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Phoneutria nigriventer Tx3-3 peptide toxin reduces fibromyalgia symptoms in mice

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ABSTRACT

Fibromyalgia is characterized by the amplification of central nervous system pain with concomitant fatigue, sleep, mood disorders, depression, and anxiety. It needs extensive pharmacological therapy. In the present study, Swiss mice were treated with reserpine (0.25 mg/kg, s.c.) over three consecutive days, in order to reproduce the pathogenic process of fibromyalgia. On day 4, the administrations of the Tx3-3 toxin produced significant antinociception in the mechanical allodynia (87.16% ±12.7%) and thermal hyperalgesia (49.46% ± 10.6%) tests when compared with the PBS group. The effects produced by the classical analgesics (duloxetine 30 mg/kg, pramipexole 1 mg/kg, and pregabalin 30 mg/kg, p.o., respectively) in both of the tests also demonstrated antinociception. The administrations were able to increase the levels of the biogenic amines (5-HTP and DE) in the brain. The treatments with pramipexole and pregabalin, but not duloxetine, decreased the immobility time in the FM-induced animals that were submitted to the forced swimming test; however, the Tx3-3 toxin (87.45% ± 4.3%) showed better results. Taken together, the data has provided novel evidence of the ability of the Tx3-3 toxin to reduce painful and depressive symptoms, indicating that it may have significant potential in the treatment of FM.

1. Introduction

Fibromyalgia (FM) is described as chronic musculoskeletal widespread pain, which is associated with notable characteristic symptoms, such as psychiatric disorders (depression and anxiety), fatigue, sleep disturbances, and sexual dysfunction (especially in women). In addition, FM represents the second most common disorder that is observed by rheumatologists (Ruggiero et al., 2018; Häuser et al., 2019).

Patients with FM have high levels of substance P, glutamate, NGF, and BDNF, while they present low levels of the serotonin metabolites, noradrenaline, and dopamine (biogenic amines) in the cerebrospinal fluid (CSF) (Dadabhoy et al., 2008; Russell et al., 1992). The imbalance between these mediators can lead to increased sensory processing, with a consequent increase in the pain (Ledermann et al., 2017; Smith and Harris, 2011).

Despite it being a recurrent pathology, there is no definitive treatment for FM. The pharmacological intervention for FM is to relieve pain, to induce a better quality of sleep, to control the associated psychiatric symptoms, and to improve the physical functions. According to the FDA (Food and Drug Administration, USA), the drugs that are most effective for this condition when compared to other pharmacological agents are duloxetine and pregabalin. Carville et al. (2008) stated that the use of pramipexole is endorsed by the European League Against Rheumatism (EULAR), but that the dosing strategy is different from the doses that are used in Parkinson's disease. Although it is speculative, it is conceivable that agents, such as pramipexole, may possess beneficial effects for those

Abbreviations: 5-HTP, 5-Hydroxytryptophan; DE, Dopamine; FM, Fibromyalgia; PWT, Paw Withdrawal Threshold; CSF, Cerebrospinal Fluid.

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patients with FM (Smith et al., 2011). However, the currently available therapies exhibit limited efficacy and they can trigger several undesirable effects (Bennett et al., 2007; Choy et al., 2011; Macfarlane et al., 2017). Besides, according to the Brazilian Consensus for the treatment of fibromyalgia, pramipexole is recommended for a decrease in pain and it is frequently prescribed in clinical practices in the country (Heymann et al., 2010).

The Tx3-3 toxin is a peptide that is purified from the poison of the *Phoneutria nigriventer* spider. This toxin blocks the voltage-gated calcium channels, not selectively the P/Q and R types, and it inhibits the release of glutamate in the preparations of the encephalon synaptosomes (Gomez et al., 2002; Grishin, 1999; Peigneur et al., 2018). Studies have shown that the Tx3-3 toxin produces antinociception in neuropathic pain models (Dalmolin et al., 2017; Dalmolin et al., 2011). However, there are no investigations in the literature that mention these toxin effects in fibromyalgia models. Thus, the present study evaluated the antidepressant and antinociceptive effects of the Tx3-3 toxin that is isolated from the venom of the *Phoneutria nigriventer* spider in FM-induced mice. The dosages of the biogenic amines validated the animal model and this was a parameter for evaluating the toxin's efficacy.

2. Materials and methods

2.1. Animals

The experiments were conducted when using 85 Swiss male mice (35–40 g) that were obtained from the animal house of the Lutheran University of Brazil (ULBRA). All of the animal experiments complied with the ARRIVE guidelines and they were carried out and followed in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for Animal Experiments, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). They were also approved by the Institutional Committee for Animal Care and Use from ULBRA under Protocol n° 2015-52P. Five (Can et al., 2012) mice per box were maintained with food and water ad libitum in a temperature-controlled room (24 ± 2 °C), while under a 12-h light/dark cycle.

2.2. Drugs

Reserpine, pregabalin, and pramipexole were obtained from Sigma-Aldrich (USA). Duloxetine was acquired from the Lilly Laboratory (Brazil). Before the experiments, reserpine was dissolved in acetic acid and then diluted to a final concentration of 0.5% acetic acid with distilled water. The control drugs of pregabalin, pramipexole, and duloxetine were diluted in a sterile saline solution (NaCl 0.9%).

The Tx3-3 toxin purification was performed by a combination of chromatographic steps, according to the method of Cordeiro Mdo et al. (1993). The purification presented a purity that was better than 95%. The amino acid sequence of the toxin was GKCADAWESCDNYPCCVVNGYSRTCMCSANRCNCDDTKTLREHFG and its molecular weight was 510,000 Da. The Tx3-3 toxin isolation, purification, and amino acid sequencing were carried out by the Ezequiel Dias Foundation (FUNED, Brazil). The Tx3-3 toxin was received in a lyophilized form and then dissolved in phosphate-buffered saline (PBS, mmol/L composition: NaCl 137, KCl 2.7, and phosphate buffer 10, pH 7.4).

2.3. Fibromyalgia model (FM)

FM was induced in accordance with the methodology as confirmed by Nagakura et al., (2018). For the biogenic amine depletion, the selected mice received the reserpine injection (0.25 mg/kg) by an s.c. route, once a day, over three consecutive days. The control animals received the vehicle (saline 10 ml/kg) at the same schedule of administration.

2.4. Intrathecal injections

The intrathecal injections were performed according to the previously described method for mice (Hylden and Wilcox, 1980). Briefly, a volume of 5 μL was administered between the lumbar vertebrae (L4-L5) by using a 28-gauge needle that was connected to a 10 μL Hamilton Microsyringe, while the animal was gently restrained, in order to maintain the position of the needle. The puncture of the dura was indicated behaviorally by a slight flick of the tail.

2.5. Experimental groups

On the 4th day after the reserpine injections (0.25 mg/kg), the mice were randomized into six groups, according to their treatment: 1) saline (10 ml/kg, p.o., $n = 10$); 2) duloxetine (30 mg/kg, p.o., $n = 13$); 3) pramipexole (1 mg/kg, p.o., n = 13); 4) pregabalin (30 mg/kg, p.o., n = 13); 5) PBS (5 μL/site, i.t., n = 13); and 6) Tx3-3 toxin (30 pmol/site, i.t., n = 13). Saline and PBS were used as controls for the drugs and the Tx3-3 toxin. There were two groups that received either saline (10 ml/kg, s.c., and p.o.), or saline (10 ml/kg) and PBS (5 μL/site, i.t.). All of the treatments were administered 1 h before the behavioral tests.

2.6. Behavioral tests

2.6.1. Mechanical allodynia (von Frey test)

The mechanical allodynia threshold was measured by using the up-down paradigm, as described by Dixon (1980). Following the appropriate treatments, the animals were acclimatized for 1 h in individual acrylic compartments with a mesh floor, in order to allow for access to the plantar surface of the hind paws. Von Frey filaments of increasing stiffness (0.02–4.0 g) were directed perpendicular to the right-hind paw plantar surface of the animals, with a high enough pressure to bend the filament. The sessions began with an application of the 0.4 g filament. If the tension-response was harmful (paw withdrawal), a filament with less value in (g) was used. However, if the voltage-response was innocuous, the filament with the highest subsequent value in (g) was tested from the last response. The von Frey filaments were applied for six sessions and the thresholds for a 50% mechanical paw withdrawal threshold (PWT) response were recorded for the subsequent calculations.

2.6.2. Hot plate test

Each mouse was submitted to the hot plate test for evaluating thermal hyperalgesia, as described by Hunskaar and Hole (1987). The metal surface of the plate was heated to a constant temperature of 52 ± 0.1 °C. The time in s that elapsed to the first pain response, such as a hind paw licking, a sudden raising of or jumping, was measured as a nociceptive response. A 30-s cut-off was used, in order to avoid tissue injury.

2.6.3. Forced swimming test

The forced swimming test was carried out, as described by Nomura et al. (1982). In order to investigate the antidepressive activities, the mice were placed inside a polyvinyl chloride (PVC) cylinder that contained at least 30 cm of water (24 °C - 30 °C) for recording their mobility (an expression of some movement, except for motions to preserve the mouse's head above the water) over a period of 6 min.

2.6.4. Open field test

For this test, the study used the methodology as previously described by Pietropaolo (2010). The mice were individually placed in the center of an acrylic open box (40 × 60 × 50 cm), with the background equally divided into 12 squares by black lines. This test occurred in a sound-attenuated room and under low-intensity light. The horizontal locomotor activity (segments crossed by the four legs) and the vertical activity (number of erected movements with the front legs) of the animals were evaluated over a period of 5 min.

2.7. Biochemical assays

After the behavioral tests, the animals were euthanized by an isoflurane overdose, followed by cervical dislocation for the collection of their brains. A pool of 4 to 5 brains was performed for each sample.

2.7.1. Measurement of the serotonin (5-HTP) and dopamine (DE) neurotransmitters

The levels of 5-HTP and DE were measured in the brain samples of the reserpine-treated mice, according to the method as described by González et al. (2011), with minor modifications. Briefly, all of the brains were harvested and stored at -80 °C until the moment of extraction. The samples were moved into an ice-containing box during homogenization in a 10-fold volume of formic acid (0.1 M) and then centrifuged at 20,000 xg for 20 min at 4 °C. Afterward, the supernatant was filtered (0.22 μm filter) and then injected into a UHPLC 1290/MS TQ-Agilent 6460 system (all of the HPLC components and the MassHunter software were from Agilent Technologies®, Santa Clara, CA, USA). The chromatographic separations were performed by using a Zorbax Eclipse Plus C18 2.1 × 50 mm 1.8-μm column, at a flow rate of 0.2 ml/min, with a column temperature of 30 °C. Methanol (eluent A) and 0.05% formic acid with 1 mM of heptafluorobutyric acid (HFBA) (eluent B) were used as the mobile phase. A gradient was used, starting at 95% of eluent B, constant for 0.5 min, and subsequently decreasing to 0% in 3.5 min. Five microliters of the samples were injected into the UHPLC system. The monitored transitions were DE (154 > 137 and 154 > 91) and 5-HTP (177 > 160). The results were expressed in nanograms/g of tissue for DE and 5-HTP, and micrograms/g of tissue for GLU.

2.8. Statistical analysis

The data is expressed as mean + SEM. The statistical analyses were carried out by a One-Way Analysis of Variance (ANOVA), followed by the Student-Newman-Keuls post-hoc test (GraphPad Prism 5.0, USA). The values of p that were less than 0.05 were considered significant.

3. Results

3.1. Mechanical antiallodynic effects of TX3-3 in the fibromyalgia model

The researchers first evaluated the responses to reserpine (0.25 mg/kg) when it was subcutaneously applied once daily over three consecutive days. As illustrated in Fig. 1, the mice in the Reserpine/Saline group that were submitted to the von Frey test on the 4th day presented a significant decrease (1.1 ± 0.14 g; $p < 0.01$) in the PWT when in comparison to the Saline/Saline group (3.8 ± 0.25 g). This evidenced the nociceptive effect and the development of symptoms that mimic FM. The oral treatments with duloxetine 30 mg/kg (2.6 ± 0.28 g), pramipexole 1 mg/kg (3.0 ± 0.34 g), and pregabalin 30 mg/kg (2.7 ± 0.27 g) increased the mean threshold to a value that was different from that of the control animals ($p < 0.01$). Hypersensitivity to the von Frey filaments was confirmed by intrathecal administrations of PBS (5 μL/site) in the reserpine-induced mice (1.12 ± 0.13 g). Conversely, the Tx3-3 toxin (30 pmol/site) produced antiallodynic effects, with mean threshold values that were significantly different (3.5 ± 0.47 g; $p < 0.001$) to the Saline/PBS animals (3.9 ± 0.45 g; $p < 0.001$) (Fig. 1), thus resulting in an inhibition of 87.16 ± 12.7%.

3.2. Thermal antihyperalgesic effects of TX3-3 in the fibromyalgia model

In the hot plate test, when reserpine (0.25 mg/kg) was injected for three consecutive days, it produced thermal hyperalgesia on the 4th day in both of the vehicle groups, saline (7.6 ± 0.5 s; $p < 0.001$) or PBS (8 ± 0.68 s; $p < 0.001$), when compared to the control groups Saline/Saline (17.6 ± 0.5 s) or Saline/PBS (17 ± 0.91 s) (see Fig. 2). The treatments with duloxetine (14.3 ± 0.99 s; $p < 0.05$), pramipexole (13.22 ± 0.99 s; $p < 0.01$), and pregabalin (13.22 ± 0.99 s; $p < 0.01$) increased the latency time that the mice remained on the hot plate. However, their permanency was significantly lower than that of the mice in the Saline/Saline control. The animals that were administered with the Tx3-3 toxin (17 ± 0.91 s) manifested thermal antihyperalgesic effects to the thermal stimulus, with their nociceptive responses statistically different ($p < 0.001$) from the Reserpine/PBS group.

3.3. TX3-3 decreased the mice immobility times in the fibromyalgia model

According to Fig. 3, the mice models with the establishment of depression were detected in the forced swimming test by the high immobility times that were verified in the reserpine-induced animals when injected with both saline vehicles (96.1 ± 9.98 s) ($p < 0.05$ from the control groups) or PBS (160.3 ± 37.74 s). The duloxetine (98.8 ± 16.41 s) administration did not affect the immobility times of the

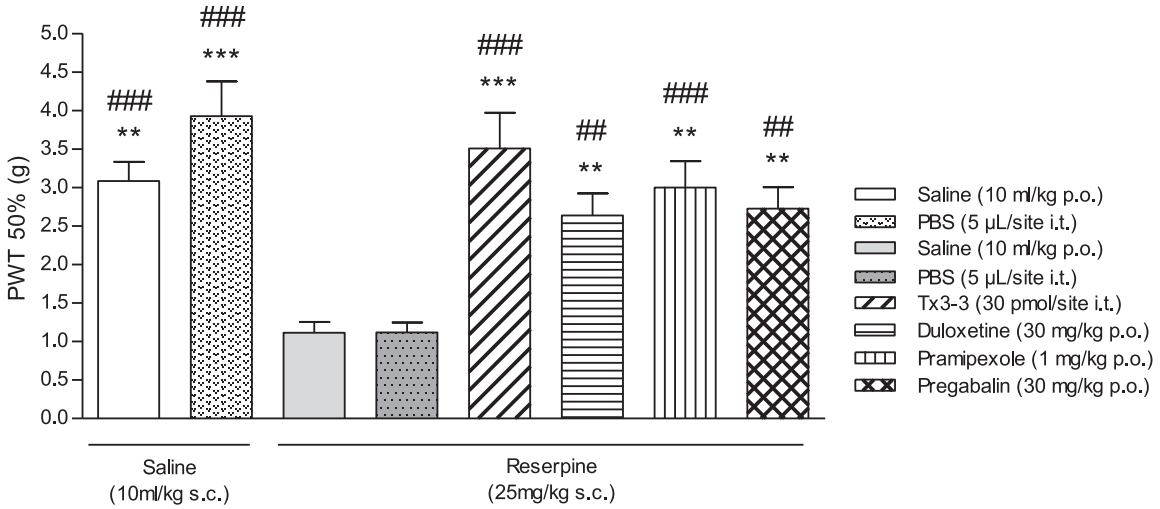

Fig. 1. Effects of the treatments in the mechanical allodynia test (von Frey filaments). The animals received reserpine (0.25 mg/kg, s.c.) for three consecutive days and they were then treated with the Tx3-3 toxin (30 pmol/site, i.t.), duloxetine (30 mg/kg, p.o.), pramipexole (1 mg/kg, p.o.), pregabalin (30 mg/kg, p.o.), PBS (5 μl/site, i.t.), or saline (10 ml/kg, p.o.) on the 4th day. Each bar represents the mean + SEM of 10–13 animals. ***$p < 0.001$ and **$p < 0.01$ represent the significance level when compared to the Reserpine/Saline group. ###$p < 0.001$ and ##$p < 0.01$ represent the significance level when compared to the Reserpine/PBS group. The statistical analyses were performed by One-Way ANOVA, followed by the Student-Newman-Keuls post-hoc test.

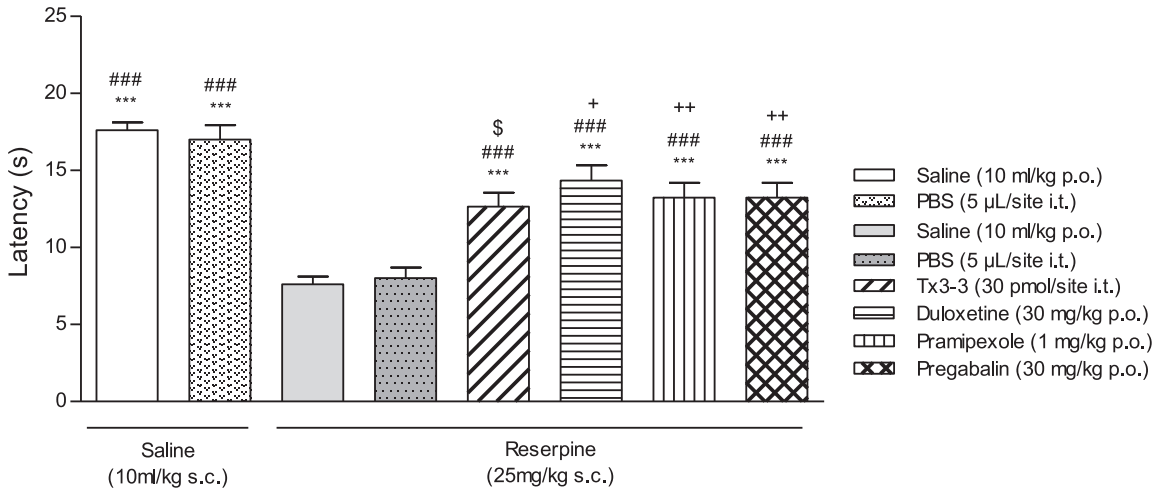

Fig. 2. Effects of the treatments for thermal hyperalgesia in the hot plate test. The animals received reserpine (0.25 mg/kg, s.c.) for three consecutive days and they were then treated with the Tx3-3 toxin (30 pmol/site, i.t.), duloxetine (30 mg/kg, p.o.), pramipexole (1 mg/kg, p.o.), pregabalin (30 mg/kg, p.o.), PBS (5 µl/site, i.t.), or saline (10 ml/kg, p.o.) on the 4th day. Each bar represents the mean + SEM of 10–13 animals. ***p < 0.001 represents the significance level when compared to the Reserpine/Saline group. ###p < 0.001 represents the significance level when compared to the Reserpine/PBS group. $$p < 0.05$ represents the significance level when compared to the Saline/PBS group. +p < 0.05 and ++$p < 0.01$ represent the significance level when compared to the Saline/Saline group. The statistical analyses were performed by One-Way ANOVA, followed by the Student-Newman-Keuls post-hoc test.

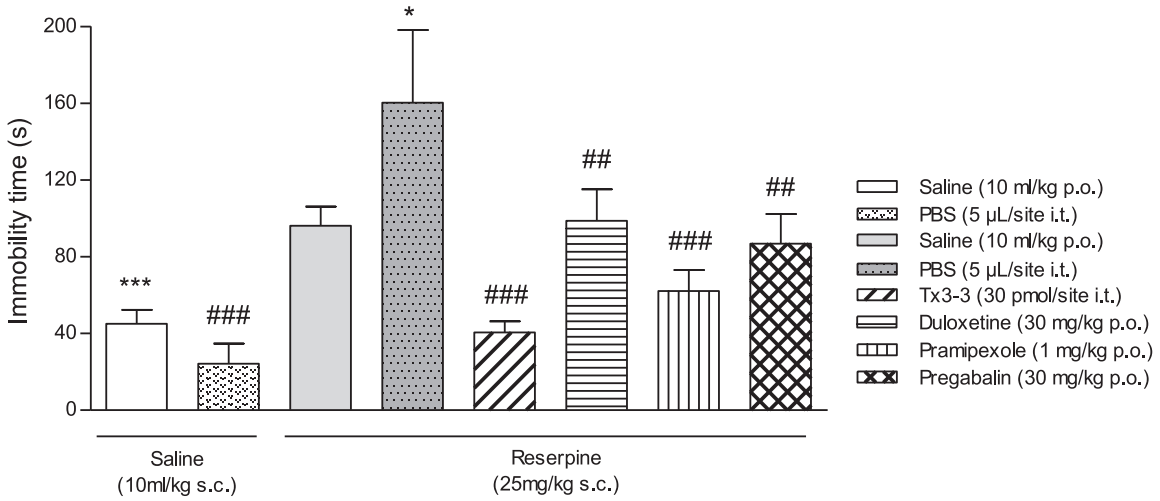

Fig. 3. Effects of the treatments on the depressive-like behavior in the forced swimming test. The animals received reserpine (0.25 mg/kg, s.c.) for three consecutive days and they were then treated with the Tx3-3 toxin (30 pmol/site, i.t.), duloxetine (30 mg/kg, p.o.), pramipexole (1 mg/kg, p.o.), pregabalin (30 mg/kg, p.o.), PBS (5 µl/site, i.t.), or saline (10 ml/kg, p.o.) on the 4th day. Each bar represents the mean + SEM of 10–13 animals. *$p < 0.05$ and ***$p < 0.001$ represent the significance level when compared to the Reserpine/Saline group. ##$p < 0.01$ and ###p < 0.001 represent the significance level when compared to the Reserpine/PBS group. The statistical analyses were performed by One-Way ANOVA, followed by the Student-Newman-Keuls post-hoc test.

reserpine-induced mice when compared to the Reserpine/Saline group. Conversely, the pramipexole (62.1 ± 11.05 s) drug demonstrated mobility when in comparison with the other classical control drugs that were utilized in this study (pregabalin 86.8 ± 15.41 s).

The immobility time responses of the Tx3-3-treated mice (40.55 ± 5.76 s; $p < 0.001$) were closer to the value of the Saline/PBS group (24.2 ± 10.49 s). They were statistically different from the PBS vehicle group, evidencing the toxin effects on mobility.

3.4. Tx3-3 altered the locomotor and exploratory activities of the reserpine-treated mice

The present results exhibited a marked decrease in the open field test parameters with the number of segments crossed (Fig. 4A) between the reserpine-induced mice that were treated with saline (15.5 ± 4.09) and PBS (18.2 ± 6.69) ($p < 0.001$) and the control mice in the Saline/Saline (74.5 ± 9.76) and Saline/PBS groups (63.8 ± 8.16). These parameters were also detected statistically by the classical drugs when compared with the saline control groups ($p < 0.001$), especially with the pregabalin applications (20.3 ± 2.13). The Tx3-3 toxin (30.6 ± 5.74) showed similar values to duloxetine (29.67 ± 5.0). However, the best result was with pramipexole (38.5 ± 4.41). Nevertheless, none of the treated groups was statistically different from the Reserpine/Saline group.

When related to the number of rearings (Fig. 4B), the same situation happened. Pramipexole (16.2 ± 2.66; p < 0.001), pregabalin (14.7 ± 2.91; p < 0.001), and duloxetine (13.0 ± 2.12; $p < 0.01$) showed values that were statistically different to the Saline/Saline control (34. 7 ± 3.56) while showing values that were similar to the reserpine-induced animals that were treated with saline (6.9 ± 1.75). Tx3-3 (7.45 ±

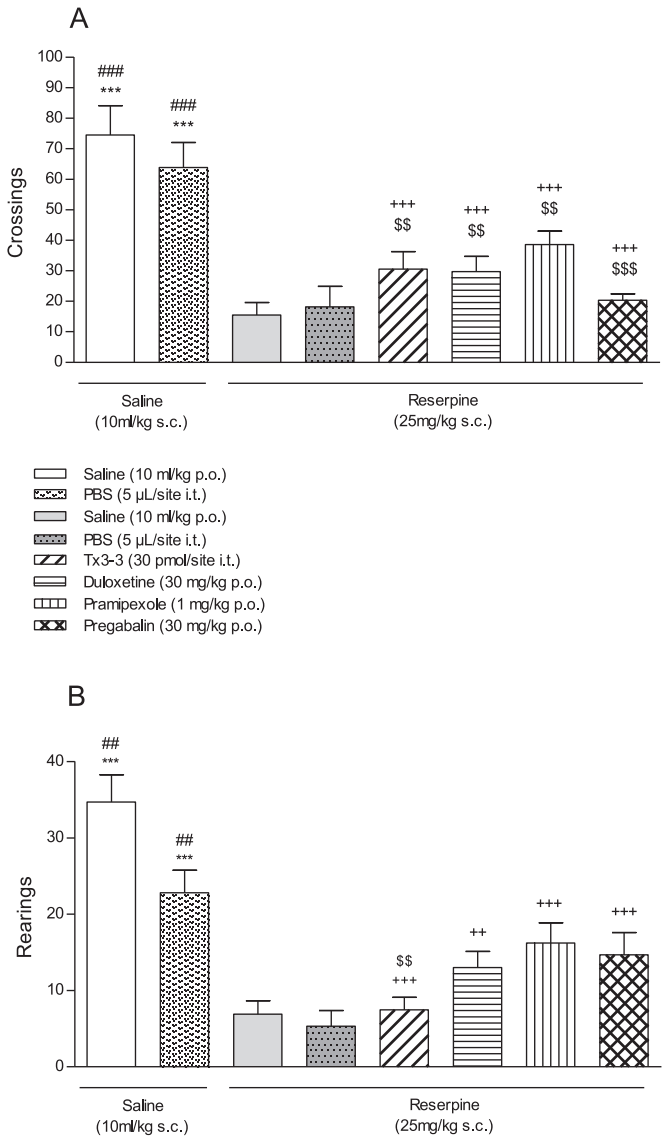

Fig. 4. Effects of the treatments on the horizontal locomotor activities and on the vertical exploratory activities in the open field test. The animals received reserpine (0.25 mg/kg, s.c.) for three consecutive days and they were then treated with the Tx3-3 toxin (30 pmol/site, i.t.), duloxetine (30 mg/kg, p.o.), pramipexole (1 mg/kg, p.o.), pregabalin (30 mg/kg, p.o.), PBS (5 µl/site, i.t.), or saline (10 ml/kg, p.o.) on the 4th day. Each bar represents the mean + SEM of 10–13 animals; ***$p < 0.001$ represents the significance level when compared to the Reserpine/Saline group. ###p < 0.001 represents the significance level when compared to the Reserpine/PBS group. $$$p < 0.05$ represents the significance level when compared to the Saline/PBS group. ++$p < 0.01$ and +++p < 0.001 represent the significance level when compared to the Saline/Saline group. The statistical analyses were performed by One-Way ANOVA, followed by the Student-Newman-Keuls post-hoc test.

1.65) and Reserpine/PBS (5.33 ± 2.04). They were also statistically different from the Saline/PBS animals (22.8 ± 2.94; $p < 0.001$).

There were no expressive differences in the locomotor activities and in the rearings between the depressive-like animals and the ones that were treated with the Tx3-3 toxin ($p > 0.05$) when evaluated for 5 min. In this study, therefore, no treatment values were fully effective for the reversion of the locomotor activities.

3.5. Increases in the 5-HTP and the DE brain levels of the fibromyalgia model mice

The present data has revealed that the 5-HTP (51.6 ± 3.1 ng/g tissue) and the DE (3.07 ± 0.87 ng/g tissue) brain levels were significantly decreased in the animal model of depression ($p < 0.001$, when compared to the Saline control group). As can be observed in Fig. 5A and B, the treatments with the Tx3-3 toxin were able to slightly reverse the reserpine administration effects of the 5-HTP (61.3 ± 0.61 ng/g tissue; $p < 0.05$) and the DE (8.1 ± 1.10 ng/g tissue; $p < 0.01$) neurotransmitter levels in the brain.

4. Discussion

It is suggested that the primary disorder of FM is a change in some of the central mechanisms of pain control, resulting in dysfunction that induces a deficiency in the inhibitory neurotransmitters (such as serotonin, enkephalin, and norepinephrine) at the spinal or supraspinal levels (Littlejohn, 2015; Littlejohn and Guymer, 2018). Thus, the main symptoms of fibromyalgia (heightened pain perception, fatigue, sleep disturbances, and depression, as well as anxiety-related symptoms) are closely linked to alterations in the neurotransmitter systems (Becker and Schweinhardt 2012). Besides, the fibromyalgia model, as proposed by Nagakura et al. (2018), and reproduced in the current work, promoted a depletion of the biogenic amines in the spinal cord and in the prefrontal cortex. Since serotonin contributes to the hyperfunction of the glutamatergic system (Benson et al., 2015), the present study's results have shown that the Tx3-3 toxin was significantly able to elevate the dopamine and the 5-HTP levels in the brains of the fibromyalgic animals. These results may be related to the activity in the glutamatergic system at the spinal and supraspinal levels; however, the mechanisms by which this occur still needs to be elucidated.

Behaviorally, the mice that were treated with the Tx3-3 toxin presented similar results to the control animals in the forced swimming test. This showed that the animals without depressive-like behavior presented floating times equal to the animals that were treated with the Tx3-3 toxin. This was, even when behavioral despair was induced, the mice never lost hope of escaping the stressful environment, proving the antidepressive effect of the drug (Can et al., 2012). Pramipexole also demonstrated an antidepressant effect. However, the action of the Tx3-3 toxin was superior to all of the control drugs. On the other hand, it should be noted that the animals that were treated in this study did not present locomotor activities similar to the control groups in the open field test. Even with their impaired exploratory activities, the animals showed themselves to be active, indicating an absence of sedation. In both of the evaluations in the open field test, pramipexole scored slightly better. It is known that serotonin, mainly, modulates neuroplasticity and it contributes to the pathophysiology of depression (Kraus et al., 2017). In this way, it has also been suggested that decreased dopamine activity may contribute to patients' pain symptomatology (Becker and Schweinhardt, 2012). Although pramipexole is a dopaminergic agonist, with a D2 receptor affinity, and a drug with no known affinity for norepinephrine or the 5-HTP neuronal elements, it exerts a facilitating action on serotonergic neurotransmission (El Mansari et al., 2010).

The venom of the *Phoneutria nigriventer* spider has been increasingly relevant in the scientific community for its ability to alter a large number of physiological systems, particularly those that are related to pain (Prado et al., 1996; Guatimosim et al., 1997; Gomez et al., 2002; Agostini et al., 2011). Leão et al. (2000) explained that the Tx3-3 toxin blocked the voltage-dependent calcium channels (VGCC) in the presynaptic terminals, with the consequent inhibition of glutamate release. According to Dalmolin et al. (2017), the Tx3-3 toxin exhibited a higher potency after nerve damage since its action profile was blocking the R-type voltage-gated calcium channels. Still related to depression, firstly, Boldrini et al. (2013) demonstrated a reduction in the number of granular neuron cells in the postmortem hippocampus and in the dentate

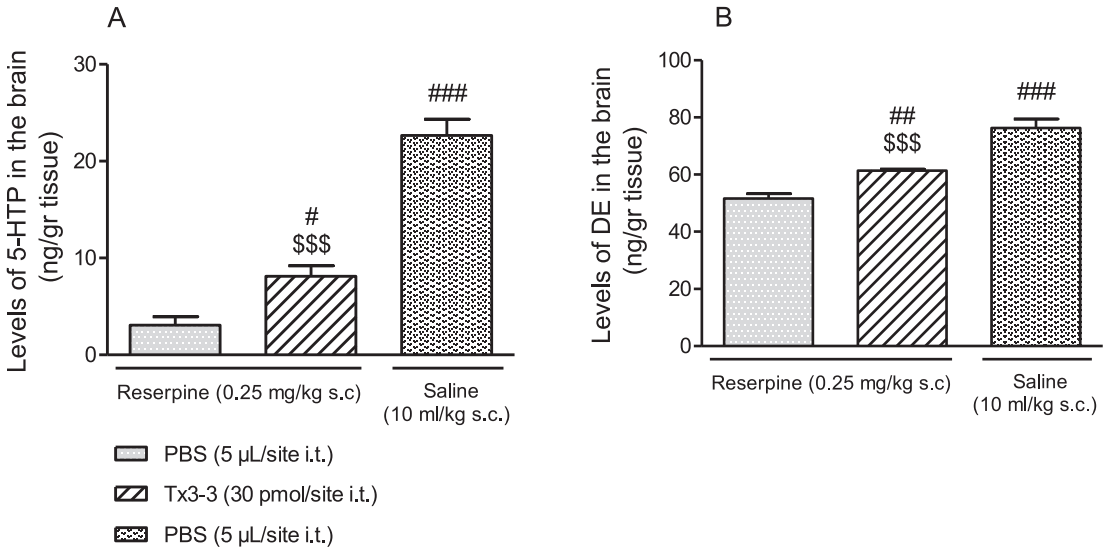

Fig. 5. The effects of the Tx3-3 toxin on the levels of 5-HTP (A) and DE (B) in the brains of the mice. The animals received reserpine (0.25 mg/kg, s.c.) for three consecutive days and they were then treated with the Tx3-3 toxin (30 pmol/site, i.t.), duloxetine (30 mg/kg, p.o.), pramipexole (1 mg/kg, p.o.), pregabalin (30 mg/kg, p.o.), PBS (5 µl/site, i.t.), or saline (10 ml/kg, p.o.) on the 4th day. Each bar represents the mean + SEM of 10–13 animals. #$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$ represent the significance level when compared to the Reserpine/PBS group. \$\$\$$p < 0.001$ represents the significance level when compared to the Saline/PBS group. The statistical analyses were performed by One-Way ANOVA, followed by the Student-Newman-Keuls post-hoc test.

gyrus of depressive patients. Secondly, these cells mostly have P/Q and R-type calcium channels, respectively (Randall and Tsien, 1995). Thus, in the current study, the probable blockade of the R-type calcium channels that was promoted by the Tx3-3 toxin in the brain regions with granular neurons may be related to a decrease in the depressive symptoms that were manifested in the fibromyalgia mice. However, more studies when directed at the spinal cord, the cerebellum, and the prefrontal cortex should be developed, in order to confirm this hypothesis.

Studies on the calcium channel blockers and the *N*-methyl-D-aspartate (NMDA) antagonists, which have the ultimate aim of rendering a decrease in glutamate, have shown antidepressant (Murrough et al., 2017) and analgesic effects (Mochida, 2018; Patel et al., 2018; Saegusa et al., 2000). Individuals with fibromyalgia present diffuse hyperalgesia (increased pain to normally painful stimuli) and/or allodynia (pain to normally non-painful stimuli) (Sluka and Clauw, 2016). The antinociceptive effects of the Tx3-3 toxin are evident in other studies (Dalmolin et al., 2011; Dalmolin et al., 2017; Silva et al., 2015). In this current research, for the fibromyalgia model with reserpine, the results have shown that the Tx3-3 toxin produced a significant response of increased latency in the thermal hyperalgesia (hot plate test) and threshold mechanical allodynia (von Frey filaments test) tests when compared with the reserpine-induced animals. These results were as effective as the pregabalin drug. The duloxetine drug showed slightly higher levels than did the Tx3-3 toxin in the hot plate test, as well as pramipexole in the mechanical allodynia test.

All of the data that was acquired in this study has demonstrated that the treatments with the Tx3-3 toxin triggered significant antinociceptive effects and decreased the depressive-like behavior in most of the behavioral tests when compared to the control animals. The Tx3-3 treatments were fully effective for the biochemical assay and this showed that the toxin reversed the biogenic amine levels in the brain. It is also likely that this toxin will not have an antidepressant effect. The increased mobility, as shown in the forced swimming test, may have possibly occurred due to the antinociceptive effects of the toxin. In this way, the animals without pain would have a decrease in immobility time (de Souza et al., 2014). In this study, the Tx3-3 toxin was shown to be an excellent candidate for further studies from a pharmacological perspective for treating pain, together with the relief of depressive symptoms in those patients with fibromyalgia.

Policy and ethics disclosure

The work described in this article has been carried out in accordance with EC Directive 86/609/EEC for animal experiments.
https://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm

Author contributions

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

References

Agostini, R.M., do Nascimento Pinheiro, A.C., Binda, N.S., et al., 2011. Phoneutria spider toxins block ischemia-induced glutamate release and neuronal death of cell layers of the retina. Retina. 31 (7), 1392–1399. https://doi.org/10.1097/IAE.0b013e318205b249.

Becker, Susanne, Schweinhardt, Petra, 2012. Dysfunctional Neurotransmitter Systems in Fibromyalgia. Their Role in Central Stress Circuitry and Pharmacological Actions on These Systems, 741746. https://doi.org/10.1155/2012/741746.

Bennett, R.M., Jones, J., Turk, D.C., et al., 2007. An internet survey of 2,596 people with fibromyalgia. BMC Musculoskelet. Disord. 8, 27. Published 2007 Mar 9. https://doi.org/10.1186/1471-2474-8-27.

Benson, C., Mifflin, K., Kerr, B., et al., 2015. Biogenic amines and the amino acids GABA and glutamate: relationships with pain and depression. Mod Trends Pharmacopsychiatr. 30, 67–79. https://doi.org/10.1159/000435933.

Boldrini, M., Santiago, A.N., Hen, R., et al., 2013. Hippocampal granule neuron number and dentate gyrus volume in antidepressant-treated and untreated major depression. Neuropsychopharmacology. 38 (6), 1068–1077. https://doi.org/10.1038/npp.2013.5.

Can A, Dao DT, Arad M, et al. The mouse forced swim test. J. Visualiz. Exp., 2012;(59), e3638. https://doi.org/10.3791/3638.

Carville, S.F., Arendt-Nielsen, L., Bliddal, H., et al., 2008. EULAR evidence-based recommendations for the management of fibromyalgia syndrome. Ann. Rheum. Dis. 67, 536–541. https://doi.org/10.1136/ard.2007.071522.

Choy, E., Marshall, D., Gabriel, Z.L., et al., 2011. A systematic review and mixed treatment comparison of the efficacy of pharmacological treatments for fibromyalgia. Semin. Arthritis Rheum. 41 (3) https://doi.org/10.1016/j.semarthrit.2011.06.003, 335–45.e6.

Cordeiro Mdo, N., de Figueiredo, S.G., Valentim Ado, C., et al., 1993. Purification and amino acid sequences of six Tx3 type neurotoxins from the venom of the Brazilian 'armed' spider *Phoneutria nigriventer* (Keys). Toxicon. 31 (1), 35–42. https://doi.org/10.1016/0041-0101(93)90354-l.

Dadabhoy, D., Crofford, L.J., Spaeth, M., et al., 2008. Biology and therapy of fibromyalgia. Evidence-based biomarkers for fibromyalgia syndrome. Arthritis Res. Ther. 10 (4), 211. https://doi.org/10.1186/ar2443.

Dalmolin, G.D., Silva, C.R., Rigo, F.K., et al., 2011. Antinociceptive effect of Brazilian armed spider venom toxin Tx3-3 in animal models of neuropathic pain. Pain. 152 (10), 2224–2232. https://doi.org/10.1016/j.pain.2011.04.015.

Dalmolin, G.D., Bannister, K., Gonçalves, L., et al., 2017. Effect of the spider toxin Tx3-3 on spinal processing of sensory information in naive and neuropathic rats: an in vivo electrophysiological study. Pain Rep. 2 (4), e610. Published 2017 Jul 6. https://doi.org/10.1097/PR9.0000000000000610.

de Souza, A.H., da Costa Lopes, A.M., Castro Jr., C.J., et al., 2014. The effects of Phα1β, a spider toxin, calcium channel blocker, in a mouse fibromyalgia model. Toxicon. 81, 37–42. https://doi.org/10.1016/j.toxicon.2014.01.015.

Dixon, W.J., 1980. Efficient analysis of experimental observations. Annu. Rev. Pharmacol. Toxicol. 20, 441–462. https://doi.org/10.1146/annurev.pa.20.040180.002301.

El Mansari, M., Guiard, B.P., Chernoloz, O., et al., 2010. Relevance of norepinephrine-dopamine interactions in the treatment of a major depressive disorder. CNS Neurosci Ther. 16 (3), e1–e17. https://doi.org/10.1111/j.1755-5949.2010.00146.x.

Gomez, M.V., Kalapothakis, E., Guatimosim, C., et al., 2002. *Phoneutria nigriventer* venom: a cocktail of toxins that affect ion channels. Cell. Mol. Neurobiol. 22 (5–6), 579–588. https://doi.org/10.1023/a:1021836403433.

González, R.R., Fernández, R.F., Vidal, J.L., et al., 2011. Development and validation of an ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method for the simultaneous determination of neurotransmitters in rat brain samples. J. Neurosci. Methods 198 (2), 187–194. https://doi.org/10.1016/j.jneumeth.2011.03.023.

Grishin, E., 1999. Polypeptide neurotoxins from spider venoms. Eur. J. Biochem. 264 (2), 276–280. https://doi.org/10.1046/j.1432-1327.1999.00622.x.

Guatimosim, C., Romano-Silva, M.A., Cruz, J.S., et al., 1997. A toxin from the spider *Phoneutria nigriventer* that blocks calcium channels coupled to exocytosis. Br. J. Pharmacol. 122 (3), 591–597. https://doi.org/10.1038/sj.bjp.0701381.

Häuser, W., Sarzi-Puttini, P., Fitzcharles, M.A., 2019. Fibromyalgia syndrome: under-, over- and misdiagnosis. Clin Exp Rheumatol. 116 (1), 90–97, 37 Suppl.

Heymann, R.E., Paiva, E.S., Helfenstein Junior, M., et al., 2010. Consenso brasileiro do tratamento da fibromialgia. [Brasilian Consensus for the treatment of the fibromyalgia]. Rev. Bras. Reumatol. 50 (1), 56–66. https://doi.org/10.1590/S0482-50042010000100006.

Hunskaar, S., Hole, K., 1987. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain. 30 (1), 103–114. https://doi.org/10.1016/0304-3959(87)90088-1.

Hylden, J.L., Wilcox, G.L., 1980. Intrathecal morphine in mice: a new technique. Eur. J. Pharmacol. 67 (2–3), 313–316. https://doi.org/10.1016/0014-2999(80)90515-4.

Kraus, C., Castrén, E., Kasper, S., et al., 2017. Serotonin and neuroplasticity - links between molecular, functional, and structural pathophysiology in depression. Neurosci. Biobehav. Rev. 77, 317–326. https://doi.org/10.1016/j.neubiorev.2017.03.007.

Leão, R.M., Cruz, J.S., Diniz, C.R., et al., 2000. Inhibition of neuronal high-voltage activated calcium channels by the omega-*Phoneutria nigriventer* Tx3-3 peptide toxin. Neuropharmacology. 39 (10), 1756–1767. https://doi.org/10.1016/s0028-3908(99)00267-1.

Ledermann, K., Jenewein, J., Sprott, H., et al., 2017. Altered dopamine responses to monetary rewards in female fibromyalgia patients with and without depression: a [11C] Raclopride bolus-plus-infusion PET study. Psychother. Psychosom. 86 (3), 181–182. https://doi.org/10.1159/000455927.

Littlejohn, G., 2015. Neurogenic neuroinflammation in fibromyalgia and complex regional pain syndrome. Nat. Rev. Rheumatol. 11 (11), 639–648. https://doi.org/10.1038/nrrheum.2015.100.

Littlejohn, G., Guymer, E., 2018. Neurogenic inflammation in fibromyalgia. Semin. Immunopathol. 40 (3), 291–300. https://doi.org/10.1007/s00281-018-0672-2.

Macfarlane, G.J., Kronisch, C., Dean, L.E., et al., 2017. EULAR revised recommendations for the management of fibromyalgia. Ann. Rheum. Dis. 76 (2), 318–328. https://doi.org/10.1136/annrheumdis-2016-209724.

Mochida, S., 2018. Presynaptic calcium channels. Neurosci. Res. 127, 33–44. https://doi.org/10.1016/j.neures.2017.09.012.

Murrough, J.W., Abdallah, C.G., Mathew, S.J., 2017. Targeting glutamate signaling in depression: progress and prospects. Nat. Rev. Drug Discov. 16 (7), 472–486. https://doi.org/10.1038/nrd.2017.16.

Nagakura, Y., Ohsaka, N., Azuma, R., et al., 2018. Monoamine system disruption induces functional somatic syndromes associated symptomatology in mice. Physiol. Behav. 194, 505–514. https://doi.org/10.1016/j.physbeh.2018.07.007.

Nomura, S., Shimizu, J., Kinjo, M., et al., 1982. A new behavioral test for antidepressant drugs. Eur. J. Pharmacol. 83 (3–4), 171–175. https://doi.org/10.1016/0014-2999(82)90248-5.

Patel, R., Montagut-Bordas, C., Dickenson, A.H., 2018. Calcium channel modulation as a target in chronic pain control. Br. J. Pharmacol. 175 (12), 2173–2184. https://doi.org/10.1111/bph.13789.

Peigneur, S., de Lima, M.E., Tytgat, J., 2018. Phoneutria nigriventer venom: a pharmacological treasure. Toxicon. 151, 96–110. https://doi.org/10.1016/j.toxicon.2018.07.008.

Pietropaolo, S., 2010. Mood and anxiety-related phenotypes in mice: characterization using Behavioral tests - Edited by TD. Gould. Genes Brain Behav. 9, 544. https://doi.org/10.1111/j.1601-183X.2010.00592.x.

Prado, M.A., Guatimosim, C., Gomez, M.V., et al., 1996. A novel tool for the investigation of glutamate release from rat cerebrocortical synaptosomes: the toxin Tx3-3 from the venom of the spider *Phoneutria nigriventer*. Biochem J. 314 (Pt 1), 145–150. https://doi.org/10.1042/bj3140145.

Randall, A., Tsien, R.W., 1995. Pharmacological dissection of multiple types of Ca2+ channel currents in rat cerebellar granule neurons. J. Neurosci. 15 (4), 2995–3012. https://doi.org/10.1523/JNEUROSCI.15-04-02995.1995.

Ruggiero, L., Manganelli, F., Santoro, L., 2018. Muscle pain syndromes and fibromyalgia: the role of muscle biopsy. Curr Opin Support Palliat Care. 12 (3), 382–387. https://doi.org/10.1097/SPC.0000000000000355.

Russell, I.J., Vaeroy, H., Javors, M., et al., 1992. Cerebrospinal fluid biogenic amine metabolites in fibromyalgia/fibrositis syndrome and rheumatoid arthritis. Arthritis Rheum. 35 (5), 550–556. https://doi.org/10.1002/art.1780350509.

Saegusa, H., Kurihara, T., Zong, S., et al., 2000. Altered pain responses in mice lacking alpha 1E subunit of the voltage-dependent Ca2+ channel. Proc. Natl. Acad. Sci. U. S. A. 97 (11), 6132–6137. https://doi.org/10.1073/pnas.100124197.

Silva, R.B., Sperotto, N.D., Andrade, E.L., et al., 2015. Spinal blockage of P/Q- or N-type voltage-gated calcium channels modulates functional and symptomatic changes related to hemorrhagic cystitis in mice. Br. J. Pharmacol. 172 (3), 924–939 https://doi.org/10.1111/bph.12966.

Sluka, K.A., Clauw, D.J., 2016. Neurobiology of fibromyalgia and chronic widespread pain. Neuroscience 338, 114–129 https://doi.org/10.1016/j.neuroscience.2016.06.006.

Smith, H.S., Harris, R., 2011. Fibromyalgia: an afferent processing disorder leading to a complex pain generalized syndrome. Pain Physician 14 (2), 17–45.

Smith, H.S., Bracken, D., Smith, J.M., 2011. Pharmacotherapy for fibromyalgia. Front. Pharmacol. 2 (17), 1–17.