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Typical and atypical antipsychotics alter acetylcholinesterase activity and *ache* expression in zebrafish (*Danio rerio*) brain

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ABSTRACT

Antipsychotic agents are widely used for the treatment of psychotic symptoms in patients with several brain disorders. Antipsychotic drugs principally affect dopamine systems with the newer ones also affecting serotonin, norepinephrine, and histamine systems. Other transmitter systems can be involved with selected antipsychotic drugs but effects on cholinergic system are less known. Considerable evidence has shown that complex interactions between dopaminergic and cholinergic systems are critical for the proper regulation of motor control and memory. These neurotransmitter systems have been studied in zebrafish, which has recently become a focus of neurobehavioral studies. Therefore, we have evaluated the *in vitro* and *in vivo* effects of sulpiride, olanzapine, and haloperidol on acetylcholinesterase activity and *ache* expression pattern in zebrafish brain. For *in vitro* studies, all drugs were able to promote a decrease on acetylcholinesterase activity. For *in vivo* studies, olanzapine and sulpiride exposure did not change acetylcholinesterase activity. In contrast, this enzyme activity was significantly increased at 5 and 9 μM haloperidol (29.9% and 20.4%, respectively). Haloperidol exposure was able to increase acetylcholinesterase mRNA transcripts. These findings have suggested that the alterations in zebrafish acetylcholinesterase could reveal molecular mechanisms related to cholinergic signaling induced by antipsychotic treatment.

1. Introduction

Antipsychotic agents have been increasingly used for treatment of psychotic symptoms and agitation in patients with a variety of brain disorders, such as schizophrenia, bipolar disorder, Alzheimer's disease, and traumatic head injury (Bascunana et al., 2000; Daniel, 2000). Long-term treatment with some of these drugs may be associated with the development of a wide range of undesired effects, including debilitating extrapyramidal symptoms and cognitive dysfunction. The different therapeutic actions and side-effect profiles for typical and atypical antipsychotics have been explained based on preferential actions at specific receptors (Kinon and Lieberman, 1996; Richelson and Souder, 2000). Typical antipsychotics, such as haloperidol, act preferentially via dopamine D2 receptor blockade (Heusler et al., 2008). Atypical antipsychotics, such as olanzapine, cause less extrapyramidal symptoms than standard antipsychotics and have been classified based on less selective activity across several neurotransmitter receptors (moderately potent $5HT_2$ receptor antagonists with lesser and equal potency for dopamine D1, D2, and α_1-adrenergic receptors) (Wadenberg et al., 2001; Seeman, 2002). Sulpiride, another atypical antipsychotic, acts preferentially via D2 and D3 dopamine receptor blockade (Jaworski et al., 2001; Elisabetsky and Costa-Campos, 2006).

Although neuropsychiatric and neurodegenerative diseases have been attributed to deficits within a single neurotransmitter system, disease progression might be related to the deficit of the initially affected system to modulate or be modulated by other neurotransmitters. The extrapyramidal motor system, for example, relies on a balance between dopamine and acetylcholine, and disruption in the balance results in motor abnormalities in monkey brain (Tsukada et al., 2000). Acetylcholine plays an important role in motor functions and various domains of cognition, attention (Perry et al., 1999), and working memory (Winkler et al., 1995). Studies have been shown that acute administration of D2 dopaminergic agonist inhibits acetylcholine release in striatum whereas D1 agonist administration increases its release in rats (Bertorelli and Consolo, 1990). Furthermore, in the striatum, a region known to be critically involved in extrapyramidal motor control, the M4 as well as other muscarinic receptor subtypes are coexpressed with D1 and D2 dopamine receptors on mice striatal projection neurons (Gomeza et al., 1999). Studies have also shown a possible participation of nicotinic

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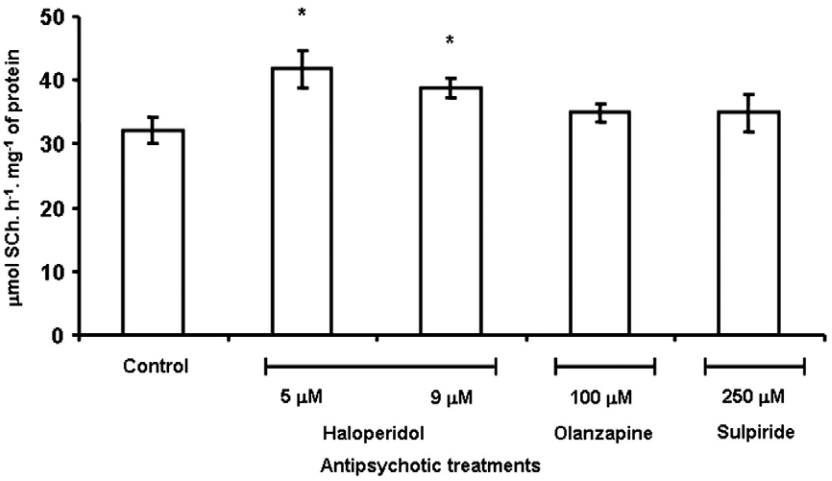

Fig. 1. Effect *in vivo* of haloperidol (5 and 9 µM), olanzapine (100 µM), and sulpiride (250 µM) on acetylcholinesterase activity in zebrafish brain. Data represent means ± S.D. of four different experiments, each one performed in triplicate. The symbol (*) indicates difference when compared to the control group. Data were analyzed statistically by one-way ANOVA followed by Tukey test as post-hoc test, considering a $P<0.05$ significant.

acetylcholine receptors in midbrain dopaminergic nuclei in mice, which may modulate reinforcement and motor behavior in different manners and may be involved in drug addiction, schizophrenia, and Parkinson's disease (Zhou et al., 2001). It has been shown that nicotine enhances cognitive functioning in zebrafish by modulating the release of dopamine in the brain or by changing the rate at which dopamine is metabolized (Eddins et al., in press). Zebrafish express functional β3, α2, and α7 nicotinic receptors and these receptors show a high degree of similarity with mammalian nicotinic receptors (Zirger et al., 2003). Proteins necessary for dopaminergic signaling have been detected in the zebrafish brain of which includes tyrosine hydroxylase and dopamine transporters (Holzschuh et al., 2001). Zebrafish also express functional D1, D2, D3, and D4 dopaminergic receptors (Boehmler et al., 2007). This demonstrates that even though there are structural differences between the zebrafish and the rodent brain, similar signaling mechanisms are important for mediating behavior.

Acetylcholine is synthesized by choline acetyltransferase, then secreted from the presynaptic nerve terminal and bound to acetylcholine receptors, which are clustered in the postsynaptic membrane. After its release, it is rapidly metabolized from the synaptic cleft by acetylcholinesterase, which belongs to the family of type B carboxylesterases and cleaves acetylcholine into choline and acetate (Soreq and Seidman, 2001). Two different types of cholinesterases are able to hydrolyze acetylcholine: acetylcholinesterase (E.C.3.1.1.7) and butyrylcholinesterase (E.C.3.1.1.8). Haloperidol has been observed to increase choline acetyltransferase and acetylcholinesterase activities in the rat striatum and hippocampus after short-term treatment (7–21 days). However, after long-term treatment (+40 days), there is a decrease only on choline acetyltransferase activity and immunoreactivity, as indicated by an apparent reduction in the size and number of stained neurons and their processes (Mahadik et al., 1988).

Zebrafish have recently become a focus of neurobehavioral studies since display learning, sleep, conditioned place preference to several drugs, as amphetamine, cocaine, and opiates (Darland and Dowling, 2001; Guo, 2004; Ninkovic and Bally-Cuif, 2006; Bretaud et al., 2007). However, reduced complexity of the zebrafish brain is a limitation to establish comparisons between this specie and human. Butyrylcholinesterase was not found in zebrafish genome and acetylcholinesterase is encoded by a single gene that was already cloned, sequenced and functionally detected in zebrafish brain (Clemente et al., 2004). This species also holds a great potential for our understanding of the genetic basis of behavior and associated behavioral disorders (Amsterdam and Hopkins, 2006; Krens et al., 2006), because zebrafish has shown genetic conservation with both mice and humans (Dooley and Zon, 2000). Fluphenazine and haloperidol, characterized for inducing severe extrapyramidal symptoms in humans, might promote movement defects in zebrafish treated with these drugs (Giacomini et al., 2006).

Considering that zebrafish may be an relevant vertebrate model system for numerous human diseases and that cholinergic and dopaminergic systems have been described in zebrafish brain, the aim of this study is to evaluate the *in vitro* and *in vivo* effects of different concentrations of haloperidol, sulpiride, and olanzapine on acetylcholinesterase activity from zebrafish brain followed by a gene expression pattern analysis.

2. Methods

2.1. Animals

Adult zebrafish (*Danio rerio*; age around 2–3 months) of both sexes were obtained from a commercial supplier (Delphis, RS, Brazil) and acclimated for at least 2 weeks in a 50-L aquarium. The fish were kept on a 12 h light/dark cycle (lights on at 7:00 h) at a temperature of 25 ± 2 °C. They were used according to the National Institute of Health Guide for Care and Use of Laboratory Animals, being healthy and free of any signs of disease. The Ethics Committee of Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS) approved the protocol under the number 014/08 – CEUA. The experiments were performed between 8:00 h and 13:00 h.

2.2. Chemicals

Haloperidol, olanzapine, sulpiride, Trizma Base, ethylenedioxy–diethylene–dinitrilo–tetraacetic acid (EDTA), ethylene glycol bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), sodium citrate, Coomassie Blue G, bovine serum albumin, acetylthiocholine, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma-Aldrich (USA). All other reagents used were from analytical grade.

2.3. In vivo treatments

For *in vivo* assay, five fish were kept in 1-L aquariums and exposed to water with haloperidol (5 and 9 µM), olanzapine (100 µM), and sulpiride (250 µM). The animals were maintained in the test aquarium during 2 h and, immediately after the exposure, the fish were euthanized. The drug solutions were changed for each experiment. For the control group, the animals were exposed only to water in a test

aquarium during 2 h and, after this time period the fish were euthanized. The haloperidol dose and time of treatment *in vivo* were chosen based on previous studies with zebrafish (Giacomini et al., 2006). The other concentrations of antipsychotic agents used in this study were chosen based on drug potencies observed in human (McClelland et al., 1990; Kiang et al., 2003) and rat (Ichikawa et al., 1998; Parikh et al., 2004) studies.

2.4. In vitro treatments

For the *in vitro* assays, haloperidol, olanzapine, and sulpiride at the final concentrations of 1, 10, 50, 100, and 250 μM were added to reaction medium (described below), pre-incubated for 10 min with the homogenized and maintained throughout the enzyme assay. For the control group, the enzyme assay was performed in the absence of antipsychotics (no drug added in the reaction medium).

2.5. Determination of AChE activity

Zebrafish were euthanized by decapitation, their brains were removed from the cranial skull by the dissection technique. A pool of five total brains of zebrafish was used for each experiment. The brains were homogenized on ice in 60 vol (v/w) of Tris-citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4, with citric acid) in a motor driven Teflon-glass homogenizer. The rate of acetylthiocholine hydrolysis (0.8 mM) was determined in a final volume of 2 ml with 100 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB using a method previously described (Ellman et al., 1961). Before the addition of substrate, samples containing protein (10 μg) and the reaction medium described above were preincubated for 10 min at 25 °C. Thiocholine released because the cleavage of acetylthiocholine by acetylcholinesterase is allowed to react with the −SH reagent DTNB, which is a reduced to thionitrobenzoic acid, a yellow coloured anion with absorption maxima at 412 nm for 2–3 min (30-second intervals). Controls without the homogenate preparation were performed in order to determine the non-enzymatic hydrolysis of acetylthiocholine. The linearity of absorbance related to time and protein concentration was previously determined. Acetylcholinesterase activity was expressed as micromole of thiocholine (SCh) released per hour per milligram of protein. Four different experiments were performed and the assays were run in triplicate.

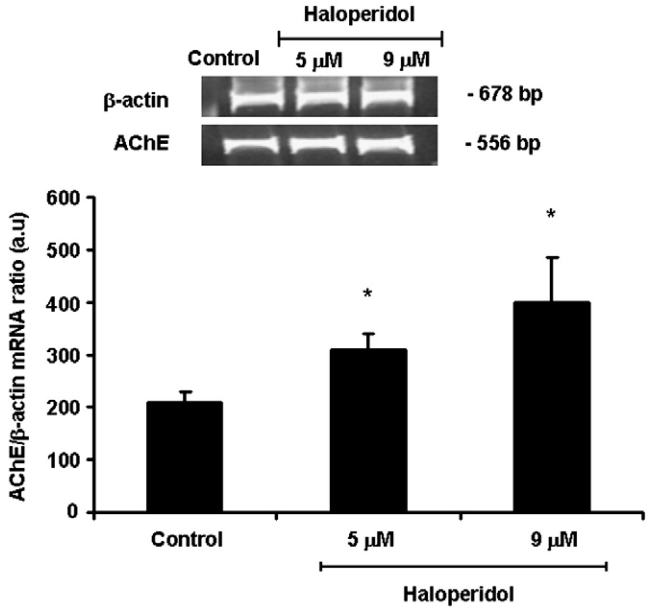

Fig. 2. Acetylcholinesterase and β-actin mRNA expression in adult zebrafish brain. Fish were exposed to haloperidol concentrations (5 and 9 μM), the brains were excised and RT–PCR experiments were conducted. The PCR products were subjected to electrophoresis on a 1% agarose gel, using β-actin as constitutive gene. The figure shows a representative gel and the *ache*/β-actin mRNA ratio (expressed as arbitrary units) obtained by optical densitometry analysis of three independent experiments, with entirely consistent results. The symbol (*) indicates difference when compared to the control group. The data were expressed as means ± S.D. and analyzed statistically by one-way ANOVA followed by Tukey test as post-hoc test, considering a $P<0.05$ significant.

2.6. Protein determination

Protein was measured using Coomassie Blue as color reagent and bovine serum albumin as standard (Bradford, 1976).

2.7. Molecular analysis

Forward (5′-CCAAAAGAATAGAGATGCCATGGACG-3′) and reverse (5′-TGTGATGTTAAGCAGACGAGGCAGG-3′) *ache* primers and optimal conditions for RT–PCR (reverse transcription–polymerase chain reaction) were used according to Rico et al. (2006). The β-actin primers forward (5′-GTCCCTGTACGCCTCTGGTCG-3′) and reverse (5′-GCCGGACTCATCGTACTCCTG-3′) were used as described previously (Chen et al., 2004).

Immediately after *in vivo* treatment with haloperidol (described above), the animals were euthanized by decapitation, their brains were removed from the cranial skull by the dissection technique. For each sample, a pool of five zebrafish brains was used. Total RNA was isolated from zebrafish brain using TRIzol reagent (Invitrogen) in accordance with manufacturer instructions. RNA was quantified by spectrophotometer and all samples were adjusted to 160 ng/μL. cDNA species were synthesized using SuperScript III™ First-Strand (Synthesis System for RT–PCR) Invitrogen Kit following the supplier instructions. One microliter of RT reaction mix was used as a template for each PCR. PCR for *ache* was performed in a total volume of 25 μL using 0.08 μM of each primer, 0.2 μM dNTP (Deoxyribonucleotide triphosphate), 2 mM $MgCl_2$ and 1U Taq DNA polymerase (Invitrogen). PCR for β-actin gene was performed in 20 μL using 0.1 μM of each primer, 0.2 μM dNTP, 2 mM $MgCl_2$ and 0.5U Taq DNA polymerase (Invitrogen). PCR were conducted at 1 min at 94 °C, 1 min at 60 °C (*ache*) and at 54 °C (β-actin), and 1 min at 72 °C for 35 cycles. A post-extension cycle was performed for 10 min at 72 °C. For each PCR set, a negative control was included. PCR products were analyzed on 1% agarose gel, containing GelRed® and visualized with ultraviolet light. The low DNA Mass Ladder (Invitrogen) was used as a molecular marker and normalization was performed employing β-actin as a constitutive gene. The band intensities were measured by optical densitometry analysis and the enzyme/β-actin mRNA ratios were established for each treatment using the Kodak 1D Image Analysis Software.

2.8. Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) and expressed as means ± S.D. A Tukey multiple test range as post-hoc was performed considering a significance level of 5%.

3. Results

The *in vivo* effect of acute antipsychotic treatment has been also demonstrated on zebrafish acetylcholinesterase. The experiments have been performed after a 2 h-exposure to sulpiride (250 μM), olanzapine (100 μM), and haloperidol (5 and 9 μM). Olanzapine and sulpiride did not promote a significant difference on acetylcholinesterase activity in zebrafish brain. However, this enzyme activity has been significantly increased at 5 and 9 μM haloperidol (29.9% and 20.4%, respectively) when compared to the control group (Fig. 1). These effects are in agreement with previous studies showing that

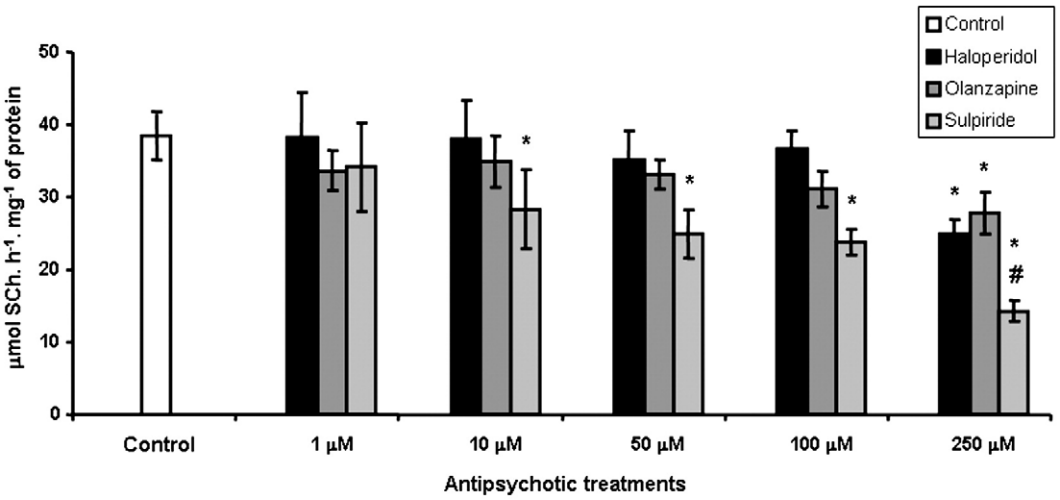

Fig. 3. Effect *in vitro* of different concentrations of sulpiride, haloperidol, and olanzapine on acetylcholinesterase activity in zebrafish brain. Data represent means ± S.D. of four different experiments, each one performed in triplicate. The symbol (*) indicates difference when compared to the control group, whereas (#) represents a significant difference when compared to other sulpiride treatments. Data were analyzed statistically by one-way ANOVA followed by Tukey test as post-hoc test, considering a $P<0.05$ significant.

haloperidol-treated fish exhibited significant erratic movements whereas olanzapine had minimal such effect (Giacomini et al., 2006).

The increase of acetylthiocholine hydrolysis promoted by haloperidol exposure could be a consequence of transcriptional control and/or post-translational regulation. RT–PCR analyses have been performed when kinetic alterations have been observed. The results have demonstrated that the relative amount of acetylcholinesterase mRNA has been significantly increased (48% and 91%) after treatment with 5 and 9 µM haloperidol, respectively (Fig. 2).

In order to evaluate if the drugs could promote a direct effect on the enzyme, we tested the *in vitro* effect of antipsychotics on acetylcholinesterase activity in zebrafish brain. All drugs were able to promote a significant decrease on zebrafish brain acetylcholinesterase activity. Haloperidol and olanzapine have significantly decreased the enzyme activity at 250 µM (39.4% and 25.1%, respectively) (Fig. 3). Sulpiride has promoted a significant inhibition of acetylcholinesterase activity ranging from 10 to 250 µM (24.5–61.8%) (Fig. 3).

4. Discussion

In the present study, we have observed significant changes on acetylcholinesterase activity and *ache* expression pattern in zebrafish brain after *in vitro* and *in vivo* exposure to antipsychotic drugs.

Considerable evidence suggests that complex interactions between dopaminergic and cholinergic systems are critical for the proper regulation of motor control (Di Chiara et al., 1994). Consistent with this notion, the severe motor deficits observed in patients suffering from Parkinson's disease and other extrapyramidal disorders are thought to reflect an imbalance between cholinergic and dopaminergic tone in the striatum (Brown and Taylor, 1996). In addition, cholinergic activity in the brain is essential to cognitive processes and motor control (Harder et al., 1998; Baxter et al., 1999). Studies have shown that deficits of working memory and attention are characteristic impairments in patients with schizophrenia (Elvevag and Goldberg, 2000). Typical antipsychotics with dopamine D2 antagonistic properties, such as haloperidol, have been shown to impair working memory in schizophrenic patients. Conversely, atypical antipsychotics with fewer dopamine D2 antagonistic properties, such as olanzapine, have improved working memory in schizophrenia (Bilder et al., 2002). Moreover, olanzapine, but not haloperidol, has improved consolidation processes in rats in a delayed radial maze task (Wolff and Leander, 2003). Interestingly, olanzapine has markedly increased acetylcholine release in rat hippocampus whereas haloperidol has produced only a slight increase (Johnson et al., 2005). These findings suggest that an increase in acetylcholine release could be involved in the ameliorative effect of olanzapine. Considering that in our study only haloperidol treatment increased acetylcholinesterase activity, it is possible to suggest that a decrease in acetylcholine levels could be involved in cognitive deficit observed after haloperidol treatment.

The alterations promoted by haloperidol in acetylcholinesterase activity could be a consequence of transcriptional control. In order to verify whether the acetylcholinesterase gene could be modulated when zebrafish were exposed to haloperidol after 2 h, we have performed semi-quantitative RT–PCR experiments after 5 and 9 µM haloperidol treatments. Interestingly, the results have demonstrated that acetylcholinesterase mRNA levels have been significantly increased after haloperidol exposure, suggesting that the increase of acetylcholinesterase activity observed in this treatment may be directly related to a higher *ache* expression.

Our results have also shown that all drugs tested were able to promote a significant decrease on acetylcholinesterase activity from zebrafish brain, when directly added in the enzyme assays, as performed for *in vitro* experiments. Differential effects promoted by haloperidol on AChE activity was observed for *in vivo* and *in vitro* experiments. These results could be related to the fact that the *in vitro* experiments evaluate the direct effect of the drug on the enzyme, without the influence of other biological systems, such as cell signaling pathways. Furthermore, therapeutic agents such as antipsychotic drugs that may interact with lipids arrangement are likely to modify membrane biological properties (Tessier et al., 2008). Since acetylcholinesterase is anchored to the outer surface of the plasma membrane by a covalently attached glycosyl-phosphatidylinositol (GPI) structure, it is possible to suggest that changes in membrane bilayer environment promoted by the interaction with antipsychotics may be able to promote the inhibitory effect observed on acetylcholinesterase activity for *in vitro* experiments. For *in vivo* studies, we cannot exclude a possible direct effect of the drug in the membrane; however, the action of signaling pathways may modulate the cholinergic system promoting an increase of AChE activity.

Studies have described that chronic exposure to haloperidol impaired spatial learning performance in rats, which was associated with a reduction in central cholinergic markers (Terry et al., 2002, 2003). In contrast to typical agents like haloperidol, atypical antipsychotics are associated with reduced (or a lack of) extrapyramidal symptoms in schizophrenic patients (Buckley, 2001). Furthermore, atypical

antipsychotics are more effective in reducing negative symptoms and improving cognitive performance (Cuesta et al., 2001). These findings are significant, particularly since a wide range of cognitive deficits (i.e. attention, learning, memory, and executive functions) is generally found in these patients even in the earlier stages of their illness (Tollefson, 1996). Since extrapyramidal symptoms are commonly induced by typical antipsychotics, such as haloperidol, and atypical drugs are characterized by their lesser occurrence, the different response induced by these drugs on acetylcholinesterase could differentially modulate the acetylcholine levels, which could be involved in the higher or lower susceptibility to these undesired effects.

Because of the different neural architecture, the relevant brain regions for neurobehavioral functions in zebrafish certainly differ from those in mammals. Lesion studies show that the teleost cerebellum is essential in classical conditioning of discrete motor responses. The lateral telencephalic pallium of the teleost fish, proposed as homologous to the hippocampus, is selectively involved in spatial learning and memory, and in trace classical conditioning. In contrast, the medial pallium, considered homologous to the amygdala, is involved in emotional conditioning in teleost fish. The optic tectum, positioned much like the neocortex in mammals and constituting a considerable portion of the zebrafish brain (Wulliman and Rink, 2002) is the major processing area for visual processing in zebrafish (Kaethner and Stuermer, 1997). Therefore, there is a remarkable parallelism between mammals and teleost fish concerning the role of different brain centers in learning and memory and cognitive processes.

Several studies have investigated the involvement of dopaminergic systems to behavioral outcomes in adult zebrafish as well as developing zebrafish larvae (Anichtchik et al., 2004; Bretaud et al., 2004; McKinley et al., 2005). Furthermore, previous studies have found that nicotine causes significant improvement in memory function as measured by a delayed task of spatial alternation (Levin and Chen, 2004) in zebrafish. This is quite similar to the effects of nicotine and other cognitive enhancing drugs in rodents, monkeys, and humans (Levin and Rezvani, 2002; Levin and Simon, 1998). There are previous studies in the literature evaluating several behavioral parameters in zebrafish after exposure to antipsychotic drugs. Giacomini et al. (2006) have showing that haloperidol and fluphenzine, both typical antipsychotics characterized to promote EPS in humans, develop erratic movements in zebrafish larvae, in contrast to olanzapine, which present minimal effects. These findings are in agreement with our results, which haloperidol promoted changes in AChE activity after *in vivo* exposure.

In summary, the findings of this study indicated that haloperidol treatment leads to changes in acetylcholinesterase activity and *ache* expression. On the other hand, atypical antipsychotics, such as olanzapine and sulpiride did not alter the acetylcholine degradation. Therefore, these findings have suggested that the alterations on zebrafish acetylcholinesterase could reveal molecular mechanisms related to cholinergic signaling induced by haloperidol treatment, which could be involved in adverse motor effects and cognitive dysfunction induced by this drug.

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