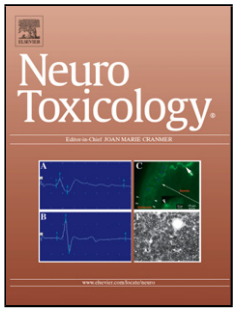

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EXPOSURE TO A GLYPHOSATE-BASED HERBICIDE DURING PREGNANCY AND LACTATION INDUCES NEUROBEHAVIORAL ALTERATIONS IN RAT OFFSPRING

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Abstract

The impact of sub-lethal doses of herbicides on human health and the environment is a matter of controversy. Due to the fact that evidence particularly of the effects of glyphosate on the central nervous system of rat offspring by *in utero* exposure is scarce, the purpose of the present study was to assess the neurobehavioral effects of chronic exposure to a glyphosate-containing herbicide during pregnancy and lactation. To this end, pregnant Wistar rats were exposed through drinking water to 0.2% or 0.4% of a commercial formulation of glyphosate (corresponding to a concentration of 0.65 or 1.30 g/L of glyphosate, respectively) during pregnancy and lactation and neurobehavioral alterations in offspring were analyzed. The

postnatal day on which each pup acquired neonatal reflexes (righting, cliff aversion and negative geotaxis) and that on which eyes and auditory canals were fully opened were recorded for the assessment of sensorimotor development. Locomotor activity and anxiety levels were monitored via open field test and plus maze test, respectively, in 45- and 90-day-old offspring. Pups exposed to a glyphosate-based herbicide showed early onset of cliff aversion reflex and early auditory canal opening. A decrease in locomotor activity and in anxiety levels was also observed in the groups exposed to a glyphosate-containing herbicide. Findings from the present study reveal that early exposure to a glyphosate-based herbicide affects the central nervous system in rat offspring probably by altering mechanisms or neurotransmitter systems that regulate locomotor activity and anxiety.

Keywords

Glyphosate-based herbicides – Perinatal exposure – Sensorimotor reflexes – Locomotor activity – Anxiety –Rats

Highlights

- Early exposure to glyphosate formulation had neurobehavioral effects in offspring.
- Glyphosate-based herbicide (Gly-BH) caused early onset of cliff aversion reflex.
- Pups exposed to Gly-BH showed early auditory canal opening.
- Gly-BH exposure caused hypoactivity and anxiety reduction in the offspring.

1. Introduction

The massive influx of genetically modified (GM) crops resistant to glyphosate (Gly) in Argentina is the main reason why the most widely marketed herbicides within this country are those containing Gly in their formula. In 1996, Gly-resistant soybean became the first GM crop approved in Argentina and since then the area dedicated to GM crops has been growing steadily, reaching 22 million hectares at present (GRAIN, 2009; Trigo, 2011). As these crops

are sprayed with 200 million liters of Gly per year (Teubal, 2009; Teubal et al., 2005) its residues are often found in the environment (soil, water and food) (Bohn et al., 2014; Peruzzo et al., 2008; Van Stempvoort et al., 2014). A study carried out in the central area of soybean sowing in Argentina revealed that Gly levels in sediments and soils range between 0.5 and 5.0 mg/Kg, while in water they 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 0.7 mg/L (US-EPA, 2011).

Little is known on the impact of sub-lethal doses of Gly on human health and the environment and although Gly is considered to be safe for living beings, its safety has been questioned worldwide.

Pesticides are postulated 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) and many of the most commonly used pesticides exert their toxic effects via oxidative stress mechanisms (Astiz et al., 2009). 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, 2010). Previous research reported the case of a 54-year-old man who, after having been accidentally sprayed with an herbicide containing Gly, developed a symmetrical Parkinsonian syndrome as well as alterations in the globus pallidus and substantia nigra, the latter being shown by magnetic resonance imaging two years after the initial exposure (Barbosa et al., 2001). More recently, Wang et al. (2011) reported the case of a healthy woman who at the age of 44 had parkinsonism as a result of chronic occupational exposure to a Gly-containing herbicide.

Experimental *in vitro* and *in vivo* studies have demonstrated the neurotoxic effects of Gly and Gly-based herbicides. For example, in mouse neuroprogenitor cells, it was observed to increase the activation of the marker of potential apoptosis p53 without producing changes in caspase 3, another indicator of apoptosis (Culbreth et al., 2012). Further recent research using the MTT mitochondrial assay showed that Gly causes cytotoxicity in human neuroblastoma cell line SH-SY5Y (Chorfa et al., 2013). It was also demonstrated that Gly alters acetylcholinesterase activity in the brain and muscle of fishes exposed either to pure Gly (Menendez-Helman et al., 2012; Sandrini et al., 2013) or to Gly formulations (Modesto and Martinez, 2010; Samanta et al., 2014). Research on the nematode *Caenorhabditis elegans* revealed that exposure to Gly-containing herbicides causes neuronal degeneration, particularly, neurodegeneration of GABAergic and dopaminergic neurons (Negga et al., 2012). Further work showed that whereas oral administration of Gly in rats decreases serotonin (5-HT) and dopamine (DA) levels in the frontal cortex, midbrain and striatum, it increases 5-HT and DA metabolites (Anadón et al., 2008). More recently, Hernández-Plata et al., (2015) have shown that rat exposure to Gly decreases locomotor activity, binding to D1-DA receptor in the nucleus accumbens, and extracellular DA levels in striatum. In pregnant rats, the oral exposure to a Gly-containing herbicide, which has been reported to have the ability to cross the placenta (Mose et al., 2008; Poulsen et al., 2009), alters the activity of brain enzymes both in mothers and offspring (Daruich et al., 2001). In addition, Cattani et al. (2014) showed that oral exposure to the Gly-based herbicide Roundup® during pregnancy and lactation in rats causes a decrease in glutamate uptake by glial cells in the hippocampus of exposed offspring, thus leading to glutamate excitotoxicity.

As pesticides are used in formulations which combine an active ingredient with adjuvants, the toxicity exerted by Gly-based herbicides 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; Mesnage et al., 2013).

Little is known to date on the effects of Gly-based herbicides on the CNS of rat offspring by *in utero* exposure. The purpose of the present study was therefore to assess the neurobehavioral effects of chronic exposure to a Gly-containing herbicide during pregnancy and lactation. To this end, pregnant Wistar rats were supplied orally with 0.2% or 0.4% of a Gly commercial formulation corresponding to a concentration of 0.65 or 1.30 g/L of Gly, respectively, during the complete gestational and lactation periods, and offspring were subjected to a series of neurobehavioral tests. The postnatal day (PND) on which each pup acquired neonatal reflexes (righting, cliff aversion and negative geotaxis) and that on which eyes and auditory canals were fully opened were recorded for the assessment of sensorimotor development. Furthermore, locomotor activity and anxiety levels were analyzed in 45- and 90-day-old offspring by means of the open field test and plus maze test, respectively.

2. Materials and methods

2.1. 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).

2.2. 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 (22º ± 1ºC) 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 were controlled by the institutional committee for the care and use of research animals of the Universidad Nacional del Sur.

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-based herbicide (Gly-BH)-treated group I (n= 10), provided with 0.65 g/L (0.065%) of Gly in drinking 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 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). 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 post-gestational day (PGD) 21. Maternal weight gain and food intake were recorded on different GDs (GD 0, 3, 6, 9, 12, 15, 18 and 20) and PGDs (PGD 1, 4, 7, 10, 13, 16, 19 and 21). Drink consumption was recorded on a daily basis. Within 24 h after delivery, all pups were weighed and litters were randomly culled to five males and five females whenever possible. The following data were analyzed: length of gestation, litter size, number of males and females, and body weight of pups on different PNDs (PND 1, 4, 7, 10, 13, 16, 19 and 21). After weaning, offspring were housed in groups of six rats according to sex and treatment, receiving tap water and food *ad libitum.* One male and one female from each litter were randomly assigned for the open field and plus maze tests. These neurobehavioral tests were performed on PNDs 45 and 90. The animals analyzed on PND 45 were different from those analyzed on PND 90. The total number of animals used in each test was 10 per group and per sex.

2.3. Sensorimotor development

Starting on PND 3, each pup was subjected to a battery of developmental tests. One trial test per day was given to the pups on each test: righting reflex, cliff aversion, negative geotaxis and eye and ear opening. The dependent variable analyzed for each test was the PND of first achieving either maturity of the reflex or the conditions listed below (Molina et al., 1987).

***2.3.1. Righting reflex*:** The pup was placed on its back on a cloth-covered supporting surface, and allowed to right itself. Time for regaining normal position was recorded. This reflex was completed if the pup performed this response within 5 s on 2 consecutive days.

***2.3.2. Cliff aversion*:** Offspring were placed with their forepaws on the edge of a wooden platform and the snout protruded beyond the edge of the same platform. Latency to retract their body 1.5 cm from the edge was registered. The cliff aversion criterion was registered as mature when the pup performed this response in less than 5 s on 2 consecutive days.

***2.3.3. Negative geotaxis*:** Each rat was placed on an inclined wire mesh ramp (angle of inclination from the base: 30º) with the head facing down. The reflex was considered to be acquired when pups performed a 180º body rotation and when they could climb up within 10 s on 2 consecutive days.

***2.3.4. Eye and auditory canal opening*:** The PNDs on which both eyes were opened and on which both auditory canals were fully opened were registered.

2.4. Open field

Motor activity, which is considered to be a test of nervous system function, shows the integrated output of the sensory, motor and associative processes of the nervous system in case of absence of systemic toxicity (Hübler et al., 2005). Behavior in the open field (OF) test is used to assess locomotor activity as well as emotionality (Walsh and Cummins, 1976). Each rat was placed in a 50 cm × 50 cm × 60 cm open area box whose floor was divided into 12 cm × 12 cm squares by black lines. The number of squares entered by each rat with all four

paws, rearings (episodes of animals standing on their hind legs), grooming episodes (face washing, forepaw licking and head stroking) and fecal boluses were scored every 5 min for 15 min. Both the number of squares crossed and rearings were recorded as parameters of locomotor activity, whereas the number of grooming episodes and the number of fecal boluses deposited were recorded as parameters of emotionality (Choleris et al., 2001). Once each animal was removed, the floor was carefully cleaned with a cloth embedded with a 10% ethanol solution. The test was always carried out between 09:00 am and 03:00 pm in a quiet room intended only for this purpose.

2.5. Plus maze

Anxiety levels in offspring after exposure to a Gly-BH during pregnancy and lactation were analyzed using the plus maze (PM) test, which represents a valid behavioral model to study the emotional response of animals (Pellow et al., 1985). The PM was made up of wood and consisted of four arms, all with the same dimensions (50 x 10 cm), which were elevated 50 cm above the floor. Two of these arms were enclosed by 40 cm high lateral walls with an open roof and were located perpendicularly to the other two opposed open arms. The four arms delimited a central area of 10 cm^2. This test exploits a rodent's natural conflict between avoidance and exploration of open and elevated areas. Rats were placed in the centre of the maze facing an enclosed arm and were allowed to explore the maze freely for 5 min. The following parameters were assessed: i) percentage of time spent in open arms, ii) percentage of entries to open arms, and iii) total number of entries in open and closed arms. An increment in parameters i) and/or ii) are consistent with a decrease in anxiety behavior whereas parameter iii) is indicative of locomotor activity (Pellow et al., 1985). The floor of the maze was wiped thoroughly with a cloth embedded with a 10% ethanol solution after each test. The test was carried out in a quiet room from 09:00 am to 03:00 pm.

2.6. Statistical analysis

The data about mothers and their litters were analyzed by one-way ANOVA and repeated-measures ANOVA. The litter was used as the statistical unit. Sensorimotor development tests were analyzed by two-way ANOVA (group x sex). The data derived from the15 min OF test were analyzed using three-way ANOVA (group x sex x age). A repeated-measures ANOVA was performed for a comparative analysis of the OF parameters evaluated every 5 min. A *t*-test for paired samples was carried out to analyze the differences in each 5 min period within each group whereas a *t*-test for independent samples was carried out in order to analyze the differences among groups in each 5 min period. The PM variables were analyzed by three-way ANOVA (group x sex x age). Differences between groups were assessed using LSD *post hoc* test. A value of $p<0.05$ was considered statistically significant. All statistical analyses were carried out using software SPSS Statistics 21 for Windows.

3. Results

3.1. Data about mothers and their litters

Compared to the control group, water and food intake during pregnancy and lactation was not affected in mothers exposed to the two Gly-BH concentrations tested (Fig 1A-D).

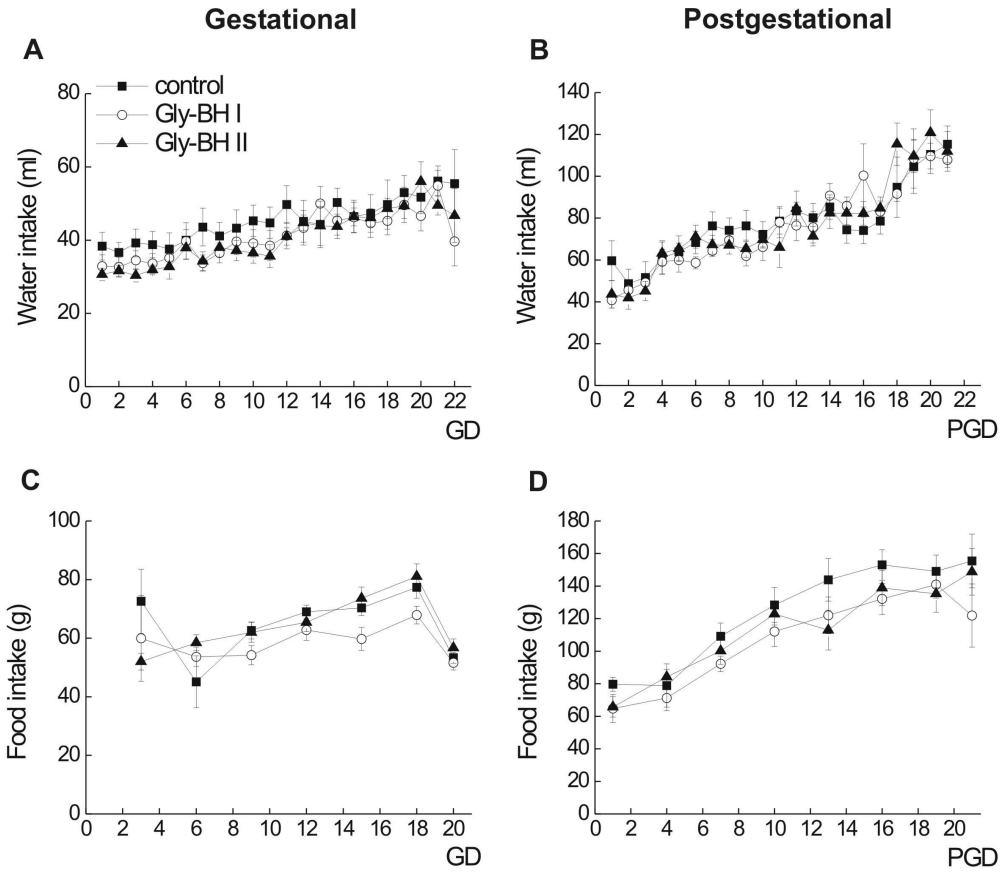

Fig. 1. Maternal consumption of water and food under control condition and under exposure to a Gly-BH. A) and C) Water and food intake on the indicated GDs. B) and D) Water and food intake during the indicated PGDs. Gly-BH I corresponds to a concentration of 0.65 g/L of Gly; Gly-BH II corresponds to a concentration of 1.30 g/L of Gly. All data are presented as mean ± SEM. n=10 for each group.

There were neither statistical differences in the dams´ body weight on GD0 nor in body weight gain during pregnancy, and gestational length and litter size were not affected by Gly-BH treatment (Table 1).

Table 1. Animal weights and characteristics of mothers and their litters in control and Gly-BH-exposed groups.

	Control (n = 10)	Gly-BH I (n = 10)	Gly-BH II (n = 10)
Body weight of dams (g)			
GD0	279.1± 16.2	256.1 ± 9.9	283.6 ± 15.7
Weight gain (g)			
GD 0-3	12.3 ± 1.4	11.5 ± 3.0	8.4 ± 3.0
GD 3-6	9.4 ± 1.8	6.4 ± 2.3	7.9 ± 1.9
GD 6-9	10.6 ± 2.1	8.0 ± 1.0	13.1 ± 1.4
GD 9-12	18.1 ± 1.9	18.0 ± 2.1	14.0 ± 1.1
GD 12-15	15.7 ± 2.6	13.4 ± 1.5	14.8 ± 1.7
GD 15-18	32.9 ± 3.9	27.1 ± 3.2	36.0 ± 3.0
GD 18-20	25.1 ± 2.6	26.5 ± 2.3	22.1± 1.8
GD 0-20	124.1 ± 5.6	110.9 ± 6.6	116.3 ± 7.2
Length of gestation (days)	22.0 ± 0.0	22.0 ± 0.5	22.1 ± 0.4
Litter size			
Female	4.9 ± 1.2	4.8 ± 0.5	5.4 ± 0.9
Male	6.4 ± 1.2	4.1 ± 1.0	5.1 ± 1.1
Total	11.3 ± 1.2	8.9 ± 1.4	10.5 ± 1.5

Gly-BH I corresponds to a concentration of 0.65 g/L of Gly; Gly-BH II corresponds to a concentration of 1.30 g/L of Gly. Values are mean ± SEM.

There were neither visible external malformations in any of the groups analyzed, nor was pups´ body weight affected as a result of Gly-BH intake by mothers during pregnancy and lactation (Fig. 2A and B).

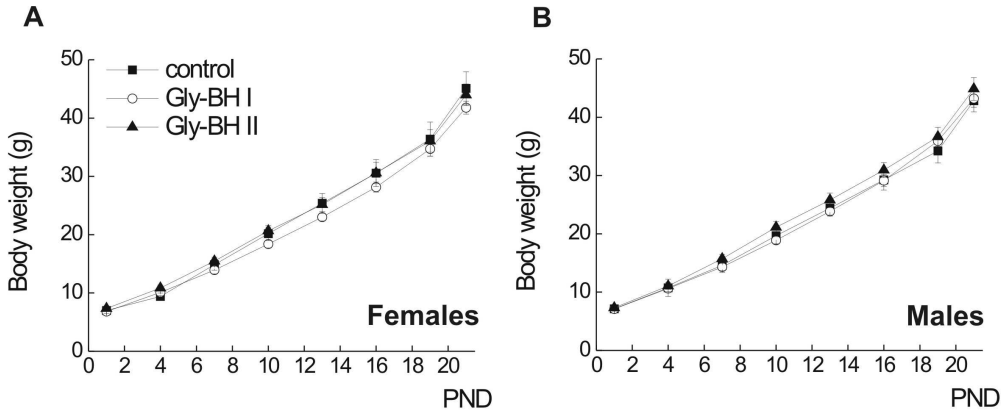

Fig. 2. Body weight of female (A) and male (B) rat pups on the indicated PNDs. Gly-BH I corresponds to a concentration of 0.65 g/L of Gly; Gly-BH II corresponds to a concentration of 1.30 g/L of Gly. All data are presented as mean ± SEM.

3.2. Sensorimotor development

Two-way ANOVA revealed significant differences between groups for the cliff aversion reflex, ($F_{(2,54)}$ = 10.34, p<0.001). *Post hoc* comparisons showed an early onset in the development of the cliff aversion reflex in Gly-BH I and II groups with respect to controls. This was significant in female pups (p<0.01 and p<0.05 for Gly-BH I and Gly-BH II, respectively) as well as in male pups (p<0.05 and p<0.01 for Gly-BH I and Gly-BH II, respectively). Two-way ANOVA revealed no statistical differences in the development of negative geotaxis and righting reflex (Table 2).

As to the day when eye and auditory canal opening occurred in offspring, differences were observed among the groups analyzed only for auditory canal opening (two-way ANOVA, $F_{(2,54)}$ = 10.74, p<0.001). *Post hoc* comparisons showed an early auditory canal opening with respect to controls in female and male pups exposed to the two Gly-BH concentrations tested (females: p<0.001 for Gly-BH I and p<0.05 for Gly-BH II; males: p<0.01 for Gly-BH I and p<0.05 for Gly-BH II) (Table 2).

Table 2. Postnatal day (PND) on which sensorimotor reflexes were acquired and physical parameters of maturation were recorded in rat pups from female rats treated orally with water or with a Gly-BH during pregnancy and lactation.

	Control		**Gly-BH I**		**Gly-BH II**	
Sensorimotor reflexes (PND)	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**
Righting Reflex	4.21 ± 0.12	4.43 ± 0.20	4.64 ± 0.13	4.45 ± 0.16	4.28 ± 0.11	4.21 ± 9.6E^{-2}
Cliff Aversion	6.37 ± 0.19	6.64 ± 0.32	5.31 ± 0.17 **	5.50 ± 0.17 *	5.72 ± 0.25 *	5.63 ± 0.26 **
Negative Geotaxis	11.26 ± 0.53	11.00 ± 0.30	11.50 ± 0.66	10.91 ± 0.39	11.50 ± 0.53	11.00 ± 0.41
Physical parameters (PND)						
Eye opening	13.89 ± 0.23	14.36 ± 0.17	14.21 ± 0.11	14.27 ± 0.14	14.00 ± 0.18	14.00 ± 0.20
Auditory canal opening	12.26 ± 0.21	12.57 ± 0.31	11.43 ± 0.17 ***	11.45 ± 0.16 **	11.78 ± 0.10*	11.79 ± 0.16*

Gly-BH I corresponds to a concentration of 0.65 g/L of Gly; Gly-BH II corresponds to a concentration of 1.30 g/L of Gly. All data are shown as mean ± SEM. n=10 per sex and per group. * p<0.05, ** p<0.01, *** p<0.001 compared to the respective control group.

3.3. Open field

Three-way ANOVA of the number of squares crossed during total 15 min OF test revealed significant differences among the factors Group ($F_{(2, 108)}$ = 26.61, p<0.001), Age ($F_{(1, 108)}$ = 33.87, p<0.001) and Sex ($F_{(1, 108)}$ = 5.68, p<0.02) as well as in the interaction of factors Age x Sex ($F_{(1, 108)}$ = 8.74, p<0.001). *Post hoc* comparisons of 45-day-old offspring showed that female rats from Gly-BH II-treated group exhibited a significant decrease in the number of squares crossed with respect to the control group (p<0.01). In contrast, no significant differences were observed in male rats (Fig. 3A). *Post hoc* comparisons in 90-day-old rat offspring showed that female and male rats exposed to the two Gly-BH concentrations tested crossed a significantly lower number of squares with respect to controls (female rats: p<0.001 for Gly-BH I and Gly-BH II; male rats: p<0.05 and p<0.001 for Gly-BH I and Gly-BH II, respectively) (Fig. 3B).

The comparison of the number of squares crossed every 5 min by ANOVA for repeated measures showed significant intra-subject differences ($F_{(2,216)}$ = 622.46, p<0.001). The number of squares crossed in each 5 min period was used to analyze the animals' habituation to the OF. All groups of rats showed greater locomotor activity during the first 5 min period which declined in the second and in the third period (p<0.05 for all groups with respect to the first 5 min period) (Fig. 3A' and B'). This gradual and significant decrease in their locomotor activity throughout the test session indicated that all animals were habituated to the OF.

As to the number of rearings performed, statistically significant differences were observed among the groups analyzed ($F_{(2, 108)}$ = 9.56, p<0.001; three-way ANOVA). *Post hoc* comparisons showed a significant decrease in the number of rearings only in 90-day-old female and male rats from the Gly-BH II-treated group in comparison with the control group (p<0.05 for both sex) (Fig. 3D).

Analysis by three-way ANOVA of emotionality parameters showed significant differences in grooming between Age ($F_{(1, 108)}$ = 11.25, p<0.001) and Sex ($F_{(1, 108)}$ = 5.29, p<0.05) and in the interaction of Age x Group ($F_{(2, 108)}$ = 3.95, p<0.05). *Post hoc* comparisons showed significant differences only in 90-day-old offspring, particularly in the male rats exposed to the two Gly-BH concentrations tested. Compared to the control group, these rats performed a higher number of grooming episodes (p<0.05 for Gly-BH I and Gly-BH II) (Fig. 3F). No statistically significant differences were observed in the number of fecal boluses (data not shown).

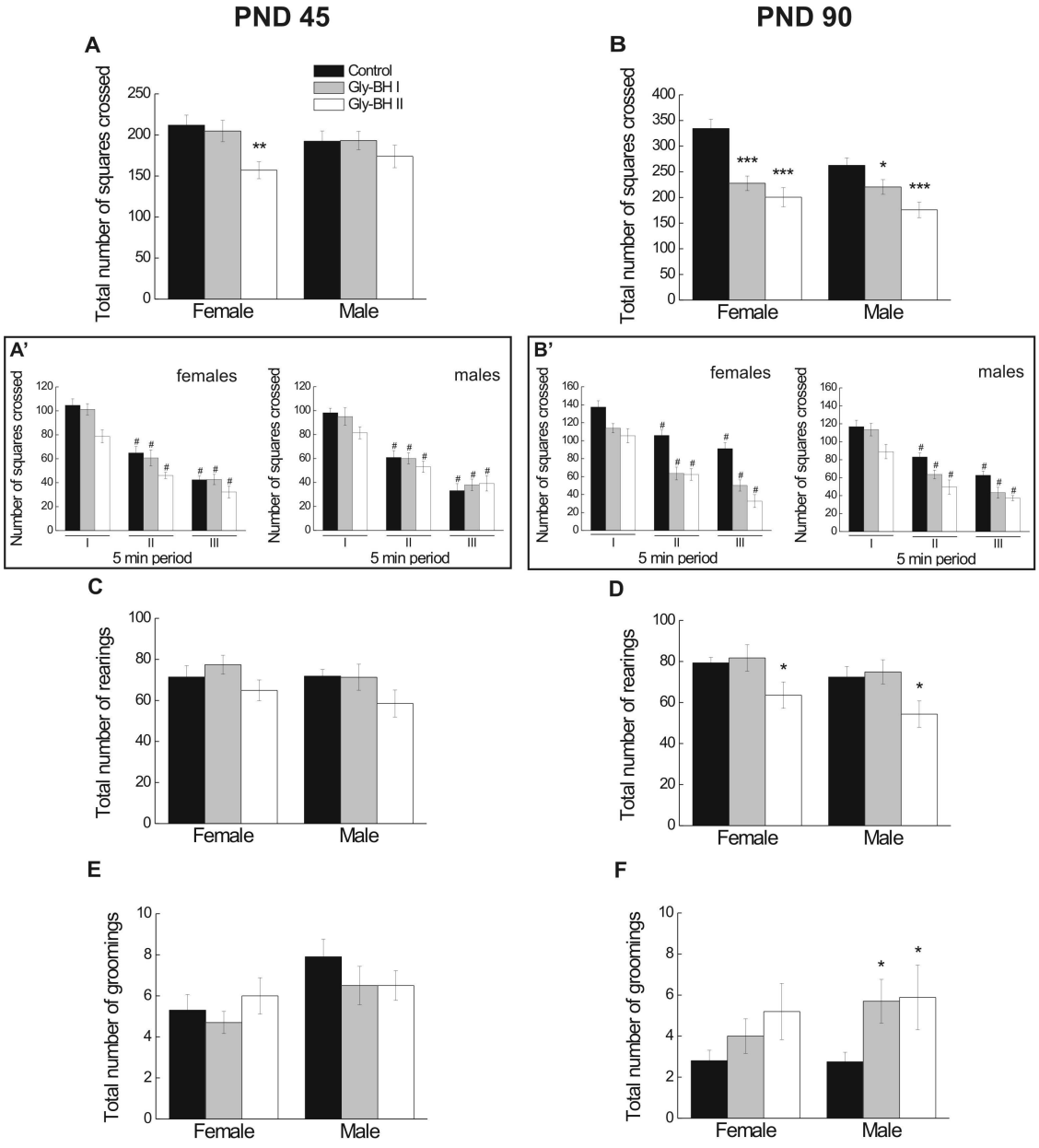

Fig. 3. Open field (OF) evaluation. Total number of squares crossed by 45- (A) and by 90-day-old (B) offspring during the 15 min test. The number of squares crossed by 45- and 90-day old offspring in each 5 min period of the OF test is shown in A') and B') respectively. Total number of rearings performed by 45- (C) and by 90-day-old (D) offspring. Total number of grooming episodes in 45- (E) and 90-day-old (F) animals. Gly-BH I corresponds to a concentration of 0.65 g/L of Gly; Gly-BH II corresponds to a concentration of 1.30 g/L of Gly. Values are mean ± SEM. n= 10 per sex and per group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to the respective control group. # $p<0.05$ compared to the respective first 5 min period.

3.4. Plus Maze

Three-way ANOVA showed significant differences in the percentage of time spent in open arms between the factors Group ($F_{(2, 108)} = 15.84$, $p<0.001$) and Age ($F_{(1, 108)} = 22.56$, $p<0.001$). *Post hoc* comparisons in 45-day-old offspring showed that female rats from Gly-BH I and Gly-BH II treated groups exhibited a significant increase in this parameter with respect to the control group ($p<0.01$ and $p<0.05$ for Gly-BH I and Gly-BH II, respectively), whereas no significant differences were observed in male rats (Fig. 4A). In PM observations of 90-day-old offspring, *post hoc* test revealed that the animals exposed to a Gly-BH spent more time in open arms than controls (female rats: $p<0.05$ and $p<0.005$ for Gly-BH I and Gly-BH II, respectively; male rats: $p<0.005$ and $p<0.05$ for Gly-BH I and Gly-BH II, respectively) (Fig. 4B).

As to the percentage of entries to the open arms, three-way ANOVA showed significant differences among the factors Group ($F_{(2, 108)} = 8.41$, $p<0.001$), Age ($F_{(1, 108)} = 25.02$, $p<0.001$) and Sex ($F_{(1, 108)} = 4.16$, $p<0.05$). For this parameter, *post hoc* comparisons showed significant differences only in 90-day-old offspring, with a higher percentage of entries to the open arms in the Gly-BH exposed groups with respect to controls (female rats: $p<0.005$ for Gly-BH II; male rats: $p<0.05$ for both Gly-BH I and Gly-BH II) (Fig. 4D).

When the total number of arm entries was analyzed, three-way ANOVA test showed no statistically significant differences (Fig. 4E and F).

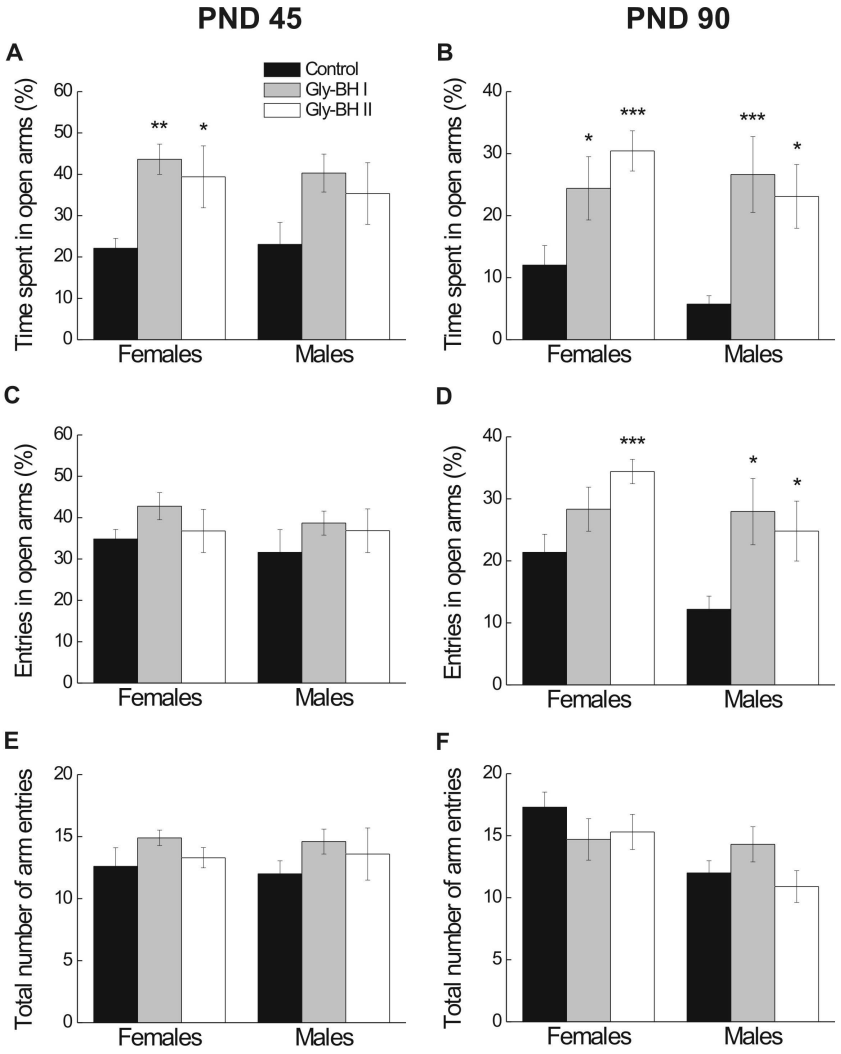

Fig. 4. Plus maze analysis of control and Gly-BH-exposed offspring. A) Percentage of time spent in open arms, C) percentage of entries to open arms, and E) total number of arm entries, on PND 45. B) Percentage of time spent in open arms, D) percentage of entries to open arms, and F) total number of arm entries, on PND 90. Gly-BH I corresponds to a concentration of 0.65 g/L of Gly; Gly-BH II corresponds to a concentration of 1.30 g/L of Gly. Data are mean ± SEM. n= 10 per sex and per group. * $p<0.05$, ** $p<0.01$, *** $p<0.005$ compared to the respective control group.

4. Discussion

The present study shows that exposure to a Gly-BH in rats during pregnancy and lactation affects neither maternal weight gain during pregnancy nor gestational length, litter

size, and body weight of rat pups on different PNDs. However, an early onset in the development of cliff aversion reflex and auditory canal opening was observed in the offspring whose mothers had been exposed to the two Gly-BH concentrations tested. In addition, the fact that neither changes in the development of negative geotaxis and righting reflex nor modifications in the age of eye opening were observed seems to indicate that exposure to a Gly-BH alters only certain restricted patterns of development. In agreement with our results, Tamburella et al. (2012) observed that rats prenatally exposed to the organic compound trimethyltin chloride (TMT) evidenced an earlier onset in the development of several neonatal reflexes as compared to the control group. These authors claimed that neonatal reflexes can be considered to be an index of brain maturation. The changes in their development and expression could represent a predictive factor of other behavioral alterations in adulthood (Fox, 1965; Iezhitsa et al., 2001) as demonstrated by impaired cognitive performance via cognitive tests in rats prenatally exposed to TMT (Tamburella et al., 2012). In line with this, our OF results showed that 45-day-old female rats from the Gly-BH II-treated group crossed significantly less squares with respect to the control group, this being indicative of a decrease in locomotor activity in the females exposed to the highest Gly-BH concentration. In contrast, no significant differences were observed in male rats. This finding was not unexpected because, compared to males, female rats are in general more sensitive to the toxic effects of chemicals (Gad and Chengelis, 1988; Mugford and Kedderis, 1998). In addition, the fact that the 90-day-old female and male rats exposed to the two Gly concentrations tested crossed a significantly lower number of squares with respect to controls could be interpreted as an indicator of an increase in the effects of the Gly-BH treatment on locomotor activity in adulthood. The statistical analysis of the number of rearings showed a significant decrease in female and male rats from the Gly-BH II-treated group with respect to the control group. The decrease in the number of squares crossed as well as in the number of rearings are positively correlated to deficits in arousal, an increase in emotional response or motor activity

impairments (Prut and Belzung, 2003). Our analysis of an emotionality parameter, such as grooming, showed that 90-day-old male rats exposed to the two Gly concentrations tested performed a higher number of grooming episodes than controls in agreement with the general assumption that male rats are more fearful than females (Aguilar et al., 2003; Gray, 1971). Under control conditions, no significant differences between females and males were observed in relation to this parameter although the exposure to a Gly-BH seemed to exacerbate the natural emotional condition of males, a phenomenon which was not observed in females.

Results derived from the PM test revealed that exposure to a Gly-BH during pregnancy and lactation significantly increased the percentage of time spent in open arms in 45-day-old female offspring with respect to the control group. In contrast, no significant differences were observed in male rats. This could be due to the increased sensitivity to toxics in females with respect to males (Gad and Chengelis, 1988; Mugford and Kedderis, 1998). In 90-day-old offspring it was observed that rats exposed to a Gly-BH not only spent more time in open arms than controls, but also registered a higher percentage of entries to open arms. These findings were observed in female and male rats at the two Gly-BH concentrations tested and they revealed a decrease in anxiety levels (Pellow et al., 1985).

Our observations from the PM test were found to be consistent with our results derived from the OF test. In line with this, OF tests carried out in previous research have also demonstrated that rats either with low emotionality or treated with anxiolytic drugs show a decrease in the total distance travelled as well as in rearing frequency (Cannizzaro et al., 2001; Denenberg, 1969). It has also been well documented that anxiety and locomotor activity are often interlinked (Courvoisier et al., 1996; Steimer et al., 1997). Therefore, taking into account our results from the OF and PM tests performed, it can be postulated that exposure to a Gly-BH during pregnancy and lactation induces a decrease in locomotor activity and anxiety levels in rat offspring. In this regard is important to highlight that in the

present work we used a formulated product instead of pure Gly. Pesticide formulations are mixtures of an active ingredient with adjuvants, many of which have intrinsic toxic effects. In the present experiments, the components of the formulation are not available, thus we could not compare our results against a control group treated with all the formulation ingredients except Gly. Because of these uncertainties, the observed results cannot be attributed solely to Gly, and could be due solely to the components of the formulation, or to interactions between Gly and other formulation ingredients (El-Shenawy, 2009; Mesnage et al., 2013). Our results certainly apply most directly to pesticide handlers and applicators, as they would be exposed to the formulated product. Further, depending on the environmental fates of the different components of the formulation, our results may also have implications for the general population.

Another important issue that we must deal with is the fact that the neurobehavioral changes observed in the offspring upon Gly-BH exposure could be a consequence of the direct action of the herbicide on the offspring's CNS, or an indirect effect, affecting brain development of the pups due to the induction of changes in the behavior of the mothers. In favor of the first possibility, we have to consider that the animals were exposed to Gly-BH so as to achieve doses of Gly that were the fifth and the tenth of the NOAEL for maternal toxicity (1000 mg/kg/day). On the other hand, although the ability of Gly to cross the placenta has been demonstrated (Mose et al., 2008; Poulsen et al., 2009), the presence of this compound in the offspring's blood was not determined in the present work. Additionally, several studies have shown the influence of maternal behavior on offspring’s brain development (Cummings et al., 2010; Ricceri et al., 2006), so the possibility that Gly-BH exposure was indirectly affecting the offspring as a result of the effects exerted on the mothers should be considered.

Locomotor activity in the OF test is positively correlated with the levels of DA and/or DA receptors (Bano et al., 2014; Gallo et al., 2015; Kim et al., 2013). Degeneration and loss

of dopaminergic neurons as well as alterations in the levels of DA and/or DA receptors have been reported in cases of locomotor disorders, such as Parkinson's disease and dystonia (Antonelli and Strafella, 2014; Giannakopoulou et al., 2010; Joyce and Millan, 2007; Michel et al., 2013). As to the neurotransmitters involved in anxiety modulation, the influence of 5-HT and GABA and of its receptors has been extensively documented (Liu et al., 2013; Liu et al., 2015; Olivier et al., 2013). Further studies have also well documented that either an increase in GABA activity or a decrease in 5-HT neurotransmission triggers anxiolytic effects (Chopin and Briley, 1987; Gray et al., 1984; Treit et al., 1993). In line with these findings, Näslund et al. (2015) found that 5-HT depletion reduced anxiety levels in the Wistar rats most inclined to avoid the open arms of the PM ("anxious" rats). In addition, Vaz et al. (2015) observed that GABA administration to Wistar rats produced an anxiolytic effect, i.e. the animals that received GABA spent more time in the open arms of the PM compared to the control group. It has also been reported that the oral administration of Gly in rats decreases 5-HT and DA levels in the frontal cortex, striatum and midbrain, whereas it increases 5-HT and DA metabolites (5-hydroxyindole-3-acetic acid, for 5-HT; 3,4-dihydroxyphenylacetic acid and homovanillic acid, for DA) (Anadón et al., 2008). In line with these findings, Hernández-Plata et al., (2015) have shown recently that exposure of rats to Gly caused hypoactivity, together with a decrease in the binding to D1-DA receptors in the nucleus accumbens and in the extracellular DA levels in striatum.

Taken together, our findings demonstrate that exposure to a Gly-BH during the gestational period and lactation produces alterations in locomotor activity, emotionality and anxiety in rat offspring. These observations could be a consequence of alterations in the GABAergic, dopaminergic and/or serotoninergic neurotransmitter systems. Further experimental research including measurements of DA, 5-HT and GABA levels as well as an analysis of the number of their corresponding receptors in specific brain areas are planned to evaluate this hypothesis.

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