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ORIGINAL ARTICLE

Perinatal Glyphosate-Based Herbicide Exposure in Rats Alters Brain Antioxidant Status, Glutamate and Acetylcholine Metabolism and Affects Recognition Memory

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Abstract

Glyphosate-based herbicides (Gly-BHs) lead the world pesticide market. Although are frequently promoted as safe and of low toxicity, several investigations question its innocuousness. Previously, we described that oral exposure of rats to a Gly-BH during pregnancy and lactation decreased locomotor activity and anxiety in the offspring. The aim of the present study was to evaluate the mechanisms of neurotoxicity of this herbicide. Pregnant Wistar rats were supplied orally with 0.2 and 0.4% of Gly-BH (corresponding to 0.65 and 1.30 g/l of pure Gly, respectively) from gestational day (GD) 0, until weaning (postnatal day, PND, 21). Oxidative stress markers were determined in whole brain homogenates of PND90 offspring. The activity of acetylcholinesterase (AChE), transaminases, and alkaline phosphatase (AP) were assessed in prefrontal cortex (PFC), striatum, and hippocampus. Recognition memory was evaluated by the novel object recognition test. Brain antioxidant status was altered in Gly-BH-exposed rats. Moreover, AChE and transaminases activities were decreased and AP activity was increased in PFC, striatum and hippocampus by Gly-BH treatment. In addition, the recognition memory after 24 h was impaired in adult offspring perinatally exposed to Gly-BH. The present study reveals that exposure to a Gly-BH during early stages of rat development affects brain oxidative stress markers as well as the activity of enzymes involved in the glutamatergic and cholinergic systems. These alterations could contribute to the neurobehavioral variations reported previously by us, and to the impairment in recognition memory described in the present work.

Keywords Glyphosate-containing herbicides · Pregnancy and lactation · Oxidative stress · Transaminases · AChE · Recognition memory

Introduction

Glyphosate (Gly) [N-(phosphonomethyl) glycine] is the active ingredient of many broad-spectrum herbicide formulations and constitutes one of the most commonly worldwide used organophosphate (OP) pesticides for weed control.

Its mechanism of action is by inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, found only in plants and certain bacteria but not in animals. Therefore, Gly is considered a safe herbicide for non-target organisms. In addition, it has been shown that Gly does not bioaccumulate, biomagnify, or persist in a biologically available form in the environment (Solomon and Thompson 2003). However, under certain circumstances, Gly could reach surface water and groundwater (Borggaard and Gimsing 2008; Peruzzo et al. 2008; Vereecken 2005). A study carried out in

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the central area of soybean sowing in Argentina revealed that Gly levels in water range from 0.10 to 0.70 mg/l (Peruzzo et al. 2008). In this respect, the highest level of Gly allowed in water for human consumption is 0.70 mg/l (US-EPA 2011).

Pesticides have been proposed as the main environmental factor associated with the etiology of neurodegenerative disorders, such as Parkinson's and Alzheimer's disease (Le Couteur et al. 1999; Richardson et al. 2014). Some human clinical reports of intoxication with commercial formulations of Gly described negative effects in the nervous system, related to Parkinsonian syndrome and alterations in the globus pallidus and substantia nigra (Barbosa et al. 2001; Wang et al. 2011), as well as anxiety and short-term memory impairments (Nishiyori et al. 2014). In vivo studies in rodents have demonstrated the neurotoxic effects of Gly and Gly-based herbicides (Gly-BHs). Intraperitoneal Gly administration decreases locomotor activity and brain dopaminergic markers in rats (Hernandez-Plata et al. 2015). In a recent work, Martinez et al. (2017) demonstrated that the oral exposure of male Wistar rats to Gly induce alteration in the levels of serotonin, dopamine, norepinephrine, and its metabolites, in different brain regions. In addition, Baier et al. (2017) demonstrated that repeated intranasal exposure of adult mice to a Gly-BH decrease locomotor activity, increase anxiety levels, and impair recognition memory.

Gly have the ability to cross the placenta (Mose et al. 2008; Poulsen et al. 2009). Oral exposure of pregnant rats to Gly-containing herbicides alters the activity of enzymes involved in NADPH generation, both in the brain of mothers and offspring (Daruich et al. 2001), and causes oxidative stress and glutamate excitotoxicity in the hippocampus of exposed offspring (Cattani et al. 2014, 2017). In line with these findings, we have recently shown that the oral exposure to a Gly-BH during pregnancy and lactation decreases locomotor activity and anxiety levels in rat offspring (Gallegos et al. 2016).

OPs herbicides such as Gly are usually much less potent acetylcholinesterase (AChE) inhibitor agents than other OPs (Balali-Mood and Saber 2012; Casida 2017). Inhibition of AChE leads to high concentrations of acetylcholine in cholinergic synapses of both central and peripheral nervous systems. Typical clinical signs of OP poisoning are consequence of excessive stimulation of muscarinic and nicotinic receptors (Marrs 1993), and measurement of AChE inhibition has been used as a biomarker of effect following exposure to OP pesticides (Lionetto et al. 2013). Multiple epidemiological studies have identified associations between occupational OP exposure and neurodegenerative diseases, psychiatric illness, and sensorimotor deficits (Voorhees et al. 2016). Inhibition of AChE activity was reported in brain and muscle of aquatic organisms (Lajmanovich et al. 2011) and several fish species exposed to sub-lethal levels of Gly or Gly-BH dissolved in their aquatic habitats (Cattaneo et al. 2011; Glusczak et al. 2007; Menendez-Helman et al. 2012; Modesto and Martinez 2010b). In addition, Larsen et al. (2016) have recently reported that Gly acts as a weak inhibitor of AChE in rats.

Many of the most commonly used pesticides exert their toxic effects via oxidative stress mechanisms (Astiz et al. 2009b). The central nervous system (CNS) is highly sensitive to free radical damage (Chong et al. 2005). In line with this, it has been extensively demonstrated that exposure to Gly (either the active ingredient or the commercial formulation) leads to oxidative stress in several tissues, including the brain (Beuret et al. 2005; Cattani et al. 2014; El-Shenawy 2009; Larsen et al. 2012; Modesto and Martinez 2010b). In particular, alterations in the activity of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), or glutathione S-transferase (GST), as well as in the levels of reduced glutathione (GSH) and of lipid peroxidation in the brain of Gly-exposed rats have been widely reported (Astiz et al. 2009a; Cattani et al. 2017; Cattani et al. 2014; Rebai et al. 2017).

Since agricultural practices are based on Gly formulations instead of pure Gly, for the present study we decided to employ a marketed Gly product (i.e., a Gly-BH). Pesticides are used in formulations which combine an active ingredient with undeclared adjuvants. For this, the toxicity exerted by Gly-BH cannot therefore be exclusively due to the active ingredient but either to the intrinsic toxicity of adjuvants or to the possible synergy between Gly and the other formulation ingredients (El-Shenawy 2009; Gallegos et al. 2016; Mesnage et al. 2013). That is, in the case of a specific commercial formulation of Gly, as the Gly-BH used in the current work, the formulation is the responsible for the effects reported, and it is not possible to determine which component(s) of the mixture was (were) responsible for the toxicity. The Gly-BH doses used in the present study were higher than the Gly-BH levels to which the population is normally exposed (Solomon 2016). However, and in agreement with Ford et al. (2017), many toxicological studies with pesticides are usually performed at higher doses in order to demonstrate a plausible drug-action mechanism.

The aim of this work was to elucidate the possible mechanisms underlying the neurotoxicity exerted by chronic exposure to a commercial Gly-containing herbicide during pregnancy and lactation. To this end, pregnant Wistar rats were supplied orally with a Gly-BH during the complete gestational and lactation periods, and biochemical and neuroconductual tests were performed on 90 days pups. Oxidative stress markers were determined in whole brain, and the activities of the enzymes AChE, transaminases, and alkaline phosphatase were assessed in specific brain areas (prefrontal cortex, striatum, and hippocampus), which are related with the neuroconductual disorders observed in previous studies. In addition, the offspring were subjected to novel object recognition test in order to analyze if Gly-BH exposure affects recognition memory.

Materials and Methods

Materials

The pesticide used in this study is a commercial formulation marketed in Argentina as Glifloglex® from Gleba S.R.L., which contains 48 g of Gly isopropylamine salt per 100 cm^3 product (equivalent to 35.6% *w*/*v* of Gly acid), together with an unspecified mix of inerts and adjuvants.

Animals

Sexually mature male and female Wistar rats (90–120 days old) from our own breeding center were used. They were maintained under constant temperature (22 ± 1 °C) and humidity (50–60%) conditions in a 12-h light-dark cycle, with food (Ganave®, Alimentos Pilar S.A., Argentina) and water ad libitum. Both animal care and handling followed the internationally accepted standard Guide for the Care and Use of Laboratory Animals (Garber et al. 2011) and the experimental protocols were approved by the Institutional Animal Care and Use Committee (CICUAE 024/2014 and 110/2017, Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, Argentina).

Nulliparous female rats at the proestrus stage were housed overnight with fertile males. The presence of spermatozoa in vaginal smears was registered as an index of pregnancy and was referred to as gestational day (GD) 0. Pregnant females were weighed and housed individually in boxes and were randomly assigned to one of the following groups: control group ($n = 10$), provided with tap water; Gly-BH-treated group I ($n = 10$), provided with 0.65 g/l (0.065%) of Gly in drinking tap water (0.2% of the commercial formulation), equivalent to 100 mg of Gly/kg/day; and Gly-BH-treated group II ($n = 10$), provided with 1.30 g/l (0.13%) of Gly in drinking tap water (0.4% of the commercial formulation), equivalent to 200 mg of Gly/kg/day. These doses were selected based on Gly no-observed adverse effect level (NOAEL) of 1000 mg/kg/day for maternal toxicity (Williams et al. 2000), and were calculated considering the average volume of water intake and the average weight of the dams during the exposure period (Gallegos et al. 2016). Although Gly half-life in water varies from 49 to 70 days (Mercurio et al. 2014), Gly solutions were prepared daily to minimize the risk of degradation.

Dams received the treatment from GD 0 to weaning on postnatal day (PND) 21 (see scheme Fig. 1). After weaning, offspring were housed in groups of six rats according to sex and treatment, receiving tap water and food ad libitum. At PND90, one male and one female from each litter were used for each biochemical assay and for memory test. The total number of animals used in each test was 5 per group and per sex for biochemical determinations, and 10 per group and per sex for the novel object recognition test.

Tissue Preparation

Animals were killed by decapitation on PND90. Brains were rapidly taken out and rinsed with ice-cold isotonic saline. At this point, brains could be destined to (1) whole brain homogenization with a dounce homogenizer; (2) brain dissection in prefrontal cortex (PFC), striatum, and hippocampus, using an acrylic coronal brain matrix (Stoelting Co, Illinois, USA) and the atlas of Paxinos and Watson (Paxinos and Watson 2007) as a guide for tissue dissection. Brain regions were homogenized with disposable homogenization pestles. For both, homogenization of the whole brain and brain areas, tissue samples were homogenized in 10 volumes (1:10, *w*/*v*) of ice-cold phosphate buffer saline (PBS; pH 7.4). Homogenates were immediately centrifuged and the resultant supernatants were kept at 4 °C until determination of enzyme activities or lipid peroxidation levels. A supernatant aliquot of each sample was reserved for protein determination.

Thiobarbituric Acid Reactive Substances Determination

Lipid peroxidation was measured as malondialdehyde (MDA), which is the main end product of lipid peroxidation. MDA reacts with thiobarbituric acid (TBA), as thiobarbituric acid reactive substances (TBARS), to produce a red colored complex that is quantified at 532 nm. We follow the method previously described by Beuret et al. (2005) with slight modifications. Briefly, 1 ml of whole brain supernatant of PND90 offspring was mixed with 1 ml 20% trichloroacetic acid (TCA), followed by 30 min incubation on ice. After centrifugation at 3000 rpm for 10 min, 1 ml of the TCA-supernatant was incubated with 1 ml of a 0.8% TBA solution for 30 min at 100 °C. Samples were allowed to cool at room temperature, and absorbance was measured spectrophotometrically at 532 nm. MDA concentrations were determined by using an acid hydrolysis product of 1,1,3,3-tetraethoxypropane (TMP) as a standard. MDA concentration was expressed as nanomole/milligram protein.

Antioxidant Enzyme Activities

The activity of the enzymes CAT and glutathione peroxidase (GPx) were determined in whole brain homogenates of PND90 offspring. CAT activity was determined by the method of Aebi (1984). Reaction was initiated by addition of 0.5 ml H_2O_2 (1/10 in PBS) to the reaction mixture containing 100 μl supernatant, 25 μl Triton-X 100 (1/10 in PBS), and 2.4 ml PBS. The decrease in absorbance was recorded for 3 min at 240 nm. The enzyme activity was expressed as the rate constant of a first-order reaction (k) per milligram protein.

GPx activity was determined following NADPH oxidation at 340 nm, according to the method of Lawrence and Burk

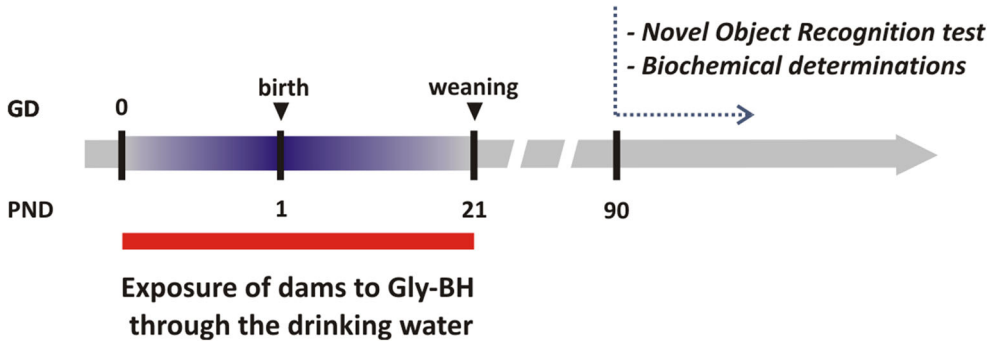

Fig. 1 Diagram of the experimental design

(1976). Reaction medium containing 100 μl GSH (10 mM), 100 μl glutathione reductase (0.24 U of enzyme activity), 10 μl of sodium azyde (100 mM), and 20 μl of supernatant was incubated at 37 °C for 10 min. Then, 100 μl of NADPH (3 mM) was added, followed by the rapid addition of 100 μl of H_2O_2 (2 mM). The decrease in absorbance was recorded for 3 min at 340 nm. The enzyme activity was expressed as micromoles of NADPH oxidized per minute per milligram protein.

Glutamate Transaminases and Alkaline Phosphatase Activities

The activities of glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), and alkaline phosphatase (AP) were evaluated in brain areas homogenates of PND90 offspring exposed to both Gly-BH concentrations, by spectrophotometric methods using the corresponding commercial kits of Wiener Lab. (Rosario, Argentina) following the manufacturers' indications.

Acetylcholinesterase Activity

The activity of the enzyme AChE was determined in supernatants of brain areas homogenates of PND90 offspring exposed to both Gly-BH concentrations following the Ellman's method (Ellman et al. 1961). Briefly, an aliquot of each supernatant was incubated with acetylthiocholine iodide (substrate) and 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) for 10 min at 30 °C. Enzymatic reaction was stopped by addition of eserine solution and incubation at 0 °C for 10 min. The absorbance was measured at 420 nm. AChE activity was calculated from a standard curve prepared with different concentrations of reduced glutathione, and expressed as micromole of thiocholine generated per minute per milligram of protein.

Protein Determination

The protein concentration of the supernatants was measured using the method of Bradford (1976). Bovine serum albumin was used as a standard.

Novel Object Recognition Test

The NOR test is a learning test for animals. In this test, the rat is presented with two similar objects during the first session (familiarization), and then one of the two objects is replaced by a new one during a second session (test) (Leger et al. 2013). The time spent exploring the novel object, relative to the familiar object, is an estimation of episodic recognition memory. The objects selected for the NOR test differed in shape and texture (plastic, glass) and the size and volume of them were similar. The test was performed according to the guidelines of Leger et al. (2013) with minor modifications (Barbieri et al. 2016; Kubota et al. 2016). Testing was performed in an open-field (OF) arena (50 cm × 50 cm × 60 cm). Before performing the Novel Object Recognition (NOR) test, each rat was habituated by 2 min/day of handling followed by another 2 min/day of free exploration of the empty OF, during 3 days.

The study consisted of two trials: a familiarization trial and a test trial carried out 24 h after the familiarization session. In the familiarization trial, two identical 250 ml plastic bottles filled with sand and parafilm-sealed were placed at opposite ends of the OF (Fig. 2). A rat was then allowed to explore the OF for 5 min while being video recorded. In the test session, 24 h later, one of the plastic bottles was replaced with a glass bottle filled with water, and the same rat was allowed to explore the OF for 5 min while being video recorded. The entire arena and objects were washed with 10% ethanol before another rat was tested.

Object exploration was defined as sniffing or touching the object with either the nose or forepaw, but not sitting or standing on the objects (Patel et al. 2014). The rat's preference for the novel object over the familiar object was measured with the automated software program developed by Patel et al. (2014). The exploratory ratio for each rat was determined as Kubota et al. (2016):

$$\textit{exploratory ratio} = \frac{\textit{time spent exploring the novel object (sec)}}{\textit{time spent exploring familiar and novel object (sec)}}$$

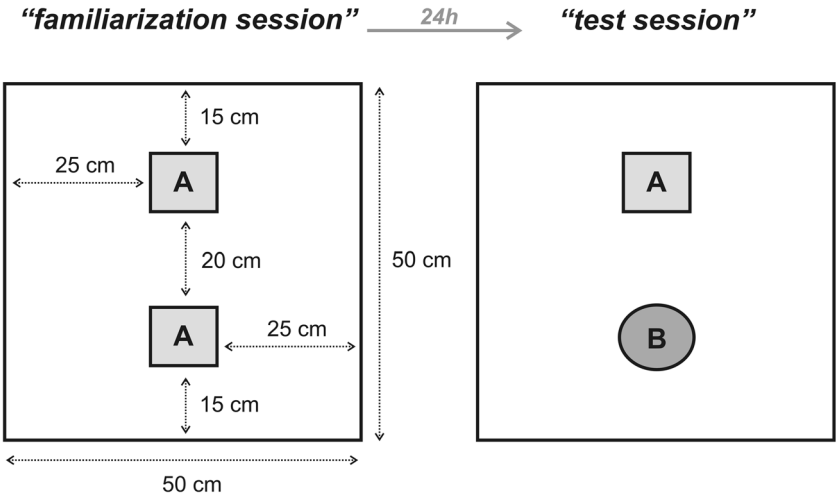

Fig. 2 Novel Object Recognition (NOR) test. Schematic representation showing dimensions and distribution of the objects in the OF during the familiarization and the test sessions of the NOR test

Statistical Analysis

Experimental biochemical data were analyzed by two-way ANOVA (group × sex). Differences between groups were assessed using LSD post hoc test. Results were expressed as mean ± S.E.M. A value of $p < 0.05$ was considered statistically significant. All statistical analyses were carried out using software SPSS Statistics 21 for Windows. For comparative analysis in the NOR test, paired samples *t* test was used. Results are expressed as mean ± S.E.M. A value of $p < 0.05$ was considered statistically significant. Statistical analyses were carried out using Origin software.

Results

Exposure to a Gly-BH Caused Oxidative Stress in the Brains of PND90 Offspring

Alteration of biochemical markers of oxidative damage was observed in whole brain homogenates of PND90 offspring exposed to Gly-BH during pregnancy and lactation (Fig. 3). Results of lipid peroxidation analyzed by two-way ANOVA showed significant differences between groups ($F_{(2,24)} = 6, 60$; $p < 0.005$). Post hoc comparisons showed that female and male rats from Gly-BH I and Gly-BH II treated groups exhibited a significant decrease in this parameter compared to the corresponding control group ($p < 0.05$) (Fig. 3a). Similar results were obtained for the activity of the enzyme CAT. Two-way ANOVA revealed significant differences between groups in the activity of this enzyme ($F_{(2,24)} = 42, 40$; $p < 0.001$). Post hoc comparisons showed a significant decrease in females as well as males from both Gly-BH concentrations tested ($p < 0.001$) (Fig. 3b). Finally, the activity of the enzyme GPx was analyzed by two-way ANOVA, showing significant differences between groups ($F_{(2,24)} = 10, 54$; $p < 0.001$). Post hoc comparisons showed a significant increase compared to controls in female and male pups exposed only to the higher concentration of Gly-BH ($p < 0.01$) (Fig. 3c).

Gly-BH Treatment Decreased the Activity of Transaminases in Specific Brain Areas of Adult Offspring

Enzymes GPT and GOT are known regulators of the metabolism of the excitatory neurotransmitter glutamate (Daikhin and Yudkoff 2000; Matthews et al. 2000). In order to investigate if Gly-BH exposure during the gestational and lactation periods affects glutamate metabolism, the activity of GPT and GOT was determined in specific brain areas (PFC, striatum, and hippocampus) of PND90 offspring exposed to Gly-BH. Statistical analysis resulted in significant differences between groups in each of the brain areas studied, both for GPT (PFC: $F_{(2,20)} = 24, 11$; $p < 0.001$; striatum: $F_{(2,18)} = 29, 09$; $p < 0.001$; hippocampus: $F_{(2,20)} = 16, 72$; $p < 0.001$) and GOT (PFC: $F_{(2,20)} = 4, 93$; $p < 0.05$; striatum: $F_{(2,19)} = 10, 71$; $p < 0.001$; hippocampus: $F_{(2,20)} = 18, 83$; $p < 0.001$). Post hoc tests showed that in female rats, the activity of GPT was significantly decreased in all brain areas and for both Gly-BH doses tested compared to control groups (Fig. 4a). In male rats, we observed a similar tendency to decrease, with exception of striatum in which GPT activity did not evidence any change (Fig. 4b). With respect to the enzyme GOT, results showed that the activity of this enzyme was also inhibited by exposure to Gly-BH, but only for the lowest concentration of the herbicide (Fig. 4c, d).

Perinatal Exposure to Gly-BH Decreased Brain AChE Activity

Enzyme AChE ends the action of the neurotransmitter acetylcholine at the synaptic cleft by hydrolyzing it into acetic acid

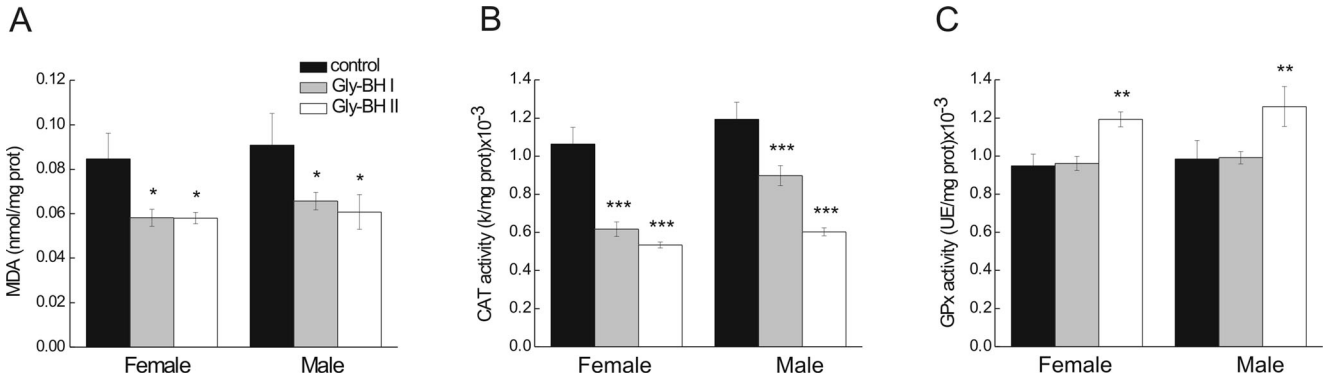

Fig. 3 Brain antioxidant status in PND90 offspring. MDA content (**a**) and activity of the enzymes CAT (**b**) and GPx (**c**) in whole brain homogenates of control and Gly-BH exposed offspring at PND90. Data are expressed as mean ± SEM. $n = 5$ per group. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$ compared to the corresponding control group (LSD post hoc test)

and choline (Thapa et al. 2017). The activity of AChE was studied in specific brain areas of control and Gly-BH exposed offspring at PND90. Two-way ANOVA showed significant differences between groups in each of the brain areas evaluated (PFC: $F_{(2,20)} = 17,\ 70$; $p < 0.001$; striatum: $F_{(2,18)} = 42,30$; $p < 0.001$; hippocampus: $F_{(2,20)} = 24,22$; $p < 0.001$). Post hoc comparisons showed that the activity of this enzyme was significantly decreased in PFC, striatum, and

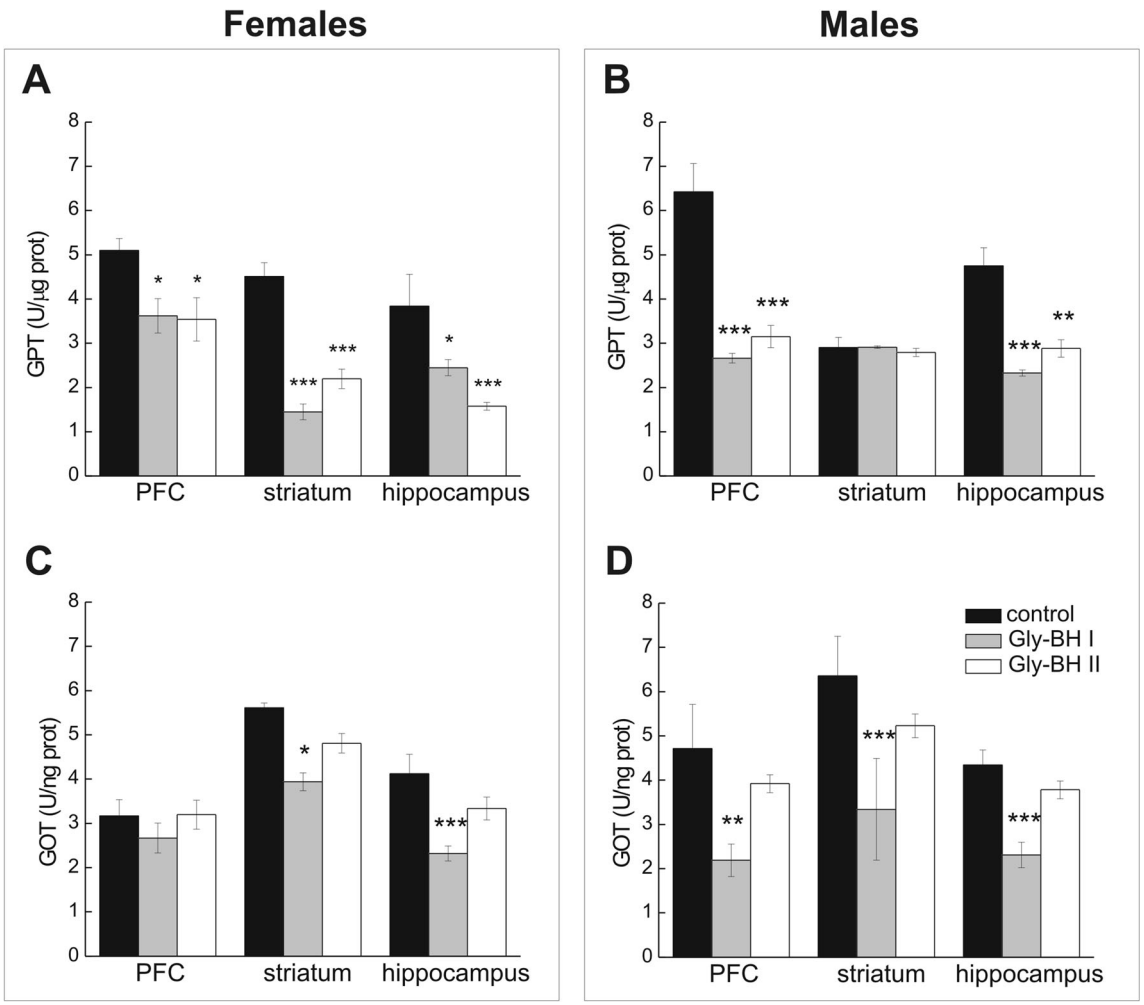

Fig. 4 Transaminases activities in PFC, striatum, and hippocampus of PND90 offspring. **a** GPT and **c** GOT activities in the indicated brain areas of female rats. **b** GPT and **d** GOT activities in the indicated brain areas of male rats. Data are expressed as mean ± SEM. $n = 4$–5 per group. *$p < 0.05$; **$p < 0.005$; and ***$p < 0.001$ compared to the corresponding control group (LSD post hoc test)

hippocampus of female, as well as male rats, exposed during gestational and suckling periods to Gly-BH (Fig. 5a, b).

Effect of Gly-BH Exposure on the Activity of the Enzyme AP in Specific Brain Areas

Tissue non-specific AP is an isozyme that has been associated with neurodegeneration and central nervous system injury (Vardy et al. 2012; Yamashita et al. 1989). The activity of AP was determined in homogenates of brain areas of female and male PND90 offspring. Two-way ANOVA revealed significant differences between groups only in PFC ($F_{(2,21)} = 6$, 61; $p < 0.05$). However, post hoc comparisons showed that Gly-BH exposure induced an increment in the activity of this enzyme in hippocampus of female rats and in PFC and striatum of male rats (Fig. 5c, d).

Administration of Gly-BH Affects Recognition Memory

To investigate whether oral administration of Gly-BH during pregnancy and lactation affects recognition memory, we performed the NOR test at PND90 in females and males offspring. Control female and male rats showed a significant increase in the exploratory ratio 24 h after the familiarization trial. This ratio was not significantly altered in female rats exposed to both Gly-BH concentrations and in male rats exposed to the higher dose of Gly-BH, indicating that Gly-BH caused impairment of recognition memory (Fig. 6).

Discussion

In a previous work from our laboratory, we demonstrated that oral exposure of rats to a Gly-containing herbicide in early stages of development (gestational and lactation periods) affects locomotor activity and decrease anxiety levels in female and male offspring (evaluated at PND45 and PND90) (Gallegos et al. 2016). The purpose of the present study was to determine the possible mechanisms through which the perinatal Gly-BH-administration could generate such behavioral alterations. Numerous studies in rats demonstrate that exposure to Gly (either the active ingredient or the commercial formulation) leads to oxidative stress in several tissues, including the brain (Beuret et al. 2005; Cattani et al. 2014, 2017; El-Shenawy 2009; Larsen et al. 2012), emphasizing oxidative damage as a basic mechanism of toxicity. Our results showed that maternal exposure to a Gly-BH during pregnancy and lactation decreased the enzymatic activity of CAT, and increased the activity of the enzyme GPx in the brains of adult offspring (PND90) (Fig. 3b, c). In this respect, it is important to denote that both an increase and/or a decrease in the expression or activity of antioxidant enzymes are indicative of oxidative stress (Khan et al. 2017). Additionally, enhanced GPx activity may act as a protective mechanism to counterbalance the oxidative insult exerted by the herbicide (Larsen et al. 2012). With respect to lipid peroxidation, TBARS levels were decreased compared to controls, for both sexes and both Gly-BH concentrations (Fig. 3a). These results are in contrast to most of the already published investigations, in which increased TBARS production is observed after exposure to pure Gly or Gly formulations (Astiz et al. 2009a; Beuret et al. 2005; El-Shenawy 2009). In this regard, it is important to highlight that in these studies, lipid peroxidation was assessed immediately after treatment, whereas in our experimental model, the animals were exposed to the herbicide from day 0 of gestation until weaning (on PND21), and evaluations were not performed until PND90, i.e., after 69 days from the last exposure to Gly-BH. It is highly probably that TBARS levels in the brain of Gly-BH exposed pups have been elevated during the time of herbicide exposure. However, in the course of PND21 to PND90, multiple defensive mechanisms could have been put in place to counteract the deleterious action of reactive oxygen species, inducing the observed decrease in TBARS generation. In line with our findings, Larsen et al. (2012) reported that in the liver and kidney of rats exposed to Gly-BH through the drinking water, the production of TBARS tended to be lower, rather than higher.

In addition to oxidative stress evaluation, we assessed the activities of glutamate metabolism related enzymes (GPT and GOT) in specific brain regions (PFC, striatum, and hippocampus). Glutamate is the main excitatory neurotransmitter in the CNS (Erecinska and Silver 1990), and once released into the synaptic cleft, it must be rapidly removed (Daikhin and Yudkoff 2000). Deleterious excess of glutamate in the brain's extracellular fluids overstimulates glutamate receptors leading to neuronal death (Leibowitz et al. 2012). Astrocytes have a crucial role removing glutamate from the synaptic cleft and metabolizing it to glutamine, a non-neuroexcitatory amino acid, which is then transferred back to neurons to be converted back to glutamate (Danbolt 2001; Hassel et al. 1997). Alternatively, glutamate could be oxidized to α-ketoglutarate by the transaminases GOT and GPT (Daikhin and Yudkoff 2000; Matthews et al. 2000). That glutamate follows one or another metabolic pathway will depend on the concentration of extracellular glutamate: at low concentrations the glutamine synthetase pathway is favored, but when the external glutamate concentration is high, oxidative processes are recruited, in order to prevent excitotoxic damage (McKenna et al. 1996; Torres et al. 2013). Our results showed that exposure to Gly-BH leads to inhibition in the activity of both transaminases (Fig. 4). Similar results were described by Cattani et al. (2014) for rat hippocampus. These authors reported that additionally to GPT and GOT inhibition, exposure to the Gly-containing herbicide Roundup increased glutamate release, reduced glutamate uptake, and decreased the activity of glutamine synthetase, leading to excessive extracellular glutamate levels and

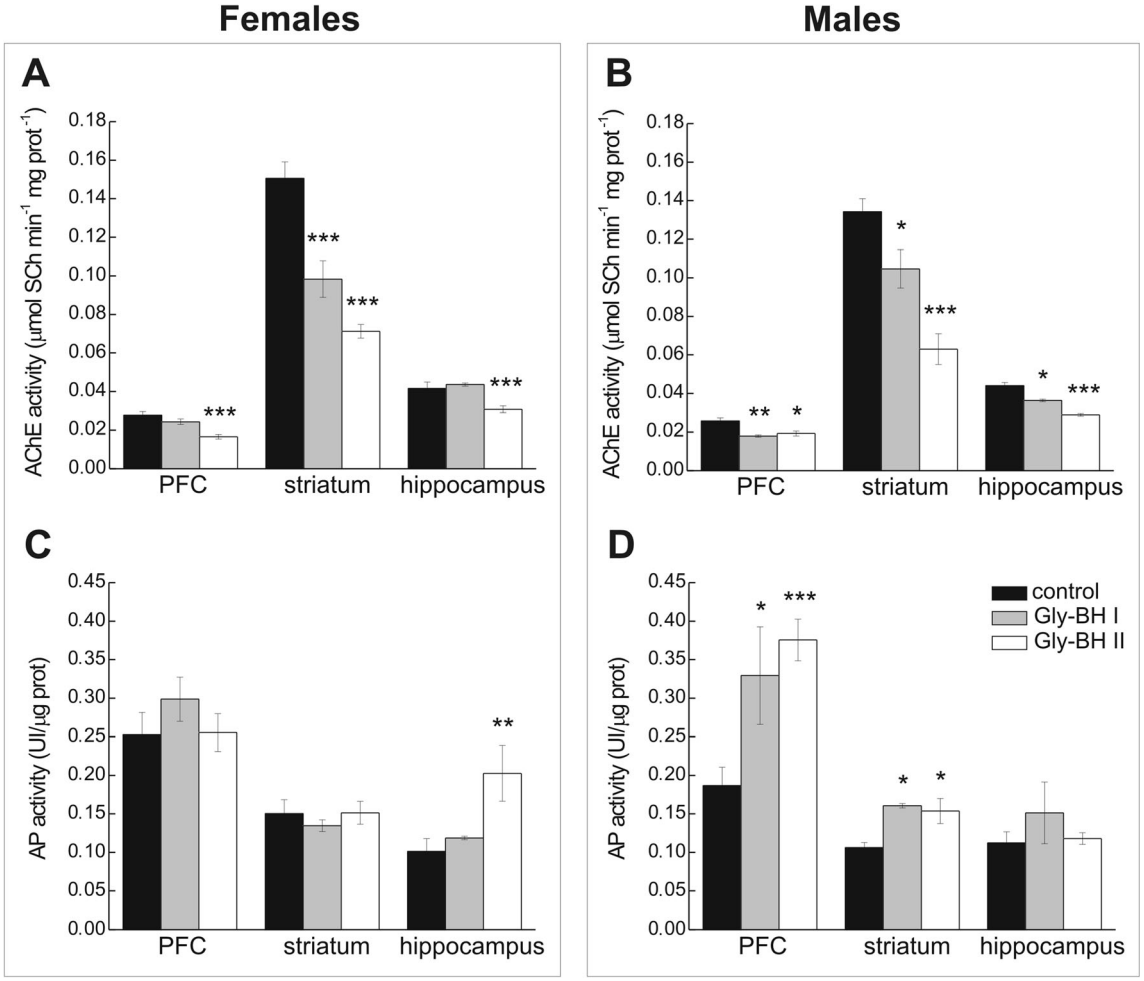

Fig. 5 Activities of the enzymes AChE and AP in PFC, striatum, and hippocampus of PND90 offspring. **a**, **b** AChE activity in female and male PND90 rats respectively. **c**, **d** AP activity in female and male PND90 rats, respectively. Data are expressed as mean ± SEM. $n = 4–5$ per group. $*p < 0.05$; $**p < 0.005$; and $***p < 0.001$ compared to the corresponding control group (LSD pot hoc test)

consequently to excitotoxic condition in rat hippocampus. The glutamate excitotoxicity is one of the main factors for reactive oxygen species generation in the brain (Bai et al. 2016; Cattani et al. 2017; Vishnoi et al. 2016).

Considering that Gly is an OP herbicide with a weak AChE inhibitory action (Larsen et al. 2016), we wondered if exposure to a Gly-BH during pregnancy and lactation could affect the AChE activity in the brain of exposed offspring at PND90. As shown in Fig. 5a, b, AChE activity was significantly reduced in the three brain areas evaluated (PFC, striatum, and hippocampus). Inhibition of AChE activity in brain and muscle of fish species by Roundup exposure has been extensively reported (Menendez-Helman et al. 2012; Modesto and Martinez 2010a, b). Moreover, Larsen et al. (2016) found that Gly produce a weak inhibition of AChE in rats. Similarly to our findings, Cattani et al. (2017) described that maternal exposure to Roundup decrease the cholinesterase activity in the hippocampus of immature and young adult-exposed offspring. Chronic inhibition of brain AChE by OPs could lead to an excess of acetylcholine at the cholinergic synapses, with prolonged excitation of postsynaptic neurons, excitotoxic damage, and degeneration of cholinergic systems (Zaganas et al. 2013). Considering that the time between the last Gly-BH exposure and AChE activity determination was of more than 2 months, probably the Gly-BH effect on AChE activity was related to enzyme expression/regulation more than a direct inhibitory effect. Future studies will be necessary to completely understand the mechanism whereby Gly-BH produces a long-term modulatory effect on AChE activity.

As mentioned above, the alteration of the enzymes that regulate both glutamate and ACh metabolism (transaminases and AChE, respectively) were observed in PFC, striatum, and hippocampus. These brain areas are critical in the regulation of locomotion, anxiety, and memory, and their regulatory functions are usually overlapped. Thus, the PFC is implicated in the control of locomotor activity, the regulation of emotional states and cognitive processes such as working memory (Thierry et al. 1994). Locomotor activity is also regulated by the striatum (Neill et al. 1974; Pisa et al. 1980). In addition, this brain area has been recently

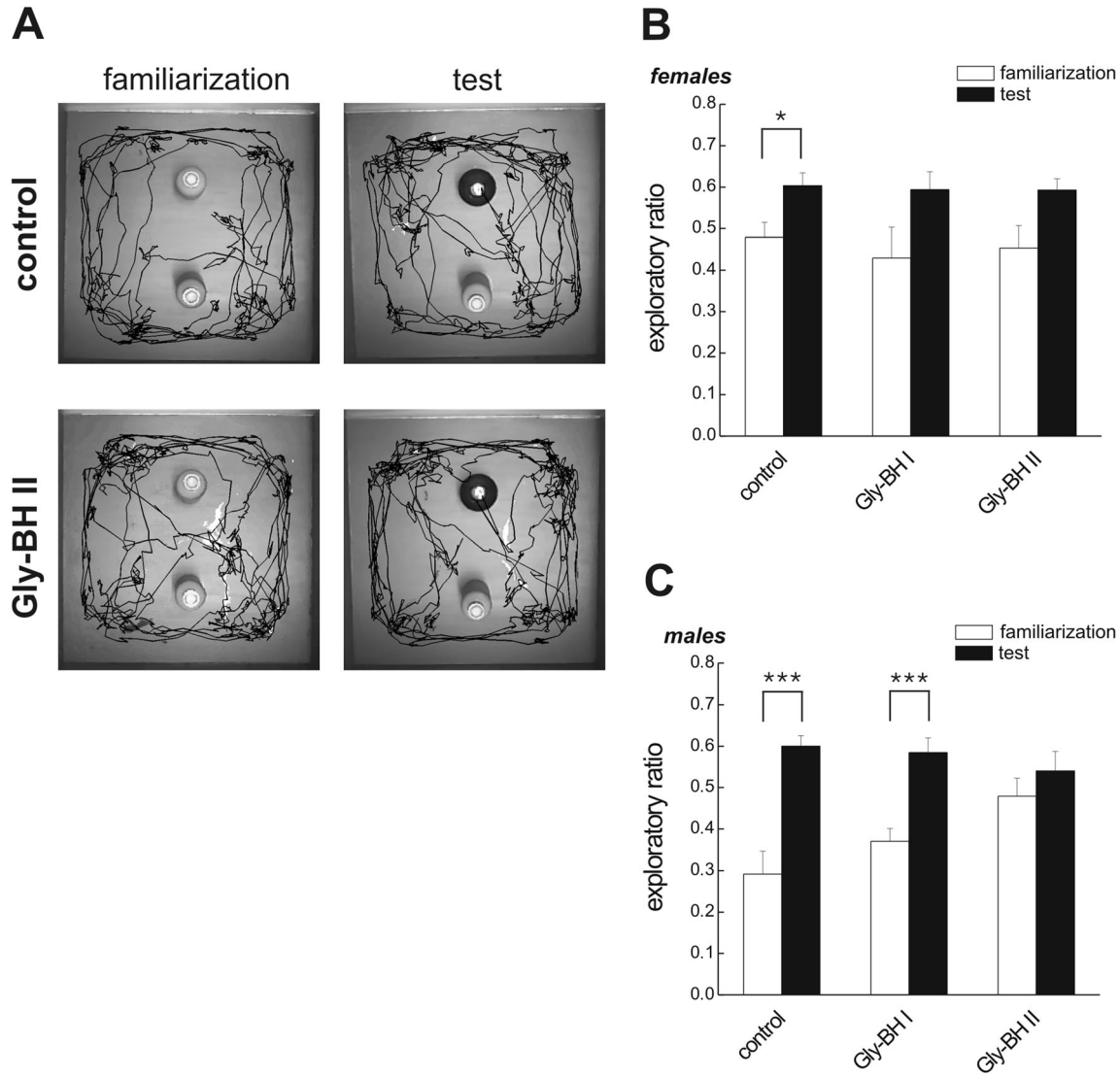

Fig. 6 Recognition memory in the Novel Object Recognition (NOR) test. **a** Cumulative trajectories of male rats from control and Gly-BH II groups, respectively, during familiarization and 24 h test, over 5 min. **b** Exploratory ratio in control and Gly-BH treated female rats. **c** Exploratory ratio in control and Gly-BH treated male rats. Data are expressed as mean ± SEM. $n = 8$–10 per group. *** and * denotes $p < 0.001$ and 0.05, respectively, statistically different from familiarization session in the same group

included to the anxiety network, integrated also by the amygdala, bed nucleus of the stria terminalis, hippocampus, and PFC (Lago et al. 2017). The hippocampus plays a key role in the regulation of memory and anxiety (Bannerman et al. 2004, 2014). In a previous work from our laboratory, we demonstrated that the oral exposure of rats to a Gly-BH during pregnancy and lactation decreased the locomotor activity and anxiety levels in the offspring (Gallegos et al. 2016). The results obtained in the present study, i.e., alteration in the activity of transaminases and AChE in specific brain areas, together with the finding of oxidative stress misbalance in whole brain homogenates, could explain, at least in part, the observed changes in locomotion and anxiety. Given that the brain areas affected by Gly-BH treatment are involved in the regulation of memory process, we decided to study if exposure to Gly-BH in early stages of rat development could alter memory retention in the adulthood. The results of the NOR test revealed a significant impairment of recognition memory. As shown in Fig. 6, control female and male rats showed a significant increase in the exploratory ratio 24 h after the familiarization trial. The recognition memory was impaired in female rats exposed to both Gly-BH concentrations and in male rats exposed to the higher dose of Gly-BH. The different results between female and male offspring could be attributed to the fact that female rats are more sensitive to the toxic effects of Gly-BH than male rats, as was described by us in a previous work (Gallegos et al. 2016).

Finally, we assessed the activity of the enzyme AP in PFC, striatum, and hippocampus. Tissue non-specific AP (TNAP)

is an isozyme expressed in tissues such as bone, kidney, liver, and brain (Brun-Heath et al. 2011; Buchet et al. 2013). TNAP activity is strongly associated with brain development and functioning (Cruz et al. 2017). In addition, TNAP has been implicated in neurodegeneration (Vardy et al. 2012). APs are responsible for cleaving phosphate groups, providing phosphates for a variety of cellular functions (Diez-Zaera et al. 2011; Millan 2006). The increased AP activity observed in our study (Fig. 5c, d) could be due to a high demand of phosphate groups required for protein production and DNA repair, as was reported by Akinrinade et al. (2015) for fluoride and aluminum neurotoxicity in rats.

In summary, the present results indicate that chronic exposure to a Gly-containing herbicide during pregnancy and lactation produced an imbalance in brain oxidative stress as well as in the activity of several brain enzymes (GOT, GPT, AChE, and AP) in specific brain areas. These disturbances could be responsible for the neurobehavioral alterations described previously, i.e., the decrease in locomotor activity and anxiety state, and in recognition memory reported in the present article.

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