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Anxiolytic activity of paraoxon is associated with alterations in rat brain glutamatergic system

Zohreh Zare[a], Mohsen Tehrani[b], Noorollah Rezaei[a], Babak Dana Ghalebarzand[c], Moslem Mohammadi[d,*]

[a] Department of Anatomical Sciences, Molecular and Cell Biology Research Center, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran
[b] Department of Immunology, Gastrointestinal Cancer Research Center, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran
[c] Department of Physiology & Pharmacology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran
[d] Department of Physiology & Pharmacology, Molecular and Cell Biology Research Center, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran

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ABSTRACT

Exposure to organophosphate (OP) compounds leads to behavioral alterations. To determine whether paraoxon has effects on anxiety, anxiety-like behaviors were assessed in paraoxon-exposed rats. Protein expression of glutamate transporters has also been measured in hippocampus and prefrontal cortex. Three doses of paraoxon (0.3, 0.7, or 1 mg/kg) or corn oil (vehicle) were intraperitoneally injected to adult male rats. At 14 or 28 days after exposure, behavioral tests were done using elevated plus-maze (EPM) or open field tests. Thereafter, animals were sacrificed and both hippocampi and prefrontal cortices were extracted for cholinesterase assay and western blotting. Animals treated with convulsive doses of paraoxon (0.7 and 1 mg/kg) showed an increase in percentage of time spent in open arms and percentage of open arm entries in the EPM. In the open field test, an increase in the time spent in central area was observed in rats treated with the same doses of paraoxon. These effects of paraoxon were independent of any changes in locomotor activity. There was an increase in both astrocytic glutamate transporter proteins (GLAST and GLT-1) in the hippocampus of animals treated with 0.7 and 1 mg/kg of paraoxon. In the prefrontal cortex, protein levels of the GLAST and GLT-1 increased in 0.7 and decreased in 1 mg/kg groups. Only a significant decrease in EAAC1 protein was observed in the prefrontal cortex at 14 days following exposure to 1 mg/kg of paraoxon. Collectively, this study showed that exposure to convulsive doses of paraoxon induced anxiolytic-like behaviors in both behavioral tests. This effect may be attributed to alterations of glutamate transporter proteins in the rat hippocampus and prefrontal cortex.

1. Introduction

Anxiety is defined as an emotional response to internal and vague stimuli and characterized by behavioral, hormonal, and autonomic reactions. Anxiety disorders are among the most common mental disorders that affect many people in different countries (Moreno-Rius, 2018; Ravindran and Stein, 2010; Tovote et al., 2015). Several neurotransmitter systems in various brain areas are involved in controlling anxiety-related behaviors. Pharmacological therapy has traditionally been concentrated on some neurotransmitters, including norepinephrine, gamma-aminobutyric acid (GABA), and serotonin (Bergink et al., 2004). Current drugs for treatment of anxiety, including benzodiazepines ($GABA_A$ receptor modulators) and selective serotonin reuptake inhibitors (SSRIs), have shown limited efficacy and several side effects such as tolerance and amnestic effect. There is a need for the development of new therapeutic agents that act through other mechanisms (Ferraguti, 2018). Recent studies indicate that anxiety disorders might originate from imbalance between excitatory and inhibitory neurotransmission in some parts of brain, such as the limbic system (Tovote et al., 2015). Therefore, agents that modulate the function of glutamatergic and/or GABAergic systems can affect the balance between excitation and inhibition and thus play a critical role in treatment of anxiety (Harvey and Shahid, 2012; Wieronska and Pilc, 2013).

Organophosphate (OP) compounds have been used as insecticides or nerve agents. Intoxication with OP insecticides is a major health problem in developing countries. Furthermore, OP threat has become increasingly important due to terrorist attacks in recent decades

* Corresponding author at: Department of Physiology & Pharmacology, Molecular and Cell Biology Research Center, School of Medicine, KM 17 Khazarabad Road, Khazar Sq, Sari, P.O. Box: 4847191971, Iran.
E-mail address: mohammadimo@yahoo.com (M. Mohammadi).

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(Albuquerque et al., 2006). OP compounds exert their acute toxicity by inhibiting acetylcholinesterase (AChE), the key enzyme in acetylcholine (ACh) breakdown. Subsequent ACh accumulation in the central and peripheral cholinergic synapse leads to a cholinergic toxicity characterized by salivation, lacrimation, muscle fasciculation, and seizure (Shih et al., 2003). Cholinergic system is closely associated with behaviors related to anxiety and depression (Gargouri et al., 2018; Mineur et al., 2013). In addition to cholinergic system, exposure to OP compounds can lead to alterations in the other neurotransmitter systems in different brain regions associated with anxiety modulation (Silva et al., 2017). Alterations in the brain serotonergic, GABAergic, and glutamatergic systems have been reported following intoxication with these compounds (Aldridge et al., 2005; Shih et al., 1991). OP-induced accumulation of acetylcholine in the synaptic cleft can modulate GABAergic and glutamatergic neurotransmission via stimulation of nicotinic presynaptic receptors (Alkondon and Albuquerque, 2004). Results obtained from our previous researches indicate the involvement of the brain GABAergic and glutamatergic systems in the acute effects of a single convulsive doses of paraoxon (Mohammadi et al., 2008; Mohammadi et al., 2016; Zare et al., 2017).

Human studies have reported a correlation between OP exposure and psychiatric disorders. Acute and chronic exposure to OP pesticides produced a consistent neurobehavioral symptoms, such as depression, anxiety, and cognitive impairments, in farm workers (Harrison and Mackenzie Ross, 2016; Reidy et al., 1992; Salvi et al., 2003). Furthermore, anxiety-related symptoms were observed in victims exposed to sarin five years after civilian terrorist attacks in Japan (Ohtani et al., 2004). Although numerous studies have been done to examine the changes in the anxiety-related behaviors following exposure to OP compounds in animal models, the results are contradictory. While some of these studies have reported increase in anxiety-like behaviors after exposure to OP compounds (Assini et al., 2005; Hashjin et al., 2013; Sanchez-Amate et al., 2001), others have demonstrated either decrease (Lopez-Crespo et al., 2007; Lopez-Crespo et al., 2009; Savy et al., 2018) or no change (Valvassori et al., 2007; Wright et al., 2010).

Given inconsistent findings mentioned above, more investigations are needed to determine the effects of OP compounds on anxiety. Paraoxon (the active metabolite of parathion(is a highly toxic chemical and used as a substitute for nerve agent in civilian laboratories (Deshpande et al., 2014a). The present study was conducted to evaluate the effects of exposure to paraoxon on anxiety-related behavior of rats using elevated plus-maze (EPM) and open field tests. Considering the importance of glutamatergic system in anxiety responses, protein expression of excitatory amino acid transporters (EAATs), including (human/rodent homolog), EAAT1/glutamate-aspartate transporter (GLAST), EAAT2/glutamate transporter-1 (GLT-1), and EAAT3/excitatory amino acid carrier 1 (EAAC1) was also investigated in hippocampus and prefrontal cortex.

2. Materials and methods

2.1. Chemicals

All chemicals were obtained Sigma-Aldrich Company, unless otherwise mentioned.

2.2. Animals

Adult male Wistar rats were housed four per cage with free access to food and water in a temperature controlled room (23 ± 2 °C) under a standard 12 h light/dark cycle (lights on at 07:00 am). All animal experiments were carried out in accordance with guide for care and use of laboratory animals approved by the Ethical Committee of the Mazandaran University of Medical Sciences, Sari, Iran (IR.MAZUMS.REC.95.2753).

2.3. Experimental protocol

Paraoxon (*O*,*O*-diethyl *O*-(4-nitrophenyl) phosphate) was dissolved in corn oil immediately before the experiment and administered via a single intraperitoneal (i.p.) injection in a volume of 1 ml/kg body weight. One sub-convulsive (0.3 mg/kg) and two convulsive (0.7 and 1 mg/kg) doses of paraoxon were used in this study, based on previous studies (Carr and Chambers, 1991; Mohammadi et al., 2016). Control animals were injected with an equal volume of corn oil as vehicle. All animals were observed for clinical signs of toxicity related to paraoxon treatment. After 14 or 28 days, animals were randomly divided into two groups to assess anxiety-like behavior and locomotor activity using EPM or open field tests. Animals were then anesthetized using diethyl ether and blood obtained via cardiac puncture in heparinized syringes. To obtain plasma, blood was centrifuged at 3000 ×*g* for 5 min. Thereafter, animals were sacrificed, brains were rapidly removed, rinsed with ice-cold saline, and hippocampi and prefrontal cortices were dissected out, one for western blotting and the other for cholinesterase assay. Tissue samples were then homogenized and stored at −70 °C until assay. To determine cholinesterase activity 1 day after exposure, another set of animals was intoxicated with the same doses of paraoxon. For each treatment group and time point, 7 rats were used for each behavioral test and 5 for biochemical assays. Animal body weights were recorded at 1, 7, 14, 21, and 28 days after exposure.

2.4. Cholinesterase activity assay

Hippocampus and prefrontal cortex were weighed and homogenized at 10% (w/v) in 0.1 M phosphate-buffered saline (PBS, pH 7.4) containing 1% Triton X-100. ChE activity was determined using the modified method of Ellman et al. (Ellman et al., 1961) as previously described (Mohammadi et al., 2008). In brief, reaction was carried out using 1.8 ml of 0.423 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) in PBS, 0.1 ml of homogenate (10 mg/ml) or diluted plasma (1:10), and 0.1 ml of 1 mM acetylthiocholine iodide (ATC) as substrate. The absorbance was spectrophotometrically measured at 412 nm for 5 min at room temperature. The ChE activity was calculated as nanomoles substrate hydrolyzed/min/ml of plasma and as nanomoles substrate hydrolyzed/min/mg protein of brain tissues. Protein concentration of the brain homogenates was determined by the method of Bradford (Bradford, 1976), using bovine serum albumin (BSA) as a standard.

2.5. Behavioral tests

Anxiety-like behaviors and locomotor activity were examined using EPM and open field tests. The behavioral tests were done either 14 or 28 days after treatment, between 08:00 am and 12:00 md under dim lighting conditions.

2.5.1. Elevated plus-maze test

Elevated plus-maze (EPM) is an experimental apparatus widely used to investigate the behavioral and neurochemical bases of anxiety (Pellow et al., 1985). EPM was made of black wood and consisted of four arms (50 × 10 cm) and a central platform (10 × 10 cm) elevated 50 cm above the floor. Two opposed closed arms had walls that were 40 cm high, whereas two opposed open arms had 1 cm edge to avoid falls. At the beginning of the test, rats were individually placed in the central platform of the maze facing an open arm and their behaviors were recorded using a camera placed above the center of the apparatus for 5 min. The following parameters were determined: the time spent in the open and closed arms and the number of entries into the open and closed arms. The percentage of time spent in the open arms, OAT% (time spent in the open arms/total time spent in the arms × 100) and the percentage of open arm entries, OAE% (number of open arm entries/number of total arm entries × 100) were calculated to assess the anxiety level of rats. Increases in the OAT% and OAE% were considered

as anxiolytic activity. In addition, total number of arm entries was measured as an index of spontaneous locomotor activity. Rats were considered to be on the central platform whenever two paws were on it and when a rat placed its all four paws into an arm, it was defined as arm entry. After each test, the maze was cleaned with 10% ethanol and dried (Hogg, 1996; Pellow et al., 1985).

2.5.2. Open field test

To further evaluate the effects of paraoxon exposure on anxiety-like behaviors and locomotor activity, another set of rats was tested in open field test at 14 or 28 days following exposure. The open field apparatus consisted of a Plexiglas box (90 cm × 90 cm) enclosed by a wall 45 cm high whose floor was divided into 16 squares. Central area defined as the 45 × 45 cm area in the center of the arena. A rat was placed in the center of the apparatus and allowed to explore it freely for 5 min. Total distance traveled (as an index of locomotor activity) and time spent in the central area (as an index of anxiety behavior) were recorded and analyzed using a video tracking system (Borj Sanat, Iran). After each test, the floor of the apparatus was cleaned with 10% ethanol.

2.6. Western blotting

Determination of glutamate transporter proteins were performed by western blotting as described previously (Mohammadi et al., 2016). Briefly, brain tissues were homogenized in radioimmunoprecipitation (RIPA) lysis buffer system (sc24948, Santa Cruz, USA). Thirty micrograms of each protein sample were loaded and run on 12% SDS polyacrylamide gels and transferred onto polyvinyl difluoride (PVDF) membranes (Santa Cruz). After blocking in PBS containing 4% BSA (Biosera, France), the membranes were incubated overnight at 4 °C with one of the following primary antibodies: rabbit monoclonal anti-EAAT1 antibody (Clone: EPR12686, Abcam, UK; 1:1000), rabbit monoclonal anti-EAAT2 antibody (clone: EPR10769(2), Abcam; 1:1000), or rabbit monoclonal anti-EAAT3 antibody (clone: EPR6774(B), Abcam; 1:10,000). After two washes with Tris-buffered saline solution with 0.1% Tween 20 (TBS-T), membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Abcam; 1:5000) as a secondary antibody. Also, mouse monoclonal anti-β-actin antibody (Santa Cruz; 1:1000) was used as a normalizer along with horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad; 1:5000) as its secondary antibody. After three washings with TBS-T, the membranes were incubated with Supersignal West Pico chemiluminescence substrate kit (Pierce, US) and imaged using the G:BOX equipped with Gene tools analysis software package (Syngene, UK). In order to remove inter-gel variability, we simultaneously loaded a single gel for one of each glutamate transporter proteins and β-actin, as a reference protein. Densities of protein bands were then normalized to those of β-actin.

2.7. Statistical analysis

Data were analyzed using the SPSS software (version 24.0). Repeated measures and two-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparison, were used for statistical analyses. Data are expressed as a mean ± S.E.M. and $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Signs of poisoning

Animals received 0.3 mg/kg of paraoxon did not clearly showed the signs of poisoning. However, within 30 min after treatment, all animals receiving two higher doses of paraoxon (0.7 and 1 mg/kg) showed clinical signs of cholinergic toxicity (such as tremor, salivation, and convulsion). Approximately 30% and 45% of animals died in 0.7 and 1 mg/kg groups, respectively. Animals that survived were selected for the experiments. Overt signs of cholinergic toxicity were disappeared by day 1.

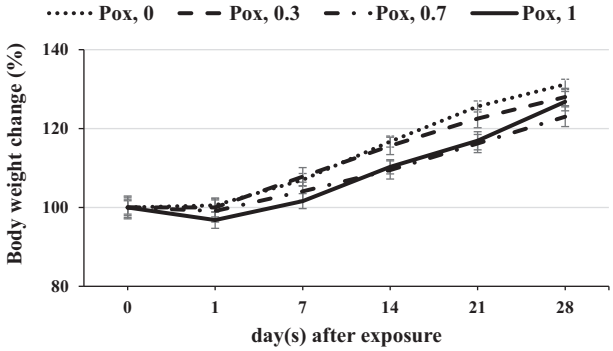

Fig. 1. Effects of treatment with paraoxon on changes in rat body weight during the experiment. Initial body weights were 215 ± 3.6, 218 ± 5.6, 222 ± 6.4, and 213 ± 4.5 in 0, 0.3, 0.7, and 1 mg/kg groups, respectively (n = 7–10 rats/group). Results are expressed as mean ± S.E.M.

3.2. Body weight

Body weight comparisons were made using repeated measures ANOVA. At the beginning of the study, there was no significant difference in body weight among groups. One day after exposure, 1 mg/kg of paraoxon caused a non-significant reduction in the body weight compared to the control group. Increasing animal body weight was significant in all groups at 14 days and thereafter ($P < 0.001$). No significant treatment-related changes were found throughout the study (Fig. 1).

3.3. Effects of paraoxon on plasma and brain ChE activity

As expected, administration of paraoxon induced a dose-dependent decrease in the plasma and brain ChE activity (Table 1). Two-way ANOVA revealed a significant main effect of treatment [$F(3, 48) = 16.5$; $P < 0.001$], time [$F(1, 48) = 81$; $P < 0.001$], and treatment × time interaction [$F(3, 48) = 13.2$; $P < 0.001$] on the ChE activity in the plasma. At 1 day after exposure with 0.3, 0.7, or 1 mg/kg of paraoxon, plasma ChE activity decreased in a dose-dependent manner by about 21%, 39%, and 52%, compared with control group, respectively. After 14 days, plasma ChE activity was completely recovered in all paraoxon treated groups. Two-way ANOVA revealed a significant main effect of treatment [$F(3, 48) = 46.4$; $P < 0.001$], time [$F(1, 48) = 106.2$; $P < 0.001$], and treatment × time interaction [$F(3, 48) = 15.6$; $P < 0.001$] on the ChE activity in the hippocampus. A significant main effect of treatment [$F(3, 48) = 69.2$; $P < 0.001$], time [$F(1, 48) = 94.7$; $P < 0.001$], and treatment × time interaction [$F(3, 48) = 15.2$; $P < 0.001$] was also observed on the ChE activity in the prefrontal cortex. Complete recovery of the brain ChE activity was observed in animals exposed to 0.3 and 0.7 mg/kg of paraoxon after 14 and 28 days, respectively. In the hippocampus, but not in the prefrontal cortex, complete recovery of the ChE activity was observed in 1 mg/kg group by day 28.

3.4. Effects of paraoxon on anxiety-related behaviors and locomotor activity

We measured anxiety-like behaviors and locomotor activity using the EPM (Fig. 2) and open-field (Fig. 3). In the EPM, there was a significant main effect of treatment [$F(3, 48) = 13.8$; $P < 0.001$], but not of time [$F(1, 48) = 0.02$; $P = 0.89$] and treatment × time interaction [$F(3, 48) = 0.23$; $P = 0.88$] on the OAT%. Tukey's post hoc comparison further revealed that at 14 days, paraoxon increased the OAT% in rats receiving 0.7 or 1 mg/kg ($P < 0.001$ and $P < 0.05$, respectively).

Table 1
Cholinesterase activity in plasma and brain of rats treated with paraoxon.

Paraoxon (mg/kg)	Time after exposure (day)		
	1	14	28
Plasma			
0	249.2 ± 10.1 A	243.2 ± 8.5 A	252.6 ± 8.9 A
0.3	198.2 ± 9.1 (21)* A	238 ± 6.3 B	255.2 ± 10 B
0.7	152.4 ± 10 (39)* A	237.6 ± 7 B	257.2 ± 9.2 B
1	119.8 ± 6.7 (52)* A	233.6 ± 7.9 B	249.6 ± 8.9 B
Hippocampus			
0	65.2 ± 2.2	64 ± 2.9	66.2 ± 3.3
0.3	45.2 ± 2.1 (31)** A	62.4 ± 2.7 B	64.4 ± 2.8 B
0.7	26.2 ± 1.9 (60)** A	52 ± 3 (19)* B	63.8 ± 1.9 C
1	18.2 ± 1.5 (72)** A	51.4 ± 3.2 (20)* B	60 ± 1.9 B
Prefrontal cortex			
0	97 ± 3.1 A	95.3 ± 2.5 A	96.4 ± 2.6 A
0.3	71.3 ± 3.9 (26)** A	93.9 ± 3.4 B	95.2 ± 5.1 B
0.7	36.1 ± 2.1 (63)** A	79 ± 5.5 (17)* B	86.4 ± 2.9 B
1	25.3 ± 1.7 (74)** A	71.5 ± 4.1 (25)** B	80.4 ± 3.9 (17)* B

Activities are expressed as nanomoles substrate hydrolyzed/min/ml plasma and nanomoles substrate hydrolyzed/min/mg protein in brain areas. Values are given as mean ± S.E.M. (n = 5 rats/group). Number in parenthesis indicates percent inhibition of cholinesterase activity. Within a row, means not followed by the same letter are significantly different ($P < 0.05$).

* $P < 0.05$ compared with the respective control groups.

** $P < 0.001$ compared with the respective control groups.

Similar significant changes were also observed in these groups after 28 days (Fig. 2A). Analysis of the OAE% also indicated a significant main effect of treatment [$F(3, 48) = 13.9$, $P < 0.001$], but not of time [$F(1, 48) = 0.88$, $P = 0.36$] and treatment × time interaction [$F(3, 48) = 0.21$, $P = 0.89$]. Further Tukey's test showed that at 14 days, OAE% increased in animals treated with 0.7 and 1 mg/kg of paraoxon ($P < 0.001$ and $P < 0.05$, respectively). Similar significant changes were also observed in these groups after 28 days (Fig. 2B). Total number of arm entries, as measures of locomotor activity, were recorded. There was no significant effect of treatment [$F(3, 48) = 1.8$; $P = 0.16$], time [$F(1, 48) = 0.14$; $P = 0.71$], and treatment × time interaction [$F(3, 48) = 0.42$; $P = 0.74$] on the total arm entries (Fig. 2C).

In the open field, there was a significant main effect of treatment [$F(3, 48) = 31$, $P < 0.001$], but not of time [$F(1, 48) = 1.5$, $P = 0.23$] and treatment × time interaction [$F(3, 48) = 1.4$, $P = 0.25$] on the time spent in the central area. Post hoc comparisons showed a significant ($P < 0.001$) increase in 0.7 and 1 mg/kg groups as compared with control group (Fig. 3A). No significant main effect of treatment [$F(3, 48) = 0.36$; $P = 0.78$], time [$F(1, 32) = 0.07$; $P = 0.8$], and treatment × time interaction [$F(3, 32) = 0.06$; $P = 0.98$] was observed in the total distance traveled (Fig. 3B).

3.5. Effects of paraoxon on glutamate transporter protein levels in hippocampus

Effects of paraoxon on the protein levels of hippocampal glutamate transporters are presented in Fig. 4. Two-way ANOVA showed a significant main effect of treatment [$F(3, 32) = 46.1$; $P < 0.001$], time [$F(1, 32) = 16$; $P < 0.001$], and treatment × time interaction [$F(3, 32) = 3.1$; $P = 0.04$] on the GLAST expression (Fig. 4A). Analysis of the GLT-1 expression indicated a significant main effect of treatment [$F(3, 32) = 31.1$, $P < 0.001$] and time [$F(1, 32) = 6.1$, $P = 0.02$], but not of treatment × time interaction [$F(3, 32) = 0.6$, $P = 0.62$] (Fig. 4B). Tukey's post hoc test revealed a significant increase in the GLAST and GLT-1 protein levels in rats treated with 0.7 or 1 mg/kg of paraoxon at both time points. There was no significant effect of treatment [$F(3, 32) = 0.1$; $P = 0.96$], time [$F(1, 32) = 1.8$; $P = 0.19$], and treatment × time interaction [$F(3, 32) = 0.4$; $P = 0.75$] on the EAAC1 protein levels (Fig. 4C).

3.6. Effects of paraoxon on glutamate transporter protein levels in prefrontal cortex

Effects of paraoxon on the glutamate transporter protein levels in the prefrontal cortex are presented in Fig. 5. Two-way ANOVA revealed a significant main effect of treatment [$F(3, 32) = 17.1$; $P < 0.001$] with no significant effect of time [$F(1, 32) = 2.8$; $P = 0.1$], as well as a significant treatment × time interaction [$F(3, 32) = 6.1$; $P < 0.005$] on the GLAST protein levels (Fig. 5A). There was a significant main effect of treatment [$F(3, 32) = 44.7$; $P < 0.001$] with no significant effect of time [$F(1, 32) = 1.4$; $P = 0.25$], as well as a significant treatment × time interaction [$F(3, 32) = 4.6$; $P < 0.01$] on the GLT-1 protein levels (Fig. 5B). Further Tukey's test showed that at 14 days, GLAST and GLT-1 protein levels increased in 0.7 mg/kg group and decreased in 1 mg/kg group as compared with control group. After 28 days, there was only a significant reduction in the level of GLT-1 protein in 1 mg/kg group. A significant main effect of treatment [$F(3, 32) = 8.3$; $P < 0.001$], but no significant effect of time [$F(1, 32) = 0.05$; $P = 0.82$] and treatment × time interaction [$F(3, 32) = 1.1$; $P = 0.35$] was observed on the EAAC1 protein levels (Fig. 5C). Decrease in EAAC1 protein level was observed in animals receiving 1 mg/kg of paraoxon at both time points, but the result was significant only at 14 days.

4. Discussion

In this study, we examined anxiety-related behaviors in rats using the EPM and open field tests as well as changes of glutamate transporter proteins in hippocampus and prefrontal cortex at 14 or 28 days after exposure to three doses of paraoxon. Results showed that 0.7 and 1 mg/kg of paraoxon induced anxiolytic-like effect in both behavioral tests and alterations in glutamate transporter proteins at both time points.

Hippocampus and prefrontal cortex were selected in this work for the following reasons: 1) There are high level of cholinergic inputs and glutamatergic innervations in these areas; 2) OP compounds have considerable effects on these brain areas (Crino et al., 2002; Francis et al., 2012); 3) These areas are known to be involved in anxiety and mood disorders (Mark et al., 1996; McHugh et al., 2004); 4) It is well recognized that cortico-hippocampal pathways to amygdala are involved in anxiety (Tovote et al., 2015).

Our results showed that following exposure to paraoxon, plasma

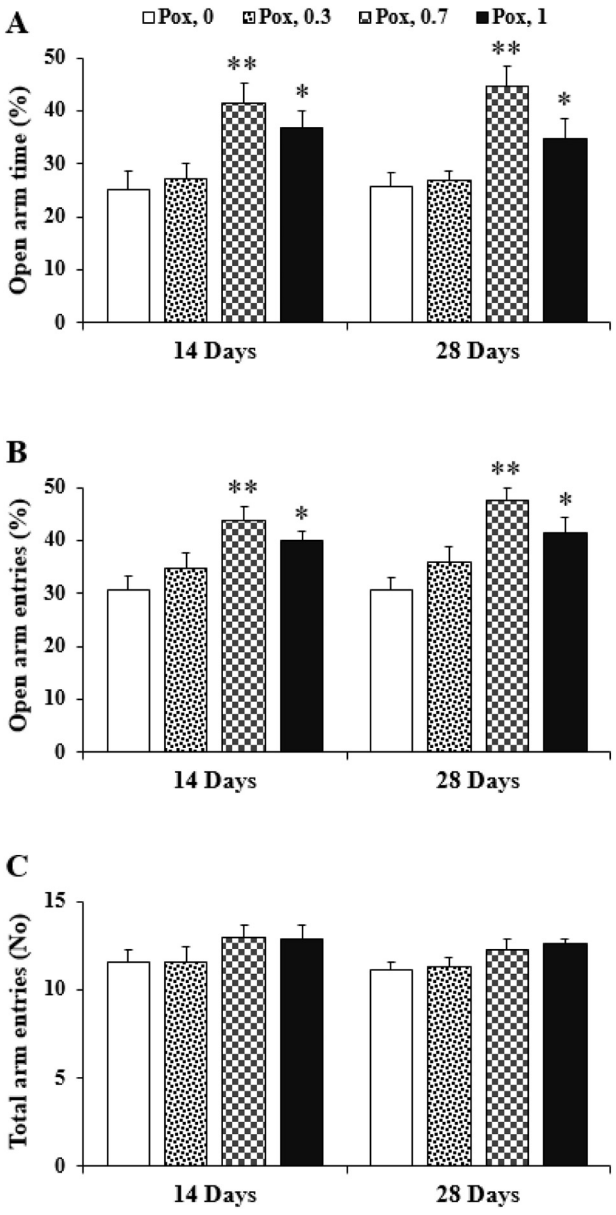

Fig. 2. Effects of paraoxon administration on the percentage of open arms time (A), open arm entries (B), and total arm entries (C) recorded on EPM 14 and 28 days after treatment (n = 7 rats/group). $^{*}P < 0.05$; $^{**}P < 0.001$ vs. control (0 mg/kg) group. Results are expressed as mean ± S.E.M.

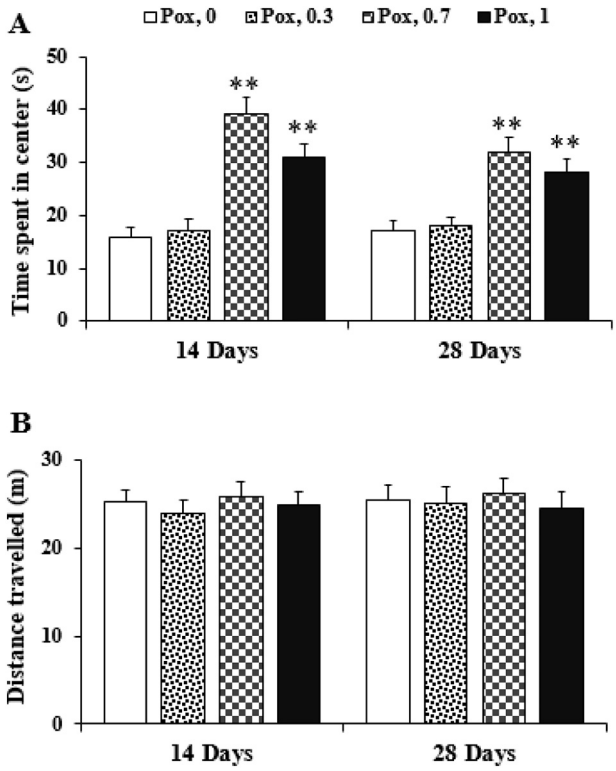

Fig. 3. Effects of paraoxon on time spent in the central area (A) and total distance traveled (B) recorded in the open field 14 and 28 days after treatment (n = 7 rats/group). Results are expressed as mean ± S.E.M. $^{**}P < 0.001$ vs. control (0 mg/kg) group.

ChE activity returned back to its basic level much faster than the brain ChE activity, indicating peripheral system recovers much faster than central nervous system. It has been reported that after systemic intoxication with OP compounds, recovery rates of ChE might vary in different brain areas (Carr and Chambers, 1991). In this study, recovery was faster in the hippocampus than in the prefrontal cortex. Due to lipophilic nature of paraoxon, storage in lipid depots must be the basis for prolonged enzyme inhibition and slow recovery of brain ChE activity after exposure to paraoxon. This is in agreement with other studies (Chambers and Carr, 1993; Chiappa et al., 1995).

EPM and open field tests were used in this study to investigate the effects of paraoxon on anxiety-related behaviors. EPM is one of the most widely used and validated devices for studying anxiety-related behaviors in rodents. Due to fear of open spaces, entry of rodents to the open arms of the maze is less likely and anxious animals refuse to enter the open arms even more. Anxiolytic agents increase the time spent in the open arms, whereas anxiogenic agents decrease it (Hogg, 1996; Pellow et al., 1985). In the current study, we observed that treatment with paraoxon at single doses of 0.7 and 1 mg/kg significantly increased the OAT% and OAE% after 14 and 28 days on the EPM test. These results demonstrated reduced anxiety-like behaviors.

To confirm the results, open field test was used, in which more anxious animals spend significantly less time in the central area of the device (Prut and Belzung, 2003). In the current study, time spent in the central area of the open field was increased in animals treated with the 0.7 and 1 mg/kg of paraoxon at both 14 and 28 days. These results were in agreement with those obtained from EPM test. Since reduced anxiety-like behaviors found only in the animals treated with 0.7 and 1 mg/kg of paraoxon, we suggested that anxiolytic effects of paraoxon is related to convulsion.

In our study, about 30 min after injection, two convulsive doses of paraoxon (0.7 and 1 mg/kg) resulted in severe signs of cholinergic toxicity, including muscle paralysis. Since OP-induced impairment of motor performance could confound anxiety-related studies, behavioral tests were done after two weeks of recovery. Concurrently, we observed that the anxiolytic effects of paraoxon were independent of any changes in locomotor activity, as no significant changes were detected in the total arm entries on the EPM and the total distance traveled on the open-field.

Contradictory results have been reported about the anxiety-related behaviors caused by various OP compounds and even with the same compound. The anxiolytic effect of paraoxon observed in our study contrasts with the previously reported anxiogenic effect of paraoxon on the EPM test (Deshpande et al., 2014b). In that study, rats were subcutaneously injected with a single high dose of paraoxon (4 mg/kg) followed by atropine sulfate and pralidoxime chloride (2-PAM) 1 min later. After 1 h, rats were rescued via administration of an optimized dose of atropine, 2-PAM, and diazepam. Surviving rats were evaluated using the EPM test for anxiety-related behaviors 3 months after exposure. The results showed that OAT% and OAE% decreased in

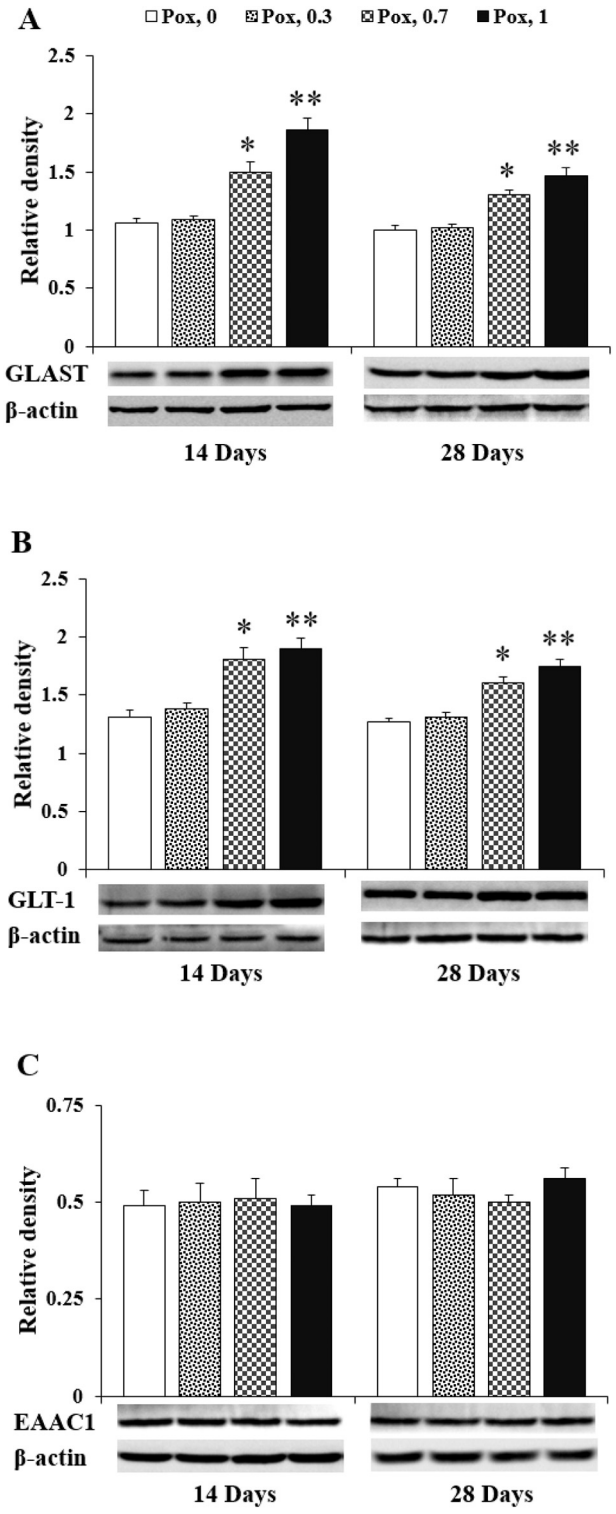

Fig. 4. Effects of paraoxon administration on the GLAST (A), GLT-1 (B), and EAAC1 (C) protein levels in rat hippocampus. Animals were treated with either vehicle (corn oil) or one of three doses (0.3, 0.7, or 1 mg/kg) of paraoxon (n = 5 rats/group). After 14 or 28 days, protein levels of glutamate transporters were measured by western blotting and densities of protein bands were normalized to those of β-actin. Results are expressed as mean ± S.E.M. $^{*}P < 0.01$; $^{**}P < 0.001$ vs. control (0 mg/kg) group.

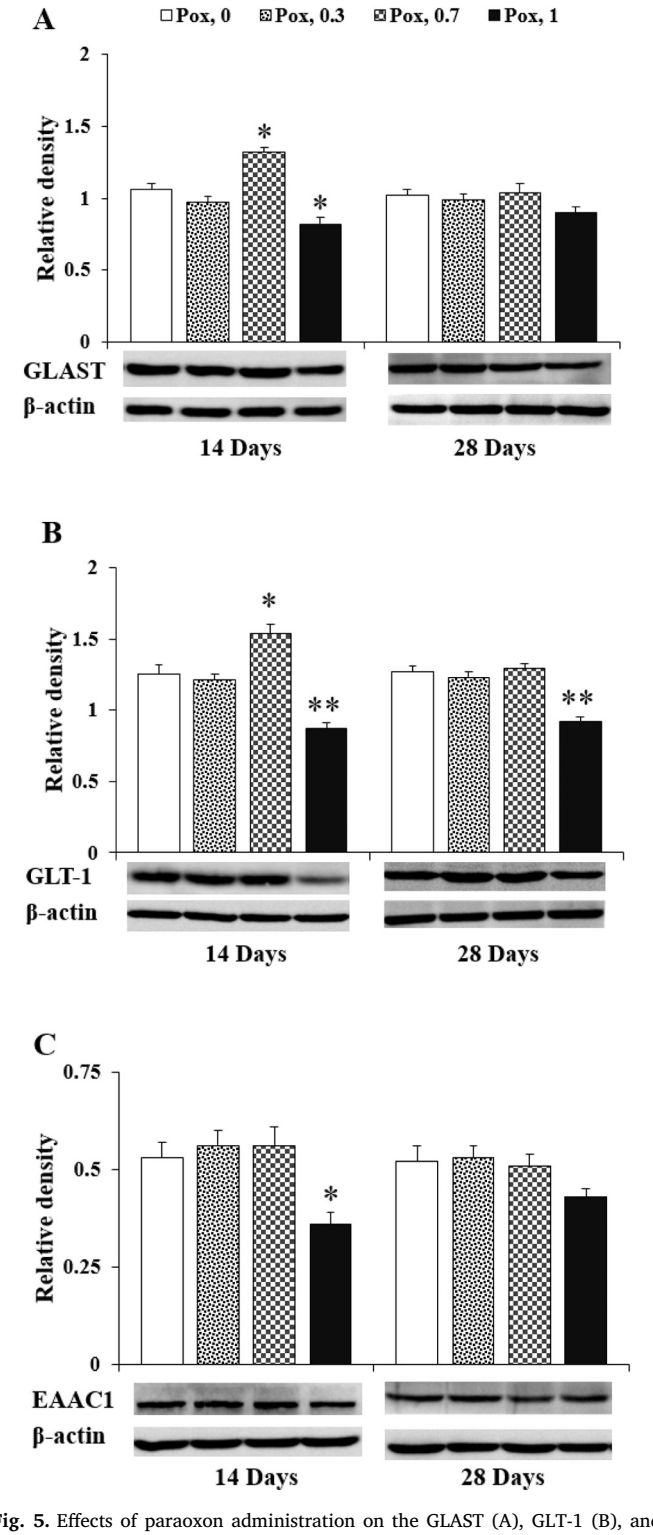

Fig. 5. Effects of paraoxon administration on the GLAST (A), GLT-1 (B), and EAAC1 (C) protein levels in rat prefrontal cortex. Animals were treated with either vehicle (corn oil) or one of three doses (0.3, 0.7, or 1 mg/kg) of paraoxon (n = 5 rats/group). After 14 or 28 days, protein levels of glutamate transporters were measured by western blotting and densities of protein bands were normalized to those of β-actin. Results are expressed as mean ± S.E.M. $^{*}P < 0.01$; $^{**}P < 0.001$ vs. control (0 mg/kg) group.

paraoxon-treated group compared to control group. This discrepancy of results between these two studies could be explained with differences in dose and route of paraoxon administration and the duration of exposure to it. The protective treatments following paraoxon administration used in Deshpande's study might be another explanation for this difference. On the other hand, chronic low level exposure to the pesticide diazinon in adult rats significantly reduced anxiety-like behaviors in marble

burying test which is in agreement with our results (Savy et al., 2018). In another study, behavioral effects of sub-chronic intoxication with low doses of methyl parathion were evaluated in male Wistar rats using the EPM test, in which animals exhibited a dose-dependent increase in time spent in open arms. The authors thus indicated a decreased anxiety level in methyl parathion-treated rats (Schulz et al., 1990). Time-dependent effects of chlorpyrifos (CPF) on the EPM have been reported in several studies. In two separate studies, anxiogenic- and anxiolytic-like effects were observed in rats after 2 and 5 days of CPF treatment, respectively (Lopez-Crespo et al., 2009; Sanchez-Amate et al., 2001). In another study, CPF administration caused anxiolytic effects on the open-field test at 2 and 5 days (Lopez-Crespo et al., 2007). Developmental exposure to CPF can alter behaviors in tasks associated with anxiety, as CPF-induced anxiolytic behaviors have been observed in rats following postnatal exposure using the EPM and emergence tests (Aldridge et al., 2005; Carr et al., 2017). However, behavioral studies have reported that gestational exposure to CPF had either anxiogenic effect in mice on EPM and light/dark box tests (Braquenier et al., 2010) or no effect in rats on EPM test (Icenogle et al., 2004). In order to investigate the possible effect of CPF on the anxiety-related behaviors of offspring rats exposed during pregnancy, female Wistar rats were orally treated with CPF on gestational days 14–20 and male offspring behaviors were examined at 21 or 70 days of life on the EPM test. The results indicated that exposure to CPF had anxiogenic effects at post-natal day 21, but not at day 70 (Silva et al., 2017). Moreover, both acute (100 mg/kg) and repeated (25 mg/kg) exposure to malathion increased anxiety-related behaviors in rats on the EPM test (Assini et al., 2005), while no significant differences in the open arm time and entry on the EPM test following acute and sub-acute exposure to different doses of malathion, suggesting that malathion had no significant effect on anxiety-like behavior in rats (Valvassori et al., 2007).

Taken together, it appears that under some conditions and depending on their chemical composition, OP compounds might induce anxiogenic or anxiolytic behaviors. Moreover, the effects of OP compounds on anxiety depend on several factors, including species and age of animals, dose and route of administration, and time of assessment. Using different devices to assess behavioral effects of OP compounds might be another reason for inconsistent results. In addition to cholinergic system, exposure to OP compounds could lead to changes in other neurotransmitter systems in the brain. Dose- and time-dependent alterations in non-cholinergic systems of the brain might be responsible for some of contradictory OP-induced behavioral effects (Assini et al., 2005; Braquenier et al., 2010; Lopez-Crespo et al., 2009; Stangherlin and Nogueira, 2014).

Increased percentage of OAT and OAE in rats treated with 0.7 and 1 mg/kg of paraoxon was associated with recovery of the plasma and brain ChE activity, indicating that non-cholinergic mechanisms could be involved in decreasing anxiety-like behaviors observed in these groups. Alteration in glutamatergic system is believed to play important roles in the pathophysiology of several neuropsychiatric diseases, such as anxiety disorders (Savy et al., 2018; Wieronska and Pilc, 2013). Moreover, cholinergic-induced seizure activity recruits brain glutamatergic system. Hyper-stimulation of glutamate receptors, especially NMDA receptors, may lead to behavioral impairments due to profound brain damages (Coubard et al., 2008). To further investigate the effect of OP exposure on anxiety mechanisms, we examined whether changes in glutamate transporter expression in the hippocampus and prefrontal cortex could affect the development of paraoxon-induced anxiolytic behaviors. In the present study, changes in the expression of glutamate transporters were found in the hippocampus and prefrontal cortex of rats 14 and 28 days after administration of convulsive doses of paraoxon. Animals exposed with 0.7 mg/kg of paraoxon expressed high levels of both astrocytic glutamate transporters (GLAST and GLT-1) compared to controls in both regions, whereas the level of neuronal glutamate transporter (EAAC1) remained unchanged. Moreover, exposure to 1 mg/kg of paraoxon increased the protein levels of GLAST and GLT-1 in the hippocampus, whereas the protein levels of all glutamate transporters decreased in the prefrontal cortex. The reduction in the level of glutamate transporters in the prefrontal cortex of animals intoxicated with 1 mg/kg might be ascribed to paraoxon-induced brain damage. Brain areas have different sensitivity to OP compounds (Wang et al., 2014) and this may explain different results obtained in hippocampus and prefrontal cortex by 1 mg/kg of paraoxon. Astrocytic glutamate transporters, especially EAAT2, play the main role in clearing glutamate from the extracellular space and limiting glutamate signaling (Danbolt, 2001). Up-regulation of astrocytic glutamate transporters following convulsive doses of paraoxon might modulate the excessive glutamate level and cell death following paraoxon-induced seizure. However, reverse transportation of glutamate by glutamate transporters following seizure might be contributed to excitotoxicity (Crino et al., 2002). EAAC1 does not seem to play a significant role in glutamate clearance, but may be more effective to limit oxidative damage by increasing cysteine uptake for glutathione synthesis by cystine/glutamate exchanger. However, efflux of glutamate through the exchanger could be harmful and contribute to excitotoxic pathology (Bridges et al., 2012).

Our findings demonstrated a relationship between number of glutamate transporter proteins and anxiety-like behaviors after exposure to paraoxon. Increasing anxiety-like behaviors following blockade of GLT-1 in the central amygdala also reinforces the association between glutamate uptake and anxiety (John et al., 2015). Reduced glutamate uptake by synaptosomes prepared from the whole brain of diphenylditoloride-treated rats has been previously reported to be associated with reduced time spent in open arms of the EPM, indicating increased anxiety behaviors (Stangherlin and Nogueira, 2014). Furthermore, decreased cerebrospinal fluid (CSF) glutamate level was correlated with anxiolytic-related behavior on the EPM in rats subjected to acute systemic guanosine administration. In addition, in hippocampal synaptosomal preparation, guanosine significantly decreased K^+-stimulated glutamate release, without affecting glutamate uptake (Almeida et al., 2017). Concurrently, patients with anxiety disorders showed increased levels of glutamate in CSF (Chakrabarty et al., 2005) and brain (Grachev and Apkarian, 2000). Taken together, an inverse correlation between anxiety and glutamate level was found in those studies.

In addition to inhibition of AChE by phosphorylating the serine hydroxyl residue, paraoxon increases protein kinase C (PKC) phosphorylation. In the study by Tian et al. (2007), paraoxon increased the phosphorylation of protein kinase C (PKC) in cultured cerebellar granule cells. Furthermore, activation of PKC produces alterations in the expression and function of all glutamate transports (Gonzalez et al., 2002; Robinson, 2006). Therefore, it can be assumed that activation of the PKC pathway by paraoxon could affect the glutamate uptake.

5. Conclusions

In summary, this study demonstrates that the anxiolytic activity of the both convulsive doses of paraoxon (0.7 and 1 mg/kg) on the EPM and open field tests was associated with alterations in the expression of glutamate transporters in the hippocampus and prefrontal cortex. Glutamate transporters, via regulation of glutamate levels in the synaptic cleft, constitute an important aspect of glutamatergic transmission. Therefore, pharmacological modulation of glutamate transporters is of significant interest and could be considered for the development a new generation of efficient anxiolytics. It remains to be investigated whether these behaviors could be attributed to changes in the glutamatergic system in other sites and other neurotransmitter systems of the brain. These topics are currently under investigation in our laboratory.

Transparency document

The Transparency document associated this article can be found, in online version.

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Conflict of interest

The authors declare no conflict of interest.

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