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Effects of acute administration of mazindol on brain energy metabolism in adult mice

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Cinara Ludvig Gonçalves[1,2,3], Giselli Scaini[1,2,3], Gislaine Tezza Rezin[1,2,3], Isabela Casagrande Jeremias[1,2,3], Gisele Daiane Bez[1,2,3], Juliana Felipe Daufenbach[1,2,3], Lara Mezari Gomes[1,2,3], Gabriela Kozuchovski Ferreira[1,2,3], Alexandra Ioppi Zugno[2,4], Emilio Luiz Streck[1,2,3]

[1]Laboratório de Bioenergética, Programa de Pós-graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil; [2]Instituto Nacional de Ciência e Tecnologia Translacional em Medicina, Porto Alegre, RS, Brazil; [3]Núcleo de Excelência em Neurociências Aplicadas de Santa Catarina (NENASC), Florianópolis, SC, Brazil; and [4]Laboratório de Neurociências, Programa de Pós-graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil

Objectives: Mazindol is a sympathomimetic amine, widely used as an anorectic agent in the treatment of obesity. This drug causes psychostimulant effects because of its pharmacological profile similar to amphetamine, acting like a monoamine reuptake inhibitor. However, the mechanisms underlying the action of mazindol are still not clearly understood.
Methods: Swiss mice received a single acute administration of mazindol (0.25, 1.25 and 2.5 mg/kg, ip) or saline. After 2 h, the animals were killed by decapitation; the brain was removed and used for the evaluation of activities of mitochondrial respiratory chain complexes, Krebs cycle enzymes and creatine kinase.
Results: Acute administration of mazindol decreased complex I activity only in the hippocampus. Complex IV activity was increased in the cerebellum (2.5 mg/kg) and cerebral cortex (0.25 mg/kg). Citrate synthase activity was increased in the cerebellum (1.25 mg/kg) and cerebral cortex (1.25 mg/kg), and creatine kinase activity was increased in the cerebellum (1.25 mg/kg).
Conclusion: We suggest that the inhibition of complex I in the hippocampus only and activation of complex IV, citrate synthase and creatine kinase occurs because of a stimulus effect of mazindol in the central nervous system, which causes a direct impairment on energy metabolism.

Keywords: brain, creatine kinase, krebs cycle, mazindol, mitochondrial respiratory chain

Prof. Emilio L. Streck, Laboratório de Bioenergética, Universidade do Extremo Sul Catarinense, Av. Universitária, 1105, Criciúma, 88806-000 SC, Brazil.
Tel: +55 48 3431 2539;
Fax: +55 48 3431 2644;
E-mail: emiliostreck@gmail.com

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Significant Outcomes

- Acute administration of mazindol affects proper functioning of the mitochondrial respiratory chain by decreasing complex I activity and increasing complex IV activity.
- The effect of acute administration of mazindol is very diverse and depends on the dose and brain area.

Limitations

- The effect of chronic administration of mazindol on brain energy metabolism should also be evaluated.
- The effect of mazindol in an animal model of obesity could also be studied.

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Introduction

Obesity has emerged as one of the principal health concerns throughout the world in modern age. According to the World Health Organization, there were about 1.6 billion overweight youngsters aged 15 years and at least 400 million adults were obese worldwide in 2005 (1). There is a range of comorbidities associated with obesity that include diabetes mellitus type 2, cardiovascular disease, hepatic steatosis, Alzheimer's disease, chronic kidney disease and certain cancers (2–4). Basically, obesity results from an imbalance between energy intake and energy expenditure. A negative energy balance is therefore essential for the treatment of obese patients to prevent such complications. Drugs can reduce food intake, alter metabolism and/or increased energy expenditure (5). Anti-obesity pharmacological treatment is indicated when body mass index (BMI) is over 30 kg/m^2, or when morbidities are associated with overweight (BMI over 25 kg/m^2), and dieting, physical activities and behavioural changes have been proved unsuccessful (6).

Mazindol [5-(p-chlorophenyl)-2,4-dihydro-3H-imidazo(2,1-a)isoindol-5-ol] is an imidazo-isoindol derivative and chemically a non-amphetamine, tricyclic compound (7), with anorectic action, which suppresses feeding in both humans (8) and animals (9) by stimulating catecholaminergic systems (10). Mazindol is prescribed to reduce appetite and has been reported to be effective for obesity (11,12). The consumption of mazindol has reached considerable levels in several countries. In Japan, mazindol is the only drug approved for long-term use in the treatment of obese individuals (13). However, the use of anti-obesity drugs to promote weight loss is widespread in the world, and obesity specialists frequently prescribe medicines in doses and for durations previously unreported in the literature. Mazindol has been developed as an anorectic drug as a short-term treatment for exogenous obesity, in combination with a diet in patients with risk factors, such as hypertension, diabetes or hyperlipidemia, demonstrating safety and efficacy (14,15); however, long-term studies in obesity treatment are absent for other drugs. For example, the efficacy and safety of obesity pharmacotherapy among the elderly is unknown.

Tissues with high-energy demands, such as the brain, contain a large number of mitochondria, therefore being more susceptible to reduction of the aerobic metabolism. Mitochondria are intracellular organelles that play a crucial role in adenosine triphosphate (ATP) production (16). Most of the cell energy is obtained by mitochondrial oxidative phosphorylation through a series of reactions in which electrons liberated from reducing substrates NADH and FADH are delivered to O_2 via a chain of respiratory proton pumps (17,18). Citrate synthase, malate dehydrogenase and succinate dehydrogenase are key enzymes involved in the Krebs cycle. Citrate synthase is the first step of the Krebs cycle, localised within cells in the mitochondrial matrix, and it catalyses the condensation of the oxaloacetate and acetyl groups of acetyl coenzyme-A. This enzyme has been used as a quantitative enzyme marker of mitochondria viability. Malate dehydrogenase catalyses the dehydrogenation of L-malate to oxaloacetate in the final step of Krebs cycle (19). Succinate dehydrogenase is one of the most reliable markers of the mitochondrial capability to supply an adequate amount of ATP, as it is part of both the Krebs cycle and the respiratory chain (complex II) (20). The mitochondrial respiratory chain is located in a special structure of the inner mitochondrial membrane. In most organisms, the mitochondrial respiratory chain is composed of four complexes. The electron transport couples with translocation of protons from the mitochondrial matrix to the intermembrane space. The generated proton gradient is used by ATP synthase to catalyse the formation of ATP by the phosphorylation of ADP (17). Another form of ATP production is through creatine kinase that acts in the brain and other tissues with high and variable rates of ATP metabolism (21–23).

Mitochondrial dysfunction has been implicated in a variety of diseases ranging from neurodegenerative diseases to diabetes and obesity. Obesity takes place in disorders that affect mitochondrial metabolism, which favours reactive oxygen species generation and oxidative stress (24,25). On the other hand, another mechanism has been proposed that involves an effect of high triglycerides on the functioning of the mitochondrial respiratory chain, in which intracellular triglycerides inhibit translocation of adenine nucleotides and promote the generation of superoxide (26,27). In addition, ultrastructural abnormalities of mitochondria have been documented in fatty hepatocytes, in the absence of overt hepatic inflammation or necrosis (28–30). Several investigators demonstrated that fatty liver mitochondria have ultrastructural abnormalities, including swelling, intramitochondrial crystalline inclusions and distorted cristae (28,29). There was also some evidence for increased rates of mitochondrial O_2 consumption (30,31).

Considering that anti-obesity drugs may modulate energy metabolism and that effects of mazindol on energy metabolism are still not clearly understood. In the present study, we evaluated the activities of enzymes of Krebs cycle, mitochondrial respiratory chain complexes and creatine kinase in the brain of adult mice subjected to acute administration of mazindol.

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Materials and methods

Animals

A total of 24 adult male Swiss mice (90 days old) weighing 40–45 g were obtained from Central Animal House of the Universidade do Extremo Sul Catarinense. They were caged in groups of five with free access to food and water and were maintained on a 12-h light–dark cycle (lights on 07:00 a.m.), at a temperature of 23 ± 1°C. All experimental procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior recommendations for animal care, with the approval of the Ethics Committee from Universidade do Extremo Sul Catarinense.

Drugs

Animals received a single acute administration of mazindol (commercial name Fagolipo®, LIBBS) dissolved in 0.9% saline (w/v) (0.25, 1.25 and 2.5 mg/kg). All the doses of mazindol were administered at a volume not exceeding 1 ml/100 g body weight of mice. Control mice received an equivalent volume of saline. The selection of this regimen was based on previous studies showing important neurochemical and anorectic effects for this drug (32). Two hours after the injection, mice were killed by decapitation, the brain was removed and the cerebral cortex, hippocampus, cerebellum and striatum were isolated and homogenised. The activities of mitochondrial respiratory chain complexes (complex I, II, II–III, IV), citrate synthase, succinate dehydrogenase, malate dehydrogenase and creatine kinase were measured.

Tissue and homogenate preparation

Two hours after the injection, the mice were killed by decapitation, the brain was removed and the hippocampus, striatum, cerebellum and cerebral cortex were homogenised (1:10, w/v) in SETH buffer, pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 IU/ml heparin). The homogenates were centrifuged at $800 \times \boldsymbol{g}$ for 10 min and the supernatants kept at –70°C until used for enzyme activity determination. The maximum period between homogenate preparation and enzyme analysis was always <5 days. Protein content was determined by the method described by Lowry et al. (33) using bovine serum albumin as standard.

Activities of Krebs cycle enzymes

Citrate synthase activity: citrate synthase activity was assayed according to the method described by Srere (34). The reaction mixture contained 100 mM Tris, pH 8.0, 100 mM acetyl CoA, 100 mM 5,5'-dithiobis-(2-nitrobenzoic acid), 0.1% triton X-100, and 2–4 μg supernatant protein and it was initiated with 100 μM oxaloacetate and monitored at 412 nm for 3 min at 25°C.

Malate dehydrogenase activity: malate dehydrogenase was measured as described by Kitto (19). Aliquots (20 μg protein) were transferred into a medium containing 10 mM rotenone, 0.2% Triton X-100, 0.15 mM NADH and 100 mM potassium phosphate buffer, pH 7.4. The reaction was started by addition of 0.33 mM oxaloacetate and absorbance was monitored at 340 nm for 5 min at 37°C.

Succinate dehydrogenase activity: succinate dehydrogenase activity was determined according to the method of Fischer et al. (35), measured by following the decrease in absorbance owing to reduction of 2,6-di-chloro-indophenol (2,6-DCIP) at 600 nm with 700 nm as reference wavelength ($\varepsilon = 19.1\ mM^{-1}cm^{-1}$) in the presence of phenazine methosulphate (PMS). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate and 8 μM 2,6-DCIP was pre-incubated with 40–80 μg homogenate protein at 30°C for 20 min. Subsequently, 4 mM sodium azide, 7 μM rotenone and 40 μM 2,6-DCIP were added and the reaction was initiated by addition of 1 mM PMS and was monitored for 5 min.

Activities of mitochondrial respiratory chain enzymes

NADH dehydrogenase (complex I) was evaluated according to the method described by Cassina and Radi (36) by the rate of NADH-dependent ferricyanide reduction at 420 nm. The activities of succinate: DCIP oxidoreductase (complex II) and succinate: cytochrome *c* oxidoreductase (complex II–III) were determined according to the method of Fischer et al. (35). Complex II activity was measured by following the decrease in absorbance owing to the reduction of 2,6-DCIP at 600 nm. Complex III activity was measured by cytochrome *c* reduction from succinate. The activity of cytochrome *c* oxidase (complex IV) was assayed according to the method described by Rustin et al. (37), measured by following the decrease in absorbance owing to the oxidation of previously reduced cytochrome *c* at 550 nm. The activities of the mitochondrial respiratory chain complexes were expressed as nmol/min/mg/protein.

Creatine kinase activity

Creatine kinase activity was measured in brain homogenates pretreated with 0.625 mM lauryl maltoside. The reaction mixture consisted of 60 mM Tris–HCl, pH 7.5, containing 7 mM phosphocreatine,

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9 mM $MgSO_4$ and ~0.4–1.2 μg protein in a final volume of 100 μl. After 15 min of pre-incubation at 37°C, the reaction was started by the addition of 3.2 mmol of ADP plus 0.8 mmol of reduced glutathione. The reaction was stopped after 10 min by the addition of 1 μmol of p-hydroxymercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Hughes (38). The colour was developed by the addition of 100 μl 2% α-naphthol and 100 μl 0.05% diacetyl in a final volume of 1 ml and read spectrophotometrically after 20 min at 540 nm. Results were expressed as units/min × mg protein.

Statistical analysis

These data were expressed as mean ± standard deviation (SD). All of the assays of biochemical analysis were performed in duplicate, and the mean was used for statistical analysis. Because the variables being analysed did not follow a normal distribution and its variance does not fulfil the assumption of homoscedasticity, they were analysed by a non-parametric test (Kruskal–Wallis test). The differences within the individual groups were analysed by Tukey's HSD *post-hoc* tests. The differences between the groups were considered significant at $p < 0.05$. All of the analyses were carried out on an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software (Armonk, New York, USA).

Results

In the present study, we investigated the effect of acute administration of mazindol on the activities of several enzymes involved in energy metabolism in homogenates from the cerebellum, hippocampus, striatum and cerebral cortex from the brain of mice. Our results showed that mazindol decreased complex I activity (Fig. 1a) in the hippocampus at all doses investigated: 0.25 mg/kg ($p = 0.034$); 1.25 mg/kg ($p = 0.014$) and 2.5 mg/kg ($p = 0.011$); the cerebellum, striatum and cerebral cortex were not affected. On the other hand, there was an increase in complex IV activity (Fig. 1d) in the cerebellum (2.5 mg/kg; $p = 0.010$) and cerebral cortex (0.25 mg/kg; $p = 0.016$). However, complexes II (Fig. 1b) and II–III activities (Fig. 1c) were not affected by mazindol. Furthermore, citrate synthase activity was increased in the cerebellum (1.25 mg/kg; $p = 0.005$) and cerebral cortex (1.25 mg/kg, $p = 0.004$; Fig. 2). A similar effect occurred in creatine kinase activity, which was increased only in the cerebellum (1.25 mg/kg, $p = 0.004$; Fig. 3). On the other hand, the acute administration of mazindol did not affect malate

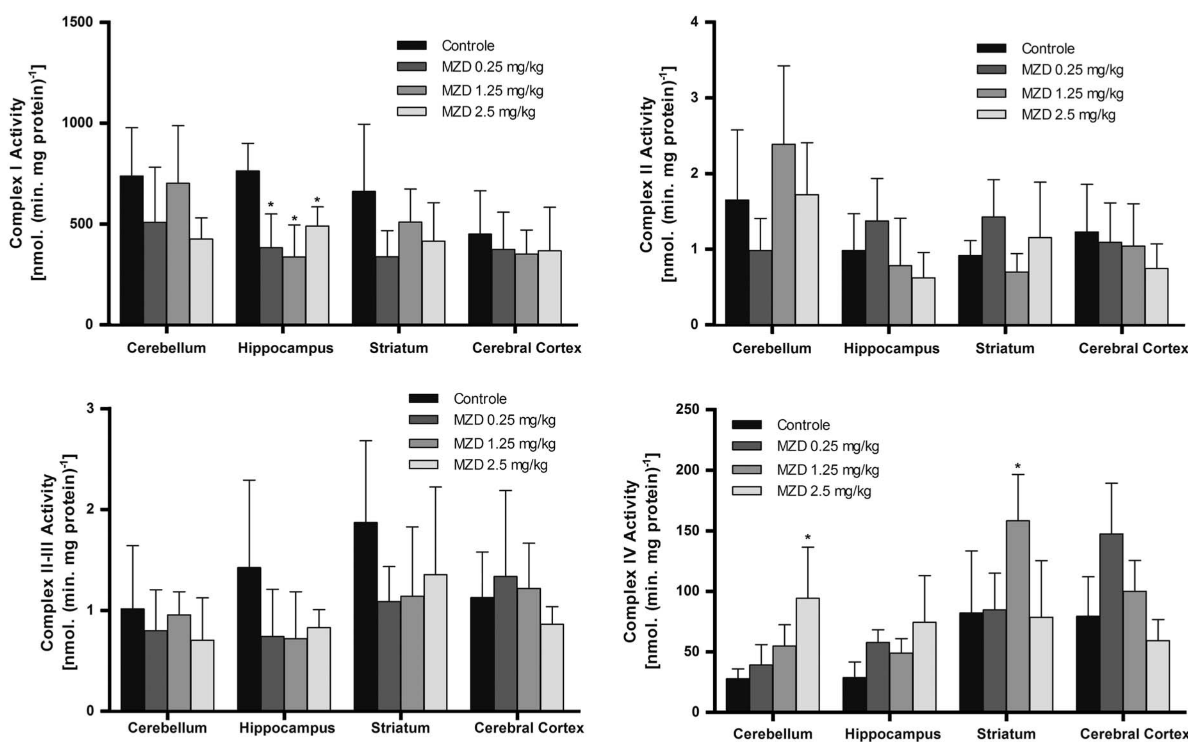

Fig. 1. Complex I activity (a), complex II activity (b), complex II–III activity (c) and complex IV activity (d) after acute administration of mazindol in the cerebellum, hippocampus, striatum and cerebral cortex of mice. Values are expressed as mean ± SD ($n = 6$). Different from control; $*p < 0.05$ (Kruskal–Wallis test followed by Tukey).

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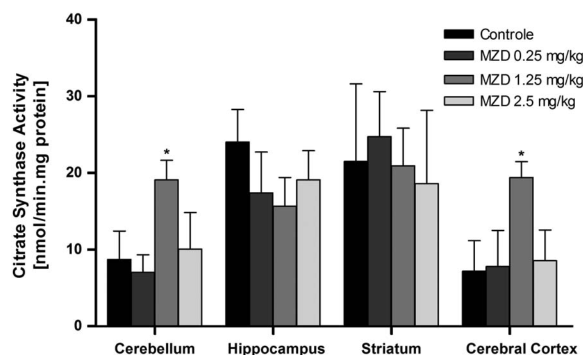

Fig. 2. Citrate synthase activity after acute administration of mazindol in the cerebellum, hippocampus, striatum and cerebral cortex of mice. Values are expressed as mean ± SD ($n = 6$). Different from control; $*p < 0.05$ (Kruskal–Wallis test followed by Tukey).

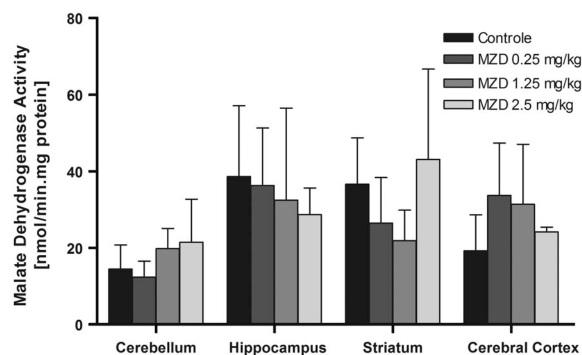

Fig. 4. Malate dehydrogenase activity after acute administration of mazindol in the cerebellum, hippocampus, striatum and cerebral cortex of mice. Values are expressed as mean ± SD ($n = 6$). Different from control; $*p < 0.05$ (Kruskal–Wallis test followed by Tukey).

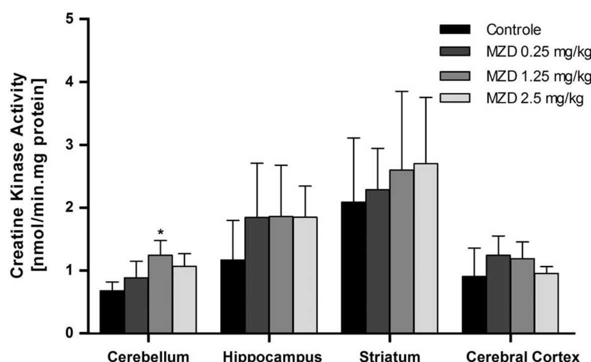

Fig. 3. Creatine kinase activity after acute administration of mazindol in the cerebellum, hippocampus, striatum and cerebral cortex of mice. Values are expressed as mean ± SD ($n = 6$). Different from control; $*p < 0.05$ (Kruskal–Wallis Test followed by Tukey).

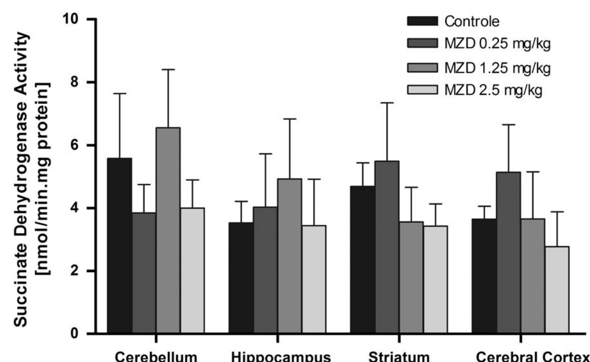

Fig. 5. Succinate dehydrogenase activity after acute administration of mazindol in the cerebellum, hippocampus, striatum and cerebral cortex of mice. Values are expressed as mean ± S.D. ($n = 6$). Different from control; $*p < 0.05$ (Kruskal–Wallis test followed by Tukey).

dehydrogenase (Fig. 4) and succinate dehydrogenase activities (Fig. 5).

Discussion

Obesity results from a prolonged imbalance of small positive energy and treatment is required to reverse this imbalance (1). The combination of mazindol – a sympathomimetic amine clinically used in the treatment of obesity – and diet therapy is effective in treating severe obesity (12,39,40). It possesses both an anti-obesity action because of inhibition of appetite and brown adipose tissue thermogenesis activation (41).

The CNS requires a high-energy supply because of its intense ATP-consuming processes. In this context, mitochondrial oxidative phosphorylation is the main ATP-producing pathway, which supplies more than 95% of the total energy requirement in the cells (17). The appropriate functioning of Krebs cycle enzymes as well as the integrity and function of respiratory chain complexes provide the major source of energy in biological systems. Our outcomes showed relevant alterations on the activity of enzymatic complexes of mitochondrial respiratory chain. Interestingly, mazindol promoted an inhibition action only in complex I of the mitochondrial respiratory chain, specifically in the hippocampus. Complex I is the first enzyme complex of the mitochondrial respiratory chain, oxidising NADH to release electrons that facilitate the translocation of protons across the inner membrane and then to promote generation of a proton gradient. It is known that the decrease in the activity of mitochondrial respiratory chain causes an increase in the generation of reactive oxygen species. It is known that the decrease in the activity of mitochondrial respiratory chain causes an increase in the generation of reactive oxygen species, mainly by complex I, because this is very sensitive and can be inhibited by reactive

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oxygen species (43,44). In fact, a number of devastating neurodegenerative disorders are associated with complex I deficiency, resulting in a decline in energy production by the respiratory chain and in increased production of reactive oxygen species (44,45). The decreased activity of complex I is determined in mitochondrial fragments as a decreased NADH-cytochrome *c* reductase activity with a simultaneously unchanged succinate-cytochrome *c* reductase activity (43) or as decreased NADH-ubiquinone reductase activity (46), and in coupled mitochondria as a decreased respiratory rate in state 3 with malate-glutamate, or other NAD-dependent substrates with a simultaneous unchanged respiratory rate with succinate as substrate (43).

Mazindol is essentially a dopamine (DA) and norepinephrine (NE) reuptake inhibitor (47) that produces an increase in a resting metabolic rate (48). This drug interacts with the DA uptake sites with a Ki value in the 10^{-7} M range (49), and because of that characteristic mazindol induces a marked labelling of noradrenaline uptake complex. The metabolism of catecholamines, such as DA and NE, is probably associated with free radical formation, and conditions associated with increased catecholamine metabolism may increase the free radical burden. Studies have shown that endogenously available DA can undergo autoxidation and form reactive quinones that attack and potentially inhibit the function of intracellular proteins. In addition to DA, autoxidation and metabolism of DA by monoamine oxidase can increase H_2O_2 production and iron-dependent reactive oxygen species production (50). In this context, we suggest that inhibition in the hippocampus can be a consequence of increased DA synthesis.

Tissues, such as the brain, and specific brain areas, such as the hippocampus, which have a slow turnover of mitochondria and mitochondrial components, show a cellular accumulation of dysfunctional mitochondria with increased apoptosis, a condition that drives the tissue to a physiological deficit (51). This host reaction determines the unfavourable outcome of the disease with neuronal injury including hippocampal apoptosis (52). This may explain why some brain areas, especially the hippocampus, are more affected than other.

Moreover, complex I is the primary source of reactive oxygen species in a variety of pathological processes (53–55). The decrease in complex I activity of the mitochondrial respiratory chain in this area compromises the use of reduced coenzyme NADH. As our results showed that complex II and succinate dehydrogenase activities are functioning properly, the pathway that is probably sustaining the respiratory chain is through FADH-complex II. Therefore, it seems justifiable to confirm the greater susceptibility of the hippocampus to drugs, in this case mazindol, and a greater susceptibility on memory and learning impairments.

Burrows et al. (56) conclude that the acute dose of psychostimulant administration produces a rapid and transient disruption of metabolic processes that is regionally selective. Psychostimulants may increase neuronal energy utilisation through the sustained activation of monoamine transporters (57). A recent study by Rezin et al. (58) showed that the activities of citrate synthase, malate dehydrogenase, succinate dehydrogenase, creatine kinase and complexes of respiratory chain were increased by acute and chronic administration of fenproporex – an anorectic drug that is converted *in vivo* into amphetamine.

The majority of ATP in the neuropil is devoted to the maintenance of ion gradients and the restoration of the membrane potential following depolarisation (59–61), and sustained activation following prolonged neurotransmitter release may lead indirectly to the depletion of substrates for the electron transport chain (62). The NE decrease neuronal reuptake promoted by mazindol had variable effects. Rothwell et al. (57) showed a slight delayed increase in Na^+, K^+-ATPase activity, which was also not sustained. It is important to bear in mind that mazindol has a very slow onset of action, which might be because of a slow build-up of NE at the synapse (62). This can be related to our results, where the increase seen on the enzymatic activity of complex IV mitochondrial respiratory chain, citrate synthase and creatine kinase is possibly a primary response after acute exposition to a drug with psychostimulant properties, such as mazindol.

It is important to remind that complex IV is the terminal enzyme of electron transport chain that transfers electrons from cytochrome *c* to oxygen while it translocates protons from the matrix into the intermembrane space. Effects on complex IV (cytochrome *c* oxidase), however, are the most severe because this is the step where oxygen is reduced to water (36). Interestingly, only the cerebral cortex was affected at the lowest dose (0.25 mg/kg) with an increase in complex IV activity. Rothwell et al. (58) showed in a study that mazindol had effects at low concentrations. Low and clinically relevant doses of stimulants exert behavioural actions that are qualitatively different from higher and behaviourally activating doses. Together these data suggest that energy metabolism impairment may occur by mazindol pharmacotherapy. In this context, several studies link energy impairment to neuronal death and neurodegeneration (63,64). The high density of the mitochondria within the neurons could provide a rationale for the sensitivity of the CNS to energy deficit because of mitochondrial dysfunction. As several studies have

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shown that stimulant effect increases the extracellular concentrations of monoamines, this class of drugs may also contribute to mitochondrial inhibition (56,65,66). On the other hand, the stimulus effect of mazindol in most of the assays analysed promotes prolonged modulating action of NE and DA, and therefore there is a greater energy demand. In addition, we demonstrated that these changes varied according to the brain structure or biochemical analysis and were not dose dependent.

It is worth mentioning that some aspects of drug–mitochondria interactions may still be underestimated because of the difficulty in foreseeing and understanding all potential implications of the complex pathophysiology of mitochondria. More studies are necessary to confirm the selective action of mazindol on CNS and its dual effect on the mitochondrial respiratory chain, where there was a significant inhibition in the activity of complex I and an activation in complex IV – if these results are repeated on a chronic treatment and as far as the complex I inhibition and complex IV activation undertakes brain metabolism.

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Author Contribution

Gonçalves CL, Rezin GT, Scaini G and Streck EL designed the experiments; Rezin GT, Ferreira GK, Bez GD, Daufenbach JF and Gomes LM conducted the experiments; Rezin GT and Jeremias IC analysed the data; Gonçalves CL, Zugno AI and Streck EL wrote the manuscript.

Conflict of Interest

The authors declare that we have no conflict of interest.

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