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Exposure to glyphosate during pregnancy induces neurobehavioral alterations and downregulation of Wnt5a-CaMKII pathway

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ABSTRACT

Glyphosate-based formulations are the most popular herbicide used around the world. These herbicides are widely applied in agriculture to control weeds on genetically modified crops. Although there is much evidence showing that glyphosate-based herbicides induce toxic effect on reproductive and hepatic systems, and also cause oxidative damage on cells, studies from recent years revealed that the nervous system may represent a key target for their toxicity. In the present work, we evaluated the effect of glyphosate (without adjuvants) in neonate rats after gestational exposure. Particularly, we examined whether glyphosate during gestation affected the nervous system function at early development. Pregnant Wistar rats were treated with 24 or 35 mg/kg of pure glyphosate every 48 h and neurobehavioral studies were performed. Our results indicated that gestational exposure to glyphosate induced changes in reflexes development, motor activity and cognitive function, in a dose-dependent manner. To go further, we evaluated whether prenatal exposure to glyphosate affected the Ca^{+2}-mediated Wnt non-canonical signaling pathway. Results indicated that embryos exposed to glyphosate showed an inhibition of Wnt5a-CaMKII signaling pathway, an essential cascade controlling the formation and integration of neural circuits. Taken together, these findings suggest that gestational exposure to glyphosate leads to a downregulation of Wnt/Ca^{+2} pathway that could induce a developmental neurotoxicity evidenced by deficits at behavioral and cognitive levels in rat pups.

1. Introduction

The global use of agrochemicals around the world has alarmingly increased during the last decade. The broad-spectrum glyphosate (N-phosphonomethyl-glycine) based herbicides (GBH) are widely used in agricultural practice, particularly in association with genetically modified varieties engineered to be glyphosate (Glyph) resistant such as soy crops. It is used as a non-selective, post emergence pesticide mostly to control broadleaf weeds by inhibiting the shikimic acid pathway, necessary for plant protein synthesis [1]. Commercial formulations contain an aqueous solution of Glyph salt as well as other adjuvant compounds, including surfactants that are necessary for an effective uptake of Glyph in plants but many may present intrinsic toxicity [2,3]. Indeed, it has been postulated that the herbicidal activity of the Glyph is potentiated by the presence of adjuvants [4], or by some synergic reaction between Glyph and the other formulation ingredients [5,6]. In this regard, it has been demonstrated that the predominant surfactant POEA (polyethoxylated tallowamine) of different formulations affects cell permeability and increases the toxicity induced by Glyph [2,3,7].

Two decades ago, Glyph was postulated as less toxic for human and animals than other pesticides and consequently, several worldwide regulatory agencies concluded that Glyph and its formulations were safe to use [2]. However, important associations have been suggested between the massive use of GBH and the increased rates of diseases such as cancer, endocrine effects and also neurodegenerative disorders [8–11]. In this context, the International Agency for Research on Cancer (IARC) concluded in March 2015 that Glyph and its formulated products are probably carcinogenic to humans [12].

It is known that the mammalian nervous system exhibits high vulnerability to pesticides. In fact, different studies have defined pesticide exposure as a risk factor for neurodegenerative disorders [13–16]. In line with this, Glyph has been detected in brain and cerebrospinal fluid after exposure to commercial formulations, suggesting that the active ingredient can cross the blood brain barrier in humans [17,18]. Reports have informed about accidental and occupational exposure with commercial formulations of Glyph and negative effects on the nervous

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system, including Parkinsonism [8,9], anxiety and short-term memory impairments [19]. Indeed, several experimental studies have demonstrated that pre- and postnatal exposure to herbicides may be related to neurotoxic effects. In this context, studies showed that GBH induces teratogenic effects in amphibians characterized by cranial deformities and eye abnormalities [10]. Similar developmental effects were observed in chicken embryos exposed to Glyph herbicides [20]. Lately, it has been demonstrated that maternal exposure to GBH induces neurotoxicity by inducing activation of NMDA receptor, changes associated with oxidative stress as well as glutamate excitotoxicity [21]. More recently, it has been shown that rats exposed to GBH during pregnancy and lactation exhibited global delay in reflexes and deficit in motor development. Furthermore, at adult ages those animals showed decrease in motor activity, learning and short- and long-term memory [22]. This evidence clearly supports that the exposure to Glyph formulations induces neurotoxicity that may be reflected by deficits in behavior and cognitive functions. Notwithstanding, there is less amount of data supporting the fact that Glyph without other components of the formulations is able to induce neurotoxicity. In fact, one study showed that adult male rats exposed to repeated doses of Glyph manifested hypoactivity and changes in dopaminergic markers [23].

We previously demonstrated that Glyph affects the ability of hippocampal neurons to differentiate their axons and get a mature phenotype. Briefly, undifferentiated pyramidal neurons exposed to a sublethal dose of Glyph evidenced a significant and irreversible delay in their development and complexity, since they showed a simple morphology characterized by short axons and unbranched dendrites. We also identified the molecular mechanism induced by Glyph to produce this morphological effect. Thus, a single dose of Glyph led to a significant decrease in Wnt5a expression (an essential factor for a proper neuronal development and maturation) and to the inhibition of its effector, CaMKII [24]. These alterations might be reflected in a subsequent neuronal dysfunction during development.

The aim of the present study was to evaluate the neurobehavioral effect of prenatal exposure to Glyph (without surfactants) during early postnatal periods. To go further, we examined whether Glyph exposure affects the activity of Wnt5a-CaMKII pathway in the hippocampus, as it was previously observed in hippocampal cultured neurons [24].

2. Materials and methods

2.1. Animals

Sexually mature male and female Wistar rats (90–120 days old) were purchased from the Vivarium of the Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario (FCByF-UNR, Argentina). They were group-housed in a properly controlled room with a 12 h light/dark cycle at 22 ± 1 °C, with food and water *ad libitum*. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals (National Institute of Health). They were also approved by the Institutional Animal Care and Use Committee at the FCByF-UNR.

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 embryonic day 1 (ED 1). Pregnant females were housed individually, and were randomly assigned to one of the experimental groups.

2.2. Drugs

Glyph was purchased from Sigma-Aldrich as a 40 % water solution of N-phosphonomethyl glycine-monoisopropylamine salt, was diluted in phosphate buffered saline (PBS) 1X and adjusted to pH 7.4 with NaOH. Two dilutions were prepared from Glyph stock to obtain 24 and 35 mg/mL solutions ready to inject to the dams, in a volume equivalent to the body weight.

2.3. Glyph treatment

Dams received i.p. injections from ED 8 until ED20, every 48 hs. They were divided in 3 experimental groups: Control (vehicle, PBS 1X, 1 mL/kg) (n = 8), Glyph 24 mg/kg (n = 8) or Glyph 35 mg/kg (n = 8). Each dam received seven injections over two weeks either of PBS solution (vehicle) or Glyph (24 or 35 mg/kg). Doses were selected based on Glyph No Observed Adverse Effect Level (NOAEL) of 1000 mg/kg/day for maternal and developmental toxicity [2,25]. For both, the doses and injection regimen, we also considered results from a previous study where adult rats received six i.p. injections of Glyph during 2 weeks. These results led us to test doses lower than 50 mg/kg i.p. (the lowest one tested by [23]) and to give them every other day. Moreover, the time between Glyph administrations would attenuate the injection stressful effect on the dams, as well as Glyph's body accumulation. In this context, the cumulative doses given to the mothers at the end of treatment was between 44 and 60 mg of the herbicide (for the lower dose, 24 mg/kg) and between 63 and 87 mg of Glyph (for the higher dose, 35 mg/kg), depending on their weight gain throughout pregnancy. During treatment, maternal weight gain was recorded every other day and, 24 h after delivery all pups were weighed, and litters were reduced to 8 when necessary. For each treatment, at least two dams were run in parallel and from each litter, 4 pups were used. However to assure data independence two of those four pups were assigned to another dam of the same treatment group during the litter reduction. After weaning, offspring were housed in groups of 4–6 male rats according to treatment and behavioral tests, receiving tap water and food *ad libitum*. The following data were analyzed: length of gestation, litter size, and body weight of pups on different postnatal days (PND) (every 48 hs from PND3 to 46).

2.4. Experimental procedures

Half of the pups were submitted to the behavioral tests run up to PND 25, that is: the righting reflex test on PND 5, 7 and 9; the negative geotactic reaction on PND 18; and the locomotor activity on PND 25 giving a total n of 16 pups for each group.

The remaining pups were divided in half and submitted to the Morris water maze test from PND 30–35; or the Conditioned Fear Test from PND 40–44 giving a total n of 8 pups for each group and each test.

2.5. Behavioral tests

2.5.1. Righting reflex

Neonates were placed in supine position on a flat surface, and the time needed to regain a position on its 4 limbs was recorded [26,27].

2.5.2. Negative geotaxis reaction

Pups were placed in a head-down position on an inclined surface (45°) covered by wire mesh and the latency to rotate 180° was measured. The test was considered successful when the pup turned 180° (head up). The cut off time was 3 min [26,27].

2.5.3. Locomotor activity

The locomotor activity apparatus consisted of 8 acrylic boxes (43 × 43 × 30 cm) equipped with eight infrared photocell beams located 3 cm above the floor. Interruption of any beam resulted in a photocell count. Pups were individually placed in the boxes, and the total activity as well as the time spent on the centre of the arena was recorded for an hour. The assessment of the total activity is a guide of the exploratory response to a novel environment [28,29]. In addition, the time spent in the centre of the arena assesses approach-avoidance toward novel stimuli, which is considered a reliable index of anxiety, response to anxiolytic agents, and is sensitive to stress-induced anxiety states [30]. The apparatus and software were developed by Laboratorio de Investigación Aplicada y Desarrollo, Facultad de Ciencias Exactas, Físicas y

Naturales (Universidad Nacional de Córdoba, Argentina).

2.5.4. Morris water maze

The water maze test was performed on a water pool (120 cm in diameter) that contains opaque water at 26 °C and a submerged platform (12 cm diameter, 1 cm below water surface) [31,32]. Four training sessions per day (10 min intervals) for 4 days were conducted on each animal. The platform and the water pool location remained in the same place while the entry points of the animals were randomly changed. Each rat was placed on the water pool and allowed to explore for 60 s. Latency time to locate the platform was recorded and in case the animals could not find it, they were guided and placed over it for 15 s. The quadrant that previously contained the hidden platform was designated as 'target quadrant'. On the fifth day (test session) the platform was removed from the pool and the time spent on the target site was recorded for 60 s.

2.5.5. Contextual fear conditioning

Fear conditioning test chamber consisted of an acrylic box (45 × 20 × 23 cm) with a transparent lid, and steel parallel grid bars on the floor connected to a scrambled shocker. During the first day rats were habituated for 10 min and on day 2 (pre-training) they were placed back in the chamber and their freezing time was measured for 5 min. On day 3 (training) animals received 7 scrambled footshocks (0.6 mA, 1−2 s, with 30 s intershock interval). Day 4 (post-training) consisted of recording the freezing time of each rat for 5 min [33,34].

2.6. Hippocampal homogenates

On ED 21, embryos from control and glyphosate-exposed pregnant rats were obtained by C-section and their whole brains were removed. The hippocampi were isolated and homogenized on chilled RIPA buffer and kept on ice for 15 min. Centrifugation at 4 °C and 15,000 × *g* for 5 min was performed and supernatant was recovered [35,36]. Samples were finally prepared for western blot experiments. For each sample, two hippocampi per mother were pooled and lysed in Ripa buffers as was described.

2.7. Electrophoresis and western blot

Protein extracts coming from total homogenates of ED21 hippocampal samples were heated to 80 °C for 5 min with Laemmli buffer as a reducing treatment. Samples (total homogenate: 10 µg/lane) were run in 10 % SDS-polyacrylamide gel and transferred to nitrocellulose membrane (GE, Amersham). Membrane was blocked with 3% BSA for 1 h and primary antibodies were incubated at 4 °C, overnight in 1% BSA while secondary antibodies (HRP conjugated) were incubated at room temperature for 1 h in 0,2% milk. Antibodies used: anti-β-tubulin III (1:10000, rabbit, Sigma-Aldrich), anti-Wnt5a (1:1500, rabbit, Abcam), anti-rabbit (1:5000, Bio-Rad Laboratories). Reactivity was detected using enhanced chemiluminescence (ECL) and quantified using ImageJ software.

2.8. mRNA extraction and RT-PCR

Fresh tissue from ED21 hippocampal samples was homogenized in TRIzol (Invitrogen, Waltham, Massachusetts) and processed according to the manufacturer's instructions. Details on cDNAs synthesis and PCR procedure can be found in the Supporting Information. Primers were selected using Primer3 free software [37] as follow: **F** 5′-TCGAC-TATGGCTACCGCTTC-3′; **R** 5′-CGACCTGCTTCATTGTTGTG-3′. PCR products were separated on 1 % agarose gel stained with ethidium bromide and then observed under UV light. Optical densities (OD) of PCR products were measured using the ImageJ software and normalized to OD values from 18S. Unless specifically stated, all RT-PCR reagents were from Promega (Madison, WI).

2.9. Statistical analysis

All graphs and statistical analyses were performed using GraphPad Prism software. Results were expressed as mean ± SEM and the significance was defined as $p < 0.05$. Either ANOVA or Student's *t*-test analysis was performed according to the experiment design. The ANOVA was followed by a Bonferroni's post hoc test.

3. Results

3.1. Physical parameters from mothers and litters

Table 1 shows the different parameters that were measured to examine physical development of litters as well as mothers, in order to evaluate the effect of Glyph during gestation. There were no differences between groups in litter size, stillbirths, or pregnancy duration. Water and food intake was not affected in mothers exposed to both Glyph doses (24 and 35 mg/kg) compared to control. More importantly, there were not significant differences between control and Glyph treated dams in body weight gain during the gestational period. At birth, pups did not show any visible malformations (or abnormality) neither in control nor in Glyph treated groups. No differences were found regarding the day when hair was appearing, or eyes were opened. However, when pups body weight was measured over time from PND3 until PND45 differences between treatments were observed. In this context, a two-way ANOVA for repeated measures on data from Fig. 1 revealed a significant effect of Glyph dose [$F_{(2, 44)} = 132.9$; $p < 0.0001$], a significant effect of time [$F_{(17, 748)} = 4970$; $p < 0.0001$] as well as interaction between Glyph dose x time [$F_{(34,748)} = 13.57$; $p < 0.0001$]. A post hoc analysis showed that from PND19 until PND23 only pups exposed during gestation to the higher dose of Glyph (35 mg/kg) displayed a significant decrease in body weight compared to control pups. While from PND26 to 45 the body weight of Glyph treated animals with the higher dose was significantly different to the remaining groups (low dose and control). Moreover, from PND29 to 45 pups exposed to the lower dose of Glyph (24 mg/kg) showed a significant decrease of body weight compared to control.

Table 1
Physical parameters from mothers and their litters in control and Glyph exposed groups.

	Control	Glyph 24 mg/kg	Glyph 35 mg/kg
Body weight of dams (g) GD 0	253.8 ± 12.0	261.3 ± 17.2	258 ± 14.6
Body weight of dams (g) GD 22	350.1 ± 8.3	358.4 ± 6.0	354.9 ± 5.1
Length of gestation (days)	22 ± 0.5	22 ± 0	22 ± 0
Litter size	12 ± 1.1	11.4 ± 2.0	11.6 ± 1.5
Hair appearance day	6.5 ± 0.3	7.2 ± 0.7	6.8 ± 0.1
Eyes opening day	14.5 ± 0.2	14.0 ± 0.5	13.9 ± 0.3

All data are presented as mean ± SEM, n = 8 dams for each treatment group.

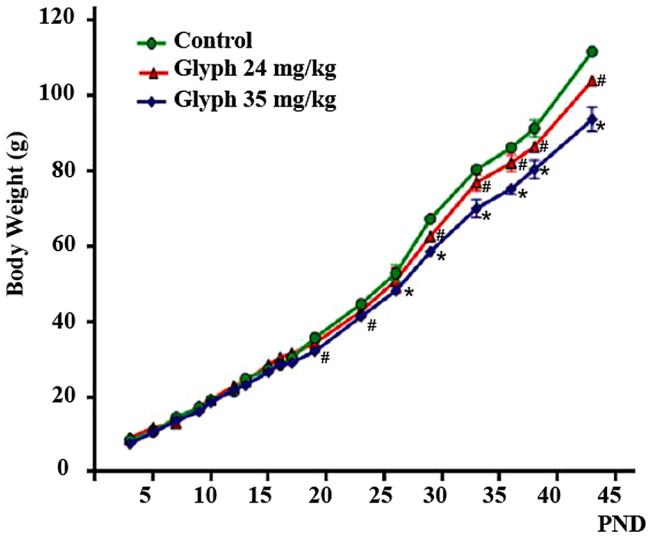

Fig. 1. Gestational Exposure to Glyph affects pups body weight. Graph shows the body weight of pups exposed to saline or Glyph solution (24 or 35 mg/kg) from PND3 to PND45. All data are presented as mean ± SEM. n = 16 for each group, # $p < 0.001$ compared to controls; * $p < 0.0001$ compared to both control and 24 mg/kg Glyph treated groups.

3.2. Glyph affected developmental reflexes

In order to examine potential signs of neurotoxicity on prenatally Glyph exposed rats, we first evaluate the expression of neonatal reflexes by applying a standard battery of tests (Fox, 1965) typically used as an index of brain maturation. The righting reflex was evaluated at PND 5, 7 and 9 in control and Glyph-treated pups. A two-way ANOVA for repeated measures applied to the data in Fig. 2A showed a significant effect of interaction time x dose $F_{(4,88)} = 4.044$, $p < 0.005$; time $F_{(2,88)} = 23.64$, $p < 0.0001$ and dose $F_{(2,44)} = 32.41$, $p < 0.0001$. When analyzed over time, control pups showed a slight but not significant decrease in the latency to return to their four limbs on PND 9 compared to PND 5. However, the reaction time shown by pups exposed to the higher dose of Glyph at PND 5 and 7 was significantly higher compared to PND 9. Moreover, reaction time showed by pups exposed to the Glyph 35 mg/kg at PND 5 and 7 was also significantly different from control and Glyph 24 mg/kg treated pups; suggesting that prenatal treatment with 35 mg/kg of Glyph could delay the acquisition of the righting reflex.

The negative geotaxis was measured on PND 18. A one-way ANOVA applied to data on Fig. 2B showed a significant effect of dose $F_{(2,44)} = 6.583$, $p < 0.005$; while a post hoc analysis revealed that pups prenatally treated with the higher dose of Glyph required a significantly longer time to rotate 180° compared to control and to the lower dose of Glyph groups.

Taken together, these observations suggest that prenatal exposure to Glyph affects the development of reflexes in neonates in a dose-dependent manner.

3.3. Neonates locomotion in response to a novel environment was affected by Glyph

We further examined the total motor activity of control and Glyph-treated pups in response to a novel environment, as well as time spent in the centre of the arena. A one-way ANOVA of the locomotor activity data showed a significant effect of treatment $F_{(2,44)} = 11.69$, $p < 0.0005$; while a post hoc analysis revealed that the higher dose of Glyph

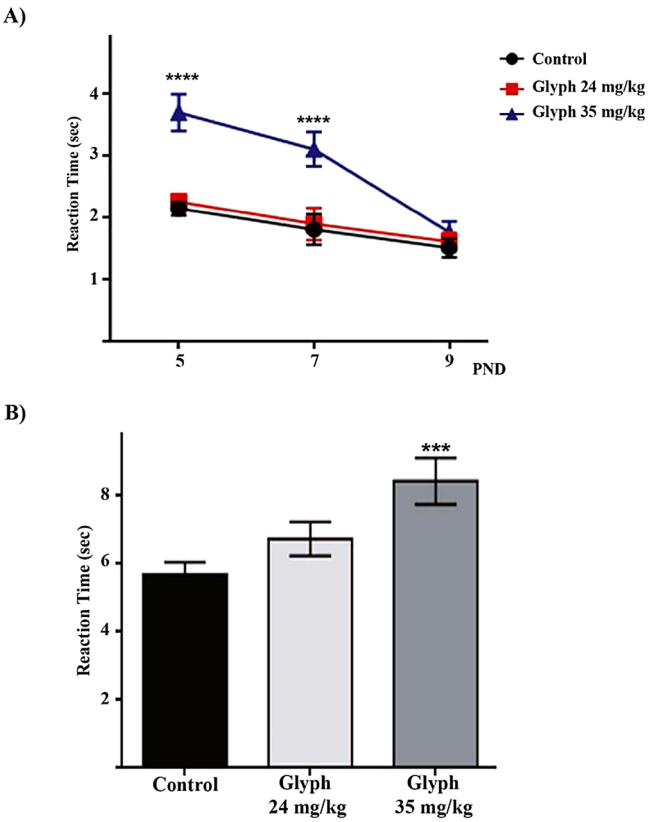

Fig. 2. Glyph affects developmental reflexes in pups. Reaction time of righting reflex (A) and negative geotaxis (B) in control or Glyph exposed pups. All data are presented as mean ± SEM, n = 16 for each group, *** $p < 0.005$ and **** $p < 0.0001$ compared to control groups.

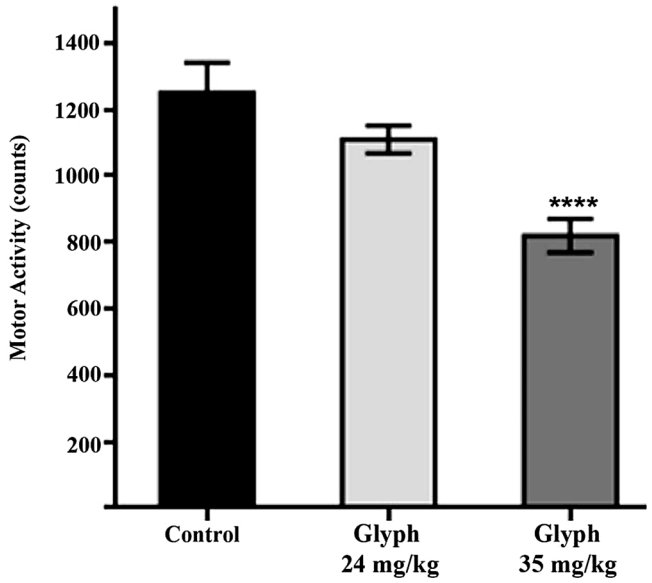

Fig. 3. Spontaneous motor activity is altered by Glyph gestational exposure. Locomotor activity of control and Glyph treated pups at PND 25. The activity was registered for 60 min. Data are presented as mean ± SEM, n = 16 for each group, **** $p < 0.0001$ compared with control group.

significantly reduced exploratory activity compared to control and lower-dose treated pups (Fig. 3). However, prenatal Glyph treatment did not affect the time spent in the centre of the arena ($F_{(2,44)} = 1.886$, $p = 0.1638$; data not shown).

3.4. Glyph impaired cognitive function in neonates exposed during gestation

The Morris water maze was used to evaluate the spatial learning and memory among controls and Glyph-treated pups (Fig. 4). As was mentioned above, the assay was performed during five consecutive days (Fig. 4A). At the training sessions (the first 4 days) the platform location remained constant. After that, the probe trial was conducted by removing the platform and allowing pups to explore the space and reach the quadrant where the platform was previously located (Fig. 4A). The percentage of time each rat spent in the target quadrant was used to assess retention performance during the probe trial. Data showed in Fig. 4B revealed that over the 4 day training session controls and both Glyph-treated (24 and 35 mg/kg) groups spent the same time to locate the hidden platform, indicating that the learning ability was not affected by prenatal Glyph. Thus, a two-way ANOVA showed a significant effect of time $F_{(3,63)} = 302.8$, $p < 0.0005$; where a post hoc analysis revealed that the time spent to reach the platform on day one was significantly higher compared to the other days on the same group, for all the treatments. However, on probe trial day when the platform was removed, the percentage of time spent in the target quadrant was significantly lower in the group exposed to the higher dose of the herbicide compared to the other two groups (control and Glyph 24 mg/kg) (Fig. 4C). A one-way ANOVA applied to data on Fig. 4C showed a significant effect of Glyph dose $F_{(2,21)} = 11.87$, $p < 0.0005$; while a post hoc revealed that the time spent in the platform quadrant by pups prenatally treated with the higher Glyph

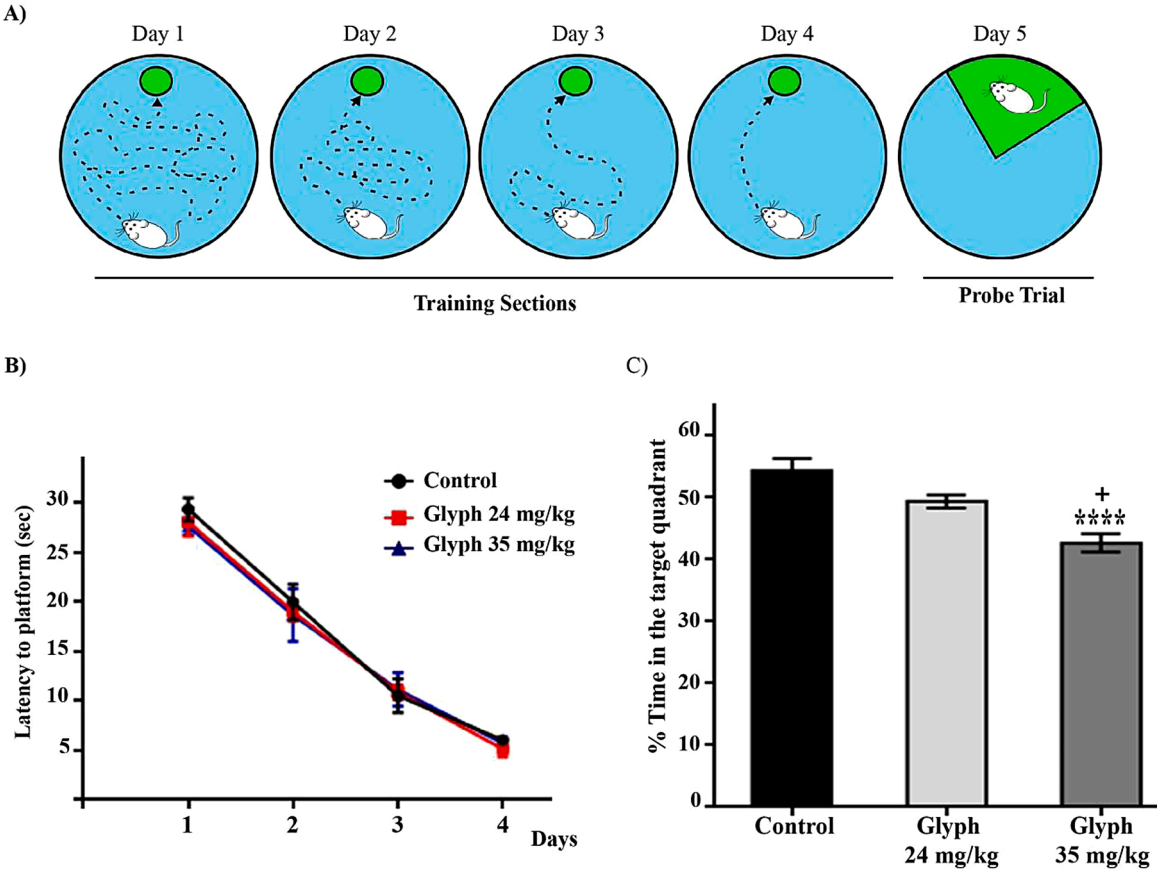

Fig. 4. Glyph exposure affects memory processes. (A) Scheme showing the steps of the Morris water maze test during the trial days. (B) Latency to find the platform during the training days for control and 24 or 35 mg/kg Glyph treated pups. (C) Percentage of time that control and treated animals spent in the quadrant where the platform was. All data are presented as mean ± SEM, n = 8 for each group. **** $p < 0.0001$ compared to control group. + $p < 0.05$ from 24 mg/kg Glyph treated pups.

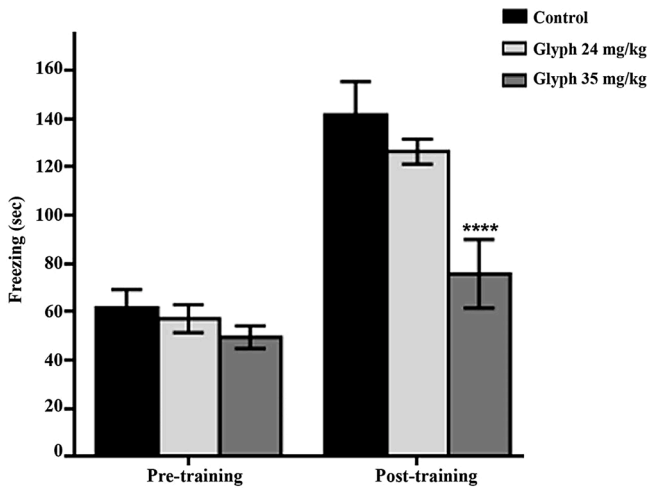

Fig. 5. The exposure to Glyph altered emotional memory. Fear conditioning test was applied for control and Glyph exposed pups (24 and 35 mg/kg) at PND 40. The freezing time of each rat was registered on day 2 (pre-training) and day 4 (post-training). Data are presented as mean ± SEM, n = 8 for each group, **** $p < 0.0001$ compared to control group.

dose was significantly different from control as well as the lower Glyph dose. In addition, the time spent in that quadrant by rats exposed to Glyph 24 mg/kg was similar to controls (Fig. 4C). These results indicated that only the higher Glyph dose induced a poor retention performance in the probe trial, suggesting that prenatal Glyph induces memory deficits in a dose-dependent manner.

Additionally, we performed the contextual fear conditioning test to evaluate the emotional memory of animals exposed to the herbicide. A two-way ANOVA for repeated measures applied on data from Fig. 5 revealed a significant effect of time $F_{(1,20)} = 58.57$, $p < 0.0005$; Glyph dose $F_{(2,20)} = 5.029$, $p < 0.05$ as well as an interaction time x dose $F_{(2,20)} = 4.885$, $p < 0.05$. The post hoc analysis showed that during the pre-training phase, control and Glyph-exposed rats displayed equivalent time of freezing, while during the test phase (post-training) the 35 mg/kg Glyph-treated pups revealed a significantly lower freezing time compared to control and Glyph 24 mg/kg treated pups. Moreover, the freezing time displayed by pups belonging to the control and Glyph 24 mg/kg groups during the test phase was significantly higher than pre-training phase suggesting that those groups acquired the contextual fear conditioning. In contrast, pups prenatally treated with the higher Glyph dose showed equal freezing times implying deficits in the acquisition of this classical conditioning type of learning. Taken together, results from both cognitive tests show that exposure to Glyph during gestation would affect neural processes associated to learning and memory in neonates in a dose-dependent manner.

3.5. Glyph induced a downregulation of Wnt5a-CaMKII pathway in hippocampus

As it was mentioned above, we previously showed that a single dose of Glyph induced a delay in the development of hippocampal cultured neurons through the inhibition of Wnt5a-CaMKII pathway [38]. Interestingly, Wnt5a is a critical factor required for neuronal growth and maturation [39]. In the present study, we evaluate whether Glyph is also able to affect Wnt5a expression *in vivo*. To that, we analyzed the level of Wnt5a mRNA and its protein expression in hippocampus from control and Glyph-treated embryos at ED 21 (Fig. 6). A simple comparison of data shown in Fig. 6 revealed that prenatal exposure to Glyph 35 mg/kg induces a decrease in the level of Wnt5a mRNA compared to control embryos ($t\ 8 = 4.08$, $p < 0.005$; Fig. 6A). Additionally, we observed a significant reduction in Wnt5a protein levels compared to controls ($t\ 10 = 5.415$, $p < 0.0005$; Fig. 6B). These findings showed that Glyph exposure affects the expression of Wnt5a factor suggesting that an intracellular pathway downstream of it could be altered by the herbicide. To go further, we examined whether Glyph treatment affects the CaMKII-mediated pathway in rat embryos. We analyzed the level of phospho-CaMKII (p-CaMKII) as a read out of CaMKII activity on the hippocampus of control and Glyph exposed embryos. Results showed that the exposure to the herbicide leads to a significant decrease in the level of p-CaMKII (around 40 %) ($t\ 4 = 4.899$, $p < 0.01$) compared to controls, whereas total CaMKII level remained unchanged (Fig. 6C). Taken together, these findings suggest that the exposure to Glyph during gestation downregulates Wnt5a-CaMKII pathway in rat embryonic hippocampus.

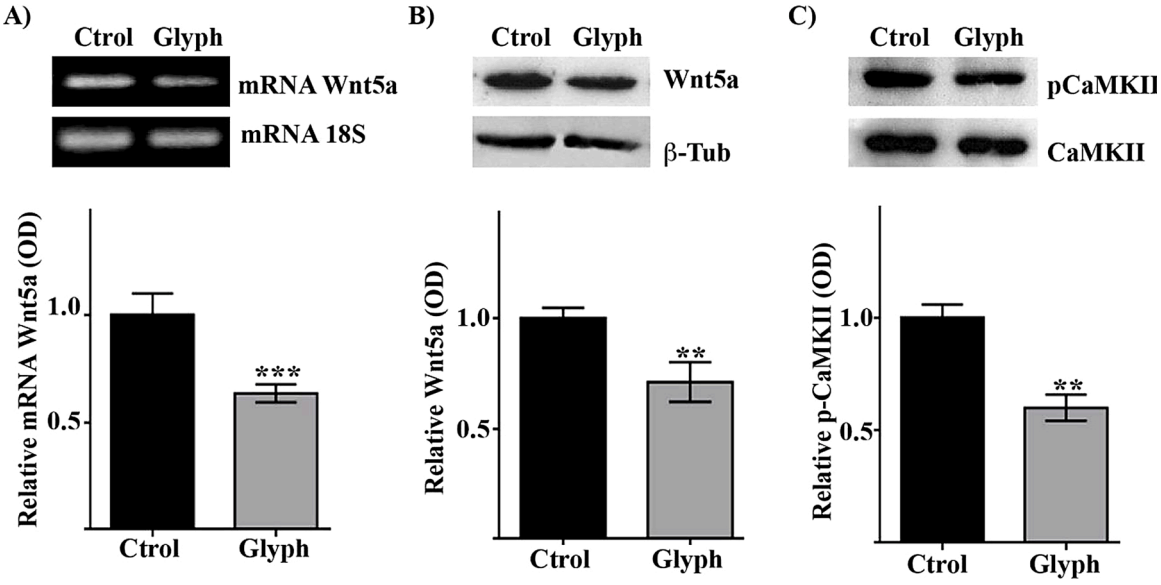

Fig. 6. Gestational exposure to Glyph induces downregulation of Wnt5a/CaMKII in rat hippocampus. The level of mRNA Wnt5a (A), Wnt5a protein (B) and the active form of CaMKII (p-CaMKII) (C) were determined on the hippocampus from control or Glyph treated (35 mg/kg) embryos at ED 21. Below panels show the quantification of relative optical density of mRNA Wnt5a, Wnt5a and p-CaMKII for each condition. As loading controls, mRNA 18S, β-Tub and CaMKII were used. *** $p < 0.001$ and ** $p < 0.05$ compared to controls, n = 5 for each group.

4. Discussion

The purpose of the present study was to describe the effects of pregnancy exposure to Glyph in rats and identify a possible mechanism through which that exposure could generate behavioral and cognitive alterations. Glyph is one of the most commonly used herbicides and the application of GBHs has increased significantly in the last decade. Although Glyph has low environmental persistence [40], its rate and frequency of use might have a negative impact on the environment [41]. Humans are indirectly exposed to Glyph by food and water contaminated with Glyph residues. According to the US Environmental Protection Agency (EPA) the estimated level of Glyph exposure for the general population is about 0.088 mg/kg/day (range 0.058-0.23 mg/kg/day) through food and water [42]. However, the potential toxicity of Glyph or GBHs is commonly described by many toxicological studies obtained from protocols based on higher doses. Indeed, the Glyph doses used in the present study, as in others' reports, are higher than levels to which the population is normally exposed [42] in order to elucidate a probable drug-action mechanism [43]. In this context, we performed treatments using two i.p. sublethal doses given to mothers every 48 h from ED8 to ED20. Doses were selected considering the level of LD50 for oral exposure to Glyph (>5000 mg/kg/day), the NOAEL for maternity and developmental toxicity (1000 mg/kg/day) [2,25] as well as the elimination half-life from plasma after a single i.v. dose of Glyph (e.g. around 10 h) [44]. Consequently, pups were exposed to doses that were thirty to forty times below the NOAEL for maternal toxicity. Moreover, Glyph was administered every other day in order to reduce the stress that injections may cause in the pregnant rats. Although, the level of Glyph in the offspring blood was not measured in our study, the ability of Glyph to cross the placental barrier has already been documented since it was found in maternal and umbilical cord serum [45–47]. In line with our work, a recent study showed that intraperitoneal exposure to higher doses of Glyph for two weeks (3 injections/week) induces neurotoxicity in adult rats [23].

Our findings revealed that pregnancy exposure to Glyph induces a delay in the development of neonatal reflexes and a reduction in locomotion as well as deficits in learning and memory processes, at different post-natal ages in a range from 5 to 45 days after the end of the treatment. Importantly, Glyph exposure affects neither maternal weight gain throughout pregnancy, nor gestational length, litter size or the age of eye opening of rat pups. However, prenatal Glyph exposure produces a 10–20% decrease on pups body weights mainly from PND 29. Moreover, pups treated with the higher dose of Glyph showed a delay in the acquisition of the righting reflex. Whereas rats in the control and lower Glyph-dose group developed it on PND 5, the higher Glyph dose-treated group did it on PND 9. Something similar happened with the negative geotaxis where pups exposed to the higher dose of Glyph need a significantly longer time to rotate compared to control and lower dose groups. Additionally, our results showed that exposure to the higher dose of Glyph induced a decrease in locomotor activity in the open field suggesting that at PND 25 rats have lower levels of exploration in a novel environment; while all pups displayed similar levels of anxiety measured by the time spent in the centre of the arena, regardless of their treatment. A previous study showed that rats orally exposed to GBH (doses equivalent to 100 and 200 mg/kg/day of Glyph) during pregnancy and lactation exhibited a reduction in locomotor activity as well as on anxiety level compared to controls at PND 45, but did show neither changes in the developmental reflexes nor pups body weights [48]. Conversely, rats orally exposed to GBH during pre and postnatal period did not show a decrease in locomotor activity even when the treatment induced a decrease in body mass gain [21] at similar postnatal ages as in our findings. In line with our results, another recent study showed that pre- and postnatal chronic exposure to GBH caused behavioral and cognitive impairment in adult mice. Particularly, it induced a delay in the development of reflexes and a deficit in motor activity in offspring [22]. These discrepancies between studies may be due to different experimental factors such as: exposure pathway (oral vs i.p.), compound used in the study (Glyph vs GBH), length of treatments and the time when assays were made regarding the end of the treatment. Importantly, development and expression of reflexes in neonates have been considered as an index of brain maturation and connectivity [49] and could represent a predictive factor for other behavioral alterations in adulthood [50,51]. Taking these into account, the delay in the development of neonatal reflexes induced by early exposure to Glyph could indicate deficits in brain maturation caused by the herbicide that would predict behavioral alterations such as motor activity and cognitive function in youth/adulthood.

Regarding the cognitive function, we additionally performed cognitive tests to evaluate the effect of gestational exposure to Glyph on spatial learning and memory. Both behavioral tests require the integrity of the hippocampus among other areas [52–54]. Firstly, we carried out the Morris water maze test from PND30 to 35 showing that all pups learnt at a similar rate where the platform was regardless of the Glyph treatment. Then, during the probe trial, when tested without the platform, pups exposed to the higher dose of Glyph spent less time in the original target quadrant than the remaining groups (control and lower Glyph dose). These observations would suggest that Glyph exposure during gestation affects memory retention in pups. Furthermore, we conducted the contextual fear-conditioning test to evaluate another type of learning that involves a classical conditioning task. Our results showed that prenatal Glyph treatment with the higher dose impaired contextual fear acquisition in pups at PND 45. In line with our work, several studies have shown the impairment of cognitive functions in rodents after Glyph or GBH exposure. For instance, exposure to GBH alters recognitionand working and contextual memory in mice [22,55]. Moreover, Gallegos et al. (2018) [56] have shown an impairment of recognition memory in adult rats prenatally exposed to GBH. These results are in agreement with reports showing that humans accidentally exposed to GBH developed short- and long-term memory impairments accompanied with hippocampal lesions [8,19]. Furthermore, it was shown that GBH exposure during pregnancy and lactation induced neurotoxicity mediated by glutamate excitotoxicity and oxidative stress in the rat hippocampus [57], that was partially associated to depressive-like behaviors [21]. Taken together, these results are demonstrating that pre- and/or postnatal exposure to Glyph or GBH might result in an impairment of the cognitive performance since learning and memory processes can be affected.

Therefore, deficits on neonatal reflexes, motor activity and cognitive function of pups exposed to Glyph during gestation could likely be the results of changes on neuronal development and maturation, which are crucial for a proper neural connectivity. Multiple cellular processes, such as, neuronal differentiation, axonal growth, dendritogenesis and synaptic function involve Wnt family proteins [35,36,58–61]. Previously, we showed that a single dose of Glyph irreversibly induces a delay in axonal outgrowth and inhibits dendrite development in hippocampal cultured neurons through downregulation of Wnt signaling pathway. Briefly, Glyph treatment decreased Wnt5a expression and CaMKII activity in hippocampal neurons [38]. In order to investigate whether Glyph affects Wnt5a-CaMKII pathway in vivo we examined the expression of Wnt5a and the level of p-CaMKII in embryos hippocampus. Our findings demonstrated that gestational exposure to the higher Glyph dose led to a decrease in Wnt5a expression and inhibition of CaMKII activity. Wnt5a is expressed in the embryonic hippocampus where it modulates the formation and function of synaptic structures while, Wnt5a-CaMKII cascade maintains hippocampal connectivity and synaptic plasticity [62]. Moreover, transgenic mice that did not express Wnt5a in the hippocampus displayed learning and memory deficits as well as profound disruptions in synaptic plasticity as adults. It was also shown that those changes were signaled through CaMKII [63]. Taken together, our findings suggest that the impairment of Wnt5a-CaMKII pathway in the embryonic hippocampus by gestational exposure to Glyph may disrupt neural circuit connectivity and functioning, inducing

behavioral deficits later on. However, it is important to consider that in our study, the prenatal exposure is systemic and Glyph might be modifying the Wnt5a-CaMKII pathway in different brain areas giving rise to a higher impact on behavior.

In summary, gestational exposure to Glyph leads to a down-regulation of Wnt-CaMKII signaling pathway in the embryonic hippocampus. This could result in a delay of axonal outgrowth and neuronal maturation, as it happened in hippocampal cell cultured [38] as well as decreased connectivity and synaptic function [63], ultimately resulting in long term learning and memory deficits expressed weeks after the exposure ends.

Finally, the results presented here demonstrate that gestational exposure to Glyph (the active ingredient of many formulated herbicides) induces short and long-term neurotoxicity, which could likely be exacerbated by the presence of surfactants in many formulations. Further studies are required to identify key synaptic molecules as potential targets of Glyph induced neurotoxicity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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