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Behavioral changes induced by long-term proline exposure are reversed by antipsychotics in zebrafish

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ABSTRACT

Hyperprolinemia is an inherited disorder of proline metabolism and patients affected by this disease may present neurological manifestations, including seizures and cognitive dysfunctions. Moreover, an association between adulthood schizoaffective disorders and moderate hyperprolinemia has been reported. However, the mechanisms underlying these behavioral phenotypes still remain unclear. In the present study, we investigated the effect of proline treatments on behavioral parameters in zebrafish, such as locomotor activity, anxiety, and social interaction. Adult zebrafish (*Danio rerio*) were exposed to proline (1.5 and 3.0 mM) during 1 h or 7 days (short- or long-term treatments, respectively). Short-term proline exposure did not promote significant changes on the behavioral parameters observed. Long-term exposure at 1.5 mM proline significantly increased the number of line crossing (47%), the total distance (29%), and the mean speed (33%) when compared to control group. A significant increase in the time spent in the upper portion of the test tank was also observed after this treatment (91%), which may be interpreted as an indicator of anxiolytic behavior. Proline at 1.5 mM also induced social interaction impairment (78%), when compared to the untreated group after long-term treatment. Moreover, these proline-induced behavioral changes in zebrafish were completely reversed by acute administration of an atypical antipsychotic drug (sulpiride), but not by a typical (haloperidol). These findings demonstrate that proline is able to induce schizophrenia-like symptoms in zebrafish, which reinforce the use of this species as a complementary vertebrate model for studying behavioral phenotypes associated with neurological dysfunctions characteristic of metabolic diseases.

1. Introduction

Hyperprolinemia is a metabolic disease that may be caused by two distinct inherited disorders of proline metabolism. The hyperprolinemia type I (HPI) is provoked by a deficiency of proline oxidase (POX; EC 1.5.1.2) activity. This enzyme is encoded by proline dehydrogenase (PRODH) gene located in the 22q11 chromosomal region. In the hyperprolinemia type II (HPII), the enzyme defect involves Δ^1-pyrroline-5-carboxylic acid dehydrogenase (P5CDh; EC 1.5.1.12) due to mutation in the P5CDH gene (Phang et al., 2001). Studies demonstrated that siblings affected by these diseases may present neurological manifestations, including seizures and cognitive dysfunctions (Di Rosa et al., 2008; Flynn et al., 1989; Phang et al., 2001). Moreover, it has been reported an association between proline metabolism and neuropsychiatric disorders, such as schizophrenia (Jacquet et al., 2005; Oresic et al., 2011). However, the mechanisms related to these behavioral phenotypes still remain unclear.

Evidence supports an influence of proline on excitatory neurotransmission in central nervous system (CNS). A brain-specific high-affinity proline transporter has been identified exclusively in a subset of glutamatergic neurons (Fremeau et al., 1992; Renick et al., 1999). It has also been shown that high proline concentrations activate NMDA and AMPA receptors, suggesting that proline may potentiate the glutamatergic neurotransmission (Cohen and Nadler, 1997; Nadler,

Abbreviations: HPI, Hyperprolinemia type I; POX, Proline oxidase; PRODH, Proline dehydrogenase gene; HPII, Hyperprolinemia type II; P5CDh, Δ^1-pyrroline-5-carboxylic acid dehydrogenase; P5CDH, Δ^1-pyrroline-5-carboxylic acid dehydrogenase gene; NMDA, N-Methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; UFRGS, Federal University of Rio Grande do Sul; COBEA, Brazilian Collegium of Animal Experimentation; CCAC, Canadian Council for Animal Care; DMSO, polymerase chain, dimethylsulfoxide; Pro, Proline; MK-801, dizocilpine; PCP, phencyclidine; DA, dopamine.

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1987; Nadler et al., 1992). This hypothesis is supported by findings that demonstrate that higher proline and glutamate levels could be found in cerebrospinal fluid of hyperprolinemic patients (Phang et al., 2001; Van Harreveld and Fifkova, 1974). Moreover, studies also demonstrated that proline impairs memory (Bavaresco et al., 2005; Delwing et al., 2006) and decreases glutamate uptake in rat brain, as well as the Na^+, K^+-ATPase, creatine kinase, and acetylcholinesterase activities, which are crucial enzymes for normal brain function (Delwing et al., 2005, 2007; Kessler et al., 2003; Pontes et al., 1999, 2001). These reports propose that high proline levels have a detrimental effect on neuronal integrity inducing changes in different neurotransmitter systems. In this sense, the identification of an effective treatment for this disease is going to require a better understanding of the proline-induced physiological and behavioral responses (Wyse and Netto, 2011).

The zebrafish have emerged as an excellent vertebrate model for assessing neurobehavioral phenotypes associated with metabolic diseases, such as hyperprolinemia. Firstly, this species is a well-established model system used in developmental biology and genetic studies because of its known biological features (Zon and Peterson, 2005). Secondly, since zebrafish have optimal absorption and internal distribution of substances added in its tank water, this small teleost is considered also as one of the most cost-effective vertebrates that can be used for high throughput screening and toxicological studies (Kari et al., 2007; Lele and Krone, 1996; Parng et al., 2002). Thus, it can be easily and continuously exposed to different concentrations of amino acids for long periods, whereas in rats the doses administered are rapidly metabolized (Moreira et al., 1989). Furthermore, this species exhibits genetic and anatomic conservation in relation to both mice and humans and a high degree of genetic homology, which is an additional attractive feature for studying genetic basis of human neurological disorders (Barbazuk et al., 2000; Ganser and Dallman, 2009; Guo, 2004; Kabashi et al., 2010). Finally, recent studies have also examined behavioral phenotypes in zebrafish including, social behavior, locomotor activity (Fontaine et al., 2008; Seibt et al., 2010), exploratory activity (Rosemberg et al., 2011), anxiety (Egan et al., 2009), stress (Champagne et al., 2010; Piato et al., 2011), and learning and memory (Blank et al., 2009; Pather and Gerlai, 2009). The effects of neuroactive drugs on behavioral parameters in zebrafish have also been evaluated (Norton and Bally-Cuif, 2010; Seibt et al., 2010; Stewart et al., 2011).

Considering that: (i) the development of novel animal models that can simulate, at least in part, human diseases is a field of growing interest, (ii) new models can contribute to a better understanding of the relevant pathways and mechanisms to the development of clinical treatments for those diseases, (iii) the zebrafish has become a promising model to many human diseases and, finally, (iv) recent studies suggest a relationship between the proline metabolism and psychiatric diseases, we sought to investigate the effects of short- and long-term proline exposure on behavioral parameters in zebrafish, such as locomotion, anxiety, and social interaction. Since antipsychotic drugs are effective in treating neuropsychiatric symptoms, we also verified the effects of typical and atypical antipsychotic drugs on proline-induced behavioral changes in the zebrafish.

2. Materials and methods

2.1. Animals

Adult males and females (approximately in the ratio 1:1) of the "wild type" (short fin — SF) zebrafish (*Danio 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 in 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 number 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.

2.2. Chemicals

Proline, haloperidol, sulpiride, and dimethylsulfoxide (DMSO) were used. The antipsychotics used were from clinical grade/suppliers, while proline and DMSO were purchased from Sigma-Aldrich (St. Louis, USA). Tank water was used as the vehicle for haloperidol and tank water with 5% DMSO was used as the vehicle to sulpiride.

2.3. Experimental protocols

Animals were exposed to two proline concentrations (1.5 and 3.0 mM). For the short-term proline exposure, animals were exposed to treatments for 1 h, while the long-term proline exposure lasted 7 days. The tank water was replaced daily, and behavioral tests were performed immediately after the period of exposure.

In order to verify the effects of antipsychotics on proline-induced behavioral changes, fish were exposed to proline (1.5 mM) during 7 days (long-term exposure). Afterwards, the following acute treatments were performed in a beaker for 15 min: (i) a control group (exposed to water); (ii) a proline group; (iii) a proline group plus DMSO (5%); (iv) a proline group plus sulpiride (250 μM); and (v) a proline group plus haloperidol (9 μM).

The short-term (1 h) and long-term (7 days) proline exposures were performed as previously described in studies with rats and also based on plasma proline levels verified in human hyperprolinemic patients (Delwing et al., 2005; Phang et al., 2001). The antipsychotic concentrations and time of exposure were chosen based on previous studies with zebrafish (Seibt et al., 2010; 2011).

2.4. Behavioral assessment

2.4.1. Locomotion and anxiety

Behavioral testing of drug effects took place during the light phase between 10:00 a.m. and 1:00 p.m. Animals were individually placed in the test tank (30 cm × 15 cm × 10 cm, length × height × width, Fig. 1) immediately after the pharmacological manipulation and kept for 30 s before the video recording as previously described (Gerlai et al., 2000). There was no drug exposure during behavioral experiments.

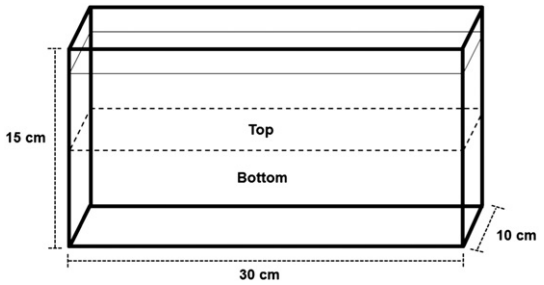

Fig. 1. The apparatus consisted in a rectangular glass tank with the specific dimensions described above and virtual divisions were used to evaluation of zebrafish swimming activity in the novel tank test, with two vertical areas (bottom and top) and eight horizontal sections with 4 sections per area.

The locomotor activity was recorded on video for 5 min after the habituation period and simultaneously analyzed using the ANY-Maze recording software (Stoelting Co., Wood Dale, IL, USA). The tank was divided into equal sections with four vertical lines and one horizontal line, and the following behavior patterns were measured: number of line crossings (vertical and horizontal lines), distance traveled and mean speed. The time spent in each tank position (bottom vs. upper levels) was considered as the index of anxiety. This task exploits the natural tendency for zebrafish to spend most of the time at the bottom when introduced into a novel environment and then gradually to extend the swimming range, over a period of minutes, to include the upper portions of the test tank (Levin et al., 2007). A longer time spent in the bottom and less time spent in the top part of the tank indicates heightened anxiety (Levin et al., 2007). Visual observations throughout the experimental periods allow the documentation of erratic movements, defined as sharp changes in direction or velocity and repeated rapid darting behaviors (Levin et al., 2007). In addition, these movements may be manifested by bouts of vertical swimming or sideways swimming, suggesting a problem with coordination (Giacomini et al., 2006).

2.4.2. Social interaction

The zebrafish is a schooling fish that may exhibit preference for its conspecifics under certain circumstances. The rationale behind using a group of five fish as subjects is that this social setting biases behavior toward schooling. Fish were placed in groups of five in a small experimental tank (30×15×10 cm, length×height×width, Fig. 1). On one side of the test tank an empty tank was placed, and on the other side, a tank of identical size held 15 zebrafish, designed as "stimulus fish". The experimental fish were kept in the experimental tank for a 30 s period, after which their behavior was video recorded during 10 s. In order to quantify their preference between the "stimulus fish" side of their tank in detriment of the empty tank, the experimental fish tank was divided in two equal sections and the amount of time the five experimental fish spent on the side of the tank closer to that the conspecific school was measured using an event recorder program (Gerlai et al., 2000).

2.5. Statistical analysis

Results were expressed as mean ± standard error of mean (S.E.M.). Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by a Tukey multiple range test. Statistically significant differences between groups were considered for a $p<0.05$.

3. Results

3.1. Effects of proline on behavioral parameters in zebrafish

The effects of proline were evaluated on behavioral parameters in zebrafish after short- (1 h) or long-term (7 days) treatments. Short-term proline exposure did not promote significant changes on behavioral parameters examined in this study. However, after long-term exposure, proline at 1.5 mM induced significant changes on parameters of zebrafish swimming activity. As indicated by the number of line crossings, locomotor activity increased (47%; $F(2,18)=10$; $p<0.01$) when compared with the control group (215.8 ± 10.4 line crossings) (Fig. 2A). Long-term proline exposure at 1.5 mM also increased the distance traveled (29%; $F(2,18)=6.2$; $p<0.05$) and the mean speed (33%; $F(2,18)=5.2$; $p<0.05$) in relation to control group (17.8 ± 0.9 m; 0.059 ± 0.003 m/s) (Fig. 2B and C). We also observed a significant increase in the time spent in the upper portion of the test tank (91%; $F(2,18)=7.9$; $p<0.05$) when compared with the control group (97.3 ± 22.2 s) (Fig. 2D), which may be interpreted as an indicator of anxiolytic behavior. The long-term proline exposure

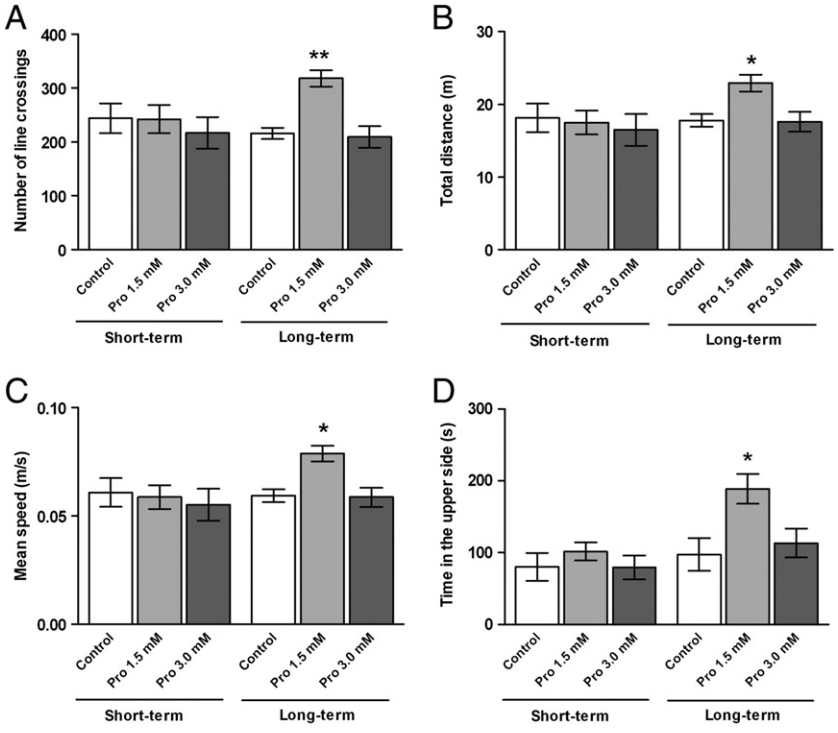

Fig. 2. Effect of short-term (1 h) and long-term (7 days) proline exposure on the number of line crossings (A), distance traveled (B), mean speed (C), and time spent in the upper zone (D) in zebrafish determined during 5 min of videorecording in the tank diving behavioral test. Fishes were exposed at two proline concentrations (1.5 and 3.0 mM) during 1 h or 7 days (short- or long-term treatments, respectively). Data were expressed as mean ± S.E.M. of at least 6 animals for each group and were analyzed by one-way ANOVA followed by followed by Tukey test as post-hoc test. The asterisks represent $p<0.05$ (*) and $p<0.01$ (**).

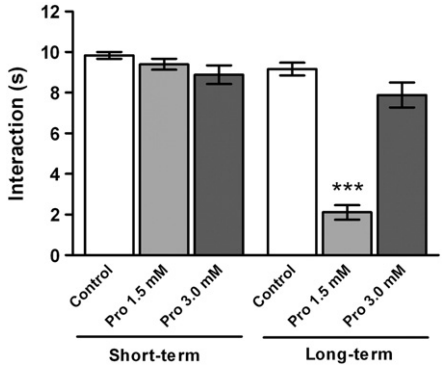

Fig. 3. Effect of short-term (1 h) and long-term (7 days) proline exposure on social behavior in zebrafish. Fishes were exposed at two proline concentrations (1.5 and 3.0 mM) during 1 h or 7 days (short- or long-term treatments, respectively). Data were expressed as mean ± S.E.M. of at least 6 animals for each group and were analyzed by one-way ANOVA followed by followed by Tukey test as post-hoc test. The asterisks represent $p<0.001$ (***).

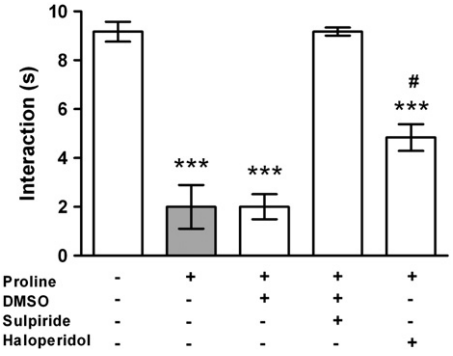

Fig. 5. Effects of haloperidol and sulpiride on proline-induced social interaction deficits in zebrafish. Fishes were exposed to proline (1.5 mM) during 7 days (long-term exposure). Afterwards, the following acute treatments were performed in a beaker for 15 min: (i) a control group (exposed to water); (ii) a proline group; (iii) a proline group plus DMSO (5%); (iv) a proline group plus sulpiride (250 μM); and (v) a proline group plus haloperidol (9 μM). Data were expressed as mean ± S.E.M. of at least 6 animals for each group and were analyzed by one-way ANOVA followed by followed by Tukey test as post-hoc test. The asterisks represent $p<0.001$ (***) compared to untreated group; and # $p<0.05$ when compared to proline group.

at 3.0 mM did not promote significant changes on parameters of zebrafish swimming activity when compared to the untreated group. Moreover, we also examined the effects of proline on social interaction after short- and long-term exposures. The results demonstrated that only long-term proline exposure at 1.5 mM induced social interaction impairment (78%; $F(2,21)=62.10$; $p<0.001$) in zebrafish when compared to the untreated group (9.2 ± 0.3 s) (Fig. 3).

3.2. Antipsychotic drugs reverse behavioral changes induced by long-term proline exposure

Since long-term proline exposure at concentration of 1.5 mM induced hyperlocomotor behavior and social interaction impairment, we also verified whether typical (haloperidol) and atypical (sulpiride) antipsychotic drugs are able to reverse these proline-induced behavioral changes. The results confirmed that long-term proline exposure significantly increased the number of line crossings ($F(4,27)=16.8$; $p<0.001$), the distance traveled ($F(4,27)=17.22$; $p<0.001$), the mean speed ($F(4,27)=13.18$; $p<0.001$), and the time spent in the upper portion ($F(4,27)=18.88$; $p<0.001$). The post-hoc test showed that only sulpiride was able to reverse these proline-induced effects compared to the untreated group ($p>0.05$) (Fig. 4A, B, C and D). Fig. 5 shows that long-term proline exposure induced social interaction impairment ($F(4,25)=41.78$; $p<0.001$), post-hoc analysis showed that sulpiride reversed the social impairment ($p>0.05$), while haloperidol was able to partially reversed this effect as compared to proline group ($p<0.05$).

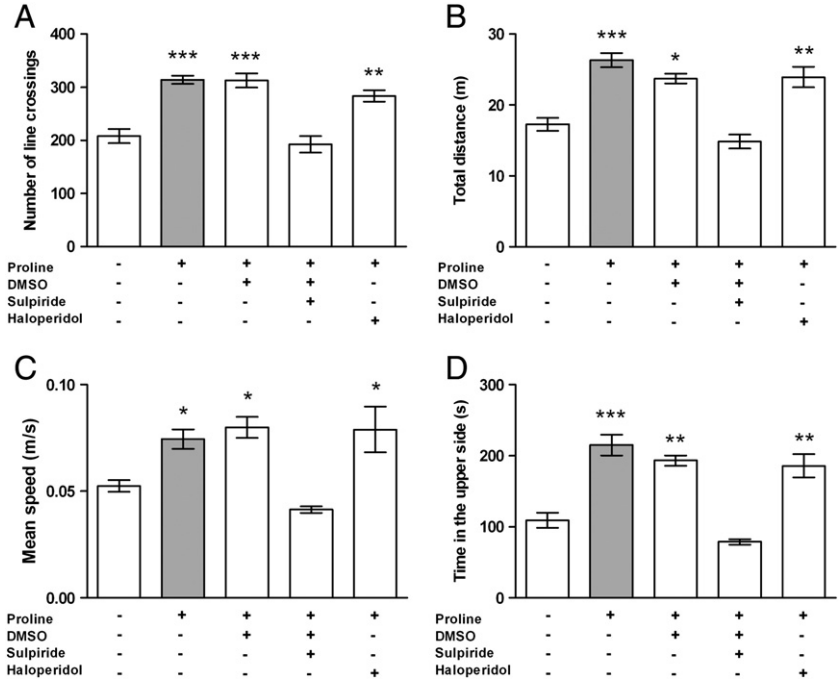

Fig. 4. Effects of haloperidol and sulpiride on proline-induced increased number of line crossings (A), distance traveled (B), mean speed (C), and time spent in the upper zone (D) in zebrafish determined during 5 min of videorecording in the tank diving behavioral test. Fishes were exposed to proline (1.5 mM) during 7 days (long-term exposure). Afterwards, the following acute treatments were performed in a beaker for 15 min: (i) a control group (exposed to water); (ii) a proline group; (iii) a proline group plus DMSO (5%); (iv) a proline group plus sulpiride (250 μM); and (v) a proline group plus haloperidol (9 μM). Data were expressed as mean ± S.E.M. of at least 6 animals for each group and were analyzed by one-way ANOVA followed by followed by Tukey test as post-hoc test. The asterisks represent $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

4. Discussion

Hyperprolinemic patients usually present neurological manifestations and the proline metabolism seems to be specifically related to psychotic disorders, such as schizophrenia (Jacquet et al., 2005; Oresic et al., 2011; Phang et al., 2001). Although the mechanisms that lead to abnormal brain function in these patients remain unclear, there is evidence that at least part of the pathology and symptomatology of hyperprolinemia results from a dysfunction of the glutamatergic neurotransmission (Vorstman et al., 2009). Nevertheless, there is no effective treatment for this disease and few studies have been conducted to identify potential therapeutic mechanisms to minimize the impact of the symptoms on patient's quality of life (Mitsubuchi et al., 2008; Wyse and Netto, 2011). Therefore, in this study, we characterized the effects of proline exposure on behavioral parameters in zebrafish, a promising vertebrate model for studying the mechanisms underlying human diseases and pharmacological treatments. Furthermore, we demonstrated that proline-induced behavioral changes were completely reversed by acute administration of an atypical antipsychotic drug (sulpiride), but not by a typical (haloperidol).

The influence of proline on glutamatergic system has become more evident over the last few years. Several studies showed that high proline concentrations activate NMDA and AMPA receptors, suggesting that proline may potentiate the glutamatergic neurotransmission and increase glutamate release (Cohen and Nadler, 1997; Nadler, 1987; Nadler et al., 1992). Interestingly, NMDA receptor antagonists, such as dizocilpine (MK-801) and phencyclidine (PCP), psychomimetic drugs used as pharmacological model of schizophrenia, also increase the glutamate release and this glutamatergic dysfunction may lead to secondary dopamine (DA) release in the prefrontal cortex (Moghaddam, 2002; Moghaddam and Adams, 1998). A putative modulatory effect of proline on glutamatergic transmission inducing DA release in the brain has been proposed (Paterlini et al., 2005; Vorstman et al., 2009). In agreement with this hypothesis, we showed that long-term proline exposure at concentration of 1.5 mM induces hyperlocomotion and social interaction impairment (schizophrenia-like symptoms) in zebrafish. Likewise, studies demonstrated that MK-801 (a NMDA receptor antagonist) induced hyperlocomotion and social deficits in zebrafish (Seibt et al., 2010, 2011).

We also demonstrated that proline at 3.0 mM was not able to induce these schizophrenia-like symptoms. Authors have reported that increased levels of DA result in a dose dependent response, which resembles an inverted U curve (Lavergne and Jay, 2010; Vijayraghavan et al., 2007). The DA inverted U dose–response curve of NMDA receptor effects seems to provide a regulatory mechanism that may be protective for the neuron, preventing toxic responses (Lavergne and Jay, 2010; Skolnick et al., 2009). Therefore, the potential effect of proline at 3.0 mM on the glutamatergic system could induce a higher dopamine release at non-responsive levels. As a result, we did not observe behavioral changes at this proline concentration.

Antipsychotic drugs are widely used for the treatment of neuropsychiatric disorders, including schizophrenia. Previous studies showed that antipsychotic drugs are able to reverse the MK-801-induced hyperlocomotion and social interaction deficits in zebrafish. In addition, the authors demonstrated that these drugs per se did not alter these behavioral parameters (Seibt et al., 2010, 2011). As we already reported, we showed in our experiment that an atypical antipsychotic drug completely reversed the proline-induced hyperlocomotion and social deficits while a typical antipsychotic was only able to attenuate the social impairment.

Several mechanisms may be involved in the inhibitory effect of antipsychotic drugs on proline-induced hyperlocomotion and social interaction deficits. Typical antipsychotics, such as haloperidol, act preferentially via dopamine D_2 receptor blockade and induce severe motor side effects (Heusler et al., 2008). Atypical antipsychotics, while less potent than their typical counterparts in blocking central D_2 receptors, have affinity for a wide range of other receptors including dopaminergic D_1 and D_4, serotonergic 5-HT2A and 5-HT6, adrenergic α1, histaminergic H1, and muscarinic M1 (Jones et al., 2008). Sulpiride, an atypical antipsychotic, acts preferentially via D_2 and D_3 dopamine receptor blockade (Jaworski et al., 2001; Tadori et al., 2011). In agreement to our data, studies have shown that atypical antipsychotics are more potent than typical antipsychotic drugs in inhibiting the locomotor activity and social impairment induced by NMDA receptor antagonists (Geyer et al., 2001; Jentsch and Roth, 1999; Seibt et al., 2010, 2011). Boulay et al. (2004) reported that haloperidol did not reverse acute NMDA antagonist-induced deficits in social investigation. On the other hand, Linck et al. (2008) showed that sulpiride completely prevented the MK-801-induced social deficits. Therefore, it is possible that sulpiride completely reverses the proline-induced hyperlocomotion and social deficit through a similar mechanism.

At last, a proline-induced anxiolytic-like effect was also observed in our study after long-term exposure at 1.5 mM. This data is in agreement with a previous study in zebrafish, in which MK-801 induced anxiolytic-like behavior (Seibt et al., 2010). Other studies conducted in mammalian models also reported that animals treated with MK-801 and submitted to the elevated plus-maze test presented an increase in time spent in the open arms, indicative of an anxiolytic-like effect (Bertoglio and Carobrez, 2003; Dunn et al., 1989). Seibt et al. (2010) also reported that antipsychotic drugs failed to reverse the anxiolytic-like effect of MK-801 in zebrafish. However, we showed that the proline-induced anxiolytic-like effect was reversed only by sulpiride, as we also verified in the other behavioral parameters analyzed in this study.

5. Conclusion

In summary, our findings demonstrated that long-term proline exposure induced schizophrenia-like behaviors in zebrafish, suggesting an influence of this amino acid on glutamatergic and dopaminergic systems. Moreover, these behavioral changes were completely reversed by acute administration of an atypical antipsychotic drug, but not by a typical antipsychotic. These data may contribute to a better understanding of the pathophysiological mechanisms that increase the susceptibility to psychotic disease in hyperprolinemic patients. In addition, such findings might facilitate the use of zebrafish as a complementary vertebrate model for studying inborn errors of metabolism and pharmacological treatments as well as for assessing behavioral phenotypes associated with these diseases.

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