 2014) and, thus, inhibition of GSK3β may also contribute to the antimanic-like effect of ANDRO observed in the present study.

5. CONCLUSION

In conclusion, the present results suggest that ANDRO possesses antimanic-like effects in the LDX-administration model of mania, preventing LDX-induced increases in 50-kHz USVs and hyperlocomotion, and its antioxidant effects may mediate these effects.

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CHAPTER 5

Quercetin reduces manic-like behavior and brain oxidative stress induced by paradoxical sleep deprivation in mice

Luiz K. S. Kanazawa,[a] Débora D. Vecchia,[a] Etiéli M. Wendler,[a] Palloma de A. S. Hocayen,[a] Francislaine A. dos Reis Lívero,[b] Maria Carolina Stipp,[b] Inara M. R. Barcaro,[a] Alexandra Acco,[b] Roberto Andreatini[a,*]

[a] *Laboratory of Physiology and Pharmacology of the Central Nervous System, Department of Pharmacology, Federal University of Paraná, Centro Politécnico, 81540-990, Curitiba, PR, Brazil*

[b] *Laboratory of Pharmacology and Metabolism, Department of Pharmacology, Federal University of Paraná, Centro Politécnico, 81540-990, Curitiba, PR, Brazil*

*Corresponding author

ABSTRACT

Quercetin is a known antioxidant and protein kinase C (PKC) inhibitor. Previous studies have shown that mania involves oxidative stress and an increase in PKC activity. We hypothesize that quercetin affects manic symptoms. In the present study, manic-like behavior (hyperlocomotion) and oxidative stress were induced by 24 h paradoxical sleep deprivation (SD) in male Swiss mice. Both 10 and 40 mg/kg quercetin prevented SD-induced hyperlocomotion. Quercetin reversed the SD-induced decrease in glutathione (GSH) levels in the prefrontal cortex (PFC) and striatum. Quercetin also reversed the SD-induced increase in lipid peroxidation (LPO) in the PFC, hippocampus, and striatum. Pearson's correlation analysis revealed a negative correlation between locomotor activity and GSH in the PFC in sleep-deprived mice and a positive correlation between locomotor activity and LPO in the PFC and striatum in sleep-deprived mice. These results suggest that quercetin exerts an antimanic-like effect at doses that do not impair spontaneous locomotor activity, and the antioxidant action of quercetin might contribute to its antimanic-like effects.

Key words: Bipolar disorder, mania, oxidative stress, paradoxical sleep deprivation, protein kinase C, quercetin.

Abbreviations: BD, bipolar disorder; CAT, catalase; CMC, carboxymethylcellulose; CNS, central nervous system; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); GPx, glutathione peroxidase; GSH, reduced glutathione; GSK-3, glycogen synthase kinase-3; LPO, lipid peroxidation; PFC, prefrontal cortex; PKC, protein kinase C; SD, sleep deprivation; SOD, superoxide dismutase.

1. INTRODUCTION

Bipolar disorder (BD) is a chronic disease that is characterized by recurrent episodes of depression and mania (Abrial et al., 2015). It is a common, disabling, and recurrent mental health condition that has various levels of severity (Price and Marzani-Nissen, 2012) and prevalence of 1-3% worldwide (Merikangas et al., 2007). Bipolar patients experience both symptoms of the disease and also impaired functioning, compromised quality of life, and stigma (Michalak et al., 2011). Lithium is the main pharmacological treatment for BD. Other treatments include the anticonvulsants valproate, carbamazepine, and lamotrigine and atypical antipsychotics quetiapine, risperidone, olanzapine, and aripiprazole (Chiu et al., 2013; Miklowitz and Johnson, 2006; Müller-Oerlinghausen et al., 2002). The currently available treatments for BD have many side effects (Price and Marzani-Nissen, 2012), such as xerostomia, polydipsia, polyuria, weight gain, insulin resistance, extrapyramidal symptoms, and sexual dysfunction, among others (Kemp et al., 2014; Chiu et al., 2013; Price and Marzani-Nissen, 2012; Müller-Oerlinghausen et al., 2002). The great number of side effects results in low treatment adherence (Miklowitz and Johnson, 2006; Sajatovic et al., 2004), and more than one-third of BD patients do not respond to treatment, even when it is adequate (Perlis et al., 2006; Judd et al., 2005).

The pathophysiology that underlies BD involves alterations in neurotransmitter levels (Lahera et al., 2013; Hashimoto et al., 2007), increases in the activity of protein kinase C (PKC) (Manji et al., 2000), and increases in oxidative stress (Berk et al., 2011; Andreazza et al., 2008). Studies have shown that chronic treatment with lithium and valproate at therapeutic concentrations exert robust antioxidant effects *in vitro* by inhibiting glutamate-induced DNA fragmentation, lipid peroxidation (LPO), and protein oxidation (Shao et al., 2005). Souza et al. (2014) reported an increase in oxidative stress in rats that exhibited ouabain-induced hyperlocomotion (i.e., an animal model of mania). Lithium also prevented manic-like behavior in rats and the ouabain-induced increase in superoxide dismutase (SOD) activity and decreases in catalase (CAT) and glutathione peroxide (GPx) activity in the cerebral cortex and hippocampus.

Another relevant mechanism of action of lithium is its inhibitory effect on the enzyme PKC, which can be related to its antimanic actions (Wang and Friedman, 1989). Wang and Friedman (1996) showed that *post-mortem* brains of BD patients had increases in the translocation of PKCγ and PKCζ to the membrane, which

indicates higher levels of the active form of these enzymes. Similarly, some of the pharmacological activities of flavonoids, such as the flavonol quercetin, have been postulated to result from inhibitory activity on kinases, such as PKC (Gamet-Payrastre et al., 1999). However, quercetin is best known for its antioxidant properties (Dajas et al., 2015; Lakhanpal et al., 2007). It appears to be one of the most powerful flavonoids with regard to protecting the body against reactive species that are produced during normal oxygen metabolism or induced by exogenous damage (De Groot, 1994; Grace, 1994). Quercetin is able to increase the levels or activity of antioxidants, such as glutathione (GSH), CAT, and SOD, and decrease in LPO (Rinwa and Kumar, 2013).

Manic patients experience sleep disturbances (Streck et al., 2015), and the model of paradoxical sleep deprivation (SD) is considered a non-pharmacological animal model of mania that induces manic-like behavior (i.e., hyperlocomotion) (Abrial et al., 2015; Streck et al., 2015; Gessa et al., 1995; Tufik et al., 1978) and increases in oxidative stress (Streck et al., 2015; Ghosh et al., 1976).

Considering the relationship between manic symptoms and increases in PKC activity and oxidative stress in BD, the objective of the present study was to evaluate the effects of the antioxidant and PKC inhibitor quercetin on manic-like behavior and oxidative stress in mice that were subjected to SD-induced hyperlocomotion.

2. MATERIALS AND METHODS

2.1. Animals

The experiments were conducted using male Swiss mice (30-40 g) housed at 22°C ± 2°C (6-8/cage) under a 12 h/12 h light/dark cycle (lights on at 7:00 AM). The animals were kept in polypropylene cages (41 cm × 34 cm × 16 cm) with food and water available *ad libitum*. All of the experiments were approved by the Committee of Animal Experimentation of the Federal University of Paraná (CEUA/BIO-UFPR, protocol no. 733).

2.2. Drugs

The mice were treated with saline (0.9% NaCl; 10 ml/kg i.p.), lithium carbonate (Eurofarma, Itapevi, Brazil; positive control; 100 mg/kg i.p., dissolved in saline, with

the pH adjusted to 7.4 with HCl), or quercetin (Sigma, St. Louis, MO, USA; 10 or 40 mg/kg i.p., suspended in 0.5% carboxymethylcellulose [CMC]). All of the drugs were administered in a volume of 10 ml/kg of body weight. The doses were based on data from the literature (Silva et al., 2004) and previous studies by our research group (Bhutada et al., 2010; Sabioni et al., 2008).

2.3. Paradoxical sleep deprivation protocol

Prior to SD, basal spontaneous locomotor activity was measured in an automated activity box for 20 min. Based on the animals' basal locomotor activity, they were divided by stratified randomization into sleep-deprived and non-sleep deprived groups and further subdivided into vehicle, lithium, and 10 and 40 mg/kg quercetin groups. The mice were treated with the respective drugs and sleep-deprived for 24 h according to an adaptation of the multiple platform protocol (Ghosh et al., 1976).

Briefly, in the SD procedure, groups of six animals were placed in polypropylene cages (41 cm × 34 cm × 16 cm). Each cage contained 12 platforms (3 cm diameter, 5 cm height), surrounded by water up to 1 cm below the surface of the platforms. The animals could move freely, jumping from one platform to another. Food and water were available during the entire procedure.

Thirty minutes before the end of the deprivation period (i.e., 23.5 h after beginning the SD period), the animals were treated again with the respective drugs. After the deprivation period of 24 h, the animals were immediately placed in the automated activity box for 20 min to evaluate locomotor activity (Figure 1).

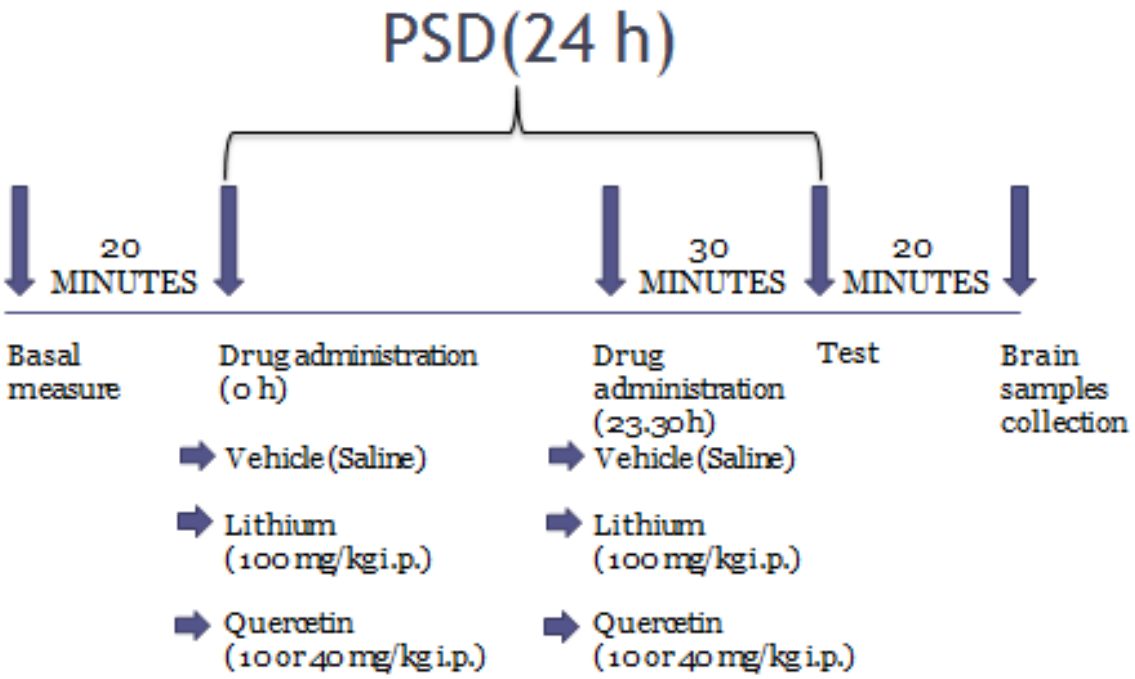

Fig 1. Timeline of the paradoxical sleep deprivation protocol.

2.4. DPPH assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay is used to evaluate the free radical scavenging activity of antioxidants. The assay is based on the principle that DPPH, upon accepting a hydrogen atom from a scavenger molecule (e.g., an antioxidant), is reduced, and the purple color of the solution changes to yellow, concomitant with a decrease in absorbance (Pereira et al., 2011).

Following the protocols described by Blois (1958) and Chen et al. (2004), with minor modifications, different concentrations of either quercetin or lithium (0.001, 0.003, 0.01, 0.03, 0.1, 0.3 and 1 μg/ml) were mixed with DPPH methanolic solution (10 μg/ml). Ascorbic acid (50 μg/ml) was used as a positive control. Distilled water with 0.5% CMC was used as a negative control. Absorbance was measured at 517 nm using a multi-mode microplate reader (BioTek Synergy HT, BioTek Instruments, Highland Park, VT, USA).

2.5. Evaluation of oxidative stress parameters in the mouse brain

2.5.1. Brain samples

The mice were euthanized by decapitation immediately after being exposed to the automated activity box. The prefrontal cortex (PFC), hippocampus, and striatum were dissected, frozen in liquid nitrogen, and stored at -80ºC until further analysis. The brain samples were homogenized in potassium phosphate buffer (0.1 M, pH 6.5) in a 1:10 dilution. One part of the homogenate was used to determine GSH levels, and the other was centrifuged at 9000 × *g* in a micro-high-speed refrigerated centrifuge (VS-15000 CFNII, Vision Scientific, Daejeon, South Korea) for 20 min. The supernatant was used to evaluate LPO.

2.5.2. Evaluation of GSH levels

To evaluate GSH levels, 100 μl of the homogenate was mixed with 80 μl of 12.5% trichloroacetic acid and centrifuged at 6000 rotations per minute (rpm) for 15 min at 4ºC. Afterwards, 20 μl of the supernatant was mixed with 280 μl of Tris buffer (0.4 M, pH 8.9) and 5 μl of DTNB (5,5'-dithiobis-[2-nitrobenzoic acid] or Ellman's reagent; 0.01 M) according to the protocol that was originally described by Sedlak and Lindsay (1968), with minor modifications. Absorbance was read at 415 nm using a multi-mode microplate reader (BioTek Synergy HT, BioTek Instruments, Highland Park, VT, USA). The individual values were interpolated in a standard curve of GSH (0.375-3 μg) to verify the linearity of the reaction (r^2 must be > 0.99), and the values were divided by a correction factor. The results are expressed as μg/g of tissue, representing the quantity of GSH (μg) in the tissue (g).

2.5.3. Evaluation of LPO levels

LPO was measured according to the method described by Jiang et al. (1992), with minor modifications. First, 100 μl of the supernatant was suspended in 100 μl of methanol, vortexed, and centrifuged at 5000 rpm for 5 min at 4ºC. Afterwards, 100 μl of the supernatant was added to 900 μl of FOX2 reagent (Wolff's reagent; 4 mM BHT, 250 μM $FeSO_4$, 250 mM H_2SO_4, and 100 mM xylenol orange). The samples were then vortexed and incubated for 30 min at room temperature in the dark. Absorbance was

read at 560 nm using a multi-mode microplate reader (BioTek Synergy HT, BioTek Instruments, Highland Park, VT, USA). The results are expressed as mmol of hydroperoxides/mg of tissue.

2.6. Statistical analysis

For all of the experiments, two-way analysis of variance (ANOVA) was used, with the exception of the DPPH assay, followed by the Newman-Keuls *post hoc* test if significant main effects or interactions were found in the ANOVA. The DPPH assay data were analyzed using one-way ANOVA followed by the Newman-Keuls *post hoc* test. The data are expressed as mean ± SEM. Values of $p < 0.05$ were considered statistically significant. Pearson's correlation analysis was performed to identify possible relationships between the behavioral and oxidative stress parameters (all mice, $n = 38\text{-}42$).

3. RESULTS

3.1 Effect of quercetin on 24 h SD-induced hyperlocomotion

The two-way ANOVA revealed significant main effects of SD ($F_{1,50} = 4.21$, $p < 0.05$) and treatment ($F_{3,50} = 19.52$, $p < 0.001$) and a SD $\times$ treatment interaction ($F_{3,50} = 4.80$, $p < 0.01$). The Newman-Keuls *post hoc* test revealed that SD increased locomotor activity compared with the control group (non-sleep-deprived + vehicle; $p < 0.001$) and all of the other groups (all $p < 0.01$; Figure 2). Treatment with lithium and 10 and 40 mg/kg quercetin blocked SD-induced hyperlocomotion ($p < 0.001$). Sleep-deprived mice that were treated with 40 mg/kg quercetin exhibited a decrease in locomotor activity compared with control mice ($p < 0.05$). Non-sleep-deprived mice that were treated with 40 mg/kg quercetin exhibited a tendency toward a decrease in locomotor activity ($p = 0.055$).

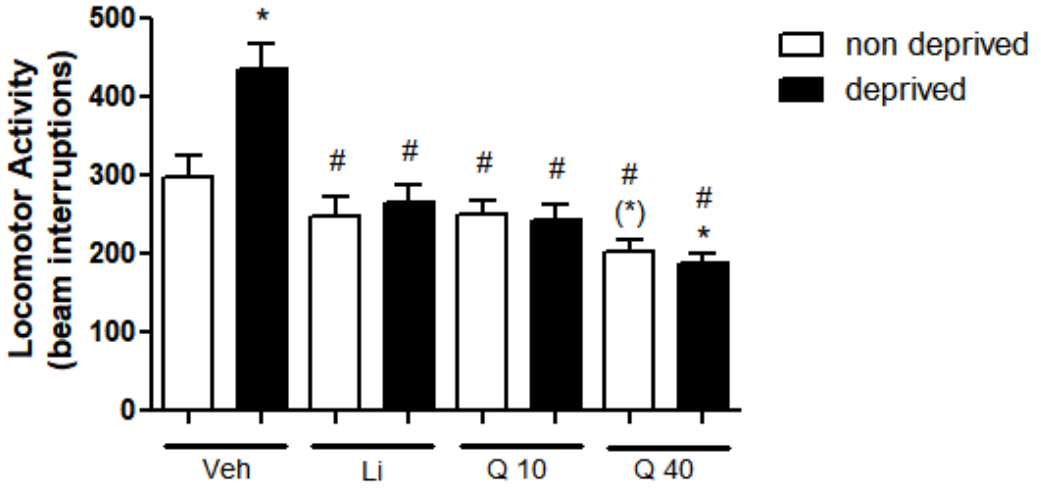

Fig 2. Effect of acute quercetin (Q; 10 and 40 mg/kg i.p.) and lithium (Li; 10 mg/kg i.p.) administration on 24 h SD-induced hyperlocomotion. Control: non-deprived mice. SD: sleep-deprived mice. Veh, vehicle. The data are expressed as the mean ± SEM number of beam breaks over 20 min. *n* = 7-8 mice/group. **p* < 0.05, (*) 0.05 < *p* < 0.10, compared with non-sleep-deprived mice treated with vehicle; #*p* < 0.05, compared with sleep-deprived mice treated with vehicle (two-way ANOVA followed by Newman-Keuls *post hoc* test).

3.2. Oxidative stress

3.2.1. DPPH assay

The one-way ANOVA of the free radical scavenging activity of lithium and quercetin in the DPPH assay revealed an antioxidant effect ($F_{8,18}$ = 137.39, p < 0.001, and $F_{8,18}$ = 52.07, p < 0.001, respectively; Figure 3A, B). The *post hoc* comparisons showed that all concentrations of lithium, quercetin (except 0.001 and 0.003 µg), and ascorbic acid reduced DPPH levels (p < 0.001).

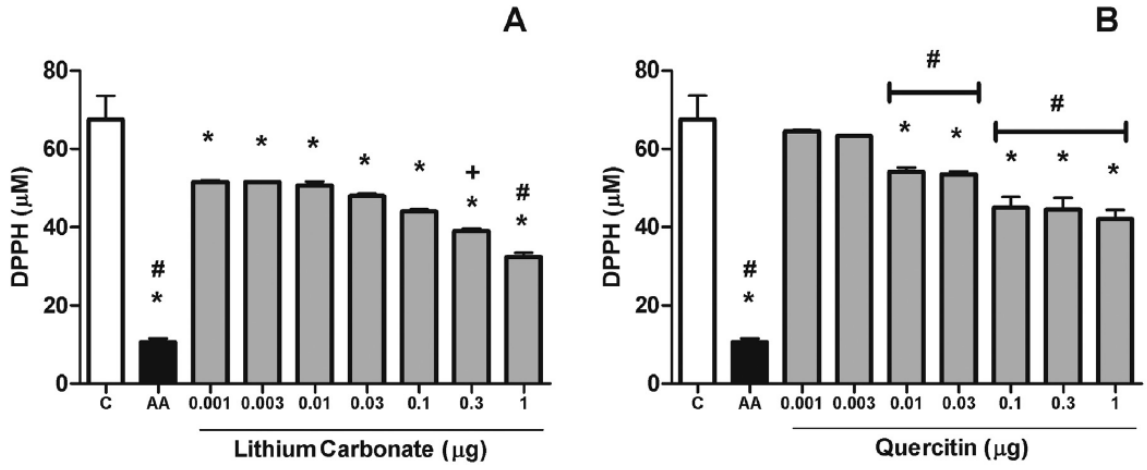

Fig 3. Effects of lithium (A), quercetin (B), vehicle (C; negative control), and ascorbic acid (AA; positive control) on free radical scavenging activity in the DPPH assay. The data are expressed as mean ± SEM. The tests were performed in triplicate. *$p < 0.001$, compared with control (C). $^{\#,}p < 0.05$, compared with all other groups; MW: Li_2CO_3=42.39 (1 µg=23.6 nmol); Quercetin ($C_{15}H_{10}O_7$): 302.24 (1 µg=3.3 nmol).

3.2.2. Effect of acute administration of quercetin on glutathione levels after SD

The two-way ANOVA revealed an effect of treatment ($F_{3,31} = 16.93$, $p < 0.001$) on GSH levels in the PFC and a SD × treatment interaction ($F_{3,31} = 12.11$, $p < 0.001$). In the hippocampus, there was an effect of treatment ($F_{3,31} = 3.33$, $p < 0.05$) on GSH levels. The two-way ANOVA also revealed significant effects of SD ($F_{1,30} = 8.72$, $p < 0.001$) and treatment ($F_{3,30} = 12.26$, $p < 0.001$) on GSH levels in the striatum and a SD × treatment interaction ($F_{3,30} = 8.70$, $p < 0.001$). The *post hoc* tests revealed that SD decreased GSH levels in the PFC (Figure 4A) and striatum (Figure 4C) compared with non sleep deprived mice ($p < 0.05$, PFC; $p < 0.01$, striatum).

Treatment with 10 and 40 mg/kg quercetin and lithium blocked the SD-induced decrease in GSH levels in the PFC ($p < 0.001$; Figure 4A). The *post hoc* comparisons revealed that mice treated with 10 and 40 mg/kg quercetin (non deprived + deprived) exhibited an increase in GSH levels in the hippocampus (Figure 4B) compared with the vehicle group (non deprived + deprived; $p < 0.05$). The *post hoc* comparisons revealed that sleep-deprived mice that were treated with 10 mg/kg quercetin exhibited an increase in GSH levels in the striatum compared with the sleep deprived mice that were treated with vehicle ($p < 0.001$; Figure 4C). Sleep-deprived mice that were treated with lithium exhibited lower GSH levels in the striatum compared with the control group ($p < 0.001$) and non-sleep-deprived mice that were treated with lithium ($p < 0.05$). Sleep-deprived mice that were treated with 40 mg/kg quercetin did not differ from either group that was treated with vehicle ($p > 0.05$).

3.2.3. Effect of acute administration of quercetin on LPO after SD

The two-way ANOVA revealed effects of SD ($F_{1,34} = 44.15$, $p < 0.001$) and treatment ($F_{3,34} = 7.92$, $p < 0.001$) on LPO in the PFC and a SD × treatment interaction ($F_{3,34} = 4.53$, $p < 0.001$). In the hippocampus, there were effects of SD ($F_{1,34} = 64.48$, $p < 0.001$) and treatment ($F_{3,34} = 6.89$, $p < 0.001$) on LPO. The two-way ANOVA

revealed an effect of SD ($F_{1,30}$ = 25.23, p < 0.001) on LPO in the striatum and a SD × treatment interaction ($F_{3,30}$ = 9.58, p < 0.001).

SD increased LPO in the PFC (p < 0.05), hippocampus (p < 0.05), and striatum (p < 0.05) compared with non sleep deprived mice that were treated with vehicle. Sleep-deprived mice that were treated with lithium (p < 0.05), 10 mg/kg quercetin (p < 0.001), and 40 mg/kg quercetin (p < 0.01) exhibited a decrease in LPO in the PFC compared with sleep-deprived mice that were treated with vehicle (Figure 5A). Sleep-deprived mice that were treated with 10 mg/kg quercetin exhibited a significant increase in LPO compared with non-sleep-deprived mice that were treated with 10 mg/kg quercetin (p < 0.05). Sleep-deprived mice that were treated with 10 mg/kg quercetin and lithium exhibited an increase in LPO compared with non sleep deprived mice that were treated with vehicle (p < 0.05).

The *post hoc* comparisons showed that sleep-deprived mice that were treated with lithium (p < 0.05), 10 mg/kg quercetin (p < 0.001), and 40 mg/kg quercetin (p < 0.05) exhibited a decrease in LPO in the hippocampus compared with sleep-deprived mice (Figure 5B). Sleep-deprived mice that were treated with lithium and 40 mg/kg quercetin exhibited an increase in LPO compared with the control group (p < 0.05) and non-sleep-deprived mice that were treated with the respective drugs (p < 0.01).

The *post hoc* comparisons revealed that sleep-deprived mice that were treated with lithium (p < 0.01) and 10 mg/kg quercetin (p < 0.05) exhibited a decrease in LPO in the striatum compared with sleep-deprived mice that were treated with vehicle (Figure 5C). Treatment with 40 mg/kg quercetin did not affect the SD-induced increase in LPO (p > 0.05).

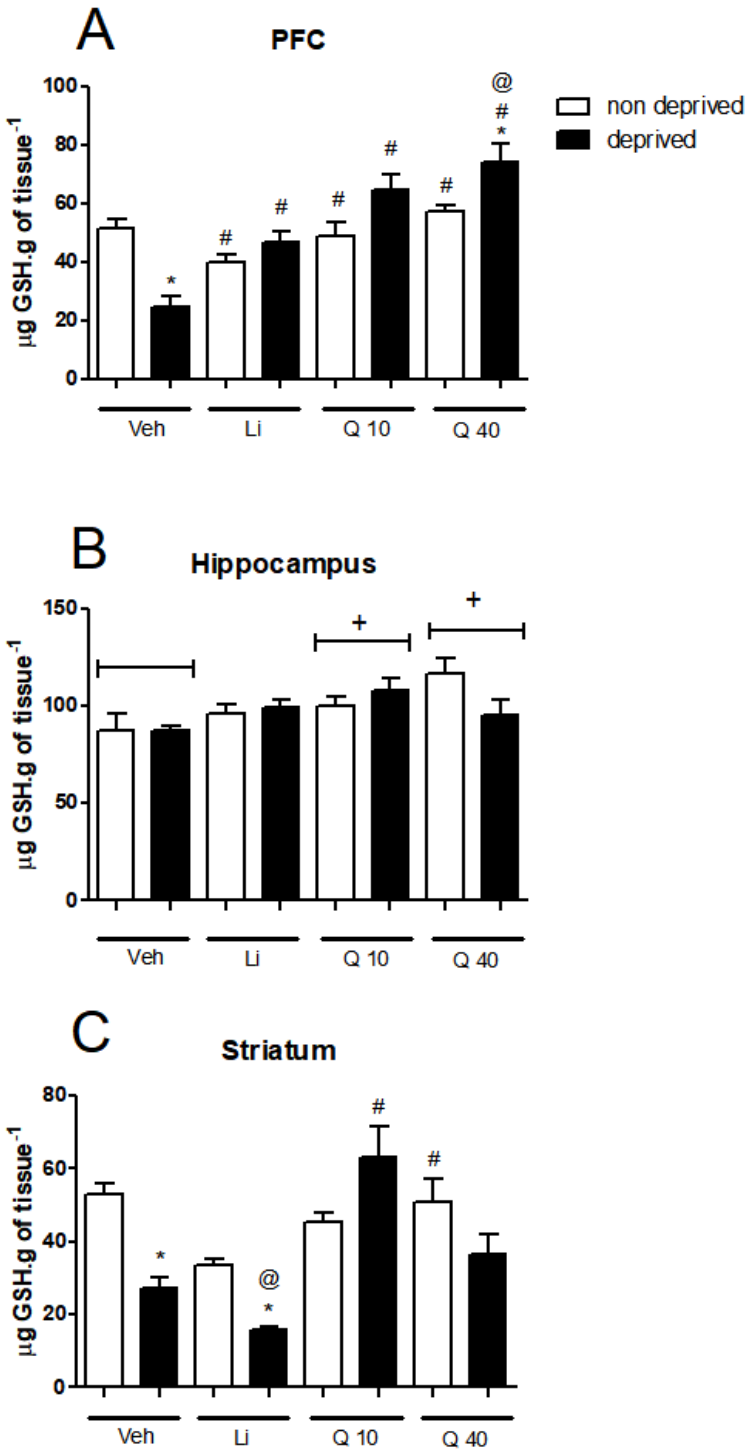

Fig 4. Effect of acute administration of quercetin (Q; 10 and 40 mg/kg i.p.) and lithium (Li; 100 mg/kg i.p.) on GSH levels in the PFC (A), hippocampus (B), and striatum (C) in mice subjected or not to 24 h SD. The data are expressed as mean ± SEM. n = 6 mice/group. $^{*}p < 0.05$, compared with non-sleep-deprived mice treated with vehicle; $^{\#}p < 0.05$, compared with sleep-deprived mice treated with vehicle; $^{@}p < 0.05$, compared with non-sleep-deprived mice treated with the same drug; $^{+}p < 0.05$, compared with sleep-deprived and non-sleep-deprived mice that were treated with vehicle. Control: non-deprived mice. SD: sleep-deprived mice.

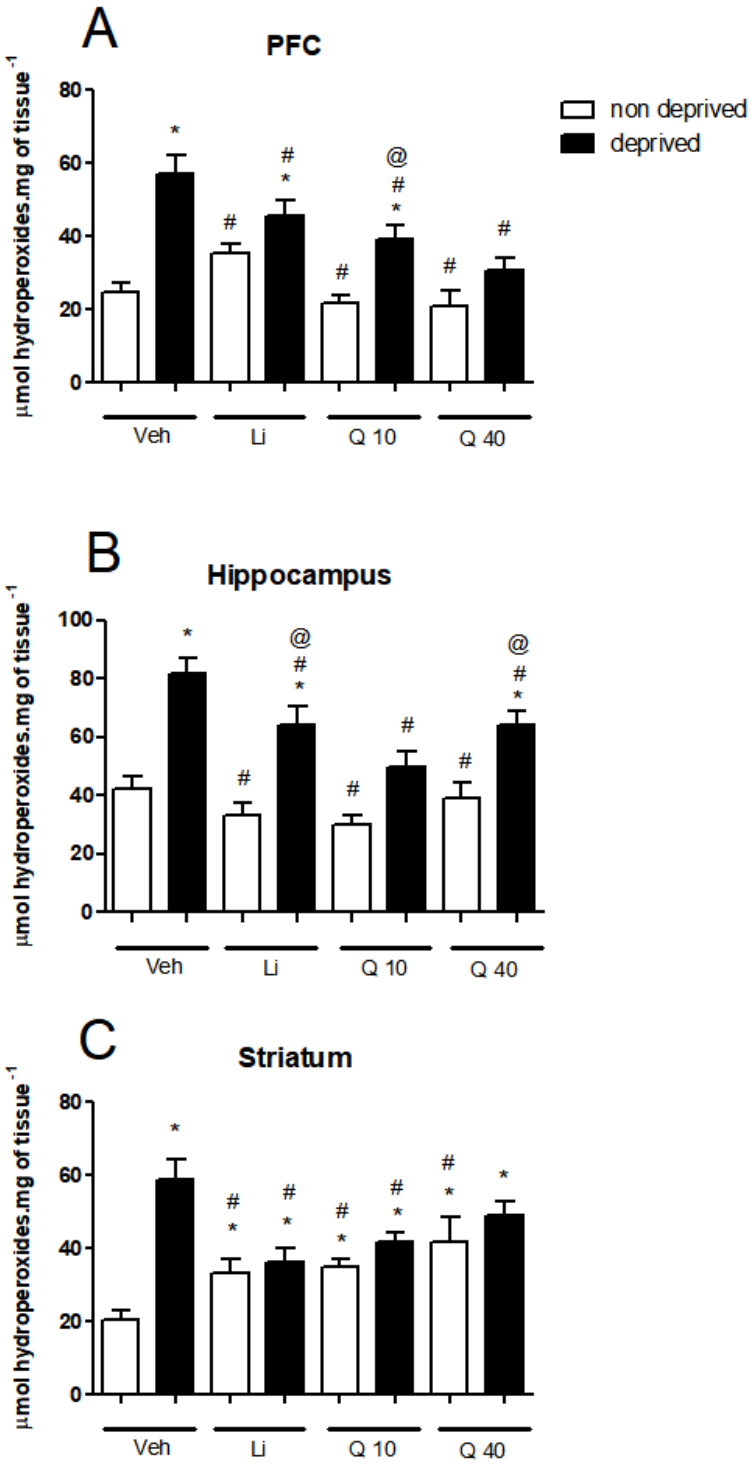

Fig 5. Effect of acute administration of quercetin (Q; 10 and 40 mg/kg i.p.) and lithium (Li; 100 mg/kg i.p.) on LPO in the PFC (A), hippocampus (B), and striatum (C) in mice that were or were not subjected to 24 h SD. The data are expressed as mean ± SEM. n = 6 mice/group. *$p < 0.05$, compared with non-sleep-deprived mice treated with vehicle; $^{\#}p < 0.05$, compared with sleep-deprived mice treated with vehicle; $^{@}p < 0.05$, compared with non-sleep-deprived mice treated with the same drug. Control: non-deprived mice. SD: sleep-deprived mice.

3.2.4. Correlation between SD-induced hyperlocomotion and indices of oxidative stress

The correlation analysis revealed a negative correlation between locomotor activity and GSH levels in the PFC in sleep-deprived mice (r = -0.76, $p < 0.001$) and a positive correlation between locomotor activity and LPO in the PFC (r = 0.53, $p < 0.05$) and striatum (r = 0.49, $p < 0.05$) in sleep-deprived mice (Table 1).

When considering all of the animals together, a negative correlation was found between locomotor activity and GSH levels (r = -0.57, $p < 0.001$), and a positive correlation was found between locomotor activity and LPO (r = 0.44, $p < 0.01$) in the PFC. The correlation analysis also revealed a tendency towards a positive correlation between locomotor activity and LPO in the hippocampus (r = 0.30, $0.01 < p < 0.05$) and striatum (r = 0.32, $0.01 < p < 0.05$). In the sleep-deprived group, a tendency toward a positive correlation was found between locomotor activity and LPO in the hippocampus (r = 0.39, $0.01 < p < 0.05$; Table 1).

Table 1. Correlation coefficients (Pearson's *r*) between SD-induced hyperlocomotion and GSH and LPO in the hippocampus, striatum, and PFC.

	Hippocampus		**Striatum**		**Prefrontal Cortex**	
Locomotor Activity	*GSH*	*LPO*	*GSH*	*LPO*	*GSH*	*LPO*
All mice (n = 38-42)	-0.14	0.30(*)	-0.14	0.32(*)	-0.57***	0.44**
Sleep-deprived (n = 17-22)	-0.30	0.39(*)	-0.15	0.49*	-0.76***	0.53*
Non-sleep-deprived (n = 19-21)	0.10	0.12	0.04	-0.10	-0.05	0.07

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, (*) $0.01 < p < 0.05$.

4. DISCUSSION

The present study showed that 24 h sleep-deprived mice exhibited hyperlocomotion compared with control non-sleep-deprived mice. Acute treatment with quercetin at doses that did not impair spontaneous locomotor activity and acute treatment with lithium blocked SD-induced hyperlocomotion. Thus, our findings demonstrated a potential antimanic-like effect of quercetin, indicating that this flavonoid may be a promising therapeutic agent for the treatment of manic symptoms.

Animal models of mania frequently focus on a specific aspect of the disorder, namely hyperactivity. This feature can be easily induced and monitored. Non-

pharmacological or environmental methods, such as SD, are widely used to induce manic-like behavior in animals (Einat, 2006). Gessa et al. (1995) showed that SD results in a short period of hyperactivity that is responsive to lithium, which validates the model for screening novel antimanic drugs. In the clinical context, during a manic episode, 69-99% of patients experience a reduced need for sleep (Harvey et al., 2009). Indeed, many studies have shown that BD patients present sleep disturbances (Rumble et al., 2015; Harvey et al., 2009), and SD can predict manic episodes (Colombo et al., 1999). An important validation of SD-induced manic-like symptoms as a model for the human condition is its sensitivity to lithium (Gessa et al., 1995). In the present study, the SD model was sensitive to lithium treatment, in which sleep-deprived animals that were treated with lithium exhibited a reduction of hyperlocomotion compared with sleep-deprived animals that were treated with vehicle.

Ghosh et al. (1976) reported that SD caused a marked increase in dopaminergic neurotransmission and concomitant increase in locomotor activity. In humans, amphetamine, which increases dopaminergic neurotransmission, was shown to increase motor activity in a human open field test (Minassian et al., 2016). Likewise, the increase in dopamine levels that is caused by SD is a source of oxidative stress in the brain, which might be related to the establishment of manic symptoms (Berk et al., 2007; Rees et al., 2007). The central nervous system (CNS) is vulnerable to oxidative stress. The brain utilizes great amounts of oxygen, consequently promoting the formation of oxygen free radicals and reactive oxygen species. Corroborating the notion that oxidative stress may indeed be related to the pathophysiology of BD and manic-like behavior, Souza et al. (2014) showed that melatonin treatment decreased ouabain-induced hyperlocomotion in rats. Their study also showed a positive correlation between manic-like behavior and indices of oxidative stress in the PFC and hippocampus.

In the present study, the antioxidant property of quercetin was demonstrated in the DPPH assay (Figure 3B). Our results also indicated that 10 mg/kg quercetin increased GSH levels in the PFC and striatum, attenuating the SD-induced decrease in GSH levels in these brain areas. Treatment with 40 mg/kg quercetin blocked the SD-induced decrease in GSH levels in the PFC. Lithium also exerts neuroprotective effects against oxidative stress (Frey et al., 2006), which is consistent with our DPPH assay results (Figure 3A). Our results showed that acute lithium treatment increased GSH levels in the PFC. However, in the striatum, sleep-deprived mice treated with

lithium exhibited a decrease in GSH levels after SD compared with the control group (non-sleep-deprived + vehicle) and non-sleep-deprived mice that were treated with lithium. Previous studies have shown that the antioxidant effect of lithium varies, depending on the specific brain region and treatment regimen (Frey et al., 2006; Bhalla and Dhawan, 2009).

The present study found a negative correlation between locomotor activity and GSH levels in the PFC in sleep-deprived animals. When considering all of the animals together, a negative correlation was found between locomotor activity and GSH levels in the PFC. The CNS has a limited antioxidant capacity, and GSH is the main antioxidant in the brain (Jornada et al., 2011). Gawryluk et al. (2011) showed that the *post mortem* PFC in BD patients had lower levels of GSH, which can lead to higher susceptibility to neuronal oxidation, interfere with neuronal activity, and may contribute to the establishment of BD symptoms (Dringen, 2000). In the present study, SD-induced hyperlocomotion was related to lower GSH levels in the PFC. Other studies have shown that the replenishment of GSH diminishes oxidative cellular damage and ameliorates the symptoms of BD (Magalhães et al., 2011; Dean et al., 2009). Our results also suggest that the hippocampus is less vulnerable to SD-induced GSH depletion compared with the PFC and striatum.

Another parameter of oxidative stress that was analyzed in the present study was LPO. Lipids are the major components of cellular membranes and myelin sheaths that allow the conduction of neuronal signaling. LPO can dramatically compromise brain function (Joshi and Praticò, 2014). High levels of oxidative damage to membrane phospholipids or the aggregation of oxidized proteins alters fluidity, which can induce cell death by apoptosis (Mahadik et al., 2001). Under certain conditions, LPO causes structural disturbances, alterations in integrity, fluidity, and permeability, the functional loss of biomembranes, and the generation of potentially toxic products (Greenberg et al., 2008). Hydroperoxides are the major products of the free radical-mediated LPO of polyunsaturated fatty acids, and they are assumed to be pathogenic and contribute to the etiology of many diseases, including neurodegenerative diseases (Niki, 2009; Sultana et al., 2006). Previous studies have reported that uncompensated oxidative stress increases LPO throughout the course of BD (Andreazza et al., 2007; Machado-Vieira et al., 2007). In the present study, acute lithium administration and 10 and 40 mg/kg quercetin reversed the increases in LPO in the PFC, hippocampus, and striatum that were induced by SD. These findings demonstrate the antioxidant and

neuroprotective effects of both lithium and quercetin, suggesting antimanic-like effects of both drugs.

In the present study, a positive correlation was found between locomotor activity and LPO in the PFC and striatum in sleep-deprived mice. Considering all of the animals together, a positive correlation was found between locomotor activity and LPO in the PFC. Our findings indicate that SD-induced hyperlocomotion is correlated with higher LPO in the PFC and striatum. Brüning et al. (2012) also reported a positive correlation between hyperlocomotion and indices of LPO.

Other studies have shown that antioxidant substances, such as a-lipoic acid and N-acetyl-cysteine, are capable of reducing levels of oxidative stress, manic-like behavior and manic symptoms (Pereira et al., 2014; Andreatini et al., 2013). Our research group has shown that the flavonoid myricitrin, which possesses antioxidant and PKC-inhibiting activities, is capable of reducing levels of oxidative stress and manic-like behavior (Pereira et al., 2014; Andreatini et al., 2013). However, Valvassori et al. (2014) reported that tamoxifen reduced levels of oxidative stress, although it does not possess antioxidant properties. Furthermore, trials have assessed well-known antioxidant agents, such as vitamin E, have reported either negative or controversial results (La Fata et al., 2014; Bjelakovic and Gluud, 2007. This indicates that different mechanisms are involved in the lower levels of oxidative stress, which does not necessarily require antioxidant effects. Therefore, we can only hypothesize that the antioxidant effect of quercetin might be responsible for the reduction of the SD-induced increase in LPO levels. Likewise, it is only possible to imply that the antioxidant effect of this flavonoid might contribute to its antimanic-like effect.

Our results indicate that quercetin decreases SD-induced hyperlocomotion and displays an antimanic-like effect. Our results also indicated an antioxidant effect of quercetin, which may be related to its antimanic-like effect. Similar results were found for lithium, which is currently the treatment of choice for BD. The antioxidant and PKC inhibitor quercetin may be an alternative therapeutic agent for the treatment of manic symptoms.

5. CONCLUSION

Overall, the present study showed that quercetin prevented SD-induced hyperlocomotion in mice at doses that did not impair locomotor activity, suggesting an

antimanic-like effect. This flavonoid also reversed the SD-induced decrease in GSH levels in the PFC and striatum and SD-induced increase in LPO in the PFC, hippocampus, and striatum. These behavioral and neurochemical effects appeared to be correlated, suggesting that quercetin may exert its actions by reducing oxidative stress.

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CHAPTER 6

Effects of acute and chronic quercetin administration on methylphenidate-induced hyperlocomotion and oxidative stress

Luiz K. S. Kanazawa,[a] Débora D. Vecchia,[a] Etiéli M. Wendler,[a] Palloma de A. S. Hocayen,[a] Paulo S. Beirão Júnior,[a] Manuela L. de Mélo,[a] Francislaine A. dos Reis Lívero,[b] Claudia Rita Corso,[b] Maria Carolina Stipp,[b] Alexandra Acco,[b] Roberto Andreatini [a, *]

[a] *Laboratory of Physiology and Pharmacology of the Central Nervous System, Department of Pharmacology, Federal University of Paraná, Centro Politécnico, Curitiba, PR, 81540-990, Brazil*

[b] *Laboratory of Pharmacology and Metabolism, Department of Pharmacology, Federal University of Paraná, Centro Politécnico, Curitiba, PR, 81540-990, Brazil*

*Corresponding author

ABSTRACT

Increases in protein kinase C (PKC) and oxidative stress have been related to mania. Drugs with antioxidant effects or inhibitory actions on PKC may have antimanic effects. The flavonoid quercetin has antioxidant and PKC-inhibiting effects that resemble those of lithium, the first-line treatment for mania in bipolar disorder. We hypothesized that quercetin may have antimanic-like effects in an animal model. In the present study, we investigated the effects of acute and chronic treatment with quercetin (2.5, 5, 10, and 40 mg/kg i.p.) in male Swiss mice that were subjected to methylphenidate (5 mg/kg i.p.)-induced hyperlocomotion, an animal model of mania. Lithium (100 mg/kg i.p.) and diazepam (5 mg/kg i.p.) were used as positive and negative controls, respectively. We also evaluated the effects of these treatments on methylphenidate-induced oxidative stress in the brain by measuring reduced glutathione (GSH) and lipid peroxidation (LPO) levels in the prefrontal cortex, hippocampus, and striatum. Acute and chronic (21-day) treatment with lithium and diazepam reduced methylphenidate-induced hyperlocomotion. Chronic but not acute treatment with quercetin (10 and 40 mg/kg) blocked methylphenidate-induced hyperlocomotion. These effects of lithium and quercetin occurred at doses that did not alter spontaneous locomotor activity, whereas diazepam reduced spontaneous locomotor activity. Chronic treatment with lithium and quercetin blocked the methylphenidate-induced increase in LPO levels in the striatum. These results suggest that chronic quercetin treatment has antimanic-like and antioxidant effects, thus encouraging further studies of quercetin as a putative new antimanic drug.

Key words: Bipolar disorder, hyperlocomotion, mania, oxidative stress, protein kinase C, quercetin.

Abbreviations: BD, bipolar disorder; CMC, carboxymethylcellulose; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); GSH, reduced glutathione; LPO, lipid peroxidation; PFC, prefrontal cortex; PKC, protein kinase C.

1. INTRODUCTION

Manic episodes in bipolar disorder (BD) are treated with mood stabilizers (e.g., lithium), atypical antipsychotics (e.g., risperidone), and anticonvulsants (e.g., sodium valproate), but their management in clinical settings remains a challenge (Hoertel et al., 2013; Nivoli et al., 2012). Mania has been related to oxidative stress (Berk et al., 2011; Andreazza et al., 2008) and increased activity of protein kinase C (PKC) (Armani et al., 2014; Manji and Lenox, 2000). Lithium and sodium valproate, which are the most frequently used antimanic drugs, exert antimanic effects via PKC inhibition and/or antioxidant activity (Abrial et al., 2015; Armani et al., 2014; Macêdo et al., 2012; Frey et al., 2006 Wang and Friedman, 1989).

One animal model that is employed to induce manic-like behavior involves the administration of psychostimulants, such as methylphenidate. The pharmacological induction of manic-like behavior (e.g., hyperlocomotion) is relatively easy to generate and test and has reliability and validity. Locomotor activity is known to increase in manic patients (Young et al., 2011). Mines et al. (2013) showed that methylphenidate, which blocks dopamine and noradrenaline transporters (DAT and NET respectively) enhancing dopamine and norepinephrine synaptic levels, increased locomotor activity in mice, and this effect was blocked by sodium valproate, carbamazepine, and lithium at doses that did not impair spontaneous locomotor activity (Nogoceke et al., 2016; Souza et al., 2016; Arunagiri et al., 2014; Tonelli et al., 2013; Barbosa et al., 2011; Pereira et al., 2011).

Quercetin is a flavonoid that possesses antioxidant properties (Dajas et al., 2015) and inhibits PKC (Gamet-Payrastre et al., 1999). We hypothesized that quercetin might also have antimanic-like effects. A previous study found that acute administration of quercetin blocked both hyperlocomotion and oxidative stress that were induced by sleep deprivation (Kanazawa et al., 2016). However, this previous study administered quercetin only acutely and employed only one model, which may have resulted in false-positive results. Quercetin has already been tested in a clinical trial of its anti-inflammatory effects (Boots et al., 2011), indicating its therapeutic utility. The objective of the present study was to evaluate the effects of acute and chronic quercetin administration on methylphenidate-induced hyperlocomotion and oxidative stress in the brain in mice.

2. MATERIALS AND METHODS

2.1. Animals

The study included male Swiss mice, weighing 30-40 g, that were housed at 22ºC ± 2ºC under a 12 h/12 h light/dark cycle (lights on at 7:00 AM). The animals were kept in polypropylene cages (41 cm x 34 cm x 16 cm) with food and water available *ad libitum*. All of the experiments were approved by the Committee of Animal Experimentation of the Federal University of Paraná (CEUA/BIO-UFPR, protocol no. 733) and conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health).

2.2. Drugs

Mice were treated with saline (0.9% NaCl; 10 ml/kg i.p.), lithium carbonate (Eurofarma, Itapevi, Brazil; positive control; 100 mg/kg i.p. dissolved in saline, with the pH adjusted to 7.4 with hydrochloric acid), diazepam (Cristália, Itapira, Brazil; 5 mg/kg i.p., dissolved in distilled water), or quercetin (Sigma, St. Louis, MO, USA; 2.5, 5, 10, or 40 mg/kg i.p., suspended in 0.5% carboxymethylcellulose [CMC]). Methylphenidate (Novartis, São Paulo, Brazil) was dissolved in saline and administered subcutaneously (s.c.) at a dose of 5.0 mg/kg. The drugs were administered in a volume of 10 ml/kg of body weight. The doses were based on data from the literature (Bhutada et al., 2010) and previous studies by our research group (Kanazawa et al., 2016; Pereira et al., 2011; Sabioni et al., 2008). Chronic treatment with lithium, diazepam, and quercetin was performed once per day for 21 days.

2.3. Methylphenidate-induced hyperlocomotion protocol

In the acute treatment protocol, the animals were pretreated with lithium, diazepam, quercetin, or vehicle 15 min before the administration of either vehicle or methylphenidate (Figure 1, top). In the chronic treatment protocol, the animals were pretreated with lithium, diazepam, quercetin, or vehicle once per day for 21 days. On the test day, vehicle or methylphenidate was administered 15 min after the last administration of lithium, diazepam, quercetin, or vehicle (Figure 1, bottom).

Twenty minutes after vehicle or methylphenidate administration, the animals were individually placed in an automated activity box (40 cm x 20 cm x 26 cm) that was constructed from wood with a wire mesh floor. The box had three photoelectric sensors (10 cm apart) on the two longer lateral walls. The number of crossings was cumulatively recorded by photoelectric sensors over a 20 min period. The number of crossings was considered an index of locomotor activity. An increase in the number of crossings after methylphenidate administration indicated a stimulant effect. The blockade of the stimulant effect of methylphenidate at a dose that did not decrease spontaneous locomotor activity indicated an antimanic-like effect (Sabioni et al., 2008; Gould et al., 2001).

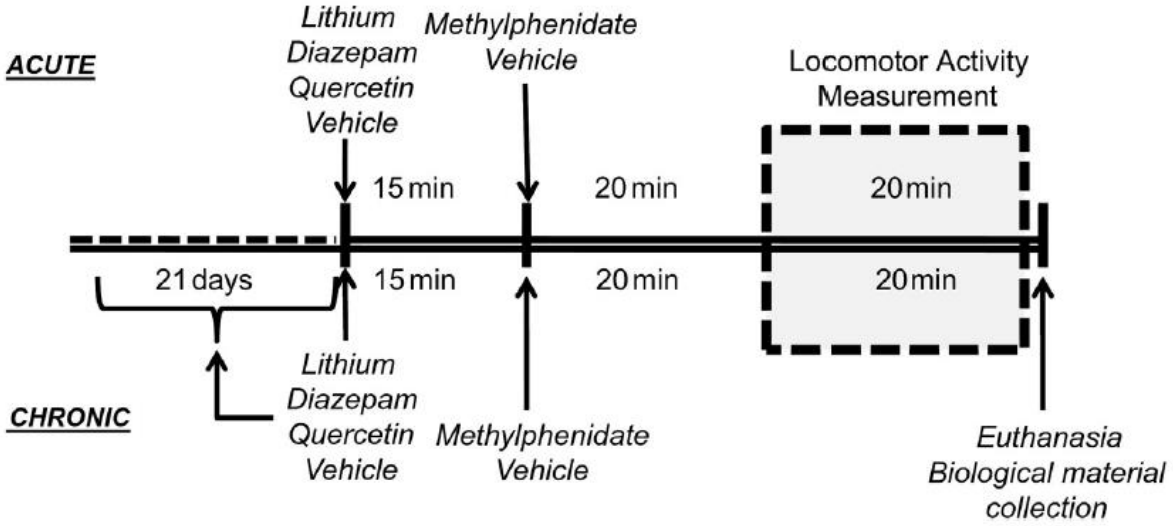

Fig 1. Timeline of treatment schedule and methylphenidate-induced hyperlocomotion model of mania in mice.

2.5. Evaluation of oxidative stress parameters in the mouse brain

2.5.1. Brain samples

The mice were euthanized by decapitation immediately after being exposed to the automated activity box. The prefrontal cortex (PFC), hippocampus, and striatum were dissected, frozen in liquid nitrogen, and stored at -80ºC until further analysis. The samples were homogenized in potassium phosphate buffer (0.1 M, pH 6.5) in a 1:10 dilution. One part of the homogenate was used to evaluate reduced glutathione (GSH) levels, and the other part was centrifuged at 9700 rotations per minute (rpm) in a micro-

high-speed refrigerated centrifuge (VS-15000 CFNII, Vision Scientific, Daejeon, South Korea) for 20 min. The supernatant was used to evaluate lipid peroxidation (LPO) levels.

2.6. Evaluation of GSH levels

To evaluate GSH levels, 100 µl of the homogenate was mixed with 80 µl of 12.5% trichloroacetic acid and centrifuged at 6000 rpm for 15 min at 4ºC. Afterwards, 20 µl of the supernatant was mixed with 280 µl of Tris buffer (0.4 M, pH 8.9) and 5 µl of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB; 0.01 M) according to the protocol of Sedlak and Lindsay (1968), with minor modifications. Absorbance was read at 415 nm using a multi-mode microplate reader (BioTek Synergy HT, BioTek Instruments, Winooski, VT, USA). The individual values were then interpolated in a standard curve of GSH (0.375-3 µg) to verify the linearity of the reaction (r^2 must be > 0.99), and the values were divided by a correction factor. The results are expressed as µg of GSH per g of tissue.

2.7. Evaluation of LPO levels

LPO levels were measured according to the method of Jiang et al. (1992), with minor modifications. Initially, 100 ml of the supernatant was suspended in 100 µl of methanol, vortexed, and then centrifuged at 5000 rpm for 5 min at 4ºC. Afterward, 100 ml of the supernatant was added to 900 µl of FOX2 reagent (4 mM BHT, 250 µM $FeSO_4$, 250 mM H_2SO_4, and 100 mM xylenol orange). The samples were vortexed and incubated in the dark for 30 min at room temperature. Absorbance was read at 560 nm using a multi-mode microplate reader (BioTek Synergy HT, BioTek Instruments, Winooski, VT, USA). The results are expressed as mmol of hydroperoxides per mg of tissue.

2.9. Statistical analysis

For all of the experiments, two-way analysis of variance (ANOVA) was used. Significant main effects or interactions in the ANOVA were followed by the Newman-Keuls *post hoc* test. The Pearson correlation coefficient (r) was performed to measure

the correlation between locomotor activity and oxidative stress parameters. The data are expressed as mean ± SEM. Values of $p < 0.05$ were considered statistically significant. All analysis were performed using Statistica 7.0 (Statsoft Inc., Tulsa, USA) software.

3. RESULTS

3.1. Behavioral test

3.1.1. Effect of acute quercetin administration on methylphenidate-induced hyperlocomotion

There are significant main effects of methylphenidate ($F_{1,42} = 127.56$, $p < 0.0001$) and acute treatment ($F_{6,42} = 17.24$, $p < 0.0001$) and a methylphenidate ´ acute treatment interaction ($F_{6,42} = 3.85$, $p < 0.01$). Lithium and diazepam blocked the effects of methylphenidate ($p < 0.05$). Diazepam alone reduced spontaneous locomotor activity ($p < 0.05$, compared with vehicle + vehicle). No effect of quercetin was observed at any dose tested (Figure 2).

3.1.2. Effect of chronic quercetin treatment on methylphenidate-induced hyperlocomotion

There are significant main effects of methylphenidate ($F_{1,42} = 131.90$, $p < 0.001$) and chronic treatment ($F_{6,42} = 14.90$, $p < 0.001$) and a methylphenidate chronic treatment interaction ($F_{6,42} = 9.96$, $p < 0.001$). Methylphenidate increased locomotor activity compared with the control (vehicle + vehicle; $p < 0.01$; Figure 4). Treatment with lithium, diazepam, and 10 and 40 mg/kg quercetin blocked methylphenidate-induced hyperlocomotion (all $p < 0.05$). Treatment with 2.5 and 5 mg/kg quercetin attenuated methylphenidate-induced hyperlocomotion ($p < 0.05$, compared with vehicle + vehicle and vehicle + methylphenidate). No effect of lithium or quercetin on spontaneous locomotor activity was observed. Treatment with diazepam alone significantly reduced spontaneous locomotion ($p < 0.001$).

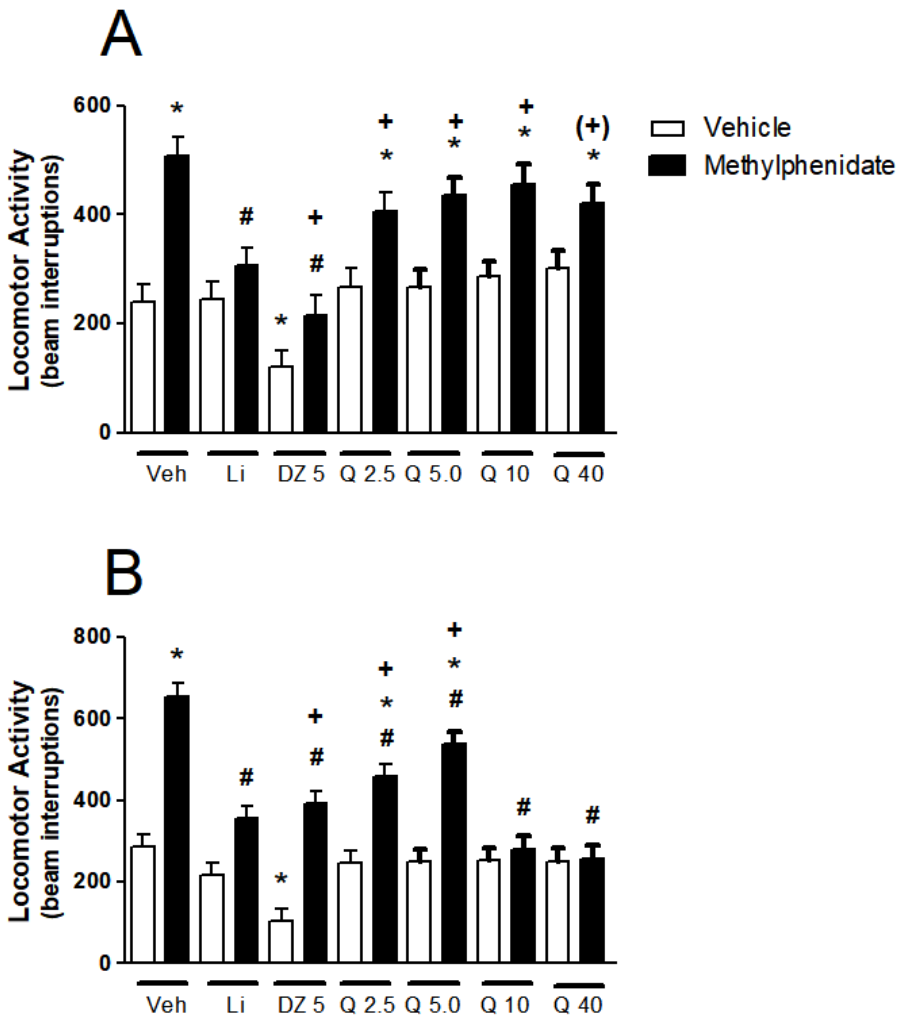

Fig 2. Effect of acute (A) and chronic (21-day) (B) treatment with quercetin (Q; 2.5-40 mg/kg i.p.), lithium (Li; 100 mg/kg i.p.), and diazepam (DZ; 5 mg/kg i.p.) on methylphenidate-induced hyperlocomotion. Veh, vehicle; MPH, methylphenidate (5.0 mg/kg i.p.). The data are expressed as the mean ± SEM number of beam breaks over 20 min. *n* = 4 mice/group (acute) and *n* = 10-12 mice/group (chronic). **p* < 0.05, compared with vehicle+vehicle; $^{\#}p$ <0.05, compared with vehicle+methylphenidate; ^{+}p < 0.05, compared with same drug+vehicle; $^{(+)}0.05 < p < 0.10$, compared with same drug+vehicle (Two-way ANOVA followed by Newman-Keuls test).

3.2. Oxidative stress

3.2.1. Effect of acute administration of quercetin on GSH levels after methylphenidate-induced hyperlocomotion

There are significant effects of methylphenidate ($F_{1,42}$ = 75.73, $p < 0.001$) and acute treatment ($F_{6,42}$ = 5.72, $p < 0.001$) on GSH levels in the PFC and a methylphenidate x acute treatment interaction ($F_{6,42}$ = 9.54, $p < 0.001$). Methylphenidate increased GSH levels compared with the vehicle + vehicle group (*p*

< 0.001). Lithium and 2.5, 5.0, and 40 mg/kg quercetin also increased GSH levels (p < 0.05, compared with vehicle + vehicle). None of the treatments affected the methylphenidate-induced increase in GSH levels (Table 1).

There are significant effects of methylphenidate ($F_{1,40}$ = 19.95, p < 0.001) and acute treatment ($F_{6,40}$ = 4.70, p < 0.01) but no methylphenidate x acute treatment interaction ($F_{6,40}$ = 1.22, p > 0.05; Table 1). Independent of treatment, methylphenidate increased GSH levels (p < 0.01). Quercetin (2.5 and 5.0 mg/kg) increased GSH levels independent of methylphenidate treatment (both p < 0.05).

There is a significant main effect of methylphenidate ($F_{1,33}$ = 9.45, p < 0.05) but no effect of acute treatment ($F_{6,33}$ = 1.42, p > 0.05) and no methylphenidate x acute treatment interaction ($F_{6,33}$ = 0.13, p < 0.05; Table 1). Methylphenidate decreased GSH levels independent of treatment (p < 0.01).

Table 1. Effects of acute quercetin (Q: 2.5-40 mg/kg i.p.), lithium (Li: 100 mg/kg i.p.) and diazepam (DZ: 5 mg/kg i.p.) administration on LPO levels in the PFC, hippocampus, and striatum in mice in the methylphenidate-induced hyperlocomotion model.

Index	Area		Treatment						
			Veh	Li	DZ	Q 2.5	Q 5	Q 10	Q 40
GSH	Hippocampus	Veh	23 ± 1	32 ± 3	23 ± 4	29 ± 2 [2]	37 ± 3 [2]	24 ± 4	30 ± 3
		MPH	31 ± 2	34 ± 4	28 ± 2	41 ± 2 [2]	40 ± 2 [2]	38 ± 4	36 ± 3
	PFC	Veh	81 ± 6	286 ± 24	153 ± 9	242 ± 11*	182 ± 18*	130 ± 13	168 ± 27*
		MPH	302 ± 36*	250 ± 22*	358 ± 6*	298 ± 19*	202 ± 20*	255 ± 25*	281 ± 36*
	Striatum	Veh	33 ± 3	32 ± 1	30 ± 1	32 ± 2	31 ± 1	33 ± 1	28 ± 2
		MPH	30 ± 1	29 ± 1	27 ± 3	30 ± 1	29 ± 2	28 ± 1	25 ± 3
LPO	Hippocampus	Veh	12 ± 1	14 ± 1	14 ± 1	15 ± 2	22 ± 2*	22 ± 2*	19 ± 2*
		MPH	22 ± 1*	15 ± 1[#]	15 ± 1[#]	19 ± 1*	22 ± 1*	27 ± 2*	18 ± 1*
	PFC	Veh	32 ± 1	28 ± 1	49 ± 2	35 ± 4	45 ± 4	40 ± 3 [2]	43 ± 1 [2]
		MPH	39 ± 5	29 ± 3	51 ± 5	39 ± 1	37 ± 2	45 ± 5 [2]	59 ± 4 [2]
	Striatum	Veh	14 ± 1	16 ± 1	15 ± 1	18 ± 1	15 ± 2	14 ± 2	14 ± 1
		MPH	15 ± 1	16 ± 2	18 ± 1	16 ± 2	15 ± 2	15 ± 1	17 ± 1

Veh, vehicle; Q, quercetin (2.5-40 mg/kg i.p.); Li, lithium (100 mg/kg i.p.); DZ, diazepam (5 mg/kg i.p.).; MPH: methylphenidate. The data are expressed as mean + SEM. n = 4 mice/group.

* p < 0.05, compared with vehicle + vehicle

[#] p < 0.05, compared with vehicle + methylphenidate

[1] p < 0.05, compared with vehicle (independent of acute treatment factor)

[2] p < 0.05, compared with vehicle (independent of methylphenidate factor)

3.2.2. Effect of acute quercetin treatment on LPO levels after methylphenidate-induced hyperlocomotion

There are significant main effects of methylphenidate ($F_{1,41}$ = 10.32, p < 0.01) and acute treatment ($F_{6,41}$ = 13.13, p < 0.001) but no methylphenidate x acute treatment interaction ($F_{6,41}$ = 1.11, p > 0.05; Table 1). Independent of treatment, methylphenidate increased LPO levels (p < 0.01). Quercetin (10 and 40 mg/kg) increased LPO levels independent of methylphenidate treatment (both p < 0.01).

There are significant main effects of methylphenidate ($F_{1,41}$ = 16.14, p < 0.001) and acute treatment ($F_{6,41}$ = 13.28, p < 0.001) and a significant methylphenidate x acute treatment interaction ($F_{6,41}$ = 3.92, p < 0.001). Methylphenidate increased LPO levels compared with the vehicle + vehicle group (p < 0.001). Treatment with 5.0, 10, and 40 mg/kg quercetin alone increased LPO levels (5 and 10 mg/kg, p < 0.001; 40 mg/kg, p < 0.05) but did not block the methylphenidate-induced increase in LPO levels. Lithium and diazepam blocked the effect of methylphenidate (p < 0.05; Table 1).

There is no significant effect of methylphenidate ($F_{1,41}$ = 1.61, p > 0.05) or acute treatment ($F_{6,41}$ = 0.88, p > 0.05) and no methylphenidate x acute treatment interaction ($F_{6,41}$ = 0.84, p > 0.05; Table 1).

3.2.3. Effect of chronic quercetin treatment on GSH levels after methylphenidate-induced hyperlocomotion

There is a significant effect of chronic treatment ($F_{6,42}$ = 18.65, p < 0.001) but no effect of methylphenidate ($F_{1,42}$ = 2.92, p > 0.05) and no methylphenidate x chronic treatment interaction ($F_{6,42}$ = 1.18, p > 0.05; Figure 3). Lithium and 5.0 and 40 mg/kg quercetin increased GSH levels independent of methylphenidate administration.

There are significant effects of methylphenidate ($F_{1,40}$ = 42.78, p < 0.001) and chronic treatment ($F_{6,40}$ = 4.77, p < 0.001) but no methylphenidate x chronic treatment interaction ($F_{6,40}$ = 2.18, p = 0.064; Figure 3). Independent of treatment, methylphenidate increased GSH levels (p < 0.001). No significant difference was observed between vehicle and any other treatment in chronic treatment factor.

There are significant effects of methylphenidate ($F_{1,40}$ = 71.38, p < 0.001) and chronic treatment ($F_{6,40}$ = 5.03, p < 0.001) and a methylphenidate chronic treatment interaction ($F_{6,40}$ = 7.35, p < 0.001). The *post hoc* test indicated that the vehicle +

methylphenidate group exhibited a tendency toward a decrease in GSH levels compared with the vehicle + vehicle group ($p = 0.06$). Chronic treatment with 2.5 mg/kg quercetin increased GSH levels in the striatum compared with the vehicle + vehicle group ($p < 0.05$). The 2.5 mg/kg quercetin + methylphenidate group exhibited a decrease in GSH levels compared with the vehicle + vehicle group ($p < 0.05$). The methylphenidate + quercetin groups exhibited a reduction of striatal GSH levels compared with the vehicle + quercetin group at all quercetin doses tested, except for the 5.0 mg/kg dose (all $p < 0.05$; Figure 3).

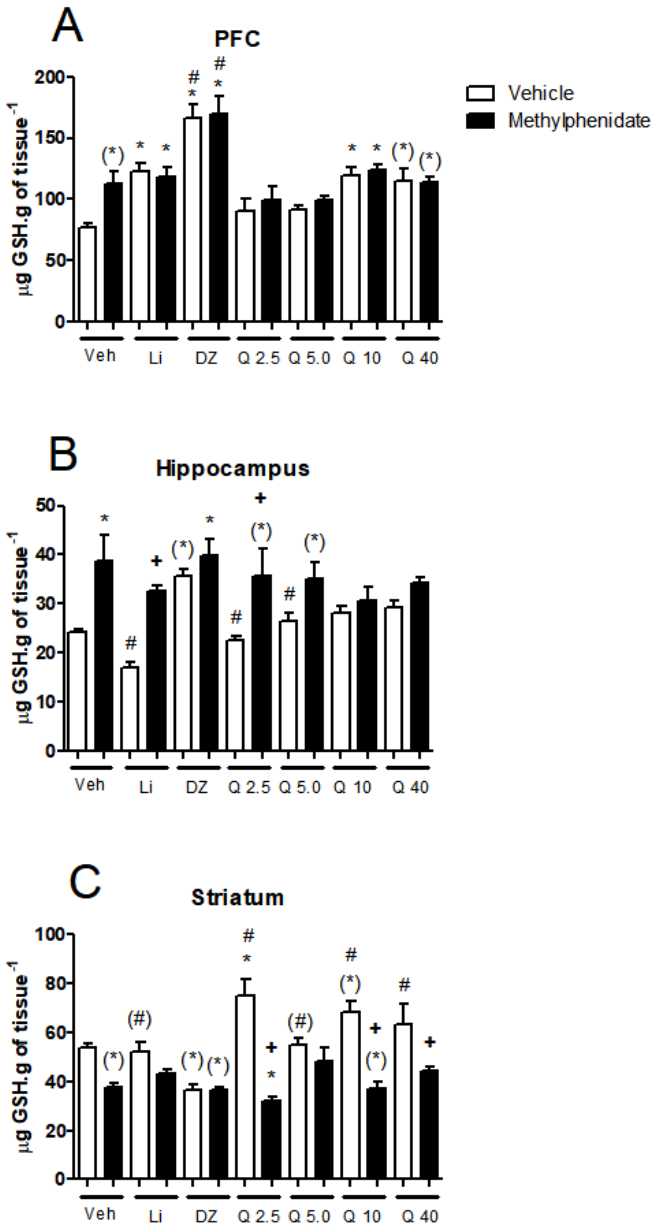

Fig 3. Effects of chronic quercetin (Q; 2.5-40 mg/kg i.p.), lithium (Li; 100 mg/kg i.p.), and diazepam (DZ; 5 mg/kg i.p.) administration on GSH levels in the PFC (A), hippocampus (B), and striatum (C) in mice in the methylphenidate-induced hyperlocomotion model. The data are expressed as mean ± SEM. *n* =

4 mice/group. *p < 0.05, compared with vehicle+vehicle; #p < 0.05, compared with vehicle+methylphenidate; (*)0.05 < p < 0.10, compared with vehicle+vehicle; (#)0.05 < p < 0.10, compared with vehicle+methylphenidate; +p < 0.05, compared with same drug+vehicle (Two-way ANOVA followed by Newman-Keuls test).

3.2.4. Effect of chronic quercetin treatment on LPO levels after methylphenidate-induced hyperlocomotion

There are significant effects of methylphenidate ($F_{1,41}$ = 41.49, p < 0.001) and chronic treatment ($F_{6,41}$ = 3.96, p < 0.01) but no methylphenidate x chronic treatment interaction ($F_{6,41}$ = 1.93, p > 0.05; Figure 4A). Independent of treatment, methylphenidate increased LPO levels (p < 0.001). Quercetin (40 mg/kg) increased LPO levels independent of methylphenidate treatment (p < 0.05).

There are significant effects of methylphenidate ($F_{1,41}$ = 16.89, p < 0.001) and chronic treatment ($F_{6,41}$ = 3.59, p < 0.01) and a methylphenidate x chronic treatment interaction ($F_{6,41}$ = 4.77, p < 0.001). The *post hoc* test indicated that methylphenidate increased LPO levels in the hippocampus compared with the vehicle + vehicle group (p < 0.05). No dose of quercetin blocked the methylphenidate-induced increase in LPO levels in the hippocampus. Treatment with diazepam alone increased LPO levels compared with vehicle + vehicle (p < 0.05), whereas the diazepam + methylphenidate group did not differ from the vehicle + vehicle group (Figure 4B).

There are significant effects of methylphenidate ($F_{1,39}$ = 33.88, p < 0.001) and chronic treatment ($F_{6,39}$ = 8.22, p < 0.001) and a methylphenidate x chronic treatment interaction ($F_{6,41}$ = 3.24, p < 0.05). The *post hoc* test indicated that methylphenidate increased LPO levels compared with the vehicle + vehicle group (p < 0.001). Treatment with lithium, diazepam, and 2.5, 5.0, 10, and 40 mg/kg quercetin decreased LPO levels compared with the vehicle + methylphenidate group (all p < 0.05; Figure 4C).

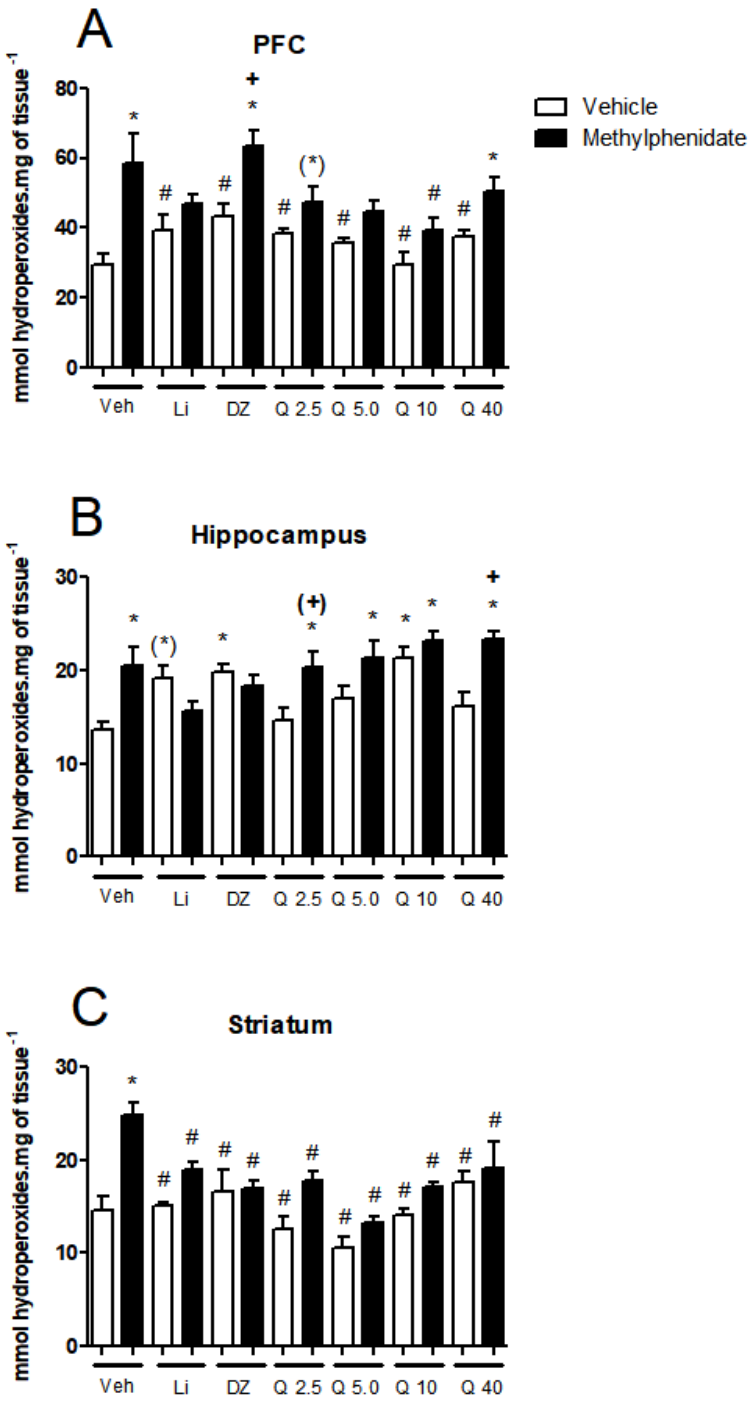

Fig 4. Effects of chronic quercetin (Q; 2.5-40 mg/kg i.p.), lithium (Li; 100 mg/kg i.p.), and diazepam (DZ; 5 mg/kg i.p.) administration on LPO levels in the PFC (A), hippocampus (B), and striatum (C) in mice in the methylphenidate-induced hyperlocomotion model. The data are expressed as mean ± SEM. $n = 4$ mice/group. $^{*}p < 0.05$, compared with vehicle+vehicle; $^{\#}p < 0.05$, compared with vehicle+methylphenidate; $^{+}p < 0.05$, compared with same drug+vehicle; $^{(+)}0.05 < p < 0.10$, compared with same drug+vehicle (Two-way ANOVA followed by Newman-Keuls test).

3.2.5. Correlation between methylphenidate-induced hyperlocomotion and GSH and LPO levels in the hippocampus, striatum, and PFC

Considering all animals from the acute treatment protocol, a positive correlation was found between hyperlocomotion and LPO levels in the PFC (r = 0.48, $p < 0.001$) and hippocampus ($r = 0.56$, $p < 0.001$), and a positive correlation was found between hyperlocomotion and GSH levels in the PFC ($r = 0.32$, $p < 0.05$) and hippocampus ($r = 0.46$, $p < 0.001$) (Table 2).

Considering all animals from the chronic treatment protocol, a positive correlation was found between hyperlocomotion and GSH levels in the hippocampus ($r = 0.37$, $p < 0.01$), and a positive correlation was found between hyperlocomotion and LPO levels in the PFC ($r = 0.26$, $p < 0.05$) and striatum ($r = 0.32$, $p < 0.05$). A negative correlation was found between hyperlocomotion and GSH levels in the striatum ($r = -0.36$, $p < 0.01$) (Table 2).

Table 2. Correlation coefficients (Pearson's *r*) between methylphenidate-induced hyperlocomotion and GSH and LPO levels in the hippocampus, striatum, and PFC.

	Hippocampus		Striatum		Prefrontal cortex	
Locomotor activity	GSH	LPO	GSH	LPO	GSH	LPO
Acute treatment						
All mice (*n* = 47-56)	0.46*	0.56***	-0.17	-0.01	0.38**	0.48***
Chronic treatment						
All mice (*n* = 53-56)	0.37***	0.09	-0.36**	0.32*	-0.03	0.26***

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, (*) $0.01 < p < 0.05$.

4. DISCUSSION

The present study showed that chronic but not acute treatment with 10 and 40 mg/kg quercetin blocked methylphenidate-induced hyperlocomotion. Both acute and chronic lithium treatment blocked methylphenidate-induced hyperlocomotion, which is consistent with its clinical antimanic effect. These effects were seen at doses that did not alter spontaneous locomotor activity. The results with lithium indicate the sensitivity and validity of the procedure. The effects of quercetin on methylphenidate-induced

hyperlocomotion indicated an antimanic-like effect of chronic quercetin administration. Diazepam was used as a negative control, but acute and chronic diazepam treatment also blocked methylphenidate-induced hyperlocomotion. However, in both experiments, diazepam alone reduced locomotor activity, suggesting a sedative effect instead of an antimanic-like effect of diazepam (Young et al., 2011).

Psychostimulant-induced hyperlocomotion is the most frequently used animal model of mania (Einat, 2006). This pharmacological induction of manic-like behavior is reliable and has face, construct, and predictive validity (Einat, 2006; Machado-Vieira et al., 2004). Psychostimulants that are able to increase the levels of dopamine induce behavioral effects that resemble mania, such as hyperlocomotion (Huey et al., 1981). Mania has been related to an increase in dopaminergic activity (Cousins et al., 2009). A reduction of dopaminergic activity ameliorates the symptoms of mania (Post et al., 1980). Lithium is able to decrease dopaminergic transmission (Dziedzicka-Wasylewska et al., 1996). Methylphenidate, which blocks dopamine reuptake, was shown to induce hyperlocomotion, which was prevented by lithium, valproate carbamazepine, and antipsychotic drug administration (Nogoceke et al., 2016; Souza et al., 2016; Arunagiri et al., 2014; Tonelli et al., 2013; Barbosa et al., 2011; Pereira et al., 2011). Moreover, methylphenidate can induce manic-like symptoms in humans (Ekinci et al., 2016; Chakraborty and Grover, 2011; Huey et al., 1981; Smith and Davis, 1977).

However, other factors are also involved in manic-like behavior that is induced by methylphenidate, such as oxidative stress. Burrows et al. (2000) reported that the administration of psychostimulants that increase dopaminergic neurotransmission can also cause oxidative stress. Shanthakumar et al. (2013) showed that mice that were treated with methylphenidate exhibited hyperlocomotion and increased oxidative stress, which were blocked by lithium treatment.

The main antioxidant molecule in the brain is GSH, which participates in many cellular reactions. Reduced glutathione is involved in the regulation of lipid, glucose, and amino acid utilization. It is also involved in several chemical reactions that are associated with liver function, immunity, cellular physiology, and biosignaling pathways. However, the main function of GSH is its antioxidant activity. It effectively scavenges free radicals and other reactive oxygen species, removing hydrogen and lipid peroxides and preventing the oxidation of biomolecules (Wu et al., 2004). Therefore, the depletion of GSH levels may have deleterious effects. Gawryluk et al.

(2011) found lower levels of GSH in the *post mortem* PFC of BD patients compared with brain samples from individuals with no psychiatric illnesses. Macêdo et al. (2013) found that lisdexamphetamine dimesylate induced manic-like behavior in rats and decreased GSH content in the PFC, hippocampus, and striatum in rats, and these effects were reversed by lithium.

In the present study, methylphenidate increased GSH levels in the PFC and hippocampus but decreased GSH levels in the striatum. Pearson's correlation indicated a negative correlation between hyperlocomotion and GSH levels in the striatum in all of the animals that were subjected to the chronic treatment protocol. The heterogeneous effects of methylphenidate on GSH levels are not unusual or uncommon. Other research groups also reported diverse results regarding the effects of drugs that depend on dose, brain site, the specific oxidative stress parameter (Shanthakumar et al., 2013; Jornada et al., 2011; Bhalla and Dhawan, 2009; Frey et al., 2006). Our results showed that quercetin did not reverse the methylphenidate-induced decrease in GSH levels, but in some cases quercetin increased GSH levels compared with controls (vehicle + vehicle).

Emerging data indicate that oxidative stress may play a role in psychiatric illnesses, including BD. Low levels of free radicals or reactive oxygen/nitrogen species are considered normal, but high levels can damage and oxidize nucleic acids, carbohydrates, and lipids (Joshi and Praticò, 2014). Lipids are the major components of cell membranes, including neuronal membranes, and their peroxidation and alterations that generate hydroperoxides can greatly affect brain function (Joshi and Praticò, 2014). The levels of LPO increase during manic episodes. Lithium, which is used to treat mania in bipolar disorder, inhibits LPO and protein oxidation, suggesting that it has neuroprotective effects against oxidative stress that may be related to its antimanic effect (Cui et al., 2007). LPO that results from uncompensated oxidative stress is an important finding in BD, regardless of the stage of the illness (Andreazza et al., 2007; Machado-Vieira et al., 2007). Macêdo et al. (2013) also found an increase in LPO levels in the PFC, hippocampus, and striatum in rats in an animal model of psychostimulant-induced hyperlocomotion. Lithium treatment blocked this increase in oxidative stress.

In the present study, the effects of methylphenidate on oxidative stress were heterogeneous. Pearson's correlation indicated a positive correlation between hyperlocomotion and LPO levels in the PFC and hippocampus. Overall,

methylphenidate administration increased LPO levels in the PFC, hippocampus, and striatum. Acute lithium administration blocked the methylphenidate-induced increase in LPO only in the hippocampus. Acute diazepam administration blocked the methylphenidate-induced increase in LPO in the hippocampus, although at a dose that also decreased spontaneous locomotor activity. Chronic treatment with 10 mg/kg quercetin blocked the methylphenidate-induced increase in LPO levels in the PFC. Chronic treatment with lithium, diazepam, and all doses of quercetin blocked the methylphenidate-induced increase in LPO levels in the striatum. These results suggest that treatments that prevent methylphenidate-induced hyperlocomotion also have antioxidant effects.

However, such effects have been inconsistent. Arunagiri et al. (2014) showed that methylphenidate decreased GSH levels and increased LPO levels in whole brain homogenates, and lithium treatment restored GSH levels and reduced LPO levels. Various other studies reported heterogeneous results concerning the effects of drugs on oxidative stress in the brain (Shanthakumar et al., 2013; Jornada et al., 2011; Bhalla and Dhawan, 2009; Frey et al., 2006). The results have varied, depending on the treatment protocol, drug, dose, and brain site, among other factors. Martins et al. (2006) found that methylphenidate administration reduced LPO levels in the PFC and striatum in rats. At lower doses, methylphenidate decreased LPO levels in the hippocampus. At higher doses, methylphenidate increased LPO levels in the hippocampus. Although our study found heterogeneous results, chronic treatment with lithium and quercetin reduced antimanic-like behavior and reduced LPO levels in the striatum. The present and previous findings reinforce the putative role of oxidative stress in mania.

Overall, the present results indicated that chronic quercetin decreased methylphenidate-induced oxidative stress and hyperlocomotion, whereas acute treatment did not. This partially agrees with previous data that showed that acute quercetin treatment attenuated the increase in oxidative stress that was induced by sleep deprivation, which also led to an increase in locomotor activity (Kanazawa et al., 2016). The disagreement between these studies may be related to the different animal models of mania that were used, which reinforces the importance of using more than one model to evaluate the potential antimanic-like effects of drugs. Altogether, these data indicate an acute and chronic effect of quercetin on manic-like behavior.

Previous studies have shown that increases in dopaminergic transmission and consequent hyperlocomotion involve the activation of signaling pathways that are related to such enzymes as PKC (Abrial et al., 2015). Both lithium and quercetin exert antioxidant effects, and both are known to exert inhibitory effects on the activity of PKC (Manji and Lenox, 2000). Our research group showed that myricitrin, a flavonoid that has antioxidant and PKC-inhibitory actions, blocked oxidative stress and manic-like behavior (Pereira et al., 2014; Andreatini et al., 2013). The pathophysiology of mania in bipolar disorder involves increases in both PKC activity and oxidative stress (Andreazza et al., 2007; Machado-Vieira et al., 2007; Friedman et al., 1993). We hypothesized that quercetin might have antimanic properties. Indeed, chronic treatment with 10 and 40 mg/kg quercetin blocked methylphenidate-induced hyperlocomotion. In some cases, quercetin also restored GSH levels and decreased LPO levels after methylphenidate administration, similar to the effects of lithium. This indicates that the antimanic-like effect of quercetin may be linked to a decrease in oxidative stress.

PKC regulates several potential targets related to mood disorders, such as DAT, SERT, GSK3β, adenylate cyclase, NMDA receptors, GAP-43, BDNF and neurogenesis (Abrial et al., 2015). Specifically to dopamine system, PKC regulates DAT trafficking to cell surface, modulating dopamine neurotransmission (Vaughan and Foster, 2013; Chen et al., 2009) Moreover, PKC also affects drug induced dopamine efflux (Mikelman et al., 2016) Thus, an increase in PKC activity is associated with enhanced dopaminergic activity. It was proposed that dopamine transmission is increased in mania (Cousins et al., 2009), which can result in an augment in dopamine metabolism, generating dopamine metabolites rising. Quinone oxidative products of dopamine metabolites can generate toxic effects and increase oxidative stress (Segura-Aguilla et al., 2014). In this line, psychostimulants (amphetamine and fenproporex) act by augmenting dopamine transmission and they induce hyperlocomotion and oxidative stress increasing (Model et al., 2014; Young et al., 2011). Thus, quercetin can exert its antimanic-like effect reducing oxidative stress and PKC activity.

Quercetin has already been tested in clinical trials with regard to its anti-inflammatory effect. The effective dose range in the present study was within the dose range (40-240 mg/day) that was used in clinical trials (Boots et al., 2011).

5. CONCLUSION

Chronic quercetin treatment blocked methylphenidate-induced hyperlocomotion in mice, indicating an antimanic-like effect. Moreover, chronic quercetin treatment blocked the methylphenidate-induced increase in oxidative stress indices. These results corroborate previous data that indicated that quercetin may have antimanic-like effects that are associated with an antioxidant effect. Furthermore, the present findings extend previous data that showed that no tolerance to this effect develops after repeated administration, which is an important issue for the treatment of mania. The putative anti-inflammatory and antioxidant effects of quercetin have been tested in clinical trials, and quercetin may be an interesting candidate as a new antimanic drug.

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CHAPTER 7

General discussion

The reduced number of efficient and safe pharmacological options for the management of bipolar disorder (BD) makes the research for alternative drugs necessary. Considering that lithium as the gold standard antimanic drug inhibits the enzyme glycogen synthase kinase 3 beta (GSK3β), we hypothesized that GSK3β inhibitor andrographolide (ANDRO), a component of the plant *Andrographis paniculata*, could possess antimanic-like properties. Moreover, considering that brain oxidative stress plays a pivotal role in several psychiatric disorders including BD, and that lithium shows antioxidant effects, we hypothesized that the flavonoid and antioxidant quercetin could also possess antimanic-like properties.

Our findings show that chronic treatment with 0.5 and 2.0 mg/kg ANDRO and 100 mg/kg lithium prevented 24h sleep deprivation (SD)- and methylphenidate-induced hyperlocomotion. These are, respectively, non-pharmacological and pharmacological models of induction of manic-like behavior. The induction of SD and the administration of methylphenidate are considered to be valid animal models of mania, as they show face, construct and predictive validity (Einat, 2006; Machado-Vieira et al., 2004). After 24h SD, animals show insomnia, hyperactivity, irritability, aggressiveness, hypersexuality, and stereotypy, which are behaviors also seen in humans after SD (Pereira et al., 2014; Gessa et al., 1995), and increases in the emission of 50-kHz ultrassonic vocalizations (USVs) (Wendler et al., 2019). Methylphenidate administration in animals mimic the effects of psychostimulant administration in humans, which induce manic behavior in healthy humans and BD patients, such as decreased need for sleep, elevated mood, hypersexuality, affecting sensorimotor function, and learning and memory (Corp et al., 2014; Cousins et al., 2009; Berk et al., 2007; Asghar et al., 2003; Jacobs and Silverstone, 1986). This underlines the face validity of both models of mania.

The 24h SD model also involves several neurochemical alterations, such as increase in the expression of D_2 receptors in the striatum and supersensibility of dopaminergic receptors, leading to increased dopaminergic signaling in animals, which is also seen in BD patients (Lima et al., 2007; Tufik et al., 1978). Methylphenidate administration affects several neurotransmitter systems, as it

increases synaptic dopamine and norepinephrine through inhibition or reversing reuptake mechanisms, which is in line with the fact that these neurotransmitters are affected in patients with BD. This shows the construct validity of both models (Beyer and Freund, 2017; Berk et al., 2007).

Lithium and valproate, which are drugs frequently used in the therapy of BD, can attenuate the SD- and methylphenidate-induced mania-relevant behavior, showing predictive validity of the models (Einat, 2006; Machado-Vieira et al., 2004; Gessa et al., 1995). Thus, the fact that ANDRO treatment prevented SD- and methylphenidate-induced hyperlocomotion denotes that ANDRO appears to possess antimanic-like properties.

In parallel, we evaluated the effects of ANDRO on the levels of GSK3β and p-GSK3β in the prefrontal cortex (PFC) and striatum of mice submitted to SD- or methylphenidate-induction of hyperlocomotion. Our results showed that SD decreased Ser^9 phosphorylation of GSK3β in the PFC of mice, indicative of increased GSK3β activity. Chronic treatment with lithium or 2.0 mg/kg ANDRO were able to reverse this SD-induced decrease in p-Ser^9-GSK3β. Methylphenidate induced decreased Ser^9 phosphorylation of GSK3β in the striatum, which was prevented by 2.0 mg/kg ANDRO and lithium.

GSK3β is also considered to be a proinflammatory molecule, stimulating the production of various inflammatory cytokines and tumor necrosis factors. The inhibition of GSK3β has been proven to be beneficial in many inflammatory conditions (Jope et al., 2007). The impact of GSK3β on neurotransmission is unclear, but studies show that GSK3β interferes with neurotransmission as it affects the functioning of calcium and potassium channels. It is also involved in the phosphorylation of clock proteins and the regulation of the periodicity of the endogenous clock mechanism (Luca et al., 2016). Higher levels of GSK3β have been reported in blood among patients experiencing manic episodes compared to healthy subjects (Luca et al., 2016). After lithium treatment, the levels did not change, but the inhibitory Ser^9-phosphorylation of GSK3β increased (Li et al., 2010). Li and Jope (2010) reported a dysregulated activity of GSK3β, in terms of hyperactivity, due to a lower inhibitory Ser^9 phosphorylation among BD. Dal-Pont et al. (2019) showed that SD induced manic-like behaviors in mice and decreases in neurotrophic factors in the PFC and hippocampus, which were reversed by treatment with lithium or valproate. Xue et al. (2019) showed that SD inhibited the PI3K/Akt/GSK3β pathway, suggesting an activation of GSK3β,

neuroinflammation and oxidative stress. Acute psychostimulant administration activates GSK3β by reducing its inhibitory Ser^9-phosphorylation in mouse striatum and cerebral cortex, which is required for certain behavioral effects of psychostimulants, such as increased locomotor activity (Enman and Unterwald, 2012). By ablating GSK3β in the striatum of mice using a CRISPR-Cas9 system, Kim et al. (2019) showed that it suppressed amphetamine-induced hyperlocomotion. Mines and Jope (2012) demonstrated that 8-day intraperitoneal administration of 2 mg/kg amphetamine or 20 mg/kg methylphenidate led to decreased levels of p-Ser^9-GSK3β in the striatum of C57BL/6J mice, which is similar to our observation in the methylphenidate model. They also showed that the effects of psychostimulants are brain region-selective as was also shown by our divergent PFC effects. Clearly more research is needed into the effects of GSK3β and their link to the mechanism of action of ANDRO in order to explain its antimanic-like affect.

In the present study, we also evaluated the antimanic-like effect of ANDRO by inducing manic-like behavior (hyperlocomotion) by lisdexamfetamine (LDX) administration, which also leads to increases in 50-kHz USVs. LDX is a long-acting *d*-amphetamine prodrug (Ermer et al., 2016) and its administration can be used as a model for mania (Eger et al., 2016; Wendler et al., 2016). The emission of USVs can be analyzed as a reflection of hedonic states of the rats (Burgdorf et al., 2011). Studies show that SD induces hyperlocomotion and increases in rearings and 50-kHz USV emission in rats, which denotes manic-like behaviors, showing face validity of the model (Wendler et al., 2019). Psychostimulants seem to induce increased 50-kHz USVs by D_1 and D_2 receptor activation (Rippberger et al., 2015), and this agrees with the pivotal role of increased dopaminergic neurotransmission in mania (Ashok et al., 2017). Intra-accumbens application of quinpirole, a D_2 and D_3 receptor agonist, induces increases in 50-kHz USV emission (Brudzynski et al., 2012). LDX administration induces manic-like behaviors by enhancing dopaminergic neurotransmission, as well as inducing oxidative stress, and both parameters are involved in the pathophysiology of BD, thus showing the construct validity of the LDX administration model of mania (Macêdo et al., 2013). LDX administration induces increases in the number of 50-kHz USVs, which can be prevented by lithium treatment (Wendler et al., 2016). Pereira et al. (2014) demonstrated that lithium reversed amphetamine-induced increases in 50-kHz USV emission, and these studies demonstrate the predictive validity of the model as a model of mania. In our study,

chronic treatment with 2.0 mg/kg ANDRO prevented LDX-induced increases in the number of USVs and in the total time of USVs, as did chronic treatment with lithium. Both drugs also prevented LDX-induced hyperlocomotion.

In parallel to the effects of ANDRO on LDX-induced hyperlocomotion and increases in 50-kHz USVs, we also evaluated the antioxidant effects of ANDRO. It has also been reported that both ANDRO and lithium possess antioxidant properties. Lithium reduces the superoxide dismutase and catalase ratio, thus reducing oxidative stress. In addition, lithium seems to exert positive effects on mitochondrial dysfunctions and endoplasmic reticulum stress response (Machado-Vieira et al., 2009; Maurer et al., 2009). The inhibition of GSK3β due to lithium administration has been found to be responsible for the resistance of oxidative stress of murine hippocampal neuronal cells (Schäfer et al., 2004). Zhang et al. (2019) showed that bupivacaine increased the levels of reactive oxygen species and decreased reduced glutathione (GSH) levels in human neuroblastoma SH-SY5Y cells, which was prevented by preincubation with ANDRO. Thakur et al. (2016) showed that streptozotocin-induced diabetic rats had increased lipid peroxidation (LPO) levels in the PFC, and this was reduced by ANDRO treatment. In our study, LDX administration induced increases in LPO in rat striatum, which were prevented by repeated treatment with ANDRO and lithium. There was a positive correlation between increased LPO levels in rat striatum and hyperlocomotion as well as increases in 50-kHz USVs.

We also evaluated the antimanic-like properties of the flavonoid quercetin, a powerful antioxidant molecule and a protein kinase C (PKC) inhibitor, similar to lithium. Several studies link oxidative stress and PKC activity to the pathophysiology of BD (Valvassori et al., 2020; Garzón-Niño et al., 2017; Saxena et al., 2017). Therefore, we hypothesized that the antioxidant and PKC inhibitor quercetin might possess antimanic-like properties. In the present study, treatment with 10 and 40 mg/kg quercetin prevented SD-induced hyperlocomotion, as did lithium, the positive control. In addition, quercetin reversed SD-induced decreases in GSH levels in the PFC and striatum of mice, as well as reversing SD-induced increases in LPO in the PFC, hippocampus and striatum of mice. We also tested the effects of the acute and chronic treatment with 2.5, 5, 10 and 40 mg/kg quercetin on mice after methylphenidate-induced hyperlocomotion. Our findings showed that chronic (but not acute) treatment with 10 and 40 mg/kg quercetin reversed methylphenidate-induced hyperlocomotion. Quercetin also reversed methylphenidate-induced increases in LPO levels in the

striatum of mice. Arunagiri et al. (2014) showed that methylphenidate decreased GSH levels and increased LPO levels in whole brain homogenates, and lithium treatment restored GSH levels and reduced LPO levels. Various other studies reported heterogeneous results concerning the effects of drugs on oxidative stress in the brain (Minassian et al., 2016; Berk et al., 2007; Einat, 2006; Müller-Oerlinghausen et al., 2002). The results have varied, depending on the treatment protocol, drug, dose, and brain site, among other factors. Martins et al. (2006) found that methylphenidate administration reduced LPO levels in the PFC and striatum in rats. At lower doses, methylphenidate decreased LPO levels in the hippocampus. At higher doses, methylphenidate increased LPO levels in the hippocampus. Although our study found heterogeneous results, chronic treatment with lithium and quercetin reduced antimanic-like behavior and reduced LPO levels in the striatum. The present and previous findings reinforce the putative role of oxidative stress in mania.

Gawryluk et al. (2011) showed that the *post mortem* PFC in BD patients had lower levels of GSH, which can lead to higher susceptibility to neuronal oxidation, interfere with neuronal activity, and may contribute to the establishment of BD symptoms (Dringen, 2000). In the present study, SD-induced hyperlocomotion was related to lower GSH levels in the PFC. Other studies have shown that the replenishment of GSH diminishes oxidative cellular damage and ameliorates the symptoms of BD (Magalhães et al., 2011; Dean et al., 2009). High levels of oxidative damage to membrane phospholipids or the aggregation of oxidized proteins alters fluidity, which can induce cell death by apoptosis (Mahadik et al., 2001). Under certain conditions, LPO causes structural disturbances, alterations in integrity, fluidity, and permeability, the functional loss of biomembranes, and the generation of potentially toxic products (Greenberg et al., 2008). Previous studies have reported that such uncompensated oxidative stress increases LPO throughout the course of BD (Andreazza et al., 2007; Machado-Vieira et al., 2007). All of this underlines the need to further study the antioxidant effect of ANDRO.

Despite the numerous pharmacological activities of ANDRO, poor solubility and relatively low potency are still the major drawbacks of ANDRO to achieve therapeutic effect (Sharma et al., 2017). Further modification of the chemical structure and optimization of delivery system could enhance the pharmacological activity and increase the therapeutic index of ANDRO and its derivatives (Dai et al., 2018).

With regards to quercetin, studies also report poor oral bioavailability, however some studies indicate that quercetin glycosides are converted into aglycones in the intestine, by β-glycosidases, and then are adequately absorbed (Walle et al., 2005). The use of quercetin in the pharmaceutical industry is limited because of its poor permeability, poor solubility in water and poor bioavailability. However, several studies demonstrate that these parameters can be improved by modifications in the quercetin molecular structure. New quercetin formulations have been created, such as quercetin-loaded nanoparticles and quercetin-loaded polymeric micelles, or combinations with ions, such as calcium-phosphate-quercetin nanocomplex (Xu et al., 2019). Thus, there is a perspective for the usage of ANDRO and quercetin as drugs, as they are already used as supplements and other preparations by the general population worldwide, and their safety has been attested.

In conclusion, chronic treatment with ANDRO prevented hyperlocomotion induced by SD, methylphenidate and LDX, while increasing the p-Ser^{9}-GSK3β/GSK3β ratio in the PFC or striatum of mice. ANDRO also prevented the increases of 50-kHz USVs induced by LDX, while preventing LDX-induced hyperlocomotion and lipid peroxidation in rats. Quercetin also prevented SD- and methylphenidate-induced hyperlocomotion and decreases in GSH or increases in LPO levels (oxidative stress parameters) in mice. Thus, both ANDRO and quercetin appear to possess antimanic-like effects and are promising agents to be thoroughly investigated for the management of mania in BD.

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SUMMARY

Increased activity of the enzyme glycogen synthase kinase 3 beta (GSK3β) is shown to play a pivotal role in the pathophysiology of several psychiatric disorders, including bipolar disorder (BD). Lithium, the prototype mood stabilizer, is an inhibitor of GSK3β. In animal models of mania, GSK3β inhibitors reproduce behaviors that mimic the effects of lithium. The pharmacological management of BD consists of administration of mood stabilizers or antipsychotics associated to antidepressants and it often involves a myriad of adverse effects or non-responsiveness, which affect medication adherence and quality of life. The search for new therapeutic agents for BD is therefore necessary. Considering that the inhibition of GSK3β activity may display antimanic-like effects, we tested the effects of andrographolide (ANDRO), the major bioactive compound isolated from *Andrographis paniculata*, which is an inhibitor of GSK3β. Studies showed that ANDRO possesses several therapeutic properties, including anti-inflammatory, antioxidant, antibacterial, hepatoprotective, neuroprotective, among others. Both ANDRO and lithium have been shown to decrease GSK3β levels and increase the levels of p-Ser^{9}-GSK3β, the phosphorylated and inactive form of GSK3β .

Taking into consideration that GSK3β is an enzyme involved in the pathophysiology of BD and that GSK3β inhibition, such as induced by lithium, ameliorates manic symptoms, the effects of the chronic treatment with the GSK3β inhibitor ANDRO were tested in different animal models of mania, such as sleep deprivation (SD)-, methylphenidate- and lisdexamfetamine (LDX)-induced hyperlocomotion, increases in 50-kHz ultrassonic vocalizations (USVs) and oxidative stress. We also evaluated the effects of ANDRO in the levels of GSK3β and p-Ser^{9}-GSK3β in the prefrontal cortex (PFC) and striatum of mice. A summary of the results is given below.

SD resulted in hyperlocomotion and treatment with lithium, 0.5 mg/kg ANDRO, and 2.0 mg/kg ANDRO blocked SD-induced hyperlocomotion. SD decreased the p-Ser^{9}-GSK3β/GSK3β ratio in the PFC. Both lithium and 2.0 mg/kg ANDRO increased the p-Ser^{9}-GSK3β/GSK3β ratio in the PFC.

Methylphenidate administration increased locomotor activity compared to the control group and treatment with lithium, 0.5 mg/kg ANDRO, and 2.0 mg/kg ANDRO

blocked methylphenidate-induced hyperlocomotion. Methylphenidate reduced the p-Ser^{9}-GSK3β/GSK3β ratio in the striatum. Both lithium and 2.0 mg/kg ANDRO increased the p-Ser^{9}-GSK3β/GSK3β ratio in the striatum.

LDX increased locomotor activity in rats, which was prevented by chronic treatment with lithium or 2.0 mg/kg ANDRO. LDX administration increased the number of 50-kHz USVs, which was also prevented by chronic treatment with lithium or 2.0 mg/kg ANDRO. Lithium and 2.0 mg/kg ANDRO also prevented LDX-induced increases in lipid peroxidation (LPO), an oxidative stress parameter in the striatum. There was a positive correlation between LDX-induced hyperlocomotion and LDX-induced increases in 50-kHz USVs and LPO.

Both 10 and 40 mg/kg quercetin prevented SD-induced hyperlocomotion. Quercetin reversed the SD-induced decrease in glutathione (GSH) levels in the PFC and striatum. Quercetin also reversed the SD-induced increase in LPO in the PFC, hippocampus, and striatum. Pearson's correlation analysis revealed a negative correlation between locomotor activity and GSH in the PFC in sleep-deprived mice and a positive correlation between locomotor activity and LPO in the PFC and striatum in sleep-deprived mice.

Chronic but not acute treatment with quercetin (10 and 40 mg/kg) blocked methylphenidate-induced hyperlocomotion. Chronic treatment with lithium and quercetin blocked the methylphenidate-induced increase in LPO levels in the striatum.

Overall, the results show that chronic treatment with ANDRO prevented hyperlocomotion induced by SD and methylphenidate, while increasing the p-Ser^{9}-GSK3β/GSK3β ratio in the PFC and striatum of mice, respectively. ANDRO also prevented LDX-induced hyperlocomotion and increases in the number of 50-kHz USVs, while also preventing LDX-induced LPO in the striatum of rats. Quercetin also prevented SD and methylphenidate-induced hyperlocomotion, while also preventing SD-induced decreases in GSH in the PFC and striatum, and LPO in the PFC, hippocampus and striatum. Quercetin also blocked methylphenidate-induced hyperlocomotion, and methylphenidate-induced increase in LPO levels in the striatum. Thus, both ANDRO and quercetin appears to possess antimanic-like effects and they are promising agents to be thoroughly investigated for the management of mania in BD.

VALORIZATION

Despite the numerous pharmacological options for bipolar disorder (BD), treatment still shows inadequate response in acute manic or depressive episodes or in long-term preventive maintenance treatment (Gitlin, 2006). Established first-line treatments include lithium, valproate and second-generation antipsychotics in acute mania, and lithium and valproate for maintenance treatment, as well as anticonvulsants. Combining multiple agents is the most commonly used clinical strategy, especially with antidepressants (López-Muñoz et al., 2018; Gitlin, 2006). Common adverse effects that result from the use of these drugs include weight gain, akathisia, nausea, vomiting, somnolence, tremors, dizziness, asthenia and blood dyscrasias, among many others (Bai et al., 2019; López-Muñoz et al., 2018). These aspects, such as low self-efficacy for medication-taking behavior, fear of dependence on medications, concern about medication adverse effects affect treatment adherence in BD (Levin et al., 2020; Levin et al., 2016; Chang et al., 2015; Devulapalli et al., 2010).

In this perspective, the research for new alternative drugs is relevant. We investigated the possible antimanic-like effects of andrographolide (ANDRO) and quercetin. We hypothesized that ANDRO could possess antimanic-like properties as it shares common mechanisms of action as the mood stabilizer lithium, for instance, inhibitory activity over the enzyme glycogen synthase kinase 3 beta (GSK3β) and antioxidant properties. We also hypothesized that the flavonoid quercetin could exert antimanic-like properties as it also has similar mechanisms of action as lithium, such as inhibitory activity over protein kinase C (PKC) and antioxidant effects.

ANDRO is already sold and consumed worldwide as dietary supplement as *Andrographis paniculata* pills (powdered plant) or Kalmegh pills, mainly for its anti-inflammatory and antioxidant properties (Kataky and Handique, 2010). Safety has been widely tested preclinically. Bothiraja et al. (2012) showed that up to 5 g/kg or 500 mg/kg oral doses of ANDRO given daily for up to 14 days and 21 days, respectively, had no observable adverse effects in rats. Handa and Sharma (1990) showed that the LD_{50} of ANDRO given via intraperitoneal injection to mice is 11.46 g/kg. Prakash and Manavalan (2011) showed that the acute administration of 2000 mg/kg ANDRO p.o. in mice did not alter body or liver weight, kidney and heart. It neither altered creatinine,

total cholesterol or blood sugar levels, nor hematological parameters, such as platelet counts, hemoglobin or red/white blood cells of mice treated with ANDRO when compared to the control group. Al Batran et al. (2013) showed that acute oral administration of 500 mg/kg ANDRO did not induce toxic effects in the liver or kidney in rats. These studies show that ANDRO appears to be relatively safe, and for this reason, there is an increasing interest in its therapeutic use (Lu et al., 2019; Tan et al., 2017).

In addition, scientific research involving ANDRO does not rely solely upon pre-clinical tests, but several clinical studies have already been performed and are still being developed. In a randomized, double-blind, placebo-controlled trial, the therapeutic efficacy of tablets of *A. paniculata* extract (170 mg of *A. paniculata* containing 85 mg of ANDRO) was evaluated in subjects with relapsing-remitting multiple sclerosis receiving interferon therapy significantly improved multiple sclerosis-associated fatigue (following the Fatigue Severity Scores), in 44% compared to the placebo group (Bertoglio et al., 2016). A phase II randomized double-blind placebo-controlled clinical study for the evaluation of *A. paniculata* oral tablets in patients with multiple sclerosis was completed in 2015 (NCT02280876), but the results are yet to be published. Thus, there are great perspectives for the research involving ANDRO and its therapeutic effects.

Quercetin is also taken as a dietary supplement as quercetin pills, due to its antioxidant properties (Vida et al., 2019). This flavonoid is widely distributed in nature in plants and vegetables as quercetin glycosides, while dietary supplements usually contain quercetin in its free form, as aglycones (Andres et al., 2017). In dietary supplements, recommended daily doses of quercetin aglycones can range up to 1000 mg (Andres et al., 2017). Studies show that no adverse effects were reported by volunteers after repeated daily intake of 500 mg quercetin for 4-8 weeks (Javadi et al., 2017), 730 mg quercetin for 4 weeks (Edwards et al., 2007), or 1000 mg for 5 days to 12 weeks (Rezvan et al., 2017), showing the safety in the administration or intake of quercetin. Ferry et al. (1996) showed that 945 mg/m² intravenous quercetin injection was still a safe dose of quercetin, demonstrating its safety, although studies suggest that excessive consumption of quercetin, above the recommended daily intake, can lead to nephrotoxicity and carcinogenesis (Singh et al., 2010; Dunnick and Hailey, 1992).

Clinical trials are in course to test the therapeutic effects of quercetin. A randomized double-blind placebo-controlled trial has shown that the combination of quercetin (500, 1000 or 2000 mg), vitamin C (350 mg) and niacin (10 mg) is beneficial for patients with chronic obstructive pulmonary disease (NCT01708278). Another randomized double-blind placebo-controlled clinical study to evaluate the effects of quercetin on sarcoidosis is taking place at Maastricht University, the Netherlands, where the antioxidant and inflammatory status of the participants is analyzed after 24 h from taking 1000 mg of quercetin (NCT00402623). Another research group is evaluating the effects of dietary supplement with luteolin (100 mg/capsule), quercetin (70 mg/capsule) and rutin (30 mg/capsule) on 50 children with autism spectrum disorders (NCT01847521). This shows a future perspective in the employment of quercetin as a drug in the management of various health problems.

BD patients have increased risk of many general-medical disorders, with increased morbidity, disability and diminished longevity (Baldessarini et al., 2020). BD patients have more adverse clinical outcomes and diminished life-expectancy, with all-cause mortality up to 15-times above general population rates (Ösby et al., 2018; Staudt-Hansen et al., 2019). Thus, it is very important to continue the research for new antimanic drugs that can be included in the pharmacological arsenal for the management of BD. Actually, our studies showed that ANDRO and quercetin exert antimanic-like effects and are promising candidates for further development and testing of new antimania drugs with a safe therapeutic window.

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CURRICULUM VITAE

Luiz Kae Sales Kanazawa was born on January 27th, 1990, in São Paulo, Brazil. He completed the secondary education at the Colégio Estadual do Paraná, in Curitiba, in 2006. During the following year, he studied to enroll for the Federal University of Paraná (UFPR), where he started his Bachelor in Pharmacy, in 2008. In 2014, he was admitted to the Master Degree in the Post-Graduation Program in Pharmacology of UFPR, under the supervision of Dr. Roberto Andreatini. During his Masters, Luiz studied the effects of quercetin on animal models of mania and brain oxidative stress. During his PhD (2016-2020), he continued to perform research regarding mania, by evaluating the effects of andrographolide in animal models of mania. With funding from the CAPES/NUFFIC (88887.199578/2018-00) Program, he performed experiments as part of his PhD at Maastricht University, in Maastricht, Netherlands. Luiz will receive a double doctoral degree of the Federal University of Paraná and Maastricht University, since his project was financially supported by both institutions.

PUBLICATIONS

Journal articles

1. Vecchia, D. D.; **Kanazawa, L. K. S**.; Wendler, E. M.; Hocayen, P. A. S.; Vital, M. A. B. F.; Takahashi, R. N.; Miyoshi, E.; Andreatini, R. Ketamine reversed short-term memory impairment and depressive-like behavior in animal model of Parkinson's disease. Brain Research Bulletin. 2020.

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3. Coelho, D. G.; **Kanazawa, L. K. S.**; Vecchia, D. D. Schizophrenia: effects of aripiprazole in metabolic syndrome. Brazilian Journal of Pharmaceutical Sciences. v. 55, 2019.

4. **Kanazawa, Luiz K.S.**; Vecchia, Débora D.; Wendler, Etiéli M.; Hocayen, Palloma De A.S.; Berão, Paulo S.; De Mélo, Manuela L.; Dos Reis Lívero, Francislaine A.; Corso, Claudia Rita; Stipp, Maria Carolina; Acco, Alexandra; Andreatini, Roberto. Effects of acute and chronic quercetin administration on methylphenidate-induced hyperlocomotion and oxidative stress. Life Sciences (1973), v. 171, p. 1-8, 2017.

5. **Kanazawa, Luiz K.S**.; Vecchia, Débora D.; Wendler, Etiéli M.; Hocayen, Palloma De A. S.; Dos Reis Lívero, Francislaine A.; Stipp, Maria Carolina; Barcaro, Inara M.R.; Acco, Alexandra; Andreatini, Roberto. Quercetin reduces manic-like behavior and brain oxidative stress induced by paradoxical sleep deprivation in mice. Free Radical Biology & Medicine, v. 99, p. 79-86, 2016.

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pectin from acerola (*Malpighia emarginata*). International Journal of Biological Macromolecules, 2017.

8. Wendler, Etieli; De Souza, Camila Pasquini; Vecchia, Debora Dalla; **Kanazawa, Luiz Kae Sales**; De Almeida Soares Hocayen, Palloma; Wöhr, Markus; Schwarting, Rainer K.W.; Andreatini, Roberto. Evaluation of 50-kHz ultrasonic vocalizations in animal models of mania: Ketamine and lisdexamfetamine-induced hyperlocomotion in rats. European Neuropsychopharmacology, v. 26, p. 1900-1908, 2016.

9. Martynhak, Bruno Jacson; **Kanazawa, Luiz Kae Sales**; Nascimento, Guilherme Messias Do; Andreatini, Roberto. Social interaction with rat exposed to constant light during lactation prevents depressive-like behavior induced by constant light in adulthood. Neuroscience Letters (Print), v. 588, p. 7-11, 2015.

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Publications in progress

1. **Kanazawa, L. K. S**.; Nelissen, E.; Prickaerts, J.; Andreatini, R. Overview of the effects of andrographolide on disorders of the Central Nervous System.

2. **Kanazawa, L. K. S.**; Nelissen, E.; Aguiar, R. P.; Prickaerts, J.; Andreatini, R. Andrographolide prevents sleep deprivation- and methylphenidate-induced manic-like behavior mediated via GSK3β inhibition.

3. **Kanazawa, L. K. S.**; Radulski, D. R.; Pereira, G. S.; Prickaerts, J.; Schwarting, R. K. W.; Acco, A.; Andreatini, R. Andrographolide prevents increases in 50-kHz ultrassonic vocalizations, hyperlocomotion and oxidative stress induced by lisdexamfetamine in rats, an animal model of mania*. Submitted to Progress in Neuropsychopharmacology & Biological Psychiatry*.

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