Prolonged acetylcholinesterase inhibition and impairment in object recognition memory in rats subjected to chronic hyperprolinemia

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Prolonged acetylcholinesterase inhibition and impairment in object recognition memory in rats subjected to chronic hyperprolinemia

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Abstract
Previous studies show that hyperprolinemia impairs spatial memory. Rats received subcutaneous injections of proline (Pro) twice a day at 10 hours intervals from the 6th to the 28th days of age and equivalent volume of 0.9% saline solution (control). The animals were killed 3 hours, 12 hours, or 30 days after the last injection by decapitation without anesthesia to determine acetylcholinesterase (AChE) activity or the animals remained at the animal facility until the 60th day of life to assess the effect of Pro administration on non-spatial memory. Results showed that rats subjected to experimental chronic administration of Pro present a significant non-spatial memory deficit (short- and long-term memory). Prolonged alterations in AChE activity were observed in the hippocampus and cerebral cortex of rats subjected to Pro administration. Data indicate that chronic hyperprolinemia alters AChE activity, interfering in acetylcholine levels, which could participate in inducing the memory deficit observed in hyperprolinemic rats.

Keywords: Hyperprolinemia; non-spatial memory; short- and long-term memory.

Introduction
Type II Hyperprolinemia (HP II) is a rare inherited autosomal recessive disorder of amino acid metabolism characterized by the hepatic deficiency of Δ-1-pyrroline-5-carboxylic acid dehydrogenase activity, which leads to tissue accumulation of proline (Pro) (Phang et al., 2001). Affected patients present neurological manifestations including seizures and mental retardation. While many hyperprolinemic individuals remain asymptomatic, high concentrations of Pro and neurological symptoms have been demonstrated in patients with this disease (Phang et al., 2001). Although neurological dysfunction is found in a considerable number of hyperprolinemic patients, the mechanisms by which this occurs are poorly understood. In this context, (Moreira et al., 1989) have developed an experimental model of hyperprolinemia, in which the Pro levels are similar to those found in human HP II (Phang et al., 2001). Using this model, we have reported that rats subjected to this experimental model of hyperprolinemia present oxidative stress, impairment of spatial memory in the Morris water maze and alterations in the activities of (Na⁺,K⁺)-ATPase and creatine kinase (Bavaresco et al., 2005; Delwing et al., 2003a; Delwing et al., 2003c; Kessler et al., 2003; Pontes et al., 2001). In addition, acute administration of Pro reduces acetylcholinesterase (AChE) activity in cerebral cortex of rats sacrificed 1 hour after administration, while no significant alteration was observed on the enzyme activity when rats were sacrificed 12 hours after chronic administration of Pro (Delwing et al., 2003b; Delwing et al., 2005). On the other hand, studies have associated the cholinergic input to the perirhinal cortex in object recognition memory (Abe et al., 2004; Warburton et al., 2003; Winters and Bussey, 2005; Winters et al., 2006).

Impairments in learning, memory, and behavior observed in patients with dementia are suggested to be caused, at least in part, by
changes in cholinergic system function (Ballard et al., 2005; Fodale et al., 2006), because there is consistent evidence that low levels of acetylcholine (ACh) in the brain are associated with cognitive dysfunction (Mesulam, 2004).

Cholinergic transmission is mainly terminated by ACh hydrolysis by the enzyme AChE (EC 3.1.1.7). This enzyme is widely expressed in tissues that receive cholinergic innervations, such as neurons and muscle cells (Massoulié et al., 1993). On the other hand, reports suggest that AChE substantially contributes to synaptic transmission, both in cholinergic and other types of synapses, such as dopaminergic and glutamatergic synapses (Zimmerman and Soreq, 2006).

Excitotoxic properties have also been demonstrated for Pro, which at higher concentrations activates N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, indicating that Pro might potentiate glutamate transmission (Nadler et al., 1992). Accordingly, Pro decreases glutamate uptake in slices of cerebral cortex and the hippocampus of rats (Delwing et al., 2007). In addition, NMDA receptor blockade deteriorated the performance of the spontaneous object recognition task (Abe et al., 2004). Therefore, since hyperprolinemia provokes deficits in spatial memory in rats, the aim of the present study was to investigate the effects of chronic hyperprolinemia on the ability of rats to recognize objects (using a test of non-spatial memory), as well as its effects on AChE activity in the hippocampus and cerebral cortex of rats. We used hippocampus and cerebral cortex, because these cerebral structures are involved in functions such as memory and learning (Liu and Bilkey, 1998; Roef et al., 1993), which are impaired in hyperprolinemic patients (Phang et al., 2001) and rats (Bavaresco et al., 2005).

Materials and Methods

Animals and reagents
Wistar rats obtained from the Central Animal House of the Regional University of Blumenau, Blumenau, Brazil, were used in the experiments. The animals from our own breeding stock were maintained on a 12 hours light/12 hours dark cycle at a constant temperature (22 ± 1°C), with free access to water and commercial protein chow. The “Principles of Laboratory Animal Care” (NIH publication 85-23, revised 1985) were followed in all the experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the local Institution. All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA.

Proline administration
For chronic treatment, a Pro solution was administered subcutaneously twice a day at 10 hours intervals from the 6th to the 28th days of age, as described by (Moreira et al., 1989). During the first 8 days of treatment, the rats received 12.8 µmol of Pro/g body weight; from 14 to 17 days the rats received 14.6 µmol of Pro/g body weight; from 18 to 21 days of life the rats received 16.4 µmol of Pro/g body weight; and from 22 to 28 days the rats received 18.2 µmol of Pro/g body weight. Rats subjected to this treatment attained plasma Pro levels of between 1.0 and 2.0 mM, similar to those found in HP II patients (Phang et al., 2001). Control animals received saline injections in the same volumes as those applied to Pro-treated rats. The animals were killed 3 hours, 12 hours, or 30 days after the last injection by decapitation without anesthesia for determination of AChE activity or the animals remained at the animal facility until the 60th day of life for assessment of non-spatial memory.

Object recognition test
The apparatus consisted of a circular wooden arena (40 cm in diameter and 50 cm high). All animals were habituated to the experimental arena in the absence of any specific behavioral stimulus for 20 minutes/days during 4 days. The objects, made of metal or glass, were fixed to the arena’s floor with adhesive ribbon. On the first day (training session) the animals were placed in the arena containing two different objects (M and N) and left to explore them freely for 5 minutes. The test was repeated 180 minutes later to test short-term memory (STM) or 24 hours later to evaluate long-term memory (LTM) after the subjectted to Pro administration. In the tests, one of the objects was changed for a new object (P, for STM or R, for LTM) and the rat was introduced to the arena for a further 5 minutes. The positions of the objects (familiar or novel) were randomly permuted for each experimental animal and the arena was cleaned between trials. Exploration was defined as snifing or touching the object with the nose and/or forepaws. Sitting on or turning the object around was not considered exploratory behavior. The main dependent measure
was the investigation ratio of discrimination, the proportion of total object-investigation during the test phase that was spent investigating the novel object $[t_{\text{novel}}/(t_{\text{novel}} + t_{\text{familiar}})]$ (Ennaceur and Delacour, 1988).

**Open field test**
The open field test consisted of the measurement of behavioral variables of experimental subjects, placed in an arena bounded by a circular wall of 50 cm high and 40 cm in diameter (Walsh and Cummins, 1976). To test the locomotor and exploratory activity, the animals were placed in the open field to freely explore for 5 minutes, where times were recorded in the following behavioral categories: time of motility (voluntary movement), immobility time (remaining motionless), time of rearing (raising the upper body, supported by the lower), and time of grooming (moving arms over head).

**Tissue preparation**
Rats were killed by decapitation without anesthesia and hippocampus and cerebral cortex were rapidly removed. Hippocampus and cerebral cortex were homogenized in 10 volumes 0.1 mM potassium phosphate buffer, pH 7.5, and centrifuged for 10 minutes at 1,000 g. The supernatant was used for the enzymatic AChE analyses.

**AChE activity assay**
AChE activity was determined according to (Ellman et al., 1961), with some modifications. Hydrolysis rates were measured at an acetylthiocholine concentration of 0.8 mM in 1 mL assay solutions with 30 mM phosphate buffer, pH 7.5, and 1.0 mM 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) at 25°C. About 50 µL of rat hippocampus or cerebral cortex supernatant was added to the reaction mixture and pre-incubated for 3 minutes. The hydrolysis was monitored by the formation of thiolate dianion of DTNB at 412 nm for 2–3 minutes (intervals of 30 s). All samples were run in duplicate.

**Protein determination**
Protein was measured by the Bradford (1976) method, using serum bovine albumin as standard.

**Statistical analysis**
Data were analyzed by Student’s $t$-test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC compatible computer. Values of $p < 0.05$ were considered to be significant.

**Results**

**Effect of chronic hyperprolinemia on a test of non-spatial memory**
We first studied the effect of chronic hyperprolinemia on a test of non-spatial memory in order to assess short- and long-term memory. To this end, we evaluated the ratio of discrimination 3 hours and 24 hours after the pre-test. Statistical analyses showed that rat treated with saline showed a higher ratio of discrimination than the rats subjected to chronic administration of Pro $[t(20) = 3.550, p < 0.01]$ and $[t(20) = 7.082, p < 0.001]$, respectively; characterizing the presence of the short- and long-term memory (Figure 1A). Results also showed significant differences in the time to explore the new object at 3 hours after pre-test, since Pro-treated rats spent more time exploring the familiar object $[t(20) = 3.790, p < 0.01]$ than the new object $[t(20) = 3.159, p < 0.01]$, when compared with the controls. In addition, the same was observed for the time to explore the new object at 24 hours after pre-test, since the Pro-treated rats spent more time exploring the familiar object $[t(20) = 4.305, p < 0.001]$ than the new object $[t(20) = 3.936, p < 0.001]$, when compared with the controls (Figure 1B).

**Effect of chronic hyperprolinemia on the open field test**
Next, we evaluated the effect of chronic hyperprolinemia on motor activity. As can be observed, no significant change was observed in the time of motility $[t(20) = 0.7813, p > 0.05]$ and in the time of immobility $[t(20) = 0.1183, p > 0.05]$. Conversely, the data showed a significant reduction in rearing time $[t(20) = 3.638, p < 0.05]$ and grooming $[t(20) = 2.323, p < 0.05]$ for rats treated with Pro, when compared with the rats treated with saline (control) (Figure 2).

**Effect of chronic administration of Pro on AChE activity**
We also examined the effect of chronic administration of Pro on AChE activity in the hippocampus and cerebral cortex of rats sacrificed at 3 hours, 12 hours, and 30 days after the last injection of Pro. As can be observed in Figure 3A, chronic administration of Pro significantly...
reduced AChE activity in the hippocampus of rats sacrificed 3 hours and 12 hours after the last injection of Pro \( t(11) = 2.490, p < 0.05 \) and \( t(12) = 2.530, p < 0.05 \), respectively. On the other hand, chronic administration of Pro increased AChE activity at 30 days after the last injection \( t(14) = 3.157, p < 0.01 \), when compared with the control group. In addition, Figure 3B shows a decrease in AChE activity in the cerebral cortex of rats sacrificed 3 hours after the last injection of Pro \( t(12) = 5.775, p < 0.001 \). Conversely, no statistical change was observed in AChE activity in the cerebral cortex of rats sacrificed at 12 hours \( t(12) = 887, p > 0.05 \) and 30 days \( t(12) = 427, p > 0.05 \) after the last injection of Pro.

**Discussion**

Tissue accumulation of Pro occurs in HP II, an inherited disorder of amino acid metabolism caused by a severe deficiency of \( \Delta \)1-pyrroline-5-carboxylic acid dehydrogenase activity. Most
**Figure 2:** Behavioral responses in the open field of rats subjected to chronic administration of saline or proline. Vertical lines represent standard errors of the mean and bars the average of the subgroups of rats ($n = 10$; $^* p < 0.05$, Student’s $t$-test).

**Figure 3:** Effect of chronic administration of proline on acetylcholinesterase (AChE) activity in homogenates of rat hippocampus and cerebral cortex at 3 hours, 12 hours, and 30 days after the last injection. (A) Hippocampus AChE activity. (B) Cortical AChE activity. The specific activity of the enzyme is expressed in mmol of acetylcholine per hour per milligram of protein. The data represent mean $+/-$ SD for 6–7 independent experiments (animals) performed in duplicate (Student’s $t$-test). Different from control, $^* p < 0.05$, $^{**} p < 0.001$. 
patients detected so far have neurological dysfunction manifested as seizures and mental retardation, with a pathophysiology that is poorly understood (Phang et al., 2001).

Animal models are useful to better understand the pathophysiology of diseases. We have used a chemical experimental model of hyperprolinemia (Moreira et al., 1989), in which the Pro levels are similar to those found in human HP II (Phang et al., 2001). Studies using this experimental model of hyperprolinemia showed that Pro impairs memory (Bavareseco et al., 2005), induces free radical generation and reduces antioxidant defenses in rat cerebrum, suggesting that Pro elicits oxidative stress (Delwing et al., 2003a, Delwing et al., 2003c). In addition, Pro reduces glutamate uptake in slices of cerebral cortex and hippocampus of rats (Delwing et al., 2007) and decreases the activities of (Na⁺,K⁺)-ATPase, AChE, and creatine kinase, which are considered critical enzymes for normal central nervous system function (Delwing et al., 2003b; Delwing et al., 2005; Kessler et al., 2003; Pontes et al., 2001).

In the present study, we investigate the effect of chronic hyperprolinemia on a test of non-spatial memory in order to assess long- and short-term memory. To this end, we evaluated the time of exploration of a familiar and a new object at 3 hours and 24 hours after the pre-test. Results showed significant differences in time to explore the new object at 3 hours and 24 hours after the pre-test, since Pro-treated rats spent more time exploring the familiar object than the new object, when compared with the controls. These data suggest that Pro interferes in the mechanisms of memory formation, both in the short- and long-term.

We also tested the possible effect of the chronic administration of Pro on motor activity. However, no significant change was observed in the time of motility and in the time of immobility on tests performed. Thus, we may suggest that Pro causes no motor deficit. Conversely, the data showed a significant reduction in rearing time and grooming for rats treated with Pro, when compared with the control group. It has been suggested that rearing behavior and locomotor activity do not necessarily reflect the same physiological mechanisms (Pawlak and Schwarting, 2002). Decreased rearing and grooming, but with preservation of motility levels, possibly represents an altered cognitive response to the chronic administration of Pro. As a consequence, obvious reference memory deficits are present in Pro-treated animals, but they are, however, able to preserve the time of motility. In contrast, the treatment showed a reduction in rearing and grooming. However, this condition does not correlate to learning success, i.e. more time exploring the new object. In addition, while AChE would be expected to be present in cholinergic synapses, its presence is no guarantee that the systems with which it is associated are necessarily cholinergic.

Since ACh is an important neurotransmitter involved in the mechanisms of memory, we also verified the effect of chronic hyperprolinemia on AChE activity in the hippocampus and cerebral cortex of rats sacrificed at 3 hours, 12 hours, and 30 days after the last injection of Pro. Results showed that chronic administration of Pro significantly reduced AChE activity in the hippocampus and cerebral cortex of rats sacrificed at 3 hours after the last injection, and also reduced the activity of this enzyme in the hippocampus of rats at 12 hours after the last injection. In contrast, chronic administration of Pro increased AChE activity at 30 days after the last injection of Pro in the hippocampus, when compared with the control group. Conversely, no statistical change was observed in AChE activity in the cerebral cortex of rats at 12 hours or 30 days after the last injection of Pro, corroborating the studies of (Delwing et al., 2005), where no alteration was verified in this enzyme’s activity in the cerebral cortex of rats sacrificed at 12 hours after chronic administration of Pro.

AChE, a highly conserved enzyme in the animal kingdom, is distributed throughout a wide range of vertebrate tissues. It is essential for the normal function of the nervous system, since it rapidly terminates the action of ACh released into the synapse. The involvement of cholinesterase in modulating glial activation, cerebral blood flow, amyloid cascade, and tau phosphorylation has also been described (Ballard et al., 2005; Lane et al., 2006). It is currently speculated that actions of this enzyme could affect underlying processes in Alzheimer’s disease (Ballard et al., 2005), making AChE an important therapeutic target. In this context, reversible inhibitors of this enzyme have been used as cognitive enhancers for the treatment of patients with Alzheimer’s and other neurodegenerative disorders (Ballard, 2002; Lane et al., 2006). Furthermore, based on these data and on our results showing that chronic hyperprolinemia increases AChE activity at 30 days after the last injection of Pro, we may expect that the constant stimulation of this
enzyme by Pro or its metabolites might decrease ACh levels, which could be associated with memory deficits observed in hyperprolinemic rats. In addition, since a reduction in this enzyme activity was observed at 3 and 12 hours after the last injection of Pro in the hippocampus, the increase in AChE activity verified at 30 days after the last injection, may constitute a compensatory effect to normalize ACh levels.

In conclusion, the present study demonstrated that rats subjected to experimental chronic administration of Pro present a significant non-spatial memory deficit and alterations in AChE activity in the hippocampus and cerebral cortex of rats. Thus, it is possible that an alteration in ACh levels helps to provoke the memory deficit observed in this study. Object recognition memory, is agreed to be dependent on the perirhinal cortex. Whether it is a hippocampal or non-hippocampal dependent task is still in debate. While some studies reported that the task does not require the hippocampus, several studies support the fact that the hippocampus contributes to learned object familiarity (Mumby et al., 2002). Consequently, we may conclude from these behavioral data that the effects of Ach may be specific to particular cognitive processes, especially those involving the hippocampus, as has been previously suggested. Although it is difficult to extrapolate our findings to human hyperprolinemia, without in vivo data, it is tempting to speculate that these consequences may contribute at least in part to the pathophysiological characteristics present in the affected patients.

Ethical Approval

The study was approved by the institutional ethical committee of the Department of Natural Sciences, Regional University of Blumenau.

Conflict of Interests

Authors have no conflicting interests.

References


