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PROLINE-INDUCED CHANGES IN ACETYLCHOLINESTERASE ACTIVITY AND GENE EXPRESSION IN ZEBRAFISH BRAIN: REVERSAL BY ANTIPSYCHOTIC DRUGS

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Abstract—Hyperprolinemia is an inherited disorder of proline metabolism and hyperprolinemic patients can present neurological manifestations, such as seizures, cognitive dysfunctions, and schizoaffective disorders. However, the mechanisms related to these symptoms are still unclear. In the present study, we evaluated the *in vivo* and *in vitro* effects of proline on acetylcholinesterase (AChE) activity and gene expression in the zebrafish brain. For the *in vivo* studies, animals were exposed at two proline concentrations (1.5 and 3.0 mM) during 1 h or 7 days (short- or long-term treatments, respectively). For the *in vitro* assays, different proline concentrations (ranging from 3.0 to 1000 μM) were tested. Long-term proline exposures significantly increased AChE activity for both treated groups when compared to the control (34% and 39%). Moreover, the proline-induced increase on AChE activity was completely reverted by acute administration of antipsychotic drugs (haloperidol and sulpiride), as well as the changes induced in *ache* expression. When assessed *in vitro*, proline did not promote significant changes in AChE activity. Altogether, these data indicate that the enzyme responsible for the control of acetylcholine levels might be altered after proline exposure in the adult zebrafish. These findings contribute for better understanding of the pathophysiology of hyperprolinemia and might reinforce the use of the zebrafish as a complementary vertebrate model for studying inborn errors of amino acid metabolism.

Key words: zebrafish, acetylcholinesterase, proline, inherited diseases, hyperprolinemia, haloperidol, supiride.

INTRODUCTION

Hyperprolinemia can be caused by two distinct inherited disorders of proline metabolism. Hyperprolinemia type I (HPI) occurs due to the deficiency of proline oxidase (POX; EC 1.5.1.2). The hyperprolinemia type II (HPII) is caused by deficiency of Δ^1-pyrroline-5-carboxylic acid dehydrogenase (P5CDh; EC 1.5.1.12) activity. These enzymatic defects cause proline accumulation in blood and others tissues, such as the brain (Phang et al., 2001). As a result, some hyperprolinemic patients can present epilepsy and cognitive dysfunctions whereas others are asymptomatic (Flynn et al., 1989; Phang et al., 2001; Di Rosa et al., 2008). Although proline metabolism seems to be specifically related to psychotic disorders, such as schizophrenia (Phang et al., 2001; Jacquet et al., 2005; Oresic et al., 2011), the mechanisms underlying these neurological manifestations still remain poorly understood.

Several reports proposed that high proline levels have a detrimental effect on neuronal integrity, inducing changes in different neurotransmitter systems. Studies showed that proline may activate NMDA and AMPA receptors, suggesting that it potentiates the glutamatergic neurotransmission (Nadler, 1987; Nadler et al., 1992; Cohen and Nadler, 1997). Moreover, high proline levels were able to decrease glutamate uptake in the rat brain, as well as the Na^+, K^+-ATPase and creatine kinase activities, which are crucial enzymes for normal brain function (Pontes et al., 1999, 2001; Kessler et al., 2003; Delwing et al., 2007). Additionally, proline also impaired memory (Bavaresco et al., 2005; Delwing et al., 2006) and altered the

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Abbreviations: AChE, acetylcholinesterase; ANOVA, analysis of variance; BuChE, butyrylcholinesterase; DA, dopamine; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; EDTA, diethylene-dinitrilo-tetraacetic acid; EGTA, ethylene glycol bis(betaaminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HPI, Hyperprolinemia type I; HPII, hyperprolinemia type II; RT-qPCR, quantitative real-time reverse transcription polymerase chain reaction; SCh, thiocholine; S.E.M., standard error of mean.

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acetylcholinesterase activity in the rat brain (Delwing et al., 2005; Ferreira et al., 2011).

It is currently accepted that the cholinergic neurotransmission plays an important role in the CNS by regulating many biological processes such as learning, memory, sensory perception, and cortical organization of movement (Mesulam et al., 2002; Sarter and Bruno, 2004). At synaptic cleft, acetylcholine triggers muscarinic (metabotropic) and nicotinic (ionotropic) acetylcholine receptors. The inactivation of cholinergic signaling is promoted by the cholinesterases, which cleave acetylcholine into choline and acetate. Two different types of cholinesterases hydrolyze acetylcholine: acetylcholinesterase (AChE) (EC 3.1.1.7) and butyrylcholinesterase (BuChE) (EC 3.1.1.8) (Soreq and Seidman, 2001).

Zebrafish (*Danio rerio*) have gained popularity as an organism for neurobehavioral studies. This species has several features that complement the existing mammalian models such as low maintenance, translucent embryos, rapid development, and high fecundity. Zebrafish has also been used for drug screening and toxicological assays (reviewed in Lele and Krone, 1996; Parng et al., 2002; Kari et al., 2007; Mathur and Guo, 2010). In this sense, it can be easily and continuously exposed to different concentrations of amino acids for different periods (Rosemberg et al., 2010; Savio et al., 2012a). Furthermore, zebrafish genes present a high degree of conservation sharing a 70–80% homology with human genes, which is an additional attractive feature to study genetic and biochemical mechanisms of neurological diseases (Barbazuk et al., 2000; Dooley and Zon, 2000; Best and Alderton, 2008). Parameters of cholinergic signaling have already been characterized in the zebrafish brain (Clemente et al., 2004; Rico et al., 2006). It has been shown that AChE is encoded by a single gene, while BuChE has not been detected in the zebrafish genome (Clemente et al., 2004; Ninkovic et al., 2006). Thus, the effects of high amino acid concentrations on the gene expression and neurochemical changes can be evaluated in this species, as well as several parameters of neurotoxicity during development, including teratogenicity, cell death, and selected neuronal subtypes (Ton et al., 2006; Parng et al., 2007; David and Pancharatna, 2009; Long et al., 2011; Pan et al., 2011). Previous study from our group had already characterized the effects of proline exposure on behavioral parameters in the zebrafish (Savio et al., 2012a). We demonstrated that proline-induced behavioral changes are reverted by acute administration of antipsychotic drugs in this species; however, there is no evidence regarding the neurochemical mechanisms that may contribute to these behavioral responses.

Considering that: (i) the hyperprolinemic patients can present neurological dysfunctions, (ii) the cholinergic system is associated with several neurological disorders, (iii) recent studies suggest an influence of proline on cholinergic neurotransmission, and, finally, (iv) the zebrafish has become a prominent vertebrate to study neurological disorders related to human inherited diseases, here, we sought to investigate the effects of short- and long-term proline exposure on AChE activity and gene expression in the zebrafish brain. Furthermore, we also verified whether typical and atypical antipsychotic drugs, such as haloperidol and sulpiride, are able to revert the proline-induced changes in biochemical and molecular parameters of cholinergic signaling.

EXPERIMENTAL PROCEDURES

Animals

Adult males and females (approximately in the ratio of 1:1) of the ‘‘wild type’’ (short fin – SF) zebrafish (*D. rerio*) strain (6–8-months-old) were obtained from a commercial supplier (Redfish, RS, Brazil). Animals were kept in 50 L housing tanks with tap water previously treated with Tetra's AquaSafe® (to neutralize chlorine, chloramines, and heavy metals present in the water that could be harmful to fish) and continuously aerated (7.20 mgO_2/L) at 28 ± 2 °C, under a 14–10 h-light/dark photoperiod. The fish were kept at a density of up to five animals per liter (Westerfield, 2007). Animals were acclimated for at least 2 weeks before the experiments and fed three times a day to satiety with TetraMin Tropical Flake Fish®. All protocols were approved by the Ethics Committee of Federal University of Rio Grande do Sul (UFRGS) under License No.: 19636 and followed Brazilian legislation, the guidelines of the Brazilian Collegium of Animal Experimentation (COBEA), and the Canadian Council for Animal Care (CCAC) Guide on the care and use of fish in research, teaching, and testing.

Chemicals

L-Proline, Trizma Base, EDTA, EGTA, sodium citrate, Coomassie Blue, bovine serum albumin, acetylthiocholine, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) were obtained from Sigma–Aldrich (St. Louis, MO, USA). All reagents used were of analytical grade.

In vivo treatments

For the *in vivo* studies, animals were exposed at two proline concentrations (1.5 and 3.0 mM) during 1 h (short-term exposure) or 7 days (long-term exposure). During the treatments, the animals were maintained in 4 L test tanks (30 × 15 × 10 cm, length × height × width) with 3 L of water (control group) or water plus proline (1.5 or 3.0 mM) and were kept in the same environmental conditions of the housing tanks. To ensure a similar amount of amino acid present in the aquarium, the tank water was replaced daily. Immediately after the treatments, the fish were cryoanesthetized and further euthanized by decapitation. The whole brains were dissected and the homogenates were prepared.

In order to verify the effects of antipsychotics on proline-induced effects on AChE activity and gene expression, fish were exposed to proline (1.5 and 3.0 mM) during 7 days (long-term exposure) or water (control group). Afterward, the following acute