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Methylphenidate treatment causes oxidative stress and alters energetic metabolism in an animal model of attention-deficit hyperactivity disorder

Comim CM, Gomes KM, Réus GZ, Petronilho F, Ferreira GK, Streck EL, Dal-Pizzol F, Quevedo J. Methylphenidate treatment causes oxidative stress and alters energetic metabolism in an animal model of attention-deficit hyperactivity disorder

Objectives: To evaluate oxidative damage through the thiobarbituric acid-reactive species (TBARS) and protein carbonyl groups; antioxidant enzymatic system – superoxide dismutase (SOD) and catalase (CAT); and energetic metabolism in the brain of spontaneously hypertensive adult rats (SHR) after both acute and chronic treatment with methylphenidate hydrochloride (MPH).
Methods: Adult (60 days old) SHRs were treated during 28 days (chronic treatment), or 1 day (acute treatment). The rats received one i.p. injection per day of either saline or MPH (2 mg/kg). Two hours after the last injection, oxidative damage parameters and energetic metabolism in the cerebellum, prefrontal cortex, hippocampus, striatum and cortex were evaluated.
Results: We observed that both acute and/or chronic treatment increased TBARS and carbonyl groups, and decreased SOD and CAT activities in many of the brain structures evaluated. Regarding the energetic metabolism evaluation, the acute and chronic treatment altered the energetic metabolism in many of the brain structures evaluated.
Conclusion: We observed that both acute and chronic use of methylphenidate hydrochloride (MPH) in adult spontaneously hypertensive rats (SHRs) was associated with increased oxidative stress and energetic metabolism alterations. These data also reinforce the importance of the SHR animal model in further studies regarding MPH.

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Keywords: energetic metabolism, methylphenidate, oxidative stress, SHR rats

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Significant outcomes

- Acute and chronic MPH treatment increases TBARS and carbonyl groups > acute and chronic MPH treatment decreases superoxide dismutase (SOD) and catalase (CAT) activities in the brain > acute and chronic MPH treatment alters energetic metabolism in the brain.

Limitations

- The animal model used in this study is an important limitation because it is used as an animal model for other diseases such as schizophrenia.

Introduction

Attention-deficit hyperactivity disorder (ADHD) is among the most commonly diagnosed neuropsychiatry disorder and is characterised by excessive levels of inattentiveness, impulsivity and hyperactivity. ADHD is a worldwide and highly prevalent disorder, estimated to affect 5–10% of children (1) and 4% of adults (2). Most of the ADHD patients benefit from treatment with methylphenidate hydrochloride

(MPH), irrespective of the disorder's aetiology, effectively reducing symptoms in up to 70% of patients (3). MPH blocks the dopamine transporters and this indirect dopamine agonist effect may be critical for its therapeutic effects. However, the mechanisms underlying the stimulant therapeutic efficacy or possible enduring neuro-adaptational consequences of methylphenidate long-term drug exposure are poorly understood (4). Thus, like many other drugs with stimulant properties – including cocaine, amphetamine, morphine and nicotine – MPH increases extracellular concentrations of dopamine within key portions of rat brain reward circuits, including the nucleus accumbens and related regions (5–8).

Most of the cell energy is obtained through oxidative phosphorylation, a process requiring the action of various respiratory enzyme complexes located in a special structure of the inner mitochondrial membrane, the mitochondrial respiratory chain. Tissues with high-energy demands, such as the brain, contain a large number of mitochondria and are therefore more susceptible to reduction of the aerobic energy metabolism (9). Porrino and Lucignani (10) measured rates of local cerebral glucose utilisation following acute administration of MPH in doses ranging from 1.25 to 15.0 mg/kg. This study showed significant dose-dependent alterations in the metabolic activity in the components of extra-pyramidal system, nucleus accumbens and olfactory tubercle (10). Taken together, the normal brain metabolises 20% of total body oxygen and has a limited amount of antioxidant capacity, resulting in a particular vulnerability to reactive oxygen species (ROS) production. In situations where the generation of free radicals exceeds the capacity of antioxidant defense, oxidative stress may lead to membrane degradation, cellular dysfunction and apoptosis (9). Studies suggest that the formation of ROS and reactive nitrogen species may play a role in the behavioural changes and neurodegeneration after MPH use (11,12).

Repeated exposure to stimulant drugs has also been linked to the development of psychomimetic-like effects in rats (13). Once established, these behavioural adaptations can endure for remarkably long periods without drug treatment, suggesting that they may be caused by stable and long-lasting molecular adaptations (14,15). Preclinical studies raise the possibility that repeated exposure to stimulant drugs causes enduring neuroadaptations that contribute to other neuropsychiatric disorders (16). However, the knowledge that ADHD may persist into adulthood has led to an increased use of MPH in adult patients (17) and its extended use may have chronic effects on the organism. In this context, to help understand the pathophysiology of ADHD, the spontaneously hypertensive rat (SHR) – one of the most extensively studied models of ADHD (18,19) – was used. It presents impaired dopamine release in the prefrontal cortex, nucleus accumbens system and striatum (20). Considering that recent studies showed that children (21) and adults (22) with ADHD exhibited higher oxidant levels, and that MPH is widely used in children, there is a lack of studies that investigate the effects of short- and long-term MPH treatment in the central nervous system (CNS) of adults. Thus, the main objective of the present study was to investigate the effects of both acute and chronic MPH administration on some oxidative stress parameters and enzyme activities in the prefrontal cortex, cerebellum, hippocampus, striatum and cortex of adults SHR.

Methods

Animals

Adult (60 days old) male SHRs obtained from our breeding colony were housed five to a cage with food and water available *ad libitum* and maintained on a 12-h light/dark cycle (lights on at 07:00 a.m.). All experimental procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behaviour recommendations for animal care. The local Ethics Committee approved this study.

Experimental protocols

Acute treatment. MPH (2 mg/kg intraperitoneal, i.p.) or saline injections were given to rats on postnatal day (PD) 60. Each group had six to eight animals (23–25).

Chronic treatment. MPH (2 mg/kg i.p.) or saline injections were given to adults rats starting on PD 60 once daily for 28 days (last injections on PD 88). Each group had six to eight animals (23–25).

Two hours after the last injection, the animals were killed by decapitation, and the striatum, cerebellum, prefrontal cortex, hippocampus and cerebral cortex (without the prefrontal cortex) were immediately dissected and stored at –80°C for posterior biochemical analyses.

Biochemical analysis

Oxidative parameters. As an index of ROS production, we used the formation of thiobarbituric acid-reactive species (TBARS) during an acid-heating

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reaction, which is widely adopted as a sensitive method for the measurement of lipid peroxidation, as previously described (26). Briefly, the samples were mixed with 1 ml of trichloroacetic acid 10% (TCA) and 1 ml of thiobarbituric acid 0.67% (TBA), and then heated in a boiling water bath for 15 min. TBARS were determined by the absorbance at 535 nm. Results are expressed as malondialdehyde (MDA) equivalents (nmol/mg protein). The oxidative damage to proteins was assessed by the determination of carbonyl groups on the basis of the reaction with dinitrophenylhidrazine (DNPH), as previously described (27). Briefly, proteins were precipitated by the addition of 20% TCA, redissolved in DNPH and the absorbance read at 370 nm.

To determine catalase (CAT) activity, the brain tissue was sonicated in 50 mmol/l phosphate buffer (pH 7.0), and the resulting suspension was centrifuged at $3000 \times g$ for 10 min. The supernatant was used for an enzyme assay. CAT activity was measured by the rate of decrease in hydrogen peroxide absorbance at 240 nm (28). SOD activity was assayed by measuring the inhibition of adrenaline auto-oxidation, as previously described (29). All the results were normalised by the protein content (30).

Energetic metabolism

Brain structures were homogenised in SETH buffer for the determination of mitochondrial respiratory chain enzyme activities (Complexes I, II, II–III and IV). NADH dehydrogenase (Complex I) was evaluated by the rate of NADH-dependent ferricyanide reduction at 420 nm. Complex II activity was measured by following the decrease in absorbance due to the reduction of 2,6-dichloroindophenol (DCIP) at 600 nm. Complex II–III activity was measured by cytochrome c reduction from succinate. The activity of cytochrome c oxidase (Complex IV) was assayed by following the decrease in absorbance due to the oxidation of previously reduced cytochrome c at 550 nm. The activities of the mitochondrial respiratory chain complexes are expressed as nmol/min mg protein (31).

Statistical analyses

All data were presented as mean ± SD and were analysed by Student's t test. We considered $p < 0.05$ to be significant. Statistical Package for the Social Sciences 17.0 was used for the statistical analysis.

Results

Figure 1 shows the oxidative damage in the cerebral tissue in both acute and chronic treatments with MPH. The lipid peroxidation was evaluated by the formation of TBARS. The acute treatment with MPH demonstrates an increase in lipid peroxidation in the cerebellum ($t = -3.924$; df = 5; $p = 0.011$) and prefrontal cortex ($t = -4.148$; df = 3.083; $p = 0.024$) (Fig. 1a); the chronic treatment also increased lipid peroxidation in the prefrontal cortex ($t = -2.307$; df = 7; $p = 0.049$), hippocampus ($t = -2.662$; df = 8; $p = 0.029$) and striatum ($t = -4.994$; df = 7; $p = 0.002$) when compared with controls (Fig. 1b). The acute treatment with MPH increased the formation of protein carbonyl groups in the cerebellum ($t = -12.622$; df = 6; $p = 0.0001$) and cortex ($t = -8.839$; df = 6; $p = 0.0001$) in relation to the control group (Fig. 1c), whereas the chronic treatment increased protein damage in the cerebellum ($t = -5.854$; df = 3.829; $p = 0.005$) and hippocampus ($t = -5.753$; df = 6; $p = 0.001$) compared with controls (Fig. 1d).

Figure 2 shows the antioxidant activity (CAT and SOD) in the brain after acute and chronic MPH treatments. CAT activity was decreased in the cerebellum ($t = 5.920$; df = 4; $p = 0.004$) after acute treatment with MPH (Fig. 2a) and in the hippocampus ($t = 6.687$; df = 4; $p = 0.003$) after chronic treatment, when compared with controls (Fig. 2b). However, the activity of SOD was decreased in the striatum ($t = -5.142$; df = 6; $p = 0.002$) after acute treatment (Fig. 2c) and increased in the cerebellum ($t = -3.153$; df = 6; $p = 0.020$) after the chronic treatment (Fig. 2d), when compared with the control group.

The energetic metabolism is demonstrated in Fig. 3. In the acute treatment, there was a decrease of complex I activity in the prefrontal cortex ($t = 4.208$; df = 8; $p = 0.003$) and hippocampus ($t = 4.752$; df = 4; $p = 0.003$) (Fig. 3a); an increase of complex II activity in the prefrontal cortex ($t = -8.174$; df = 4; $p = 0.001$), hippocampus ($t = -1.917$; df = 4; $p = 0.048$), striatum ($t = -13.837$; df = 5; $p = 0.0001$) and cortex ($t = -3.440$; df = 4; $p = 0.026$) (Fig. 3b); an increase of complex II–III activity in the hippocampus ($t = -4.136$; df = 6; $p = 0.028$) (Fig. 3c); a decrease of complex IV activity in the prefrontal cortex ($t = 4.181$; df = 6; $p = 0.006$); and an increase in the striatum ($t = -5.756$; df = 5; $p = 0.002$) (Fig. 3d) when compared with controls. In the chronic treatment, a decrease of complex I activity was observed in the striatum ($t = 2.986$; df = 8; $p = 0.019$) (Fig. 3e); an increase of complex II activity in the cerebellum ($t = -2.560$; df = 4; $p = 0.049$); a decrease in the hippocampus ($t = 8.450$; df = 5; $p = 0.0001$) and striatum ($t = 1.930$; df = 6; $p = 0.049$) (Fig. 3f); a decrease of complex III activity in the prefrontal cortex ($t = -3.282$; df = 8; $p = 0.011$) and an

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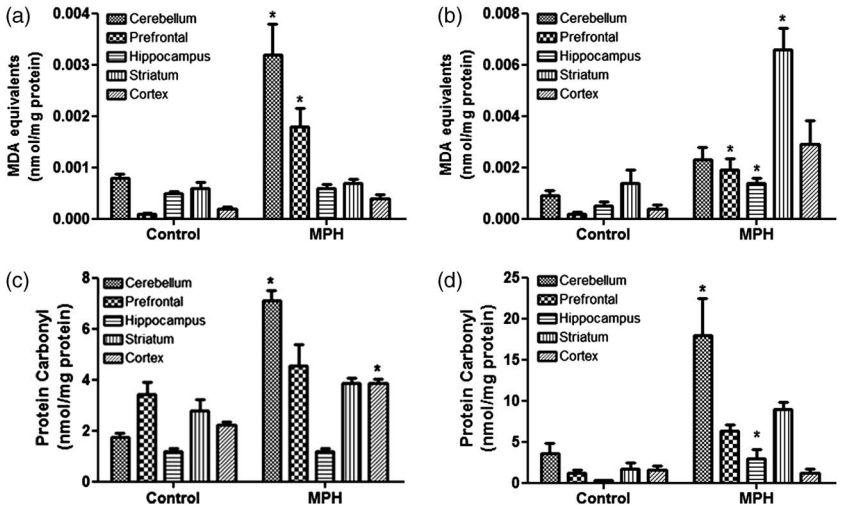

Fig. 1. Oxidative variables in the brain spontaneously hypertensive rat (SHR). Thiobarbituric acid-reactive species (TBARS)–MDA equivalents in acute (a) and chronic treatment (b); carbonyl protein in acute (c) and chronic treatment (d). Bars represent means ± SD of six to eight rats. $*p < 0.05$ versus control, according to Student's *t* test.

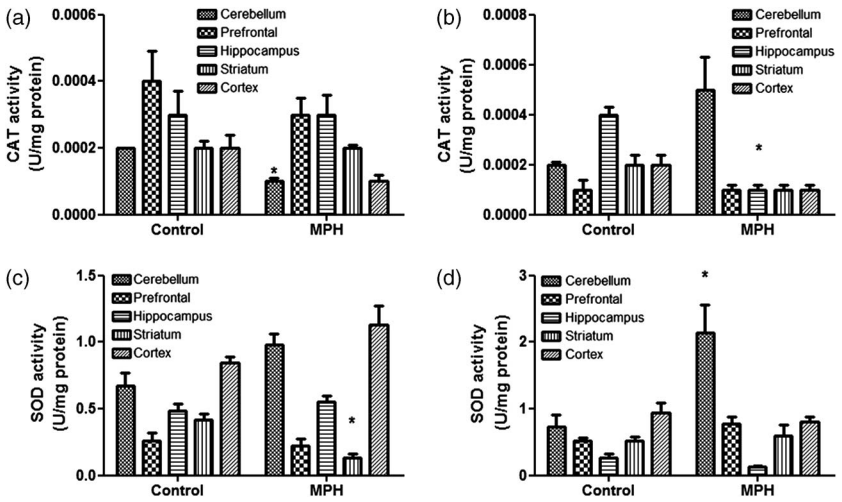

Fig. 2. Antioxidant enzymes activity in the brain spontaneously hypertensive rat (SHR). Catalase (CAT) activity in acute (a) and chronic treatment (b), and superoxide dismutase (SOD) activity in acute (c) and chronic treatment (d). Bars represent means ± SD of six to eight rats. $*p < 0.05$ versus treatment with saline, according to Student's *t* test.

increase in the striatum ($t = 4.107$; df = 8; $p = 0.003$) (Fig. 3g); and an increase of complex IV activity in the cerebellum ($t = -2.890$; df = 6; $p = 0.012$), prefrontal cortex ($t = -3.818$; df = 5; $p = 0.012$), striatum ($t = -6.029$; df = 6; $p = 0.001$) and cortex ($t = -5.234$; df = 5; $p = 0.003$) (Fig. 3h) when compared with controls.

Discussion

In this study, we observed that there was an imbalance between oxidative and antioxidant activity and an important alteration in the energetic metabolism in the different brain structures evaluated during both acute and chronic treatments with MPH. The difference in the structures evaluated is due to the distribution of MPH in the brain, which is heterogeneous, and the maximum concentration occurred in the striatum, cortex and cerebellum (32). Prefrontal cortex is also one target of MPH therapy (33). According to pharmacokinetics of MPH, we showed here an oxidative damage in the cerebellum and cortex (acute treatment), and the cerebellum and hippocampus (chronic treatment), caused by an increase in both lipid peroxidation and protein carbonylation in the cerebellum and prefrontal cortex (acute treatment), and the prefrontal cortex, hippocampus and striatum (chronic treatment).

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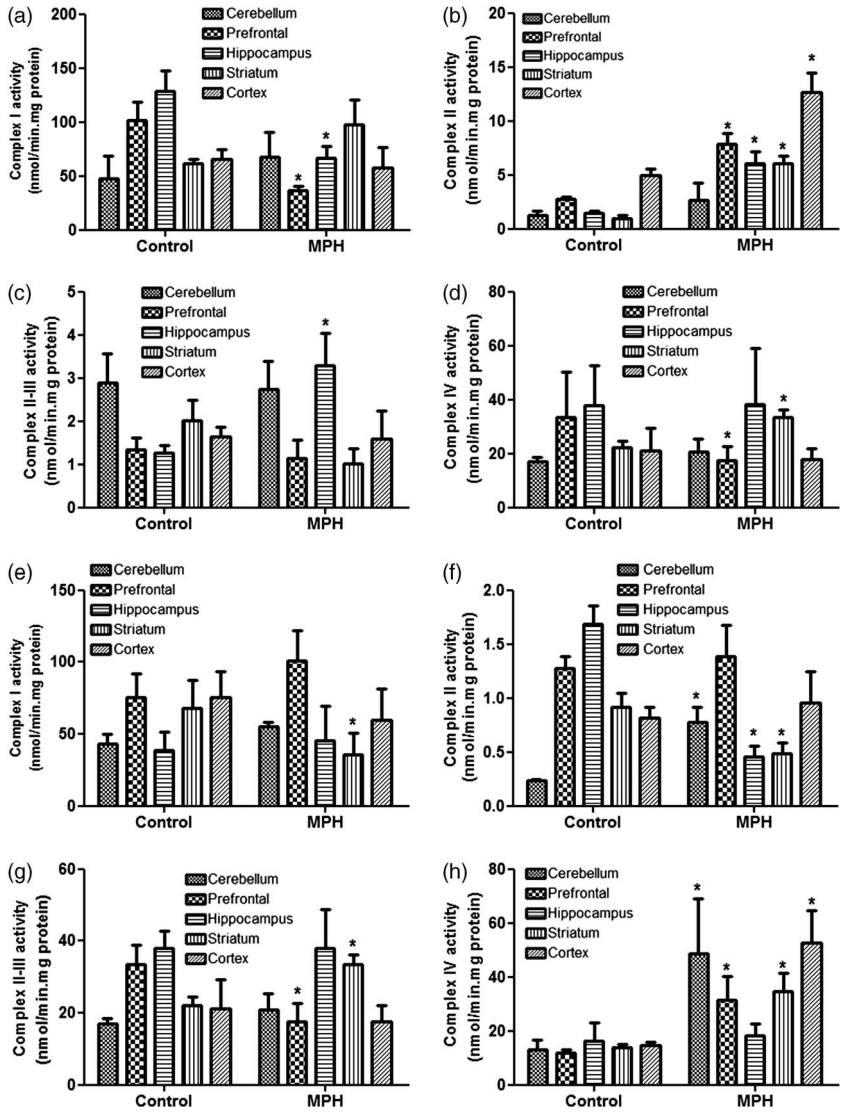

Fig. 3. Energetic Metabolism in the brain SHR. Complex I (a), II (b), III (c) and IV (d) activity after acute treatment with MPH and Complex I (e), II (f), III (g) and IV (h) activity after chronic treatment with MPH. Bars represent means ± SD of six to eight rats. *$p < 0.05$ versus control, according to Student's *t* test. MPH, methylphenidate hydrochloride; SHR, spontaneously hypertensive rat.

On the other hand, acute treatment caused a decrease of the CAT activity in the cerebellum as well as a decrease of the SOD activity in the striatum. The chronic treatment decreased the CAT activity in the hippocampus, but increased the SOD activity in the cerebellum. When we evaluated the energetic metabolism during the acute treatment, we found a decrease of complex I activity in the prefrontal cortex and hippocampus; an increase of complex II activity in the cerebellum, prefrontal cortex, hippocampus, striatum and cortex; an increase of complex II–III in the hippocampus; and a decrease of complex IV in the prefrontal cortex and an increase in the striatum. Regarding the chronic treatment, there was a decrease of complex I activity in the striatum; an increase of complex II activity in the cerebellum and a decrease in the hippocampus and striatum; a decrease of complex II–III activity in the prefrontal cortex and a increase in the striatum; and an increase of complex IV activity in the cerebellum, prefrontal cortex, striatum and cortex.

Prefrontal lesions are associated with social disinhibition, impulse dyscontrol, organisational planning, working memory, attentional dysfunctions, dysfluency and slowing of spontaneous behaviours. Striatum damage is possibly associated with the aetiology of ADHD (34). Experimental striatal lesions in animals produced hyperactivity and poor performance on working memory and response inhibition tasks (35). The striatum is also one of the richest sources of dopaminergic synapses (36), and dopamine is important in the regulation of striatal

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functions. Thus, stimulant medications, commonly used to treat ADHD, have effects on the striatum (4). Interestingly, although the cerebellum has traditionally been thought to be primarily involved in motor control, both clinical and research findings over the past 20 years have shown cerebellar involvement in a number of cognitive and affective processes. Schmahmann and Sherman (37) coined the term 'cerebellar cognitive affective syndrome' after recognising cognitive and affective disturbances in cerebellar lesion patients. In addition, Middleton and Strick (38) have demonstrated cerebellar–cortical connections that provide an anatomic substrate for a cerebellar–prefrontal cortex circuit in the pathophysiology of ADHD.

Several research studies have been focused on MPH effects in the CNS during childhood and adulthood exposure, despite the alterations in the dopaminergic function (39). Basic and clinical studies utilising psychomotor stimulants such as cocaine and amphetamine, with similar effects to the cellular and behavioural activities (17), have shown that MPH has psychomotor stimulant-like properties: it blocks the dopamine transporter, induces dose-dependent increases in dopamine levels in the brain pathways activated by drugs of abuse (8) and may produce long-term deficits in dopaminergic and serotonergic systems. These 'toxicities' may result from dopamine- and glutamate-generated ROS that inhibit mitochondrial function to further increased ROS and decreased ATP production (40–42). It is possible that MPH alone does not induce oxidative stress, but it induces an increase in extracellular catecholamine levels by affecting the nitrergic system via glutamatergic activity. In the CNS, nitric oxide has a role in nonsynaptic communication between glutamatergic and monoaminergic neurons (43). The oxidative stress, if not too severe, might also cause adaptation. There are several suggestions that mild oxidative stress upon astrocytes causes them to upregulate synthesis of nerve growth factors, which may then exert protective effects upon adjacent neurons (44). It is also well known that alterations in brain metabolism are related to various disorders. Energy impairment has been linked to neuronal death and neurodegeneration. We speculate that MPH may enhance ATP production by activating mitochondrial respiratory chain, possibly by neurotransmitters' reuptake and ionic gradient reestablishment. Consequently, more oxygen is consumed resulting in increased ROS production. However, it should be understood that the age of death of animals was different, an important limitation of the study.

Recently, our group demonstrated that, after chronic administration of MPH in the adult rats, the complexes I, II, III and IV were inhibited in the hippocampus, prefrontal cortex, striatum and cerebral cortex (45) and there were not alterations in the oxidative parameters in the same structures listed above (46). Nevertheless, it is imperative to note that MPH treatment in these studies was derived from 'healthy' animals. Thus, we may obtain different results using established animal models for ADHD. It is important to remember that some authors showed that the effects of psychostimulants in the SHR model on the behavioural alterations are controversial (47–49). Others researchers demonstrated that SHR is a good animal model to study several aspects of schizophrenia (49). These authors consider MPH as a psychostimulant that induces psychosis and an increase in oxidative stress and a possible relationship between them. The differences observed from one structure to another are owing to the different roles that each plays, as well as the antioxidant capacity and susceptibility that vary from one structure to another. This process may have happened because there were increases in the oxidative stress parameters and a decrease or a lack of increase of the antioxidant defense mechanisms. However, only the chronic treatment altered the energetic metabolism, more specifically the activity of complex II. Furthermore, as this stimulant has been largely indicated to treat both children and adults with ADHD, the present study also showed the importance of using the SHR animal model for studying the molecular effects of MPH. However, the present study showed the importance of more clear research on the use of MPH, mainly because this stimulant is also indicated for adults.

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