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Mechanisms of action of paraoxon, an organophosphorus pesticide, on *in vivo* dopamine release in conscious and freely moving rats

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ABSTRACT

Paraoxon is the active metabolite of parathion, an organophosphorus pesticide which can cause neurotoxic effects in animals and humans. In the present work, we investigated the effects of 5 mM paraoxon on striatal dopamine, DOPAC and HVA levels in conscious and freely moving rats, after treatment with TTX, reserpine, nomifensine, KCl, Ca^{++}-free/EDTA medium, AP-5 or L-NAME. The intrastriatal administration of paraoxon for 60 min, through the microdialysis probe, significantly produced an increase of the dopamine to 1066 ± 120%, relative to basal levels. Administration of paraoxon to 20 µM TTX, 10 mg/kg reserpine or Ca^{++}-free/EDTA medium-pretreated animals decreased the dopamine levels to 73%, 81%, and 70%, respectively, when compared with the effect of 5 mM paraoxon. Infusion of 50 µM nomifensine induced a maximal increase in extracellular dopamine levels to 1435 ± 387%, and when nomifensine was coadministered with paraoxon, striatal dopamine levels increased to 2429 ± 417%, an increase that was ~230% higher that observed with the administration of the pesticide alone. Coinfusion of KCl and paraoxon produced an increase in extracellular dopamine to 1957 ± 445%, that was significantly higher than that observed with POX or KCl (1104 ± 220%) administered individually. Pretreatment with 650 µM AP-5 or 100 L-NAME reduced the effect of paraoxon on extracellular dopamine levels by 49.1% and 53.7%, respectively. Our results suggest that paraoxon induces dopamine release by a vesicular-, Ca^{++}-, and deporalization-dependent mechanism, being independent of dopamine transporter. In addition, the paraoxon-induced dopamine release is mediated by glutamatergic and nitrergic neurotransmitter systems.

1. Introduction

Organophosphorus compounds are esters of phosphoric acid and their derivatives are used as pesticides, as well as chemical weapons and paradoxically as therapeutic agents in some disorders Alzheimer's disease and glaucoma (Gupta, 2004).

Organophosphate (OP) pesticides are used extensively worldwide, and the poisoning by these agents is a serious public health problem, particularly in developing nations. The toxicokinetics and toxicodynamics of organophosphate poisoning vary not only with the route and extent of exposure, but also with the chemical structure of the agent. The main mechanism of toxicity of OPs is the inhibition of acetylcholinesterase (AChE), resulting in an accumulation of the neurotransmitter acetylcholine what causes the continued stimulation of acetylcholine receptors (Kwong, Tai, 2002).

OPs pesticides are highly toxic and until the 21st century they were among the most widely used insecticides. Thirty-six of them are presently registered for use in the United States, and all can potentially cause acute and subacute toxicity. So, OPs are used in agriculture, homes, gardens and veterinary practice. However, in the last decade its use was reduced in favor of other groups of pesticides especially neonicotinoids, pyrethroids and non-neuroactive compounds (Roberts and Reigart, 2013).

Paraoxon is the active metabolite of parathion, a highly neurotoxic OP pesticide to animals and humans. Its main action in the organism is the irreversible inhibition of the AChE in the Central Nervous System (CNS) and in the neuromuscular junction (Edwards and Tchounwou, 2005; Costa, 2006).

Acetylcholine is the major neurotransmitter in the peripheral nervous system and CNS and it is involved in numerous processes, especially in cognitive functions such as memory and learning. AChE inhibition causes accumulation of acetylcholine, overestimulation of cholinergic receptors and consequent clinical signs of cholinergic toxicity (Jamal, 1997; Costa, 2006; Ray et al., 2009).

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Several experimental studies have suggested that the dopaminergic system may be a potential target for OPs in humans and animals (Karen et al., 2001; Sun et al., 2006; Moreno et al., 2008; Eells and Brown, 2009; Binukumar et al., 2010; Torres-Altoro et al., 2011; Lee et al., 2012). However, it has always linked the cholinergic system as responsible for the acute effects of pesticides, while the dopaminergic system would be rather associated with a chronic and prolonged toxicity. This toxicity could be responsible for numerous cognitive disorders and also for causing dopaminergic neurodegeneration, although the neurochemical mechanisms for which these pesticides acts on the dopaminergic system are still unknown.

It has been found that both ventral and dorsal striatum expressing high levels of nicotinic acetylcholine receptors (nAChRs). These receptors provide cholinergic modulation of dopaminergic neurotransmission (Lester et al., 2010). In dopaminergic neurons, nAChRs are primarily composed by α4 subunitis (α4β2) heteromeric receptors, and α7 subunits homomeric receptor, whose activation facilitates dopamine release in the striatum (Xiao et al., 2009). Thus, a cholinergic stimulation induced by OPs pesticides could stimulate the dopamine release through AChRs on dopaminergic terminals (Drennan et al., 2010).

It is well known that dopaminergic neurotransmision in the nigrostriatal pathway is regulated by some neurotransmitters including glutamate, acetylcholine, GABA and nitric oxide (NO). In the striatum, glutamate is released from glutamatergic neurons whose somas are in the cerebral cortex, constituting the cortico-striatal pathway. Several studies demonstrated the presence of glutamatergic receptors localized on dopaminergic terminals, and the activation of those receptors by glutamate or glutamate analogues can change the dopamine levels (David et al., 2005, for review). There is also strong evidence for the presence of nAChRs on glutamatergic terminals, capable of inducing the release of glutamate (Desce et al., 1992; Pittaluga and Raiteri, 1992; Pittaluga et al., 2001; Risso et al., 2014).

Given that paraoxon increases acetylcholine levels, by inhibiting AChE, the glutamate levels can also be altered. It has been suggested that cholinergic overstimulation induces an increase of glutamatergic transmission. The pattern of neuronal activation associated with seizures caused by cholinergic stimulation is similar to that observed in the case of kainic acid-induced seizures (Lothman and Collins, 1981; Lothman et al., 1985). N-methyl-D-aspartate (NMDA) receptors antagonists protect against neuronal loss caused by cholinergic seizures (Fujikawa et al., 1994).

Nitric oxide is a gaseous neurotransmitter that, once produced, diffuses to neighboring cells, where it produces its physiological effects. Dopaminergic neurons are an important target for this neurotransmitter, being the dopaminergic terminal a place where NO increased dopamine extracellular levels (West and Galloway, 1997; Kiss and Vizi, 2001; Kiss et al., 2004).

Brain microdialysis is an analytical technique designed to obtain neurotransmitters and other endogenous substances. It also allows the administration of drugs or toxic substances in conscious and freely moving animals. Therefore, it is a technique that allows measure with relative precision the release of neurotransmitters and its variations after pharmacological manipulation. The fact that the animals are conscious and freely moving allows monitoring brain neurochemical activity in an organism that can interacts with the environment. Thus, the neurochemical measures can then be related to sensory stimulation, spontaneous or induced behavior, or effects of treatment with certain drugs or toxic. It has been shown that brain microdialysis is a good tool to study the effects and neurochemical mechanisms of action of various drugs, toxins and pesticides on the release of dopamine in the striatum of rats (Faro et al., 2002; Campos et al., 2008; Vidal et al., 2007; Nunes et al., 2010; Machado et al., 2010).

In previous striatal *in vivo* studies using cerebral microdialysis, we observed that intrastriatal administration of paraoxon increased the spontaneous release of dopamine from rat striatum in a concentration-dependent manner, being this release dependent on AChE inhibition (5 mM paraoxon inhibited the striatal AChE activity about 52%) and mediated predominantly by the activation of striatal muscarinic receptors, once the muscarinic antagonist atropine partially blocks the paraoxon-induced dopamine release (Faro et al., 2018).

In this work, we continue the previous study in order to evaluate if the effects of paraoxon on the extracellular dopamine levels could be due to an exocytotic-, vesicular-, Ca^{++}- and voltage-dependent mechanism, and/or to a cytosolic, Ca^{++}- and voltage-independent mechanism. To do so, we used several perfusion mediums, high K^+ medium; Ca^{++}-free medium, tetrodotoxin (TTX), a voltage-sensitive Na^+ channel blocker, reserpine (RES), a vesicular dopamine depletor agent, and nomifensine (NOM), a dopamine transporter (DAT) inhibitor. Beside it was also considered interesting to investigate the effect of the competitive NMDA receptors antagonist, [2R]-amino-5-phosphonovaleric acid (AP5) on the paraoxon-induced *in vivo* dopamine release from rat striatum. In addition, to provide evidence for the proposed mechanism of glutamate action, the inhibitor of nitric oxide synthase (NOS), N_w-nitro-L-arginine methyl ester (L-NAME), was also examined.

2. Methods

2.1. Animals, drug treatments and experimental groups

To carry out all the experiments, adult female rats of the Sprague-Dawley breed, with a weight between 250 and 300 g, obtained from the Breeding Facility of the RIAIDT (Network of Infrastructures Supporting Research and Technological Development), University of Santiago de Compostela, Spain, were used. Commercial food and tap water were available *ad libitum*.

The animals were kept in a standard laboratory cage in conditions of constant temperature 22 ± 2 °C, and with controlled light-dark periods (14 and 10 h, respectively). All the experiments were carried out under the current European Directive 2010/63/EU and the current legislation of the Spanish State (Royal Decrete 53/2013) for the use of experimental animals and with the authorization of the Ethics Committee and Animal Wellness of the University of Vigo for the use of laboratory animals. All possible efforts were made to avoid animal suffering and distress.

Animals were divided into the following twelve groups:

1. 5 mM paraoxon (n = 16).
2. 5 mM paraoxon + 50 μM TTX (n = 5).
3. 5 mM paraoxon + 10/mg/kg reserpine (n = 5).
4. 5 mM paraoxon + Ca^{++}-free/EDTA (n = 5).
5. 50 μM nomifensine (n = 5).
6. 5 mM paraoxon + 50 μM nomifensine (n = 5).
7. 50 mM KCl (n = 5).
8. 5 mM paraoxon + 50 mM KCl (n = 7).
9. 650 μM AP5 (n = 5).
10. 5 mM paraoxon + 650 μM AP5 (n = 5).
11. 100 μM L-NAME (n = 5).
12. 5 mM paraoxon + 100 μM L-NAME (n = 5).

Paraoxon [O,O-diethyl O-4-nitrophenyl phosphate] was purchased from Pestanal®, (Fluka-Sigma-Aldrich St. Louis, USA); nomifensine maleate and reserpine were purchased from Sigma (St. Louis, USA), and tetrodotoxin citrate was purchased from Tocris (Bristol, RU). The substances used as standards, dopamine, dihydroxyphenylacetic acid (DOPAC) and homovanilic acid (HVA), the reagents for chromatography, sodium octanesulfonate (SOS), potassium phosphate (KH_2PO_4), ethylenediaminetetraacetic acid (EDTA), and chloral hydrate were supplied by Sigma (St. Louis, USA). All other chemicals and reagents were of analytical grade.

All drugs, including paraoxon, were dissolved in a perfusion medium and infused locally to the striatum via microdialysis probe,

except the reserpine which was dissolved in glacial acetic acid and made up to a final volume with 5.5% glucose solution before injecting the animal via i.p. (Elverfors et al., 1997).

2.2. Microdialysis procedure

The microdialysis technique was realized according the previous studies made in our laboratory (Vidal et al., 2007; Ferreira-Nunes et al., 2010; 2013, 2018; Alfonso et al., 2015; Justo et al., 2016). Briefly, after anesthetizing the animal, a guide-cannula (CMA/11, CMA/Microdialysis, Sweden) was implanted into the striatum using stereotaxic surgery. After 24 h of implantation of the cannula, a microdialysis probe (CMA/12, 3 mm membrane length, CMA/Microdialysis, Sweden) was introduced through the cannula and perfused with a ringer medium (147 NaCl, 4 mM KCl, 3.4 mM $CaCl_2$; pH 7.4), at a flow of 1.5 μL/min, and the dialysate was collected every 20 min for 3 or 4 h.

For the studies of mechanism of action, after collecting three basal samples (60 min) with normal Ringer, 20 mM TTX or Ca^{++} free-medium/EDTA was perfused during 60 min. Then, in the third hour of experiment, 5 mM paraoxon was mixed with TTX or Ca^{++} free-medium and administered during 60 min. Next, the medium was then switched back to the unmodified Ringer's solution, and sampling was continued for an additional period of 60 min. Reserpine (10 mg/kg) was injected i.p. 120 min before the start of paraoxon perfusion, that was administered during 60 min in reserpenized rats. Nomifensine (50 μM) or 50 mM KCl was perfused during 60 min alone or together with paraoxon. The administration protocol for glutamatergic antagonist or NOS inhibitor was as follows: AP5 (650 μM) or L-NAME (100 μM) was perfused during 60 min alone or, together with paraoxon (60 min).

2.3. HPLC conditions

The dialysates samples (30 μL) were collected by a CMA/142 microsampler (CMA/Microdialysis, Sweden) and the dopamine, DOPAC, and HVA were quantified by High-Performance Liquid Chromatography (HPLC) with electrochemical detection according to Durán et al. (1998). Briefly, a Jasco pump and a Reodyne 7125 injector were used, and a mobile phase (70 mM KH_2PO_4, 1 mM octanesulfonic acid, 1 mM EDTA (Ca^{++} chelating agent), and 14% methanol was eluted through a 25 cm Spherisorb ODS-1 reverse phase column with 10 μ particle size. The dopamine and metabolites detection was achieved using an ESA Coulochem III 5100A electrochemical detector at a potential of +400 mV.

2.4. In vitro recovery of dopamine, DOPAC, HVA and paraoxon

Before implantation of a probe into the brain, the recovery of dopamine, metabolites and paraoxon across the dialysis membrane, was determined *in vitro* (Vidal et al., 2007; Ferreira-Nunes et al., 2010; 2013, 2018; Alfonso et al., 2015; Justo et al., 2016). The dialysis probes were placed in a standard solution of dopamine, DOPAC or HVA (50 pg/μL) or paraoxon (5 mM) followed by flush with Ringer's solution at the same flow rate than that of the experiments (1.5 μL/min) for 20 min and the levels of substances in the dialysates were determined. Recoveries were calculated from the concentration of substrate in the perfusion fluid which was divided by the concentration in the standard solution. The recovery of dopamine, DOPAC, HVA and paraoxon across the microdialysis membrane were: 18.1 ± 0.72%, 15.6 ± 0.8%, 21.6 ± 0.6%, respectively.

The diffusion rate (recovery) of paraoxon was 6.4 ± 0.3% and the total amount of paraoxon administered to the animals during 1 h was 29 pmol.

2.5. Expression of results and statistics

All data shown are mean ± SEM values from 5 to 16 experiments. Averaged values of basal dopamine, DOPAC, and HVA levels (defined as 100%) were determined from two dialysate samples before the addition of any drug. Results were calculated as percentages of this averaged basal release. Concentration 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, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, respect to the basal levels; $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$, with respect to 5 mM paraoxon control group.

3. Results

3.1. Effects of paraoxon on dopamine, DOPAC, and HVA levels

The basal levels of dopamine and its metabolites were stable in the group control of rats to which only the perfusion medium was administered.

The average values obtained in basal striatal dopamine, DOPAC and HVA for every sample were, 0.49 ng/20 μL, 38.5 ng/20 μL and 6.8 ng/20 μL, respectively.

The intrastriatal administration of 5 mM paraoxon for 60 min, through the microdialysis probe, significantly changed extracellular dopamine levels, being the greatest increase in dopamine levels of 1066 ± 120%, in comparison with basal values. The highest increase was observed 20 min after the paraoxon perfusion was initiated, and basal values were recovered 100 min after the end of pesticide administration. The results also show the effect of paraoxon on the extracellular levels of DOPAC and HVA, relative to basal levels. The maximum increases were: 197 ± 9.2% and 176 ± 13.3%, for DOPAC and HVA, respectively. The highest levels were observed 60 min after the beginning of paraoxon perfusion, and values remained high until the end of experiment (Figs. 1–7).

3.1.1. Behavioural signs

Intrastriatal administration of paraoxon (5 mM) or other drugs or mediums did not produce seizures, tremors or other types of apparent behavioural or physiological dysfunctions.

3.2. Characterization of paraoxon-induced dopamine release from the striatum

With the purpose of investigating the possibility that paraoxon-induced dopamine overflow could be due to an exocytotic Ca^{++}, vesicular and voltage-dependent mechanism and/or a non-exocytotic mechanism through DAT, modified perfusion mediums, Ca^{++}-free medium and different pharmacological treatments (TTX, reserpine and nomifensine) were used.

3.2.1. Effects of TTX treatment in paraoxon-induced dopamine, DOPAC and HVA release

To investigate if the paraoxon-induced dopamine release was dependent on voltage-sensitive sodium channels (VSSC), 20 μM TTX was infused through the dialysis probe (Fig. 1). Intrastriatal infusion of TTX during 60 min decreased the extracellular dopamine levels to 77.6 ± 8.9% ($p < 0.05$), when compared with basal values. This value was considered as basal for the measurement of paraoxon effects on dopamine release in TTX-pretreated rats. When paraoxon was infused in TTX-treated animals, the striatal dopamine levels increased to 291 ± 50% ($p < 0.001$), compared with basal (Fig. 1A). As can be seen, the effect of paraoxon in rats pretreated with TTX is 72.7% lower than in animals treated with paraoxon alone. These results suggesting that the paraoxon induced dopamine release is partially dependent on VSSC and terminal depolarization.

The administration of TTX significantly decreased the extracellular

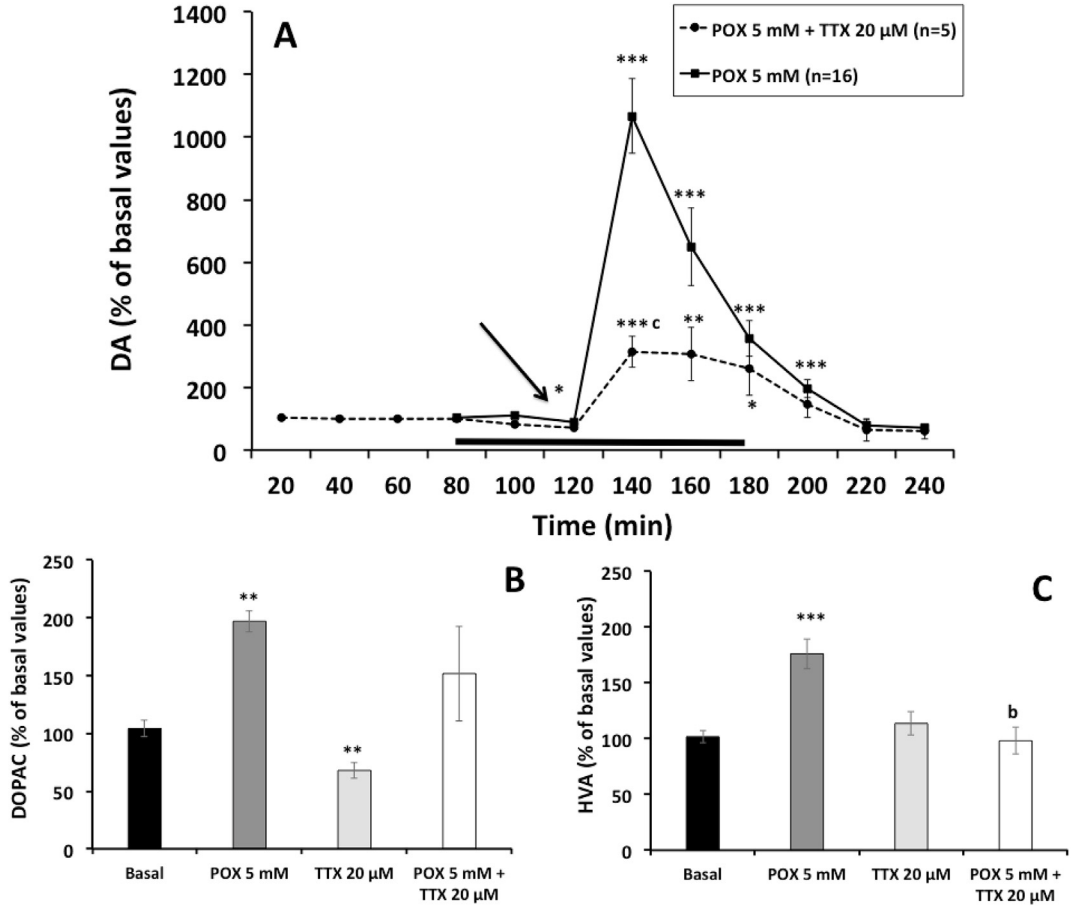

Fig. 1. Effects of 5 mM paraoxon infusion in TTX pretreated rats on the dopamine (A), DOPAC (B), and HVA (C) extracellular levels from rat striatum. Infusion of 20 µM TTX is shown by the black bar and paraoxon infusion started at the time indicated by the arrows (60 min). The results are shown as the 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 treatment 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, with respect to the basal levels, and ^{b}P < 0.01 and ^{c}P < 0.001, with respect to 5 mM paraoxon control group.

DOPAC levels to 67.9 ± 8.9% (p < 0.001), when compared with basal levels, and it had no effect on HVA extracellular levels. Infusion of paraoxon in TTX pretreated animals did not change the DOPAC levels, but significantly decreased HVA levels to 98.1 ± 11.8% (p < 0.01), when compared with 5 mM paraoxon treatment (Fig. 1B and C, respectively).

3.2.2. Effects of reserpine pretreatment on paraoxon-induced dopamine, DOPAC and HVA release

In order to verify the mediation of vesicular stores of neurotransmitter on paraoxon-induced increase in extracellular dopamine levels, rats were pretreated with 10 mg/kg reserpine, 60 min before the beginning of the experiment (Fig. 2). Two hours after i.p. reserpine injection, extracellular dopamine levels decreased to 72.3 ± 12.1% (p < 0.01), when compared with basal values. This value was considered as a basal level for the measurement of paraoxon effects in reserpinized animals. Fig. 2A shows that infusion of 5 mM paraoxon in reserpine-pretreated animals increased striatal dopamine levels to 205 ± 78% (p < 0.05), when compared with basal values. These data show that reserpine pretreatment significantly decreased the paraoxon-induced dopamine release by 81%. This result can indicate that paraoxon induces increases in extracellular dopamine levels through an exocytotic release of vesicular content.

The administration of reserpine or paraoxon to reserpinized animals did no change the DOPAC or HVA extracellular levels, when compared with basal values or 5 mM paraoxon effect (Fig. 2B and C, respectively).

3.2.3. Effect of paraoxon on dopamine, DOPAC and HVA release in a Ca^{++}-free ringer solution

To investigate if paraoxon-induced dopamine release was Ca^{++}-dependent, a Ca^{++}-free Ringer solution (added with EDTA) was infused through the dialysis probe. Fig. 3A shows the effect of Ca^{++}-free Ringer solution infusion on dopamine extracellular levels. After removing Ca^{++} from the perfusion medium, dopamine levels significantly decreased during three consecutive dialysates, levelling-off at 63 ± 8.5% (p < 0.05) of basal levels. This value was considered as basal for the measurement of paraoxon's effects on dopamine release in a Ca^{++}-free medium. When 5 mM paraoxon was perfused in a Ca^{++}-free medium/EDTA, the striatal dopamine levels increased to 322.8 ± 24.5% (p < 0.05), when compared with basal values, being this increase 70% smaller than that observed with paraoxon in normal Ringer solution (Fig. 3A). This significant decrease in dopamine overflow suggests that paraoxon-induced striatal dopamine release can be Ca^{++}-dependent.

The administration of a Ca^{++}-free + EDTA significantly increased the DOPAC and HVA levels to 142 ± 19.4% (p < 0.05) and 152.5 ± 14% (p < 0.05), when compared with basal levels, respectively. Infusion of paraoxon to Ca^{2+}-free/EDTA pretreated animals did no change the DOPAC or HVA levels, when compared to basal values, but significantly decreased DOPAC and HVA levels to 84 ± 3.7% (p < 0.05) and 90 ± 6% (p < 0.05), respectively, when compared with 5 mM paraoxon effect (Fig. 3B and C, respectively).

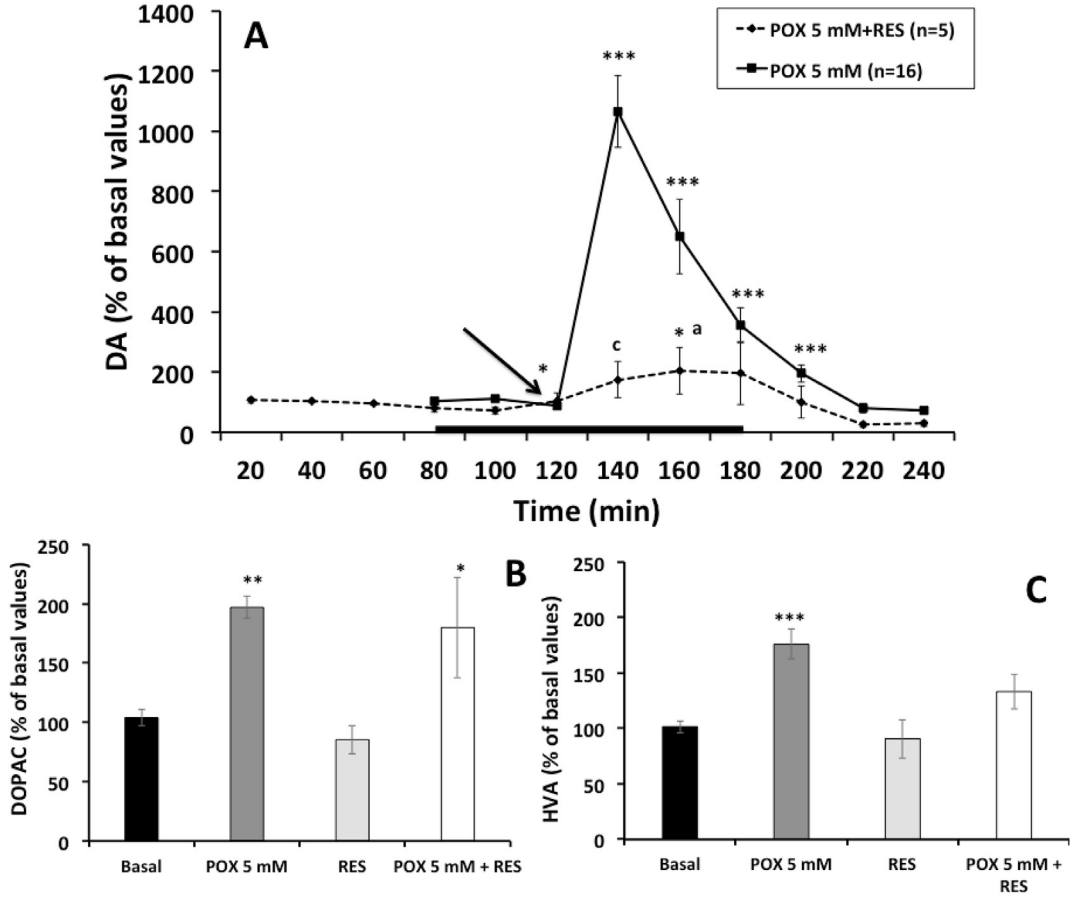

Fig. 2. Effect of 5 mM paraoxon infusion in reserpine pretreated rats on the dopamine (A), DOPAC (B), and HVA (C) extracellular levels from rat striatum. Animals received an i.p. RES injection 10 mg/kg 60 min before the beginning of the experiment. Paraoxon infusion started at the time indicated by the arrows (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 treatment 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, with respect to the basal levels, and ᶜP < 0.001, with respect to paraoxon control group.

3.3. Effect of nomifensine on the release of dopamine, DOPAC and HVA induced by paraoxon

In order to evaluate a possible role of DAT in paraoxon-induced dopamine release, nomifensine, an inhibitor of the dopamine uptake, was coinfused with 5 mM paraoxon. Fig. 4A shows the effect of 50 μM nomifensine infusion (60 min) on dopamine extracellular levels. Nomifensine induced a maximal increase in extracellular dopamine levels to 1435 ± 387%, when compared with basal values ($p < 0.001$). When nomifensine was coadministered with paraoxon, striatal dopamine increased to 2429 ± 417% ($p < 0.001$). This significant increase shows a additive effect of paraoxon and nomifensine on striatal dopamine release.

The administration of 50 μM nomifensine did not change significantly the DOPAC or HVA levels, when compared with basal. The coadministration of nomifensine and 5 paraoxon significantly increased the DOPAC and HVA levels to 182.1 ± 9% ($p < 0.05$) and 169.3 ± 9.2% ($p < 0.05$), when compared with basal levels, respectively (Fig. 4B and C).

3.4. Effects of paraoxon on the release of dopamine DOPAC and HVA induced by depolarization

We investigate the effect of paraoxon on depolarization K^+-evoked dopamine release. For this, we infused 50 mM KCl through the dialysis probe into the striatum during a period of 60 min. Fig. 5A shows that perfusion of KCl increased dopamine levels to 1104 ± 220.4% ($p < 0.001$), compared to basal values at 20 min after KCl infusion was initiated. This increase was not statistically different from that observed by the administration of 5 mM paraoxon and a similar release profile induced by both treatments could be observed.

On the other hand, when KCl was coadministered with paraoxon, striatal dopamine levels increased to 1957 ± 445% ($p < 0.001$), when compared with basal values. So, the coinfusion of KCl and paraoxon produced an increase in extracellular dopamine levels that was statistically higher than that observed with paraoxon or KCl administration alone (Fig. 5A). These results suggest that paraoxon increases the release of dopamine stimulated by depolarization.

The administration of KCl or the coadministration of KCl mixed with paraoxon did not change significantly the DOPAC or HVA levels, when compared with basal, but these levels were significantly decreased when compared with 5 mM paraoxon group (140 ± 20.4%, $p < 0.001$, and 70 ± 4.3%, $p < 0.001$), respectively (Fig. 5B and C).

3.5. Effect of intrastriatal AP5 perfusion on paraoxon-induced dopamine release

To examine the role of NMDA receptors in the paraoxon-induced increase in dopamine overflow, we perfused the specific NMDA receptors antagonist AP5 through the dialysis probe alone and mixed with the pesticide One-hour infusion of 650 μM AP5 had no significant effects on striatal dopamine levels when compared with basal values.

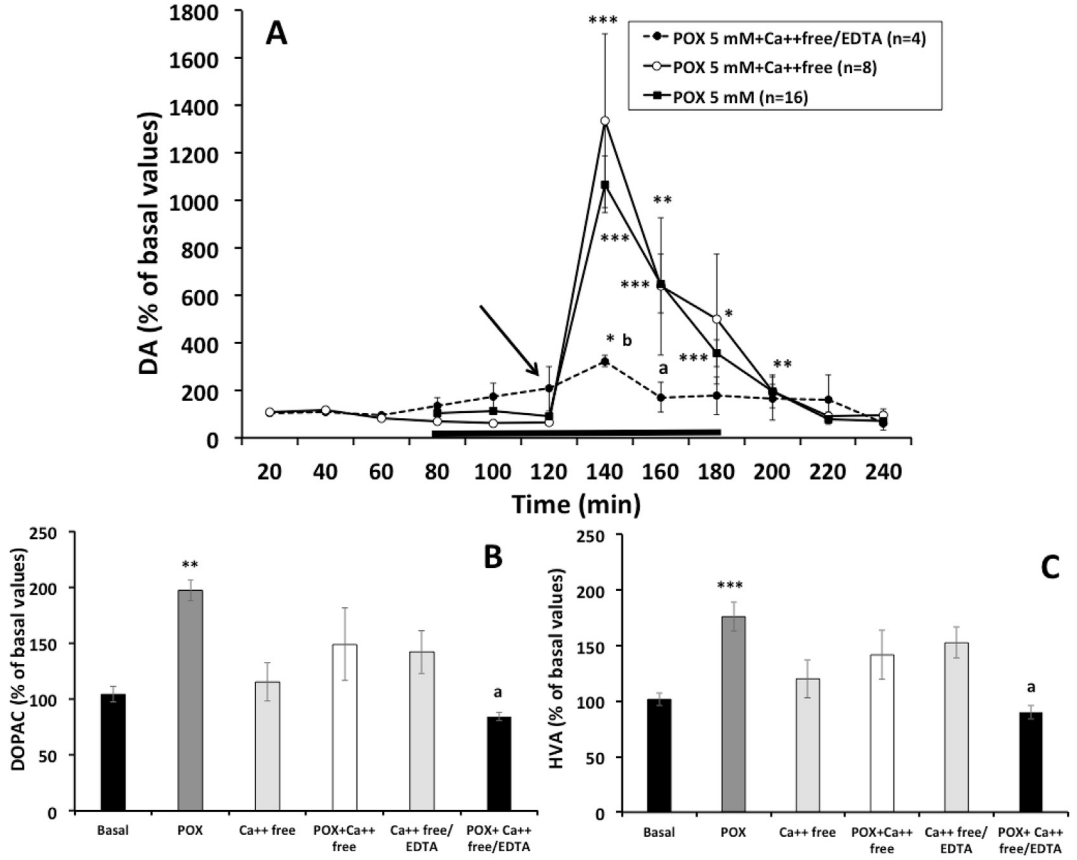

Fig. 3. Effects of 5 mM paraoxon in Ca^{++} free plus EDTA Ringer solution on the dopamine (A), DOPAC (B), and HVA (C) extracellular levels from rat striatum. Infusion of modified Ringer is shown by the black bar and paraoxon infusion started at the time indicated by the arrows (60 min). The results 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 treatment 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, with respect to the basal levels, and $^{a}P < 0.05$ and $^{b}P < 0.01$, with respect to paraoxon control group.

Under AP5 pretreatment, infusion of 5 mM paraoxon significantly increased striatal dopamine levels with respect to basal (maximal increase: 543 ± 80%, P < 0.001), being this increase 49% smaller than that one observed with paraoxon in non-pretreated animals (Fig. 6).

3.6. Effect of intrastriatal L-NAME perfusion on paraoxon-induced dopamine release

With the aim of investigating the implication of NO production in the effects produced by paraoxon on striatal dopamine levels, as well as the possible protective action of NOS inhibitors on paraoxon-induced dopamine release, we infused L-NAME through the microdialysis probe (Fig. 7). One-hour infusion of 100 μM L-NAME had no significant effects on striatal dopamine levels when compared with basal values. Under 100 μM L-NAME pretreatment, infusion of 5 mM paraoxon significantly increased striatal dopamine levels with respect to basal (maximal increase: 494 ± 24.8%, P < 0.001), being this increase 53.7% smaller than that one observed with paraoxon in non-pretreated animals (Fig. 7).

4. Discussion

Several experimental studies have suggested that the dopaminergic system could be a potential target for OPs in humans (Karen et al., 2001; Sun et al., 2006; Moreno et al., 2008; Eells and Brown, 2009; Binukumar et al., 2010; Torres-Altoro et al., 2011; Lee et al., 2012). In addition, based on several comprehensive epidemiological studies, pesticide exposure appears to be a risk factor for Parkinson's disease (Kamel and Hoppin, 2004; Dick, 2006; Firestone et al., 2005; Hatcher, 2008; Gatto et al., 2009; Goldman, 2014).

In a recent study, we have shown that the intrastriatal administration of paraoxon increases the release of dopamine in the striatum of conscious and freely moving rats in a dose-dependent way (Faro et al., 2018).

One of the first studies that has linked exposure to OPs with dopaminergic acute toxicity was the work of Jacobsson et al. (1997). Using an experimental design very similar to the present study, these authors demonstrated that acute and intrastriatal administration of 10 mM of the OP pesticide soman, produced a significant increase in dopamine release in rats. These authors also noted that soman induced seizures and they attributed this effect to changes in dopamine levels. On the other hand, Jacobsson et al. (1997) also described that, in parallel with the increase in dopamine release, soman induced an increase in the levels of excitatory amino acids, which could indicate a cascade of pathological events that would lead to important neuronal damage. This raises the possibility that both dopamine and glutamate released may act synergistically in the production of oxidative stress and neurotoxicity after administration of soman (Jacobsson et al., 1997; Jacobsson and Fowler, 1999).

Our current work confirms that, just like the soman, paraoxon also increases *in vivo* dopamine release in rat striatum. However, different from the study made by Jacobsson et al. (1997), we have not observed seizures. Nevertheless, the oxidative nature of dopamine itself has been identified as a susceptibility factor, and an abnormal increase in its

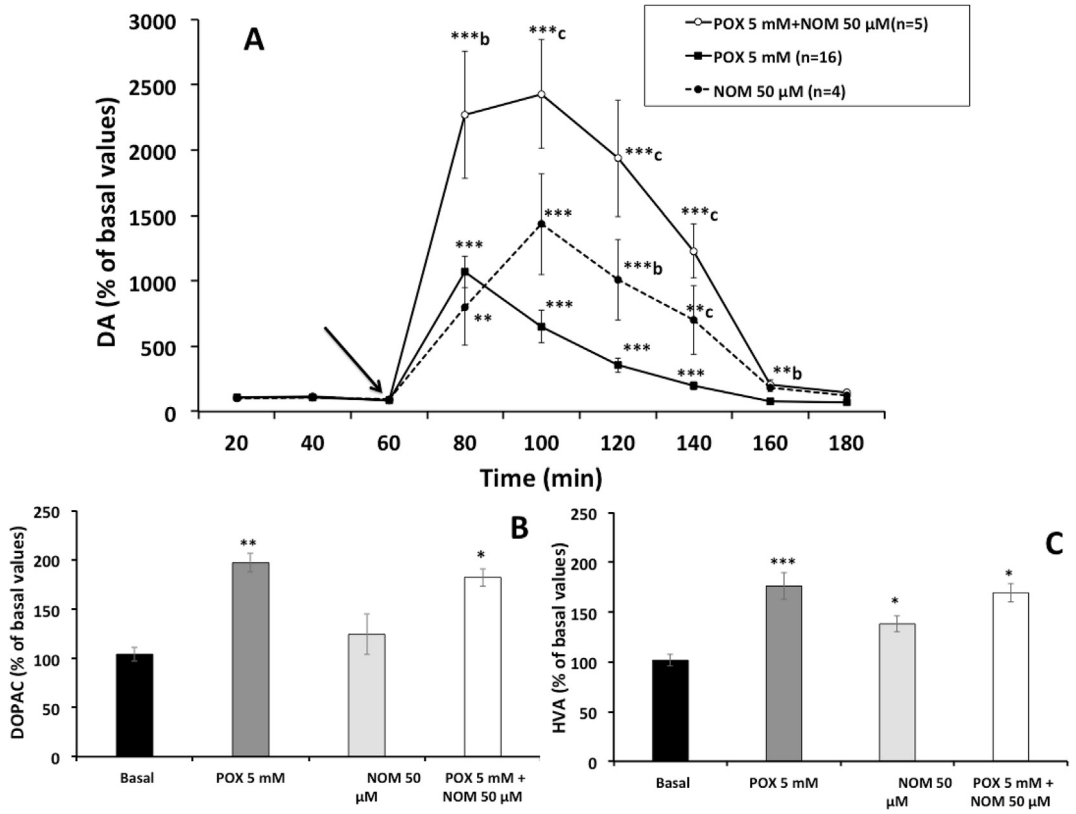

Fig. 4. Effects of 5 mM paraoxon infusion in nomifensine treated rats on the dopamine (A), DOPAC (B), and HVA (C) extracellular levels from rat striatum. Nomofensine and paraoxon infusion started at the time indicated by the arrows (60 min). The results are shown as the mean ± S.E.M., expressed as a percentage of basal levels 100%. Basal levels were considered as the mean of dopamine and its metabolites concentrations in the two samples before treatment 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 the basal levels, and ^{b}P < 0.01 and ^{c}P < 0.001, with respect to paraoxon control group.

extracellular levels, like those observed in the present study, can cause inhibition of mitochondrial respiration and, consequently, the death of dopaminergic neurons (Gluck and Zeevalk, 2004). This may initiate the symptoms that characterize the Parkinson's disease.

Like other neurotransmitters, dopamine release can occur from two processes, either through an exocytotic release mechanism Ca^{++}, vesicular, and voltage-dependent (Grace, 1991; Mac Dermott et al., 1999; Olivier et al., 1999) or through a cytoplasmic release mechanism mediated by reversion of DAT (Leviel, 2001). So, to determine by which of these two mechanisms paraoxon induces striatal dopamine release, the effect of different pharmacological treatments on dopaminergic terminal as well as the changes in the perfusion medium were studied.

To evaluate the role of voltage-dependent sodium channels in dopamine release induced by paraoxon, TTX, a selective antagonist of these channels (Taylor and Narasimhan, 1997; Penzotti et al., 1998), was administered together with the pesticide. As expected, the administration of TTX significantly decreased the dopamine release, with respect to basal levels. Additionally, intrastriatal administration of paraoxon to rats pretreated with TTX increased extracellular dopamine levels approximately 3-times over basal levels. This effect was 73% lower than the effect observed with the administration of 5 mM paraoxon without TTX. These results seem to indicate that dopamine release evoked by paraoxon is dependent on depolarization mediated by voltage-dependent sodium channels.

In this study, the effects of vesicular dopamine depletion on paraoxon response were also investigated. The effect of paraoxon on dopamine release was studied in animals pretreated with reserpine, a vesicular dopamine transporter inhibitor (Tolwani et al., 1999). As expected, reserpine induced a decrease in basal extracellular dopamine levels in agreement with the results observed in other *in vivo* studies (Heeringa and Abercrombie, 1995; Kannari et al., 2000). Intrastriatal administration of paraoxon in animals pretreated with reserpine produced an increase in dopamine release that was about 81% lower than that observed with paraoxon administration in non-reserpinized animals. This result suggests that paraoxon induced a vesicular-dependent increase in dopamine extracellular levels.

It is well known that extracellular calcium plays a key role in the mechanism of exocytotic release of dopamine. Membrane depolarization causes an activation of voltage-dependent calcium channel allowing the entry of Ca^{++} into the intracellular medium. These ions activate intracellular reactions that lead to the release of dopamine (Olivier et al., 1999; Leviel, 2001).

In order to verify the role of Ca^{++} present in the extracellular medium on the paraoxon-induced dopamine release, the pesticide was administered dissolved in a Ca^{++} free Ringer medium with EDTA, a Ca^{++} chelating agent. Thus, with this experimental group we would be removing most of extracellular calcium ions. In these experimental conditions, *in situ* infusion of paraoxon produced increases in the extracellular dopamine levels of about 30%, compared with baseline, representing a decrease of about 7-fold of the effect produced by paraoxon alone. This, would indicate that the effect of paraoxon on dopamine release is dependent on extracellular Ca^{++}.

Taken together, these results are compatible with the idea that *in vivo* dopamine release evoked by paraoxon was Ca^{++}-, vesicular-, and depolarization-dependent, and occur as a consequence of a vesicular exocytotic process.

To test if dopamine overflow may also occur through a cytoplasmic mechanism, the possible involvement of DAT on the paraoxon-induced dopamine release was evaluated. The infusion of paraoxon together with nomifensine produced an increase in the dopamine levels of about

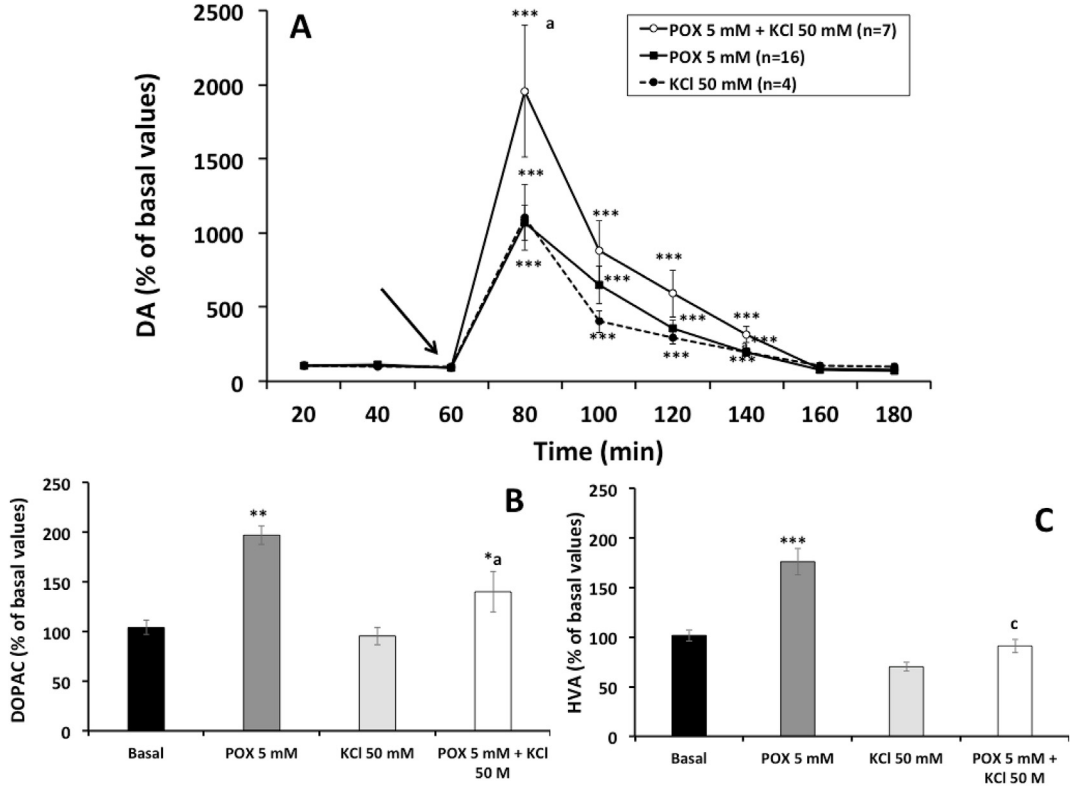

Fig. 5. Effects of KCl 50 mM infusion on the dopamine (A), DOPAC (B), and HVA (C) extracellular levels from rat striatum in the absence or presence of 5 mM paraoxon. KCl and paraoxon infusion started at the time indicated by the arrows (60 min). The results are shown as the mean ± S.E.M., expressed as a percentage of basal levels (100%). Basal levels were considered as the mean of dopamine and its metabolites concentrations in the two samples before treatment administration. Statistical analysis of the results was performed by means of ANOVA and Student–Newman–Keuls multiple range test, considering the following significant differences, $^{**}P < 0.01$ and $^{***}P < 0.001$, respect to the basal levels, and $^{a}P < 0.05$ and $^{c}P < 0.001$, with respect to 5 mM paraoxon control group.

24 times, when compared with baseline, an increase that was significantly higher than that observed to the effects of both substances administered separately (14 and 12 times to nomifensine and paraoxon, respectively). This apparent additive effect (paraoxon + nomifensine) could mean that both substances could act through different mechanisms at the dopaminergic terminal, nomifensine acting on DAT and paraoxon acting on AChE to induces increases in acetylcholine levels, which would increase the release of dopamine by activation of the cholinergic receptors present in the dopaminergic terminal, corroborating the results from the other experimental groups (Faro et al., 2018).

Another interesting hypothesis comes from the observation of dopamine release profiles following administration of paraoxon, nomifensine or coadministration of both. The paraoxon induces a maximal increase in dopamine release 20 min after the start of pesticide administration and, after this, dopamine levels decrease rapidly to basal values. On the other hand, the nomifensine produces the maximum increase at 40 min and dopamine levels it will take more time to reach baseline. When the two substances are coadministered, the dopamine levels remain high to almost the end of the experiment. Based on these release profiles, we could suggest that inhibition of DAT is increasing

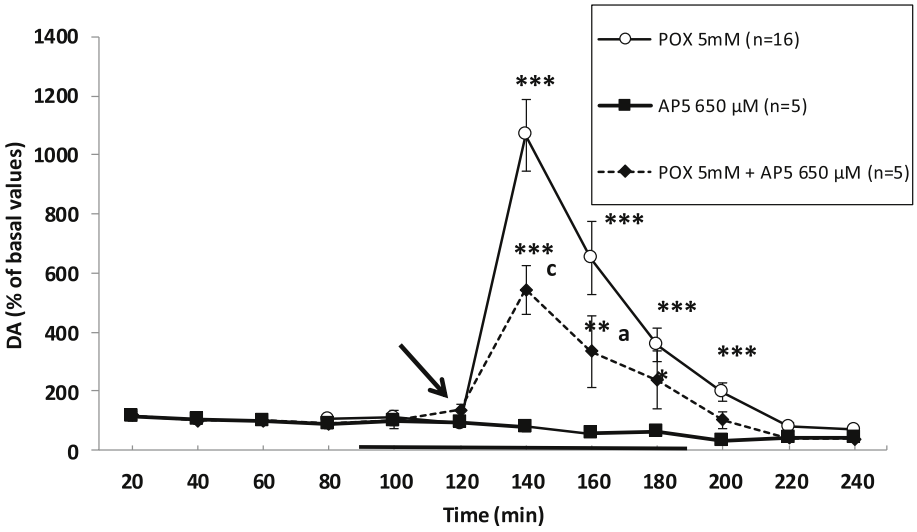

Fig. 6. Effect of AP-5650 µM on the striatal levels of dopamine (A) DOPAC (B) and HVA (C), induced by 5 mM paraoxon. The results are shown as means ± S.E.M. expressed as a percentages with respect to the basal levels (100%). Basal levels were considered as the average of the two samples prior to the administration of the treatments. The black bar on the X axis indicates the infusion time of the AP-5120 min and the arrow indicates the start of the paraoxon perfusion (60 min). 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 levels; $^{a}P < 0.05$, in relation to the paraoxon group.

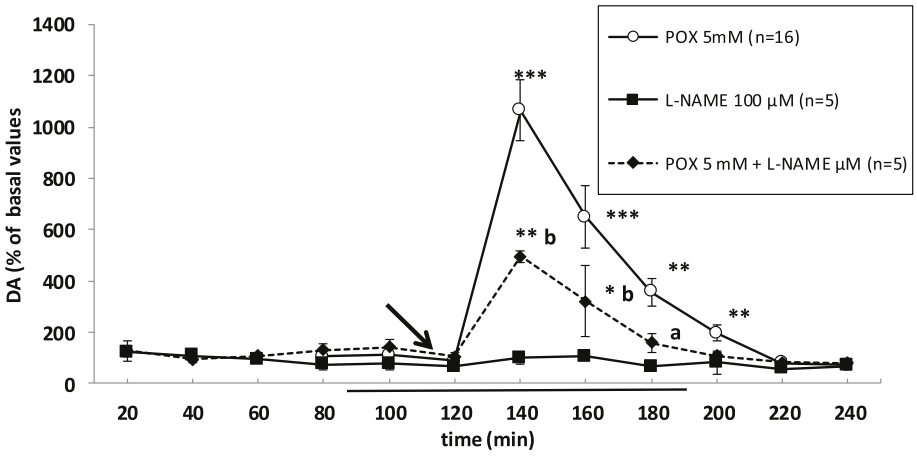

Fig. 7. Effect of L-NAME 100 µM on the striatal levels of dopamine A DOPAC B and HVA C, induced by 5 mM paraoxon. The results are shown as means ± S.E.M. expressed as a percentages with respect to the basal levels (100%). Basal levels were considered as the average of the two samples prior to the administration of the treatments. The black bar on the X axis indicates the infusion time of the L-NAME 120 min and the arrow indicates the start of the paraoxon perfusion (60 min). 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 levels; $^{a}P < 0.05$, in relation to the paraoxon group.

the half-life of striatal dopamine released in response to paraoxon.

Regarding the behavior of dopamine metabolites, we observed significant increases in both DOPAC and HVA levels in response to paraoxon administration. Levels of both metabolites are slower indicators of neuronal activity, representing long-term changes in dopamine release (Westerink, 1995), but variations in its extracellular levels are often difficult to interpret. Synthesis, metabolism, conjugation, and efflux rate of dopamine are stages that may change the extracellular levels of both metabolites and paraoxon could act on some of the stages.

Considering that extracellular DOPAC, primary source comes from metabolism, via monoamine oxidase (MAO), of the newly synthetized cytoplasmic dopamine pool (Zettreström et al., 1986; Gazzara and Anderson, 1994), a possible hypothesis that could explain those results would be that the paraoxon, by stimulation of exocytotic release of dopamine, also stimulates its synthesis from tyrosine, increasing the intracellular stocks of newly synthetized dopamine. In this way, the increase of its metabolite levels could be explained.

Finally, as our results show that paraoxon stimulates basal dopamine release, to assess whether paraoxon also influences the release of dopamine stimulated by depolarization, this pesticide was coadministered together 50 mM KCl. It has been shown that dopamine levels are increased considerably after the perfusion of a medium with high concentration of KCl (Westerink and De Vries, 1988; Herrera-Marschitz et al., 1990). Thus, KCl depolarizes nerve terminals, which leads to calcium influx into the neuron and neurotransmitter release by an exocytotic mechanism (Herrera-Marschitz et al., 1990). In our experimental conditions, administration of KCl resulted in an increase in extracellular dopamine levels of about 11 times, compared to baseline levels, whereas perfusion of paraoxon in high KCl medium resulted in an increase of about 20 times. These results show that paraoxon significantly increases striatal dopamine release, both basal and stimulated by depolarization.

It is well known that the striatum is a central region that receives inputs from the cerebral cortex, thalamus and *substantia nigra*, and that glutamate is released from the terminals of the cerebral cortex neurons and in smaller quantity by terminals of the thalamic neurons (Morari et al., 1998). *In vivo* and *in vitro* studies suggest that activation of striatal NMDA glutamatergic receptors facilitates the release of dopamine in the striatum of rats. Several authors (Hernández et al., 2003; Segovia et al., 1997) have shown that the administration of glutamatergic agonists AMPA or NMDA, increase the release of dopamine in the striatum *in vivo* and that these effects are reversed by the action of their antagonists. Other groups of researchers have found that the administration of competitive NMDA receptor antagonists, as the used in the present study (AP-5), produced a decrease in the levels of dopamine in the striatum, indicating that NMDA receptors could control over the release of dopamine and that NMDA receptors maintain a facilitating action on striatal dopaminergic neurons (Kashihara et al., 1990; Whitton et al., 1992, 1994).

In our experiments, we assessed the potential involvement of NMDA glutamatergic receptors in paraoxon-induced striatal dopamine release. Thus, administration of paraoxon in animals pretreated with AP5, reduced about 54% the extracellular dopamine levels induced by the pesticide. These results may indicate that the *in vivo* release of dopamine induced by paraoxon is partially dependent on the activation of NMDA glutamatergic receptors.

In another study, we also observed that the administration of atropine, antagonist of cholinergic nicotinic receptors, also reduces the release of striatal dopamine induced by paraoxon (Faro et al., 2018). Considering that the glutamatergic afferents and the cholinergic terminals constitute the main projections towards the striatal dopaminergic terminals, we can conclude that the results of these experimental groups would confirm an action of the paraoxon at presynaptic level on the striatal dopaminergic terminals, since the blockade of the two main aferences reduces the effect of paraoxon. Moreover, the paraoxon could act at the cholinergic neurons, increasing the levels of acetylcholine, which would act on the cholinergic receptors in the dopaminergic terminals.

In the work of Jacobson et al. (1997), it is shown that somam another OP pesticide, increases the levels of glutamate, which would contribute to the release of dopamine. The results indicated above agree with the effects of other OP pesticide (glufosinate) studied in our laboratory. So, we observed that glutamatergic antagonists reduce the release of dopamine induced by the intrastriatal administration of glufosinate (Faro et al., 2013).

There are *in vitro* and *in vivo* evidences that the dopaminergic system is influenced by NO (Hanbauer et al., 1992; Lorrain, Hull, 1993; West and Galloway, 1997; Iravani et al., 1998; Kiss et al., 1999). Furthermore, the striatum is characterized as an area with a high density of NMDA glutamatergic receptors (Greenamyre, 1995) and with a high activity of NOS (Bredt, 1991). In the present work, we assessed the potential involvement of NO on striatal dopamine release induced by paraoxon. To do this, we used L-NAME, a non-selective inhibitor of NOS. The results would indicate that the action of paraoxon is also partially dependent on the activation of NOS.

Taken together, our results may indicate that the *in vivo* release of dopamine induced by paraoxon is partially dependent on the activation of NMDA glutamatergic receptors and the production of NO in rat striatum. So, activation of NMDA receptors in nitrergic neurons induces calcium input into the terminal; calcium binds to calmodulin, and calcium-calmodulin complex activates the NOS and inducing the production of NO (Garthwaite and Boulton, 1995; Hong et al., 2005). NO is a neuromediator which diffuses from the neuron that produces it and enters in surrounding neurons to produce their effects. One of its target cells is the dopaminergic neuron (West and Galloway, 1997), in which NO stimulates the dopamine release (Kiss and Vizi, 2001; Kiss et al.,

2004). Inhibition of NOS will produce lower levels of NO and a decrease the release of dopamine. Consequently, the release of dopamine induced by paraoxon would be less.

The mechanism through which NO increases dopamine levels is not yet well established. Some authors have proposed that NO causes a reduction in DAT activity, thus increasing extracellular dopamine levels (Lonart and Johnson, 1994; Kiss et al., 1999, 2004). This could suppose a new way of interneuronal communication without a synaptic communication mediated by receptors (Kiss et al., 2004).

Other authors have suggested that the main interneuronal effect of NO is the activation of the guanylate cyclase, which triggers an increase in cyclic guanosine monophosphate (cGMP) which, in turn, produces an increase in dopamine release by activating the calcium channels (Phillips and Stamford, 2000; Khan and Hare, 2003; Trimmer and Rhodes, 2004; Rocchitta et al., 2005).

However, both the inhibition of NMDA receptors and the blockade of NO production partially decreased and did not completely block the action of paraoxon on striatal release of dopamine. This would indicate that both mechanisms would be complementary, in order to produce the effect of paraoxon on dopamine release.

5. Conclusions

In the present work, we have studied the neurochemical mechanisms of action, as well as the mediation of the NMDA receptors and NO production, by which the paraoxon induces the *in vivo* striatal dopamine release in freely moving rats. The results obtained in various experimental groups show that paraoxon-induced *in vivo* dopamine release, at least at the concentration used in this study, is predominantly mediated by a vesicular, Ca^{++}-, and action potential-dependent mechanism, and probably, this is not due to the reversal of the dopamine reuptake mechanism. Paraoxon also increases the release of dopamine stimulated by depolarization. Moreover, the release of dopamine induced by the paraoxon is partially mediated by the activation of NMDA glutamatergic receptors and NO production.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.01.001.

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