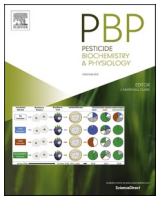
Pesticide Biochemistry and Physiology 193 (2023) 105433

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest



Neurotoxic effects of exposure to glyphosate in rat striatum: Effects and mechanisms of action on dopaminergic neurotransmission

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ARTICLE INFO

Keywords:
Glyphosate
Glyphosate-based herbicide
Dopaminergic neurotransmission
In vivo dopamine release
Mechanism of action
Rat striatum

ABSTRACT

The main objective of this study was to evaluate the effects and possible mechanisms of action of glyphosate and a glyphosate-based herbicide (GBH) on dopaminergic neurotransmission in the rat striatum. Acute exposure to glyphosate or GBH, administered by systemic (75 or 150 mg/kg, i.p.) or intrastriatal (1, 5, or 10 mM for 1 h) routes, produced significant concentration-dependent increases in dopamine release measured *in vivo* by cerebral microdialysis coupled to HPLC with electrochemical detection. Systemic administration of glyphosate also significantly impaired motor control and decreased striatal acetylcholinesterase activity and antioxidant capacity. At least two mechanisms can be proposed to explain the glyphosate-induced increases in extracellular dopamine levels: increased exocytotic dopamine release from synaptic vesicles or inhibition of dopamine transporter (DAT). Thus, we investigated the effects of intrastriatal administration of glyphosate (5 mM) in animals pretreated with tetrodotoxin (TTX) or reserpine. It was observed that TTX (10 or 20 μM) had no significant effect on glyphosate-induced dopamine release, while reserpine (10 mg/kg i.p) partially but significantly reduced the dopamine release. When glyphosate was coinfused with nomifensine (50 μM), the increase in dopamine levels was significantly higher than that observed with glyphosate or nomifensine alone. So, two possible hypotheses could explain this additive effect: both glyphosate and nomifensine act through different mechanisms at the dopaminergic terminals to increase dopamine levels; or both nomifensine and glyphosate act on DAT, with glyphosate simultaneously inhibiting reuptake and stimulating dopamine release by reversing the DAT function. Future research is needed to determine the effects of this pesticide at environmentally relevant doses.

1. Introduction

Glyphosate (*N*-phosphonomethyl-glycine) is a broad-spectrum, non-selective organophosphorus compound that is the active ingredient in some of the world's most widely used commercial herbicides (Maggi et al., 2020; Saunders and Pezeshki, 2015). The commercial interest of glyphosate lies in its ability to inhibit the shikimic acid pathway, which is absent in animals and humans, but plays a major role in the synthesis of essential aromatic amino acids in plants, fungi and some microorganisms (Santos-Sánchez et al., 2019; Van Bruggen et al., 2018).

Due to the extensive and large-scale use of glyphosate nowadays, animals and humans are chronically exposed to this pesticide through food and drinking water, as evidenced by the detection of glyphosate residues in the organs and urine of various animal species and human populations (Conrad et al., 2017; Ferreira et al., 2021; Grau et al., 2022; Monika et al., 2014; Niemann et al., 2015). This chronic exposure to glyphosate may constitute a significant health risk to animals and humans, as it has recently been associated to the development of various neurotoxic effects, including alterations in behavior, memory, learning or even an increased incidence of certain developmental disorders in humans (Ait-Bali et al., 2020; Baier et al., 2017; Coullery et al., 2020; von Ehrenstein et al., 2019; Fuhrimann et al., 2021; Wang et al., 2011).

Abbreviations: AChE, acetylcholinesterase; ANOVA, analysis of variance; DAT, dopamine transporter; DOPAC, dihydroxyphenylacetic acid; GBH, glyphosate-based herbicide; HPLC-ED, high-performance liquid chromatography with electrochemical detection; HVA, homovanillic acid; I. p., intraperitoneal; MAO-A, monoamine oxidase A; MAO, monoamine oxidase; nAChR, nicotinic acetylcholine receptor; NMDA, *N*-methyl-D-aspartate; ROS, reactive oxygen species; TTX, tetrodotoxin; VMAT-2, vesicular monoamine transporter type 2; VSSC, voltage-gated sodium channels.

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https://doi.org/10.1016/j.pestbp.2023.105433
Received 29 January 2023; Received in revised form 9 April 2023; Accepted 19 April 2023
Available online 23 April 2023

Glyphosate exposure also changes dopaminergic neurotransmission. It has been observed that exposure to glyphosate can induce alterations in extracellular levels of dopamine in some regions of the brain, such as the striatum. However, while some research has documented an increase (Faria et al., 2021; Faro et al., 2022), other studies have observed a decrease (Hernández-Plata et al., 2015; Martínez et al., 2018) in dopamine levels after pesticide exposure. However, to our knowledge, there are no studies showing the effects of glyphosate on the *in vivo* striatal dopamine release or on the possible neurochemical mechanisms involved in the effects of this pesticide on dopaminergic neurotransmission.

Therefore, the purpose of the present study was to analyze the possible effects and mechanisms by which acute glyphosate exposure exerts its neurotoxic effects on dopaminergic neurotransmission in rat striatum. To achieve this objective, adult female rats were acutely exposed to different doses of glyphosate or glyphosate-based herbicide (GBH) systemically or locally administered into the striatum. The extracellular levels of dopamine and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured by high-performance liquid chromatography with electrochemical detection (HPLC-ED). To study the possible mechanisms of action, glyphosate was administered to animals pretreated with tetrodotoxin (TTX), a blocker of voltage-gated sodium channels (VSSC), reserpine, a blocker of the vesicular monoamine transporter type 2 (VMAT2), and nomifensine and GBR 12909, two dopamine transporter (DAT) inhibitors. Additionally, motor performance, oxidative stress induction, and the activity of acetylcholinesterase (AChE) were also determined in the striatum of rats exposed to the pesticide.

2. Methods

2.1. Animals

To carry out the experiments, adult female Sprague-Dawley rats (250–300 g) obtained from the Breeding Facility of the CINBIO (*Centro de Investigaciones Biomédicas*) of the University of Vigo (Spain) were used. The animals were housed in standard laboratory cages and kept under constant humidity and temperature conditions (22 ± 2 °C) with a light/dark cycle of 14:10 h. Commercial feed and tap water used were available *ad libitum*. The experimental procedures and housing conditions used were in accordance with the Guidelines of the European Union Council (2010/63/EU) and the Spanish State (*Real Decreto* 53/2013) for the use of laboratory animals. This study was approved by the "*Ethics Committee on Animal Welfare*" of the University of Vigo. All possible efforts were made to avoid animal suffering and distress.

The present study was carried out with female rats. The use of female rats is supported by previous data showing that no sex differences have been found in the variability of behavioral, electrophysiological, neurochemical, or histological measurements (Becker et al., 2016; Egenrieder et al., 2020). Besides, previous data from our laboratory did not show significant differences between males and females both in baseline dopamine levels and in the effects produced by different types of drugs, toxic, or toxins (Arias et al., 1998; Alfonso et al., 2019; Campos et al., 2007; Faro et al., 2022).

2.2. Drugs and treatments

Glyphosate (purity 98%, molecular weight: 169.07 g/mol) was purchased from Pestanal® (Fluka-Sigma-Aldrich St. Louis, USA). Roundup® herbicide (glyphosate concentration: 360 g/L in the form of glyphosate isopropylamine salt 486 g/L, molecular weight: 228.183 g/mol), was used in the liquid commercial form supplied by Monsanto Agriculture (Spain). TTX was purchased from Tocris (Bristol, UK). Nomifensine, GBR 12909, reserpine, dopamine, DOPAC, and HVA were obtained from Sigma-Aldrich (St. Louis, USA). All other chemicals and reagents were analytical grade. For behavioral tests and biochemical assay, the glyphosate was diluted in saline solution (0.9%) and administered by intraperitoneal (i.p.) injection. For microdialysis experiments, glyphosate, GBH, nomifensine, and TTX were dissolved in the perfusion medium and delivered locally into the striatum *via* microdialysis probe. GBR 12909 was diluted in saline solution (0.9%) and administered by i.p. injection. Reserpine was dissolved in glacial acetic acid and made up to the final volume with a 5.5% glucose solution before injecting (i.p.) the animal.

2.3. Behavioral tests and biochemical assay

For this first part of the study, we used eighteen animals that were divided into 3 experimental groups: control administered with saline 0.9% ($n = 6$), glyphosate 75 mg/kg (n = 6), and glyphosate 150 mg/kg (n = 6) (Fig. 1). At the end of the behavioral tests, the animals were anesthetized with chloral hydrate (400 mg/kg i.p.) and euthanized by a rapid decapitation, the striatum was dissected, and the tissue was frozen at −80 °C until biochemical assay.

2.3.1. Motor performance

Motor impairment was measured on rotarod apparatus following the protocol described by Barbosa and Morato (2000). Briefly, the animals were trained under continuous acceleration (1 rpm/s) in 1-min sessions on a rotarod apparatus (Ugo Basile, Italy). The rotational velocity at which the animal dropped off the rotating bar was taken as the performance score. Animals that did not reach a stable baseline (at least 15 rpm) in 12 trials were discarded. The animals that presented performances between 15 and 40 rpm were selected for the experiment. After selection, experimental and control groups were matched based on both their body weight and mean performance during the last training sessions on the rotarod. With this procedure, animals presented similar basal values in all groups.

Once the baseline measurement was obtained, animals received an i.p. injection of saline, 75 mg/kg glyphosate, or 150 mg/kg glyphosate and their performance on the rotarod was evaluated. Tests were performed at 30, 60, 120 min, and 24 h after glyphosate administration. These time intervals are based on previous studies showing that, after oral or intravenous administration, the pesticide is rapidly and widely distributed and readily penetrates extravascular tissues (Anadón et al., 2009). It has also been observed that the elimination half-life of glyphosate after intravenous or oral administration is 9.5–14.4 h, so it was decided to also evaluate what effect the pesticide would have 24 h after its administration (Anadón et al., 2009; WHO/FAO, 2017). The maximum percentage of motor impairment was calculated by comparing baseline and the lower post-treatment performance, according to:

$$Maximum\ motor\ impairment = \frac{(baseline\ score) - (lower\ test\ score)}{\text{baseline score}}\ x\ 100$$

2.3.2. Acetylcholinesterase activity

AChE activity was quantified using a cholinesterase activity assay kit according to manufacturer's protocol (MAK119, Sigma, St. Louis, MO, United States) with minor modifications. Briefly, rat striatum was homogenized (10 μL/mg) in 0.1 M phosphate buffer (pH 7.5) at 4 °C followed by centrifugation at 15000 ×*g* for 10 min (Centrifuge 5418R, Eppendorf, Hamburg, Germany). Aliquots of the cleared supernatants were added to the kit reaction mixture and the reaction proceeded at room temperature. Absorbance at 405 nm was quantified using a microplate reader (EZ Read 400 ELISA, Cambridge, UK) at 2-min intervals from time 0 (tissue sample addition) up to 10 min. AChE activity was expressed as units/L. One unit of AChE is the amount of enzyme that catalyzes the production of 1.0 μmol of thiocholine per minute at room temperature at pH 7.5.

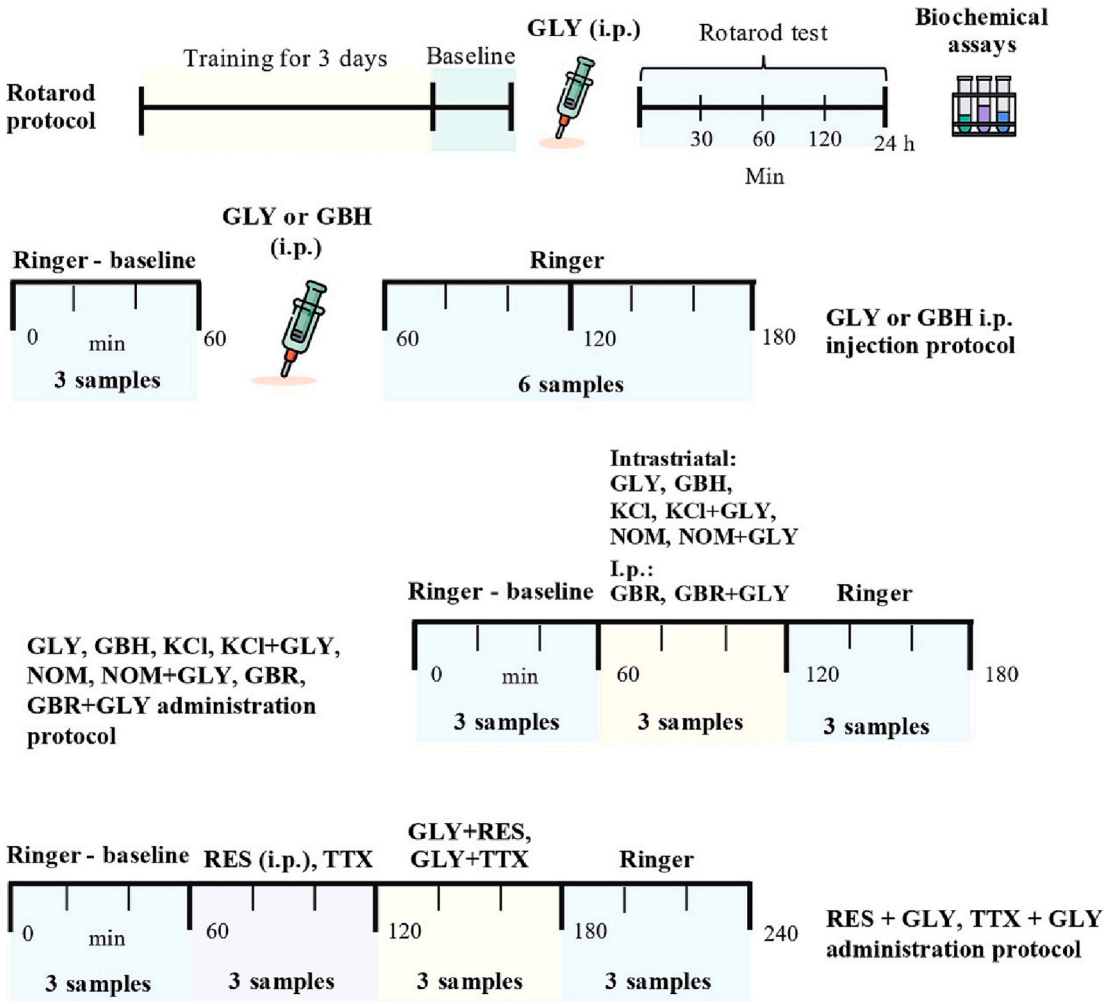

Fig. 1. Schematic representation of the different experimental designs. Abbreviations: GLY, glyphosate; i.p., intraperitoneal injection; GBH, glyphosate-based herbicide; TTX, tetrodotoxin; RES, reserpine; NOM, nomifensine.

2.3.3. Total antioxidant capacity

The total antioxidant capacity was measured using a total antioxidant capacity kit according to manufacturer's protocol (MAK 187, Sigma, St. Louis, MO, United States). Striatum was homogenized in 1 mL of distilled water by using a pestle. In the next step, 100 μL of each sample and 100 μL of the Cu^{2+} working solution were poured into wells. After the solution was incubated at room temperature (20 °C) for 90 min, samples were centrifuged (Centrifuge 5418R, Eppendorf, Hamburg, Germany) at 250 ×g for 5 min at 4 °C to separate solid tissue components. The supernatant was diluted 1:5 to bring values within range of kit standards. Subsequently, absorbance was measured at 570 nm using a microplate reader (EZ Read 400 ELISA, Cambridge, UK). Higher scores represent a greater total antioxidant capacity (measured in millimoles of trolox equivalents).

2.3.4. Statistical analysis

The motor performance was analyzed using one-way analysis of variance (ANOVA) and two-way ANOVA for repeated measures according to the experimental protocol. Maximum motor impairment, acetylcholinesterase activity, and total antioxidant capacity results were compared between the three groups (control and treated) and analyzed using one-way ANOVA, followed by a Bonferroni *post hoc* for multiple comparisons. All experimental data are represented as mean ± S.E.M., and a value of $p < 0.05$ was considered statistically significant. All statistical analyses were carried out using the SPSS software (IBM SPSS Statistics 24).

2.4. In vivo microdialysis

2.4.1. Surgery and microdialysis

The microdialysis technique was carried out according to previous studies performed in our laboratory (Alfonso et al., 2019; Faro et al., 2020, 2022). Briefly, rats were anesthetized with chloral hydrate (400 mg/kg i.p.) and stereotaxically implanted with a guide canula (CMA/12, CMA/Microdialysis, Sweden). The coordinates of the implantation were A/P + 1.0, L/M + 3.0 and V/D 6.0 from bregma (Paxinos and Watson, 1998). Microdialysis was performed the next day. Probes with a 3 mm active dialysis membrane (CMA/12, CMA/Microdialysis, Sweden) were perfused with Ringer solution at 1.5 μL/min (147 mM NaCl, 4 mM KCl y 2.4 mM $CaCl_2$, pH = 7.4), and fractions were collected each 20 min.

The experiments were carried out for periods of three or four hours, according to different experimental schemes shown in Fig. 1 and Table 1. In the first group of experiments, following a period for equilibration, baseline samples were collected for 60 min. Glyphosate or GBH was then systemically administered (75 or 150 mg/kg i.p.) and samples were collected for 120 min. In the following experimental group, made to evaluate the effects of intrastriatal administration of the glyphosate or GBH, the pesticide was diluted in the Ringer solution and directly infused into the striatum by retrodialysis (1, 5, or 10 mM) for 60 min, and sampling continued until the end of the experiment (60 min). For the study of the mechanisms of action of glyphosate, the experimental scheme was: after collecting the three basal samples (60 min), glyphosate, glyphosate+KCl, KCl, glyphosate+nomifensine, nomifensine,

Table 1
Experimental groups in which glyphosate or drugs were dissolved in the perfusion fluid and administered into striatum through the dialysis probe. Abbreviations: GLY, glyphosate; GBH, glyphosate-based herbicide; RES, reserpine; NOM, nomifensine.

Group	Treatment protocol				
	3 h microdialysis experiment				
	4 h microdialysis experiment				
	n	*60 min*	*60 min*	*60 min*	*60 min*
1-Control	4	Ringer	Ringer	Ringer	–
2-GLY 75 mg/kg (i.p.)	6	Ringer	Ringer (GLY i.p)	Ringer	–
3-GLY 150 mg/kg (i.p.)	6	Ringer	Ringer (GLY i.p)	Ringer	–
4-GBH 75 mg/kg (i.p.)	4	Ringer	Ringer (GBH i.p)	Ringer	–
5-GLY 1 mM	5	Ringer	GLY	Ringer	–
6-GLY 5 mM	6	Ringer	GLY	Ringer	–
7-GLY 10 mM	5	Ringer	GLY	Ringer	–
8-GBH 5 mM	4	Ringer	GBH	Ringer	–
9-KCl 50 mM	4	Ringer	KCl	Ringer	–
10-GLY 5 mM + KCl 50 mM	5	Ringer	KCl + GLY	Ringer	–
11-GLY 5 mM + TTX 10 μM	5	Ringer	TTX	TTX+ GLY	Ringer
12-GLY 5 mM + TTX 20 μM	5	Ringer	TTX	TTX+ GLY	Ringer
13-GLY 5 mM + RES 10 mg/kg	6	Ringer (RES 60 min before)	Ringer	GLY	Ringer
14-NOM 50 μM	5	Ringer	NOM	Ringer	–
15-GLY 5 mM + NOM 50 μM	5	Ringer	NOM+ GLY	Ringer	–
16-GBR 12909 5 mg/kg (i.p.)	5	Ringer	Ringer (GBR i.p)	Ringer	–
17-GLY 5 mM + GBR 5 mg/kg	6	Ringer	GLY + GBR (i.p)	Ringer	–

glyphosate+GBR 12909, or GBR 12909, was perfused during 60 min. After that, the medium was switched back to unmodified Ringer solution and sampling continued until the end of the experiment (60 min). In four hours-long experiments, TTX was perfused before the administration of glyphosate in TTX solution, and reserpine was injected i.p. 120 min before glyphosate perfusion; then, sampling was conducted under normal conditions (Fig. 1). A total of eighty-five animals were used in this second part of the study, that were divided into seventeen groups as presented in Table 1. Animals were awake, conscious, and freely moving in all the experiments.

2.4.2. HPLC conditions

Microdialysis samples were analyzed for dopamine, DOPAC, and HVA as previously described (Durán et al., 1998) with minor modifications. After collecting a fraction, samples were injected (20 μL) immediately into a HPLC system using a Rheodyne 7125 injection valve. The isocratic separation of dopamine and its metabolites was achieved using Dionex C18 reversed phase columns (5 μm particle size). The mobile phase (pH 3.4) was prepared as it follows: 100 mM KH_2PO_4, 1 mM octanesulfonic acid, 1 mM EDTA and 14% methanol. Elution was carried out at a flow rate of 1.0 mL/min using a Jasco PU 1580 pump. The dopamine, DOPAC and HVA detection was achieved using an ESA Coulochem III electrochemical detector (ESA, USA) at a potential of oxidation of +400 mV. All the data were analyzed by the chromatographic software Cromanec XP 1.0.4 (Micronec, Spain).

2.4.3. In vitro recovery of dopamine, DOPAC and HVA

Before probe implantation into the brain, the recoveries of dopamine, DOPAC and HVA across the dialysis membrane were determined *in vitro*. The dialysis probes were placed in a standard solution of substances (50 pg/μL) and flushed with Ringer's solution at the same flow as the experiments (1.5 μL/min) for 20 min. The levels of dopamine, DOPAC and HVA in the dialysates were determined. Recoveries were calculated from the concentration of substrate in the perfusion fluid divided by the concentration in the standard solution. The different recoveries across the microdialysis membrane were: 13.7 ± 4.1%, 19.7 ± 5.5% y 21 ± 3.6%, for dopamine, DOPAC and HVA, respectively.

2.4.4. Statistical analysis

All the data shown are mean ± S.E.M. Results were calculated as percentages of the average basal release. The average of basal levels of dopamine and its metabolites (defined as 100%) was determined from two dialyzed samples before the addition of any drug or modified perfusion medium. Data of dopamine and its metabolites were corrected using the percentage of *in vitro* recovery for every microdialysis probe.

Statistical analysis of the results was performed by means of ANOVA and Student-Newman-Keuls multiple range test, considering the following significant differences: $^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.001$, with respect to basal; $^{a}p < 0.05$, $^{b}p < 0.01$, and $^{c}p < 0.001$, with respect to 5 mM glyphosate or control group.

3. Results

Systemic or intrastriatal administration of glyphosate or GBH at the tested doses (75 or 150 mg/kg i.p.) or concentrations (1, 5, or 10 mM *in situ*) or other drugs or medium did not produce seizures, tremors, or other types of apparent behavioral or physiological dysfunctions.

3.1. Effects of glyphosate on the motor performance

The effects of glyphosate on the motor performance are shown in Fig. 2. The two-way repeated measures ANOVA showed a significant effect for treatment with glyphosate ($F_{(2,18)} = 5.554$, $p = 0.013$) but not for time ($F_{(4,72)} = 1.170$, $p = 0.332$). Likewise, the interaction between the two factors was not significant ($F_{(8,72)} = 1.170$, $p = 0.115$). The *post hoc* comparisons showed that rotarod performance was significantly reduced following 150 mg/kg glyphosate treatment at 60 and 120 min ($p < 0.01$), and 24 h ($p < 0.05$), and 75 mg/kg at 120 min ($p < 0.05$) and 24 h ($p < 0.01$), with respect to the control ($p < 0.05$) (Fig. 2A). One-way ANOVA ($F_{(2,14)} = 6.725$, $p = 0.009$) followed by Bonferroni test showed that the maximum impairment was achieved with glyphosate treatment (Fig. 2B).

3.2. Effects of glyphosate on AChE activity and total antioxidant capacity

We performed the assay of AChE activity 24 h after exposure to a single administration of glyphosate to test its potency to inhibit the activity of this enzyme in the adult rat striatum. One-way ANOVA analysis showed significant differences between groups ($F_{(2,14)} = 8.02$; $p = 0.005$). *Post hoc* comparisons revealed that both 75 mg/kg and 150 mg/kg groups showed a significant decrease of AChE activity in the striatum (75 mg/kg *vs* control: $p = 0.028$; 150 mg/kg *vs* control: $p = 0.01$) (Fig. 3A).

Exposure to glyphosate also changed the antioxidant capacity of striatum (Fig. 3B). One-way ANOVA analysis showed significant differences between groups ($F_{(2,17)} = 11.02$; $p = 0.001$). *Post hoc* comparisons revealed that both 75 mg/kg and 150 mg/kg groups showed a dose-dependent decrease in the striatal antioxidant capacity (75 mg/kg *vs* control: $p = 0.011$; 150 mg/kg *vs* control: $p = 0.002$).

3.3. Effects of glyphosate or GBH on the in vivo dopamine, DOPAC, and HVA release from rat striatum

Periodical control experiments have been carried out under our microdialysis conditions to confirm the basal values and the adequation of our conditions. Basal levels of dopamine and its metabolites in dialyzed samples were stable in control animals (non-treated rats). The mean of dopamine, DOPAC and HVA concentrations in the two samples

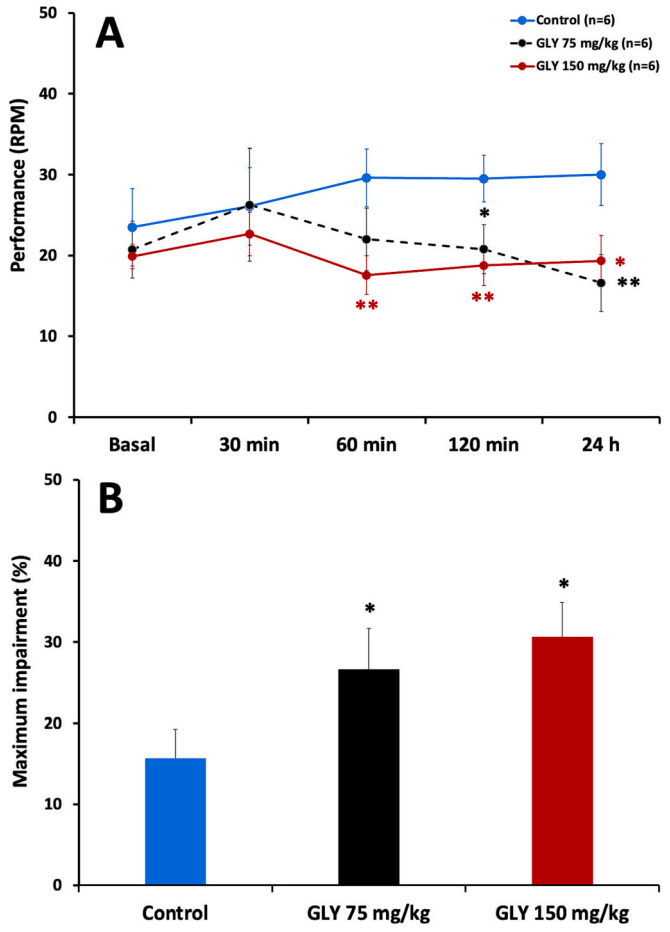

Fig. 2. Acute effects of exposition to glyphosate (GLY) on rat motor coordination measured in the rotarod test. A: Time-course of effect of glyphosate recorded at 30, 60, and 120 min, and 24 h after injections of 75 or 150 mg/kg glyphosate, or saline i.p. B: Maximum motor impairment. The results are shown as mean ± SEM of 8 animals per group. Significant differences: $*p < 0.05$, compared with the control saline group.

collected before glyphosate or drugs administration was considered as the basal levels: 0.065 ± 0.008, 1.56 ± 0.05, and 0.10 ± 0.01 ng/μL, respectively.

Systemic administration of glyphosate or GBH significantly changed the dopamine, DOPAC and HVA extracellular levels (Fig. 4). Administration of 75 or 150 mg/kg glyphosate increased extracellular levels of dopamine to 194 ± 31.2% ($p < 0.001$) and 283 ± 38% ($p < 0.001$), respectively, and administration of 75 mg/kg GBH increased dopamine levels to 269 ± 16% ($p < 0.001$), when compared to basal values. Although these maximal increases were observed 40 or 60 min after glyphosate or GBH administration, dopamine levels remained high and did not return to baseline until the end of the experiments (Fig. 4A).

Fig. 4B and C show that treatment of animals with 75 or 150 mg/kg glyphosate or GBH caused significant increases in extracellular levels of DOPAC and HVA that became statistically significant 60 and 80 min after the pesticide injection, respectively. The maximal increases observed in the DOPAC and HVA levels were: 75 mg/kg glyphosate: 170 ± 43% ($p < 0.05$) and 134 ± 13% ($p < 0.001$); 150 mg/kg glyphosate: 211 ± 18% ($p < 0.001$) and 194 ± 26% ($p < 0.001$), when compared with basal levels, respectively. Administration of 75 mg/kg GBH significantly increased HVA [144 ± 3% ($p < 0.001$)] but had no significant effect on DOPAC levels.

The intrastriatal administration of glyphosate or GBH, through the microdialysis probe, also significantly changed the dopamine, DOPAC, and HVA extracellular levels (Fig. 5).

Infusion of 1, 5, or 10 mM glyphosate or 5 mM GBH for 60 min increased extracellular levels of dopamine to 203 ± 72.7% ($p < 0.05$), 1080 ± 240% ($p < 0.001$), 3414 ± 341% ($p < 0.001$), and 2502 ± 275% ($p < 0.001$), when compared to baseline, respectively. The maximum increase was obtained 40 min after the beginning of 1 mM glyphosate and GBH perfusion and 20 min after the beginning of 5 or 10 mM glyphosate administration. Basal values recovered 120 min later (Fig. 5A).

Perfusion of 1 mM glyphosate or 5 mM GBH significantly increased the extracellular concentration of DOPAC, which reached maximum values of 247 ± 20% ($p < 0.001$) and 175 ± 25% ($p < 0.001$), respectively (Fig. 5B). Regarding HVA, only the highest concentration of glyphosate (10 mM) significantly reduced the extracellular levels of this metabolite, for which a minimum value of 58 ± 10.3% ($p < 0.001$) was recorded at 60 min from the start of the pesticide administration (Fig. 5C).

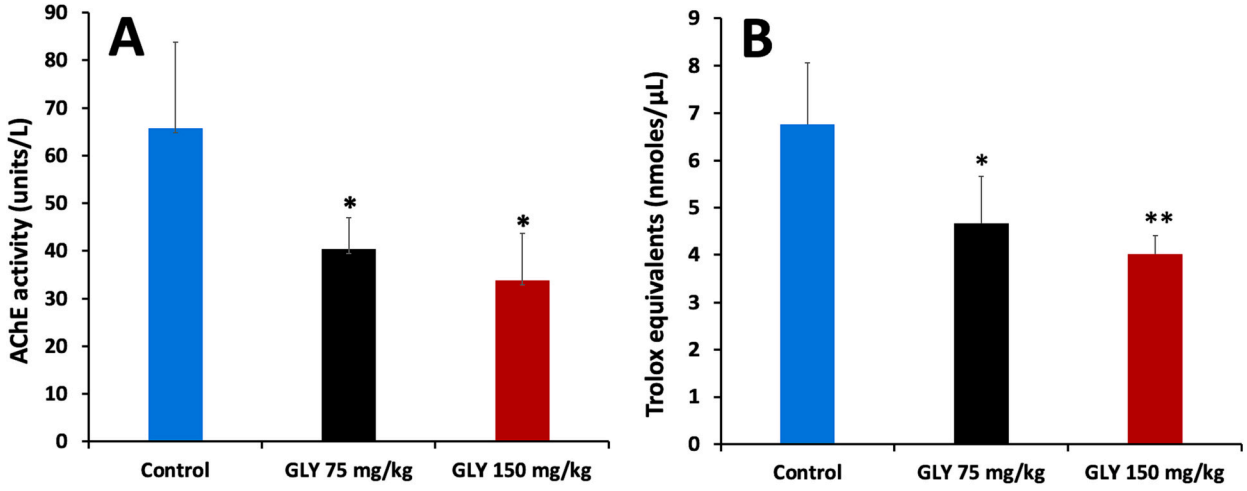

Fig. 3. A) Effect of systemic administration of 75 or 150 mg/kg glyphosate (GLY) on acetylcholinesterase (AChE) activity in rat striatum. Data represent mean ± S.E.M. of enzyme activity expressed as units of AChE/L. B) Effect of administration of 75 or 150 mg/kg glyphosate on total antioxidant capacity in rat striatum. Data represent mean ± S.E.M. of the antioxidant capacity of the tissue that is expressed as trolox equivalent in nmol/μL. Significant differences: $*p < 0.05$ and $*p < 0.01$, compared with the control saline group.

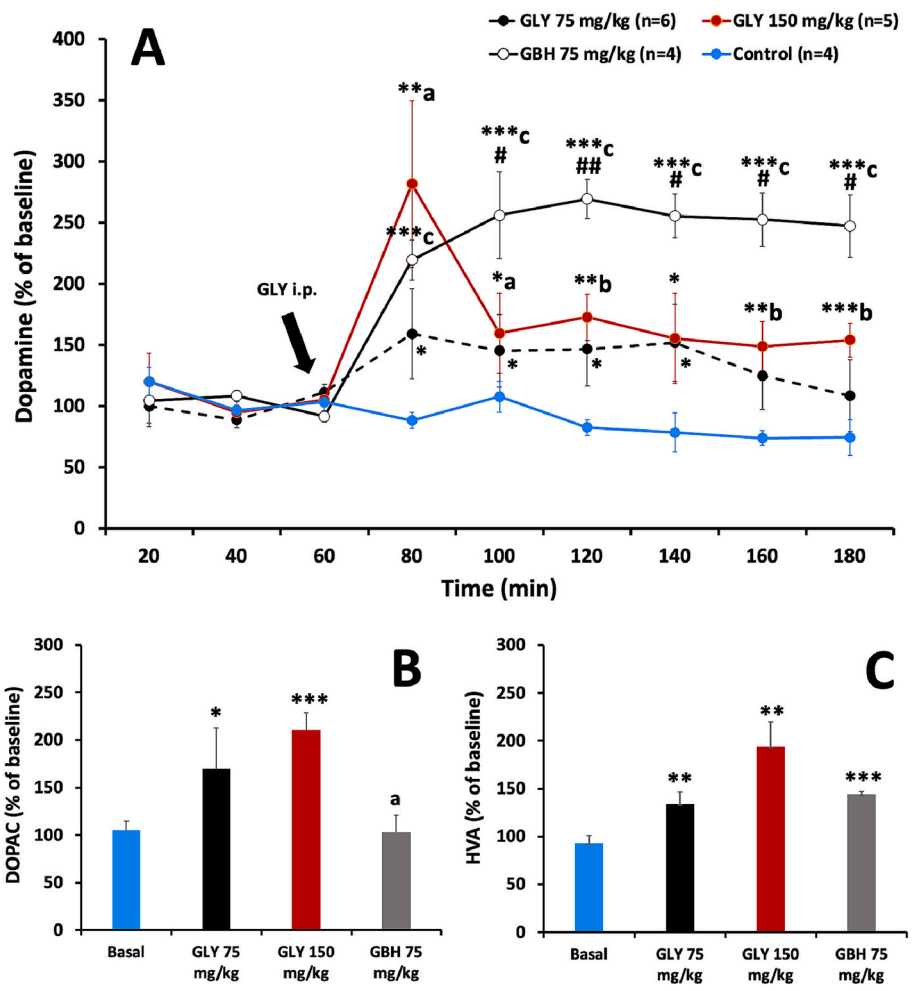

Fig. 4. Effects of systemic administration of 75 or 150 mg/kg glyphosate (GLY) or 75 mg/kg glyphosate-based herbicide (GBH) on *in vivo* dopamine (A), DOPAC (B), and HVA (C) release from rat striatum. Arrow denotes the i.p. injection of glyphosate or GBH 60 min after the beginning of microdilaysis experiment. The results are shown as means ± S.E.M., expressed as a percentage of the basal levels (100%). Basal levels were considered as the mean of dopamine and its metabolites concentration in the two samples collected before pesticide administration. Significant differences were: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, with respect to basal levels; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, with respect to 5 mM glyphosate; $^{a}p < 0.05$, $^{b}p < 0.01$, and $^{c}p < 0.001$, with respect to control group in the same times.

3.4. Neurochemical characterization of glyphosate-induced dopamine, DOPAC, and HVA release from the striatum

With the purpose of investigating the possibility that increases in the dopamine, DOPAC, and HVA levels induced by glyphosate could be due to an exocytotic mechanism (depolarization-, vesicular-, and voltage-dependent) or a voltage-independent mechanism mediated by an action of this pesticide on DAT, a modified perfusion medium and different pharmacological treatments (TTX, reserpine, nomifensine, and GBR 12909) were used. Since the concentration of 5 mM induced significant increases in the dopamine levels (10 times), this concentration of glyphosate was selected for these subsequent experiments. The data for the effects of 5 mM glyphosate on dopamine, DOPAC and HVA levels are plotted in Figs. 6–10 in order to compare them with the data obtained under other experimental conditions.

3.4.1. Effects of glyphosate on K^{+}-stimulated dopamine, DOPAC and HVA release

In this first experimental group, the effect of glyphosate on depolarization-evoked dopamine, DOPAC and HVA release was investigated (Fig. 6). For this, 50 mM KCl was perfused directly into the striatum for 60 min through the microdialysis probe.

Fig. 6A shows that 20 min after the start of KCl perfusion, dopamine levels increased to 1104 ± 220% ($p < 0.001$), compared to baseline values. This effect was similar to that induced by treatment with glyphosate 5 mM and a similar dopamine release profile could be observed for both treatments administered individually. However, when KCl was coadministered together with glyphosate, striatal dopamine levels increased to 2648 ± 478% ($p < 0.001$), when compared to basal values. These results show a significant additive effect of glyphosate infused together with KCl on dopamine release from rat striatum.

Fig. 6B and C showed the effects of KCl and/or glyphosate on DOPAC and HVA levels. The administration of KCl had no significant effect on DOPAC levels, while decreased HVA to 58 ± 9.1% ($p < 0.01$), compared to baseline. Administration of KCl together with glyphosate significantly increased DOPAC to 208 ± 48% ($p < 0.01$) and had no effect on HVA levels.

3.4.2. Effects of TTX pretreatment on glyphosate-induced dopamine, DOPAC and HVA release

To investigate whether glyphosate-induced dopamine, DOPAC and HVA release was dependent on VSSC activation, 10 or 20 μM TTX was infused through the dialysis probe into the dorsal striatum (Fig. 7).

Fig. 7A shows how intrastriatal perfusion of 10 or 20 μM TTX for 60 min significantly reduced the dopamine levels to 62.5 ± 12.6% ($p < 0.05$) and 49 ± 7.8% ($p < 0.05$) compared to baseline values, respectively (bar graph in Fig. 7A). When 5 mM glyphosate was administered to animals pretreated with 10 μM TTX the dopamine levels increased to 1250 ± 244% ($p < 0.001$), compared to baseline levels, this increase being not significantly different from that induced by glyphosate alone. As this TTX concentration had no significant effect on glyphosate-induced dopamine release, we used a higher concentration of the blocker to assess whether this lack of effect was due to the low concentration used. Intrastriatal administration of 5 mM glyphosate to animals pretreated with 20 μM TTX increased dopamine levels to 1561 ± 252% ($p < 0.001$). Likewise, this increase was not statistically different

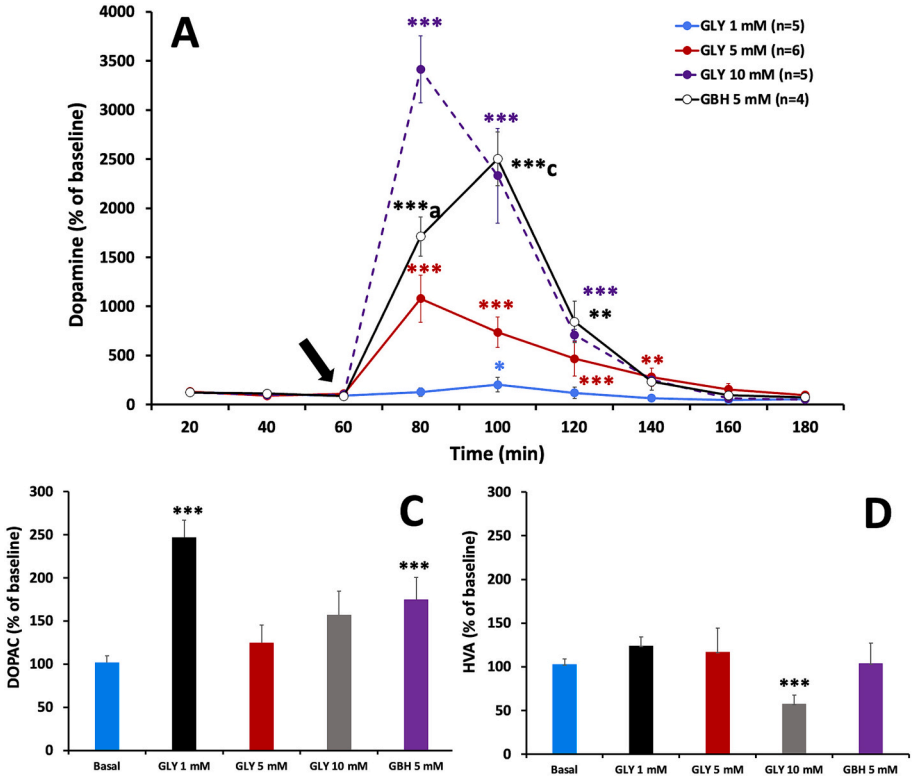

Fig. 5. Effects of intrastriatal administration of 1, 5, and 10 mM glyphosate (GLY) or 5 mM glyphosate-based herbicide (GBH) on dopamine (A), DOPAC (B), and HVA (C) release from rat striatum. Arrow denotes the infusion of pesticide during 60 min. The results are shown as means ± S.E.M., expressed as a percentage of the basal levels (100%). Basal levels were considered as the mean of dopamine and its metabolites concentration in the two samples collected before pesticide administration. Significant differences were: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, with respect to basal levels; $^{a}p < 0.05$, $^{b}p < 0.01$, and $^{c}p < 0.001$, with respect to 5 mM glyphosate group in the same times.

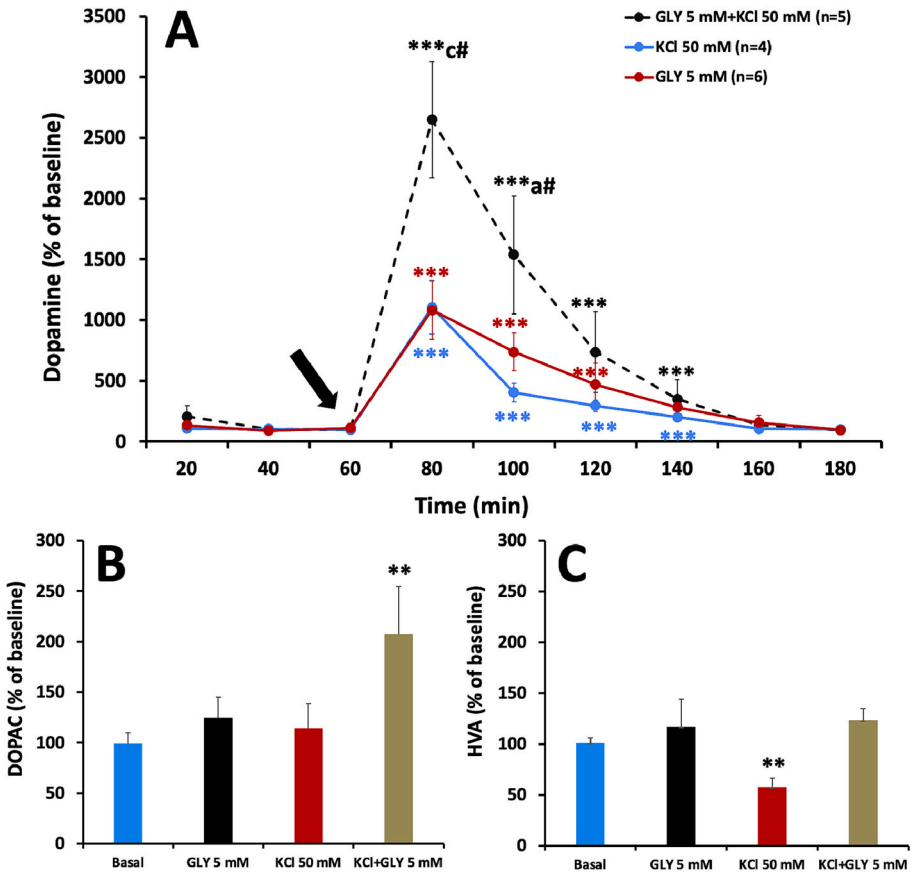

Fig. 6. Effects of KCl (50 mM) infusion on dopamine (A), DOPAC (B), and HVA (C) release from rat striatum in the absence or presence of 5 mM glyphosate (GLY). KCl and glyphosate infusion started at the time indicated by the arrow over 60 min. The results are shown as the mean ± S.E.M., expressed as a percentage of basal levels (100%). Basal levels were considered as the mean of dopamine and its metabolites concentrations in the two samples before glyphosate, glyphosate+KCl or KCl administration. Significant differences were: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, with respect to basal levels, and $^{a}p < 0.05$, $^{b}p < 0.01$, and $^{c}p < 0.001$, with respect to 5 mM glyphosate control group.

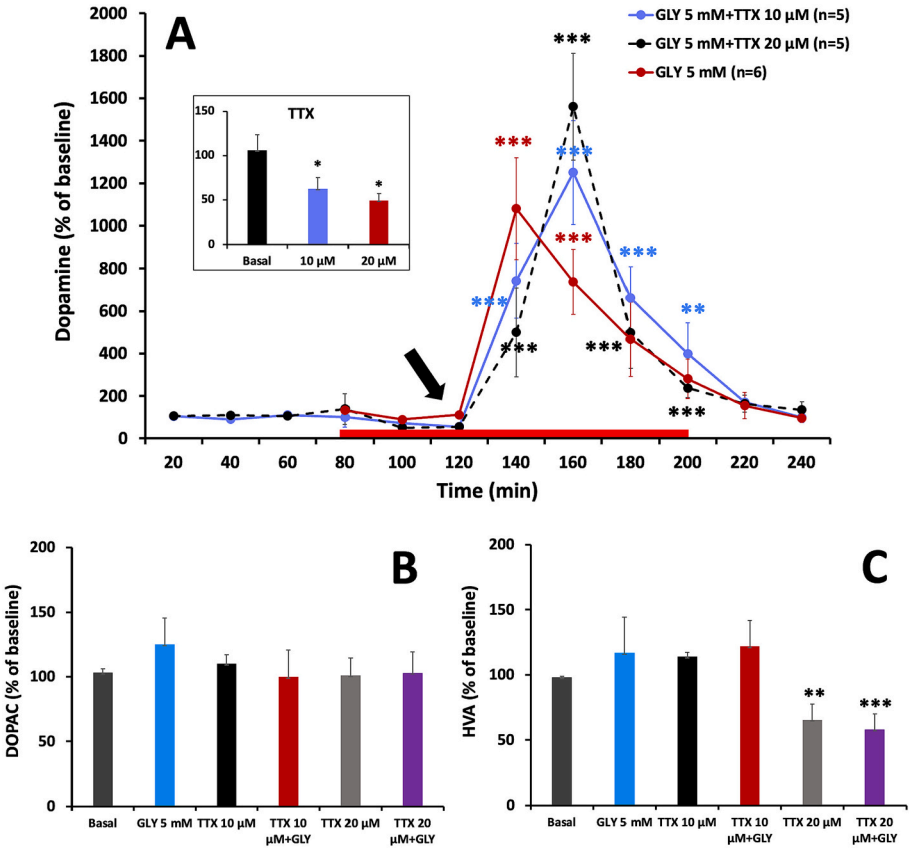

Fig. 7. Effects of 5 mM glyphosate (GLY) infusion in tetrodotoxin (TTX) (10 or 20 µM) pretreated animals on dopamine (A), DOPAC (B), and HVA (C) release from rat striatum. The effect of TTX on dopamine levels is shown in the box. Infusion of TTX is shown by the red bar and glyphosate infusion started at the time indicated by the arrow over 60 min. The results are shown as the mean ± S.E.M., expressed as a percentage of basal levels (100%). Basal levels were considered as the mean of dopamine and its metabolites concentrations in the two samples before glyphosate, glyphosate+TTX or TTX administration. Significant differences were: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, with respect to basal levels; $^{a}p < 0.05$, $^{b}p < 0.01$, and $^{c}p < 0.001$, with respect to 5 mM glyphosate group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

from that produced by glyphosate alone. These findings shows that both concentrations of TTX were not able to inhibit the effect of the glyphosate on dopamine release.

The effects of TTX and glyphosate together with TTX on the DOPAC and HVA concentrations are showed in Fig. 7B and C. Infusion of 10 or 20 µM TTX did not produce significant changes in the metabolite levels. Alike, administration of 5 mM glyphosate to animals pretreated with 10 or 20 µM TTX did not produce significant changes in the DOPAC but significantly decreased HVA levels to 65.4 ± 12% ($p < 0.01$) and 58 ± 12% ($p < 0.001$), respectively.

3.4.3. *Effects of reserpine pretreatment on glyphosate-induced dopamine, DOPAC, and HVA release*

To verify the mediation of vesicular stores on the glyphosate-induced increases in extracellular dopamine, DOPAC, and HVA levels, rats were pretreated with reserpine (10 mg/kg i.p.) 60 min before the beginning of the experiment and 120 min before glyphosate administration (Fig. 8).

Two hours after reserpine injection, extracellular dopamine levels decreased to 49 ± 7.6% ($p < 0.01$), with respect to the baseline. This value was considered as basal level for the measurement of glyphosate effects in reserpinized animals. Fig. 8A shows that infusion of 5 mM glyphosate in reserpine-pretreated animals increased striatal dopamine levels to 653 ± 223% ($p < 0.001$), with respect to basal. It should be noted that this maximum increase was observed 40 min after the start of the glyphosate infusion in the pretreated animals with reserpine, while the maximum increase induced by the pesticide was observed 20 min after the start of its administration.

Fig. 8B and C show the effects of glyphosate administration to reserpine pretreated animals. Reserpine treatment did not produce significant alterations in the levels of both metabolites. Glyphosate infusion to pretreated animals significantly decreased DOPAC [96 ± 6.2% ($p < 0.001$)] and HVA [72 ± 11.2% ($p < 0.001$)] levels both relative to baseline and relative to the effect of glyphosate alone.

3.4.4. *Effect of nomifensine or GBR 12909 on glyphosate-induced dopamine, DOPAC, and HVA release*

To evaluate a possible role of DAT in the glyphosate-induced dopamine, DOPAC and HVA release, nomifensine or GBR 12909, both inhibitors of the dopamine uptake, was co-infused with 5 mM glyphosate (Figs. 9 and 10).

Fig. 9A shows the effect of nomifensine (50 µM) infusion, for 60 min, on the dopamine extracellular levels. Nomifensine administration induced a maximum increase in extracellular dopamine levels of 1215 ± 183% ($p < 0.001$) at 40 min after starting drug infusion. When nomifensine (50 µM) was co-administered with glyphosate, striatal dopamine levels were increased to 2080 ± 180% ($p < 0.001$), when compared to basal values, at 40 min after starting of treatment. This increase is significantly higher from those produced by nomifensine or glyphosate alone.

The intrastriatal administration of nomifensine did not produce significant alterations in the DOPAC and HVA levels (Fig. 9B and C). The co-administration of 50 µM nomifensine with 5 mM glyphosate significantly increased DOPAC levels to 150 ± 18.7% ($p < 0.001$), compared to baseline levels, and had no significant effect on HVA levels.

The effects of exposure to GBR 12909 or GBR 12909 together with glyphosate on dopamine, DOPAC, and HVA release from rat striatum are shown in Fig. 10. Systemic administration of GBR 12909 (5 mg/kg i.p.) significantly increased the dopamine and HVA levels to 198 ± 48% ($p < 0.05$) and 208 ± 26% ($p < 0.01$), respectively, at 80 min after starting drug infusion (Fig. 10A and C), without significantly altering DOPAC levels. Fig. 10A shows that administration of glyphosate together with GBR 12909 increased dopamine levels to 1162 ± 376 ($p < 0.001$). Regarding dopamine metabolites, we observed that coadministration of GBR 12909 + glyphosate significantly decreased HVA levels to 76.5 ± 15.2 ($p < 0.05$) (Fig. 10C) and had no significant effect on DOPAC extracellular levels.

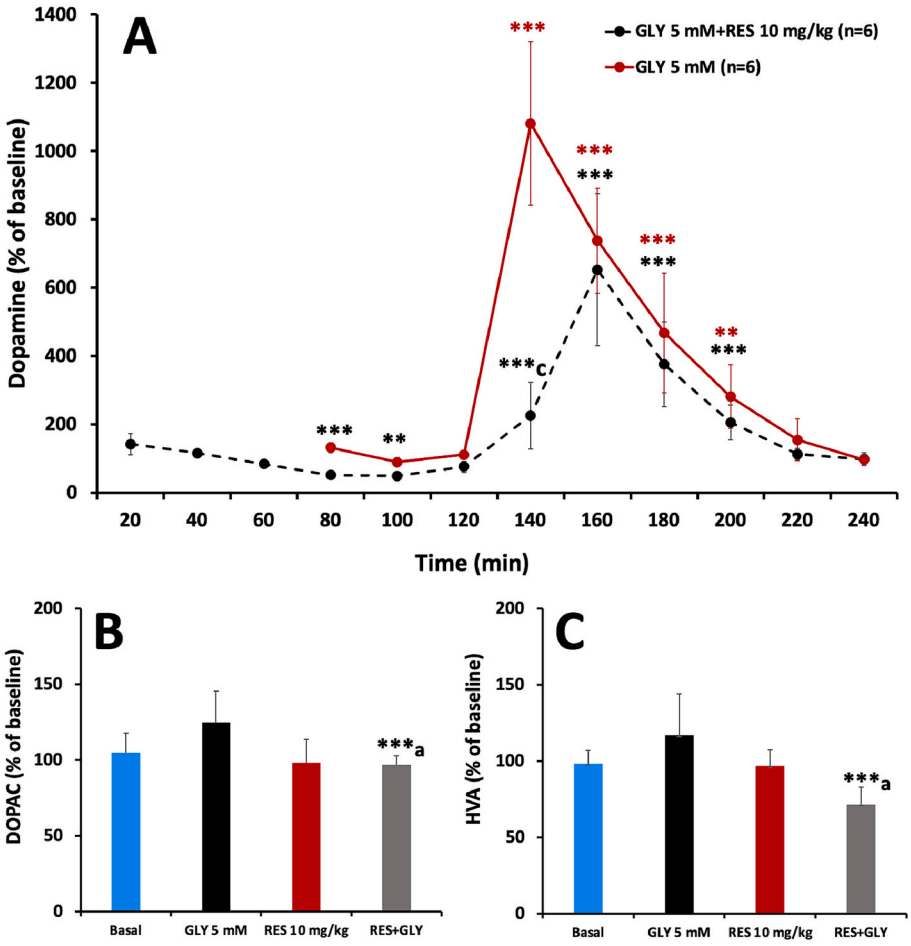

Fig. 8. Effect of 5 mM glyphosate (GLY) infusion in reserpine (RES, 10 mg/kg)-pretreated animals on the dopamine (A), DOPAC (B), and HVA (C) release from rat striatum. Reserpine was administered 60 min before the start of the experiment. Glyphosate infusion started at the time indicated by the arrow over 60 min. The results are shown as the mean ± S.E.M., expressed as a percentage of basal levels (100%). Basal levels were considered as the mean of dopamine and its metabolites concentrations in the two samples before glyphosate, glyphosate+reserpine or reserpine administration. Significant differences were: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, with respect to basal levels; $^{a}p < 0.05$, $^{b}p < 0.01$, and $^{c}p < 0.001$, with respect to 5 mM glyphosate group.

4. Discussion

The results observed in the present study show that acute exposure to glyphosate produced a series of neurotoxic effects that include: 1) impaired motor activity that was maintained 24 h after exposure to the pesticide; 2) decreased AChE activity and total antioxidant capacity in the striatum; 3) both glyphosate and GBH administered systemically or locally into the striatum produced significant increases in striatal dopamine release in a dose-dependent manner; 5) glyphosate increased depolarization-induced dopamine release; 6) the increase in dopamine release did not appear to depend on VSSC activation and was partially dependent on vesicular storage of the neurotransmitter and; 7) DAT blockade along with glyphosate administration potentiates the glyphosate-induced dopamine release.

4.1. Behavioral and biochemical effects

Impaired motor function after glyphosate exposure is one of the most frequently reported symptoms in rodent model studies (Ait-Bali et al., 2020, 2017; Baier et al., 2017; Coullery et al., 2020; Hernández-Plata et al., 2015). In line with these previous data, in the present study we observed that acute exposure to glyphosate affected motor performance, decreasing the motor coordination of the animals, an effect that was maintained 24 h after the administration of the pesticide. The use of the accelerating rotarod apparatus is a reliable indicator to demonstrate central/peripheral neurotoxicity in experimental animals (Barbosa and Morato, 2000). Thus, although the dose of glyphosate administered was below the NOAEL established by the regulatory agencies (Tarazona et al., 2017), in our experimental conditions it was sufficient to generate a significant decline in motor function that began 60 or 120 min after exposure to glyphosate and that was maintained for at least the following 24 h.

At the cellular level, oxidative stress is one of the key mechanisms mediating glyphosate toxicity, as it has been widely demonstrated that this pesticide promotes excessive production of reactive oxygen species (ROS) (Cattani et al., 2014; Faria et al., 2021; Gallegos et al., 2020; Martínez et al., 2020; Neto da Silva et al., 2020; Pereira et al., 2018; Sobjak et al., 2017; Wang et al., 2022). The imbalance between elevated ROS concentrations induced by glyphosate and insufficient antioxidant defense mechanisms in the cell leads to oxidative stress, which can damage lipids, proteins, and DNA, ultimately resulting in cell death. In a previous study from our laboratory, we showed that administration of antioxidants such as trolox, glutathione, dithiothreitol, or alphalipoic acid significantly inhibited glyphosate-induced dopamine release from striatum (Faro et al., 2022), indicating that this pesticide can induce oxidative stress even in periods of only 1–2 h after its administration.

In the present study we measured striatal trolox levels after a systemic administration of glyphosate, which is a more direct measure of the antioxidant capacity. Trolox is a water-soluble analogue of vitamin E which promotes the protection of membrane structure and cell function and reduce ROS levels (Wada et al., 2016). So, a decrease in its levels may be due to exposure to toxic substances that cause oxidative stress, such as pesticides. The results indicate that the acute exposure to the glyphosate reduced significantly trolox levels in a dose-dependent manner, corroborating the potential of this pesticide to cause oxidative stress in rat striatum. Furthermore, unlike our previous study, which showed that glyphosate was able to produce oxidative stress in periods of 1–2 h, in the present study we demonstrated that this oxidative stress

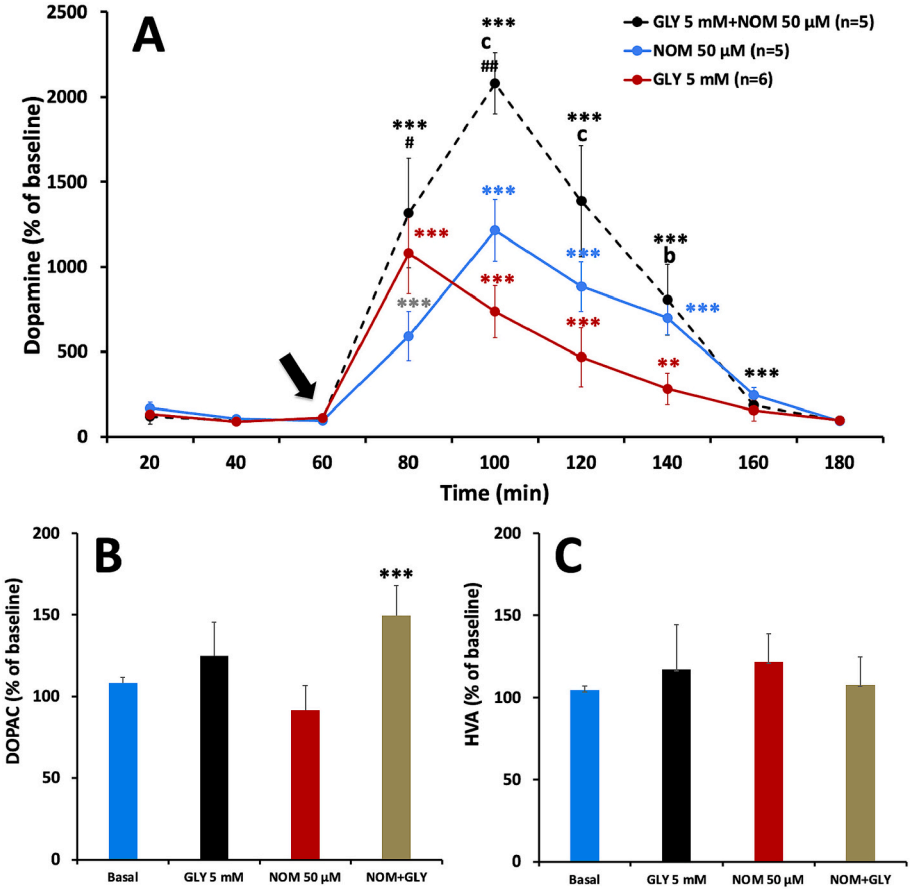

Fig. 9. Effects of nomifensine (NOM, 50 µM) infusion on the dopamine (A), DOPAC (B), and HVA (C) release from rat striatum in the absence or presence of 5 mM glyphosate (GLY). Nomifensine and glyphosate infusion started at the time indicated by the arrow over 60 min. The results are shown as the mean ± S.E.M., expressed as a percentage of basal levels (100%). Basal levels were considered as the mean of dopamine and its metabolites concentrations in the two samples before glyphosate, glyphosate + nomifensine or nomifensine administration. Significant differences were: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, with respect to basal levels, and $^{a}p < 0.05$ and $^{b}p < 0.01$, with respect to 5 mM glyphosate group.

is maintained for up to 24 h after acute systemic exposure to the pesticide. This period would be sufficient to allow extensive distribution and metabolization of glyphosate in the body (Anadón et al., 2009; WHO/FAO, 2017), with the oxidative damage observed 24 h later reflecting the harmful consequences of exposure to small amounts of glyphosate in the brain.

Although glyphosate is an organophosphorus, a group of substances whose main characteristic is irreversible inhibition of AChE, this pesticide does not induce strong inhibition of the enzyme and is considered a weak inhibitor of AChE in the mammalian central nervous system (Ait-Bali et al., 2020, 2019; Cattani et al., 2017; Gallegos et al., 2020; Larsen et al., 2016). Thus, it appears that higher concentrations of glyphosate than those normally observed in the environment are necessary to achieve an effective AChE inhibition. Our results show a significant inhibition of AChE activity (38.5% and 48.6% for the 75 and 150 mg/kg doses, respectively) in the striatum that was maintained at least 24 h after administration of the pesticide. It is well known that an inhibition of AChE leads to increases in the extracellular acetylcholine levels with consequent activation of its receptors, both presynaptic and postsynaptic. Furthermore, it has also been shown that prolonged AChE inhibition can promote oxidative damage, which could contribute to glyphosate-induced brain neurotoxicity (Kazi and Oommen, 2012).

4.2. *Effects on in vivo dopamine, DOPAC, and HVA release from striatum*

In the present study, we demonstrate that acute exposure of rats to glyphosate or GBH significantly changed extracellular levels of dopamine and its metabolites DOPAC and HVA measured *in vivo* by cerebral microdialysis. We observed that systemic administration of glyphosate alone (75 mg/kg or 150 mg/kg) or GBH (75 mg/kg), as well as local administration of the pesticide (1, 5, or 10 mM) or GBH (5 mM) dose-dependently increased dopamine release from striatum of awake and freely moving animals.

Other studies have evaluated the effects of different doses of glyphosate on the total content or *in vivo* release of dopamine and its metabolites from rat striatum (Hernández-Plata et al., 2015; Martínez et al., 2018). Regarding the *in vitro* data, only a very high dose of glyphosate (800 mg/kg/day for 6 days) was able to significantly reduce the total content of dopamine (intra and extracellular) in the striatum, without altering the DOPAC or HVA levels (Martínez et al., 2018). Like our experimental design, the *in vivo* study by Hernández-Plata et al. (2015), also evaluated by brain microdialysis the effect of acute glyphosate exposure in Sprague-Dawley rats. These authors demonstrated that intraperitoneal injection of a single dose of glyphosate (150 mg/kg) decreased both basal and KCl-stimulated dopamine release from striatum. Thereby, our results seem to contradict those obtained by Hernández-Plata et al. (2015), since we observed significant increases in striatal dopamine levels induced by glyphosate under very similar experimental conditions. However, these authors measured the dopamine, DOPAC, and HVA in animals anesthetized with isoflurane (1.5–2%), while in our study dopamine was measured in awake and freely moving animals.

Isoflurane, by itself, increases the levels of dopamine and its metabolites in the striatum in a concentration-dependent manner (Adachi et al., 2008, 2005). However, when these authors administered substances that increase dopamine release, such as clozapine, risperidone, or pargyline, to animals anesthetized with isoflurane, they observed an attenuation of the effects of these substances on dopamine, DOPAC, and HVA release. That is, isoflurane antagonized the effects of clozapine, risperidone, or pargyline on dopamine release from the striatum (Adachi et al., 2008, 2005). Based on this information, we believe that an inhibitory effect of isoflurane on glyphosate-induced dopamine release

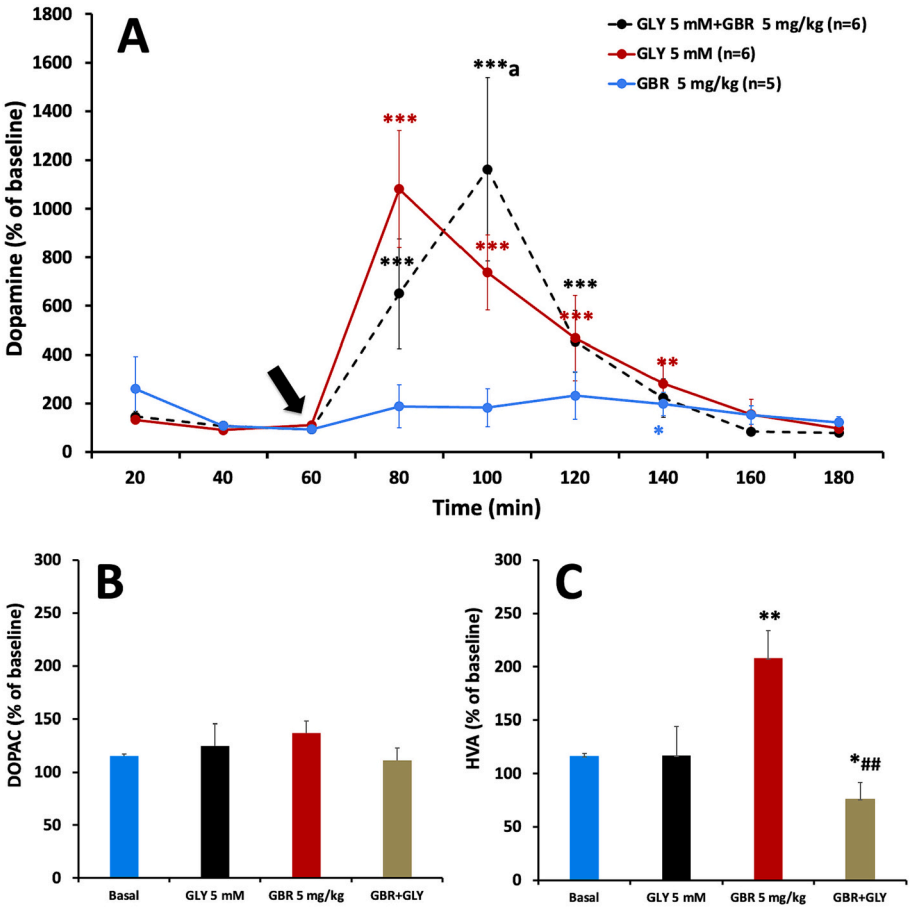

Fig. 10. Effects of GBR 12909 (5 mg/kg) infusion on the dopamine (A), DOPAC (B), and HVA (C) release from rat striatum in the absence or presence of 5 mM glyphosate (GLY). Administration of GBR 12909 and glyphosate started at the time indicated by the arrow over 60 min. The results are shown as the mean ± S.E.M., expressed as a percentage of basal levels (100%). Basal levels were considered as the mean of dopamine and its metabolites concentrations in the two samples before glyphosate, glyphosate+GBR 12909 or GBR 12909 administration. Significant differences were: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, with respect to basal levels, and $^{a}p < 0.05$ and $^{b}p < 0.01$, with respect to 5 mM glyphosate group.

may have occurred in the study by Hernández-Plata et al. (2015). In our study we observed that all doses of glyphosate, as well as GBH, clearly increased the extracellular levels of dopamine and its metabolites in rat striatum. Furthermore, we observed that the stimulatory effect of glyphosate on dopamine release also occurs under conditions of KCl-induced depolarization (Fig. 6).

When comparing the effect of glyphosate with that of a commercial formulation containing 36% of the pesticide and other substances, we observe that the effect of GBH on dopamine release, at the same doses, is stronger than the effect of glyphosate alone (Figs. 4 and 5). These results confirm that, although glyphosate by itself increases dopamine levels, this effect is enhanced by some other component present in the formulation. However, the main objective of our study was to evaluate the effects and mechanisms of action of glyphosate and our results show that this substance has a powerful neurotoxic effect on the nigrostriatal dopaminergic system, significantly increasing striatal dopamine release. On the other hand, it would also be necessary to evaluate the effects of its commercial formulations, since the presence of adjuvants could alter the mechanisms of action of glyphosate and, consequently, modify its effects.

Some case studies of people intoxicated with high doses or chronically with glyphosate show a correlation between exposure to the pesticide and the development of parkinsonism (Barbosa et al., 2001; Eriguchi et al., 2019; Zheng et al., 2018), but the underlying mechanism is unclear. Experimental studies with models of parkinsonism show that the oxidative nature of dopamine itself has been identified as a susceptibility factor (Gluck and Zeevalk, 2004), and an abnormal increase in its extracellular levels, like those induced by glyphosate exposure, can induce neuronal damage through oxidative stress mechanisms. Intracellularly, dopamine is degraded by monoamine oxidase A (MAO-A) in the mitochondrial outer membrane or by autoxidation (Hald and Lotharius, 2005). The metabolism of dopamine by MAO-A leads to the production of DOPAC and hydrogen peroxide by consuming oxygen and water. So, intracellular autoxidation of dopamine generates hydrogen peroxide and dopamine-quinone, which leads to neurotoxicity (Gluck and Zeevalk, 2004; Hald and Lotharius, 2005). This may initiate the symptoms that characterize the Parkinson's disease. Thus, abnormal increases in striatal dopamine levels, like those observed in the present study, may constitute a risk factor for the generation of oxidative stress and a probable development of parkinsonism in cases of exposure to the pesticide.

4.3. Possible neurochemical mechanism by which glyphosate induces striatal dopamine release

Glyphosate-induced increases in the extracellular dopamine levels in the striatum can occur from two processes, either through an exocytotic release mechanism Ca^{2+}-, vesicular-, and voltage-dependent (Klein et al., 2019; Liu and Kaeser, 2019) or through a cytoplasmic release mechanism mediated by an inhibition and/or reversion of DAT (Mulvihill, 2019). So, to determine by which of these two possible mechanisms glyphosate induces dopamine overflow, the effect of different pharmacological treatments on dopaminergic terminal was studied.

In the first set of experiments, we studied the effects of TTX, a blocker of VSSC, on dopamine release evoked by glyphosate. Intrastriatal administration of TTX decreased, in a concentration-dependent manner, the basal dopamine levels in the striatum. However, blocking VSSCs with TTX did not produce a significant effect on glyphosate-induced dopamine release (Fig. 7). These results seem to indicate that glyphosate-induced dopamine release from rat striatum does not depend on VSSC activation and action potential generation.

The effects of vesicular dopamine depletion on glyphosate effects

were also investigated. The effect of this pesticide on dopamine release was studied in animals pretreated with reserpine, a vesicular dopamine content depletor (Tolwani et al., 1999). Thus, intraperitoneal injection of reserpine induced a significant decrease in basal extracellular dopamine levels that resembled the observed in other *in vivo* studies (Heeringa and Abercrombie, 1995; Kannari et al., 2000). Pretreatment with reserpine also significantly reduced the striatal dopamine release induced by glyphosate (Fig. 8). The glyphosate-induced increase in the dopamine in animals pretreated with reserpine was 40% less than that seen with glyphosate alone, indicating that reserpine pre-treatment partially blocked the action of glyphosate on dopamine release. This result suggest that the effect of the pesticide could be mediated, at least partially, by an exocytotic release of vesicular contents. However, an exocytotic release of vesicular content may not be the only mechanism involved in the effect of glyphosate on dopamine levels.

In another experiment, we studied the involvement of the dopamine uptake system in the effects of glyphosate by measuring the effect of administration of glyphosate together with nomifensine on extracellular dopamine levels. When nomifensine or glyphosate were administered, the dopamine release increased 1215% and 1080%, with respect to the basal levels, respectively. When glyphosate was coinfused with nomifensine, the increase was 2215% with respect to the basal levels, an increase significantly higher than that observed with glyphosate or nomifensine alone. Two possible hypotheses could explain this additive effect of both substances:

1) Nomifensine and glyphosate could act through different mechanisms at the dopaminergic terminal: as an inhibitor of DAT, nomifensine decreases dopamine reuptake (Harsing et al., 2022), and glyphosate, acting on exocytotic release, would also increase extracellular levels of the neurotransmitter. However, blocking VSSCs with TTX did not inhibit the effect of glyphosate, and reserpine had only a partial effect on pesticide-induced dopamine release. This could indicate that, although glyphosate can induce exocytotic dopamine release, this would not be the only mechanism that would explain the increases in the extracellular levels of the neurotransmitter observed by us.

2) Another possibility is that both nomifensine and glyphosate act on DAT to increase extracellular dopamine levels: nomifensine as a DAT inhibitor and glyphosate would act as a releaser in a similar way to amphetamine (Khoshbouei et al., 2003; Vaughan and Foster, 2013). We hypothesized that glyphosate might have an amphetamine-like effect, simultaneously inhibiting reuptake and stimulating dopamine release by reversing DAT functioning. This can produce very large increases in extracellular dopamine levels, similar to those observed in our work. However, this is only a hypothesis since we did not compare the effects of glyphosate with those induced by amphetamine in the present study.

In an attempt to further clarify the possible role of DAT in the effects of glyphosate on dopamine release, we conducted an additional experimental group by administering GBR 12909, another DAT inhibitor, together with glyphosate. However, the results do not allow us to clarify the possible role of DAT on the glyphosate action since the effect of GBR 12909 (5 mg/kg i.p.) was very small when compared to the effect of glyphosate, which was locally administered into the striatum.

On the other hand, due to the architecture of the striatum, with an extensive functional interconnection between dopaminergic, glutamatergic, cholinergic, and GABAergic terminals, we must also consider the possible participation of these neurotransmitter systems in the effects of glyphosate on striatal dopaminergic neurotransmission. Glyphosate, directly or indirectly, could alter the activity of certain receptors located at the dopaminergic terminals and cause dopamine release. In line with this hypothesis, various studies have shown that exposure to glyphosate increases glutamate levels, by decreasing both its reuptake and metabolism (Cattani et al., 2014, 2017). Furthermore, it has been shown that glyphosate itself can bind to the glycine and glutamate cavities at the *N*-methyl-D-aspartate (NMDA) receptors and cause them to open (Cattani et al., 2014, 2017). Thus, both high levels of glutamate and glyphosate could cause overstimulation of NMDA receptors and the consequent Na^{+} and Ca^{2+} influx into the neuron. This assumption is consistent with previous studies that have suggested that glutamate can locally stimulate striatal dopamine release (Borland and Michael, 2004; Krebs et al., 1991).

Another possibility would be that the inhibition of AChE induced by glyphosate, observed in the present study, would increase the extracellular levels of acetylcholine and the consequent stimulation of its striatal postsynaptic receptors. Striatal dopaminergic terminals express a wide variety of nicotinic acetylcholine receptors (nAChRs), and their stimulation by acetylcholine or agonists increase dopamine release (Abudukeyoumu et al., 2019; Iarkov et al., 2021). We believe that this may be a possible mechanism used by glyphosate to induce the dopamine overflow observed in our study. Furthermore, it has been shown that some types of nAChRs are also present at striatal glutamatergic terminals (Assous, 2021). In this way, an increase in acetylcholine levels could also stimulate the nAChRs present in the glutamatergic terminals, increasing the release of glutamate and, indirectly, the dopamine release (Stone, 2021).

Regarding the behavior of dopamine metabolites, significant increases in DOPAC levels have been observed both after intraperitoneal and intrastriatal administration of glyphosate, while changes in HVA levels were more uncertain. Variations in the extracellular levels of these metabolites are indicative of long-term changes in dopamine synthesis and release (Westerink, 1995). Since the levels of these metabolites vary during dopamine synthesis, metabolism, conjugation, and rate of efflux, it is possible that glyphosate acts at one of these steps. In fact, since extracellular DOPAC comes mainly from the metabolism of newly synthesized dopamine by the action of monoamine oxidase (MAO) (Faro et al., 2021), the elevation of extracellular levels of this metabolite observed in our experiments may suggest that glyphosate could stimulate dopamine synthesis and thus increase intracellular stores of newly synthesized dopamine.

5. Conclusions

The findings described in the present study show that acute exposure to both pure glyphosate and GBH administered systemically or locally produced alterations in rat striatum. The pesticide dose-dependently increased both basal and depolarization-stimulated *in vivo* dopamine overflow.

The large increase in the neurotransmitter levels is likely responsible for glyphosate-induced neurotoxicity in the nigrostriatal pathway, altering motor behavior and generating oxidative stress. Furthermore, by inhibiting AChE activity, glyphosate can also alter other neurotransmission systems, such as the cholinergic one.

In the study of the possible mechanisms of action, we observed that the effects of glyphosate on the nigrostriatal dopaminergic terminals could occur through at least two mechanisms: 1) stimulating the exocytotic release of the vesicular content of dopamine, once reserpine partially but significantly inhibited the effect of the pesticide on dopamine release; 2) by acting on the DAT by either inhibiting it, stimulating its reverse function, or both. This is because when administered together with nomifensine, glyphosate potentiated its effect, increasing dopamine levels well above what was observed with nomifensine alone. However, future research needs to evaluate the potential interactions between glyphosate and the receptors or ionic channels, in order to identify specific mechanisms of action by which this pesticide can alter the nigrostriatal dopaminergic system.

CRediT authorship contribution statement

LFF and RD designed the study. CCF conducted the experiments and participated data interpretation. CCF and LFF drafted the manuscript. All authors read the manuscript and approved the final manuscript.

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.

Acknowledgements

C. Costas-Ferreira acknowledges University of Vigo (Spain) for a scholarship (CO53819572-20220201ALD74). The research was supported by grant from University of Vigo (Contrato-Programa con Grupos de Investigación 2022) and Xunta de Galicia (C18-GPC-2019).

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