Int. J. Morphol.,
43(4):1122-1127, 2025.

Glyphosate-Based Herbicide Exposure Increases GFAP-Immunoreactivy in Ventral Lateral Hypothalamic and Supraoptic Nuclei of Male Rats

Exposición a un Herbicida Basado en Glifosato Incrementa la Immunoractividad GFAP en el Núcleo Hipotalámico Ventral Lateral y en el Núcleo Supraóptico de Ratas Macho

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HURTADO-GIRALDO, H.; ROCHA-MUÑOZ, L. P.; ROMERO-OSORIO, N. I.; CONDE-OCAZIONEZ, S.; COVEÑAS, R. & DUQUE-DÍAZ, E. Glyphosate-based herbicide exposure increases gfap-immunoreactivy in ventral lateral hypothalamic and supraoptic nuclei of male rats. *Int. J. Morphol.,43(4)*:1122-1127, 2025.

SUMMARY: The effects of glyphosate, aminomenthylphosphoric acid (AMPA) and Roundup® Active on the glial fibrillary acidic protein (GFAP) immunoreactivity area were studied in male rats in the following hypothalamic nuclei: anterior hypothalamic, lateroanterior hypothalamic, ventrolateral hypothalamic (VLH), preoptic area, paraventricular, periventricular, suprachiasmatic and supraoptic (SO). No difference in water intake containing these xenobiotics was observed ($p > 0.05$). A significant increase in the GFAP immunoreactivity area was only observed in VLH in animals treated with glyphosate or Roundup® Active, whereas in SO, only the exposure to Roundup® Active resulted in a larger GFAP immunoreactivity area. AMPA did not affect the GFAP immunoreactivity areas of the hypothalamic nuclei. Small/medium size astrocytes containing GFAP, and typical cytoplasmic processes were observed in most cases, but large and overlapping astrocytic processes were visualized in SO. GFAP immunoreactivity increase suggests a neuro-inflammatory reaction due to xenobiotics. These results are compatible with a pronounced effect of adjuvants and surfactants present in glyphosate commercial formulations.

KEY WORDS: Hypothalamus; Astrogliosis; Neuroinflammation; Xenobiotics

INTRODUCTION

Glyphosate based herbicides (GBH) have been widely used for decades in agriculture and current evidences suggest that these compounds act as endocrine disruptors altering the hypothalamic-pituitary-endocrine axis (Maddalon *et al*., 2021; Serra *et al*., 2021). After exposure to GHB, increase in the number of mastocytes, changes in metamorphosis, alterations in the expression of Trhab, Dio2, and Dio2 genes and altered hormonal levels have been described (Romano *et al*., 2012; Popoola & Sakpa, 2018; Fu *et al*., 2021). Neurotransmitters levels of dopamine and homovanillic acid were also affected after glyphosate exposure (Martínez *et al*., 2018). Commercial glyphosate formulations were more disruptive than glyphosate alone (de Araújo-Ramos *et al*., 2021; Reis *et al*., 2022). Altogether, the data show that GHB promote important physiological changes.

Astrocytes are sensitive to central nervous system insults, leading to increased glial fibrillary acid protein (GFAP) and morphological changes (Giovannoni & Quintana, 2020; Baxter *et al*., 2021; Maddalon *et al*., 2021). It has recently published that glyphosate, AMPA and Roundup® Active increased the GFAP immunoreactivity areas in neuroendocrine hypothalamic nuclei of male p21 rats (Duque-Díaz *et al*., 2022). However, no detailed description is available on the effects of glyphosate, AMPA and Roundup® Active on GFAP levels in the adult male rat hypothalamic nuclei after a long-term exposure. The main aim of this work is to determine the levels of immunoreactivity for GFAP in the male offspring rat hypothalamus after 150-days of exposure to these compounds, to gain a better understanding of its effects on the mammalian neuroendocrine system.

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FUNDING. This work has been supported by the Ministerio de Ciencia y Tecnología (MINCIENCIAS) (129977758110/Cont-595-18), the Universidad de Santander from Bucaramanga, Colombia

Received: 2025-01-28 Accepted: 2025-05-08

MATERIAL AND METHOD

Adult (250 g body weight) Wistar rats were housed under standard conditions (light from 07:00 h to 19:00 h, 24° C; ad libitum water and food). The xenobiotic concentration chosen in this work is the same as that used in a similar study performed in early postnatal rats (P10) (Duque-Diaz *et al*, 2022), and is lower than that in which no-observed adverse effect are found (500 mg/kg/day). Male rats were exposed to xenobiotics from birth (P0) to postnatal day 21 (P21) via lactation (ten pups/mother/cage), weaned, and exposed to water containing xenobiotics up to postnatal day 150 (3 rats/cage).

Four experimental groups with 10 male pups each were used: 1) group exposed to glyphosate (5 mg/L dissolved in water); 2) group exposed to Roundup® Active (5 mg/L glyphosate plus unknown adjuvants dissolved in water); 3) group exposed to AMPA (5 mg/L dissolved in water), and 4) control group which received tap water. Water was changed every two days. The design and proceedings of the experiments were conducted in accordance with Colombian laws (Resol. 8430/1993 and 1774/2016 law). This work was also approved by the Research Commission of the Universidad de Santander (Bucaramanga, Colombia) under act n° 009-VII. All efforts were made to minimize animal suffering.

Rats were heparinized intraperitoneally (1000 IU/rat) and deeply anesthetized (ketamine 90 mg/kg and xylazine 0.7 mg/kg). They were then perfused through the ascending aorta with 300 ml of saline (0.9 % NaCl) followed by 250 mL of 4 % formaldehyde in phosphate buffer (PB) 0.1 M (pH 7.4). Brains were removed and processed as described by Duque-Díaz *et al*. (2022).

Free-floating sections were processed as described by Duque-Díaz *et al*. (2022). Primary antibody incubation was carried overnight a 4º C, using rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) antibody (1:400, Sigma-G9269). The specificity of the immunostaining was controlled by omission of the primary antibody. In all cases, no residual immunoreactivity was found.

The GFAP-immunoreactive area was studied in the following hypothalamic nuclei involved in the hypothalamus-adrenal-gonadal axis: anterior hypothalamic nucleus (AHA), lateral anterior hypothalamic nucleus (LA), periventricular nucleus (Pe), ventrolateral hypothalamic nucleus (VLH), preoptic nucleus (PON), paraventricular nucleus (PaAP), suprachiasmatic nucleus (SchN), and supraoptic nucleus (SO). Sections were analyzed taking as reference the stereotaxic atlas of Paxinos & Watson (2009). Photomicrographs were obtained with an Olympus DP 22 digital camera attached to an Olympus BX43 microscope. Brightness and contrast of the images were adjusted using Adobe Photoshop CS6 Software. Immunoreactive areas were obtained with Fiji imageJ software (developed by the NIH), available free of charge on internet (https://imagej.net/software/fiji/). Images were calibrated (27 pixels by micron, for 400x magnification). Color channels (RGB) were split, and the immunoreactive area was measured for all sections.

A MatLab R2021b software was used. Fulfilment of ANOVA conditions were tested first. If such was the case, a one-way ANOVA test was carried out, followed by a Holm-Sidak comparison test. If not, a rank Kruskal-Wallis's test was applied, followed by a media comparison test: if 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

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

Table I. Cumulative water consumption (ml) per animal.

Xenobiotic	Mean ± SD
Control	3596 ± 678
Roundup® Active	3375 ± 526
AMPA	3844 ± 710
Glyphosate	3916 ± 679

Neuroanatomical location of GFAP-immunostaining cells is reported in Figures 1A and B. A low/moderate density of small/medium size astrocytes containing GFAP and showing typical cytoplasmic extensions was observed in exposed and unexposed animals (Fig. 2 A, B, D). Similar immunoreactive area values were observed in AHA, LA, PON, PaAP, Pe or SchN in both exposed and unexposed animals; however, in comparison to unexposed animals, higher GFAP-immunoreactivity area values were detected in SO and VLH in those animals exposed to glyphosate or Roundup® Active (Fig. 2 B-D). Cells with fusiform nuclei and extensive and overlapping processes, forming a glial scar, were observed in the SO nucleus of animals exposed to Roundup® Active (Fig. 2 C).

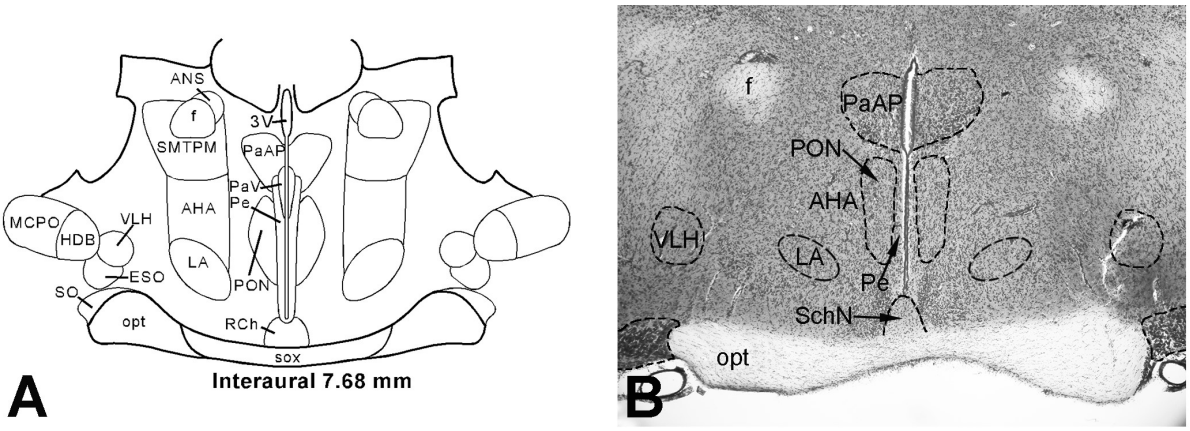

Fig. 1. Coronal section (left) and transversal section (right, Nissl staining) in the interaural 7.68 mm plane of the rat hypothalamus. 3V: third ventricle; AHA: anterior hypothalamic nucleus; ANS: accessory neurosecretory nucleus; ESO: episupraoptic nucleus; f: fornix; HDB: horizontal limb diagonal nucleus; LA: lateroanterior hypothalamic nucleus; MCPO: magnocellular preoptic nucleus; opt: optic tract; PaAP: paraventricular hypothalamic nucleus; PaV: paraventricular thalamic nucleus; Pe: periventricular nucleus; PON: preoptic area nucleus; SchN: suprachiamatic nucleus; SMTPM: bed nucleus striate medial division; SO: supraoptic nucleus; sox: supraoptic decussation; VLH: venrolateral hypothalamic nucleus.

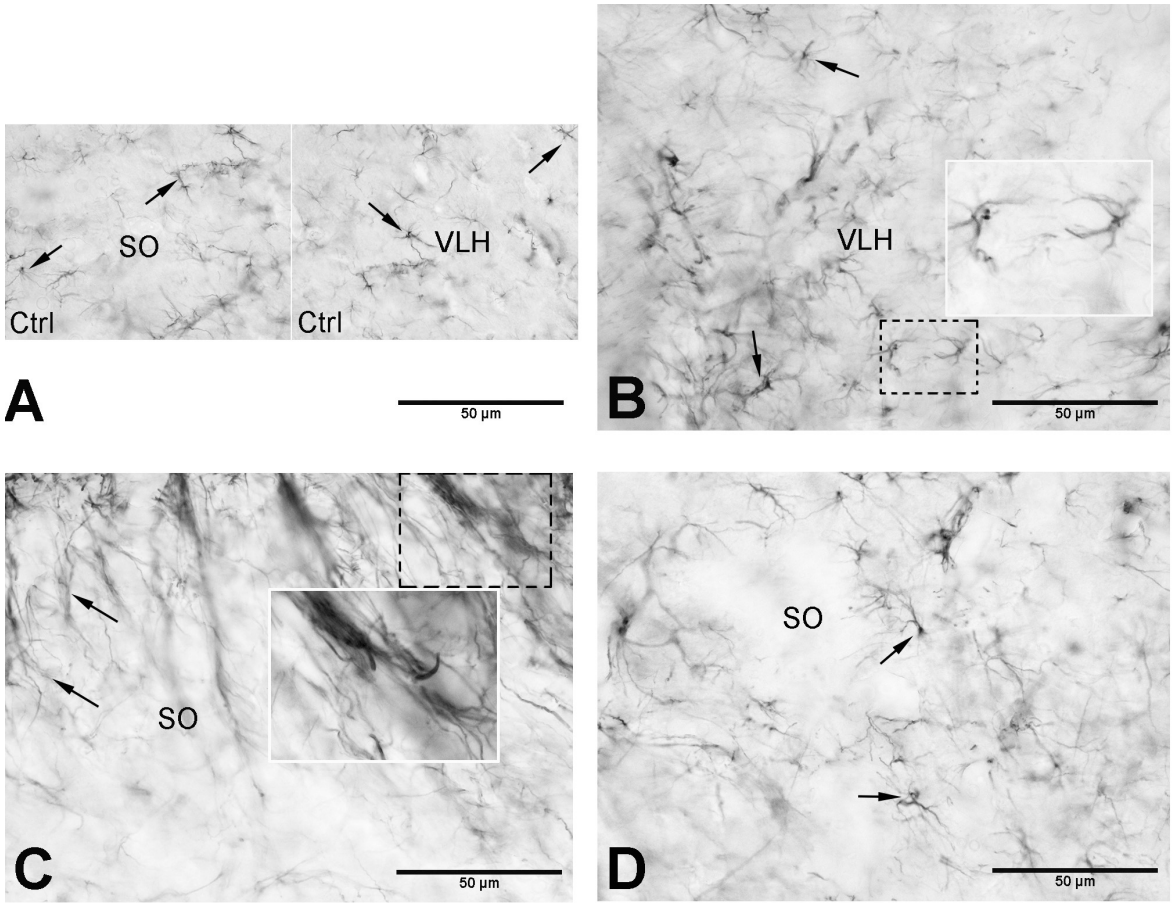

Fig. 2. GFAP-containing immunoreactive structures in the hypothalamus. (A) GFAP-containing immunoreactive astrocytes (arrows) in the supraoptic nucleus (SO) and the lateroventral nucleus (VLH) in unexposed offspring. (B) Glyphosate-treated animal: high immunoreactivity in GFAP-positive cells (arrows) located in the lateroventral nucleus (VLH). The white outline rectangle is a high-power image of the region bounded by the dashed rectangle. (C) Roundup® Active-treated animal: GFAP-positive cells in the supraoptic nucleus (SO). Arrows show long cytoplasmic processes; arrowheads show glial scarring. (D) AMPA-treated animal: GFAP-immunoreactive astrocytes (arrows). Scale bar: 20 µm.

Table II shows the immunoreactivity area (μm2) in the nuclei studied. No significant effect of xenobiotic exposure was observed in AHA, PON, PaAP, Pe and SchN nuclei ($p > 0.05$). However, an increased GFAP immunoreactivity area was observed in VLH after exposure to glyphosate ($p < 0.05$) or Roundup® Active ($p < 0.01$). In comparison to unexposed animals ($p < 0.01$), an increase immunoreactivity was also observed in SO after exposure to Roundup® Active. No changes in the GFAP immunoreactivity areas were observed after AMPA exposure.

Table II. Immunoreactivity area (μm^2), Mean ± standard deviation.

Nucleus	Control	Glyphosate	AMPA	Roundup® Active
AHA	112.2 ± 82.7	193.5 ± 82.9	108.4 ± 55.7	118.3 ± 59.8
LA	355.4 ± 183.4	455 ± 206.3	319 ±113.2	620 ± 202.3
VLH	122.2 ± 70.1	352.5 ± 168	135 ± 115.7	433.8 ± 86
PON	393 ± 185.2	409.4 ± 236.1	326.4 ± 128.9	206.7 ± 118.5
PaAP	656 ± 177.8	805.2 ± 272.6	505.2 ± 65.1	649.8 ± 117.2
Pe	118.7 ± 278.3	937.5 ± 191.2	1075.2 ± 345.2	1020.5 ±233.1
SchN	770.4 ± 265.5	1053 ± 542.1	939.2 ± 126	1218.2 ± 390.4
SO	609.8 ± 126	838.2 ± 302.8	570.4 ± 152.5	1441.4 ± 209.4

DISCUSSION

Astrocytes are involved in the transport of molecules across the blood-brain barrier, in the regulation of the levels of neurotransmitters and in the control of gliotransmitters release (Stein *et al*., 2020). Glyphosate commercial formulations can impair neuronal and glial differentiation in vitro (Reis *et al*., 2022); central nervous system rodent models have shown alterations in GFAP labeling due to injuries (Glushakova *et al*., 2018); focal gliosis have been reported after glyphosate exposure (Turkmen *et al*., 2019), as well as an increased GFAP expression in the prefrontal cortex and hippocampus (Ait-Bali *et al*., 2020). The intranasal administration of glyphosate resulted in larger GFAP-immunoreactive cells in the anterior olfactory nucleus (Gallegos *et al*., 2020). Increased GFAP immunoreactivity has been postulated as a marker for neuro-inflammation; this agrees with a microglia increase which is detected by an increase in Iba1 immunoreactivity (Ait-Bali *et al*., 2020) and, in terms of microglia activation, caspase activation was relevant to neuro-inflammation as well as an increased GFAP expression (Goshi *et al*., 2020).

SO regulates the release of the antidiuretic hormone (Pop *et al*., 2018) and changes in the level of GFAP altered the release of gonadotrophin-releasing hormone (Vanacker *et al*., 2021). In this work, we have detected in SO, not only a higher GFAP immunoreactivity area, but also astrocyte overlapping processes (glial scar) (Fig. 2C), suggesting that neuro-inflammatory mechanisms are occurring and that altered physiological actions could also occur (e.g., osmotic regulation, intake (water/food) or satiety) (Wang *et al*., 2021). VLH and the ventromedial hypothalamic nucleus are involved in glucose and energy homeostasis (Cotero & Routh, 2009). We have demonstrated a higher GFAP-immunoreactive area in VLH, suggesting possible physiological implications on neuron metabolism, since astrocytes plays a key role in neuron metabolic regulation, by glutamate intake, aerobic glycolisis and lactate excretion (Beard *et al*., 2022).

In this work, a higher effect of Roundup® Active was observed in SO. GBH include, not only glyphosate, but other unidentified molecules. In vitro and in vivo studies have showed that commercial glyphosate formulations are more toxic than pure glyphosate, suggesting a stronger toxicity due to adjuvants: memory deficiencies, increased anxiety levels in mice (Ait-Bali *et al*., 2020), changes in monoaminergic markers and serotonin immunoreactivity (Ait-Bali *et al*., 2020), diminished acetylcholinesterase activity (Cattani *et al*., 2017), and changes in hippocampal synaptophysin (Dechartres *et al*., 2019). These reports agree with our findings because, in comparison with a pure glyphosate exposure, a stronger alteration on GFAP immunoreactivity was observed after Roundup® Active exposure.

CONCLUSIONS

Exposure to glyphosate-based herbicides results in changes compatible with astrocytosis and neuroinflammation in some hypothalamic nuclei. Currently regulatory agencies are not including commercial formulations in safety tests, adjuvant effects are not being measured, and no definition exists on maximal adjuvant levels. Clearly, these regulation deficiencies must be corrected, to gain a clear understanding on the real effects of glyphosate commercial formulations on the environment.

ACKNOWLEDGEMENTS. This work has been supported by the Ministerio de Ciencia y Tecnología (MINCIENCIAS) (129977758110/Cont-595-18), the Universidad de Santander from Bucaramanga, Colombia. The authors thank the Language Service, University of Salamanca, Salamanca, Spain, for supervising the English text.

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RESUMEN: Se estudiaron los efectos del glifosato, el ácido aminomentilfosfórico (AMPA) y Roundup® Active sobre el área de inmunorreactividad de la proteína ácida fibrilar glial (GFAP) en ratas macho en los siguientes núcleos hipotalámicos: hipotálamo anterior, hipotálamo lateroanterior, hipotálamo ventrolateral (VLH), área preóptica, paraventricular, periventricular, supraquiasmático y supraóptico (OS). No se observaron diferencias en la ingesta de agua con estos xenobióticos ($p > 0,05$). Solo se observó un aumento significativo del área de inmunorreactividad de GFAP en VLH en animales tratados con glifosato o Roundup® Active, mientras que en OS, solo la exposición a Roundup® Active resultó en un área de inmunorreactividad de GFAP mayor. El AMPA no afectó las áreas de inmunorreactividad de GFAP de los núcleos hipotalámicos. En la mayoría de los casos se observaron astrocitos de tamaño pequeño/mediano que contenían GFAP y procesos citoplasmáticos típicos, pero en el SO se visualizaron procesos astrocíticos grandes y superpuestos. El aumento de la inmunorreactividad de GFAP sugiere una reacción neuroinflamatoria debida a xenobióticos. Estos resultados son compatibles con un efecto pronunciado de los adyuvantes y surfactantes presentes en las formulaciones comerciales de glifosato.

PALABRAS CLAVE: Hipotálamo; Astrogliosis; Neuroinflamación; Xenobióticos.

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