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Mediation of a glutamate antagonist, a NOS inhibitor and antioxidants with –SH groups on striatal dopamine release induced by clothianidin

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Abstract. Is Clothianidin is a neonicotinoid insecticide with selective action on nicotinic acetylcholine receptors. The aim of this study was to determine if the administration of a glutamate antagonist (APV), a NOS inhibitor (L-NAME) or two antioxidants (glutathione, and dithiothreitol,) prevent the increase in the striatal dopamine levels induced by clothianidin, using the microdialysis technique in freely moving and conscious rats. Intrastriatal administration of clothianidin (3.5 mM) produced an increase in striatal dopamine levels of 2462 ± 627%, with respect to basal levels. Coadministration of 0.65 mM APV and 3.5 mM clothianidin generated an increase in extracellular dopamine levels of 1089 ± 243.5%, being this increase 55.7% lower than the generated by clothianidin alone. Coadministration of 0.1 mM L-NAME and 3.5 mM clothianidin generated a significant increase in extracellular dopamine levels of 836.5 ± 150.6%., this increase is 70% lower than the generated by clothianidin alone. Coadministration of 3.5 mM clothianidin in combination with 0.4 mM glutathione induced an increase in striatal dopamine levels of 465.6 ± 126.8% , indicating that the administration of glutathione results in an inhibition of 81% of the effect generated by the infusion of clothianidin alone. Administration of 3.5 mM clothianidin associated with 0.005 mM dithiothreitol induced an increase in extracellular dopamine levels in the striatum of 693.8 ± 117.8% with respect to basal levels, being this increase 72% lower that the generated by clothianidin alone. Our results suggest that the effect of clothianidin on striatal dopamine release can be reduced by the administration of a glutamate antagonist, a NOS inhibitor or antioxidants with –SH groups, which suppose a simple protection mechanism against the damage caused for clothianidin.

Key words: Clothianidin, striatal dopamine, APV, L-NAME, antioxidants, microdialysis.

Resumen: La clotianidina es un insecticida neonicotinoide con actividad selectiva sobre los receptores de acetilcolina. El objetivo de este estudio es comprobar si un inhibidor de los receptores glutamatérgicvos (APV), un inhibidor de la óxido nítrico sintetasa (L-NAME) y dos antioxidantes como el glutatión y el dithiotreitol previene la liberación de dopamina inducida por la clotianidina, usando la técnica de microdiálisis en ratas conscientes y en libre movimiento. La administración intraestriatal de clothianidina (3.5 mM) produce un aumento de 2462 ± 627%, de los niveles estriatales de dopamina respecto a los niveles basales. La coadministracion de 0.65 mM de APV y 3.5 mM d clothianidina genera un a aumento de 1089 ± 243.5% de los niveles estriatales de dopamina, siendo este incremento 55.7% más bajo que el generado por la clotianidina sola. La Coadministration de.0,1 mM de L-NAME y3.5 mM de clotianidina genera un aumento de 836.5 ± 150.6% de los niveles extracelulares de dopamina, siendo este aumento un 55.7% más bajo que el generado por la clotianidina sola. La coadministracion of 3.5 mM clothianidina en combinación con 0.4 mM de glutatión induce un aumento de 465.6 ± 126.8% de los niveles estriatales de dopamina, indicando que la administración de glutatión provoca una inhibición del 81% del efecto generado por la infusión de clotianidina sola. La administración de 3.5 mM de clothianidina junto con 0.005 mM de diithiothreitol induce un aumento de 693.8 ± 117.8% en los niveles extracelulares de dopamina en el estriado, siendo este incremento 72% más bajo que el generado por la clotianidina sola. Nuestros resultados sugieren que el efecto de la clotianidina sobre la liberación estriatal de dopamina pueden ser reducidos por la administración de un antagonista glutamatérgico, un ihibibidor de la NOS o por antioxidantes con grupo –SH, lo cual supone un simple mecanismo de protección contra el daño causado por la clotianidina.

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Introduction

The neonicotinoid insecticide clothianidin is a broad spectrum pesticide, with selective action on nicotinic receptors of insects but with low toxicity in mammals [1]. Clothianidin is a metabolite of the neonicotinoid insecticide thiamethoxam [2], but it is also commercialized as an insecticide. Exposure to clothianidin and other neonicotinoids produced neurological and behavioural changes in mammals. Several authors [3-5] have observed changes in motor activity, discoordination of walking, ataxia, tremors, etc. These alterations are probably related to an action of neonicotinoids or their metabolites on the cholinergic receptors present in different brain regions that control motor activity in animals, such as the striatum. Striatal dopamine is a neurotransmitter that coordinates motor activity in mammals.

In previous studies of our laboratory, we found that clothianidin induced an increase in striatal dopamine levels [6]. This dopamine release was caused by an exocytotic, vesicular, and calcium- and depolarization-dependent mechanism [7]. Furthermore, dopamine release induced by clothianidin was mediated by activation of nicotinic cholinergic receptors [6].

Glutamate is one of the neurotransmitters involved in striatal dopamine release. On the other hand, the activation of NMDA glutamatergic receptors induces a rise in the intracellular Ca^{2+} concentration. This Ca^{2+} binds to calmodulin and the Ca^{2+} / calmodulin complex activates the nitric oxide synthase (NOS) and produces nitric oxide. The use of glutamate antagonists or NOS inhibitors could prevent the effect of clothianidin on the dopamine release.

Also, other authors have studied the beneficial effect of some antioxidants (such as glutathione, GSH) on the toxic effects of various pesticides. GSH is the major low molecular weight thiol present in the mammalian organism and plays a fundamental role in cellular resistance against oxidative damage [8-9]. At present, the glutathione is the most studied antioxidant [9-10], being one of its main roles the storage and transport of cysteine. Glutathione acts detoxifying *reactive oxygen species* (ROS) and recycling thiols of oxidized proteins [11].. Another molecule that acts as a reducing agent and as a cellular antioxidant is dithiothreitol (DTT) [12-13]. DTT acts reducing the disulfide groups S-S to sulfhydryl groups -SH [12]. The preservation of sulfhydryl groups of a protein in a reduced status is critical for maintaining the function of many proteins, being DTT one of the exogenous thiols most commonly used for this purpose [14].

The aim of this work is to study if the administration of glutamate antagonist (DL-2-amino-5-phosphonovaleric acid, APV), NOS inhibitor (L-nitro-arginine methyl esther, L-NAME) or antioxidants (GSH and DTT) prevent the increased levels of striatal dopamine induced by clothianidin administration in conscious and freely moving rats, using the microdialysis technique.

Material and methods

Female adult Sprague–Dawley rats (240 260 g) were used in all the experiments. Animals were housed under controlled conditions of temperature (22±2 °C) and ilumination (light:dark 14:10 h), with

free access to food and water. The experiments were performed according with the Guidelines of the European Union Council (2010/63/CEE) and the Spanish Government (R. D. 53/2013) for the use of laboratory animals.

Clothianidin [(E)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine], 99,9%, was purchased by Pestanal® (Sigma-Aldrich, USA). DL-2-amino-5- phosphonovaleric acid (APV), L-nitro-arginine methyl esther (L-NAME), glutathione (GSH), and dithiothreitol (DTT) were purchased by Sigma-Aldrich (St. Louis, USA). All other chemicals were of analytical grade.

Animals were anesthetized with chloral hydrate (400 mg/kg i.p.) and placed in a stereotaxic apparatus (Narishige SR-6). The skin above the skull was cut, the top of the skull was exposed, and a small hole was drilled at the following coordinates from Bregma according to the atlas of Paxinos and Watson A/P +1.0 mm, L, +3.0 mm, V, +3.0 mm. A CMA12 guide cannula (CMA/Microdialysis, Sweden) was implanted through the skull just over the striatum. The cannula was anchored to the skull using steel screws and acrylic cement. Then, the rats were housed in individual cages. After microdialysis experiments, rats were sacrificed by cervical dislocation and brains were removed for subsequent histological confirmation of probe placement.

The experiments were carried out 24 h after implantation of the guide-cannula. Continuous perfusion was performed with a Ringer solution (147 mM NaCl, 4 mM KCl, 3.4 mM $CaCl_2$; pH 7.4) using a CMA/102 infusion pump (CMA/Microdialysis, Sweden) at a flow rate of 1.5 µl/min. All experiments were conducted in awake, conscious, and freely moving animals. The experiments were carried out over periods of 3 or 4 h, and samples were collected every 20 min (30 µl). After three basal samples collected (60 min), clothianidin (3.5 mM) was infused for 60 min. After this, the medium was switched back to the unmodified Ringer solution and sampling was continued for an additional period of 60 min. In the groups pretreated with APV (0.65 mM), L-NAME (0.1 mM), GSH (0.4 mM), or DTT (0.005 mM), three basal samples were collected and after, the drug was infused for 60 min. Then, clothianidine together with the drug was infused during 60 min more. Finally, the medium was switched back to the unmodified Ringer solution and sampling was continued for an additional period of 60 min.

The samples obtained from the microdialysis procedure (30 µl) were collected by means of a CMA/142 microsampler (CMA/Microdialysis, Sweden) and dopamine levels were quantified by High-Performance Liquid Chromatography (HPLC) with electrochemical detection {15]. For this purpose, we used a Jasco PU-980 pump. The dialysates were injected (20 µl) using a Rheodyne 7125 injection valve. The isocratic separation of dopamine was achieved using Spherisorb ODS-1 reversed phase columns (5 µm particle size). The eluent (pH 4.0) was prepared as follows: 100 mM KH_2PO_4, 1 mM octanesulfonic acid, 1 mM EDTA, and 10 % methanol. Elution was carried out at a flow rate of 1.5 ml/min. The dopamine detection was achieved using an ESA Coulochem 5011A electrochemical detector at a potential of +400 mV.

Before probe implantation into the brain, the recovery of dopamine through the dialysis membrane was determined *in vitro*. The dialysis probes were placed in a standard solution of dopamine (50 pg/µL) and were flushed with Ringer solution at a flow of 1.5 µL/min for 20 minutes. Then, the levels of dopamine in the dialysates were determined. So, the recovery rate for dopamine across the microdialysis membrane was 13.2 ± 0.7%

To assess the amount of clothianidin that cross the dialysis membrane, the recovery rate for this substance through the microdialysis probe was also estimated *in vitro*. In this case, clothianidin (3.5 mM) was perfused through the dialysis probe at a flow rate of 1.5 µL/min. The probe was placed in an Eppendorf tube containing 1 mL of Ringer solution. The collected samples were measured by HPLC using ultraviolet (UV) detection. 20 µl of clothianidin solution was injected into the HPLC-UV system, which was equipped with a Jasco PU-1580 HPLC pump and a UV detector (HP series 1050). The separation of clothianidin was achieved using Dionex C18 reversed-phase columns, under isocratic conditions. Column was eluted with a mobile phase consisting of 40% acetonitrile in a 1 mmol sodium acetate buffer (pH = 6.7) at a flow rate of 2 mL/min. The recovery rate for clothianidin was 19.0 ± 0.9% for 20 minutes. So, the amount of clothianidin that crosses the microdialysis membrane is 1.05 nmol / min.

Results

The participation of NMDA glutamatergic receptors on clothianidin induced dopamine release was evaluated by administration of APV, a competitive antagonist of these receptors.

Intrastriatal administration of clothianidin (3.5 mM) produced an increase in striatal dopamine levels of 2462 ± 627%, with respect to basal levels.

APV administration (0.65 mM) has not produced statistically significant changes on extracellular dopamine levels in the striatum. In the experimental group, coadministration of 0.65 mM APV and 3.5 mM clothianidin generated an increase in extracellular dopamine levels of 1089 ± 243.5% ($P \leq 0.001$), compared to the basal levels. This increase was55.7%lower than the generated by clothianidin alone(Figure 1).

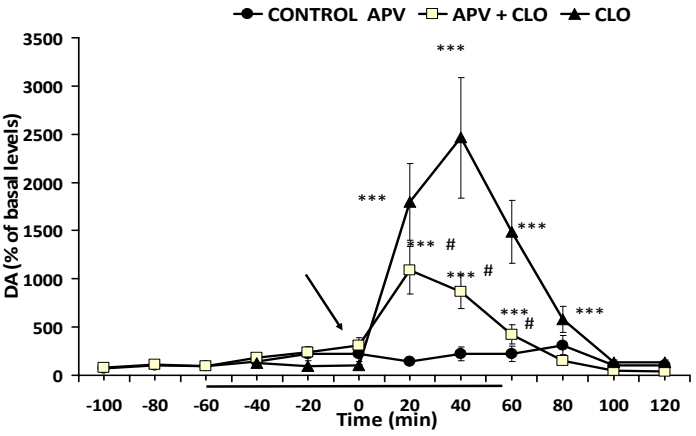

Figure 1. Effect of perfusion of 3,5 mM clothianidin and 0,65 mM APV on dopamine extracellular levels in the striatum. Infusion of APV is shown by the black bar and clothianidin infusion started at the time indicated by arrow. Dopamine levels are expressed as the variation of porcentage with respect to basal (100%), calculated as the mean of the two fist samples before the administration of the treatments. Values are mean ± S.E.M. (n = 5-7). Signification levels: ****P ≤ 0.001* respect to basal levels and $^{\#}P \leq 0.05$ respect to clothianidin group.

To investigate if the production of nitric oxide may be involved in the effects of clothianidin on the release of dopamine, we administered L-NAME, a NOS inhibitor, through the microdialysis probe.

The intrastriatal administration of L-NAME (0.1 mM) did not alter significantly the dopamine levels in the striatum. Coadministration of 0.1 mM L-NAME and 3.5 mM clothianidin generated a significant increase in extracellular dopamine levels of 836.5 ± 150.6% ($P \leq 0.001$), compared to the basal levels. This increase is 70% lower than the generated by clothianidin alone (Figure 2).

To test if reduced GSH (endogenous antioxidant) and DTT (with reactive -SH groups) could have any effect on dopamine release induced by clothianidin, each one of those substances was administered together with clothianidin through the microdialysis probe.

Administration of 0.4 mM GSH has not generated statistically significant changes on extracellular dopamine levels in the striatum. Coadministration of 3.5 mM clothianidin in combination with 0.4 mM GSH induced an increase in striatal dopamine levels of 465.6 ±

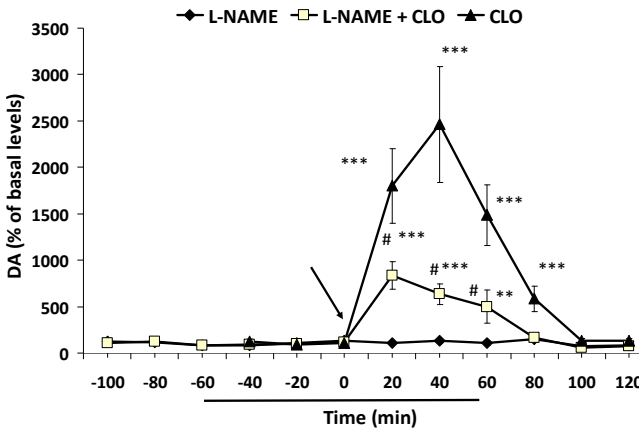

Figure 2. Effect of perfusion of 3,5 mM clothianidin and 0,1 mM on dopamine extracellular levels in the striatum. Infusion of L-NAME is shown by the black bar and clothianidin infusion started at the time indicated by arrow. Dopamine levels are expressed as the variation of porcentage with respect to basal (100%), calculated as the mean of the two fist samples before the administration of the treatments. Values are mean ± S.E.M. (n = 5-7). Signification levels: ***$P \leq 0.001$ respect to basal levels and #$P \leq 0.05$ respect to clothianidin group.

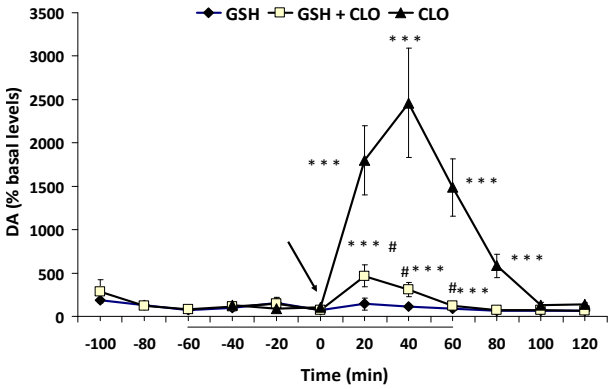

Figure 3. Effect of perfusion of 3,5 mM clothianidin and 0,4 mM glutathione on dopamine extracellular levels in the striatum. Infusion of glutathione is shown by the black bar and clothianidin infusion started at the time indicated by arrow. Dopamine levels are expressed as the variation of porcentage with respect to basal (100%), calculated as the mean of the two fist samples before the administration of the treatments. Values are mean ± S.E.M. (n = 5-7). Signification levels: ***$P \leq 0.001$ respect to basal levels and #$P \leq 0.05$ respect to clothianidin group.

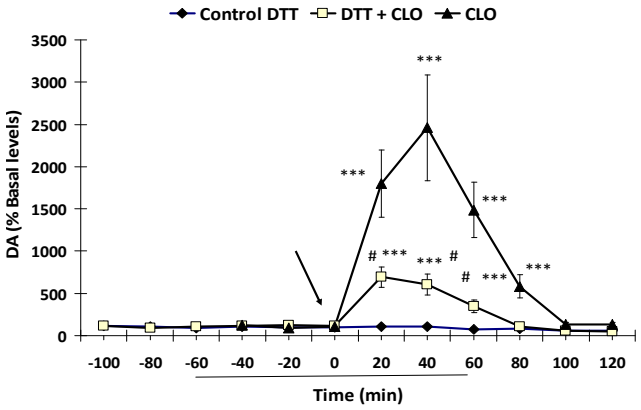

Figure 4. Effect of perfusion of 3,5 mM clothianidin and 0,005 mM dithiothreitol on dopamine extracellular levels in the striatum. Infusion of dithiothreitol is shown by the black bar and clothianidin infusion started at the time indicated by arrow. Dopamine levels are expressed as the variation of porcentage with respect to basal (100%), calculated as the mean of the two fist samples before the administration of the treatments. *Values are mean ± S.E.M. (n = 5-7). Signification levels:* ***$P \leq 0.001$ respect to basal levels and #$P \leq 0.05$ respect to clothianidin

126.8% ($P \leq 0.001$), with respect to basal levels. This result indicates that the administration of GSH results in an inhibition of 81% in the effect generated by the infusion of clothianidin alone (Figure 3).

Administration of 0.005 mM DTT has not produced significant alterations in the striatal dopamine levels. Administration of 3.5 mM clothianidin associated with 0.005 mM DTT induced an increase in extracellular dopamine levels in the striatum of 693.8 ± 117.8% ($P \leq 0.001$), with respect to basal levels. This increase is 72% lower that the generated by clothianidin alone (Figure 4)

Discussion

It is 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 and in a less amount in the thalamus [16]. *In vivo* and *in vitro* studies suggest that activation of NMDA glutamatergic receptors facilitates the release of dopamine in the striatum of rats [17-18] and that stimulation of these receptors in the dopaminergic terminals induces release of dopamine [19].

In our experiments, we assessed the potential involvement of NMDA glutamatergic receptors in clothianidin-induced dopamine release in the striatum. Thus, administration of clothianidin in animals pre-treated with APV, a competitive antagonist of NMDA receptors [20-22], reduced a 55.7 % the extracellular dopamine levels induced by clothianidin. These results may indicate that the *in vivo* release of dopamine induced by clothianidin is partially dependent on the activation of NMDA glutamatergic receptors.

Moreover, there are *in vitro* and *in vivo* evidences that the dopaminergic system is influenced by nitric oxide. These results were obtained from *in vitro* [23-26] and *in vivo* studies (27 [26-30]. Furthermore, the striatum is characterized as an area with a high density of NMDA glutamatergic receptors [31] and with a high activity of NOS [32].

Therefore, in this work, we also evaluated the possible involvement of nitric oxide in the effect of clothianidin on striatal dopamine release using a NOS inhibitor. So, the administration of clothianidin in animals pretreated with L-NAME (an inhibitor of NOS), reduced a 70 % the extracellular dopamine levels induced by clothianidin.

Both results obtained by us, taken together, may indicate that the *in vivo* release of dopamine induced by clothianidin is partially dependent on the activation of NMDA glutamatergic receptors and the production of nitric oxide in rat striatum. So, activation of NMDA receptors in nitrergic neurons induces calcium input into the terminal; calcium binds to calmodulin, originating calcium-calmodulin complexes, activating the NOS and inducing the production of nitric oxide ([33]. Nitric oxide 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 [29], in which nitric oxide stimulates dopamine release [25-26].

Based on our results we can infer that clothianidin may be inducing the release of dopamine in the striatum via two mechanisms:

1) Clothianidin may be acting on cholinergic receptors located on glutamatergic terminals, stimulating the release of glutamate. Glutamate released would act on NMDA receptors present on dopaminergic terminals enabling and facilitating the release of dopamine.

2) Clothianidin may be inducing the release of glutamate in glutamatergic terminals by activating cholinergic receptors present in these terminals. Glutamate, in turn, would act on NMDA receptors in nitrergic neurons, activating NOS and producing nitric oxide, which would act directly on the dopaminergic neuron facilitating dopamine release.

Therefore, blocking NMDA receptors or inhibiting NO production it would be produced a decrease in the clothianidin effect on dopamine release.

The mechanism by which nitric oxide increases dopamine levels is not well clarified. Some authors have suggested that nitric oxide causes a reduction in the activity of the dopamine transporter (DAT), thereby increasing extracellular dopamine levels [33], which represents a new way in the interneuronal communication without any communication mediated by synaptic receptors [34] By contrast, others authors have suggested that the main interneuronal effect of nitric oxide is the activation of the enzyme guanylate cyclase, which triggers an increase in the cyclic guanosine monophosphate (cGMP), which, in turn, causes increased levels of dopamine by activation of Ca^{2+} channels [35 -38].

However, the inhibition of NMDA receptors, as well as the inhibition of NO production, partially decreased but does not completely block the effect of clothianidin on the release of dopamine in the striatum. This would indicate that such mechanisms would be complementary and not the only ones generating the effect of clothianidin on dopamine release.

GSH is an essential tripeptide in mammalian cells, which plays an important role in cellular resistance to oxidative damage, through the provision of enzymes involved in the metabolism of ROS. GSH eliminates potentially toxic products originated from the oxidative reactions, by reducing their oxidized thiol groups to disulfide groups, in the presence of oxygen. It is considered an important agent for protection against aggressions produced by chemical agents [8]

Because a common approach to oxidative stress research is exogenous administration of compounds that have antioxidant properties, to test if GSH and DTT could have any effect on striatal dopamine release induced by clothianidin, each one of two substances were given together with the pesticide through the microdialysis probe.

The results showed that the administration of GSH produced a reduction (81%) of dopamine release induced by clothianidin. Similarly, DTT administration together with clothianidin generated an increase in extracellular dopamine levels 72% lower than that generated by clothianidin alone.

These results are consistent with other *in vivo* microdialysis studies that demonstrated the protective effects of GSH on the striatal dopamine release in animals treated with pesticides or other neurotoxic substances, such as mercury [39-40].

So, the decrease in dopamine release observed after pretreatment with antioxidants could occur through a direct binding of these compounds with clothianidin. This would result in a reduced availability of the pesticide to interact with nicotinic receptors, generating a smaller effect on dopamine release.

An interaction between antioxidant and toxics as a possible mechanism of protection against neurotoxicity was observed in a comparable study conducted by Faro et al (2005). They observed a decrease in the mercury-induced *in vivo* release of striatal dopamine in animals pretreated with GSH and cysteine. However, the observed decrease was significantly greater when GSH was given with mercury (without pretreatment) that when administered prior to mercury (pre-treatment). According to these authors, the greatest protective effect observed when mercury was coadministered with GSH may mean that the two substances interact before reaching the striatum (still in the syringe of microdialysis), which would reduce the effective concentration of the toxic [39]

Another possibility to explain the protective effect observed by us is that the antioxidants could interact with the cholinergic receptors. In this regard, in a study conducted in superior cervical ganglia of rat, Kwiatkowski and Brown (1976) observed that DDT acts on nicotinic receptors altering their properties, decreasing the membrane depolarization induced by carbacol, a neuronal nicotinic agonist. Similarly, the antioxidants used in this study, including DDT, could be altering, in some way, certain properties of nicotinic receptors. Because the alteration of these receptors, the pesticide can present difficulties when binding to those receptors, and consequently it can induce a lower neuronal membrane depolarization and decrease the release of dopamine in the striatum.

Our study demonstrates that neonicotinoids not only generate effects on the neurological system of mammals (dopaminergic neurotransmission) but also these effects appear to be significantly reversed by exogenous administration of certain antioxidants, which suppose a simple protection mechanism against the damage caused for such type of insecticides.

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