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Role of voltage-sensitive Ca^{2+} channels in the *in vivo* dopamine release induced by the organophosphorus pesticide glufosinate ammonium in rat striatum

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ABSTRACT

The possible role of voltage-sensitive calcium channels (VSCC) activation in the glufosinate ammonium (GLA)-induced dopamine release was investigated using selective VSCC blockers and the dopamine levels were measured by HPLC from samples obtained by *in vivo* cerebral microdialysis. While pretreatment with 10 μM flunarizine (T-type VSCC antagonist) or nicardipine (L-type VSCC antagonist) had no statistically significant effect on dopamine release induced by 10 mM GLA, pretreatment with 100 μM of both antagonists, or 20 μM ω-conotoxin MVIIC (non-selective P/Q-type VSCC antagonist) significantly decreased the GLA-induced dopamine release over 72.2%, 73%, and 70.2%, respectively. Administration of the specific antagonist of neuronal N-type VSCCs, the ω-conotoxin GVIA (20 μM), produced an almost complete blockade of *in vivo* dopamine release induced by GLA. These results show that GLA-induced dopamine release could be produced by the activation of a wide range of striatal VSCC located at the synaptic terminals and axons of striatal dopaminergic neurons, especially N-type VSCC.

1. Introduction

Glufosinate ammonium (GLA) is the ammonium salt of the amino acid phosphinothricin (D,L-homoalanin-4-[methyl] phosphinate) and the active component of a broad-spectrum herbicide used to control weeds in agriculture, domestic areas and public domains (Meme et al., 2009). GLA targets glutamine synthetase (GS), an enzyme that plays an essential role in nitrogen metabolism, by catalyzing the condensation of glutamate to ammonia to produce glutamine (Takano and Dayan, 2020). In plants, irreversible inhibition of this enzyme leads to a rapid accumulation of ammonia and concomitant depletion of glutamine and other intracellular amino acids (Hoerlein, 1994; Lacuesta et al., 1989), thus causing plant death. In the mammals Central Nervous System (CNS), this enzyme is located in glial cells, where it plays a fundamental role in the metabolic regulation of glutamate levels and in ammonia detoxification.

GLA exposure has been linked to the development of various neurological symptoms, including loss of consciousness, memory impairment, seizures, or behavioral changes (Calas et al., 2008, 2016; Dong et al., 2020; Farahat et al., 2003; Park et al., 2006; Watanabe and Sano, 1998). Given the structural analogy of GLA with glutamate, it is considered that some of the neurological effects caused by this pesticide could be related to its possible action on the N-methyl-D-aspartate receptors (NMDAR) (Droge-Laser et al., 1994; Nakaki et al., 2000). Therefore, it is likely that some of the signs of neurotoxicity resulting from GLA exposure are due to its action on the glutamatergic system, as has been shown in some previous research (Lapouble et al., 2002; Matsumura et al., 2001).

However, GLA also affects dopaminergic neurotransmission in the rat brain. It has been shown in nigrostriatal dopaminergic terminals that GLA increases the dopamine overflow in freely moving rats in a concentration-dependent way, being this effect partially dependent on extracellular Ca^{2+}, since the removal of Ca^{2+} from the perfusion medium significantly decreased the effect of pesticide (Ferreira-Nunes et al., 2010). In addition, it has also been observed that GLA-induced

Abbreviations: ANOVA, analysis of variance; AUC, area under the curve; Ca^{2+}, calcium; DOPAC, 3,4-dihydroxyphenylacetic acid; GLA, glufosinate-ammonium; GS, glutamine synthetase; HPLC, high performance liquid chromatography; HVA, homovanillic acid; NMDAR, N-methyl-D-aspartate receptor; VSCC, voltage-sensitive calcium channels.

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dopamine release depends on the activation of striatal NMDARs and nitric oxide (NO) production, since the blockage of the NMDARs and inhibition of the nitric oxide synthase (NOS) activity significantly inhibited the effect of the pesticide (Faro et al., 2013).

The mechanism of synaptic transmission at chemical synapses begins with Ca^{+2} influx into neurons through voltage-sensitive calcium channels (VSCC). There are several types of VSCC with differentiated electrophysiological and pharmacological properties (Dolphin, 2021). Generally, VSCCs are grouped into low-voltage-gated channels (T-type) and high-voltage-gated channels (N-, L-, and P/Q-types) (Zamponi et al., 2015). Based on their different properties and functions, these channels show different locations. Specifically, N-, L-, and P/Q- type VSCCs have been identified in the substantia nigra *pars compacta* and striatum, and N- and P/Q-types have been associated with dopamine release (Bergquist et al., 1998; Turner et al., 1993).

In accordance with this previous information, we hypothesize that GLA induces an exocytotic dopamine release by increasing Ca^{+2} influx from the extracellular medium through the different types of VSCC present in the nigrostriatal dopaminergic terminal. So, in the present study, we aimed to investigate the possible involvement of VSCC on the GLA-induced *in vivo* dopamine release from rat striatum, using selective L-, T-, N-, and P/Q-type Ca^{2+} channels blockers. To achieve this purpose, we measured the *in vivo* dopamine release from rat striatum using a microdialysis technique coupled to high performance liquid chromatography coupled to electrochemical detection (HPLC-EC). The extracellular levels of 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), both metabolites of dopamine, have also been quantified. It has been widely assumed that the levels of these substances reflect the activity of dopaminergic systems in the brain. Therefore, an alteration in the levels of DOPAC and HVA caused by a pesticide such as glufosinate could reflect not only changes in the release and metabolism of dopamine, but also an alteration in the functioning of the enzymatic systems responsible for its synthesis and catabolization in the neuron (Meiser et al., 2013; Soares-da-Silva and Garrett, 1990).

2. Materials and methods

2.1. Animals, drugs treatments and experimental groups

For the microdialysis experiments, female adult Sprague-Dawley rats (250–300 g) were obtained from the Breeding Facility of the CINBIO (*Centro de Investigaciones Biomédicas*) of the University of Vigo, Spain. All animals were group-housed in standard laboratory cages and kept in a constant temperature and humidity-controlled colony room (22 ± 2 °C) with a 14:10 h light/dark cycle. Commercial food and tap water were available *ad libitum*. The experimental procedures were in accordance with the Guidelines of the European Union (2010/63/EU) and the Spanish Government (*Real Decreto* 53/2013) for the use of animals for experimental purposes. 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.

Glufosinate ammonium (purity 98%) was purchased by Pestanal® (Sigma-Aldrich, Germany). Flunarizine, nicardipine, dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were obtained from Sigma-Aldrich (St. Louis, USA). The ω-conotoxin MVIIC was purchased by Abcam plc (Cambridge, UK) and the ω-conotoxin GVIA was purchased by Tocris (Bristol, UK). All other chemicals were of analytical grade. Water used for the preparation of reagents, solvents and chromatographic solutions was obtained from a MilliQ system (Millipore).

GLA and VSCC blockers concentrations were selected based on available literature and previous work carried out in our laboratory (Bergquist et al., 1998; Burley and Dolphin, 2000; El Ayadi et al., 2001; Faro et al., 2013, 2018; Ferreira-Nunes et al., 2010; Furukawa et al., 1999; Kato et al., 1992; Lohr et al., 2005; Ye et al., 2011a, 2011b; Rocchitta et al., 2004; Thaler and Brehm, 2001). The pesticide and the antagonists were dissolved in perfusion medium and administered locally to the striatum through a microdialysis probe.

Fifty-nine rats were divided into the following eight groups: 10 mM GLA (n = 12), control group (Ringer) (n = 5); 10 μM flunarizine+ 10 mM GLA (n = 7), 100 μM flunarizine+ 10 mM GLA (n = 6); 10 μM nicardipine+ 10 mM GLA (n = 10); 100 μM nicardipine+ 10 mM GLA (n = 7); 20 μM ω-conotoxin MVIIC+ 10 mM GLA (n = 7); 20 μM ω-conotoxin GVIA+ 10 mM GLA (n = 5).

2.2. Microdialysis procedure

To carry out the microdialysis sampling, a guide cannula (CMA/12, CMA/Microdialysis, Sweden) was implanted in the left striatum. Animals were anesthetized with chloral hydrate (400 mg/kg i. p.) and placed in a stereotaxic apparatus (Nashigire SR-6, Japan). The skin above the skull was cut off, the top of the skull was exposed, and a small hole was drilled according to the following coordinates established by the stereotaxic atlas by Paxinos and Watson (1998): A /P + 1.0 mm; L + 3.0 mm; V + 6.0 mm; taking the Bregma as a reference point.

At the start of the experiment, the microdislysis probe (CMA/12, CMA/Microdialysis, Sweden) was introduced into the striatum and perfused with a Ringer solution (147 mM NaCl, 4 mM KCl and 2.4 mM $CaCl_2$, pH = 7.4) using a microdialysis pump (CMA /402, CMA/Microdialysis, Sweden) at a constant flow of 1.5 μL/min. Samples resulting from the microdialysis process were collected every 20 min (30 μL) using a fraction microcollector (CMA/142, CMA/Microdialysis, Sweden). The experiments had a total duration of 3 or 4 h, during which the animals were awake, conscious and in free movement.

Different protocols were used to carry out the experiments: 1) after the stabilization time and the collection of three basal samples (60 min), GLA was infused for 60 min. Subsequently, the medium was switched back to the Ringer solution and the sampling continued for an additional period of 60 min. 2) For the study of the effects of VSCC antagonists after collecting three basal samples, flunarizine or nicardipine (10 or 100 μM), was infused for 60 min and then mixed with GLA, which was infused throughout the third hour of the experiment. The next step was switching the medium back to the Ringer solution (60 min). 3) Due to the high molecular weight of the ω-conotoxin MVIIC and ω-conotoxin GVIA, they do not cross the membrane of the microdialysis probe (20 kD cut off). In this way, to investigate the effect of these toxins on the GLA-induced dopamine release, they were administered intrastriatally at the beginning of the experiment through a microdialysis probe without a membrane. The concentration used was 20 μM and the total volume administered was 2 μL. Three baseline samples were then collected over a period of 60 min, then GLA was infused (60 min) and sampling with unmodified Ringer solution was continued for another 60 min. The three protocols described above are illustrated in Fig. 1. After microdialysis experiments, rats were sacrificed by cervical dislocation and brains were removed for subsequent histological confirmation of probe placement. Only data from animals with correct probe placements in the dorsal striatum were used.

2.3. HPLC conditions

Dopamine, DOPAC, and HVA levels were quantified by High-Performance Liquid Chromatography (HPLC) with electrochemical detection, according to Durán et al. (1998). After collecting a fraction, samples were injected (20 μL) immediately into a HPLC system using a Rheodyne 7125 injection valve (Rheodyne, USA). 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 1.2 mL/min using a Jasco PU 1580 pump (Jasco, Japan). The dopamine, DOPAC, and HVA detection was achieved using an ESA Coulochem III electrochemical detector (ESA, USA) at a potential of oxidation of

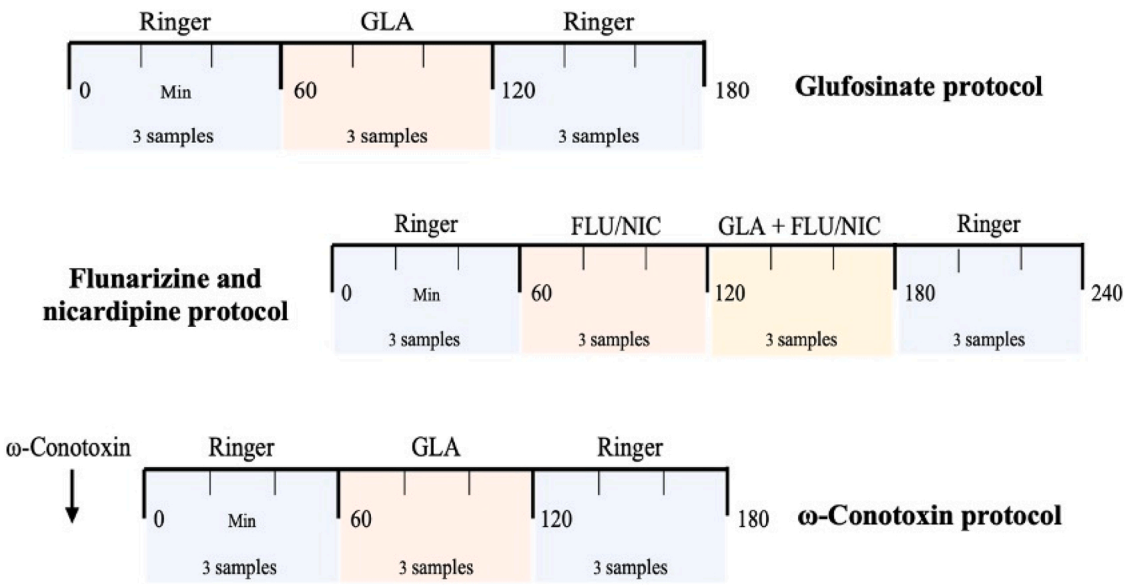

Fig. 1. Schematic representation of experimental design.

+ 400 mV. All the data were analyzed by the chromatographic software Cromanec XP 1.0.4 (Micronec, Spain).

2.4. Data analysis

All values of the effects of GLA and VSCC antagonists on extracellular levels of dopamine, DOPAC, and HVA were expressed as mean ± SEM of 4–12 animals in each group. Average of basal levels of dopamine, DOPAC, and HVA (defined as 100%) was determined from the two dialysate samples before addition of GLA or any drug. Results were calculated as percentages of this average basal release. Data of dopamine, DOPAC, and HVA 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: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, with respect to the basal level or control group; $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$, with respect to 10 mM GLA group; and $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$, and $^{\#\#\#}$ $P < 0.001$, with respect to ω-conotoxin MVIIC or ω-conotoxin GVIA effects.

The comparative effects of the VSCC blockers on extracellular dopamine levels are presented as values of area under the curve (AUC), represented as the dopamine concentration measured during the administration of glufosinate+antagonist and compared with the baseline values and with the effect of the pesticide alone. AUC was calculated by using a standard trapezoid method and were analyzed by using a two-way ANOVA followed by the *post hoc* Bonferroni test to determine statistical differences between groups.

3. Results

3.1. Effects of GLA on the dopamine, DOPAC, and HVA extracellular levels

Basal levels of dopamine, DOPAC, and HVA in dialyzed samples were stable in control animals (non-treated rats). The mean of substance concentrations in the two samples collected before GLA administration was considered as the basal level: dopamine: 0.84 ± 0.24 ng/20 min; DOPAC: 21.3 ± 4.5 ng/20 min; HVA: 13.7 ± 2.8 ng/20 min

The intrastriatal infusion of 10 mM GLA for 60 min, through the microdialysis probe, significantly increased the extracellular dopamine levels to 791 ± 90.1% ($P < 0.001$), compared with basal values. The maximum increases were observed 40 min after the beginning of GLA perfusion, and basal values were recovered 40 min after the end of GLA administration. The administration of GLA also induced significant increases in the DOPAC and HVA levels to a maximum of 150.5 ± 27.5% ($P < 0.001$) and 140.0 ± 18.7% ($P < 0.001$), respectively. The maximum increases were observed 40 min after the beginning of GLA perfusion, and basal values were recovered 60 min after the end of GLA administration. These data for the effects of 10 mM GLA are plotted in Figs. 2–5 in order to compare them with the data obtained under other experimental conditions (treatments with different VSCC blockers together with GLA).

3.2. Effect of flunarizine on the GLA-induced striatal dopamine, DOPAC, and HVA release

To investigate if the T-type VSCC could be implicated in the dopamine release induced by GLA, we infused 10 μM or 100 μM flunarizine, a blocker of these VSCC type, before and then together with 10 mM GLA through the microdialysis probe (Fig. 2). It has been shown that at these concentrations, flunarizine significantly reduced calcium currents in both neurons and glial cells (Lohr et al., 2005; Ye et al., 2011a, 2011b). Intrastriatal administration of 10 μM flunarizine (for 60 min) had no significant effect on dopamine levels, while perfusion of 100 μM of this blocker significantly reduced extracellular dopamine levels to 53.6 ± 11.3% ($P < 0.001$), compared to basal values (Faro et al., 2018; Kato et al., 1992, Tamura et al., 1995). As shown in Fig. 2A, when 10 mM GLA was administered to animals pretreated with 10 μM flunarizine, the dopamine levels increased to 949.6 ± 154.4% ($P < 0.001$), being this increase not statistically different from that observed with perfusion of GLA only. On the other hand, the administration of GLA to animals pretreated with 100 μM flunarizine increased the dopamine levels up to 221 ± 45.9% ($P < 0.001$), compared to basal levels. This increase was significantly lower (72.1%) than that observed with perfusion of GLA alone.

Administration of 10 or 100 μM flunarizine significantly increased DOPAC levels to 123.7 ± 7.2% ($P < 0.05$) and 146.8 ± 11.4% ($P < 0.001$), compared to basal values, respectively. These maximum increases were observed 40 and 60 min after the beginning of 10 and 100 μM flunarizine administration, respectively. However, no effect on extracellular HVA levels was observed for either antagonist concentration. Infusion of GLA to animals pretreated with 10 or 100 μM flunarizine did not produce significant changes in either DOPAC or HVA levels

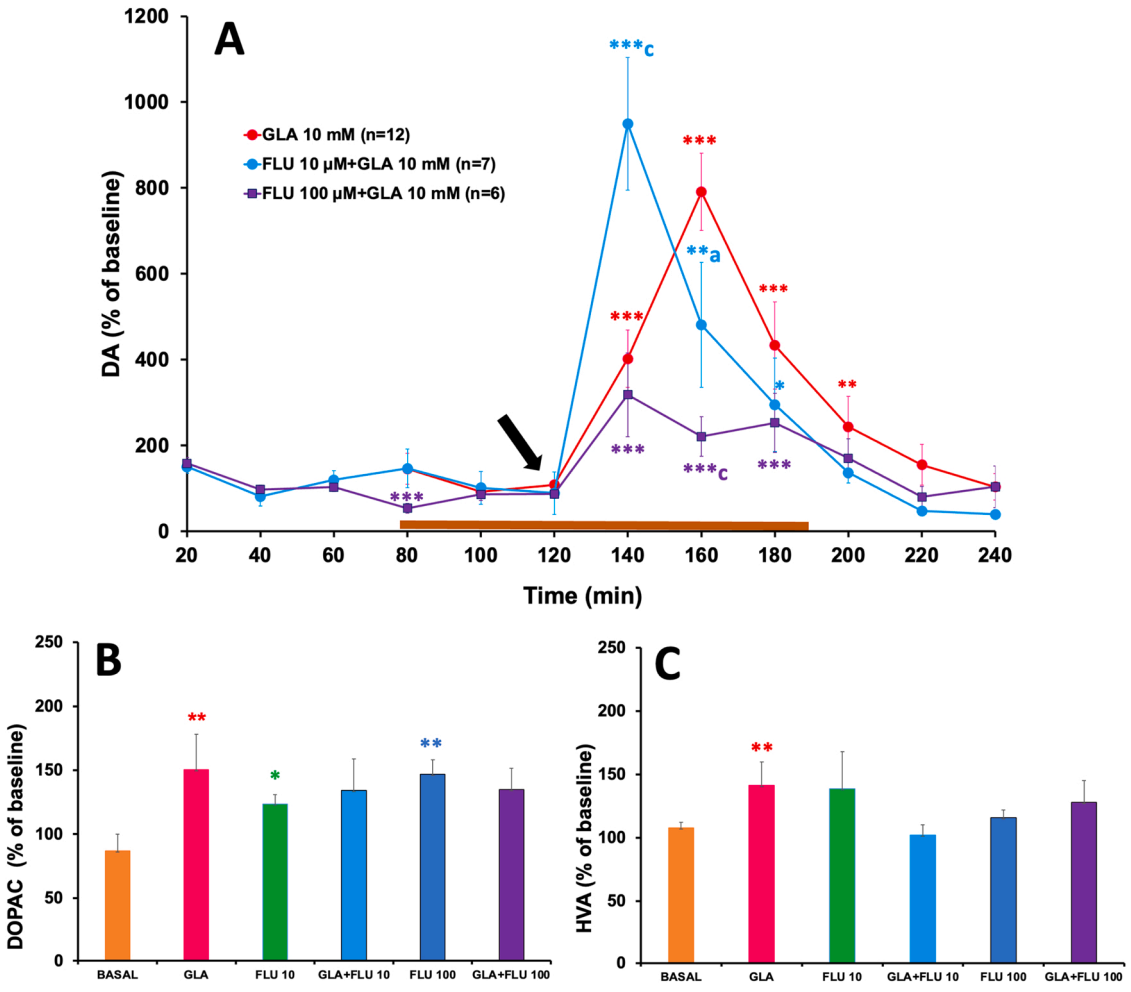

Fig. 2. Effects of 10 mM glufosinate ammonium (GLA) infusion in flunarizine (FLU) pretreated rats on the dopamine (A), DOPAC (B), and HVA (C) extracellular levels in rat striatum. The FLU infusion is show by the orange bar and GLA infusion started at the time indicated by the arrow over 60 min. Basal levels were considered as the mean of dopamine, DOPAC, and HVA concentrations in the two samples collected before drug administration. Statistical analysis of the results was performed by means of ANOVA and Student–Newman–Keuls multiple range test, considering the following significant differences: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, respect to basal levels; [a] $P < 0.05$, and [b] $P < 0.01$, respect to 10 mM GLA group.

(Fig. 2B and C).

3.3. Effect of nicardipine on the GLA-induced striatal dopamine, DOPAC, and HVA release

To investigate if the L-type VSCC could be implicated in the dopamine release induced by GLA, we infused 10 µM or 100 µM nicardipine (Burley and Dolphin, 2000; Furukawa et al., 1999), a blocker of these VSCC type, before and then together with 10 mM GLA through the microdialysis probe (Fig. 3). Intrastriatal administration of 10 µM nicardipine (for 60 min) had no significant effect on dopamine levels, while perfusion of 100 µM of this blocker significantly reduced extracellular dopamine levels to 66.1 ± 18.5% ($P < 0.05$), compared to basal values (Faro et al., 2018; Inazu et al., 2001; Kato et al., 1992). As shown in Fig. 3 A, when 10 mM GLA was administered to animals pretreated with 10 µM flunarizine, the dopamine levels increased to 926.9 ± 192.3% ($P < 0.001$), being this increase not statistically different from that observed with perfusion of GLA only. On the other hand, the administration of GLA to animals pretreated with 100 µM flunarizine increased the dopamine levels up to 213.2 ± 86.2% ($P < 0.05$), compared to basal levels. This result shows that the highest concentration of this antagonist significantly reduced the effect of the pesticide by 73% ($P < 0.05$).

Administration of 10 µM nicardipine induced a slight but significant increase in DOPAC and HVA levels to 120.7 ± 11.5% ($P < 0.05$) and 136.2 ± 22% ($P < 0.05$), relative to baseline values, respectively. The maximum increases in DOPAC and HVA levels were observed 40 and 60 min after the beginning of 10 µM nicardipine administration, respectively. However, no significant changes in the levels of the metabolites were observed at the 100 µM concentration of the antagonist. Similarly, when 10 mM GLA was administered in combination with 10 or 100 µM nicardipine, neither DOPAC nor HVA levels underwent significant changes (Fig. 3B and C).

3.4. Effect of ω-conotoxin MVIIC on the GLA-induced striatal dopamine, DOPAC, and HVA release

In the next set of experiments, we administered GLA to animals pretreated with ω-conotoxin MVIIC, a non-selective antagonist of P/Q-like VSCC. In the present study we administered 20 µM ω-conotoxin MVIIC, a concentration that we consider sufficient to produce partial or total blockade of P/Q-type VSCC. In the concentration range of 0.01–10,000 nM, both ω-conotoxin MVIIC and ω-conotoxin GVIA have been shown to block calcium current through P/Q and N-type channels (Okada et al., 1998; Thaler and Brehm, 2001). Fig. 4 A shows that intrastriatal perfusion of ω-conotoxin MVIIC (20 µM) significantly decreased extracellular dopamine levels to 51.3 ± 8.5% ($P < 0.01$), relative to the control group (Bergquist et al., 1998; El Ayadi et al., 2001;

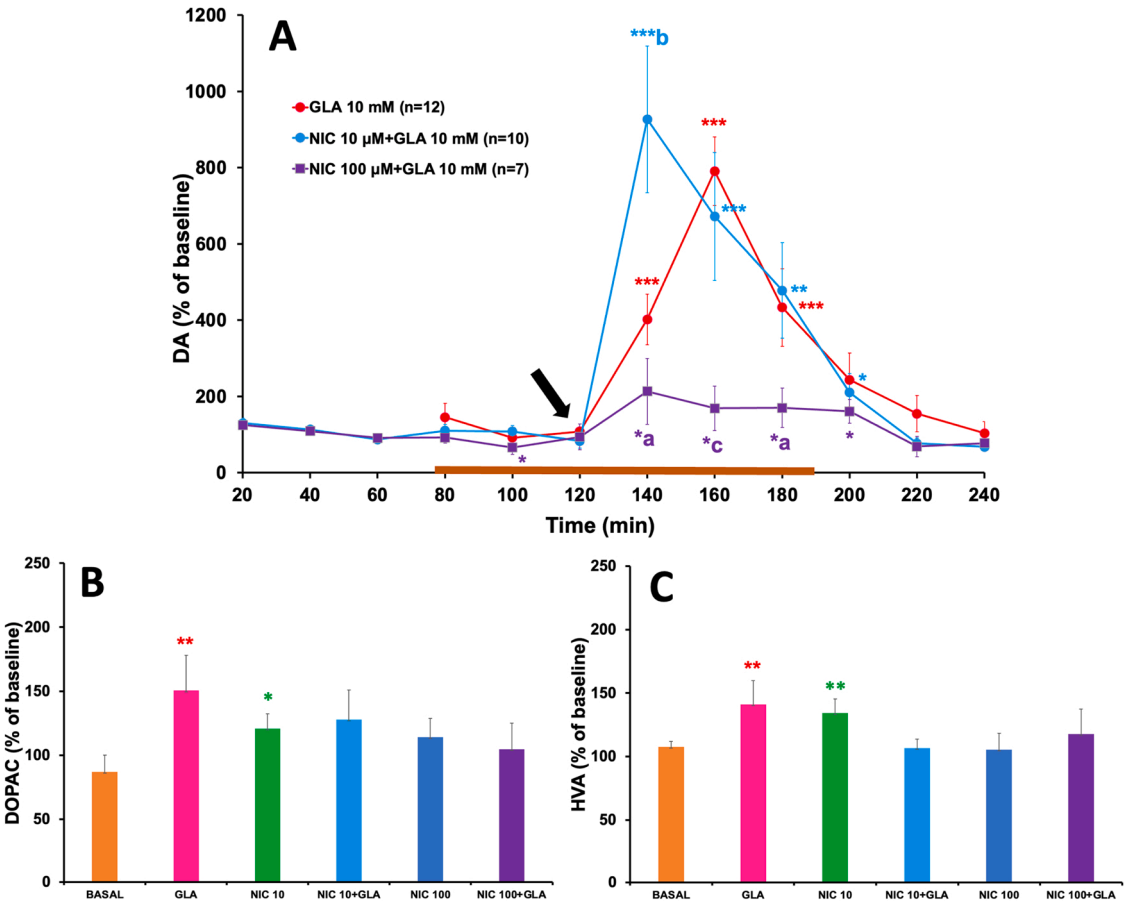

Fig. 3. Effects of 10 mM glufosinate ammonium (GLA) infusion in nicardipine (NIC) pretreated rats on the dopamine (A), DOPAC (B), and HVA (C) extracellular levels in rat striatum. The NIC infusion is show by the orange bar and GLA infusion started at the time indicated by the arrow over 60 min. Basal levels were considered as the mean of dopamine, DOPAC, and HVA concentrations in the two samples collected before drug administration. Statistical analysis of the results was performed by means of ANOVA and Student–Newman–Keuls multiple range test, considering the following significant differences: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, respect to basal levels; [a] $P < 0.05$, and [b] $P < 0.01$, respect to 10 mM GLA group.

Faro et al., 2018; Phillips and Stamford, 2000; Rocchitta et al., 2004). Infusion of 10 mM GLA in rats pretreated with 20 µM ω-conotoxin MVIIC increased extracellular dopamine levels to 235.6 ± 79% ($P < 0.01$). Thus, administration of ω-conotoxin MVIIC significantly reduced the effect of 10 mM GLA by 70.2% ($P < 0.001$).

Regarding dopamine metabolites, 40 min after infusion of ω-conotoxin MVIIC (20 µM) a significant decrease in DOPAC and HVA levels was observed to 47.4 ± 13.2% ($P < 0.001$) and 36.9 ± 8.7% ($P < 0.001$), compared to the control group, respectively. 40 min after the beginning of 10 mM GLA administration in toxin-pretreated rats, DOPAC and HVA levels increased significantly to 156.1 ± 26.7% ($P < 0.001$) and 153.4 ± 31.7% ($P < 0.001$), respectively, these increases being significantly greater than those observed for the control group and for the effect of the toxin alone (Fig. 4B and C).

3.5. *Effect of ω-conotoxin GVIA on the GLA-induced striatal dopamine, DOPAC, and HVA release*

In the last set of experiments, the role of N-type VSCCs in GLA-evoked *in vivo* dopamine release was investigated. For this, the effect of GLA was studied in the presence of 20 µM ω-conotoxin GVIA (Okada et al., 1998; Thaler and Brehm, 2001), a specific blocker of neuronal N-type VSCC.

Fig. 5 A shows that intrastriatal perfusion of ω-conotoxin GVIA (20 µM) significantly reduced extracellular dopamine levels to 45.2 ± 15.4% ($p < 0.01$), compared to the control group (Bergquist et al., 1998; El Ayadi et al., 2001; Faro et al., 2018; Phillips and Stamford, 2000; Rocchitta et al., 2004). Administration of 10 mM GLA to animals pretreated with 20 µM ω-conotoxin GVIA decreased dopamine levels to 31.8 ± 6.1% ($P < 0.05$), compared to baseline levels. This result shows that treatment with ω-conotoxin GVIA completely inhibited the effect of GLA on the dopamine release from rat striatum.

Fig. 5B-C shows that treatment with the ω-conotoxin GVIA significantly reduced DOPAC and HVA levels to 55.4 ± 14.4% ($P < 0.001$) and 35.5 ± 6.0% ($P < 0.001$), relative to the control group, respectively. The maximum decreases in HVA and DOPAC levels were observed 40 and 60 min after toxin infusion, respectively. The administration of GLA to the animals pretreated with the toxin did not produce significant variations in the levels of both metabolites when compared to the control group. However, a decrease in DOPAC levels was observed when compared to the GLA group (Fig. 5B-C).

Fig. 6 shows a comparative bar graph of the effects of VSCC blockers on dopamine release induced by GLA. All the antagonists used significantly reduced the effects of GLA on striatal dopamine overflow, although they did not completely inhibit this release. These data show that GLA-induced dopamine release from rat striatum appears to be largely dependent on N-type VSCC activation since pretreatment of animals with the ω-conotoxin GVIA completely blocked the effect of the pesticide.

4. Discussion

VSCC have been typically divided between high-voltage activated (P, Q, R, L- and N-types) and low-voltage activated channels (T-type)

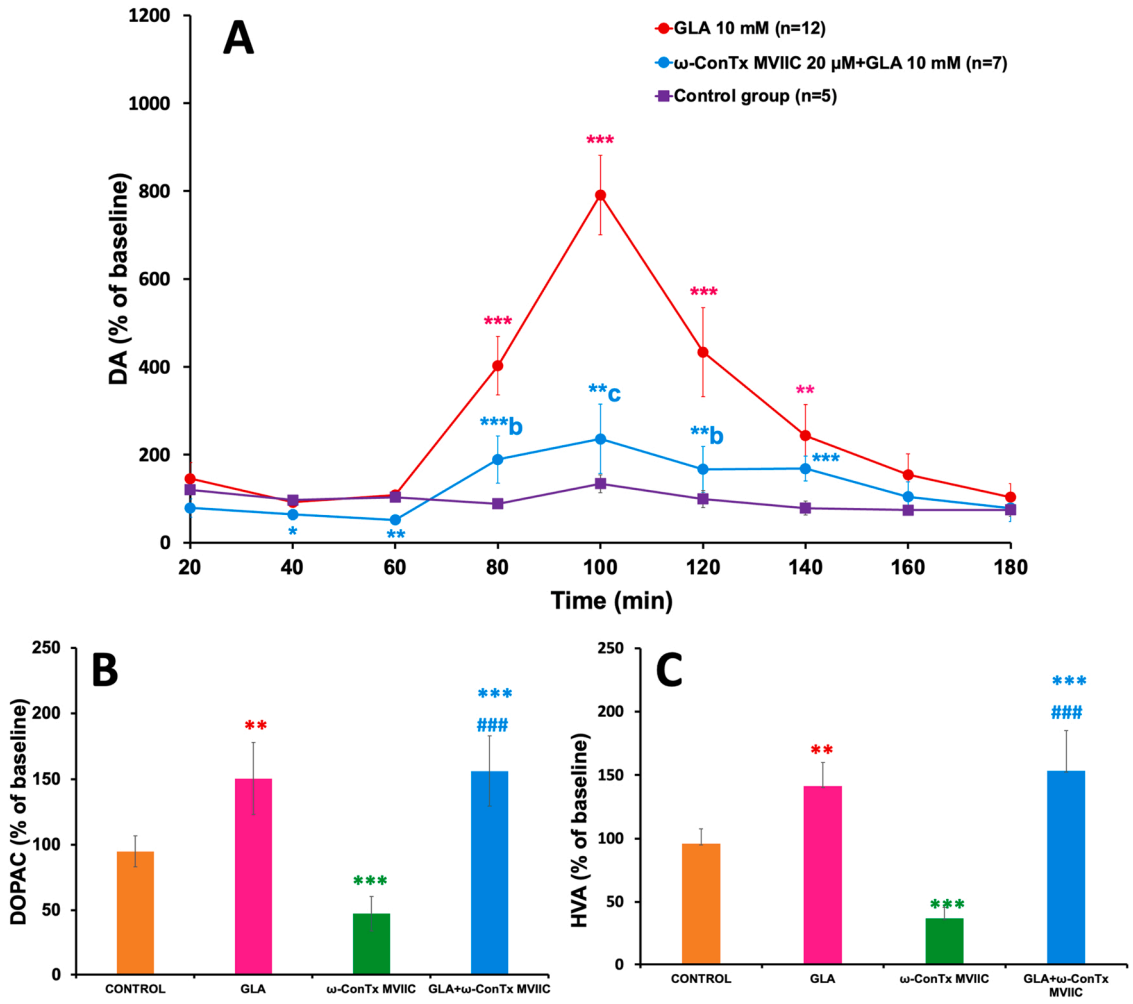

Fig. 4. Effects of 10 mM GLA infusion in ω-conotoxin MVIIC (ω-ConTx MVIIC) pretreated rats on the dopamine (A), DOPAC (B), and HVA (C) extracellular levels in rat striatum. GLA infusion started at the time indicated by arrow. In the pretreated group, ω-ConTx MVIIC was administered since the beginning of experiment. Statistical analysis of the results was performed by means of ANOVA and Student–Newman–Keuls multiple range test, considering the following significant differences: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, respect to baseline or control group; ^{b} $P < 0.01$, and ^{c} $P < 0.001$, respect to 10 mM GLA group; and $^{\#\#\#}$ $P < 0.001$, respect to ω-ConTx MVIIC control group.

(Catterall, 2000, Perez-Reyes, 2003). Several studies show that the main VSCC that regulate the dopamine release from striatal terminals are the N- and P/Q-type channels (Bergquist et al. 1998; Dobrev and Andreas, 1997; Herdon and Nahorski, 1989; Phillips and Stamford, 2000; Turner et al. 1993) but axons of dopaminergic neurons use a wider range of VSCC to support dopamine release. In addition, there are also other channels that participate in the regulation of Ca^{2+} levels and neuronal activity, such as the channels associated with metabolism. In the present study, we examined whether L-, T-, P/Q, and N-type VSCC regulate the *in vivo* dopamine release induced by GLA in rat striatum. For this, we use classic antagonists for each type of calcium channel, which have been extensively used as research tools to help define some physiological roles of these channels in the CNS.

Although there is no evidence of the presence of T-type VSCC on the dopaminergic terminals (Duda et al., 2016), some studies show that the dopamine release is regulated by these channels in the striatum (Brimblecombe et al., 2015; Chen et al., 2006). In line with this, we found an important role for striatal T-channels in GLA-induced dopamine release, as blockade of these channels by flunarizine produced a significant decrease in dopamine levels induced by GLA. On the other hand, it should also be considered that although flunarizine acts mainly as an antagonist of T-type VSCCs, its blocking capacity seems to extend to other channels closely related to synaptic transmission, including L-, N-, and P/Q-type VSCCs, and voltage-sensitive sodium channels (Geer et al., 1993; Pauwels et al., 1991; Ye et al., 2011a, 2011b). Because of this unspecific blockade, there would be a significant decrease in dopamine release, as observed after pretreatment with the highest dose of this antagonist.

Nicardipine is an antagonist of L-type VSCCs, a set of channels that plays a critical role in maintaining neuronal basal activity in the adult substantia nigra *pars compacta* (Dragicevic et al., 2015; Takada et al., 2001). Although there is no solid evidence of their presence in the dopaminergic terminals, some current works indicate that the activity of the L-type VSCC located at the axons can actively contribute to the dopamine release from striatum (Brimblecombe et al., 2020; Okita et al., 2000), which could explain some of the effects observed after treatment with nicardipine observed by us. In addition, some studies have found that when nicardipine is administered in concentrations greater than 10 μM it also appears to exert an antagonistic effect on N-type VSCCs (Burley and Dolphin, 2000; Furukawa et al., 1999). Therefore, since in the present study only the highest concentration of nicardipine (100 μM) significantly reduced GLA-induced dopamine release, this could indicate a possible non-specific effect of nicardipine on N-type VSCCs in our experimental conditions.

Our findings show that pretreatment with the ω-conotoxin MVIIC significantly decreased the release, while the ω-conotoxin GVIA

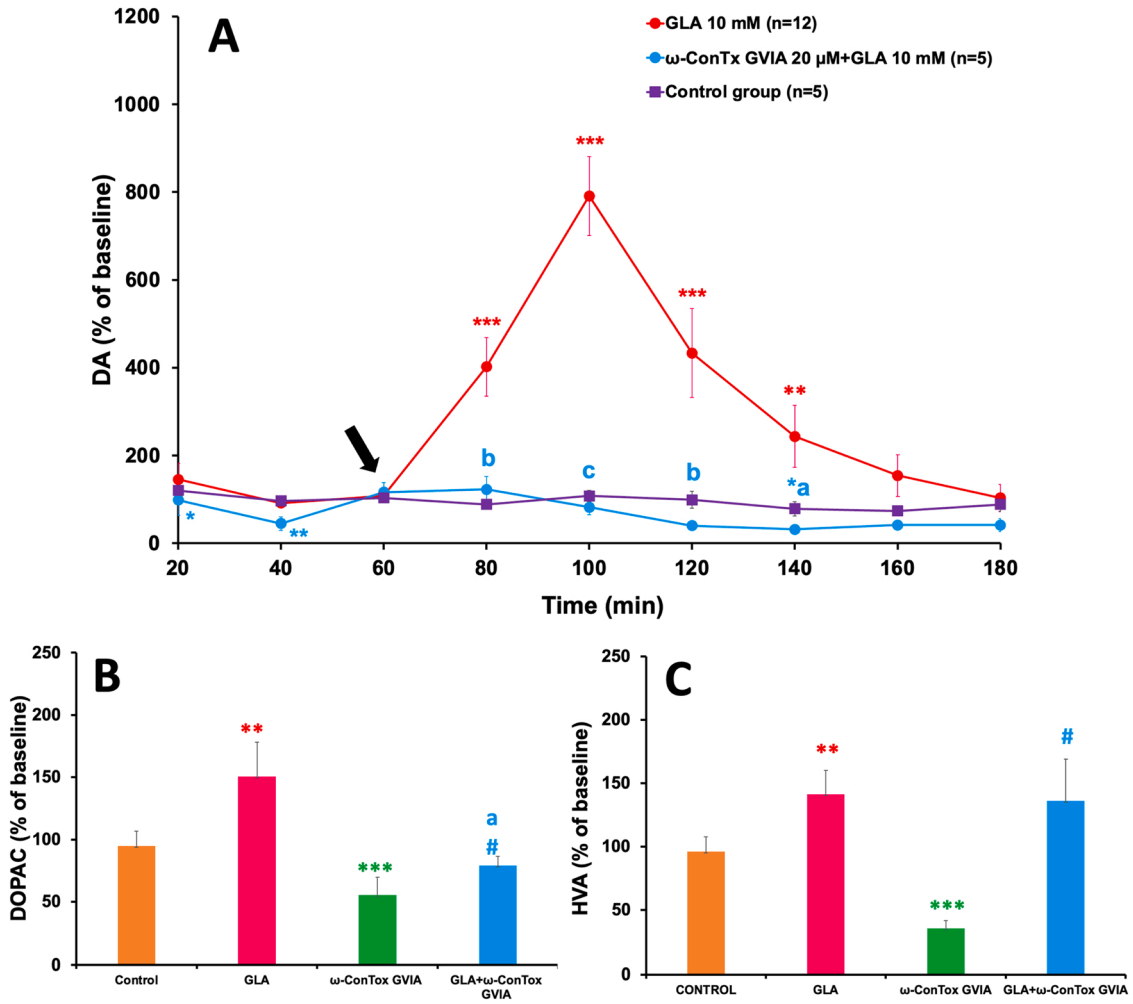

Fig. 5. Effects of 10 mM GLA infusion in ω-conotoxin GVIA (ω-ConTx GVIA) pretreated rats on the dopamine (A), DOPAC (B), and HVA (C) extracellular levels in rat striatum. GLA infusion started at the time indicated by arrow. In the pretreated group, ω-ConTx GVIA was administered since the beginning of experiment. Statistical analysis of the results was performed by means of ANOVA and Student–Newman–Keuls multiple range test, considering the following significant differences: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, respect to baseline or control group; [a] $P < 0.05$, [b] $P < 0.01$, and [c] $P < 0.001$, respect to 10 mM GLA group; and [#] $P < 0.05$, respect to ω-ConTx GVIA control group.

completely blocked the effect of GLA on dopamine release. The ω-conotoxin GVIA is a known irreversible antagonist of N-type VSCCs, while ω-conotoxin MVIIC appears to selectively block P/Q-type VSCCs (Schroeder and Lewis, 2006). N- and P/Q-type VSCCs are the main Ca^{2+} channels present in nerve terminals, where they play a crucial role in the vesicle exocytosis and rapid release of neurotransmitter (He et al., 2018; Rusakov, 2006). Since the entry of Ca^{+2} through these channels triggers the machinery responsible for vesicular fusion, its blockade will seriously affect dopamine release, which is consistent with the findings of this study.

Taken together, our results could indicate that *in vivo* dopamine release induced by GLA occurs mainly as a consequence of an exocytotic process dependent on Ca^{2+} entry through VSCCs. The data obtained in the present study agree with those previously described that dopamine release depends on the presence of Ca^{2+} ions in the extracellular medium (Ferreira-Nunes et al., 2010). Here, we showed that the GLA-induced dopamine release appears to be strongly dependent on the activation of the striatal L-, T-, and P/Q-type VSCC, and completely dependent on N-type channel activation.

It is important to note that the contribution of the different VSCCs to dopamine release is not fixed but appears to vary dynamically depending on factors such as neuronal activity or the probability of dopamine release (Brimblecombe et al., 2015). In this sense, it has been shown that L-type VSCC can increase their contribution to the exocytosis when there is a higher frequency of stimulation (Trueta and De-Miguel, 2012). Therefore, under overstimulation conditions, such as that observed with GLA exposure, it is possible that L- and T-types may play a more active role in the release of neurotransmitter than has traditionally been attributed to them. Likewise, our results also show that the administration of each antagonist reduced the effect of the pesticide by approximately 70–80% but did not completely inhibit the dopamine release. This could be because, despite the blockade of each VSCC type individually, the Ca^{2+} entry through the other VSCC subtypes acting in cooperation could allow the exocytosis of some proximal vesicles.

As previously mentioned, GLA can inhibit the degradation of glutamate by GS in glial cells, which leads to the accumulation of this neurotransmitter in the extracellular medium and the consequent stimulation of its receptors. At the same time, the pesticide itself could act as an NMDA receptor agonist, a hypothesis that is supported by several studies in which the administration of NMDA receptor antagonists inhibited the main effects produced by GLA in the CNS (Faro et al., 2013; Kim et al., 2019; Matsumura et al., 2001; Nakaki et al., 2000). The overstimulation of glutamate receptors at the striatal dopaminergic terminals could trigger Ca^{+2} entry through the different VSCCs (types N, P/Q, L and T) and the consequent activation of the exocytotic machinery for dopamine release. This observation is consistent with previous

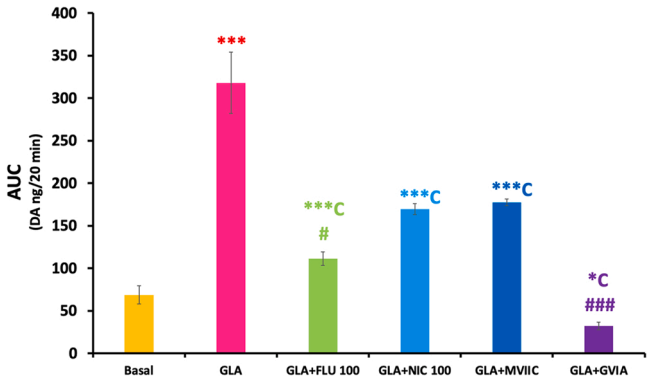

Fig. 6. Comparative effects of flunarizine (FLU, 100 μM), nicardipine (NIC, 100 μM), ω-conotoxin MVIIC (MVIIC, 20 μM), and ω-conotoxin GVIA (GVIA, 20 μM) on striatal dopamine release induced by glufosinate (GLA, 10 mM). Dopamine concentrations are expressed as Area Under Curve (AUC), calculated before treatments (basal values), during the GLA administration and during coadministration of GLA+blockers. The graph shows that ω-conotoxin GVIA completely blocked, while the other antagonists induced a partial inhibition of GLA-induced dopamine release. Significant differences: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, respect to basal levels; $^{c}P < 0.001$, respect to GLA group; $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$, respect to FLU and NIC group.

studies suggesting that the action of glutamate on striatal dopaminergic terminals could locally stimulate dopamine release, without affecting nerve impulse activity (Borland and Michael, 2004; Clow and Jhamandas, 1989; Krebs et al. al., 1991).

In general, our data seems to indicate that exposure to GLA can induce a significative influx of Ca^{2+} into the neuron through multiple pathways, such as VSCC. An excess Ca^{2+} inside neurons can generate not only an increase in the release of dopamine and other neurotransmitters, but also activate other biochemical pathways inside neurons that can lead to oxidative stress contributing to increase the toxicity of the pesticide in the rat CNS.

Regarding the behavior of dopamine metabolites, we observed that the administration of 10 mM of GLA significantly increased the levels of DOPAC and HVA, which corroborates previous data from our laboratory (Ferreira-Nunes et al., 2010). According to these authors, GLA could increase the enzymatic dopamine degradation and, therefore, the levels of DOPAC and HVA in the extracellular medium, as observed in the present study. On the other hand, it was also observed that both flunarizine and nicardipine increased DOPAC and HVA levels, while both ω-conotoxins induced decreases in these levels. However, in general, pretreatment with these antagonists did not significantly change the effect of the GLA on metabolite levels. The most important thing to note is that, due to the oxidative nature of dopamine and the free radicals that are released during its metabolism, abnormal increases in the dopamine, DOPAC, or HVA levels, such as those observed after exposure to GLA, could favor the appearance of oxidative stress and the consequent death of dopaminergic neurons (Gluck and Zeevalk, 2004; Meiser et al., 2013).

5. Conclusions

In the present study we evaluate whether the organophosphorus pesticide GLA increases *in vivo* dopamine release by activating different types of VSCC present in the nigrostriatal dopaminergic terminal using selective L-, T-, N-, and P/Q-type Ca^{2+} channels blockers. Our findings show that GLA-induced dopamine release was significantly reduced by the L-, T-, and P/Q-channels block and completely inhibited by the N-channels block in the striatum. These results support the idea that GLA can activate a wide range of striatal VSCC to induce dopamine release from rat striatum, which in turn could lead to an excessive Ca^{2+} influx contributing to an increase in the toxic effects of the pesticide.

Author contributions

C. Costas-Ferreira has carried out the experiments, analyzed the data, wrote, and reviewed the manuscript; T. Romero has carried out part of the experiments and analyzed the data. Rafael Durán designed the study and reviewed the manuscript; L. R. F. Faro designed the study, analyzed the data, wrote, and reviewed the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data Availability

Data will be made available on request.

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