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Old mice present increased levels of succinate dehydrogenase activity and lower vulnerability to dyskinetic effects of 3-nitropropionic acid

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ABSTRACT

Huntington's disease (HD) is a neurodegenerative disorder, with an age-related onset and a progressive development, characterized by choreiform movements. 3-Nitropropionic acid (3NP) induces the inhibition of succinate dehydrogenase (SDH), an increase in oxidative stress and anatomic changes that are related to the pathophysiology of HD. Hence, this toxin is a useful tool to study this pathology. This study compares the effects of 3NP on the development of orofacial dyskinesia (OD) and on SDH activity in young and old mice. Treatment with 3NP (5, 10, 15 or 20 mg/kg once a day, for four days) induced OD in young mice. Old mice presented an increase in the basal level of orofacial movement that was not potentiated by any dose of 3NP. Histochemical analyses showed that old mice presented an increase in the SDH activity. Finally, 3NP induced a decrease in SDH activity at both ages. We suggest that the 3NP-induced OD in young mice is related to the inhibition of SDH activity. In parallel, an enhancement in the basal activity of SDH could be related to the absence of a further increase in the OD presented by old mice treated with 3NP.

1. Introduction

HD is a progressive neurodegenerative disorder characterized by cognitive impairment, emotional disturbance and movement abnormalities (Barbeau et al., 1981) that starts in midlife and progressively leads to death (Vonsattel and DiFiglia, 1998). The first motor sign is changes in eye movements, which is followed by progressive orofacial dyskinesia (Brouillet et al., 1999). Despite the discovery of the genetic mutation of HD (Huntington's Disease Collaborative Research Group, 1993), the mechanisms of the pathogenesis of HD are still not understood. Oxidative stress and mitochondrial dysfunction (Petersén et al., 1999), have been proposed and seem to contribute to the appearance of motor alterations (Coyle and Puttfarcken, 1993; Browne et al., 1999; Turrens, 1997; Barja, 2004). Since mitochondria is responsible for the increase in oxidative stress, the production of reactive oxygen species (ROS) can affect the physical and chemical structures of the mitochondrial inner membrane itself, compromising cell respiration (Boveris and Chance, 1973) and energy supplies. Moreover, abnormalities in mitochondrial enzymatic activities, such as the deficiency of succinate dehydrogenase (SDH), an enzyme of the complex II of the mitochondrial respiratory chain (Gu et al., 1996; Panov et al., 2002), have also been related to a decrease in cellular energy and neuronal irreversible injury (Beal, 1998) that occur in caudate nuclei of HD patients.

Besides all these features, aging, a multi-faceted process, is an important factor to be considered. The increase of ROS production with aging causes defects in the mitochondrial DNA (mtDNA) and damages to the mitochondrial components (Sato and Tauchi, 1982; Meccocci et al., 1994; Lopes et al., 2004). Some of the consistent changes are the decreased activity of several proteic complexes of the electron transport chain (ETC) that lead to a decrease in energy production and a decline of the physiological conditions (Ozawa, 1997; Beckman and Ames, 1998; Mattson, 2000; Szibor and Holtz, 2003). As a result, these processes can accelerate cell death and the degenerative mechanisms (Harman, 1999; Cadenas and Davies, 2000). Therefore, the age-related onset and progressive course of HD may be due to a cyclic process involving the impairment of energy metabolism.

3-Nitropropionic acid (3NP), the metabolic product of 3-nitropropanol, is known to be responsible for an irreversible and progressive inhibition of SDH (Ludolph et al., 1992; Palfi et al., 1996), as well as cellular and metabolic dysfunction and motor deficits (Guyot et al., 1997) similar to HD (Brouillet et al., 1999). Because of these facts, the treatment with 3NP became a relevant experimental model for the study of this disease (Brouillet et al., 1999; Rosenstock et al., 2004). 3NP treatment induces, for example, the development of orofacial dyskinesia in rodents, one of the first motor signs of HD (Brouillet et al., 1999) appearing as vacuous chewing movement (Andreassen and Jorgensen, 1995; Brouillet et al., 1999; Rosenstock et al., 2004). This behavioral parameter seems to be related to an increase in oxidative stress (Abílio et al., 2002, 2003, 2004; Faria et al., 2005), one of the consequences of 3NP treatment (Rosenstock et al., 2004). Moreover, 3NP can also lead to alterations in mitochondrial

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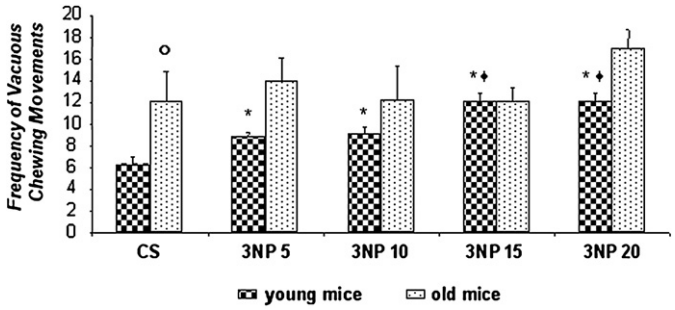

Fig. 1. Behavioral alterations in one- and eighteen-month-old mice treated with different doses of 3NP. Frequency of vacuous chewing movement in B6xCBA/F1 mice treated with control solution (CS) or 5, 10, 15 or 20 mg/kg 3-nitropropionic acid (3NP). Data are reported as the mean ± standard error. Two-Way ANOVA followed by Duncan's test. °$p<0.05$ compared to young mice with the same treatment; *$p<0.05$ compared to CS treated mice of same age; ♦$p<0.05$ compared to 3NP 5 and 3NP 10 mg/kg treated mice of same age.

homeostasis, as a decrease in mitochondrial membrane potential (Rosenstock et al., 2004), that is also related to an increase in the generation of free radicals due to an impairment of energy supply (Beal, 1995). Furthermore, these processes can contribute to the release of substances such as apoptosis-inducing factors and cytochrome *c* that culminate with apoptotic cell death (Ellerby et al., 1997; Kroemer, 1997; Zamzami et al., 1998).

The aim of this study was to compare the effects of 3NP treatment on orofacial dyskinesia as well as SDH activity (through histochemical analysis) in young and old mice.

2. Materials and methods

2.1. Animals

The experiments were performed with one-month-old (young) and eighteen-month-old (aged) male B6CBA/F1 mice (the first generation of female B6 and male CBA mice). Groups of five or six animals were kept in cages with free access to food and water. They were housed in the Center of Development of Animal Models for Medicine and Biology (CEDEME) at the Federal University of São Paulo, under conditions of controlled temperature (21 ± 2 °C) and under a 12-h light/dark cycle with lights on at 07:00 h. On the days of the experiments, animals were removed from CEDEME and transported to the experimental room where they were allowed to habituate for at least 1 h. This room had controlled temperature and sound (maximal level of noise is 40 dB). Animals used in this study were maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources, National Research Council, USA.

2.2. Drugs

3-Nitropropionic acid (3NP), purchased from Sigma (St. Louis, MO, USA), was dissolved, one day before the beginning of the treatment, in distilled water at the concentrations of 0.5, 1, 1.5 or 2 mg/ml. In subsequent days, solutions remained in the refrigerator (4 °C). The control solution used in the experiments was a saline solution (NaCl 0.9%). All the solutions were administered intraperitoneally (i.p.) and all animals received the same number of injections in a volume of 10 ml/kg of body weight. Succinate and Nitroblue tetrazolium, used in histochemical analysis, were also purchased from Sigma.

2.3. Experimental procedures

For the behavioral analysis, young and old B6xCBA/F1 male mice were randomly divided into five groups according to their treatment ($n=12$ for each group of young mice and $n=8$ for each group of old mice). The protocol treatment was chosen based on previous studies (Rosenstock et al., 2004; Kumar et al., 2006) and consisted of daily injections, once a day, for four days, of control solution (CS) or 5, 10, 15 or 20 mg/kg of 3NP (3NP 5, 3NP 10, 3NP 15 and 3NP 20, respectively). On day 5, the animals were observed for orofacial movement quantification, as described below. All the tests were performed during the morning to avoid changes in the animal's activity due to different activity phases.

To histochemical studies, young and old B6xCBA/F1 male mice ($n=3$ for each group) were used to evaluate the SDH activity. Animals were submitted to the same treatment described above, with daily injections for four days, of control solution (CS) or 3NP 20 mg/kg. Twenty-four hours after the last injection (day 5), the animals were sacrificed, the brains were rapidly removed, frozen in dry ice-cooled isopentane with temperature varying from −20 °C to −30 °C and stored at −80 °C until the day of the experiment.

2.4. Behavioral testing

Mice were placed individually in wire mesh cages (16 cm × 30 cm × 19 cm). Immediately after, they were observed for quantification of vacuous chewing movement frequency for 10 min, scored by a hand-operated counter. In the present study, vacuous chewing movements were

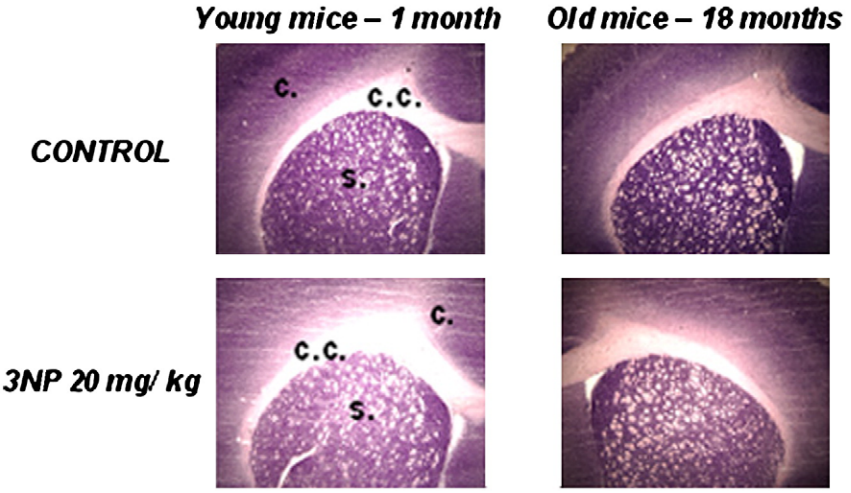

Fig. 2. Histochemical staining for SDH activity. Mice B6xCBA/F1 treated with CS or 3NP 20 mg/kg/day for four days. The images were obtained and analyzed in Olympus BX 60 Microscopy (4× objective). (S) striatum; (CC) corpus callosum; (C) cortex.

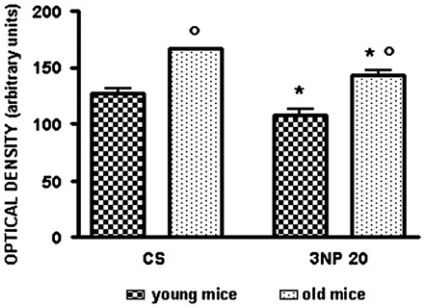

Fig. 3. Quantification of SDH activity in brain slices of B6xCBA/F1 mice. Representative histogram of SDH activity in mice with one and eighteen months of age treated with CS or 3NP 20 mg/kg. The data are expressed in mean ± standard error. Two-Way ANOVA followed Duncan's test. °$p<0.05$ compared to young mice with the same treatment; *$p<0.05$ compared to CS treated mice of same age.

considered to be single mouth openings in the vertical plane not directed toward physical material. Mirrors were placed under the floor and behind the back wall of the cage to permit observation of vacuous chewing movement when the animal was facing away from the observer. The results were expressed as mean ± standard error.

2.5. Histochemical analysis

To evaluate SDH activity by histochemistry (Seligman and Rutenburg, 1951; Brouillet et al., 1998), frozen brains were cryosectioned (Leica EG 1120) at 20 µm at a temperature of −20 °C. The brain sections were transferred to glass slides and air-dried for 60 min before histochemical staining. Each tissue section was then incubated for 1 h at 37 ° C in a dark and humidified chamber in 0.2 M phosphate buffer (PB: Na_2HPO_4 0.2 M and KH_2PO_4 0.2 M, pH 7.4) containing succinic acid (as a substrate) (200 mM) and Nitroblue tetrazolium (NBT; 1.34 mM) (Seligman and Rutenburg, 1951; Tanji and Bonilla, 2001; Kiyomoto et al., 2008). Endogenous SDH activity resulted in blue diphormazan deposits from the NBT reduction through succinate oxidation. No blue deposits are formed in the absence of succinate substrate or in the presence of 3NP in the incubation mix. After this procedure, the slices were rinsed with distilled water to stop the chemical reaction. The slides were then coverslipped in glycerine jelly.

To quantify SDH, the image of each brain section was captured using a cooled CCD camera connected to an optical microscope (Olympus BX 60) with 4× objective lens. The relative optic density (OD) deposited in tissues (dark-blue stain produced by the formazan product in SDH histochemistry) was quantified simultaneously in all groups of mice by the program Scion Image (Scion Corporation). The analyses were made considering the following regions of the brain section: cortex (C), striatum (S) and corpus callosum (CC) and the results were expressed as mean ± standard error.

2.6. Statistical analysis

Data were treated by Two-Way ANOVA followed by Duncan's test. A probability of $p<0.05$ was considered to show significant differences for all comparisons made.

3. Results

3.1. Behavioral testing

The effect of several doses of 3NP on orofacial dyskinesia of young and old mice is shown in Fig. 1. Two-Way ANOVA revealed a significant effect of age [F (1, 90) = 18.12, $p<0.05$] and treatment [F (4, 90) = 3.92, $p<0.05$] and no interaction between these factors. Post-hoc analysis revealed that 3NP significantly increased the frequency of vacuous chewing movement in a dose dependent manner in adult but not in old mice. Furthermore, old mice presented an increase in vacuous chewing movements that reached statistical significance in the CS group.

3.2. Histochemical analysis

Figs. 2 and 3 show histochemical staining and analysis of SDH activity in young and old B6xCBA/F1 mice treated with CS or 3NP (20 mg/kg/day). Fig. 2 shows brain slices stained for SDH in the striatum (S), corpus callosum (CC) and cortex (C). The representative histogram of the analysis of the SDH staining, representing SDH activity in all these regions, is shown in Fig. 3. Two-Way ANOVA revealed a significant effect of age [F (1, 8) = 17.69, $p<0.05$] and treatment [F (1, 8) = 6.81, $p<0.05$] and no interaction between these factors. Post-hoc analysis showed that old mice presented an increase in SDH activity independently of the treatment. In addition, 3NP induced a decrease in SDH activity at both ages.

4. Discussion

The present study compares the development of orofacial dyskinesia as well as alterations in the SDH activity in young and old mice treated with 3NP. In agreement with the literature, 3NP induced an increase in vacuous chewing movement in a dose dependent manner in young mice (Andreassen and Jorgensen, 1995). These results are also in accordance with clinical data showing that among motor alterations, the earliest one is the progressive appearance of orofacial dyskinesia (Brouillet et al., 1999). In this respect, in the striatum, one of the main areas related to motor behaviors, dopaminergic neurons have been well studied (Reynolds et al., 1998; Jakel and Maragos, 2000; Bywood and Johnson, 2003; Blum et al., 2004; Napolitano et al., 2004), and display a greater sensitivity to the 3NP presence.

Regarding SDH activity, 3NP induced a significant decrease in young mice. In this respect, the decrease of SDH could lead to an increase in oxidative stress and also to a decrease in ATP production, due to a mitochondrial dysfunction, which could result in alteration in orofacial dyskinesia (Andreassen and Jorgensen, 1995), a behavioral alteration extensively related to oxidative stress (Abílio et al., 2002, 2003, 2004; Calvente et al., 2002; Faria et al., 2005; Burger et al., 2005; Ferreira and Rocha, 2005; Fachinetto et al., 2005; Naidu et al., 2002, 2003, 2006; Colpo et al., 2007).

Despite the effect of 3NP in young mice, old mice treated with the same doses of 3NP did not present any behavioral changes. The absence of any differences in orofacial dyskinesia could be related to a maximal level of basal oxidative stress at this age (Levine and Stadtman, 2001; Sohal et al., 2002; Rebrin and Sohal, 2004; Navarro and Boveris, 2004). This increase in oxidative stress is possibly related to alterations in mitochondrial homeostasis (Beal, 1995) that may contribute to cause a maximal expression of this behavior. Our data is not in agreement with others indicating that aging increases the effects of 3NP. However, the aforementioned data are not conclusive since most of them were obtained from in vitro or ex vivo studies in which the goals were to characterize the processes related to the induction of cell death and associated mechanisms rather than to study behavioral alterations (Bossi et al., 1993; Beal et al., 1993; Brouillet et al., 1993; Kim and Chan, 2001; Blum et al., 2004; Galas et al., 2004). On the other hand, the increase in vacuous chewing movement in old mice treated with CS (control solution), when compared to young animals, is in accordance with the literature, showing that the aging process itself can cause an increase in orofacial dyskinesia due to an increase in oxidative stress (Bergamo et al., 1997; Abílio et al., 2002, 2004; Burger et al., 2004a,b). In fact, several studies have indicated that orofacial movements increase with aging (Rupniak et al., 1984; Waddington et al., 1985; Johansson et al., 1986; Bergamo et al., 1997; Waddington, 1990; Abílio et al., 2002; Burger et al., 2004a,b) and that in the aged brain there is an increase in oxidative stress, accompanied by free-

radical-induced damage. Burger et al. (2004a,b), for example, have shown that the increase of vacuous chewing movements with aging is associated with an increase in TBARS levels. In addition, the development and persistence of vacuous chewing movements were presented in old rats treated with haloperidol which increased oxidative stress and caused nigral degeneration (Andreassen et al., 2003). Abilio et al. (2002) have shown that the treatment with the free radical scavenger melatonin (Reiter, 1998) attenuates the age-induced orofacial dyskinesia and prevents the enhancement in striatal lipid peroxidation. The use of vitamin E attenuates dyskinesia and increases striatal oxidized/total glutathione ratio (Abilio et al., 2003), corroborating the hypothesis that OD is related to an increase in oxidative stress. These findings support the idea that the accumulation of reactive oxygen species leads to oxidative stress which is a common mechanism present either in dyskinesia or aging (Casey, 2000; Hensley and Floyd, 2002; Lohr et al., 2003; Wickens, 2001).

Regarding basal activity of SDH in old mice, it was observed that its activity is significantly higher when compared to young mice. Considering previous results, the work presented by Kwong and Sohal (2000), for example, shows that mice with 12–14 months of age have an increase in SDH, while Haripriya et al. (2004) describe a decrease in SDH in 22 months-old rats. Actually, the 3NP treatment may vary and cause different alterations in SDH inhibition, striatal lesions, brain damage and behavioral changes depending on animal species, strain and age (Alexi et al., 1998; Brouillet et al., 1998, 2000; Ouary et al., 2000) or just age (Brouillet et al., 1993). In addition, the effect of 3NP on SDH inhibition may also be related to differences in blood–brain barrier permeability (Nishino et al., 1995, 1997), to the toxin's peripheral metabolism or SDH turnover (Ouary et al., 2000). 3NP induced a decrease in SDH activity in both age groups. However, considering the higher level of SDH in old mice, the decrease in SDH activity induced by 3NP might not be enough to induce any further behavior alterations. These data do not decline the fact that alterations in other cellular function are occurring. The increase in the enzyme activity in old mice could be explained by a compensatory response to mitochondrial dysfunction and ROS production induced by aging (Miquel et al., 1980; Toescu and Verkhratsky, 2004; Lopes et al., 2004, 2006). In fact, ROS production may cause mitochondrial DNA (mtDNA) damage (Ozawa, 1997; Beckman and Ames, 1998; Toescu et al., 2000; Lee and Wei, 2001; Droge, 2002; Nicholls, 2002; Genova et al., 2004). Therefore, alterations in the transport of electrons can occur, since mtDNA encodes most part of mitochondria complexes (Ozawa, 1997; Beckman and Ames, 1998). As a consequence, changes in proton flux, ATP production and oxidative stress levels ultimately contribute to the disruption of cellular homeostasis, such as increase of intracellular calcium and the induction of cell death (Beal, 1995; Ellerby et al., 1997; Kroemer, 1997; Zamzami et al., 1998; Rosenstock et al., 2004). Thus, to supply these deficits, the cells could induce a compensatory increase in the SDH activity, since human mtDNA encodes 13 subunits of oxidative phosphorylation enzymes of the complexes, except complex II (Olgun and Akman, 2007). In fact, during aging, when mutation surpassed 90% of the total mitochondrial genomes in muscle fibers, there is an increase in SDH activity (Herbst et al., 2007). Similar results are described to an aging model of *Saccharomyces cerevisiae* (Samokhvalov et al., 2004). Interestingly, other pathophysiological conditions have also shown an elevation of SDH activity such as hyperadrenergic status (Khunderyakova et al., 2008) and hypertensive neurocirculatory dystonia (Vasin et al., 2002). Finally, Battaglia et al. (2007) have reinforced the idea of an increase in SDH activity as a compensation for deficiency in complex I in a model for Parkinson disease.

Therefore, damaged mitochondrial complexes may have their deficiency supplied by another complex or functional elements of the chain as a compensation to maintain cellular homeostasis (Kwong and Sohal, 2000; Schagger, 2002; Battaglia et al., 2007). Three mechanisms could be associated with this compensation, namely a) damage of one mitochondrial complex, which could be compensated by the increased activity of the other elements of the electron transport chain in order to maintain cellular homeostasis (Battaglia et al., 2007; Kwong and Sohal, 2000; Schagger, 2002); b) alteration in the expression of the genes of the mitochondrial electron transport chain, which were shown to be regulated by aging (Manczak et al., 2005; Reddy et al., 2004); c) loss of deficient mitochondria that might be substituted by resistant organelles (Brustovetsky et al., 2005; Panov et al., 2002).

Taken together, our results indicate that an increase in SDH activity in old mice could act as a compensatory mechanism to counteract the increase in oxidative stress related to the aging process, which would decrease the vulnerability of the behavioral alterations induced by 3NP. Nevertheless, this possible compensatory increase in SDH activity is not sufficient to attenuate the multi-faceted alterations related to the aging process (Haripriya et al., 2004; Nicholls, 2002; Szibor and Holtz, 2003), which causes an increase in the severity of neurodegenerative diseases. In this respect, several factors, besides SDH function, may cause neurodegeneration such as bioenergetic alterations, oxidative stress and mitochondrial dysfunction, changes in Ca^{2+} homeostasis and decrease in ATP synthesis (Ozawa, 1997; Beckman and Ames, 1998; Harman, 1999; Cadenas and Davies, 2000; Mattson, 2000; Szibor and Holtz, 2003).

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