



The Chronic Oral Administration of Clobenzorex or Amphetamine Decreases Motor Behavior and Induces Glial Activation in the Striatum Without Dopaminergic Degeneration

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Abstract

Described as amphetamine-like due to their structural and stimulant similarities, clobenzorex is one of the five most commonly used drugs in Mexico for the treatment of obesity. Various studies have shown that amphetamines induce dopaminergic neurotoxicity and neuroinflammation in the striatum, symptoms which are associated with motor damage. For this reason, the present study aimed to evaluate the effect of chronic clobenzorex administration on motor behaviors, TH immunoreactivity, gliosis, and the neurodegenerative process in the striatum and substantia nigra pars compacta (SNpc). The present research was conducted on three experimental groups of male Wistar rats: the vehicle group, the amphetamine group (2 mg/kg), and the clobenzorex group (30 mg/kg). All groups were subject to oral administration every 24 h for 31 days. Motor activity and motor coordination were evaluated in the open field test and the beam walking test, respectively. The animals were euthanized after the last day of treatment to enable the extraction of their brains for the evaluation of tyrosine hydroxylase (TH) levels, the immunoreactivity of the glial cells, and the neurodegeneration of both the striatum and SNpc via amino-cupric-silver stain. The results obtained show that amphetamine and clobenzorex administration decrease motor activity and motor coordination in the beam walking test and cause increased gliosis in the striatum, while no significant changes were observed in terms of immunoreactivity to TH and neurodegeneration in both the striatum and SNpc. These results suggest that the chronic administration of clobenzorex may decrease motor function in a manner similar to amphetamine, via the neuroadaptive and non-neurotoxic changes caused to the striatum under this administration scheme.

Keywords Amphetamine · Clobenzorex · Motor behaviors · Striatum · Gliosis · Neurodegeneration

Introduction

Clobenzorex hydrochlorate, trade name Asenlix, is an N-substituted compound of S (+) - amphetamine. In Mexico, it is one of the five main anorectic drugs used to treat

obesity (Espinoza-Franco and Morín-Zaragoza 2013). Although the US Food and Drug Administration withdrew clobenzorex from the market some years ago, there is evidence of its availability along the USA-Mexico border (Tong et al. 2002). A controlled drug sold only by prescription, clobenzorex is available on the illicit market, where it is sold as a recreational drug (Cornaert et al. 1986; Baumevieuille et al. 1997). Athletes have been reported to use it to improve their performance (Docherty 2008), while, in Brazil, it has been identified in pills seized from truck drivers who consume them to remain awake and reduce fatigue when driving long distances (Moreira et al. 2016).

Clobenzorex and amphetamine derivatives are metabolized into amphetamine and excreted in the urine (Glasson et al. 1971; Valtier and Cody 2000). Some studies have shown that the consumption of clobenzorex produces hepatotoxicity (Tong et al. 2002) and cardiovascular side effects such as heart failure, arterial hypertension, and pulmonary artery

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hypertension (Cornaert et al. 1986; Seferian et al. 2013; García-Alonso et al. 2019). However, its toxic effects on the central nervous system are unknown. Young et al. showed that clobenzorex exerts stimulating effects on the central nervous system in a manner similar to that exerted by amphetamines (Young et al. 1997). Furthermore, it has been shown that, after oral administration, up to 15% of the initial clobenzorex dose is metabolized to d-amphetamine (Valtier and Cody 2000), which could contribute to the prolongation of its stimulating effects.

Amphetamines and their derivatives (methamphetamine, or METH, and methylenedioxi-methamphetamine, or MDMA) are neurotoxic psychostimulant drugs that induce short- and long-term effects. In the short term, amphetamines cause euphoria, increased heart rate, hypertension, hyperthermia, excitement, alertness, and wakefulness (Homer et al. 2008; Moratalla et al. 2017). The chronic administration of amphetamine in laboratory animals leads to dopaminergic neurotoxicity, a finding which has generated considerable concern about the use of these compounds both in clinical practice and recreationally (Eisch et al. 1992). The alteration of the function of the vesicular monoamine transporter (VMAT) and the dopamine reuptake protein (DAT) promote increased dopamine (DA) levels (Sulzer et al. 2005; German et al. 2015). Furthermore, amphetamines can increase serotonergic (5HT) and noradrenergic neurotransmission (Heal et al. 2013), while the long-term use of amphetamines has been associated with cognitive dysfunction and motor deficits in both humans and animal models (Ornstein et al. 2000; Volkow et al. 2001; Moratalla et al. 2017). Amphetamine-induced motor behavior deficits are mainly related to neurotoxicity in the nigrostriatal dopamine system (Walsh and Wagner 1992; Ares-Santos et al. 2014). This dopaminergic neurotoxicity is characterized by a decrease in DA and its metabolites and a decrease in the levels of the enzyme tyrosine hydroxylase (TH) and the dopamine transporter in the striatum (Quinton and Yamamoto 2006). Acutely high amphetamine doses have been shown to induce neurodegeneration in the striatum and SNpc (Ricaurte et al. 1982; O'Callaghan et al. 1995; Ares-Santos et al. 2014).

Studies conducted on the neurotoxicity mechanism of amphetamines have involved oxidative stress, excitotoxicity, mitochondrial dysfunction, energy failure, and neuroinflammation (Cadet et al. 2007; Yamamoto et al. 2010; Halpin et al. 2014). Most of said studies show damage to the dopaminergic nerve terminals in METH or MDMA binge-dosing models (Krasnova and Cadet 2009; Moratalla et al. 2017). Various studies have shown that amphetamine exposure induces neuroinflammation (Krasnova and Cadet 2009; Northrop and Yamamoto 2014), glial reactivity (which includes the release of cytokines, chemokines, reactive oxygen, and nitrogen species), and blood-brain barrier dysfunction (Kousik et al. 2012;

Clark et al. 2013). The activation of microglia and astrocytes is a well-characterized response to amphetamines that induce damage to the monoamine nerve terminals of the striatum (O'Callaghan et al. 1995; Thomas et al. 2004; Friend and Keefe 2013). To date, the long-term effects of clobenzorex on cellular mechanisms and motor behavior are unknown, despite it being an amphetamine-type drug and subject to illicit use. The present study aimed to evaluate the effects of the chronic administration of clobenzorex on motor behaviors, TH immunoreactivity, glial cell reactivity, and the neurodegenerative process in the striatum and SNpc.

Materials and Methods

Animals

Adult male Wistar rats (350–450 g) ($n = 42$) were obtained from the *Claude Bernard* animal facilities at the *Benemérita Universidad Autónoma de Puebla* (BUAP). The animals were individually housed in groups of three–four per cage under constant temperature conditions ($22 \pm 2^\circ\text{C}$) and a 12-h light/dark cycle (lights on at 08.00), with food and water given ad libitum. All experimental procedures complied with the Guide for the Care and Use of Laboratory Animals set out in the corresponding Official Mexican Standard (NOM-062-ZOO-1999) and were approved by the BUAP bioethics committee.

Drug Treatments

(+) Amphetamine hydrochloride (Sigma-Aldrich Ltd., St. Louis, MO, USA) and clobenzorex hydrochloride were dissolved in isotonic saline solution (ISS) to obtain final concentrations of 1 mg/mL and 9 mg/mL, respectively. The amphetamine and clobenzorex doses were 2 mg/kg and 30 mg/kg, respectively. The clobenzorex dose was selected in accordance with that reported by Young et al. (1997), who found that clobenzorex is 20 times less potent than amphetamine. The amphetamine dose was selected based on that reported by Armstrong et al. (2004), who observed that it was the minimum repeated amphetamine dose for which an astrocytosis response has been observed. The pharmacological treatments prepared shortly before daily oral administration for 31 days from 14:00 to 16:00 pm (Fig. 1).

Rectal Temperature

The rectal temperature of the animals was monitored using a digital thermometer (Citizen CTA301) lubricated with Vaseline and inserted into the rectum of the animals up to a depth of 2 cm. The basal temperature was recorded prior to the administration of the drugs and every 2 h post-administration.

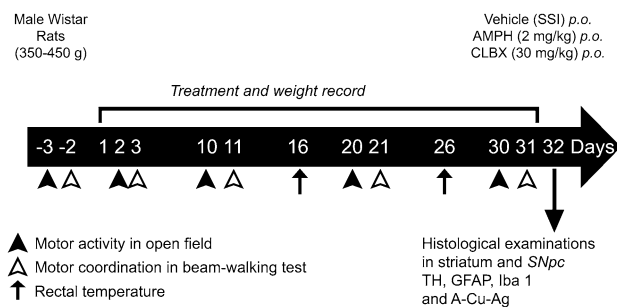


Fig. 1 Scheme of the experimental sequence. Three groups were obtained: vehicle (SSI); clobenzorex (30 mg/kg); and amphetamine (2 mg/kg). Every 10 days, the animals were tested for motor activity and motor coordination prior to the administration of the drugs. Finally, the histological examinations were performed in the striatum and SNpc

Motor Behaviors

The time sequences for the behavioral tests are shown in Fig. 1. The motor behaviors presented by the animals were evaluated before the daily administration of each drug, in order to assess the consequences of the previous doses administered on motor function. Motor activity in the open field test was evaluated on days 2, 10, 20, and 30 post-treatment, while the narrow beam walking test was conducted on days 3, 11, 21, and 31 post-treatment. All the behaviors were carried out at 08:00–12:00 h and a temperature of 22 ± 2 °C, in a sound-insulated room.

Motor Activity in the Open Field Test

The animals were subject to a motor activity test four times over the course of the open field model experiment (Opto-Varimex 5, Columbus Instruments, OH, USA). This test evaluates the distance travelled (cm), the average speed (cm/s), and the number of rearings made over a 15-min period. The present study used transparent acrylic boxes, 44 cm long by 40 cm wide and 20 cm high, for this test, with the data generated then captured, recorded, and analyzed in the software Auto-Track.

Assessment of Motor Balance and Coordination in the Narrow Beam Walking Test

The motor coordination of the animals was evaluated in the narrow beam walking test in accordance with the protocol previously reported by Patricio et al. (2019). The narrow beam consisted of a wooden beam, 120 cm long, 2.5 cm wide, and 2.5 cm high and suspended 43 cm off the ground on wooden supports. A dark box, 15 cm × 30 cm × 20 cm was placed at the end of the beam. Each animal was trained to use the beam for one day four days prior to the

administration of each drug, undergoing five trials, with a 1-h interval between each trial, until it could cross the beam and reach the dark box. If the rat stopped on the beam and reached the dark box. If the rat stopped on the beam to look around, the researcher pushed it gently from behind with their fingers. Once the animals had been trained on the beam, the administration of each of the drugs of interest began on day 1, with motor coordination analyzed again on days 3, 11, 21, and 31 of administration. Evaluating the latency to cross the beam and the number of errors, each test comprised three trials with a maximum duration of 90 s, with a 1-h interval applied between trials. An error was defined as any incident of slipping on the surface of the beam, loss of balance, or a fall from the beam (Lezcano 2009; Biernaskie 2004), with a fall recorded as the maximum time permitted. The task was considered as completed when the rat had completely placed all four limbs in the dark box at the opposite end of the beam.

Histological Examinations

One day after the final administration of each drug, all animals ($n = 14$ per group) were anesthetized and intracardially perfused with 200–250 mL 4% paraformaldehyde in borate buffer (pH = 7.4). The brains were removed and stored in a 30% sucrose solution, after which 50- μ m coronal sections of the striatum and the SNpc were obtained in a vibratome (Leica VT1000S). The sections intended for A-Cu-Ag staining were kept in 4% paraformaldehyde in borate buffer, while the sections to be used for the immunohistochemical analysis for TH, GFAP, and Iba-1 were kept in phosphate-buffered saline (PBS, pH 7.4). The striatum sections were taken, approximately, at 1.7 to -0.4 mm, while the SNpc sections were taken, approximately, -5.2 to -6.0 mm from the bregma.

Immunohistochemistry Analysis for Tyrosine Hydroxylase

The coronal sections taken from each subject were washed three times in 0.2% PBS-Triton X-100 for 10 min in order to impregnate the tissue. The endogenous peroxidase was deactivated via H_2O_2 (3%) and methanol (10%) incubation for 20 min, with the tissue samples then washed three times with PBS-Triton for 10 min each wash. The non-specific binding sites were blocked by means of incubation in IgG-free 2% bovine serum albumin (BSA, Sigma) for 60 min, with the sections then incubated for two nights at 4 °C with the monoclonal mouse antibody anti-tyrosine hydroxylase (TH) (Merck Millipore, USA), diluted to 1:1000. After 48 h of incubation with the primary antibody at 4 °C, the brain tissue sections were incubated in biotinylated mouse secondary antibody (Vector Laboratories, Burlingame, CA) and exposed to an avidin-biotin

complex solution (Thermo Fisher Scientific). Finally, the tissue sections were incubated with 0.05% diaminobenzidine and 0.03% hydrogen peroxide and then mounted on gelatin-coated slides, dehydrated in an ascending series of alcohols, cleared in xylene, and coverslipped using PolyMount (PolyScience, USA).

The images of the striatum and SNpc were observed using a Leica DM/1000 microscope (Leica DM/1000 Microsystems) at 1.5× and 10×, respectively, while the photographs were taken using a Jenoptik ProgRes C14 plus digital camera and ProgRes software.

The TH levels in the striatum were quantified using the ImageJ program to graph the percentage of the area that had been stained. The number of TH-positive neurons in the SNpc was established using an image analysis tool based on the ImageJ program. The data are represented as the number of TH immunoreactive neurons in the SNpc.

Immunohistochemistry for GFAP and Iba-1

Immunoreactivity to glial fibrillary acidic protein (GFAP) and ionized calcium-binding adaptor molecule 1 (Iba1) were used to identify astrocytes and microglia cells, respectively. The presence of GFAP and Iba1 was determined by means of the use of specific antibodies in free-floating brain sections, which were permeated with 0.2% Triton X-100 in PBS for three wash cycles, each comprising three 10-min washes. The non-specific binding sites were blocked via incubation in IgG-free 2% bovine serum albumin (BSA, Sigma) for 60 min. The coronal brain sections were incubated overnight at 4 °C in the corresponding antibody—either polyclonal anti-GFAP rabbit antibody, diluted to 1/500 (Dako, Denmark A/S), or rabbit anti-Iba1 monoclonal antibody, diluted to 1/500 (Wako Pure Chemical Industries, Ltd. Osaka, Japan). Primary antibody labeling was undertaken using a secondary conjugated rhodamine (GFAP) and visualized in the red channel, while the primary antibody was recognized using a secondary conjugated FICT (Iba1) and visualized in the green channel. The sections were mounted on gelatin-subbed glass slides and covered with Mowiol-DABCO resin (Sigma-Aldrich). The immunostained cells in the dorsomedial and dorsolateral striatum were observed through a Leica DM/1000 microscope (Leica DM/1000 Microsystems) at 20×, while the photographs were taken using a Jenoptik ProgRes C14 plus digital camera and the ProgRes software.

The digitized images were converted into Tagged Image File Format files to facilitate subsequent analysis. For GFAP and Iba1, solely the total area of the photomicrograph that presented fluorescent staining was analyzed, with the threshold then chosen from the vehicle group and applied to all experimental groups.

Amino-cupric-silver (A-Cu-Ag) Stain

Neuronal degeneration was analyzed by means of A-Cu-Ag staining, applied in accordance with the protocol previously reported by Ares-Santos et al. (2014), which specifically stains the dendritic trees and cell bodies of damaged neurons and axons during the early or later stages of neurodegeneration (de Olmos et al. 1994; Switzer 2000). The immunostained cells of the striatum were observed through a Leica DM/1000 microscope at 20× and those of the SNpc at 1.5×. The percentage of the area that was stained was calculated using the ImageJ image analysis program to convert color intensities to grayscale, with the threshold chosen for the vehicle group.

Statistical Analysis

The data were analyzed by means of a Shapiro-Wilk normality test, with the data presenting parametric behavior analyzed using a one-way or two-way ANOVA and presented here as mean ± standard error of the mean (SEM). Relevant differences were analyzed pairwise via a Student's *t*-test or a post hoc Tukey test. The data presenting non-parametric behavior were analyzed using a Kruskal-Wallis test and Dunn's multiple comparisons test. The criterion for significance was $p < 0.05$.

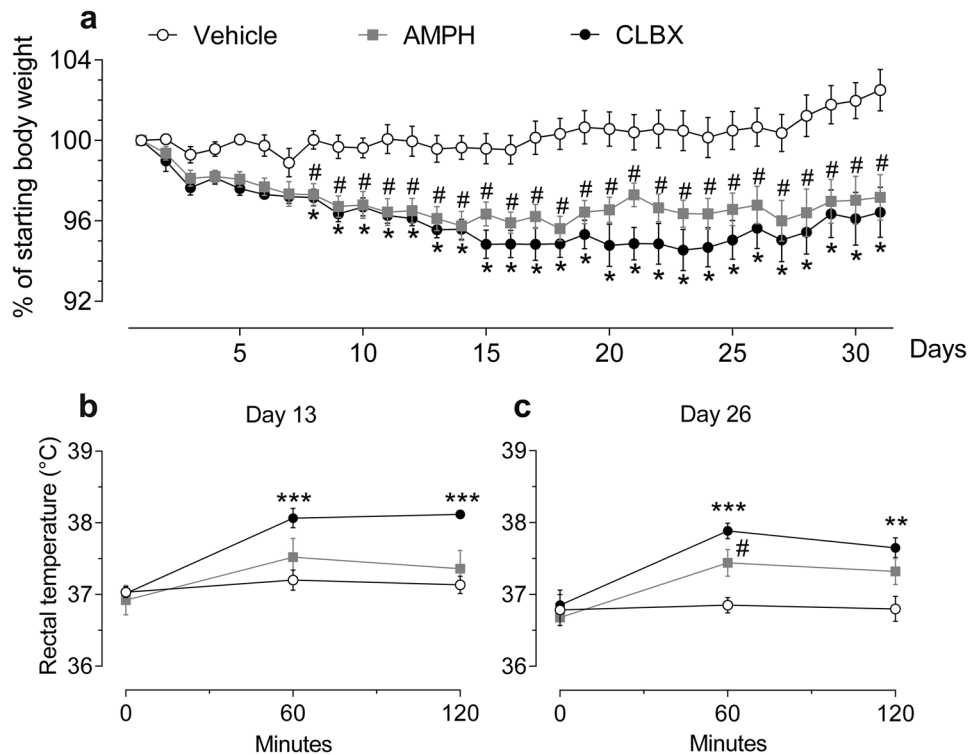
Results

The Chronic Oral Administration of Clobenzorex or Amphetamine Decreases Body Weight and Increases Rectal Temperature

Clobenzorex or amphetamine treatment was administered for 31 days, with the results obtained showing that both treatments decreased the body weight of treated animals significantly more than that of the control group ($p < 0.05$) (Fig. 2a).

The rectal temperature of the subjects was measured at 0, 60, and 120 min on the 13th and 26th days post-treatment. On day 13, the rats administered with amphetamine did not show significant changes in rectal temperature; however, those administered with clobenzorex showed a significant increase in temperature from minute 60 onwards (mean 38.0), which remained until minute 120 (mean 38.11) and was higher than that recorded for the control group (mean 37.2; 37.1) (Fig. 2b). By day 26, it was found that both the amphetamine and clobenzorex groups presented a significantly higher rectal temperature, at minute 60 (mean 37.4; 37.8), than that found for the vehicle group (mean 36.8). At minute 120, only the clobenzorex group presented a higher temperature than the vehicle group (Fig. 2c).

Fig. 2 Chronic administration of clobenzorex or amphetamine decreases body weight and modifies rectal temperature. **(a)** Clobenzorex and amphetamine decrease body weight. Data represent mean \pm SEM, $n = 14$ per group. **(b, c)** Clobenzorex induces increased body temperature on days 13 and 26 of treatment. Data represent mean \pm SEM. Statistical analysis was conducted via two-way ANOVA for repeated measures and a Tukey post-test. * $p < 0.05$ clobenzorex vs vehicle; ** $p < 0.01$ clobenzorex vs vehicle; *** $p < 0.001$ clobenzorex vs vehicle; # $p < 0.05$ amphetamine vs vehicle



Similar to Amphetamine, Oral Clobenzorex Administration Decreases Locomotor Activity

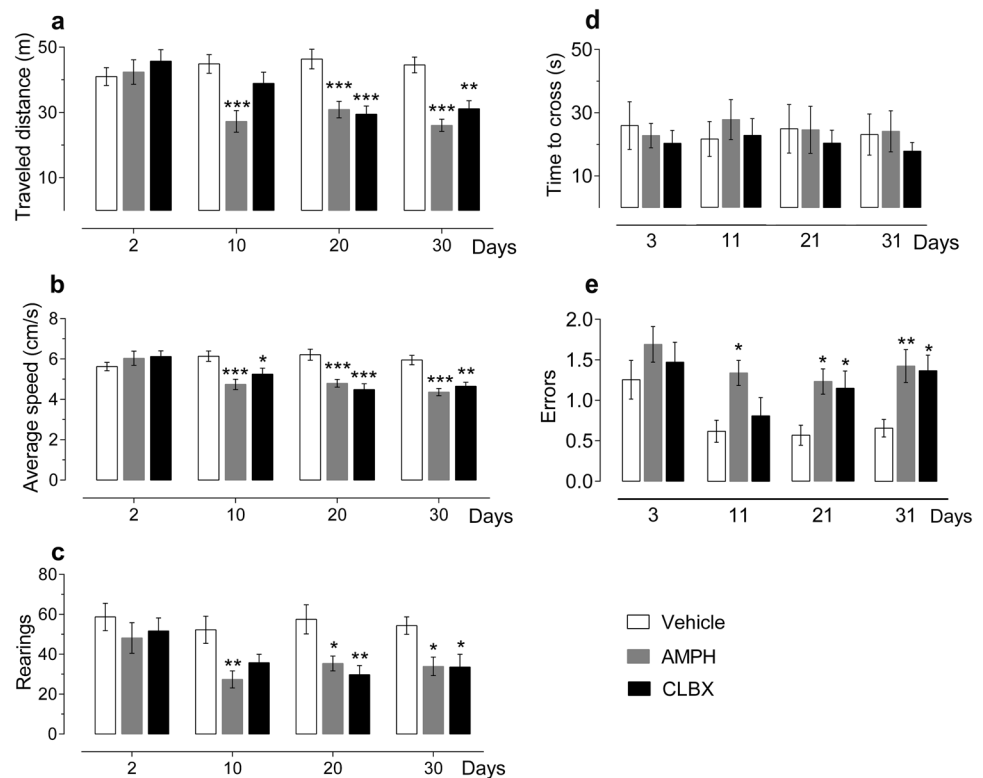
Motor activity was evaluated in the open field test on days 2, 10, 20, and 30 post-treatment prior to the daily administration of clobenzorex or amphetamine. The results obtained show that clobenzorex or amphetamine treatment decreases horizontal and vertical locomotor activity from the tenth day post-treatment onwards. The distance traveled in meters on day 2 did not change for the vehicle (mean 41.0), amphetamine (mean 42.4), and clobenzorex (mean 45.7) groups. The amphetamine treatment was found to achieve a significantly lower distance traveled in the open field model, on days 10 (mean 27.3; $p < 0.001$), 20 (mean 30.9; $p < 0.001$), and 30 (mean 26.1; $p < 0.001$), than that observed for the vehicle group (mean 44.9; 46.4; and 44.6). Similarly, treatment with clobenzorex was found to achieve a significantly lower distance traveled, by days 20 (mean 38.9; $p < 0.001$) and 30 (31.1; $p < 0.01$), than that observed for the vehicle group (Fig. 3a). The average speed (cm/s) observed on day 2 did not change for the vehicle (mean 5.6), amphetamine (mean 6.0), and clobenzorex (mean 6.1) groups. The amphetamine treatment was observed to have reduced the average speed by days 10 (mean 4.7; $p < 0.001$), 20 (mean 4.8; $p < 0.001$), and 30 (mean 4.4; $p < 0.001$) significantly more than that observed for the vehicle group (mean 6.1; 6.2; and 5.9). The clobenzorex treatment had decreased the average speed by days 10 (mean 5.2; $p < 0.05$), 20 (mean 4.5; $p < 0.001$), and

30 (4.6; $p < 0.01$) significantly more than that observed for the vehicle group (Fig. 3b). The number of rearings recorded on day 2 did not change for the vehicle (mean 58.7), amphetamine (mean 48.2), and clobenzorex (mean 54.6) groups. The amphetamine treatment had reduced the number of rearings by days 10 (mean 27.4; $p < 0.01$), 20 (mean 35.4; $p < 0.05$), and 30 (mean 33.6; $p < 0.05$) significantly more than that observed for the vehicle group (mean 51.1; 52.2; and 54.3). Similarly, the clobenzorex treatment had decreased the number of rearings by days 20 (mean 29.8; $p < 0.01$) and 30 (33.6; $p < 0.05$) significantly more than that observed for the vehicle group (Fig. 3c). The findings of the present study show that clobenzorex induces a deficit in locomotor activity in a similar way to amphetamine.

Similar to Amphetamine, the Oral Administration of Clobenzorex Impairs Motor Coordination

Motor coordination was evaluated in the beam walking test on days 3, 11, 21, and 31 post-treatment, prior to the daily administration of clobenzorex or amphetamine. The time taken by each of the experimental groups to cross the beam was quantified, although no significant differences were found for the vehicle, clobenzorex, and amphetamine groups (Fig. 3d). On the other hand, in terms of the number of errors made by the animals, the amphetamine and clobenzorex groups had not shown significant changes by day 3. Treatment with amphetamine was found to have increased the

Fig. 3 Chronic clobenzorex or amphetamine administration decreases locomotor activity in the open field test and motor coordination. Motor activity was evaluated by (a) distance travelled, (b) average speed, and (c) number of rearings. (d) Time to cross beam and (e) number of errors in the narrow beam test. Data show mean \pm SEM, $n = 14$ per group. Statistical analysis was conducted via two-way ANOVA for repeated measures and a Tukey post-test. * $p < 0.05$ vs vehicle; ** $p < 0.01$ vs vehicle; and *** $p < 0.001$ vs vehicle



number of errors by days 11 (mean 1.3, $p < 0.05$), 21 (mean 1.2, $p < 0.05$), and 31 (mean 1.4; $p < 0.01$) significantly more than that observed for the vehicle group (mean 0.6, 0.6, and 0.7). The group administered with clobenzorex had shown an increase, in the number of errors by days 21 (mean 1.1, $p < 0.05$) and 31 (mean 1.4; $p < 0.05$) post-administration, that was higher than that observed for the vehicle group (Fig. 3e). The results indicate that motor behavior is affected by clobenzorex and amphetamine treatment.

Chronic Oral Administration of Clobenzorex or Amphetamine Modifies Neither Tyrosine Hydroxylase Levels Nor Neurodegeneration

The present study aimed to ascertain whether the motor effects induced by clobenzorex or amphetamine were related to damage sustained to the dopaminergic system. To this end, on day 31, the percentage area of the striatum that was stained and, thus, the level of TH were established, while both the number of TH-positive cells in the SNpc and the level of neurodegeneration were evaluated via A-Cu-Ag staining (Figs. 4 and 5). The results obtained show that treatment with either amphetamine (mean 83.1) or clobenzorex (mean 83.0) does not modify the percentage area stained for the TH enzyme in the striatum to a greater degree than that observed for the control group (mean 82.5) (Fig. 4d). The evaluation of the SNpc revealed that treatment with amphetamine (mean 171.3) or clobenzorex (mean 154.0) did not

modify the number of +TH cells to a greater degree than that observed for the control group (mean 153.2) (Fig. 5d). Furthermore, treatment with amphetamine (mean 0.041, 0.34) or clobenzorex (mean 0.12, 0.35) did not increase neurodegeneration in the striatum and SNpc significantly more than the control group (mean 0.05, 0.50) (Figs. 4e and 5e).

The Chronic Oral Administration of Clobenzorex or Amphetamine Increases Gliosis in the Striatum

Immunohistochemical staining via GFAP and Iba-1 was applied to determine astrocyte and microglial activation (Figs. 6 and 7). The results obtained showed that 31 days after the oral administration of clobenzorex, the levels of GFAP and Iba-1 in the dorsomedial striatum (DMS) increased by 11.8% and 3.4%, respectively, while the vehicle group presented a GFAP-stained area of 1.7% and an Iba-1-stained area of 1.7% ($p < 0.001$). The amphetamine group showed larger percentage areas of GFAP and Iba-1 staining in the DMS, 4.4% and 2.9%, respectively, than those observed for the vehicle group ($p < 0.05$). Moreover, it was observed that the clobenzorex group presented a larger percentage area stained by GFAP than that observed for the amphetamine group ($p < 0.01$) (Fig. 6).

The immunoreactivity of GFAP and Iba-1 in the dorsolateral striatum (DLS) was also analyzed (Fig. 7). The results obtained found that the oral administration of clobenzorex caused a significantly higher percentage of the area of the

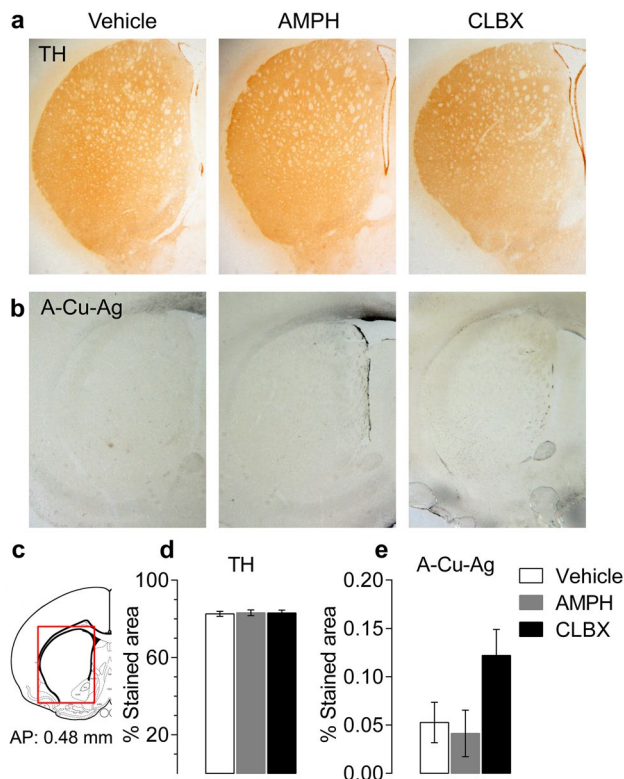


Fig. 4 Chronic clobenzorex or amphetamine administration does not modify TH expression and degeneration in the striatum. Photomicrographs show (a) the immunoreactivity of TH, (b) amino-cupric-silver (A-Cu-Ag) staining, and (c) scheme of striatum localization in a rat brain coronal section. (d) Histograms show the percentage of the total area stained for TH in the striatum. The values show mean \pm SEM, $n = 6$ per group. The statistical analysis was conducted via one-way ANOVA and a post hoc Tukey test. (e) Histograms show the percentage of A-Cu-Ag staining in the striatum. The values show mean \pm SEM, $n = 4$ per group. Statistical analysis was conducted via a Kruskal-Wallis test and Dunn's multiple comparisons

DLS that was stained, 6.9% and 3.9% for GFAP and Iba-1 ($p < 0.001$, $p < 0.01$), respectively, than that observed for the vehicle group (1.7% for the GFAP-stained area and 2.1% for the Iba-1-stained area). The amphetamine group showed greater percentages of GFAP and Iba-1-stained areas in the DLS, 3.9% and 3.3%, respectively, than those observed for the vehicle group ($p < 0.05$). The findings of the present study show that the chronic administration of clobenzorex or amphetamine increased GFAP and Iba-1 levels in both the DMS and DLS.

Discussion

Clobenzorex is a drug used for both therapeutic purposes and recreationally (Cornaert et al. 1986; Baumeville et al. 1997; Tong et al. 2002; Docherty 2008; Espinoza-Franco and Morín-Zaragoza, 2013; Moreira et al. 2016).

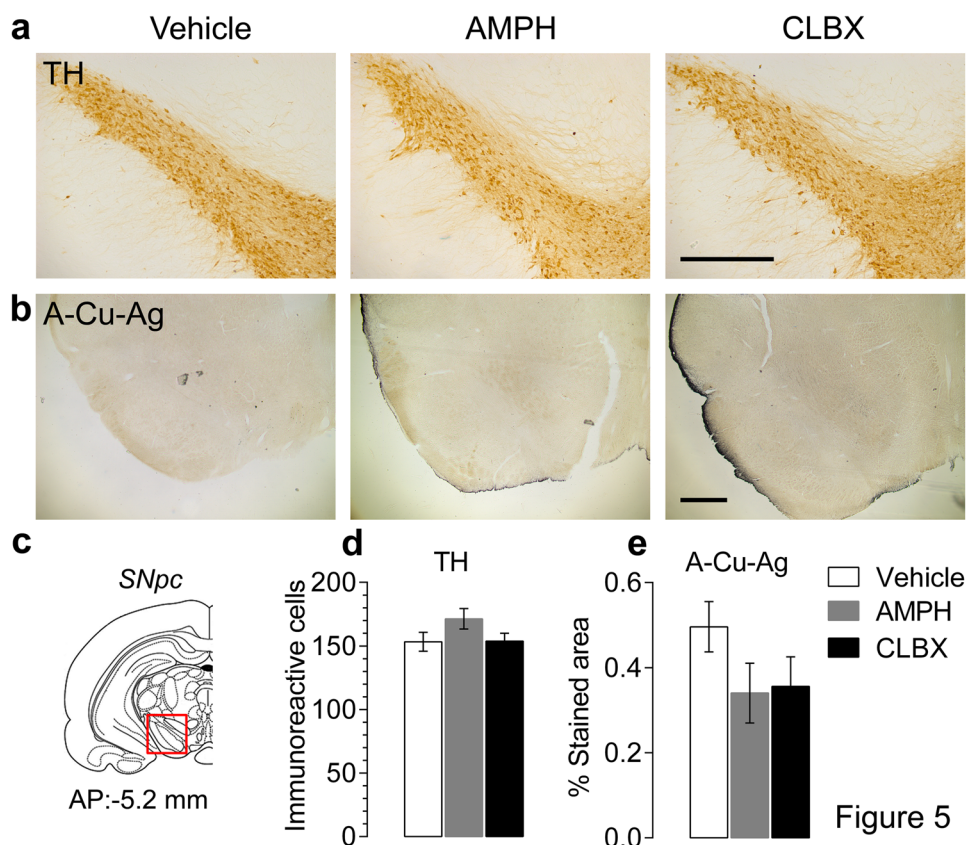
Therefore, there is a critical need to understand the cellular mechanisms involved in triggering both toxic effects in the brain and motor behavior. The present study found that chronic treatment with clobenzorex or amphetamine causes a decrease in locomotor activity and an alteration in motor coordination. The alterations in motor function were accompanied by the activation of glial cells in the dorsal striatum, with no evidence found of nigrostriatal dopaminergic neurotoxicity after chronic exposure. In addition, a decrease in body weight and an increase in body temperature were observed.

The results obtained indicate that treatment with clobenzorex or amphetamine reduces the subject's body weight and coincide with the findings of other research groups that show that the chronic administration of METH at low doses causes a decrease in body weight (Lloyd et al. 2017; Thanos et al. 2016). It has been proposed that the decrease in body weight observed with the administration of amphetamine is attributed to the decreased food intake caused by the appetite suppression associated with this drug (Shu-Chen et al. 2014). This suggests that the doses of amphetamine and clobenzorex used in the present study have anorexic effects. The appetite suppression induced by amphetamines (including clobenzorex) is mediated by increased levels of monoamines in the paraventricular nucleus of the hypothalamus, while the activation of the noradrenergic (Wellman et al. 1993) and serotonergic (Bovetto and Richard 1995) systems is involved in suppressing food intake. Acute exposure to amphetamines has been shown to cause weight loss, which has been associated with changes in peripheral hormones such as ghrelin, leptin, and the neuropeptide Y (Kobeissy et al. 2008a, b). Both peripheral and central mechanisms could be used to mediate the decrease in body weight induced by the chronic administration of amphetamine or clobenzorex, as evidenced in the present study.

Additionally, the daily intraperitoneal administration of METH at 4 and 8 mg/kg in rats for 16 weeks has been shown to decrease both body weight and food intake (Thanos et al. 2017) due to changes in the peripheral signals that control satiety. A decrease in food intake has been found with the use of METH in humans and rodents and is related to a modification in leptin, ghrelin, and neuropeptide Y signaling (Comer et al. 2001; Kirkpatrick et al. 2019; Kobeissy et al. 2008a, b). This finding rules out the conclusion that the reduction in locomotor activity observed in the present study is due to reduced food consumption, as the neuronal circuits involved are different.

On days 13 and 26 of the present study, the administration of clobenzorex caused an increase in body temperature 120 min after administration. The increase in body temperature induced by clobenzorex could be related to the activation of the neuroendocrine axis, as well as the release of monoamines at a central and peripheral level, an effect also induced

Fig. 5 Chronic clobenzorex or amphetamine administration does not modify the expression of TH or degeneration in the SNpc. Photomicrographs show the immunoreactivity of TH (a) and amino-cupric-silver (A-Cu-Ag) staining (b). (c) Scheme of SNpc localization in a rat brain coronal section. (d) Histograms show the number of TH immunoreactive cells in the SNpc. The values show mean \pm SEM, $n = 6$ per group. Statistical analysis was conducted via one-way ANOVA and a post hoc Tukey test. (e) Histograms show the percentage area of A-Cu-Ag staining in the SNpc. The values show mean \pm SEM, $n = 4$ per group. Statistical analysis was conducted via a Kruskal-Wallis test and Dunn's multiple comparisons. Bar indicates 500 μ m

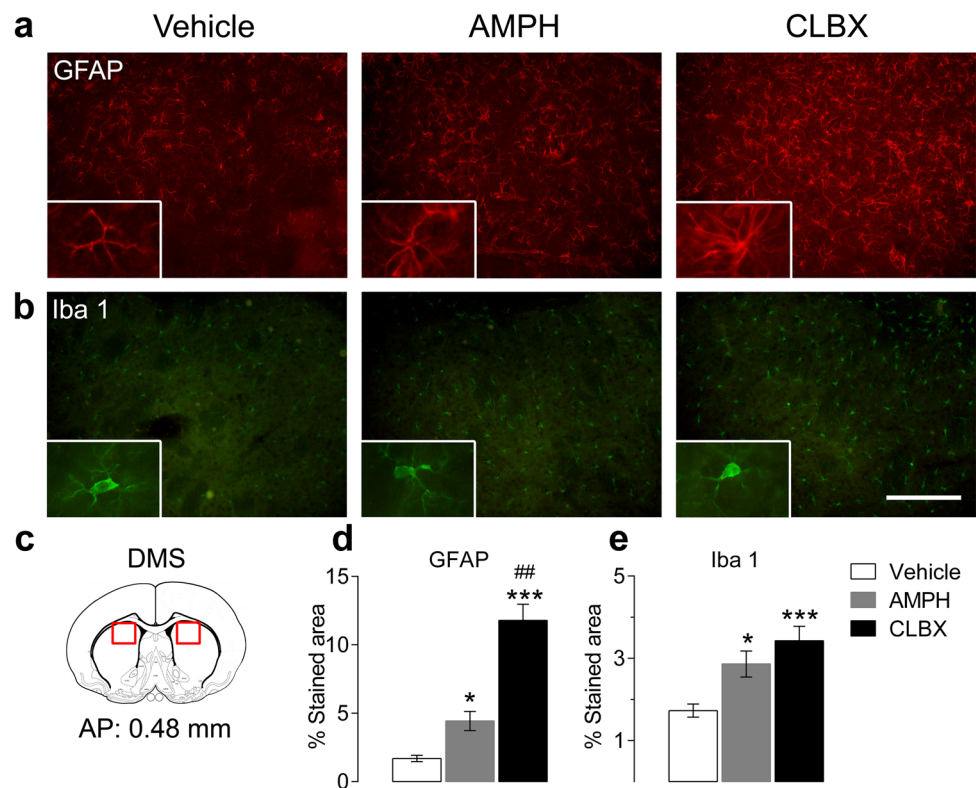


by amphetamine derivatives (Matsumoto et al. 2014). Thermogenesis induced via METH or MDMA has been shown to promote norepinephrine release in the sympathetic nervous system, the activation of the hypothalamic-pituitary-thyroid axis, and glucocorticoid secretion in the adrenal cortex (Makisumi et al. 1998; Sprague et al. 2003). Clobenzorex treatment in humans has not been associated with hyperthermia at therapeutic doses (Espinoza-Franco and Morín-Zaragoza 2013). Our results show that the administration of clobenzorex significantly increases rectal temperature without reaching hyperthermia, with, on day 26, the amphetamine group presenting an increase in rectal temperature 60 min post-administration, which is due to the low dose (2 mg/kg) administered in the present study. In binge-dosing models, the lowest dose of amphetamine or METH capable of producing hyperthermia is 3–4 mg/kg (Levi et al. 2012; McConnell et al. 2015).

It is well-described that locomotor activity is stimulated by the acute administration of amphetamines (Hirabayashi et al. 1981; Young et al. 1997; Heal et al. 2013), including clobenzorex (Young et al. 1997). The persistent administration of amphetamine stimulants has been shown to damage motor behavior in animal models (Walsh and Wagner 1992; Wallace et al. 2001; Timár et al. 2003; Ares-Santos et al. 2014; Moratalla et al. 2015). However, the foregoing studies evaluate motor impairment after the acute administration of high

doses of METH or MDMA. For the first time, the present study shows that prolonged treatment with clobenzorex has detrimental effects on locomotor activity. The chronic administration of amphetamine shows a decrease in locomotor activity that occurs even earlier than that observed for the clobenzorex group. This disparity indicates that clobenzorex takes longer than amphetamine to produce the same impairment in motor function. Furthermore, treatment with clobenzorex and amphetamine resulted in a significant increase in the number of errors observed in the beam walking test, evidencing a decrease in fine motor coordination. These results suggest that chronic treatment with clobenzorex results in impaired motor behavior similar to that observed with amphetamine treatment. Impaired motor function, specifically the impairment of both gross motor function performance and fine motor coordination, has been reported in chronic METH abusers (Volkow et al. 2001; Kalechstein et al. 2003). These findings could explain the risk of fatal traffic accidents for truck drivers who use stimulants to keep themselves awake (Logan 1996; Crifasi and Long 1996; Gates et al. 2013). Epidemiological studies conducted between 1998 and 2015 have found that amphetamines are one of the main drug types associated with traffic accidents (Gjerde et al. 2015). Furthermore, a positive relationship between blood amphetamine concentrations and impaired driving ability has been demonstrated (Gustavsen et al. 2006). Thus, the motor deficits induced by the administration of

Fig. 6 Chronic clobenzorex or amphetamine administration induces expression of GFAP and Iba-1 in the dorsomedial striatum. Photomicrographs of the dorsomedial striatum (DMS) section, stained with GFAP (a) and Iba-1 (b). (c) Scheme of the DMS in a rat brain coronal section. Histograms show the proportional GFAP-ir (d) and Iba-1-ir (e) stained areas. The values show \pm SEM, $n = 4$ per group. Statistical analysis was conducted with a Kruskal-Wallis analysis and Dunn's multiple comparisons test. * $p < 0.05$ vs vehicle; *** $p < 0.001$ vs vehicle; ## $p < 0.01$ vs amphetamine. Bar indicates 200 μ m



clobenzorex could have an impact on the motor behavior of those truck drivers who consume it.

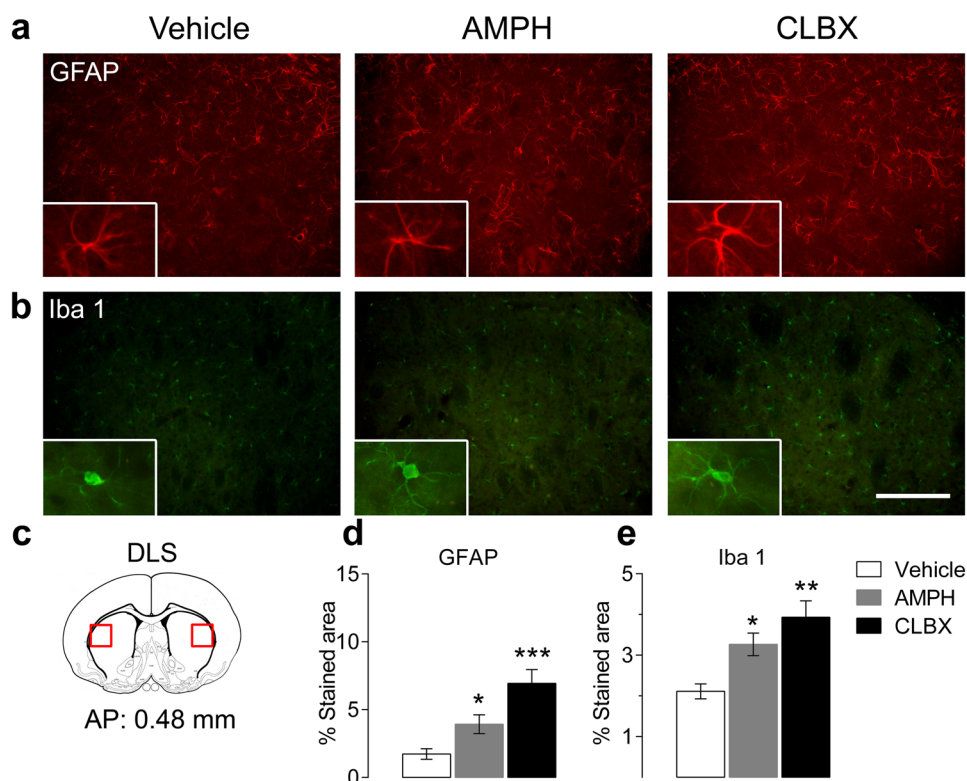
Amphetamine-induced motor disturbances mainly occur due to dopaminergic neurotoxicity in the dorsal striatum in binge-dosing models (Walsh and Wagner 1992; Moratalla et al. 2017). However, the present study shows that the 31-day administration of amphetamine or clobenzorex does not result in the loss of dopaminergic terminals in either the striatum or the respective cell bodies of the SNpc. The evaluation of neurodegeneration via A-Cu-Ag staining conducted in the present research revealed no neurodegeneration in either brain region. These results suggest that the decreased motor function caused by the chronic administration of clobenzorex is not related to a decrease in nigrostriatal dopaminergic innervation. Le Cozannet et al. (2013) showed that the intravenous self-administration of METH in rats induces hypocomotion without presenting dopaminergic decrease in the striatum. However, other studies have shown alterations in striatal dopaminergic neurotransmission after prolonged exposure to METH (Segal et al. 2005; Lacán et al. 2013; Thanos et al. 2016), without the loss of SNpc cells (Lacán et al. 2013). This suggests that neuroadaptive changes occur in the striatal dopaminergic system, in response to prolonged exposure to clobenzorex and amphetamine that result in decreased locomotor activity and motor coordination.

On the other hand, a single dose of amphetamine (10 mg/kg) has been shown to damage the dopaminergic system

via mechanisms such as excitotoxicity, oxidative stress, and energy failure (mitochondrial dysfunction) (Tung et al. 2017). Another study, which measured striatal DAT, TH, and DA levels, found that the acute administration of four 5 mg/kg amphetamine injections at 2-h intervals decreases the integrity of the dopaminergic terminals (Angoa-Perez et al. 2013). However, despite the significant evidence of the neurotoxicity induced by amphetamine, there is little evidence demonstrating its effect on neurodegeneration. Ricaurte et al. (1984) showed that the continuous administration of amphetamine or methamphetamine, at a dose of 4 mg/kg for 3 days, increases the amount of fine granular argyrophilic debris indicative of the degeneration of striatal fibers. However, it has been shown that a single injection of a high dose of amphetamine, 30 mg/kg, does not induce neuronal death in the rat striatum, as determined 72 h after treatment using the Fluoro-Jade C stain (Marques et al. 2008). The present study found no evidence of neurodegeneration in either the striatum or the SNpc after 31 days of clobenzorex or amphetamine treatment. It should be noted that Ricaurte et al. did find evidence both of the induction of the neurodegenerative process via the administration of amphetamine at a dose higher than that used in the present study and of the short-term toxic effects of amphetamine.

One possible mechanism caused by chronic exposure to clobenzorex or amphetamine is the downregulation and/or

Fig. 7 Chronic clobenzorex or amphetamine administration induces GFAP and Iba-1 expression in the dorsolateral striatum. Photomicrographs of the dorsolateral striatum (DLS) section, stained with GFAP (a) and Iba-1 (b). (c) DLS scheme in a rat brain coronal section. Histograms show the proportional area of GFA-ir (d) and Iba-1-ir (e) staining. The values show mean \pm SEM, $n = 4$ per group. Statistical analysis was conducted with a Kruskal-Wallis analysis and Dunn's multiple comparisons test. * $p < 0.05$ vs vehicle; ** $p < 0.01$ vs vehicle; *** $p < 0.001$ vs vehicle. Bar indicates 200 μ m



desensitization of DA receptors. The chronic administration of METH in rats has been shown to induce a decrease in D1 and D2 receptor levels (Segal et al. 2005; Thanos et al. 2017). Furthermore, a decrease in D2 receptor density has been demonstrated in non-human primates after chronic amphetamine treatment (Ginovart et al. 1999). The decreased D2 receptor levels induced by amphetamine seem to depend on the internalization mediated by arrestin-3 (Skinbjerg et al. 2010), while, in addition, a desensitization of the D2 receptor has been observed after chronic METH administration (Lacán et al. 2013).

It has been reported that one neurotoxic response to the administration of amphetamines or METH is glial reactivity, which triggers a neuroinflammation response (O'Callaghan et al. 1995; Thomas et al. 2004; Friend and Keefe 2013). METH increases reactive microglia levels in the striatum, hippocampus, cortex, and SNpc. METH also increases GFAP immunoreactivity in the striatum (Ares-Santos et al. 2014) and indusium griseum (Carmena et al. 2015). The present study found that chronic treatment with clobenzorex or amphetamine induces a significant increase in immunoreactivity to GFAP and Iba-1 in the dorsomedial and dorsolateral striatum, indicating the activation of astrocytes and microglia, respectively (Sofroniew and Vinters 2010; Ito et al. 1998). Although it is not clear exactly how amphetamines induce neuroinflammation, previous research has involved the activation of glutamatergic receptors and DA toxicity, as it is related to the induction of oxidative stress

through the production of ROS, quinomas, and semiquinones (Thomas and Kuhn 2005; Kuhn et al. 2006; Shah et al. 2012; Northrop and Yamamoto 2014). Increased extracellular levels of DA and glutamate have been found in response to certain amphetamines (Nash and Yamamoto 1992). Therefore, it is reasonable to postulate that the activation of astrocytes and microglia induced by the administration of clobenzorex may be a consequence of the sustained release of DA, and probably glutamate, which promotes the formation of reactive oxygen species and generates oxidative stress.

Astrocytic reactivity is likely to be triggered at an early point during clobenzorex treatment, even before motor disturbances become apparent. Data obtained by our research group show that 15 days after treatment with clobenzorex, a significant increase in the number of GFAP-positive cells occurs (Supplementary Figure 1). These findings suggest that the motor dysfunction induced by chronic clobenzorex or amphetamine treatment may be related to the neuroinflammatory response observed in the striatum. It is important to mention that astrocytes, unlike microglia, play a protective role by increasing reduced glutathione (GSH) levels, increasing growth factor and molecule levels for axonal regeneration (Ares-Santos et al. 2014). These findings are probably related to the increased GFAP immunoreactivity observed 15 days after clobenzorex treatment. In summary, the results of the present study show that chronic treatment with clobenzorex at anorectic or amphetamine doses results in decreased motor function in rats. Although, under these

treatment conditions, the nigrostriatal dopaminergic pathway was not itself affected, it did promote glial reactivity. These findings could have significant implications for the use of clobenzorex in the treatment of obesity, its consumption by truck drivers, and its deleterious effect on motor skills. However, more studies are required to explore the consequences of non-therapeutic and prolonged doses related to the abuse of clobenzorex and/or amphetamine (see Supplementary Figure [Materials and Methods](#)).

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s12640-021-00395-1>.

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