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Research report

Long-term treatment with roflumilast improves learning of fear extinction memory and anxiety-like response in a type-1 diabetes *mellitus* animal model

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ABSTRACT

Diabetic encephalopathy is related to serious damage to the Central Nervous System leading to several disturbances in memory processing and emotions. It is known that the cyclic adenosine 3′,5′-monophosphate (cAMP) responsive element-binding protein (CREB) pathway participates in neuronal plasticity and prevention of neuroinflammation, as well as the mediation of learning/memory processes and emotions in brain areas such as the hippocampus (HIP) and prefrontal cortex (PFC). We aimed to investigate the effect of acute (one injection) and long-term treatment (21 days) with roflumilast (ROF; i.p.; 0, 0.01, 0.03, 0.1 mg/kg), a drug able to inhibit the enzyme phosphodiesterase-4 (PDE-4) responsible for cAMP hydrolysis, on parameters related to the acquisition of fear extinction memory and anxiety-like responses in animals with type-1 diabetes mellitus (T1DM) induced through one injection of streptozotocin (60 mg/kg; ip; STZ animals). When we performed acute treatment, no difference was observed between all the groups when resubmitted to the same context paired with an aversive stimulus (footshock) or to a neutral context. In contrast, long-term treatment was able to improve learning of extinction fear memory and discriminating between a conditioned and neutral context. Moreover, this treatment decreased the pronounced anxiety-like response of STZ animals. In addition, there was an increase in the product of the CREB signaling pathway, the pro brain-derived neurotrophic factor, in the HIP and PFC of these animals. The treatment did not impair glycemic control, whereas it decreased the animal's blood glucose levels. To conclude, these findings suggest that ROF treatment repositioning has potential for future translational investigations involving diabetic patients considering its beneficial effects on emotional processes related to fear memory and anxiety, in addition to improvement of glycemic control.

1. Introduction

Diabetic encephalopathy is a current term that has been used to refer to serious damage to the Central Nervous System (CNS) from diabetic patients. It is important to highlight that the brain is especially sensitive to diabetes-induced damage because of the damaging consequences of hyperglycemia including increased oxidative stress and neuroinflammation, dysregulation of the neurotransmitter systems, impaired synaptic plasticity, and neurogenesis [1–12]. All this pathological neuroplasticity has been associated with various CNS dysfunctions including impairment in the processing of spatial learning, and of particular interest to the present study, fear memory [13–17].

Evidence from our laboratory and others has demonstrated in an animal model of streptozotocin (STZ)-induced type-1 diabetes *mellitus* (T1DM) that these animals show overconsolidation of aversive memory, in addition to difficulty in extinguishing it, presenting exacerbated freezing behavior [15,16]. Moreover, a more expressive anxiety-like response is also present when these animals are subjected to different

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behavioral tests [12,15,17]. These preclinical findings corroborate with clinical evidence showing that post-traumatic stress disorder (PTSD) and anxiety disorders have an important relationship with diabetes *mellitus*, with a higher prevalence of these disorders in the diabetic population compared to the general population [18–21]. PTSD is characterized by the formation and consolidation of an abnormal aversive memory related to a traumatic event and, in addition, by the difficulty and failure to extinguish this aversive memory [22,23]. These disturbances in fear memory processing can be accompanied by anxiety disorders [24,25].

The treatment of the impaired fear-associated memory processing, as well as anxiety, in patients with diabetes *mellitus* is therefore a major challenge in the clinic. Thus, the search for a treatment that induces neuroprotective action by disrupting or decreasing inflammatory processes, oxidative stress and other CNS damages would be an excellent approach. In this sense, roflumilast (ROF), a compound clinically approved for its anti-inflammatory action for the treatment of chronic obstructive pulmonary disease [26,27] and which acts by selectively inhibiting the phosphodiesterase-4 (PDE-4) enzyme, has recently been highlighted as a potential drug for the treatment of several inflammation-related conditions, including COVID-19 pathogenesis [28–30].

Besides its anti-inflammatory actions, ROF has been shown to present cognitive enhancement properties in clinical [31,32] and several preclinical [33,34] studies involving verbal word memory (clinical studies) and spatial memory (preclinical studies). Also, ROF attenuated memory deficits induced by hypertension or after transient global cerebral ischemia in rats [33,35] along with an improvement on recognition, spatial, and contextual fear memory in an animal model of Alzheimer's disease [36,37]. Concerning conditioned fear memory, a recent study demonstrated that a single peripheral injection of ROF before the acquisition of a fear extinction memory was able to impair this acquisition in the short-term period [38]. Furthermore, given that PDE-4 specifically hydrolyzes cyclic adenosine monophosphate (cAMP) and that ROF selectively inhibits PDE-4, the authors also observed that the protein kinase-A (PKA) activity-dependent ROF-induced effect was correlated with an increase in its full-length BDNF, which is actually pro-brain-derived neurotrophic factor (proBDNF) protein expression in the prefrontal cortex (PFC), a brain region necessary for memory extinction. The BDNF is crucial in the transformation of synaptic activity into long-term synaptic memories, and it is considered an instructive mediator of functional and structural plasticity in the CNS, influencing dendritic spines and, at least in the hippocampus (HIP), the adult neurogenesis (for a review see [39]). More recently, studies have demonstrated the importance of developmental proBDNF for modification of retrieval-dependent memory [40,41] For example, it was demonstrated that proBDNF into medial PFC was required for depressing fear expression, whereas it was necessary for inducing extinction of both new and old fear memories [40].

To our knowledge no study has investigated the potential of ROF in improving impaired fear memory processing, as well as the more expressive anxiety-like response in rats with experimentally induced-T1DM, which is therefore the aim of the present study. Thus, we first hypothesized that a single ROF administration would already be able to increase the acquisition of a fear extinction memory. Next, we hypothesized that prolonged treatment with ROF would restore the correct processing of the conditioned fear memory along with a disruption in the more exacerbated anxiety-like response in an animal model of T1DM. To further investigate whether the cAMP-PKA-BDNF pathway would be involved, we evaluated the proBDNF protein levels in the PFC and HIP.

2. Material and methods

2.1. Ethics statements

This study was carried out at the Federal University of Parana in accordance with the recommendations of Brazilian College of Animal Experimentation (COBEA). All animal experiments were approved by the local Ethics Committee on Animal Experimentation (license number: CEUA/BIO-UFPR; #1204). All efforts were made to reduce the number of animals used and minimize their suffering.

2.2. Animals

Ninety-eight male *Wistar* rats (aged 8–10 weeks) were used, which were provided by the *vivarium* of the Federal University of Parana. The animals were kept in groups of 4 per cage (41 × 32 × 16.5 cm) on a 12-hour light-dark cycle, in a temperature-controlled room (22 ± 2 ∘ C) and with food and water ad libitum. The experimental procedures were performed during the light phase of the cycle (7:00 am to 7:00 pm). Behavioral experiments were performed by the experimenters (APFW, ACFS, YCC), while Western Blotting analysis was performed by another experimenter (BAM). Video analyzes were performed by blinded experimenters (APFW, ACFS).

2.3. Drugs

The following drugs were used: streptozotocin (STZ, Cayman) and roflumilast (ROF, kindly provided by Dr. Jos Prickaerts, University of Maastricht, the Netherlands). STZ (60 mg/kg; administered intraperitoneally - i.p.) was diluted in citrate buffer (10 mM, pH 4.5). ROF was diluted in sterile saline (0.9% NaCl) and 10% polysorbate 80 (Tween-80®, Sigma-Aldrich, St. Louis, MO, USA; i.p.), at doses of 0.01; 0.03 or 0.1 mg/kg. The dosages selected for the drugs were based on the existing results of the literature [4,6,7,34,38,42]. The animals in control groups were treated with sterile saline only.

2.4. Experimental type-1 diabetes induction

Before the experimental T1DM induction procedure, the animals were fasted for 12 h. After this period, a single i.p. injection of STZ (60 mg/kg) was performed. Animals that went into normoglycemic groups (NGL) received a single i.p. injection of its vehicle (citrate buffer; 10 mM, pH 4.5). Diabetic condition was confirmed 72 h after STZ injection using tail vein blood samples (5 μL) added to test strips impregnated with glucose oxidase (Accu-Check Active™, Roche). Rats with blood glucose equal to or greater than 250 mg/dL were considered hyperglycemic and were maintained in the study in experimental T1DM groups (STZ groups) [4].

2.5. Contextual fear conditioning (CFC) protocol

For the investigation of behaviors related to fear memory, the CFC protocol was performed according to previous studies [16,17]. The CFC was performed in a rectangular chamber (context A; 26 ×31.5 ×21 cm; Insight, Ribeirão Preto, SP, Brazil), composed of three sides of steel and a fourth side of transparent acrylic, which allowed video recording of the tests for further analysis of the rat's behaviors. The bottom of the box consisted of small metal bars connected to an electrical stimulator that can apply electrical currents to the animals, and the top was made of clear acrylic. For the generalization test, it was used a different box (context B) made completely of transparent acrylic (30 ×30×30 cm) [15,16].

The CFC protocol consisted of the following steps:

- 1st day - familiarization: the animal was placed in the context A for 3 min only for familiarization, without the footshock (unconditioned stimulus - US).

- 2nd day - CFC session: the animal was placed in the context A (conditioned stimulus - CS). After an initial 30 s delay, he was exposed to the US (three footshocks - 1 mA, 3 s duration, 30 s intershock interval). After the last US, the animal remained in the chamber for an additional 30 s before being returned to its home cage.

- 3rd day – extinction training session: the animal was placed in the context A and remained there for 20 min without the presentation of the US.

- 4th day - extinction test session: the animal was placed in the context A for 3 min, without the presentation of the US.

- 5th day - generalization test session: this test was performed to investigate a possible fear generalization response. Thus, the animal was placed in the neutral context (context B) for 3 min without the presentation of the US.

The index used to assess context-aversive memory in the 3rd, 4th and 5th day of the procedures was the freezing behavior - considered when the animal presented a posture of complete immobility, except for breathing movements. The freezing behavior was measured in seconds (s) and expressed as the percentage (%) of total session time. For the extinction training session, the freezing time of the animals was evaluated during the total time of 20 min. However, the analyzes were conducted on the data obtained every 4 min. Thus, in the graphs the data were represented into 5 blocks of 4 min each.

The discrimination index was calculated using % freezing during the extinction test in context A by dividing by the sum of % freezing during the extinction test in context A and % freezing during the generalization test in context B, according to the following formula: [context A/ (context A + context B)] [43]. The index ranged from 0 to + 1, indicating an impairment in the discrimination between contexts when the index is closer to 0 and a better distinction between contexts with an index closer to + 1. After each procedure, the apparatus was cleaned with a solution of ethanol (20%).

2.6. Western blot analysis

Independent group of animals were submitted to a long-term treatment with ROF or VEH (21 days). The day after the last injection, the animals were euthanized, and the brains were removed. The PFC and whole HIP were dissected using a brain matrix (Insight, Ribeirão Preto, Brazil), spatulas, and tweezers. The PFC and the whole HIP were chosen for analysis taking into account that they are important structures for processing of memory and emotions [16,38]. These structures were kept at − 80 ºC until use. The collected tissues were homogenized in a buffer containing 10% glycerol, 20 mM Tris-HCl, 137 Mm NaCl (pH 7.5), and a protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA) and kept at rest for 15 min at 4 °C. The homogenized tissues were then centrifuged at 2800 rpm for 10 min at 4ºC, had the supernatant (corresponding to the cytosolic extract) separated in a new tube and centrifuged again for 15 min at 12,000 rpm. The supernatant was separated and the pellets, containing the samples, were diluted to reach protein concentrations equal to 30 μg and separated by electrophoresis in 10%, 12% or 15% polyacrylamide gel and SDS-PAGE buffer. After this step, the proteins were transferred to a nitrocellulose membrane and incubated for an hour and a half in Tris buffer (25 mM Tris, 192 mM Glycine, 200 mL of methanol, pH 8). After transfer, to perform immunoblotting, membranes were incubated with 5% powdered milk in tris-buffered saline with tween 20 (TBS-T) at room temperature for 1 h to block nonspecific binding sites. In sequence, the membranes were washed three times in TBS-T for 10 min each and incubated overnight (~16 h) with the primary pro anti-BDNF monoclonal antibody (1:300, Santa Cruz Biotechnology). Again, the membranes were washed three times for 10 min each in TBS-T and then incubated with polyclonal secondary antibody (1:2000 in TBS-T; rabbit anti-mouse IgG) for 2 h, followed by 3 washes in TBS-T of 10 min each. Finally, development was performed with an ECLplus® chemiluminescence kit (Invitrogen, Carlsbad, CA, USA) for 10 min. All blots were stripped with a harsh stripping buffer (20% SDS 10%, 12.5% Tris HCl 0.5 M and 0.8% ß-mercaptoethanol in H2O), to assess the protein control (GAPDH). Bands were visualized using Chemi-doc (Bio-Rad Laboratories Inc. Hercules, USA), and intensities of specific bands were quantified using ImageJ (NIH, Bethesda, MD, USA) and normalized to GAPDH protein levels. The results are expressed as the mean ± SEM of protein level. The technique was performed according to Bonato et al. [35].

2.7. Light-dark transition test (LDTT)

The LDTT was performed using a rectangular wooden box (56.5 cm × 26.5 cm × 28.5 cm). The box was divided into two compartments of equal size: one side painted black and covered by a black lid (this being the dark compartment) and one side painted white covered by a transparent acrylic lid (lit compartment). The two sides of the box were connected by a door (7.0 cm × 6.0 cm) that allowed transitions between the compartments. To perform the test, the animals were placed individually in the lit compartment, facing the door, and their behavior was recorded for 5 min for further analysis. The percentage of time spent in the lit compartment was used as an index of anxiety-like behavior. In addition, the total number of crossings between compartments was used to assess locomotor activity [38,44].

2.8. Open field test (OFT)

The open field consisted of a rectangular box (40 cm × 50 cm × 63 cm; divided into 9 square units) and it was used to assess locomotor/exploratory activity and some parameters related to anxiety. The animals were placed individually in the center of the apparatus for 5 min and their behaviors were recorded for further analysis. To analyze locomotor and exploratory activity, the number of crossings with all four paws from one unit to another, and the frequency of grooming (self-cleaning) and rearing (rising on the hind limbs) were analyzed. As anxiety's index, the time spent in the center of the apparatus (in seconds) was measured [45,46].

2.9. Elevated plus maze (EPM) test

The potential anxiolytic-like effect of the treatment was evaluated. The EPM was made of wood at a height of 50 cm above the floor, consisting of 4 arms (2 open and 2 closed) and at the cross between the arms there was a central area of 10 cm^2. Each animal was placed in the center of the apparatus facing a closed arm and remained freely exploring the apparatus for 5 min. The test was recorded for further analysis. The percentage of time and the number of entries in the open arms was quantified as an anxiety index. As a locomotor index, we also quantified the number of entries in the closed arms. In addition, some ethological measures were investigated, such as the frequency of head dipping (exploratory movement in open arms, so that the animal projects the head of the maze toward the floor) and risk assessment (exiting an enclosed arm with the forepaws and head and investigating the surroundings. The risk assessment was often, but not necessarily, accompanied by body stretching, that is, the animal stretches to its full length and turns back to the anterior position. Between each session, the apparatus was cleaned with a 20% ethanol solution [12,17,47,48].

2.10. Experimental protocols

At the beginning (before the induction of experimental diabetes) and at the end (after the last behavioral test) the animals had their body weight (BW) assessed to calculate the weight gain (by subtracting the BW taken from the last day of the experiment from the BW taken on day 0). Blood glucose (BG) was assessed 72 h after induction of experimental diabetes, and again at the end of all experimental protocols. In all experiments, a group of vehicle (VEH)-treated NGL animals was conducted in parallel, as a control of the diabetic condition. Importantly, for the long-term treatment, the last injection was performed at least 18 h before each behavioral test.

The rats were allowed one week to acclimate to the environment before beginning the tests and before each behavioral experiment, the animals underwent a 1-hour habituation in the experimentation room.

All behavioral testing was performed between 1 and 5 PM.

Experiment 1 - The rationale of this study was to evaluate the effect of a single injection of ROF (0, 0.01, 0.03 and 0.1 mg/kg, i.p.) on the acquisition of fear extinction memory in STZ animals. All animal groups were submitted to CFC. The animals underwent the induction of experimental T1DM on day 0. On the 25th day the CFC protocol was started following these steps: familiarization (day 25), CFC (day 26), extinction training (27), extinction test (28) and generalization test (29). Acute treatment with ROF (STZ animals) or VEH (STZ and NGL animals) occurred on day 27 of the protocol, 1 h before extinction training. At the end of the 29th day, the animals were euthanized.

Experiment 2 - This experiment was conducted to study the effect of a long-term treatment (21 days) with ROF (0, 0.01, 0.03 and 0.1 mg/kg; ip) on the impaired aversive memory processing of STZ animals. All animals were submitted to the CFC protocol. At the beginning of the experiment, the animals underwent experimental T1DM induction (day 0) and on the 7th day of the protocol, treatment with ROF (STZ animals) or VEH (STZ or NGL animals) was started for 21 days (from the 7th day to the 28th day). On the 25th day the CFC protocol was started following the steps, as described in the Experiment 1.

Experiment 3 - In this experiment, the aim was to evaluate whether the long-term treatment with ROF (0, 0.01, 0.1 mg/kg; i.p.) would induce a beneficial effect on the more expressive anxiety-like behavior in STZ animals. In addition, whether the treatment would alter the locomotor or exploratory activity of these same animals. For this, on day 0 the animals underwent the induction of experimental T1DM, and on the 7th day of the protocol, treatment with ROF (STZ animals) or VEH (STZ or NGL animals) was started for 21 days (from the 7th day to the 28th day). On the 27th day animals were submitted to the LDTT. On the following day (day 28) they were submitted to the OFT followed by EPM test (day 29). At the end of the protocol, the animals were euthanized.

Experiment 4 - To evaluate a possible recruitment of cAMP/PKA signaling, the level of PROBDNF protein was evaluated in the HIP and CPF of the animals. For this, on day 0 the animals underwent the induction of experimental T1DM, and on the 7th day of the protocol, treatment with ROF (0.01 and 0.1 mg/kg; ip. - in STZ animals) or VEH (STZ or NGL animals) was started for 21 days (from the 7th day to the 28th day). On the following day (29th day), the animals were euthanized with decapitation, the brain was removed, and the HIP and PFC were dissected and kept at − 80 ºC.

2.11. Statistical analysis

Shapiro-Wilk normality test was used to determine the distribution profile (Gaussian curvature). As the normality criteria was confirmed, the data are expressed as mean ± standard error of the mean (SEM).

For the data of extinction training in Experiments 1 and 2, the two-way repeated measure analysis of variance (two-way RM ANOVA) was performed (independent factors: time-repetition and groups). For the analysis of multiple comparisons, when there was an effect between the factors (time-repetition and groups), but no interaction between these factors, a comparison was made between column means (main column effect) and groups. In this case, it merges all 5-time blocks as a single time and considers the different groups.

Regarding the analyzes of other behavioral findings and weight gain and blood glucose, we performed Student's *t*-test between the NGL and STZ/VEH groups, in order to assess whether the experimental induction of diabetes *mellitus* actually occurred and whether the behavioral findings would be reproduced. Between all the STZ groups, we performed one-way ANOVA being the treatment (VEH or ROF) the only independent factor. When appropriate, the Newman-Keuls test was used as a *post-hoc* analysis. The results were considered statistically significant when the p value < 0.05. Statistical analyzes were performed using the GraphPad Prism software (version 8, San Diego, CA, USA).

3. Results

3.1. Effect of single or prolonged treatment with ROF on blood glucose and weight gain of STZ animals

As can be seen at Table 1, for blood glucose, Student's *t*-test showed a statistically significant difference between the NGL/VEH and STZ/VEH groups [$t = 20.28$; $df = 8$; $p < 0.05$], confirming the ability of STZ to induce experimental diabetes *mellitus* in animals. In turn, the one-way ANOVA performed between all STZ animals treated with VEH or ROF showed no statistical difference [$F\ (3, 16) = 0.7508$; $p > 0.05$]. Regarding to weight gain, Student's *t*-test also showed statistical differences between the NGL/VEH and STZ/VEH groups [$t = 27.56$; $df = 8$; $p < 0.05$], confirming one more feature of the experimental diabetes condition, the impaired weigh gain of these STZ animals. The one-way ANOVA showed that between all STZ groups there were no significant differences between the groups [$F\ (3, 16) = 2.072$; $p > 0.05$].

In the experiment 2 (see Table 1), Student's *t*-test showed significant difference between the NGL/VEH and STZ/VEH groups [$t = 18.15$; $df = 11$; $p < 0.05$] when blood glucose was evaluated, confirming that STZ injection was able to induce the experimental T1DM in animals. Then, the one-way ANOVA performed between all STZ groups showed a statistically significant effect [$F\ (3, 23) = 4.037$; $p < 0.05$]. The Newman-Keuls test showed that the three doses of ROF were able to decrease the blood glucose of the STZ animals ($p < 0,05$). Regarding to weight gain, Student's *t*-test between the NGL/VEH and STZ/VEH groups showed again a significant difference [$t = 28.75$; $df = 11$; $p < 0.05$]. Among the STZ groups, the one-way ANOVA showed no significant differences for the control group and the ROF-treated groups [$F\ (3, 23) = 0.8502$; $p > 0.05$].

In the experiment 3 (Table 1), as observed in the experiment 2 regarding to blood glucose, the Student's *t*-test showed that there is a difference between the NGL/VEH and STZ/VEH groups [$t = 17.01$; $df = 12$; $p < 0.05$] when blood glucose was evaluated, and one-way ANOVA performed between all STZ groups showed significant differences [$F\ (2, 18) = 8.461$; $p < 0.05$]. The Newman-Keuls *post-hoc* test showed that the two doses of ROF were able to decrease the blood glucose in relation to STZ/VEH animals ($p < 0.05$). Student's *t*-test showed significant differences between the NGL/VEH and STZ/VEH groups [$t = 13.62$; $df = 12$; $p < 0.05$] when weight gain was evaluated, with STZ animals gaining much less weight than control animals. One-way ANOVA demonstrated that there are differences between STZ groups [F (2, 18)

Table 1
Effect of treatment with Roflumilast (ROF; at doses 0.01, 0.03 or 0.1 mg/kg) or vehicle (VEH) on the parameters of glycemia and weight gain in streptozotocin animals (STZ) or normoglycemic animals (NGL).

Groups	Glycemia (mg/dL)	Weight gain (g)
Experiment 1		
NGL/VEH	93.71 ± 2.766	122.9 ± 4.41
STZ/VEH	542.7 ± 22.26 *	-12.5 ± 1.708 *
STZ/ROF 0.01	574.8 ± 15.38	-16 ± 1.871
STZ/ROF 0.03	539.5 ± 35.61	-15 ± 2.041
STZ/ROF 0.1	562 ± 13.24	-20 ± 2.739
Experiment 2		
NGL/VEH	96.14 ± 3.327	125 ± 4.226
STZ/VEH	546.5 ± 26.72 *	-18.33 ± 2.108 *
STZ/ROF 0.01	426.1 ± 33.29#	-21.43 ± 2.608
STZ/ROF 0.03	442 ± 19.01#	-19.29 ± 1.7
STZ/ROF 0.1	436.1 ± 26.38#	-24.29 ± 4.144
Experiment 3		
NGL/VEH	94.86 ± 1.654	64.71 ± 6.443
STZ/VEH	414.1 ± 18.69 *	-26 ± 1.69 *
STZ/ROF 0.01	351.3 ± 15.06#	-13 ± 6.966
STZ/ROF 0.1	326.1 ± 12.34#	-40.57 ± 7.425

Values were expressed as mean ± SEM of 6–8 animals/experimental group. *p < 0.05 when compared to NGL/VEH group and #p < 0.05 when compared to STZ/VEH group.

= 5.358; $p < 0.05$]; however, when applying the Newman-Keuls post hoc test it was not possible to observe differences between the STZ groups ($p > 0.05$). This result indicates that ROF treatment was not able to improve the impaired weight gain of STZ animals.

3.2. Experiment 1

3.2.1. A single injection of ROF was not able to induce the acquisition of fear extinction memory in STZ animals or context discrimination

In the extinction training session, the two-way RM ANOVA showed a significant difference in the two factors - time [$F(4, 80) = 36.15$; $p < 0.05$] and treatment [$F(4, 20) = 3.405$; $p < 0.05$], but no interaction between them [$F(16, 80) = 0.8993$; $p > 0.05$]. The Newman-Keuls test demonstrated that STZ-VEH compared to NGL-VEH presented an increase in freezing behavior (%). In turn, it did not show significant differences between all STZ groups analyzed (Fig. 1, panel A).

In the extinction test (Fig. 1, panel B), Student's *t*-test showed a significant difference between the NGL/VEH and STZ/VEH groups [$t = 2.655$; $df = 8$; $p < 0.05$]. Regarding the one-way ANOVA applied between the STZ groups treated or not with ROF, there were no significant differences between the groups [$F(3, 16) = 0.5371$; $p > 0.05$]. Regarding the generalization test (Fig. 1, panel C), Student's *t*-test showed a statistically significant difference between the NGL/VEH and STZ/VEH groups [$t = 3.037$; $df = 8$; $p < 0.05$]. However, between the STZ/VEH and STZ groups treated with ROF, the one-way ANOVA showed no significant difference [$F(3, 16) = 0.1947$; $p > 0.05$].

Finally, when analyzed the discrimination index (Fig. 1, panel D), Student's *t*-test showed a decrease in this index in the STZ/VEH group compared to NGL/VEH [$t = 4.933$; $df = 8$; $p < 0.05$], while one-way ANOVA did not show differences between the STZ groups treated or not with ROF [$F(3, 16) = 0.2715$; $p > 0.05$].

3.3. Experiment 2

3.3.1. Prolonged treatment with ROF facilitated the acquisition of fear extinction memory in STZ animals and improved the context discrimination

For the extinction training session, two-way RM ANOVA (Fig. 2, panel A) showed a statistically significant effect on both factors – time [$F(4, 116) = 8.298$; $p < 0.05$] and treatment [$F(4, 29) = 16.97$; $p < 0.05$], but showed no interaction between the two factors [$F(16, 116) = 0.732$; $p > 0.05$]. The Newman-Keuls test showed a significant difference between NGL group and STZ/VEH group ($p < 0.05$; panel A). Also, all treatments performed in STZ animals were able to decrease the freezing response, compared to STZ/VEH group ($p < 0.05$; Fig. 2, panel A).

Regarding the extinction test, Student's *t*-test showed a significant difference between the NGL/VEH and STZ/VEH groups [$t = 5.983$; $df = 11$; $p < 0.05$], indicating an increase in freezing time in STZ/VEH animals (Fig. 2, panel B). One-way ANOVA showed a significant

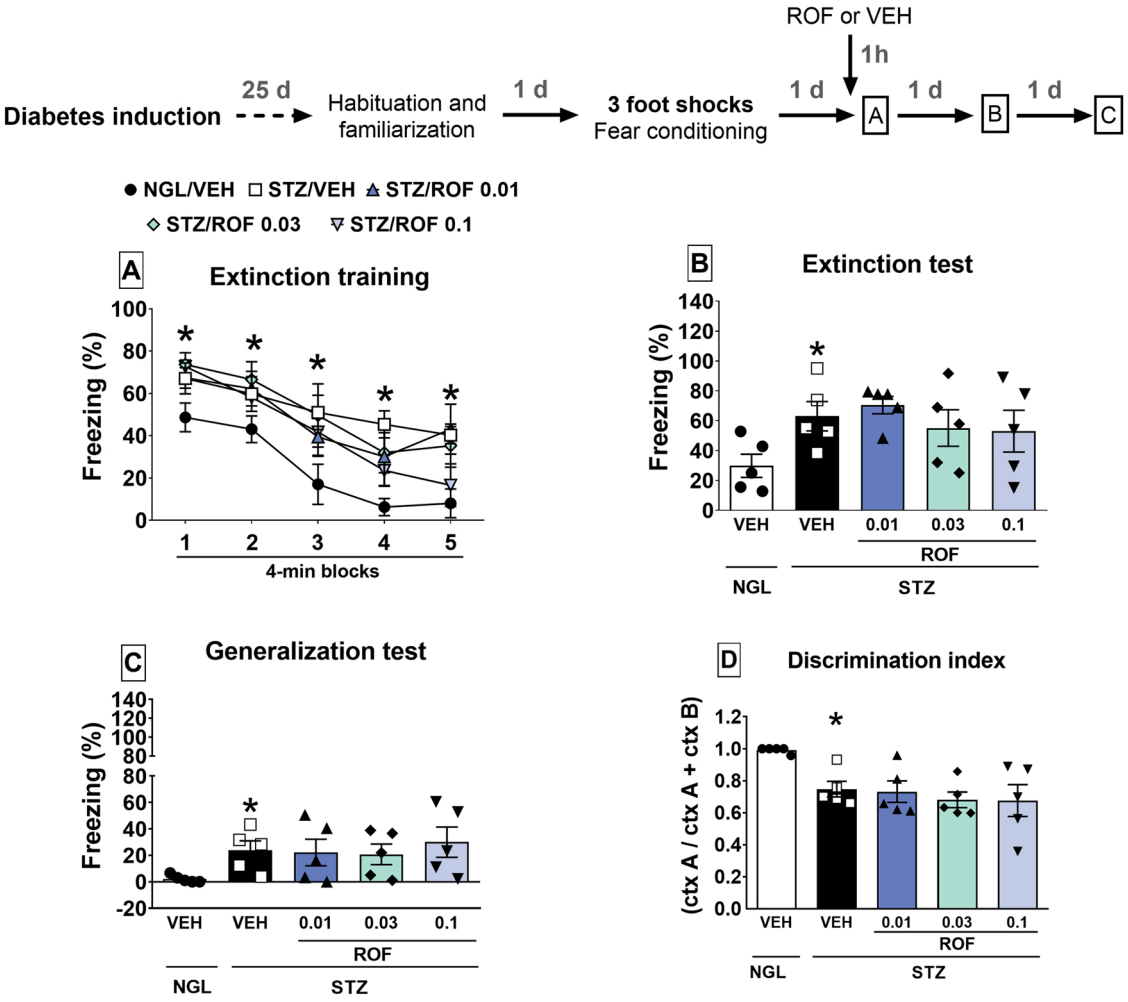

Fig. 1. Effect of a single injection of roflumilast (ROF; 0.01, 0.03 or 0.1 mg/kg) or vehicle (VEH) on acquisition of the fear extinction memory in a contextual fear conditioning paradigm. The scheme above the graphs represents the experimental design used in this experiment: (A) - Extinction training of contextual fear memory; (B) - Extinction test; (C) - Generalization test; (D) - Discrimination index between conditioning (context- Ctx A) and neural (Ctxt B). Values were expressed as mean ± SEM (n = 5/group). *$p < 0.05$ when all groups are compared to NGL/VEH group.

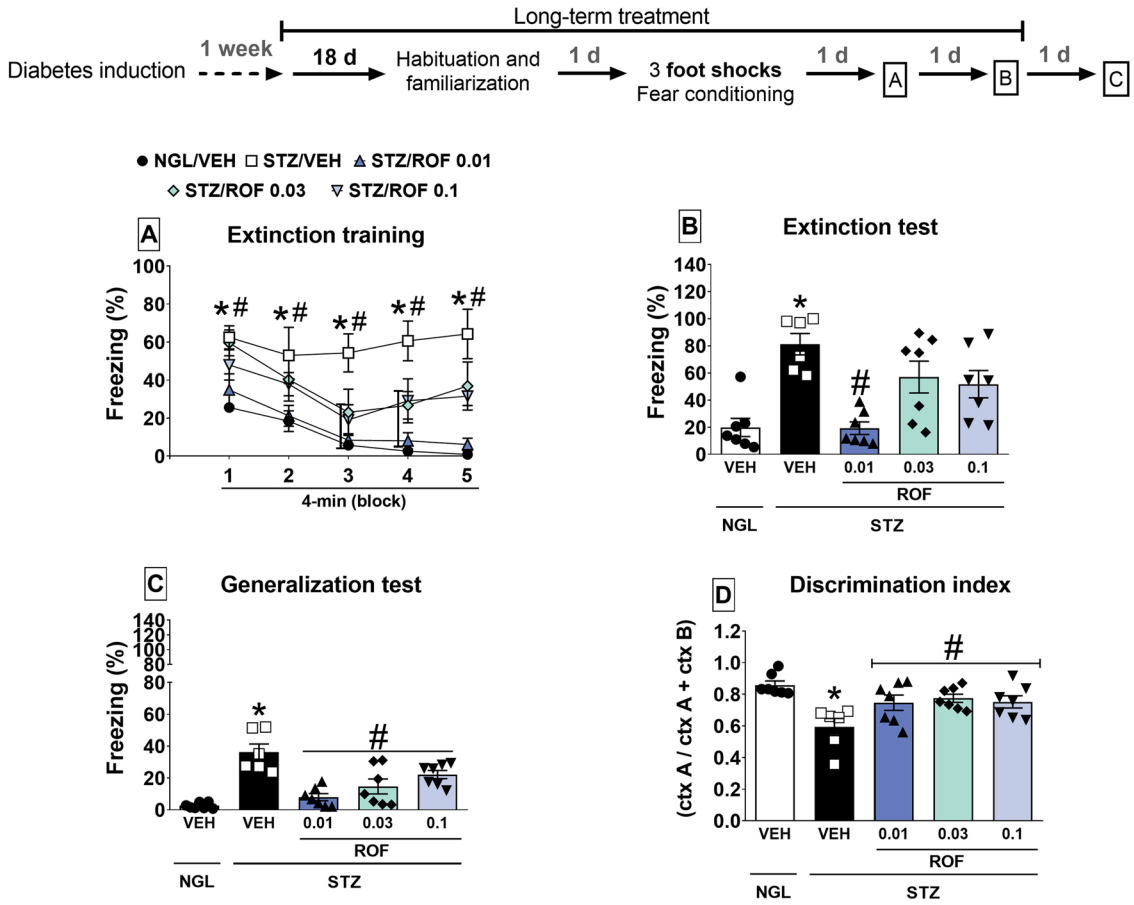

Fig. 2. Effect of long-term treatment with roflumilast (ROF; 0.01, 0.03 or 0.1 mg/kg) or vehicle (VEH) on fear extinction memory in contextual fear conditioning paradigm. The scheme above the graphs represents the experimental design used in this experiment: (A) - Extinction training of contextual fear memory; (B) - Extinction test; (C) - Generalization test; (D) - Discrimination index between conditioning (context- Ctx A) and neural (Ctxt B). Values were expressed as mean ± SEM (n = 6–7/group). *p < 0.05 when all groups are compared to NGL/VEH group and #p < 0.05 when STZ/VEH group is compared to all treated-STZ groups.

difference when applied between all STZ groups (which received the VEH and the three doses of ROF) [F (3, 23 = 7.653; p < 0.05]. The Newman-Keuls *posthoc* showed that the STZ/ROF 0.01 group has a reduced freezing time in relation to the STZ/VEH group (p < 0.05; Fig. 2, panel B). In the generalization test, the Student's *t*-test showed a significant difference between the NGL/VEH and STZ/VEH groups [t = 7.027; df = 11; p < 0.05], indicating an increase in the freezing time of STZ animals (Fig. 2, panel C). One-way ANOVA between STZ animals (VEH and ROF treated) showed a statistically significant difference [F (3, 23) = 9.825; p < 0.05]. The Newman-Keuls *posthoc* showed statistical differences in all groups treated with ROF (STZ/ROF 0.01, 0.03 and 0.1) in relation to the STZ/VEH group, indicating that ROF was able to reduce the freezing time in treated STZ animals (p < 0.05; Fig. 2, panel C).

Finally, Student's *t*-test showed significant differences between the NGL/VEH and STZ/VEH groups in the discrimination index [t = 4.667; df = 11; p < 0.05] (Fig. 2, panel D). The one-way ANOVA showed a significant difference between the STZ groups treated or not with ROF [F (3, 23) = 3.568; p < 0.05], and the Newman-Keuls *posthoc* showed differences in all ROF-treated groups (STZ/ROF 0.01, 0.03 and 0.1) in relation to the STZ/VEH group (Fig. 2, panel D).

3.4. Experiment 3

3.4.1. Prolonged treatment with ROF reduced the more pronounced anxiety-like behavior of STZ animals

Student's *t*-test showed significant differences between the NGL/VEH and STZ/VEH groups in the time spent on the lit compartment (Fig. 3, panel A) [t = 2500; df = 12; p < 0.05], on the dark compartment (Fig. 3, panel B) [t = 2.538; df = 12; p < 0.05] and on frequency of risk assessment (Fig. 3, panel D) [t = 2.229; df = 12; p < 0.05], indicative of a more pronounced anxiety-like response in these STZ animals. Regarding the number of crossings, there was no difference between the groups (Fig. 4, panel C) [t = 1.331; df = 12; p > 0.05].

When comparing all STZ groups, the one-way ANOVA showed that ROF treatment was able to increase the time spent by the animal on the lit compartment (Fig. 3, panel A) [F (2, 18) = 5.097; p < 0.05], decrease the time spent on the dark compartment (Fig. 3, panel B) [F(2, 18) = 3.892; p < 0.05], and decrease the frequency of risk assessment (Fig. 3, panel D) [F (2, 18) = 4.290; p < 0.05], indicative of anxiolytic-like effect. The Newman-Keuls post hoc showed that the two groups treated with ROF (STZ/ROF 0.01 and STZ/ROF 0.1) were able to induce these beneficial effects (p < 0.05). Regarding the number of crossings (Fig. 3, panel C), the one-way ANOVA showed no difference between the STZ groups treated or not with ROF [F (2, 18) = 1.064; p > 0.05].

Treatment with ROF for 21 days was able to improve anxiety-like behaviors of STZ animals submitted to the open field test, without improving locomotor and exploratory activity (Fig. 4). Student's *t*-test showed statistically significant differences between the NGL/VEH and STZ/VEH groups, i.e. STZ/VEH animals presented a decrease in the number of crossings (Fig. 4, panel A) [t = 3.248; df = 12; p < 0.05], in the frequency of rearing (Fig. 4, panel B) [t = 4.080; df = 12; p < 0.05] and of grooming (Fig. 4, panel C) [t = 3.703; df = 12; p < 0.05] along with a decrease in the time spent by the animals in the center of the apparatus (Fig. 4, panel D) [t = 3.568; df = 12; p < 0.05], indicative of a more pronounced anxiety-like behavior.

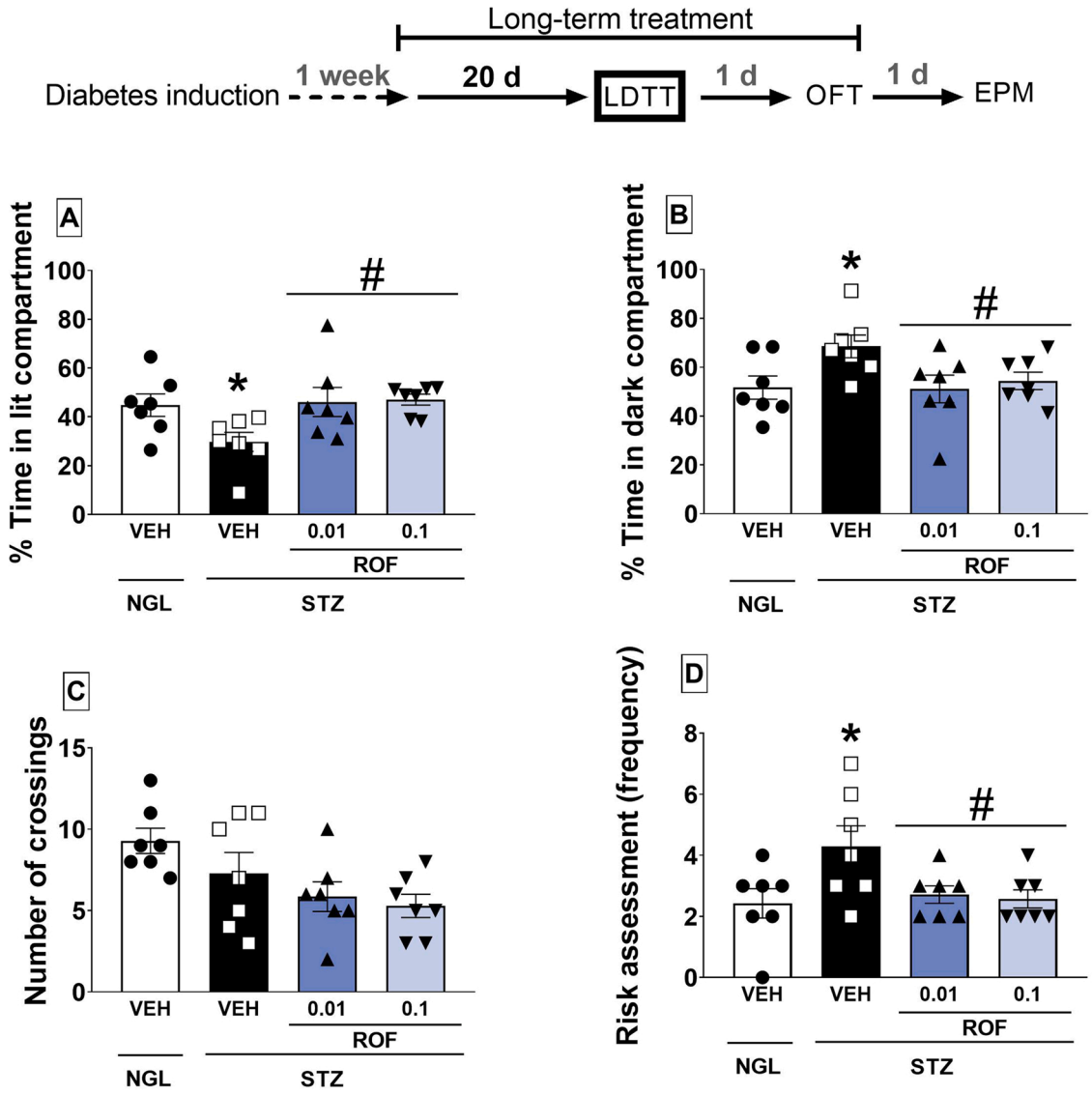

Fig. 3. Effect of long-term treatment with roflumilast (ROF; 0.01 or 0.1 mg/kg) or vehicle (VEH) on anxious-like behavior in the light-dark transition test (LDTT). The scheme above the graphs represents the experimental design used in this experiment: (A) - Time in the lit compartment (%); (B) - Time in the dark compartment (%); (C) - Number of crossings; (D) - Frequency of risk assessment. Values were expressed as mean ± SEM (n = 7). *p < 0.05 when compared to NGL/VEH group and #p < 0.05 when compared to STZ/VEH group.

One-way ANOVA performed between all STZ groups showed differences between groups in three parameters: frequency of rearing (Fig. 4, panel B) [F (2, 18) = 5.575; p < 0.05], grooming frequency (Fig. 4, panel C) [F (2, 18) = 5.776; p < 0.05] and time spent in the center of the apparatus (Fig. 4, panel D) [F (2, 18) = 4.672; p < 0.05]. For these three parameters, the Newman-Keuls post hoc test showed that the two doses of ROF (STZ/ROF 0.01 and STZ/ROF 0.1 groups) were able to increase grooming and rearing frequencies, and the time spent in the center of the apparatus, when compared to STZ/VEH animals (p < 0.05). Regarding the number of crossings, the one-way ANOVA showed no significant difference between the STZ groups treated or not with ROF (Fig. 4, panel A) [F (2, 18) = 1.158; p > 0.05].

When animals were evaluated in the EPM test, prolonged treatment with ROF was able to reduce some anxiety-like parameters in the STZ animals, but not all (Fig. 5). Student's *t*-test showed that, when compared to NGL/VEH group, STZ/VEH group presented a decrease in the time spent and in the entries number by the animals in the open arms (Fig. 5, panels A and B; t = 2.648; df = 12; p < 0.05 and t = 4.479; df = 12; p < 0.05, respectively], besides a decrease in the frequency of head dipping (Fig. 5, panel D - t = 3.753; df = 12; p < 0.05), indicating a more pronounced anxiety-like behavior. Regarding the number of entries in the closed arms, these STZ/VEH animals present a decrease in the number of entries (Fig. 5, panel C - t = 2.870; df = 12; p < 0.05). Student's *t*-test did not show differences between the NGL/VEH and STZ/VEH groups when frequency of risk assessment was measured (Fig. 5, panel E) [t = 1.016; df = 12; p > 0.05].

When one-way ANOVA was performed between the STZ groups treated or not with ROF, the only parameter that showed differences was the frequency of risk assessment (Fig. 5, panel E - F (2, 18) = 5.085; p < 0.05). The Newman-Keuls post hoc test showed differences between the two groups treated with ROF (STZ/ROF 0.01 and STZ/ROF 0.1) in relation to the STZ/VEH group, i.e. the treatment was able to reduce the frequency of risk assessment (p < 0.05), indicating an anxiolytic-like effect. For the other parameters, one-way ANOVA showed no differences between STZ groups treated or not with ROF: time spent by animals in open arms (Fig. 5, panel A) [F (2, 18) = 0.7829; p > 0.05], number of entries in the open arms (Fig. 5, panel B) [F (2, 18) = 0.6516; p > 0.05], number of entries in closed arms (Fig. 5, panel C) [F (2, 18) = 0.06936; p > 0.05] and frequency of head dippings (Fig. 5, panel D) [F (2, 18) = 0.7552; p > 0.05].

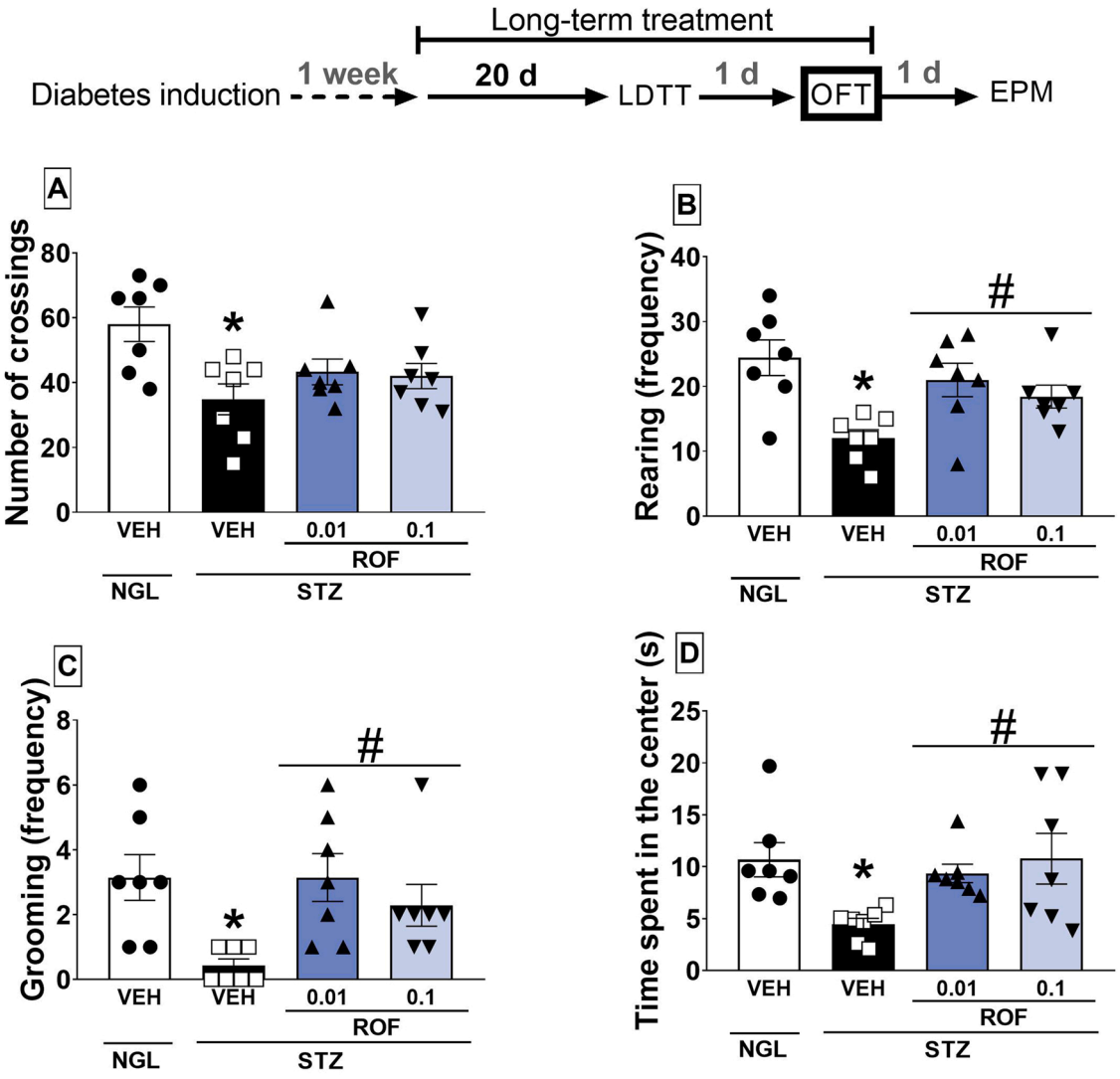

Fig. 4. Effect of long-term treatment with roflumilast (ROF; 0.01 or 0.1 mg/kg) or vehicle (VEH) on locomotor/exploratory activity, and parameters related to anxiety in the open field test (OFT). The scheme above the graphs represents the experimental design used in this experiment: (A) - Number of crossings; (B) - Rearing (frequency); (C) - Grooming (frequency); (D) - Time spent in the center. Values were expressed as mean ± SEM (n = 7/group). *p < 0.05 when compared to NGL/VEH group and #p < 0.05 when compared to STZ/VEH group.

3.5. Experiment 4

3.5.1. Prolonged treatment with ROF increased proBDNF levels in the HIP and PFC of STZ animals

Regarding to the full-length proBDNF levels in the HIP (Fig. 6, panel A), Student's *t*-test showed a significant decrease in the proBDNF levels of STZ/VEH animals, when compared to NGL/VEH animals [t = 5.887; df = 8; p < 0.05]. One-way ANOVA also showed significant differences between all STZ groups - treated or not with ROF [F (2, 12) = 4.301; p < 0.05]. However, the Newman-Keuls test showed that only the lowest dose of ROF (0.01 mg/kg) was able to increase proBDNF levels in the HIP of the STZ animals (p < 0.05; Fig. 6, panel A).

The proBDNF levels in the PFC were also evaluated (Fig. 6, panel B). The Student's *t*-test showed a statistically significant decrease in the proBDNF levels of STZ/VEH group, when compared to NGL/VEH group [t = 2.915, df = 8; p < 0.05]. One-way ANOVA revealed that STZ groups treated or not with ROF presented a significant difference between the groups [F (2, 12) = 6.056; p < 0.05]. Newman-Keuls post hoc test demonstrated that the two doses employed were able to increase proBDNF levels in the PFC in relation to the STZ/VEH group (p < 0.05; Fig. 6, panel B).

4. Discussion

The main finding of the study was that long-term treatment, yet not acute, with ROF was able to reduce the exacerbated freezing behavior in STZ animals associated with conditioned contextual fear. Importantly, this treatment seems to improve the processing of memory related to conditioned fear once there was an improvement in the discrimination index between the fear-conditioned environment, with a neutral one. This finding may also be linked to the anxiolytic-like effect induced by the treatment, observed mainly when LDTT and OFT were performed.

Considering ROF mechanism of action and its neuroprotective effects, we also observed an increase in full-length proBDNF levels in the HIP and PFC of ROF-treated STZ animals compared to VEH-treated STZ animals. The injection of STZ (via i.p.) in rats destroys pancreatic-beta cells, leading to hyperglycemia. This method has been used extensively worldwide to induce experimental T1DM in rodents [8,12,15,49,50]. It is known that this animal model of T1DM induces a series of alterations in the CNS, which has behavioral consequences as demonstrated in pre-clinical studies including more exacerbated anxiety-like, depression-like, and fear-like behaviors. Interestingly, data obtained from diabetic patients also demonstrate a higher prevalence of

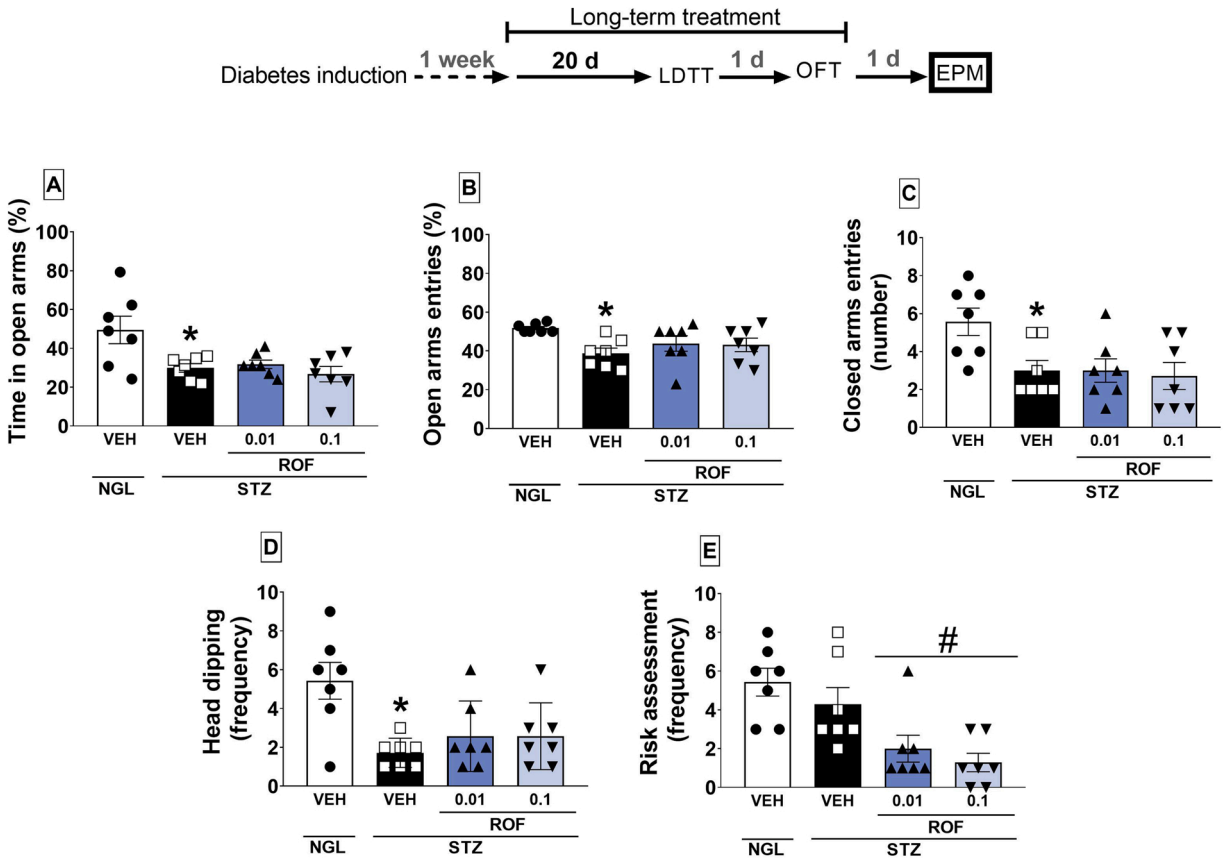

Fig. 5. Effect of long-term treatment with roflumilast (ROF; 0.01 or 0.1 mg/kg) or vehicle (VEH) on parameters related to anxiety/exploratory activity in the elevated plus maze (EPM) test. The scheme above the graphs represents the experimental design used in this experiment: (A) - Time in open arms (%); (B) - Open arms entries (%); (C) - Closed arms entries (number); (D) - Head dipping (frequency); (E) - Risk assessment (frequency). Values were expressed as mean ± SEM (n = 7/group). *p < 0.05 when compared to NGL/VEH group and #p < 0.05 when compared to STZ/VEH group.

psychiatric disorders, such as Depression and Anxiety [20,21] when compared to the general population. Furthermore, it is also well reported that these STZ animals, as well as T1DM patients, have cognitive deficits including impairments in learning and memory processing involving object recognition and its spatial location [51–53].

This impairment in the processing of these memories also extends to fear-related memories. For example, studies using animals with experimental T1DM showed exacerbated fear responses during the CFC test, i.e. they present an increased and persistent freezing behavior, indicative of overconsolidation of fear memory, and/or difficulty in extinguishing this fear memory [15,16,47,54–56]. The more expressive anxiety-like response is also present when these animals are subjected to different behavioral tests [12,15,17]. Our results are in line with all previous studies by demonstrating that STZ animals presented hyperglycemia and reduced weight gain (Table 1), increased freezing behavior during extinction and generalization test (CFC test), and a more expressive anxiety-like response (Figs. 1–5) [16,17]. Interestingly, for the first time we observed in these STZ animals an impairment in discriminating neutral environments from those previously conditioned to fear (Figs. 1 and 2), indicative of an impairment in fear-related memory processing.

In a study carried out by Sohn et al. [38], a single injection of ROF before the acquisition of extinction memory, at the same doses used in the present study (0.01, 0.03, 0.1 mg/kg, via i.p.), was able to decrease the freezing behavior in the NGL animals compared to the group of untreated NGL animals, in a contextual fear protocol being also the US the foot shock [38]. However, our data demonstrate that STZ animals showed no change in freezing behavior when they received a single injection of ROF before the acquisition of extinction memory (Fig. 1). This difference between non-diabetic and STZ animals after acute treatment with ROF is not surprising as several studies demonstrate that experimental T1DM induces encephalopathy in these animals characterized by increased oxidative stress and neuroinflammation, chronic increase in the long-term activity of the hypothalamus-pituitary-adrenocortical (HPA) axis [57,60], dysregulation of the neurotransmitter systems, impaired synaptic plasticity, and neurogenesis [1–12]. Thus, a longer treatment would be necessary for a beneficial effect to occur.

In this sense, our findings confirmed this hypothesis, that is, after a long-term treatment with ROF, STZ animals showed significant decrease in freezing behavior when submitted to the CFC protocol - during extinction and generalization test, compared to STZ/VEH animals (Fig. 2). Also, the discrimination index improved after the treatment. But, here it is important to highlight that with this experimental design, we have no way of pointing out whether long-term treatment with ROF affected contextual fear conditioning, fear memory extinction learning, extinction memory consolidation, and/or fear memory extinction retention. Despite this, it is interesting to note that these beneficial effects after long-term treatment with ROF had already been shown when other memory-related behaviors were studied. For example, it was demonstrated that long-term treatment with ROF (21 days or more) with different doses, including those in the present study, improved several memory-related behaviors in different animal models, such as transient global cerebral ischemia and even Alzheimer's disease [35,36,42,61]. Related to fear memory, a beneficial effect on this type of memory in a model of Alzheimer's disease (APP transgenic mice) had already been demonstrated in 2004, in which Gong et al. [62] used another PDE-4 inhibitor, rolipram [62].

Regarding the doses of ROF used in the study, which were chosen

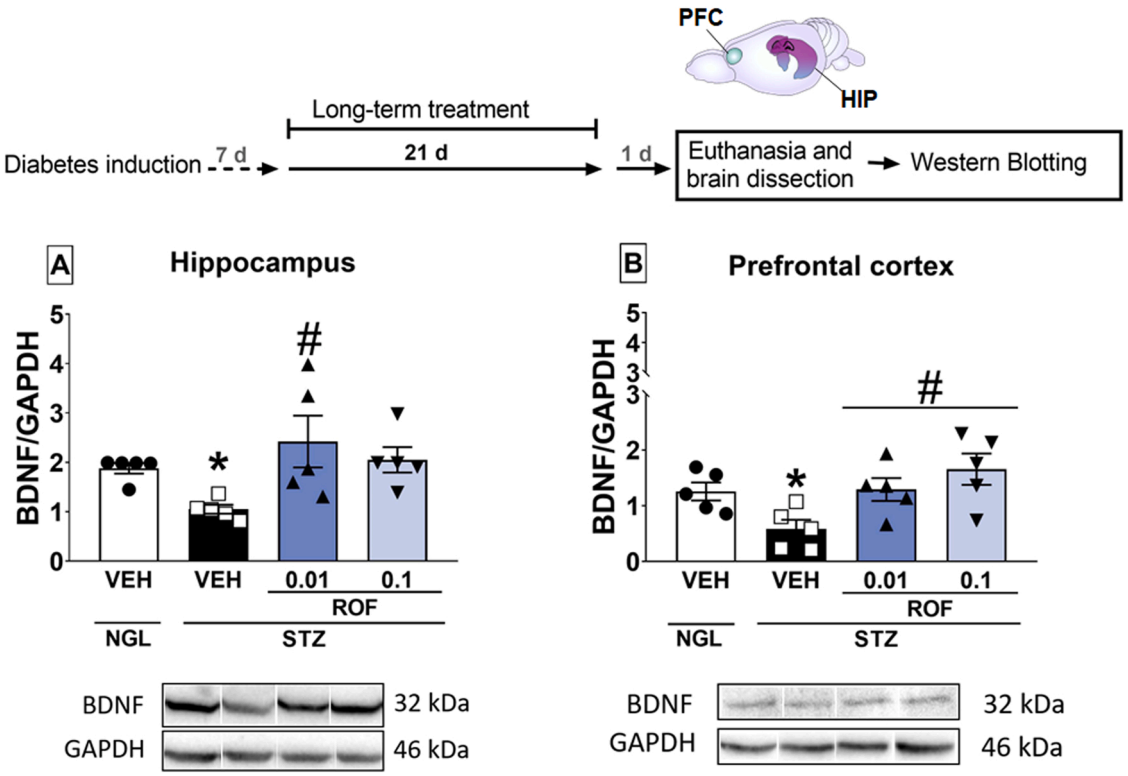

Fig. 6. Effect of long-term treatment with roflumilast (ROF; 0.01 or 0.1 mg/kg) or vehicle (VEH) on proBDNF expression in the hippocampus (A) and prefrontal cortex (B) of the animals. The scheme above the graphs represents the experimental design used in this experiment. Values were expressed as mean ± SEM (n = 5/group). *p < 0.05 when compared to NGL/VEH group and #p < 0.05 when compared to STZ/VEH group.

based on previous studies [4,6,7,34,38,42], despite ROF being the second-generation selective PDE-4 inhibitor with fewer emetic properties (10-fold lower compared to rolipram), at higher doses ROF could induce emesis (nausea and vomiting) [26,34,42]. However, the relatively low doses used in the present study do not have emetic-like potential in rodents [34]. In addition, it is important to point out that in recent clinical studies, the authors showed that a single 5 times lower COPD dose of ROF (100 μg, v.o.) in healthy young adults, as well as the elderly, did not induce any adverse effects, while this treatment was able to improve the performance of cognitive tests related to verbal memory in these experimental subjects [31,32]. Although our findings show that during the extinction test the lowest dose of ROF was effective in decreasing freezing behavior (0.01 mg/kg; Fig. 2B), when we evaluated the generalization and the discrimination index all doses are effective (Fig. 2 - panels C and D). Perhaps the large variability between groups treated with the higher doses masked the effect of lowering the fear response in the extinction test.

Considering that both the lowest and the highest dose of ROF induced an improvement in the discrimination of conditioned environment with fear and neutral environment, in the following studies we chose to treat the animals with the lowest and highest dose of ROF. The rationale of studies involving anxiety-related responses was to investigate whether the effects of prolonged treatment with ROF on improving memory processing related to CFC protocol could also be associated with anxiolytic-like effect. Our data showed a clear anxiolytic-like effect of ROF treatment at the lowest and highest dose when animals were subjected to LDTT (increased time in the lit compartment, and decreased risk assessment frequency and time in the dark compartment - Fig. 3) and OFT (increased grooming and rearing frequencies and decreased time spent in the center - Fig. 4) tests. On the other hand, in the EPM test, the treatment was only able to change the frequency of risk assessment, decreasing this frequency (Fig. 5). It is known that, in general, a decrease in risk assessment has been associated to an anxiolytic-like effect [63,64]. Here, it is important to highlight that every anxiety behavioral test has some advantages and disadvantages over other paradigms. In addition, different results have been associated to species, whether mice or rats, and differences between sexes. Several different parameters that trigger anxiety/fear behavioral reaction (aversion to open spaces, spaces with high light, spaces raised from the floor, conflict between open and closed spaces) are involved and they are used to evaluate if a drug or compound carrying an anxiolytic-like effect or not. Some paradigms can be used to evaluate more than one type of anxiety disorder (for a review see [65,66].

The anxiolytic-like effect has been previously observed in non-diabetic animals subjected to LDTT, OFT and EPM test after prolonged treatment with different PDE-4 inhibitors, such as rolipram and etazolate [67–71]. Interestingly, Li et al. [67] observed in mice exposed to LDDT and EPM test that the anxiolytic-like effect induced after the long-term treatment with rolipram seems to be associated with hippocampal neurogenesis induced by this PDE-4 inhibitor, as there was an increase in BrdU-positive neurons in an immunofluorescence protocol in the HIP of animals treated with rolipram. Moreover, when they blocked rolipram-induced neurogenesis with the use of methylazoxymethanol (toxic for proliferating cells), they observed a reversal of the anxiolytic-like effect induced by rolipram [67]. Soares et al. [70] observed along with the anxiolytic-like effect of rolipram when animals were submitted to the OFT that this rolipram treatment was able to decrease hippocampal neurodegeneration, in addition to increasing neuroplasticity via increased dendritic arborization in doublecortin immunoreactive neurons, in a model of transient global cerebral ischemia [70].

The pro-cognitive action and anxiolytic-like effects of PDE-4 inhibitors, including ROF, have been associated with its relationship between the increase in cAMP levels that occurs after PDE-4 inhibition and the consequent activation of the CREB/BDNF pathways in the rat brain in different animal models [35,37,38,61]. In addition, the inflammatory

component could be involved in the action of ROF, as several studies point out to its anti-inflammatory activity related to improvements on cognition and memory [35,37,42,58,72]. Our data confirmed previous evidence that STZ animals present a decrease in the proBDNF in the HIP and/or PFC [6,59,73]. This reduction may be involved in this impairment in the processing of fear memory, as it has already been shown that proBDNF into medial PFC is required for depressing fear expression, whereas it is also necessary for inducing extinction of both new and old fear memories [40]. Using an animal model related to type-2 diabetes mellitus, induced with a lower dose of STZ, it was demonstrated that drugs that inhibit PDE-4 increase the intracellular signaling pathway that increases BDNF expression and/or its activity in the HIP and/or PFC [58,59]. In that sense, it was observed that long-term supplementation with rolipram improved diabetes-associated cognitive decline and increased expression of CREB and pCREB, in addition to reducing the inflammatory reaction (decreased tumor necrosis factor alpha levels and increased interleukin 10 levels) in the HIP [58]. Confirming these findings, Zhong et al. [59] also observed that long-term supplementation with rolipram improved diabetes-associated cognitive decline in addition to increasing CREB levels in the HIP of these animals. Additionally, the authors observed an increase in the levels of BDNF protein in the HIP, which were reduced in these animals [59]. Thus, it seems that PDE-4 inhibitors may repair the imbalance in the CREB/BDNF pathway in addition to other neuroprotective actions, such as the reduction of neuroinflammation.

In summary, the present study - which is to our knowledge the first study to demonstrate the effect of ROF treatment in an animal model of T1DM on the parameters evaluated - demonstrated that ROF improves contextual fear-related memory processing and associated anxiety-like behavior. In addition, our findings provide insights into the mechanisms underlying this emotional processing through the activation of the CREB/BDNF signaling pathway in the HIP and PFC.

CRediT authorship contribution statement

Janaina Menezes Zanoveli designed the study conception. Material preparation and data collection was performed by Ana Paula Farias Waltrick, Ana Carolina Felipe da Silva, Bianca Andretto de Mattos, Yane Costa, and Rúbia Maria Weffort de Oliveira. Statistical analysis was performed by Janaina Menezes Zanoveli. The first draft of the manuscript was written by Ana Paula Farias Waltrick, while the following versions were made by Janaina Menezes Zanoveli with English revision made by Rúbia Maria Weffort de Oliveira and Jos Prickaerts. The roflumilast was kindly provided by Dr. Jos Prickaerts. All authors commented on previous versions of the manuscript and read and approved the final manuscript.

Declaration of Competing Interest

The author(s) declared no conflict of interest, except for Prof J Prickaerts who has a proprietary interest in the PDE4 inhibitor roflumilast for the treatment of memory impairment.

Data availability

Data will be made available on request.

Acknowledgments

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