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Glyphosate, AMPA and glyphosate-based herbicide exposure leads to GFAP, PCNA and caspase-3 increased immunoreactive area on male offspring rat hypothalamus

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ABSTRACT

Glyphosate, aminomethylphosphonic acid (AMPA), and glyphosate-based herbicides altered the neuroendocrine axis, the content of brain neurotransmitters, and behavior in experimental animal models. Glyphosate alone, AMPA or Roundup® Active were administered to postpartum female rats, from P0 to P10, and their water consumption was measured daily. The immunoreactivity for glial fibrillary acidic protein (GFAP), proliferating cell nuclear antigen (PCNA) and caspase-3 was measured in the anterior, medial preoptic, periventricular, supraoptic and lateroanterior hypothalamic nuclei of P0-P10 male pups after exposure, *via* lactation, to these xenobiotics. Puppies exposed to glyphosate had a moderate level of GFAP with no overlapping astrocyte processes, but this overlapping was observed after Roundup® Active or AMPA exposure. After being exposed to Roundup® Active or AMPA, PCNA-positive cells with strong immunoreactivity were found in some hypothalamic nuclei. Cells containing caspase-3 were found in all hypothalamic nuclei studied, but the labeling was stronger after Roundup® Active or AMPA exposure. Xenobiotics significantly increased the immunoreactivity area for all of the markers studied in the majority of cases ($p<0.05$). AMPA or Roundup® Active treated animals had a greater area of PCNA immunoreactivity than control or glyphosate alone treated animals ($p<0.05$). The effects observed after xenobiotic exposure were not due to increased water intake. The increased immunoreactivity areas observed for the markers studied suggest that xenobiotics induced a neuro-inflammatory response, implying increased cell proliferation, glial activation, and induction of apoptotic pathways. The findings also show that glyphosate metabolites/adjuvants and/or surfactants present in glyphosate commercial formulations had a greater effect than glyphosate alone. In summary, glyphosate, AMPA, and glyphosate-based herbicides altered GFAP, caspase-3, and PCNA expression in the rat hypothalamus, altering the neuroendocrine axis.

Key words: glyphosate; hypothalamus; immunohistochemistry; apoptosis; neuro-inflammation; pesticides; proliferation.

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Introduction

Glyphosate, N-(phosphonomethyl) glycine, is an herbicide showing a broad-spectrum activity. After its absorption by the plant foliage, inhibits the shikimic acid pathway and blocks the synthesis of 5-enolpyruvyl- shikimate-3-phosphate synthase, inhibiting chorismate synthesis, involved in the biosynthesis of the essential aromatic amino acids tyrosine, tryptophan, and phenylalanine; in fact, this herbicide diminished the levels of these amino acids, resulting in the death of plants after several days of exposure.[1-4] Aminomethylphosphonic acid (AMPA), the main metabolite of glyphosate in the environment, is produced by microbial degradation in the soil *via* glyphosate oxidoreductase.[5-7] Glyphosate is an active ingredient in more than 700 products that act as herbicides such as Roundup PowerMAX® or Roundup Pro®,[1,8-11] being the most used Roundup®.[1,12-14] Pesticides, including glyphosate-based herbicides, have been widely used in agriculture for weed control, grain drying and fumigation of transgenic crops.[15-17]

Data, obtained from several vertebrate species, strongly indicate that glyphosate and its commercial formulations act as endocrine disruptors by altering the hypothalamic-pituitary-endocrine axis. For example, the levels of key molecules involved in reproductive mechanisms are altered to different degrees.[11,18] It has been reported that the number of mastocytes increased in the preoptic area of the adult fish *Paracheirodon axelrodi* after exposure to a Roundup® solution containing 1-5 mg/L glyphosate.[15] In tadpoles, after exposure to glyphosate formulations (0.21 mg/L), changes in metamorphosis were observed as well as a diminished brain THR alpha and beta expression and an increased Dio2/Dio3 expression were also reported.[19] In adult rats, diminished levels of follicle stimulating hormone and testosterone coupled to increased levels of luteinizing hormone and estrogens have been reported after glyphosate exposure (400 or 2000 mg/kg/day).[20] Weaned piglets, fed with glyphosate treated pellets (10, 20, and 40 mg/kg), showed increased levels of serum luteinizing hormone-releasing hormone, gonadotropin-releasing hormone and testosterone, and diminished follicle stimulating hormone levels.[21] Moreover, hypothalamic dopamine and homovanillic acid contents were significantly decreased in rats under glyphosate treatment (35, 75, 150 or 800 mg/kg BW)[22] and changes in hypothalamic 3,4-dihydroxyphenylacetic acid/dopamine and 5-hydroxyindoleacetic acid/serotonin turnover were also observed in adult rats after treatment with glyphosate-based herbicides (50 or 150 mg/kg).[23] Due to contradictory findings, the disruptive effects of glyphosate are highly controversial; however, it seems that commercial glyphosate formulations are more disruptive than glyphosate alone.[24] It is known that astrocytes play an important role in the response to central nervous system insults, a process called reactive astrogliosis, which is characterized by morphological alterations in these cells and by the overexpression of intermediate filament proteins.[11,25-27] Astrocyte activation has been associated to the oxidative damages induced by herbicides such as rotenone.[26] Caspase-3 is a key enzyme involved in apoptotic mechanisms that exerts important functions in the nervous system under normal and pathological conditions.[28,29] *In vitro* studies, reporting the expression of several caspases (including caspase-3), have been focused on the apoptotic effects mediated by glyphosate, AMPA and glyphosate-based herbicides (Roundup® Plus, Roundup® Ultra Max or Roundup® Active).[30-35] Caspase-3 has been related to immunity mechanisms by inducing pyroptosis in microglia and macrophages.[36] In the adult brain, glial, ependymal, and endothelial cells proliferate under normal and pathological conditions.[37,38] To detect this proliferation, *in vivo* and *in vitro* experiments have reported the expression of the proliferating cell nuclear antigen (PCNA) in testes and mammary glands.[17] However, currently no detailed description is available on the effects exerted by glyphosate, AMPA and Roundup® Active on glia, apoptosis and cell proliferation in the hypothalamus of rat perinatal pups. Most of the published studies have been performed in adults; however, it is very probable that newborns are more sensitive than adult animals to the exposure of xenobiotics. Because the above xenobiotics are considered endocrine disruptors, it seems very probable that glial fibrillary acidic protein (GFAP), PCNA (marker for cell proliferative activity) and caspase-3 (an important member of the apoptotic activating chain) expressions will be altered in the central nervous system after exposure to xenobiotics. Accordingly, the aim of this study is to know in the neuroendocrine hypothalamic nuclei (periventricular nucleus (Pe), anterior hypothalamic nucleus (AHA), lateroanterior nucleus (LA), medial preoptic nucleus (MPO) and supraoptic nucleus (SO)) whether the immunoreactivity for GFAP, PCNA and caspase 3 is altered or not after exposure to xenobiotics. Our study has been performed in male rats exposed to xenobiotics (via lactation) at early stages of the postnatal development (P21). This will serve to gain a better understanding on the effects exerted by glyphosate, its metabolite (AMPA) and a commercial formulation (Roundup® Active) on the neuroendocrine system of newborn lactating mammals.

Material and methods

Animals

Male and female adult Wistar rats (3 months old) weighing 250 g were obtained from the Universidad Industrial de Santander (Bucaramanga, Colombia**).** Animals were housed in cages (25x30x25) under standard conditions of photoperiod (light from 07:00 h to 19:00 h) and temperature (24°C), with free access to water and food. The design and procedures of the experiments were conducted in accordance with the Colombian laws (Resol. 8430/1993). This work was also approved by the Research Commission of the Universidad de Santander (Bucaramanga, Colombia) under act n° 009-VII.

Exposure protocol

Females were mated with breeding males (one female for one male) for one day and examined the next day by inspection of the vaginal plug to assess mating success. Confirmed pregnant females were isolated in individual cages during gestation and this was considered as day 0 of gestation (G0). The xenobiotic concentration chosen in this work was similar to that reported in previous published studies[39,40] and it was lower than that in which no-observed adverse effect was found (500 mg/kg/day).[41] Female rats were exposed to xenobiotics from birth (P0) to postnatal day 10 (P10), followed by tap water up to postnatal day 21 (P21). Four experimental groups were studied, each one including a minimum of ten male pup rats: group 1 was exposed to glyphosate alone (5 mg/L, dissolved in water); group 2 was exposed to Roundup® Active (containing 5 mg/L glyphosate, dissolved in water); group 3 was exposed to AMPA (5 mg/L, dissolved in water) and control group which received tap water. Water, changed every two days, was intake *ad libitum* and daily consumption was measured. Pups were kept until P21; then, they were anesthetized (see below) and the brains were dissected and processed for histology and immunohistochemistry.

Tissue preparation

The procedure followed in this study has been previously published.[42,43] Rats were deeply anaesthetized with an intraperitoneal

mix of ketamine (90 mg/kg) and xylazine (0.7 mg/kg). Then, animals were heparinized (1000 IU/rat) and perfused via the ascending aorta with 200 mL of saline solution (0.9% NaCl) followed by 200 mL of formaldehyde (4% in phosphate buffer (PB) 0.1 M, pH 7.4) prepared from alkaline hydrolysis of paraformaldehyde. Brains were removed and stored for 12 h in 4% buffered formaldehyde at 4°C. For cryoprotection, brains were embedded in increasing solutions of sucrose (5-30%) until they sank. Before brains were cut in a cryostat, they were immersed in PBS containing glycerol (10%) and dimethyl sulfoxide (2%). 50 µm thick serial hypothalamic transversal sections were obtained and stored at 4°C in PBS (0.1 M, pH 7.2) containing glycerol (20%) and ethylene glycol (20%), until the immunohistochemistry was performed. Four of the five sections were used for immunohistochemistry (section 1 for anti-GFAP; section 2 for anti-PCNA; section 3 for anti-caspase-3, section 4 for negative control (omission of the primary antisera) and section 5 identification/delineation of the hypothalamic nuclei using Nissl's technique.[42,43] The study has been focused on the hypothalamic nuclei because they play a crucial role in the neuroendocrine axis and because it seems that this axis is a target for glyphosate and glyphosate-based herbicides.

Immunohistochemistry

Free-floating sections were treated with methanol containing H_20_2 (30%) for 20 min to avoid possible interference by endogenous peroxidase.[44] Sections were washed in PBS (20 min) and incubated latter in PBS containing Triton X-100 (0.3%) and normal horse serum (1%) (30 min). Then, sections were incubated in a solution containing rabbit polyclonal anti-GFAP antibody (1:400; G9269, Sigma-Aldrich, St. Louis, MO, USA), polyclonal PCNA antibody (1:400; SAB2108448, Sigma-Aldrich) or polyclonal caspase-3 antibody (1:400; C8487, Sigma-Aldrich). Sections were washed in PBS (30 min) and then incubated in PBS containing the biotinylated anti-rabbit immunogamma globulin antiserum (diluted 1:200) (60 min, room temperature). Then, sections were washed in PBS (30 min) and incubated with the avidin-biotin-peroxidase complex (diluted 1:100) (60 min, room temperature). Sections were washed in PBS (30 min) and Tris-HCl (pH 7.6) (10 min) and the tissue-bound peroxidase was developed (3, 3' diaminobenzidine was used as chromogen). Finally, sections were washed (PBS), dehydrated and mounted with Entellan®. The specificity of the immunostaining was controlled by omitting the primary antibodies. In all cases, no residual immunoreactivity was found.

Immunoreactivity area

In each rat, sections containing the AHA, LA, MPO, Pe and/or SO hypothalamic nuclei were analyzed because these nuclei are involved in the hypothalamic-pituitary-adrenal axis. The atlas of Paxinos and Watson[45] was followed for mapping and nomenclature. Photographs were obtained with an Olympus DP 22 digital camera attached to an Olympus BX43 microscope and a CellSense software. Contrast and brightness adjustments were similar for all images (Adobe Photoshop CS6 Software was used) without any further manipulation of the photographs. To determine the area covered by the immunoreactive structures, images were analysed with the Fiji imageJ software[46] (developed by the NIH), available free of charge on internet (https://imagej.net/software/fiji/). The colour images for each treatment (Roundup® Active, glyphosate, AMPA and control) were loaded into the imageJ software and a duplicate was made to work on it. Then, a scale adjustment (27 pixels/micron) was made according to the magnification (400x) with which the images were captured to calibrate the section area. For each section, colour channels (red, green, and blue) were separated; blue was taken for all measurements because labelling was clearly observed in this channel. After binarization and calibration in square microns, immunoreactive area was measured in all sections. No other image manipulation was made to keep similar analysis conditions. Before image analysis, an image with 24 black circles was binarized and analyzed to control when the imageJ software was counting black or white structures, and to adjust analysis conditions.

Statistical analysis

A Sigma Stat 3.5 software was used. Fulfilment of ANOVA conditions (normal population, variance homogeneity) was tested first. If such was the case, a one-way ANOVA test was made, followed by a Holm-Sidak comparison test. When data did not conform to ANOVA conditions, a rank Kruskal-Wallis's test was applied, followed by a media comparison test: when the number of observations was equal for all treatments, a Student Newman Keuls was carried out; if not, a Dunn test was applied. Significance was accepted for $p<0.05$ values.

Results

Water consumption

No difference was observed between the four experimental groups regarding the water intake (Figure 1, $p>0.05$). This means that any effect observed was not the result of a different amount of the xenobiotics being taken by the rats.

Brain nuclei distribution

Hypothalamic nuclei were located according to the atlas of Paxinos and Watson.[45] Figures 2 to 5A show (gray rectangles) the quantified areas in control animals (Figure 2) and in glyphosate (Figure 3), Roundup® Active (Figure 4) and AMPA (Figure 5) treated rats.

Glial fibrillary acid protein

In control rats, a low density of immunoreactive structures containing GFAP was observed in AHA, LA, SO, MPO and Pe nuclei (Figures 2 to 5B). These cells showed typical cytoplasmic extensions, and, in the case of the Pe nucleus, these extensions were mainly directed towards the third ventricle whereas, in the

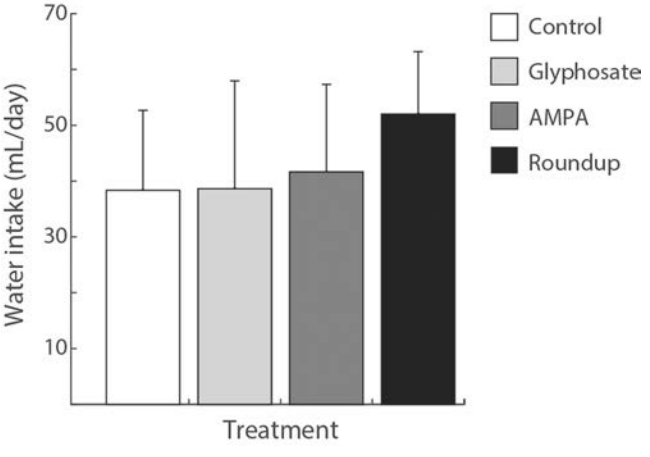

Figure 1. Quantitative analysis of water intake in adult rats upon exposure to xenobiotics. No significant differences were observed among groups [F (3,38) = 1,630; p=0.199].

SO nucleus, they were directed towards the pial surface. In pups exposed to xenobiotics, GFAP immunoreactivity was clearly observed in astrocytes showing cytoplasmic extensions in the AHA, MPO, LA, SO and Pe nuclei (Figures 3 to 5B). In the case of pups exposed to glyphosate, a moderate GFAP expression was observed when compared to control rats. However, no overlapping astrocytic processes were observed (Figure 2B). In offspring animals exposed to Roundup® Active or AMPA, a higher level of GFAP expression was observed compared to those animals treated with glyphosate (Figures 4-5B); an overlap in the territory of the astrocyte processes was also found, suggesting glial scarring. In all the hypothalamic nuclei studied, except in LA, the immunoreactivity areas were larger in animals exposed to xenobiotics than in controls rats ($p<0.05$). No difference between the xenobiotics studied was observed (Figure 6A).

Proliferating cell nuclear antigen

PCNA-positive cells showing an irregular morphology was observed in Pe, AHA, LA, MPO and SO hypothalamic nuclei (Figures 2 to 5C). Compared to control rats, in pups exposed to xenobiotics a greater number of immunolabeled structures containing PCNA was observed (Figures 3 to 5C). In animals exposed to glyphosate, the immunoreactivity observed in PCNA-positive cells was mild, but in pups exposed to Roundup® Active or AMPA was more intense (Figures 4 and 5C). The exposure to glyphosate increased the immunoreactivity for PCNA but only in the Pe nucleus. It is important to note that in the five hypothalamic nuclei studied the immunoreactivity areas for Roundup® Active or AMPA were significantly higher than those observed in control rats or in glyphosate treated animals ($p<0.05$) (Figure 6B).

Caspase-3

In pups exposed to xenobiotics and in control animals, spherical cells containing caspase-3 were observed in the Pe, MPO, SO and AHA nuclei (Figures 2 to 5D). However, in LA no immunoreactive structure containing caspase-3 was observed. Compared to animals exposed to Roundup® Active or AMPA (Figures 4 and 5D), weakly labeled structures for caspase-3 were found in offspring rats exposed to glyphosate (Figure 3D). The exposure to glyphosate increased the immunoreactivity in AHA, MPO and SO ($p<0.05$) (Figure 6C). In addition, comparing the caspase-3 immunoreactive areas observed in control rats and in glyphosate treated animals, a significant difference ($p<0.05$) was observed in offspring rats exposed to AMPA or Roundup® Active (Figure 6C). This finding suggests a higher effect of surfactants and/or adjuvants.

Discussion

In general, no differences in water intake among treatments were found, implying that the intake of xenobiotics was similar in all animals studied and that the observed effects were clearly related to xenobiotic nature. In postnatal pup rats exposed to

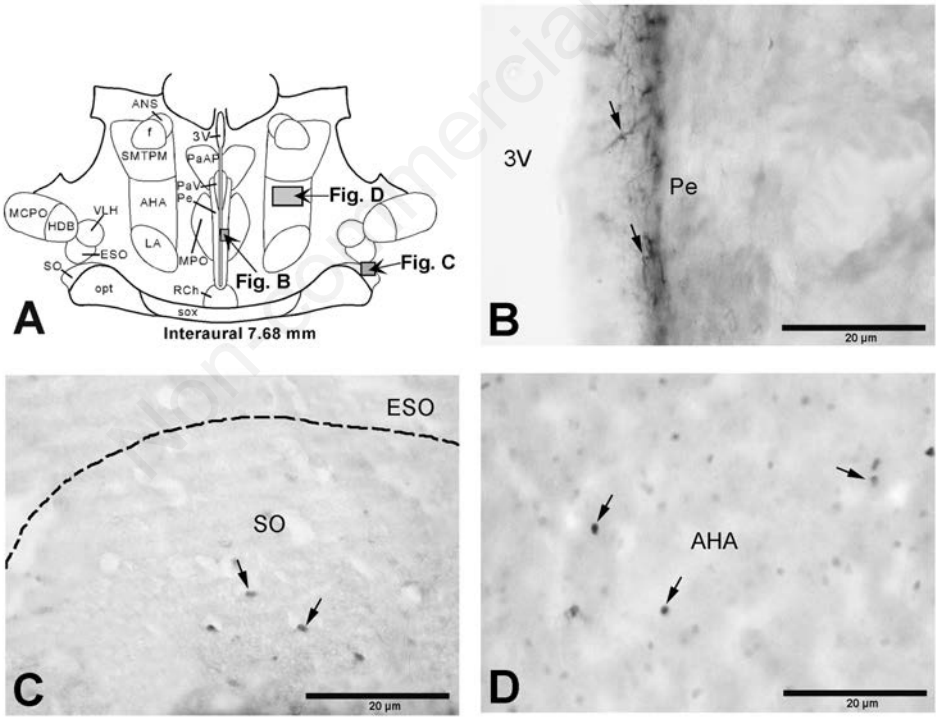

Figure 2. Immunoreactive structures in the hypothalamus of control pups. A) Frontal section of the hypothalamus; the interaural plane is indicated at the bottom center; the images in B-D were taken from the regions delimited by the rectangles in A, respectively. B) Immunoreactive astrocytes containing GFAP (arrow) in the periventricular nucleus (Pe). C) Low immunoreactivity of PCNA-positive cells (arrow) in the supraoptic nucleus (SO). D) Caspase-3-positive cells (arrow) in the anterior hypothalamic nucleus (AHA). 3V, third ventricle; ANS, accessory neurosecretory nucleus; ESO, episupraoptic nucleus; f, fornix; HDB, horizontal diagonal b nucleus; LA, lateroanterior hypothalamic nucleus; MCPO, magnocellular preoptic nucleus; MPO, medial preoptic nucleus; opt, optic tract; PaAP, paraventricular hypothalamic anterior nucleus, pars parvicellular; PaV, paraventricular hypothalamic nucleus, pars ventral; Pe, periventricular hypothalamic nucleus; RCh, retrochiasmatic area; SO, supraoptic nucleus; Sox, supraoptic decussation; STMPM, bed nucleus stria terminalis, medial division; VLH, ventrolateral hypothalamic nucleus.

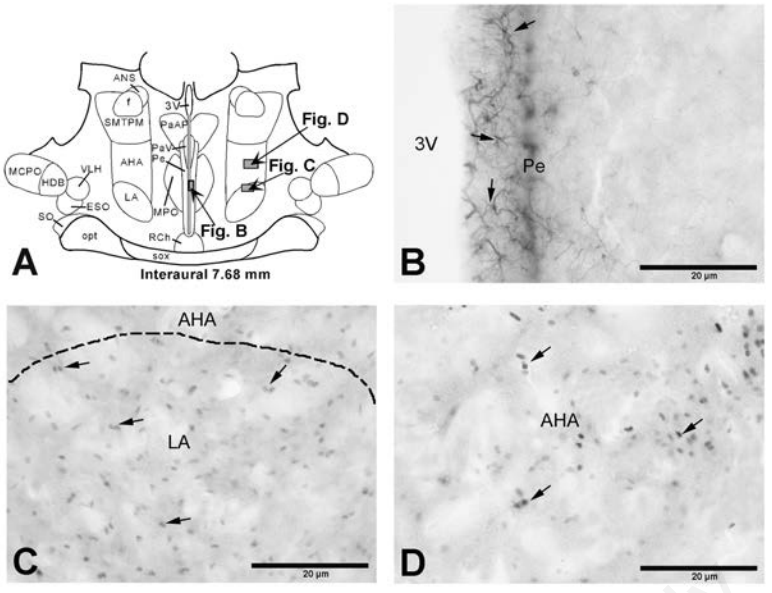

Figure 3. **Immunoreactive structures in the hypothalamus of pups exposed to glyphosate. A) Frontal section of the hypothalamus; the interaural plane is indicated at the bottom center; the images shown in B-D were taken from the regions delimited by the rectangles in A, respectively. B) Immunoreactive astrocytes containing GFAP (arrow) in the periventricular nucleus (Pe). C) PCNA-positive cells (arrow) in the lateral anterior hypothalamic nucleus (LA). D) Caspase-3-positive cells (arrow) in the anterior hypothalamic nucleus (AHA). 3V, third ventricle; ANS, accessory neurosecretory nucleus; ESO, episupraoptic nucleus; f, fornix; HDB, horizontal diagonal b nucleus; LA, lateroanterior hypothalamic nucleus; MCPO, magnocellular preoptic nucleus; MPO, medial preoptic nucleus; opt, optic tract; PaAP, paraventricular hypothalamic anterior nucleus, pars parvicellular; PaV, paraventricular hypothalamic nucleus, pars ventral; Pe, periventricular hypothalamic nucleus; RCh, retrochiasmatic area; SO, supraoptic nucleus; Sox, supraoptic decussation; STMPM, bed nucleus stria terminalis, medial division; VLH, ventrolateral hypothalamic nucleus.**

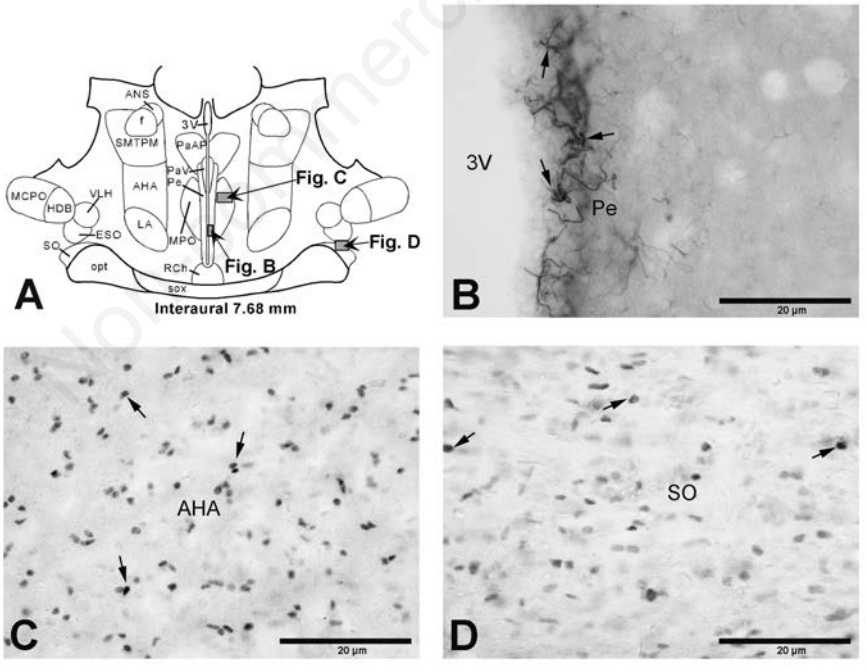

Figure 4. **Immunoreactive structures in the hypothalamus of pups exposed to Roundup® Active. A) Frontal section of the hypothalamus; the interaural plane is indicated at the bottom center; the images shown in B-D were taken from the regions delimited by the rectangles in A, respectively. B) High immunoreactive area of astrocytes containing GFAP (arrow) in the periventricular nucleus (Pe). C) PCNA-positive cells (arrow) in the anterior hypothalamic nucleus (AHA). D) Caspase-3-positive cells (arrow) in the supraoptic nucleus (SO). 3V, third ventricle; ANS, accessory neurosecretory nucleus; ESO, episupraoptic nucleus; f, fornix; HDB, horizontal diagonal b nucleus; LA, lateroanterior hypothalamic nucleus; MCPO, magnocellular preoptic nucleus; MPO, medial preoptic nucleus; opt, optic tract; PaAP, paraventricular hypothalamic anterior nucleus, pars parvicellular; PaV, paraventricular hypothalamic nucleus, pars ventral; Pe, periventricular hypothalamic nucleus; RCh, retrochiasmatic area; SO, supraoptic nucleus; Sox, supraoptic decussation; STMPM, bed nucleus stria terminalis, medial division; VLH, ventrolateral hypothalamic nucleus.**

glyphosate, AMPA or Roundup® Active we described, for the first time, an increased immunoreactivity for GFAP (astrocytes), PCNA (cell proliferation and DNA reparation) and caspase-3 (a member of the apoptotic activating chain). In addition, in some cases the effects mediated by Roundup® Active or AMPA were even higher than those observed after exposure to glyphosate.

The effects exerted by the above mentioned xenobiotics have been mainly studied in the neuroendocrine axis.[11,17,18,47] In addition, after an injury, the presence of the above mentioned markers in the central nervous system of rodents has been reported[26,37,38] and, in the rat brain, a focal gliosis was observed when a similar concentration of glyphosate to that used in this study was administered.[48] Furthermore, in the mouse prefrontal cortex and hippocampus, GFAP expression was increased after pre- and post-natal exposure to 250 mg/kg of glyphosate (about 7.5 mg/individual).[41] The intranasal administration of glyphosate based-herbicide increased the number of GFAP-immunoreactive cells in the anterior olfactory nucleus;[49] a change in GFAP immunoreactivity has been considered as a marker for neuro-inflammatory mechanisms. This agrees with the results reporting an increase for Iba-1 immunoreactivity (microglia marker)[41] and with the high density of GFAP-immunoreactive cells reported here in the Pe nucleus of rats exposed to glyphosate or Roundup® Active, which in turn can be associated with the entry of xenobiotics into the parenchyma.

PCNA immunoreactivity was markedly increased in the hepatic tissues of glyphosate exposed rats[50] and, in breast cancer cell lines, genes involved in the MCF-7 pathway (e.g., c-myc, cyclins, PCNA) exhibited expression changes after Roundup treatment.[51] In addition, low glyphosate concentrations were able to promote HaCaT keratinocytes growth, which showed an increased expression of PCNA immunoreactivity.[52] Thus, in hypothalamic nuclei such as LA, AHA or SO the data reported in our study suggest that xenobiotics promote DNA damage and that the increased PCNA expression observed could be related to DNA repair.[53]

Caspase expression is related to apoptosis.[54] *In vitro*, the activation of caspases-3/7 was observed in hepatic cell lines exposed to a glyphosate formulation, but no such effect was observed after a pure glyphosate treatment.[31] Exposure to glyphosate also altered neural-related cells; in fact, treated differentiated PC12 cells showed morphological changes compatible with apoptosis (e.g., cell shrinkage, neurite collapsation, nuclear condensation, DNA fragmentation).[55] Glyphosate increased caspases-3/7 activity in SH-SY5Y cells,[34] which resulted in an activation of the apoptotic pathway. Moreover, in the *Piaractus brachypomus* brain an increase in the immunoreactivity for caspase-6 was observed after Roundup exposure.[56] As caspase activation is relevant in microglial cell activation,[57] the increase of caspase-3 observed in our work could be related to neuro-inflammatory mechanisms or DNA fragmentation and cell death**.** This agrees with the increased expression of GFAP and PCNA in most nuclei under our experimental conditions. However, caspase-3 alone is not enough to demonstrate apoptosis activation and hence the expression of other

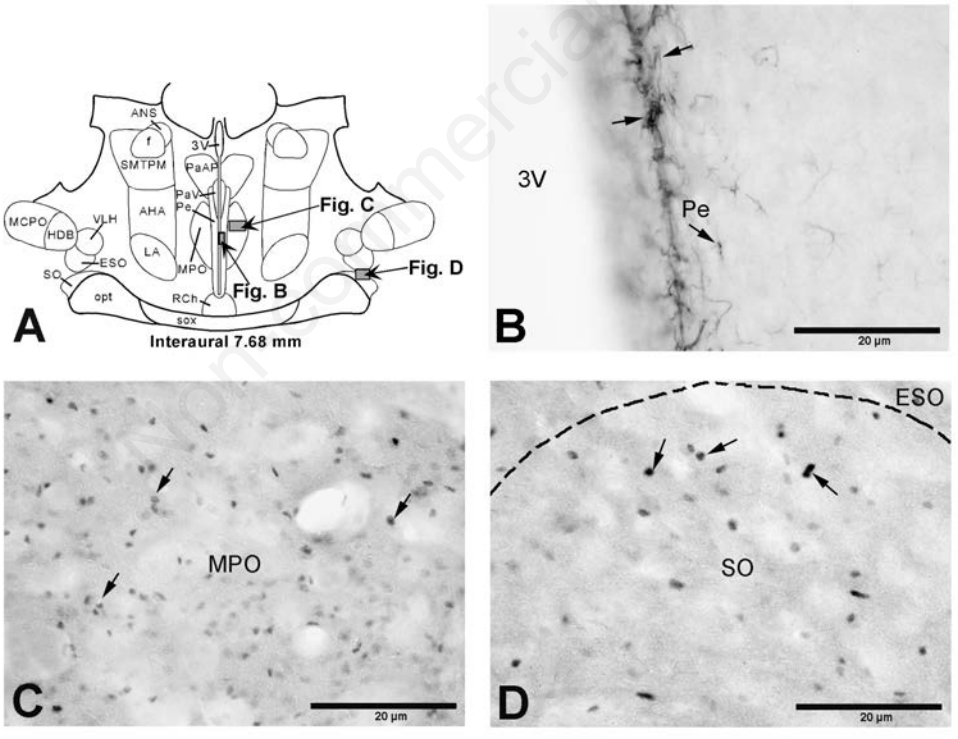

Figure 5. Immunoreactive structures in the hypothalamus of pups exposed to AMPA. A) Frontal section of the hypothalamus; the interaural plane is indicated at the bottom center; the images shown in B-D were taken from the regions delimited by the rectangles in A, respectively. B) Immunoreactive astrocytes containing GFAP (arrow) in the periventricular nucleus (Pe). C) PCNA-positive cells (arrow) in the medial preoptic nucleus (MPO). D) Caspase-3-positive cells (arrow) in the supraoptic nucleus (SO). 3V, third ventricle; ANS, accessory neurosecretory nucleus; ESO, episupraoptic nucleus; f, fornix; HDB, horizontal diagonal b nucleus; LA, lateroanterior hypothalamic nucleus; MCPO, magnocellular preoptic nucleus; MPO, medial preoptic nucleus; opt, optic tract; PaAP, paraventricular hypothalamic anterior nucleus, pars parvicellular; PaV, paraventricular hypothalamic nucleus, pars ventral; Pe, periventricular hypothalamic nucleus; RCh, retrochiasmatic area; SO, supraoptic nucleus; Sox, supraoptic decussation; STMPM, bed nucleus stria terminalis, medial division; VLH, ventrolateral hypothalamic nucleus.

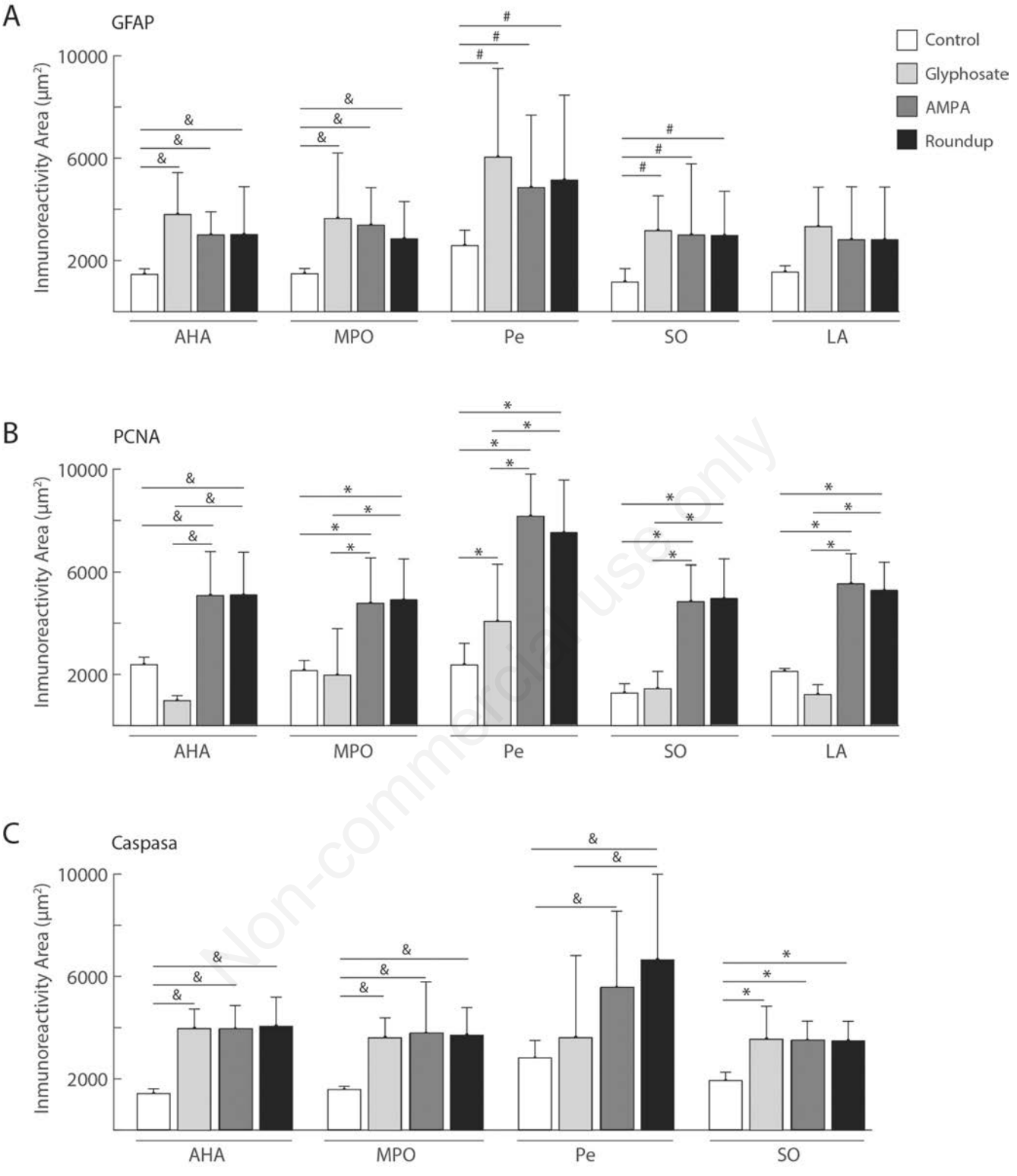

Figure 6. **Quantitative analyses demonstrating immunoreactivity area (µm²) for (A) GFAP: AHA (H (3)=17.911, p≤0.001); MPO (H(3)=21.6. p≤0.001); Pe (H (3)=2.235, p=0.007); SO (H(3)=11.043; p≤0.011); LA (F (3.16)=1.425, p=0.272). B) PCNA: AHA (H (3)=35.262, p≤0.001); MPO (F (3.40)= 2.271, p<0.001); Pe (F (3.65)=38.886, p<0.001); SO (F (3.19)= 8.19, p<0.001); LA (F (3.18)=34.495, p<0.001). C) caspase-3: AHA (H (3)=26.11, p≤0.001); MPO (H(3)=21.288, p≤0.001); Pe (H(3)=13.897, p=0.003); SO (F (3.18)=4.930, p=0.011). *ANOVA followed by a Holm-Sidak test; &Kruskal-Wallis ANOVA ranks, followed by a Dunn test; #Kruskal-Wallis ANOVA ranks, followed by a SNK test.**

molecules must be studied to fully support the role that these xenobiotics play in apoptosis.

It is important to note that Roundup® Active contains other ingredients different from glyphosate, like polyexithilenalkilamide and NN bis hydroxyethilalkilamide,[58] but no commercial information regarding the concentrations of these molecules is currently reported. No data on possible bioaccumulation of these molecules are known, and it is very difficult to estimate its plasma levels.[59] However, *in vitro* studies showed that commercial glyphosate formulations, applied at low concentrations, affected the nervous system.[60] It is also known that commercial formulations showed a higher toxic effect than pure glyphosate,[58, 61] suggesting a stronger toxicity of adjuvants. In mice, memory deficiencies, anxiety increased levels and depression-like symptoms have been reported after exposure to glyphosate-base herbicides[48] as well as a reduction in tyrosine hydroxylase and serotonin immunoreactivity.[48, 62] Moreover, in rats under prenatal or prepuberty exposure to xenobiotics, a decrease in acetylcholinesterase activity has been reported.[9] Prenatal exposure to glyphosate or its commercial formulations resulted in an increased expression of hippocampal synaptophysin.[1] Accordingly, glyphosate formulations may lead to a stronger alteration in the central nervous system due to the presence of adjuvants.

Neuro-inflammation and apoptosis can alter central nervous system functions. Xenobiotics such as glyphosate, Roundup® Active and AMPA may alter the hypothalamic-pituitary-adrenal axis by affecting the physiological actions mediated by the LA, MPO, Pe and SO hypothalamic nuclei. It is not clear how glyphosate enters into the brain parenchyma. There are contradictory findings; thus, some authors did no observed alterations in the blood-brain barrier, [63] but others have suggested that glyphosate crosses this barrier by transcellular mechanisms.[64] In our study, the high immunoreactivity observed for caspase-3 and PCNA suggests that cells containing these markers mediate a response associated with DNA damage and therefore neuronal death. Our results also suggest an affectation in the hypothalamic-adrenal axis through its effects on the hypophysiotropic neurons located in the Pe nucleus.[65] In addition, the PCNA-positive cells located in the AHA nucleus could be expressing DNA repair pathways, which is consistent with the molecular studies carried out by Ino and Chiba.[38] The high immunoreactivity for GFAP, observed in Pe, LA, AHA, MPO and SO hypothalamic nuclei in pups exposed to glyphosate, Roundup® Active and AMPA, suggests a role of astrocytes in the control of microglia inflammatory responses.[2]

Finally, maternal exposure to glyphosate-based herbicides caused dysbiosis in male murine offspring and an increased risk for autism related conditions.[66] However, mechanisms leading to autism-like syndromes are largely unknown. It can be hypothesized that alterations in GFAP, PCNA and/or caspase-3 levels may contribute to these alterations because of astrocyte functional modifications and increased levels of apoptosis in nerve and glial cells.

In conclusion, our results suggest that glyphosate, Roundup® Active and AMPA promote DNA damage and a neuro-inflammatory response in the brain. These changes can lead to increased blood-brain permeability, reactive gliosis, and apoptosis, resulting in behavioral and neurophysiological alterations. In addition, commercial glyphosate formulations are more deleterious for hypothalamic cells than glyphosate alone. However, currently the regulatory agencies are not testing the effects of adjuvants to determine the maximum permitted levels of xenobiotics, leading to an underestimation of the real effects that these molecules exert on exposed animals and human populations.

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