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Research Report

Brain apoptosis signaling pathways are regulated by methylphenidate treatment in young and adult rats

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ABSTRACT

Methylphenidate (MPH) is commonly prescribed for children who have been diagnosed with attention deficit hyperactivity disorder (ADHD); however, the action mechanisms of methylphenidate have not been fully elucidated. Studies have shown a relationship between apoptosis signaling pathways and psychiatric disorders, as well as in therapeutic targets for such disorders. So, we investigated if chronic treatment with MPH at doses of 1, 2 and 10 mg/kg could alter the levels of pro-apoptotic protein, Bax, anti-apoptotic protein, Bcl-2, caspase-3 and cytochrome c in the brain of young and adult Wistar rats. Our results showed that MPH at all doses increased Bax in the cortex; the Bcl-2 and caspase-3 were increased with MPH (1 mg/kg) and were reduced with MPH (2 and 10 mg/kg); the cytochrome c was reduced in the cortex after treatment with MPH at all doses; in the cerebellum there was an increase of Bax with MPH at all doses, however, there was a reduction of Bcl-2, caspase-3, and cytochrome c with MPH (2 and 10 mg/kg); in the striatum the treatment with MPH (10 mg/kg) decreased caspase-3 and cytochrome c; treatment with MPH (2 and 10 mg/kg) increased Bax and decreased Bcl-2 in the hippocampus; and the caspase-3 and cytochrome c were reduced in the hippocampus with MPH (10 mg/kg). In conclusion, our results suggest that MPH influences plasticity in the brain of young and adult rats; however, the effects were dependent of age and brain area, on the one hand activating the initial cascade of apoptosis, increasing Bax and reducing Bcl-2, but otherwise inhibiting apoptosis by reduction of caspase-3 and cytochrome c.

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1. Introduction

Methylphenidate (MPH) is widely used for children who have been diagnosed with attention deficit hyperactivity disorder (ADHD) (Challman and Lipsky, 2000). However, MPH treatment is also used for other indications; for example, MPH presented efficacy for apathy in Alzheimer disease (Drye et al., 2013) and prevented bulimia nervosa (Guerdjikova and McElroy, 2013). It is known that MPH blocks the dopamine transporter and increases the levels of extracellular dopamine (Carboni et al., 2003), beyond inhibits the norepinephrine transporter (Madras et al., 2005). However, the MPH mechanism of action is not fully elucidated yet. There are also other studies that show the molecular effects of MPH. Indeed, MPH treatment altered circadian clock gene product expression (Baird et al., 2013) and N-methyl-D-aspartate (NMDA) receptor composition synaptic plasticity (Urban et al., 2013). In addition, MPH has been shown to influence energy metabolism and oxidative stress (Gomes et al., 2009; Réus et al., 2013). It is well known that those alterations could lead to activation of apoptotic pathways and cell death (Hyman and Yuan, 2012). In fact, initial activation of caspases can be from plasma membrane upon ligation of death receptor or from mitochondrial damage (Tekpli et al., 2013).

The mitochondrial apoptotic pathway is initiated by release of apoptogenic factors, for example, the cytochrome c, which activates caspase-3 and initiates the apoptotic process (Saelens et al., 2004). On the other hand, there are neuroprotective factors, such as the Bcl-2 protein, that protect the cells against mitochondrial damage and inhibit apoptosis (Martinou et al., 1994). More recently, studies have pointed out an important role of caspases for the activation of mediating neuronal cell death and neuronal loss in neurodegenerative diseases, as well as mood disorders (Cui et al., 2012; Dygalo et al., 2012). However, there are few reports about the effects of MPH in apoptosis signaling pathways. So, the present study was aimed to evaluate the effects of chronic administration of MPH in pro-apoptotic protein, Bax, anti-apoptotic protein, Bcl-2, caspase-3 and cytochrome c in the cortex, cerebellum, striatum and hippocampus of young and adult Wistar rats. These brain areas were chosen because they participate in circuits involved in ADHD (Volkow et al., 2002; Li et al., 2007).

2. Results

2.1. *Effects of MPH on Bax, Bcl-2, caspase-3 and cytochrome c in young rats*

The treatment with MPH at all doses increased Bax levels (Fig. 1A; $F_{(3–15)}=7.404$; $p=0.005$) in the cortex. The Bcl-2 (Fig. 1A; $F_{(3–15)}=38.811$; $p<0.001$) and caspase-3 levels (Fig. 1A; $F_{(3–15)}=27.134$; $p<0.001$) were increased with MPH at the dose of 1 mg/kg, and were reduced with MPH at the doses of 2 and 10 mg/kg. The cytochrome c was reduced in the cortex after treatment with MPH at all doses in young rats (Fig. 1A; $F_{(3–15)}=19.478$; $p<0.001$).

In the cerebellum there was an increase of Bax levels with MPH at all doses (Fig. 1B; $F_{(3–15)}=6.821$; $p=0.006$), however, there was a reduction of Bcl-2 (Fig. 1B; $F_{(3–15)}=57.894$; $p<0.001$), caspase-3 (Fig. 1B; $F_{(3–15)}=15.630$; $p<0.001$) and cytochrome c (Fig. 1B; $F_{(3–15)}=19.646$; $p<0.001$) levels with MPH at the doses of 2 and 10 mg/kg in young rats.

In the striatum the treatment with MPH at the dose of 10 mg/kg decreased caspase-3 (Fig. 1C; $F_{(3–15)}=7.187$; $p=0.005$) and cytochrome c (Fig. 1C; $F_{(3–15)}=16.696$; $p<0.001$) levels. On the other hand the Bax (Fig. 1C; $F_{(3–15)}=0.934$; $p=0.454$) and Bcl-2 (Fig. 1C; $F_{(3–15)}=1.290$; $p=0.322$) levels did not alter in the striatum.

Treatment with MPH at the doses of 2 and 10 mg/kg increased Bax (Fig. 1D; $F_{(3–15)}=7.693$; $p=0.004$) and decreased Bcl-2 (Fig. 1D; $F_{(3–15)}=95.118$; $p<0.001$) levels in the hippocampus. The caspase-3 (Fig. 1D; $F_{(3–15)}=7.678$; $p=0.004$) and cytochrome c (Fig. 1D; $F_{(3–15)}=22.807$; $p<0.001$) levels were reduced in the hippocampus with MPH at the dose of 10 mg/kg.

2.2. *Effects of MPH on Bax, Bcl-2, caspase-3 and cytochrome c in adult rats*

In the cortex of adult rats there was an increase of Bax levels with MPH at all doses (Fig. 2A; $F_{(3–15)}=27.396$; $p<0.001$). The Bcl-2 (Fig. 2A; $F_{(3–15)}=28.766$; $p<0.001$) and caspase-3 (Fig. 2A; $F_{(3–15)}=11.262$; $p=0.001$) levels were increased with MPH at the dose of 1 mg/kg and reduced with MPH at the doses of 2 and 10 mg/kg. The cytochrome c was reduced in the cortex with MPH at all doses (Fig. 2A; $F_{(3–15)}=134.562$; $p<0.001$).

The Bax levels were increased (Fig. 2B; $F_{(3–15)}=177.188$; $p<0.001$), and the cytochrome levels were reduced (Fig. 2B; $F_{(3–15)}=70.985$; $p<0.001$) in the cerebellum after treatment with MPH at all doses. Also, in the cerebellum the Bcl-2 (Fig. 2B; $F_{(3–15)}=58.029$; $p<0.001$) and caspase-3 (Fig. 2B; $F_{(3–15)}=74.549$; $p<0.001$) levels were reduced after treatment with MPH at the doses of 2 and 10 mg/kg in adult rats.

In the striatum there was an increase of Bax levels (Fig. 2C; $F_{(3–15)}=30.979$; $p<0.001$) and a reduction of caspase-3 (Fig. 2C; $F_{(3–15)}=34.305$; $p<0.001$). The Bcl-2 (Fig. 2C; $F_{(3–15)}=26.383$; $p<0.001$) and cytochrome c (Fig. 2C; $F_{(3–15)}=34.254$; $p<0.001$) levels were reduced in the striatum after treatment with MPH at the doses of 2 and 10 mg/kg.

In adult rats there was an increase of Bax (Fig. 2D; $F_{(3–15)}=23.476$; $p<0.001$) levels at all doses in the hippocampus. On the other hand, the Bcl-2 (Fig. 2D; $F_{(3–15)}=18.289$; $p<0.001$), caspase-3 (Fig. 2D; $F_{(3–15)}=18.632$; $p<0.001$) and cytochrome c (Fig. 2D; $F_{(3–15)}=69.707$; $p<0.001$) levels were reduced in the hippocampus after treatment with MPH at the doses of 2 and 10 mg/kg.

3. Discussion

It is well known that apoptosis signaling pathways are involved with neurodegenerative diseases and mood disorders, as well as therapeutic targets for such diseases (Leonard and Maes, 2012; Hu et al., 2013). However, few studies have reported the role of these pathways in ADHD or with the classic drug for the treatment of ADHD, MPH. As the use of

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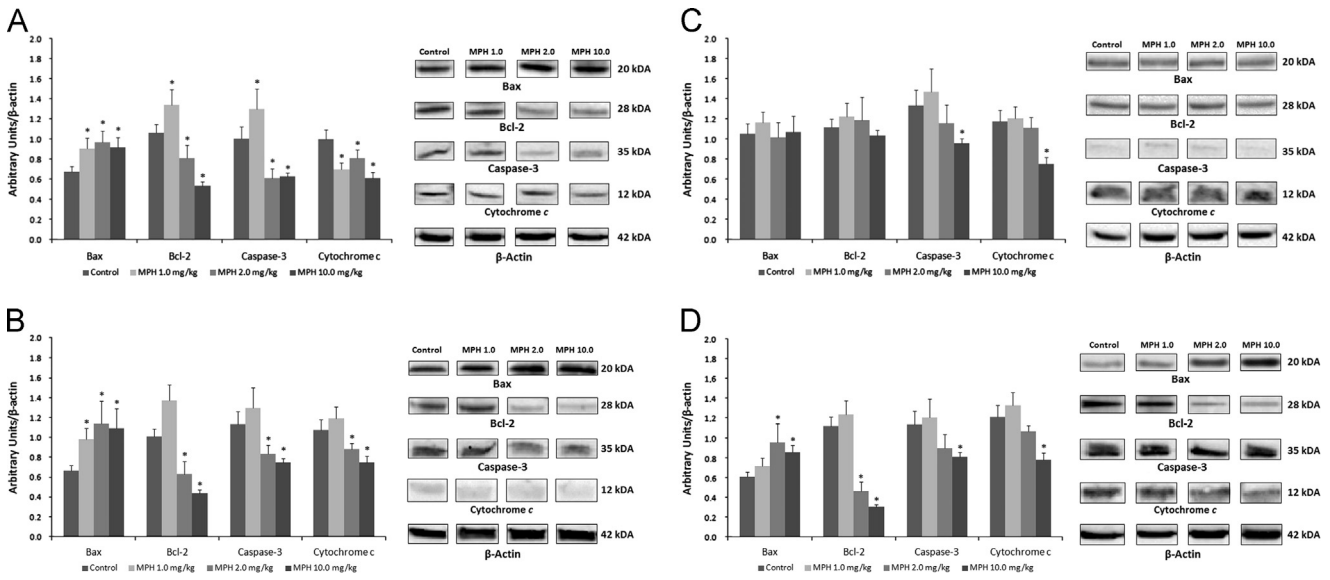

Fig. 1 – Effects of chronic administration of MPH on Bax, Bcl-2, Caspase-3 and Cytochrome c levels in the cortex (A), cerebellum (B), striatum (C), and hippocampus (D) of young rats. Bars represent means ± standard deviation ($n=4$). $^{*}P<0.05$ compared to saline group according to ANOVA followed by Tukey post-hoc test.

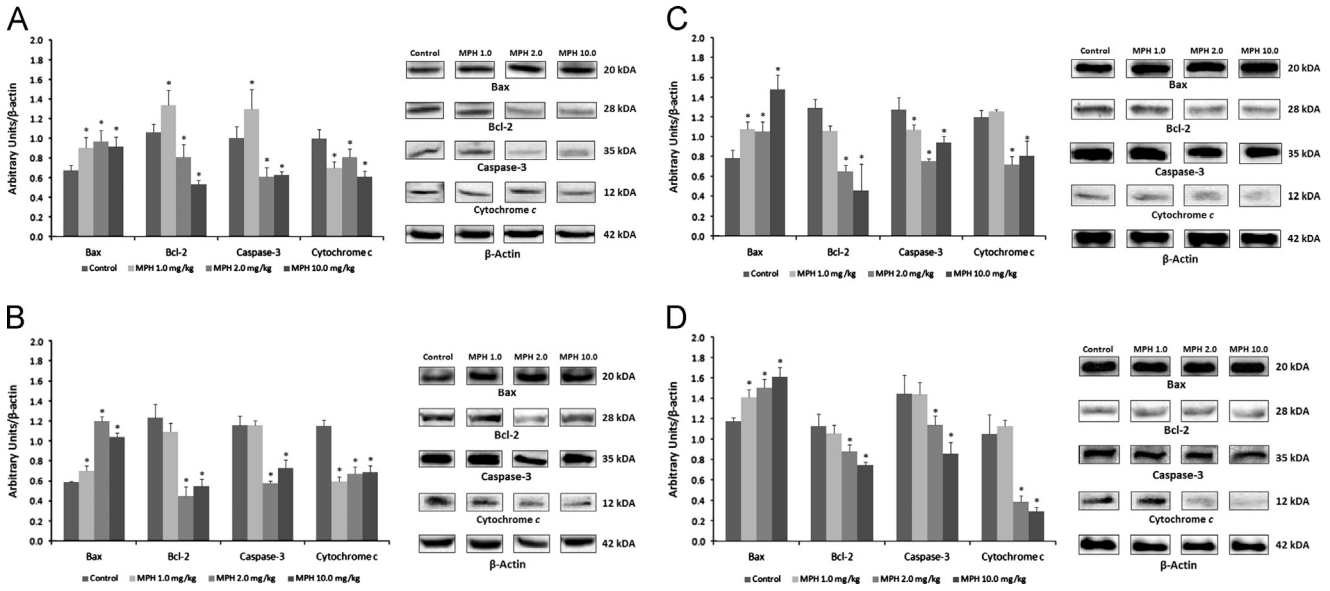

Fig. 2 – Effects of chronic administration of MPH on Bax, Bcl-2, Caspase-3 and Cytochrome c levels in the cortex (A), cerebellum (B), striatum (C), and hippocampus (D) of adult rats. Bars represent means ± standard deviation ($n=4$). $^{*}P<0.05$ compared to saline group according to ANOVA followed by Tukey post-hoc test.

this drug has dramatically increased in the last years, so, investigations are necessary to explore the potential toxicity of MPH, notably, in early stages in brain development, as well as in mature brain (Rowland et al., 2002; Husson et al., 2004). Additionally, exposure to MPH impairs long-term memory for inhibitory avoidance training and facilitated acquisition in the step down inhibitory avoidance (CMIA) in both young and adult rats (Gomes et al., 2010). Still, treatment with MPH during adolescence period led to changes in adulthood, such as depressive-like behavior and alterations on locomotor habituation (Carlezon et al., 2003; Gomes et al., 2011). In the present study we reported that the chronic treatment with MPH (1.0, 2.0 or 10.0 mg/kg) in the brain of young or adult rats led to alterations of protein involved with apoptosis signaling pathways. The doses of MPH in this study were chosen based on previous studies that showed behavioral and biochemical changes with these doses (Souza et al., 2008; Gomes et al., 2009, 2010, 2011; Réus et al., 2013). In addition, previous studies on pharmacokinetic demonstrated that plasma levels are obtained in rats after oral or intraperitoneal (IP) administration of MPH in low doses (1.0–3.0 mg/kg); for example, IP administration of MPH at the doses of 0.25 and 0.5 mg/kg resulted in plasma levels of 16 ± 3 and 36 ± 11 ng/mL, respectively; 5 min after the administration (Kuczenski and Segal, 1997; Berridge et al., 2006), similar to clinic, where psychostimulants administrated orally exert therapeutic action at

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low doses when plasma concentrations are within the range of 8–40 ng/mL (Swanson and Volkow, 2006), suggesting that when administered IP or orally at these doses in the rat, maximal concentrations of plasma MPH fall within the range associated with clinical efficacy (Berridge et al., 2006).

Cellular apoptosis can be triggered by mitochondrial abnormalities or oxidative stress (Marazziti et al., 2012). In the present study we evaluated the action of MPH on apoptosis signaling protein two hours after the last injection in chronic treatment, and the half-life of MPH is about 3–5 h. Although this may be a limitation, we believe that effects of MPH are due to the chronic treatment. Previous studies have reported that acute treatment with MPH led to increase in production of superoxide in submitochondrial particles in young rats; on the other hand, chronic treatment with MPH reduced production of superoxide in adult rats (Gomes et al., 2009). In addition, MPH treatment in young, but not adult rats caused oxidative damage in the brain (Martins et al., 2006; Schmitz et al., 2012). MPH has also been shown to alter energy metabolism, including alterations of creatine kinase, an enzyme important for normal energy homeostasis (Scaini et al., 2008), in enzymes of Krebs cycle, such as citrate synthase and isocitrate dehydrogenase (Réus et al., 2013), and in mitochondrial respiratory chain enzymes Fagundes et al., 2007) were reported. The changes caused by MPH can be, at least in part, by its blockade in dopamine transporter and by increase of dopamine levels in the brain areas, which are also activated by abused drugs(Kuczenski and Segal, 1997; Gerasimov et al., 2000). In fact, the excess in dopamine could lead to reactive oxygen species (ROS) production, which in turn directs to mitochondrial dysfunction and decrease in ATP production (Berman and Hastings, 1999; Page et al., 2001; Virmani et al., 2002; Cabezas et al., 2012). So, the impairment in energy metabolism could activate apoptosis signaling proteins, as reported in the present study. Really, MPH treatment in the present work increased the Bax levels in both young and adult rats; however, there were related alterations in all brain areas analyzed in adults: cortex, cerebellum, striatum and hippocampus, and in young rats no changes in Bax levels in the striatum were observed, as Bax is associated with the onset of the apoptotic signaling cascade which we suggest as a potential neurotoxicity effect by MPH. In fact, very recently it was shown that MPH administrated to spontaneous hypertensive rats (SHR) altered the expression of genes associated with apoptosis, cell adhesion and mitochondrial organization, beyond to alterations on neural plasticity (dela Peña et al., 2014), suggesting that these alteration may reflect to the cognitive impairment associated with chronic methylphenidate use as demonstrated in preclinical studies. Really, chronic administration of MPH in doses that span the therapeutic window (1 and 10 mg/kg) in mice induced neuroplasticity impairment, reducing expression of neurotrophic factors and basal ganglia (SNpc) dopamine neurons, and activating microglia (Sadasivan et al., 2012). The microglia is now known with an important role in regulating neural development by controlling neuronal numbers during early postnatal (and possibly embryonic) development (Eyo and Dailey, 2013). Thus, it is possibly the effects of MPH in inducing apoptosis which may be mediated by microglia activation.

Bax is one proapototic protein, which, together with antiapototic proteins, such as Bcl-2 controlled apoptotic process (Cory et al., 2003; Hyman and Yuan, 2012). In the present findings we showed that MPH treatment at low dose, 1 mg/kg, increased Bcl-2 in the cortex; however, MPH at the doses of 2 and 10 mg/kg reduced Bcl-2 in the brain of both young and adult rats; this effect induced by MPH on Bcl-2 may be related to a compensatory mechanism due the Bax elevation to protect neuronal death. A previous study related that MPH-induced autophagy was regulated by Bcl-2/Beclin1 complex, and also promoted caspase activity and suppression of autophagy accelerated by METH-induced apoptosis (Ma et al., 2014). It is reported that an increase in the relation of antiapototic vs. proaptotic proteins in outer mitochondrial membrane is linked with susceptibility to apoptosis and cell death (Lindsten et al., 2005). This imbalance can lead to cytochrome c release from mitochondria (Lakhani et al., 2006). Cytochrome c then promotes activation of caspase 3, which directs to cell death (Widlak and Garrard, 2005). In the present research, we have not shown an increase in cascades that follow for the apoptotic events. Indeed, with the exception of MPH treatment at the dose of 1 mg/kg, which increased the caspase-3 in the cortex of young and adult rats, all other doses of MPH decreased caspase-3 and cytochrome c in both young and adult rats. These results can be explained, at least in part, by the fact that in a previous study it was shown that MPH-induced reversible astrocytic activation, so it presents the absence in neurotoxicity (Suzuki et al., 2007). In fact, it was reported that the neuronal cell death in the brain is caused by excessive astrocytic activation (Takuma et al., 2004). Moreover, similar to the present study, Husson et al. (2004) reported that MPH displayed both neurotoxic and neuroprotective effects in neonatal brain of rodents, suggesting that MPH presents another action mechanism beyond its effects on the dopaminergic system, since alterations in dopaminergic neurotransmission did not result in apoptosis. In fact, more recently, have been shown that MPH facilitates NMDA receptor mediated synaptic transmission via sigma 1 receptor, and such facilitation requires PLC/IP3/PKC signaling pathway, which can explain MPH-induced addictive potential and other psychiatric adverse effects (Zhang et al., 2012). However, it is important to detach that the therapeutic doses of MPH in children with ADHD reduces the risk of substance abuse (Biederman et al., 1999). In addition, Andersen et al. (2002) related that rats exposed to MPH spent less time in environments associated with a moderate dose of cocaine (10 mg/kg), suggesting aversion to cocaine. The effect of MPH induces sensitization to substance abuse; it seems related to the developmental age and gender. Indeed, repeated administration of either 10 or 20 mg/kg of MPH produced behavioral sensitization in young, adolescent, and adult rats when assessed by stereotyped behavior, and when rats were challenged with cocaine at postnatal day (PND) 90 gender differences in locomotion and in stereotyped behavior during the cocaine challenge were observed (Torres-Reverón and Dow-Edwards, 2005). Other study related that acute treatment with MPH at the dose of 1 mg/kg decreased the surface and total protein levels of NMDA receptor subunits NR1 and NR2B in juvenile rat prefrontal cortical neurons, suggesting a role of MPH in glutamatergic receptor-mediated synaptic

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plasticity; for example, MPH increased the probability and magnitude in long-term potentiation (LTP) induction and had small effect in long-term depression (LTD) induction, which are events important in memory consolidation (Urban et al., 2013). The injection of a peptide inhibitor of caspases (z-VAD.fmk) reduced caspase activation and tissue damage, beyond the reduction of excitotoxity induced by NMDA receptor (Yuan, 2009). Therefore, we suggest, at least in part, that the inhibition of caspase-3 in our findings is by the action of MPH in NMDA receptor. Interestingly, it was showed that a low level of caspase-3 activation, as reported in the present data, cytochrome c release, and proapoptotic proteins, Bax and Bad, are necessary for some synaptic changes that lie behind memory (Li et al., 2010; Jiao and Li, 2011), suggesting that effects of MPH in LTP and LTD, as reported previously by Urban et al. (2013), might be through MPH effects in apoptosis signaling pathways.

In conclusion, this is a first study to show the effects of MPH treatment proteins involved with apoptosis signaling pathways, and that, on the one hand, MPH presented neurotoxic effects, as reported by activating the apoptotic cascade by increasing Bax and decreasing Bcl-2. In fact, MPH induced neurotoxic effects, including damage to dopaminergic and serotoninergic terminals, and endoplasmatic reticulum release (Kaushal and Matsumoto, 2011). However, otherwise, may be to a compensatory mechanism MPH showed a neuroprotective effect by reducing cytochrome c and caspase-3, suggesting that MPH influences plasticity in the brain of young and adult rats.

4. Experimental procedure

4.1. Animals

Young (25 days old) and adult (60 days old) Wistar rats were housed five in a cage with food and water available ad libitum and were maintained in a 12-h light/dark cycle (lights on at 7:00 a.m.). The total numbers of young rats were 64 and adult rats were 64 for chronic treatment and all biochemical analysis. In vivo studies were performed in accordance with the National Institutes of Health guidelines and with the approval of the ethics committee from Universidade do Extremo Sul Catarinense (UNESC) under protocol number 35/2012.

4.2. Treatment

Methylphenidate HCl (Ritalin, Novartis: dissolved in saline solution) at the dose of 1.0, 2.0 or 10.0 mg/kg or saline injections (control group) was administered intraperitoneal to young rats starting on PD 25 once a day for 28 days (last injections on PD 53). The same treatment was given to adult rats starting on PD 60 once a day for 28 days (last injection on PD 88) according to previous studies (Souza et al., 2008; Gomes et al., 2010, 2011). After the chronic treatment, the rats were sacrificed by decapitation two hours after the last injection. Cerebellum, striatum, prefrontal cortex, hippocampus and cerebral cortex (total cortex without prefrontal cortex) were quickly isolated by hand dissection using a magnifying glass and a thin brush and were immediately dissected and stored at −70 °C. The dissection was based on the histological distinctions described by Paxinos and Watson (1986).

4.3. Immunoblotting

To perform the immunoblot experiments, the samples were first homogenized in Laemmli-sample buffer (62.5 mM Tris–HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol). Equal amounts of protein (30 μg/well) were fractionated by 10–15% SDS-polyacrylamide gel and electro-blotted onto nitrocellulose membranes. The protein loading and electro-blotting efficiency were verified with Ponceau S staining. The membranes were blocked in Tween–Tris buffered saline (TTBS: 100 mM Tris–HCl, pH 7.5, containing 0.9% NaCl and 0.1% Tween-20) containing 5% albumin. The membranes were incubated overnight at 4 °C with a rabbit polyclonal antibody against Bcl-2 Antibody (Cell Signaling – 2876), Bax (Cell Signaling – 2772), caspase 3 (Cell Signaling – 9662). The primary antibody was then removed and the membranes were washed 4 times for 15 min. After washing, an anti-rabbit IgG peroxidase linked secondary antibody was incubated with the membranes for 1 h (1:10,000 dilution) and the membranes were washed again. Finally, the immunoreactivity was detected using an enhanced chemiluminescence ECL Plus kit. After exposure, the membranes were then stripped and incubated with a mouse monoclonal antibody to β-Actin (sigma – A2228) in the presence of 5% milk. An anti-mouse IgG peroxidase linked secondary antibody was incubated with the membranes for 1 h (1:10,000 dilution) and the membranes were washed again. The immunoreactivity was detected using an enhanced chemiluminescence ECL Plus kit. Densitometry was performed using Image J v.1.34 software. SeeBlue® Plus2 Prestained Standard (Invitrogen) was used as a molecular weight marker to make sure that the correct bands were analyzed for Bax, Bcl-2, caspase-3 and β-Actin.

4.4. Cytochrome c immunocontent measurement

Mitochondria were isolated from the forebrain as described by Rosenthal et al. (1987). The cerebellum, hippocampus, striatum and cerebral cortex were cut into small pieces using surgical scissors, extensively washed and then manually homogenized in a Dounce homogenizer using both a loose-fitting and a tight-fitting pestle. The homogenate was centrifuged for 3 min at 2000*g*. After centrifugation, the supernatant was again centrifuged for 8 min at 12,000*g*. The pellet was resuspended in isolation buffer containing 10 μL of 10% digitonin and recentrifuged for 8 min at 12,000*g*. The supernatant was discarded and the final pellet was gently washed and resuspended in isolation buffer devoid of EGTA. Thirty micrograms of mitochondrial protein was fractionated by 10% SDS-polyacrylamide gel and electro-blotted onto nitrocellulose membranes. The protein loading and electro-blotting efficiency were verified with Ponceau S staining. The membranes were blocked in Tween–Tris buffered saline (TTBS: 100 mM Tris–HCl, pH 7.5, containing 0.9% NaCl and 0.1% Tween-20) containing 5% albumin. The membranes were incubated overnight at 4 °C with

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a rabbit monoclonal antibody against cytochrome C Antibody (ab110325). The primary antibody was then removed and the membranes were washed 4 times for 15 min. After washing, an anti-rabbit IgG peroxidase linked secondary antibody was incubated with the membranes for 1 h (1:10,000 dilution) and the membranes were washed again. Finally, the immunoreactivity was detected using an enhanced chemiluminescence ECL Plus kit. After exposure, the membranes were then stripped and incubated with a mouse monoclonal antibody to β-Actin (sigma – A2228) in the presence of 5% milk. An anti-mouse IgG peroxidase linked secondary antibody was incubated with the membranes for 1 h (1:10,000 dilution) and the membranes were washed again. The immunoreactivity was detected using an enhanced chemiluminescence ECL Plus kit. Densitometry was performed using Image J v.1.34 software. SeeBlue® Plus2 Prestained Standard (Invitrogen) was used as a molecular weight marker to make sure that the correct bands were analyzed for cytochrome C and β-Actin.

4.5. *Statistical analysis*

Evaluation of study variables showed that normal distribution parametric tests would be most appropriate. All analyses were performed using the Statistical Package for the Social Science (SPSS) software version 16.0. All data are presented as mean ± standard deviation. Differences among experimental groups in the assessment of citrate synthase, malate dehydrogenase and isocirate dehydrogenase were determined by one-way ANOVA, followed by the Tukey post-hoc test when ANOVA was significant; P values <0.05 were considered to be statistically significant.

Author contributions

E.L.S, G.Z.R, G.S, and J.Q. designed, organized and coordinated the study. G.C.J, C.B.F, L.M.M, G.S, and M.O.S.M. participated in experimental protocols, and biochemical analysis. E.L.S, G.Z.R, G.S, and J.Q., contributed to data analysis, interpretation of data and wrote the manuscript.

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