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Research report

Widespread blunting of hypothalamic and amygdala-septal activity and behavior in rats with long-term hyperglycemia

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HIGHLIGHTS

- Glucose levels of ~500 mg/dl reduced orexin activity in hypothalamic areas.
- Blunted Fos immunoreactivity was detected in amygdala and lateral septal nucleus.
- No indicators of anxiety or anhedonia were detected in behavioral tests.
- Decreased general locomotion was observed in the open field test.
- Long-term hyperglycemia decreased neural activity rather than anxiety or anhedonia.

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ABSTRACT

Anxiety and depression in diabetic patients contributes to a poor prognosis, but possible causal relationships have been controversial. Anxiety, fear, and anhedonia are mediated by interactions between different deep structures of the temporal lobe (e.g., amygdala complex and hippocampus) and other forebrain-related structures (e.g., lateral septal nucleus). Connections between these structures and the hypothalamic orexinergic system are necessary for the maintenance of energy and wakefulness. However, few studies have explored the impact of long-term hyperglycemia in these structures on anxiety. We induced long-term hyperglycemia (glucose levels of ~500 mg/dl) in Wistar rats by injecting them with alloxan and simultaneously protecting them from hyperglycemia by injecting them daily with a low dose of insulin (i.e., just enough insulin to avoid death), thus maintaining hyperglycemia and ketonuria for as long as 6 weeks. Compared with controls, long-term hyperglycemic rats exhibited a significant reduction of Fos expression in the lateral septal nucleus and basolateral amygdala, but no differences were found in cerebellar regions. Orexin-A cells appeared to be inactive in the lateral hypothalamus. No differences were found in sucrose consumption or behavior in the elevated plus maze compared with the control group, but a decrease in general locomotion was observed. These data indicate a generalized blunting of the metabolic brain response, accompanied by a decrease in locomotion but no changes in hedonic- or anxiety-like behavior.

1. Introduction

Depression often occurs among diabetic individuals [1], but causal relationships have been controversial. Diabetes is considered a risk factor for the development of depression [2], namely among type-2 diabetic individuals [3]. A bidirectional relationship appears to exist between diabetes distress and depressive symptoms [4–6], predominantly in the elderly [7]. Depression is seemingly associated with a higher risk for developing type-2 diabetes, but type-2 diabetes is associated with only a modest increase in the risk of depression [8,9].

At the experimental level, diabetic mice exhibit more submissive social behavior, more passive avoidance in response to shock

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[10], and marked submissive behavior in the resident-intruder paradigm [11] compared with healthy controls. Among cerebral structures that have been related to emotional processes, there is considerable information about the participation of amygdala-septal areas [12]. The lateral septum is involved in the regulation of anxiety [13,14], and its neuronal activity increases after the administration of several antidepressant drugs [15,16]. Conversely, neuronal activity in the lateral septum decreases after a single session of stress [17]. Neural activity in the lateral septal nucleus appears to be opposite to neural activity in the basolateral amygdala [18]. Thus, in fear-related situations, one may expect to see an increase in amygdala activity and a decrease in septal activity. Both the amygdala complex and lateral septal nucleus are interconnected with the lateral hypothalamus [19,20], which plays a central role in the control of feeding and energy homeostasis [21]. This regulation is mediated by the liberation of two peptides: orexin-A (ORX-A) and orexin-B (ORX-B). These peptides have received substantial research attention because of their ability to stimulate food intake and their relationship with nutritional states [21,22] and variations in glucose concentrations [23]. Some affective disorders appear to be associated with hyperactivity of the orexinergic system [24].

Anhedonia (i.e., the inability to experience pleasure) is one of the main symptoms of several emotional disorders, especially depression. In behavioral models of depression in rats, anhedonia is reflected by a decrease in the responsiveness to reward, such as the intake of palatable sweet solutions [25,26]. The elevated plus maze is widely used to assess anxiety-like behavior and the anxiogenic- or anxiolytic-like effects of pharmacological agents [27–29]. Locomotion is usually evaluated in the open field test, which allows the identification of possible changes in motor activity that is associated with treatments that may influence performance in other behavioral tests. Therefore, these three experimental approaches may complement each other when exploring animal models of diabetes.

We hypothesized that long-term hyperglycemia produces changes in the amygdala-septal area and orexinergic system that regulate emotions, and such changes would be reflected by hedonic behavior, anxiety-like behavior, and locomotion. To investigate the effects of long-term hyperglycemia on emotional systems in the brain and behavior, we first injected rats with a single dose of alloxan. Animals that are subjected to alloxan-induced diabetes [30] and spontaneous genetic models of diabetes [31] may be protected by insulin. Therefore, we simultaneously injected insulin to protect the animals from lethal hyperglycemia and maintain high levels of glycemia in the long term. After 6 weeks, we evaluated Fos protein expression in the basolateral amygdala and lateral septal nucleus and orexin-A activation in the lateral hypothalamus, with cerebellar tissue as a control. Another group of rats was treated similarly and underwent behavioral testing.

2. Materials and methods

2.1. Subjects and housing conditions

Adult male Wistar rats, weighing 250–300 g and 12 weeks of age, were housed five per cage (44 cm width × 33 cm length × 20 cm height) in local housing facilities at a mean temperature of 25 °C with a 12 h/12 h light/dark (lights on at 7:00 AM) with free access to food (Harlan 2018S Teklad Lab Animal Diets, Indianapolis, IN, USA) and purified water. The animal handling procedures were conducted according to the Biomedical Research Institute Ethical Committee (Universidad Nacional Autónoma de México) in strict accordance with the National Guide for the Production, Care and Use of Laboratory Animals [32], which complies with international guidelines set forth by the National Institutes of Health. All efforts were made to minimize the number of animals used.

2.2. Experimental groups

The neurochemical study included two experimental groups: control (C; $n = 6$) and experimental long-term hyperglycemia (ELTH; $n = 6$).

2.3. Experimental long-term hyperglycemia induction

In the ELTH group, alloxan monohydrate (Sigma, St. Louis, MO, USA) was administered intraperitoneally (200 mg/kg body weight) in overnight-fasted rats. Immediately after administration, the rats received 5% dextrose solution to prevent fatal alloxan-induced hypoglycemia as a result of massive pancreatic insulin release [33]. The control group received an equivalent volume of 0.9% NaCl solution in the first session and no treatments thereafter. We verified that the control group maintained glucose levels ≤120 mg/dl. Hyperglycemia was confirmed 48 h later using a glucose meter (Accu-chek performa; Roche, Indianapolis, IN, USA). Rats with plasma glucose levels >250 mg/dl were selected for the study. Of the total number of rats, three did not meet this criterion and were excluded from the experiment. To produce controlled hyperglycemia and promote survival, the ELTH group received daily subcutaneous insulin (0.25-0.5 U/kg body weight; Lantus, Sanofi-Aventis, Paris, France). Insulin shock was rare among these rats with glycosuria. Using this procedure, we attained 84% survival while maintaining hyperglycemia >250 mg/dl.

2.4. Biological measurements

Glucose levels were monitored 3, 7, 14, 21, 28, 35, and 42 days after alloxan administration. Urinary glucose, urinary acetone, and body weight were recorded at the same time intervals (Multistix 10 SG; Siemens, Tarrytown, NY, USA). Food intake and water consumption were recorded daily.

2.5. Perfusion and fos and orexin-A immunohistochemistry

At the end of the study, the rats received a lethal dose of pentobarbital (Pisabental, PISA Agropecuaria, Atitalaquia, Hidalgo, México) and were intracardially perfused with saline solution (0.9%) followed by 4% paraformaldehyde in phosphate buffer (pH 7.4). The brains were removed immediately after perfusion, post-fixed overnight, and cryoprotected successively in 10%, 20%, and 30% sucrose in phosphate buffer. The brains were frozen at −19 °C and cut coronally into 50 μm sections with a cryostat (Leica-Jung, Nussloch, Germany) from the level of the diagonal band of Broca to the mammillary bodies. One set of sections was processed for the immunohistochemical detection of Fos-immunoreactive (IR) and Fos/ORX-A-IR cells.

For Fos immunohistochemistry, the tissue was washed three times for 5 min in phosphate buffer to remove excess aldehydes and then exposed for 10 min to 0.5% hydrogen peroxide solution to eliminate endogenous peroxidase activity. Nonspecific tissue antibody reactions were blocked by placing the sections in 3% normal horse serum (Vector Laboratories) for 1 h at room temperature. The sections were incubated in goat polyclonal Fos primary antibody (1:5000; sc-52G, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 3% normal horse serum with 0.3% Triton X-100 (Sigma, St. Louis, MO, USA) at 4 °C. After 72 h, the tissue was incubated in biotinylated horse anti-goat serum (1:250; Vector Laboratories, Burlingame, CA, USA) for 1 h and then incubated in avidin-biotin-horseradish peroxidase (HRP) complex (1:250; ABC Vectastain Elite, Vector Laboratories, Burlingame, CA, USA) for 2 h. After incubation, the tissue

was rinsed three times in phosphate buffer for 5 min each. Fos was reacted with a solution of 0.05% diaminobenzidine in the presence of nickel sulfate (10 mg/ml, Fisher Scientific, Pittsburgh, PA, USA), cobalt chloride (10 mg/ml, Fisher Scientific), and 0.01% hydrogen peroxide, which produced a black-purple precipitate. After 5 min, the tissue was transferred to phosphate buffer to stop the reaction.

For ORX-A immunohistochemistry, the sections were rinsed in phosphate buffer and incubated for 24 h at 4 °C in goat polyclonal ORX-A antibody (1:5000; sc-8070, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 3% normal horse serum with 0.3% Triton X-100 (Sigma). The sections were then sequentially incubated with biotinylated horse anti-goat and avidin-biotin-HRP complex, both diluted 1:250 in phosphate buffer for 1 h, and then incubated in avidin-biotin-HRP complex (1:250; ABC Vectastain Elite, Vector Labs) for 2 h. After incubation, the tissue was rinsed three times in phosphate buffer for 5 min each. Orexin-A antibody-peroxidase complex labeling was revealed by 0.05% diaminobenzidine, which produces a brown cytoplasmic precipitate. The sections were mounted on gelatin-coated slides, dehydrated, and coverslipped with Permount. As a control, we used tissue sections that were processed as above but without primary antibody.

2.6. Quantification of immunostaining

To quantify the staining of immunoreactive nuclei, we determined the background optical density in a nearby region that lacked immunoreactivity using Image-Pro Plus 5 software (Media Cybernetics, Silver Spring, MD, USA). Immunoreactive cells that reached five times the optical density of the background level were considered positive. Cells below this level of staining were considered negative. Using an Olympus BX41 microscope (Olympus, Tokyo, Japan) at 10× and 20× magnification, Fos immunoreactivity was identified as a black-purple precipitate from the diaminobenzidine-nickel/cobalt reaction in the cell nucleus. Orexin-A immunoreactivity was identified as a brown precipitate in the cytoplasm. Fos/ORX-A immunoreactivity was identified as a brown cytoplasm and black nucleus. Although the same secondary antibody was used, to avoid cross-reactions, double labeling was performed by considering the ubiquity of the antigen, in which Fos antibody reacts with nuclear antigens and ORX-A reacts with cytoplasmic antigens [34,35]. Immunostaining was absent in tissue that was processed without primary antibodies.

To quantify Fos-IR and Fos/ORX-A-IR cells in the brain structures, a blind observer examined five representative sections bilaterally in coronal sections in the lateral septal nucleus (Fig. 1A) and basolateral amygdala (Fig. 1B). The percentage of orexinergic (Fos/ORX-IR) cells was quantified in the lateral hypothalamus (Fig. 1B) according to the stereotaxic atlas of Paxinos and Watson [36]. The cerebellar cortex was used as a control structure.

2.7. Behavioral tests

For the behavioral part of the study, we began with 25 rats, but a final total of 21 rats was included: control group ($n = 12$) and ELTH group ($n = 9$). Five animals died soon after the alloxan injection. This second cohort of animals was subjected to the sucrose preference test. One week later, the elevated plus maze test was performed, followed 5 min later by the open field test.

2.7.1. Sucrose preference test

We followed a previously reported procedure [37]. Prior to testing, the rats were trained to adapt to a sucrose solution (1%, w/v). Two bottles of sucrose solution were placed in each cage for 24 h. One bottle of sucrose solution was then replaced with water for 24 h. After adaptation, the rats were deprived of water and food for 24 h. The sucrose preference test was performed with rats that were housed in individual cages with free access to the two bottles, one containing 100 ml of sucrose solution (1% w/v) and the other containing 100 ml of water. After 1 h, the volumes of sucrose solution and water that were consumed were recorded, and sucrose preference was calculated as the following: *sucrose preference (%) = sucrose consumption/(sucrose consumption + water consumption)*.

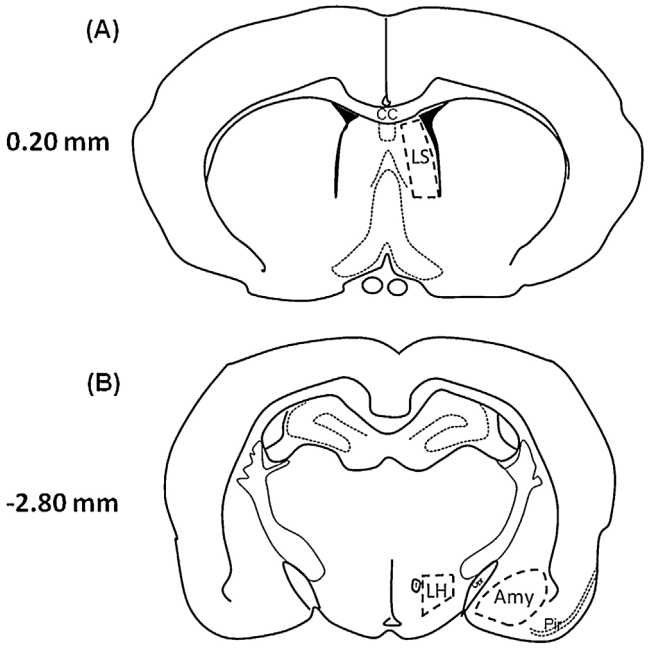

Fig. 1. Representative camera lucida drawing showing the localization of (A) the lateral septum (LS; bregma 0.20 mm) and (B) the amygdala (Amy) and lateral hypothalamus (LH; bregma −2.80 mm) where the analyses were performed. Dotted lines delimit the counted area. CC, corpus callosum; f, fornix; opt, optic tract; Pir, piriform cortex.

2.7.2. Elevated plus maze

The elevated plus maze was constructed of wood and situated in a brightly lit room (40 lx). The apparatus consisted of two opposite open and closed arms set in a plus configuration. The open and closed arms were painted white and black, respectively. The dimensions of the open arms were 50 cm length × 10 cm width. The dimensions of the closed arms were 50 cm length × 10 cm width × 40 cm height. The entire maze was elevated 50 cm above the floor. At the beginning of the test, each rat was gently placed in the center of the maze, facing an open arm, and the time spent on and numbers of entries into the open and closed arms were recorded [27]. The total number of entries (open arms + closed arms) was also recorded, and the percentage of open arm entries ([open entries]/[total entries] × 100) was calculated. Rats that fell from the maze were discarded from subsequent data analyses. In the same 5 min test, two risk assessment measures were evaluated according to Griebel et al. [38]: attempts (i.e., attempts at entries into an open arm followed by avoidance responses, including stretch-attend posture, in which the rat stretched forward and retracted to its original position) and head-dipping (i.e., protruding the head over the ledge of an open arm and down toward the floor; this response could occur while the body was in the closed arms, central square, or open arms). For these behaviors, the number of events and time spent engaged in each event were evaluated. Additionally, the Anxiety Index was calculated according to Cohen et al. [29]: *Anxiety Index = (1 − [Open arm time/Test duration] + [Open arm entries/Total number of entries]/2)*.

Table 1
Changes in blood glucose, body weight, urinary glucose, urinary acetone, and food and water intake in experimental subjects.

Biological measurements	Control group	Hyperglycemia group
Blood glucose (mg/dl)	99.3 ± 18.12	541.6 ± 18.12**
Body weight (g)	385.1 ± 11.45	296.6 ± 11.45**
Urinary glucose (mg/dl)	0.00	1777.7 ± 125.56
Urinary acetone (mg/dl)	0.00	10.2 ± 2.98
Food intake (g)	46.4 ± 3.31	77.1 ± 7.05**
Water consumption (ml/24 h)	71.7 ± 10.16	126.3 ± 17.66*

* $p < 0.01$, ** $p < 0.001$.

Table 2
Variables evaluated in the elevated plus maze that were similar between groups.

	Control group	Hyperglycemia group
Open arm time (s)	24.8 ± 10.58	56.6 ± 19.6
Closed arm time (s)	208.5 ± 16.11	180.6 ± 20.4
Open-arm entries (*n*)	2.0 ± 0.89	1.7 ± 0.59
Head dips (s)	4.1 ± 1.41	6.1 ± 1.59
Head dips (*n*)	4.3 ± 1.68	4.5 ± 1.01
Attempts (s)	31.8 ± 5.70	16.8 ± 5.36
Open-arm entries (%)	14.1 ± 5.59	22.4 ± 6.99
Anxiety Index	0.89 ± 0.03	0.85 ± 0.04

2.7.3. Open field test

To evaluate the effects of the treatment on spontaneous locomotor activity and determine possible hypoactivity or hyperactivity that was attributable to long-term hyperglycemia, a 5 min open field test was performed 5 min after the elevated plus maze test in another room (40 lx). We used an automated locomotor activity monitor (Acti-Track v2.7.10, PanLab, S.L., Instrument, Barcelona, Spain) that consisted of a Perspex box (45 cm × 45 cm) with 35 cm high walls. A total of 32 infrared beams, 16 on each perpendicular wall (3 cm above the box frame floor) were connected to an interface (LE 8811, LSI Letica Scientific Instrument, Barcelona, Spain) and subsequently to a computer. For data analysis, the floor of the cage was virtually divided into five zones (four peripheral zones and one central zone). We recorded the number of entries into each zone (crossings), total resting time, and total activity time during the 5 min test as indicators of locomotion. No other behaviors, such as rearing or grooming, were evaluated. Because of the relatively small cage, we did not compare central *vs.* peripheral exploration. After each experimental session, the elevated plus maze and locomotor activity box were carefully cleaned and deodorized with a 5% ethanol cleaning solution. Approximately 5 min elapsed between each test to allow the scent of the substances to dissipate.

2.8. Statistical analysis

One-way repeated-measures analysis of variance (ANOVA) was used to detect the effects of alloxan on blood glucose, body weight, urinary glucose, urinary acetone, and food and water intake in the ELTH and control groups. Student's *t*-test was used to compare the long-term effect of hyperglycemia on Fos-IR and Fos-/ORX-A-IR cells between the ELTH and control groups. The data from the elevated plus maze and open field test were analyzed using Student's *t*-test (SigmaStat 3.0). The results are expressed as the mean ± standard error of the mean. Values of $p \leq 0.05$ were considered statistically significant.

3. Results

3.1. Biological measurements

In the ELTH group, blood glucose levels were significantly higher than in the control group ($F_{7,95} = 58.465$, $p < 0.001$). The statistically significant difference between groups occurred 3 days after the alloxan injection ($p < 0.001$), and glucose levels remained near 500 mg/dl for 40 days. Significantly lower body weight ($F_{7,95} = 12.190$, $p < 0.001$) was observed approximately 10 days after the alloxan injection, and no weight gain was detected for as long as 30 days. The ELTH group exhibited higher urinary glucose and urinary acetone levels. Compared with the control group, the long-term hyperglycemic rats exhibited a significant increase in water intake ($t_{16} = -2.677$, $p < 0.017$) and a marked increase in food consumption ($t_{16} = -3.933$, $p < 0.001$; Table 1).

3.2. Amygdala-septal fos expression

The immunohistochemical analysis of the septal and basolateral amygdala regions (Fig. 2A, C) revealed lower neuronal activity in the ELTH group compared with the control group. Significantly lower Fos expression was observed in all septal regions in the ELTH group compared with controls ($t_{10} = 77.564$, $p < 0.001$; Fig. 2B). The expression of Fos-IR cells was also lower in the basolateral amygdala in the ELTH group compared with controls ($t_{10} = 4.852$, $p < 0.001$; Fig. 2D). The ELTH group and control group exhibited similar Fos expression in cerebellar regions (Fig. 3).

3.3. Orexinergic expression

Both groups exhibited Fos/ORX-A double-labeling (Fig. 4A). In the lateral hypothalamus in long-term hyperglycemic rats, this expression was significantly lower than in the control group ($t_{10} = 14.453$, $p < 0.001$; Fig. 4B). Quantitative analysis indicated that orexin neurons number were similar in both experimental groups ($t_{10} = -0.375$, $p = 0{,}716$; Fig. 4C).

3.4. Behavioral tests

3.4.1. Sucrose preference test

The percentage of sucrose consumption was similar between the ELTH group (65.7 ± 7.86 ml) and control group (68.4 ± 3.4 ml; $t_{19} = 0.338$, $p = 0.739$). A nonsignificant trend ($t_{19} = -1.940$, $p = 0.067$) toward higher total fluid intake (water + sucrose) was observed in the ELTH group (22.6 ± 1.02 ml) compared with the control group (18.5 ± 1.64 ml).

3.4.2. Elevated plus maze

No significant differences were observed between the control and ELTH groups in the time spent on the open arms ($t_{19} = -1.521$, $p = 0.145$), time spent on the closed arms ($t_{19} = 1.084$, $p = 0.292$), number of open-arm entries ($t_{19} = 0.264$, $p = 0.794$), number of head-dipping episodes ($t_{19} = -0.104$, $p = 0.919$), time spent head-dipping ($t_{19} = -0.944$, $p = 0.357$), or time engaged in attempts ($t_{19} = 1.862$, $p = 0.078$). No significant differences were found in the percentage of open-arm entries ($t_{19} = -0.942$, $p = 0.358$) or the Anxiety Index ($t_{19} = 0.779$, $p = 0.446$). The only significant differences in the elevated plus maze were in closed-arm entries ($t_{19} = 2.979$, $p < 0.008$), the total number of entries ($t_{19} = 2.690$, $p < 0.014$), and the number of attempts ($t_{19} = 4.870$, $p < 0.001$; Table 2, Fig. 5A).

3.4.3. Open field test

Significant differences were found between groups in the number of crossings ($t_{19} = 2.859$, $p < 0.010$), activity time ($t_{19} = 2.168$, $p < 0.043$), and resting time ($t_{19} = -2.163$, $p < 0.043$; Fig. 5B), indicating that the ELTH group exhibited lower general locomotion than the control group.

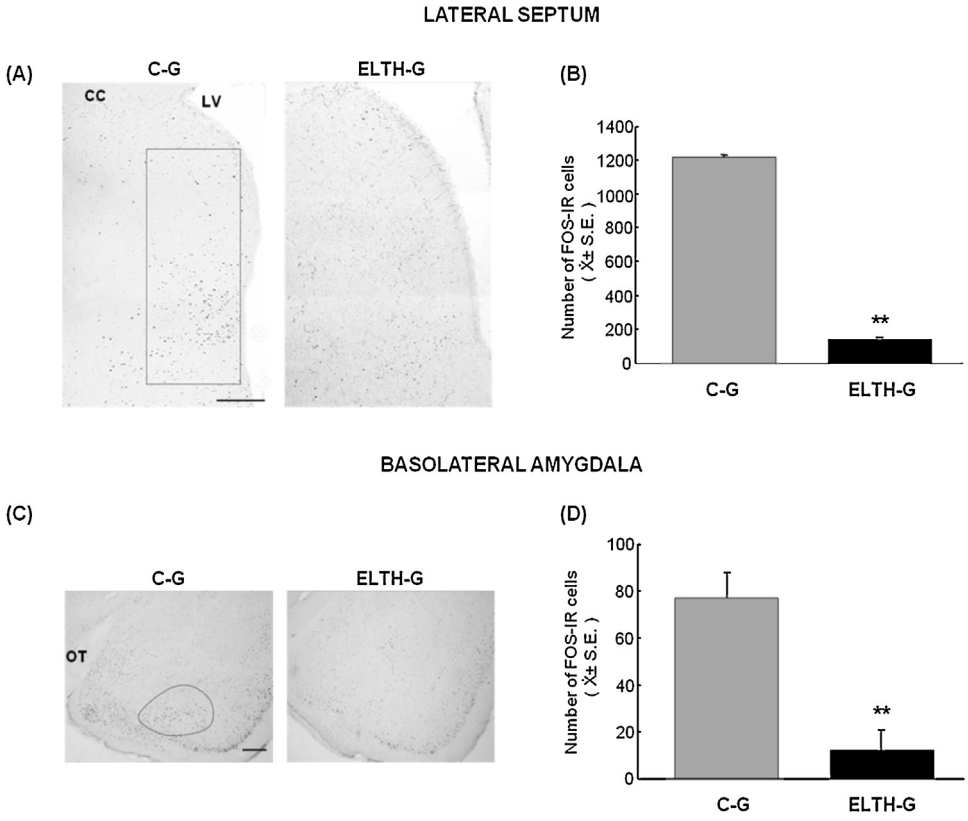

Fig. 2. Fos-IR cells in the (A) lateral septum and (C) basolateral amygdala. Dotted lines delimit the counted area. (B, D) Compared with the control group (C-G; gray bars), the experimental long-term hyperglycemia group (ELTH-G; black bars) exhibited low neuronal activity. $^{**}p < 0.001$ (Student's *t*-test). CC, corpus callosum; LV, lateral ventricle; OT, optic tract. Scale bar = 100 μm.

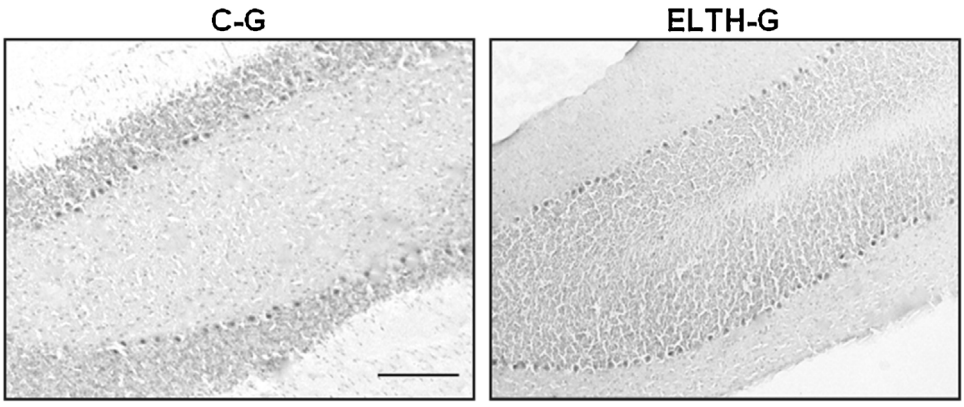

Fig. 3. Fos expression in cerebellar cells. Representative photomicrographs of Fos-IR cells in cerebellum in the experimental long-term hyperglycemia group (ELTH-G) and control group (C-G). Scale bar = 100 μm.

4. Discussion

The present study investigated whether long-term hyperglycemia leads to changes in neuronal activity in the lateral septal nucleus and basolateral amygdala and orexinergic cells in the lateral hypothalamus and whether such changes are related to changes in hedonia, anxiety, and locomotor activity. Both Fos activity and orexinergic activity appeared to be blunted after a condition in which plasma glucose was maintained at a constantly high level for 6 weeks. We did not observe any changes in tests that evaluated hedonic behavior or anxiety-like behavior, but we found decreases in all indicators of locomotion.

Animal models are unable to fully recapitulate all aspects of human diabetes and its complications [39], and genetic models merit special attention. Some signs that reflect depression (e.g., long duration of immobility in the tail suspension test) are related to a decrease in interneuronal dendritic arborization in some neurons in the medial prefrontal cortex and a decrease in γ-aminobutyric acid activity in transgenic mice 14 days after a streptozocin injection [40]. One genetic model that is used to study diabetes is db/db mice. These mice may exhibit signs of despair and anxiolysis, a reduction of locomotion, and psychosis-like symptoms [41]. In this genetic model, corticosteroids may be a precipitating factor for neuroinflammation because of a lower threshold for the release of proinflammatory cytokines [42], and db/db mice also exhibit neu-

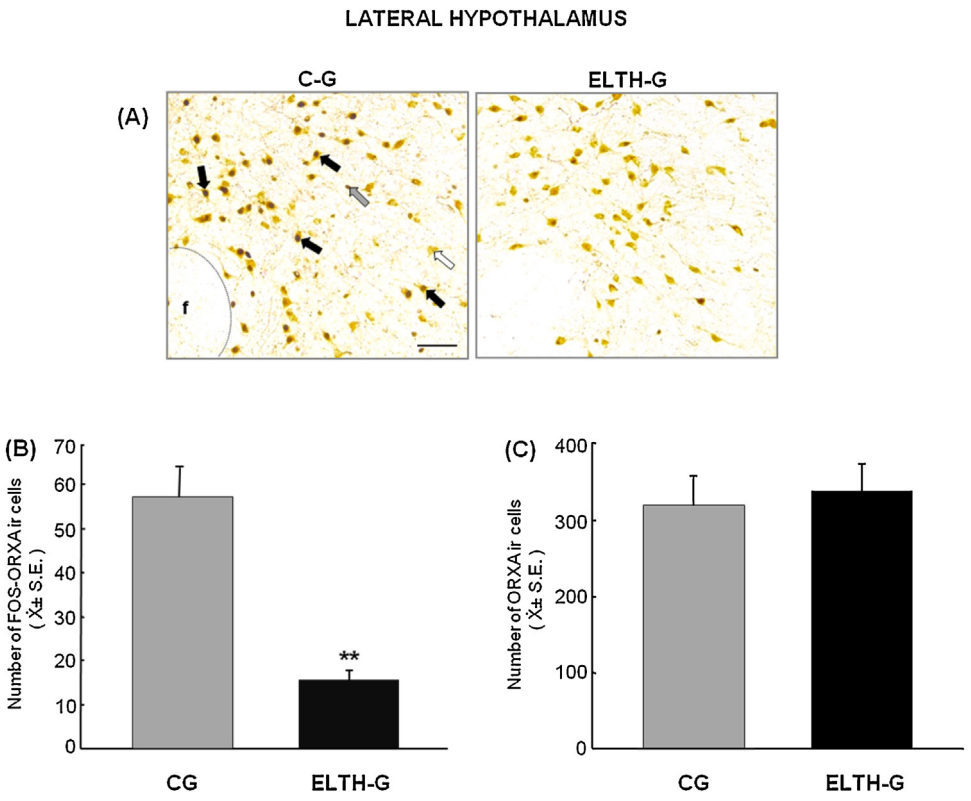

Fig. 4. (A) Fos/ORX-A-IR cells in the lateral hypothalamus. Representative photomicrographs show Fos-IR (white arrow), ORX-A-IR (gray arrow), and Fos/ORX-A-IR (black arrow) cells in the lateral hypothalamus. Scale bar = 100 μm. (B) Compared with the control group (C-G; gray bars), the experimental long-term hyperglycemia group (ELTH-G; black bars) exhibited low orexinergic activity. (C) Total orexin counts were similar between groups. $^{**}p < 0.001$ (Student's *t*-test).

rological peculiarities that are unrelated to diabetes-related neural alterations or comorbid diseases [43]. Another genetic model of diabetes has been proposed to study the relationship between diabetes and Alzheimer's disease [31]. ZDF rats represent an animal model of obesity and diabetes. In these animals, hyperglycemia is unrelated to changes in learning and long-term potentiation in hippocampal slices [44]. Although some genetic models of diabetes present behaviors that are suggestive of anxiety and depression, these alterations may only represent a part of the complex characteristics of these mice.

Alloxan is a very stable [45] toxic glucose analogue that selectively destroys beta cells in the pancreas to produce experimental diabetes [46]. Animal models of alloxan-induced type 1 diabetes are characterized by hyperglycemia and a tendency toward ketosis [30]. Using the present protocol, we observed hyperglycemia, low body weight, glycosuria, ketonuria, and an increase in water and food intake, indicating the development of type 1 diabetes.

Although cerebral energy metabolism remains stable even 8 months after streptozocin-induced diabetes [47], the effects of hyperglycemia may be different in the short and long term. In this model, alloxan is injected, hyperglycemia is verified, and effects are studied 10–20 days later [48,49]. At this time point, intravascular hemolytic events may be observed [50,51], but histological changes [52,53] and macrovascular and microvascular alterations [54], including vascular endothelia, glomerular epithelia, neurons, and glial cells that result in eye, kidney, and nerve damage [55], are only observed after a longer period of time.

In the present study, the maintenance of high plasma glucose levels for 6 weeks was related to low neuronal activity in the lateral septal nucleus and basolateral amygdala, reflected by low Fos activity. Several studies have reported low neuronal activity in the lateral septal nucleus in rodents that were exposed to tests of behavioral despair [56], and abnormalities in this structure have been associated with schizophrenia, depression, and anhedonia [57–59]. An increase in activity of the basolateral amygdala has been reported in depressive patients [60]. The volume of the prefrontal and orbitofrontal cortices, amygdala, and hippocampus [61,62] is diminished in depressive patients. Therefore, in clinical conditions, there is selective activity of the basolateral amygdala and lateral septal regions. However, in the present study, we detected lower activity in both regions, which is contrary to what was expected [18].

We also observed low activity of orexinergic cells, which contrasts with other reports [63]. The orexinergic system is sensitive to changes in glucose concentrations [64,65]. These neurons are also stimulated by insulin circulation and 2-deoxy-D-glucose [66]. Thus, hyperglycemia decreases orexinergic activity, which is consistent with the present results.

Although we reproduced many of the core symptoms of diabetes in the present study, we observed blunted activity of both Fos and ORX-A. Rodents that are subjected to chronic unpredictable mild stress exhibit decreases in body weight gain and locomotor activity in the open field and, particularly relevant for the present study, a clear decrease in sucrose preference [67], which is interpreted as anhedonia. However, we observed no changes in sucrose preference in long-term hyperglycemic rats. Therefore, at least one indicator of depressive-like behavior was absent.

Some effects in experimental models of diabetes are manifest only after a certain period of time. Streptozocin-induced diabetes is first associated with a decrease in nerve conduction (i.e., a peripheral effect) and weeks later by a central nervous system effect, reflected by a decrease in evoked potential latencies [68]. In the present study, the ELTH group did not exhibit any indicator of anxiety 6 weeks after the alloxan injection. Rodents that exhibit anxiety-like behavior in the elevated plus maze usually exhibit a reduction of the time spent on the open arms [69,70]. In this

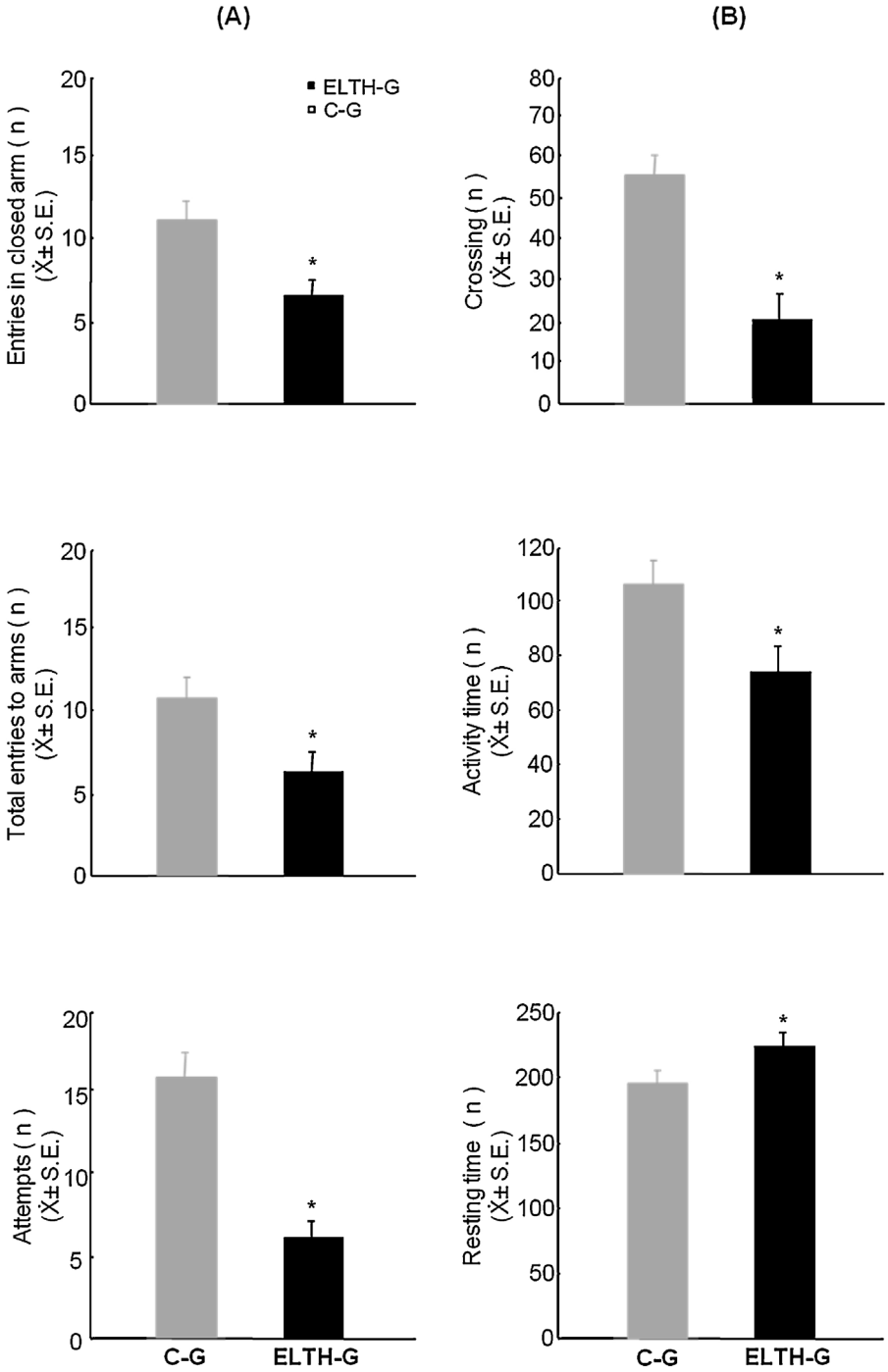

Fig. 5. (A) Elevated plus maze. The changes observed were not related to anxiety. (B) Open field test. The experimental long-term hyperglycemia group (ELTH-G) exhibited lower general locomotion than the control group (C-G). $^{*}p \leq 0.05$ (Student's *t*-test).

model, the total number of arm entries is an indicator of locomotion [27]. We observed a lower total number of arm entries in the ELTH group compared with the control group. In the open field test, the ELTH group exhibited clear reductions of the number of crossings and activity time and an increase in resting time, indicating lower locomotion. Therefore, long-term hyperglycemia produced no relevant changes in the elevated plus maze, suggesting no influence on anxiety-like behavior but a decrease in locomotion.

In addition to studies that identified emotional changes in diabetes, cognitive functions have also been explored. Diabetes in the elderly appears to involve limitations in cognitive abilities, likely because of chronic and metabolic changes [71] that lead to a higher risk of dementia [72], including the so-called diabetes-associated cognitive decline [73]. Some experimental data support this theoretical position. In streptozotocin-induced diabetic mice, 20 days after toxin injection, severe impairments were observed in learning and memory that were related to a neurodegenerative process in the hippocampus, with no changes in exploratory or non-exploratory activity in the asymmetric elevated plus maze [74]. The long-term impairments in spatial memory and long-term potentiation are seemingly related to *N*-methyl-D-aspartate receptors [75]. Delays in long-term potentiation and facilitation of long-term depression in the CA1 area of the hippocampus are detected 6 weeks after streptozocin injection [76]. These effects are likely

related to the deficits in working memory that were observed 14 weeks after the injection [77], supported by severe damage that is observed in hippocampal structures [78]. Therefore, our results suggest a deleterious effect on cognition rather than emotional disturbances.

One weakness of the present study was our failure to measure the amount of urine. Although we detected an increase in food and water consumption, we did not perform a full characterization of diabetes, including perhaps evaluations of eye fundus.

In summary, every chemical and behavioral parameter was blunted in the present study, but no signs of anhedonia or anxiety were detected. A decrease in the psychomotor response was found, with no data that were suggestive of anxiety- or anhedonic-like behavior in this alloxan-induced model of diabetes.

Conflict of interest

This study was supported by funding from CONACyT (232283) to M.L. Moreno Cortés. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Author contributions

M.L. Moreno-Cortés participated in the experimental design and experimental execution, performed the immunohistochemical analysis, and wrote and revised the article. A.G. Gutiérrez-García participated in the experimental design, performed the statistical analysis of the behavioral data, drafted the article, and revised the article. G. Guillén-Ruiz collaborated in the design and analysis of the behavioral tests and care of the experimental animals. T. Romo-González participated in the experimental design and wrote and revised the article. C.M. Contreras designed the study and experimental design and wrote and revised the article.

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References

[1] P.J. Lustman, R.J. Anderson, K.E. Freedland, M. de Groot, R.M. Carney, R.E. Clouse, Depression and poor glycemic control: a meta-analytic review of the literature, Diab. Care 23 (7) (2000) 934–942.
[2] L.P. Reagan, Diabetes as a chronic metabolic stressor: causes, consequences and clinical complications, Exp. Neurol. 233 (1) (2012) 68–78.
[3] A. Nouwen, G. Nefs, I. Caramlau, M. Connock, K. Winkley, C.E. Lloyd, et al., Prevalence of depression in individuals with impaired glucose metabolism or undiagnosed diabetes: a systematic review and meta-analysis of the European Depression in Diabetes (EDID) Research Consortium, Diab. Care 34 (3) (2011) 752–762.
[4] T. Doyle, A. Halaris, M. Rao, Shared neurobiological pathways between type 2 diabetes and depressive symptoms: a review of morphological and neurocognitive findings, Curr. Diab. Rep. 14 (12) (2014) 560.
[5] C.E. Lloyd, T. Roy, A. Nouwen, A.M. Chauhan, Epidemiology of depression in diabetes: international and cross-cultural issues, J. Affect. Disord. 142 (Suppl) (2012) S22–9.
[6] A. Nouwen, Depression and diabetes distress, Diabet. Med. 32 (10) (2015) 1261–1263.
[7] P. Demakakos, P. Zaninotto, A. Nouwen, Is the association between depressive symptoms and glucose metabolism bidirectional? Evidence from the english longitudinal study of ageing, Psychosom. Med. 76 (7) (2014) 555–561.
[8] B. Mezuk, W.W. Eaton, S. Albrecht, Golden SH: Depression and type 2 diabetes over the lifespan: a meta-analysis, Diab. Care 31 (12) (2008) 2383–2390.
[9] A. Nouwen, C.E. Lloyd, F. Pouwer, Depression and type 2 diabetes over the lifespan: a meta-analysis. Response to Mezuk et al, Diab. Care 32 (5) (2009) e57 (e56; author reply).
[10] L.J. Leedom, W.P. Meehan, A. Zeidler, Avoidance responding in mice with diabetes mellitus, Physiol. Behav. 40 (4) (1987) 447–451.
[11] W.P. Meehan, L.J. Leedom, T. Nagayama, A. Zeidler, Agonistic behavior patterns in mice with streptozotocin-induced diabetes mellitus, Physiol. Behav. 38 (3) (1986) 301–306.
[12] T. Dalgleish, The emotional brain, Nat. Rev. Neurosci. 5 (7) (2004) 583–589.
[13] J. Menard, D. Treit, Lateral and medial septal lesions reduce anxiety in the plus-maze and probe-burying tests, Physiol. Behav. 60 (3) (1996) 845–853.
[14] T.P. Sheehan, R.A. Chambers, D.S. Russell, Regulation of affect by the lateral septum: implications for neuropsychiatry, Brain Res. Brain Res. Rev. 46 (1) (2004) 71–117.
[15] C.M. Contreras, V. Alcalá-Herrera, M.L. Marván, Action of antidepressants on the septal nuclei of the rat, Physiol. Behav. 46 (5) (1989) 793–798.
[16] C.M. Contreras, J.F. Rodriguez-Landa, A.G. Gutiérrez-García, B. Bernal-Morales, The lowest effective dose of fluoxetine in the forced swim test significantly affects the firing rate of lateral septal nucleus neurones in the rat, J. Psychopharmacol. 15 (4) (2001) 231–236.
[17] C.M. Contreras, L. Chacón, J.F. Rodríguez-Landa, B. Bernal-Morales, A.G. Gutiérrez-García, M. Saavedra, Spontaneous firing rate of lateral septal neurons decreases after forced swimming test in Wistar rat, Prog. Neuropsychopharmacol. Biol. Psychiatry 28 (2) (2004) 343–348.
[18] E. Thomas, M. Dewolfe, F. Sancar, N. Todi, E. Yadin, Electrophysiological analysis of the interaction between the lateral septum and the central nucleus of the amygdala, Neurosci. Lett. 524 (2) (2012) 79–83.
[19] L.W. Swanson, W.M. Cowan, The connections of the septal region in the rat, J. Comp. Neurol. 186 (4) (1979) 621–655.
[20] P.Y. Risold, L.W. Swanson, Chemoarchitecture of the rat lateral septal nucleus, Brain Res. Brain Res. Rev. 24 (2–3) (1997) 91–113.
[21] T. Sakurai, A. Amemiya, M. Ishii, I. Matsuzaki, R.M. Chemelli, H. Tanaka, et al., Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior, Cell 92 (4) (1998) 573–585.
[22] L. De Lecea, T.S. Kilduff, C. Peyron, X. Gao, P.E. Foye, P.E. Danielson, et al., The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity, Proc. Natl. Acad. Sci. U. S. A. 95 (1) (1998) 322–327.
[23] T. Himmi, A. Boyer, J.C. Orsini, Changes in lateral hypothalamic neuronal activity accompanying hyper- and hypoglycemias, Physiol. Behav. 44 (3) (1988) 347–354.
[24] E. Mikrouli, G. Wörtwein, R. Soylu, A.A. Mathé, Å. Petersén, Increased numbers of orexin/hypocretin neurons in a genetic rat depression model, Neuropeptides 45 (6) (2011) 401–406.
[25] P. Willner, Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation, Psychopharmacology (Berl) 134 (4) (1997) 319–329.
[26] P. Willner, Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS, Neuropsychobiology 52 (2) (2005) 90–110.
[27] A.A. Walf, C.A. Frye, The use of the elevated plus maze as an assay of anxiety-related behavior in rodents, Nat. Protoc. 2 (2) (2007) 322–328.
[28] S. Pellow, P. Chopin, S.E. File, M. Briley, Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat, J. Neurosci. Methods 14 (3) (1985) 149–167.
[29] H. Cohen, M.A. Matar, Z. Joseph, Animal models of post-traumatic stress disorder, Curr. Protoc. Neurosci. 45 (2013) (Chapter 9:Unit 9).
[30] I.F. Federiuk, H.M. Casey, M.J. Quinn, M.D. Wood, W.K. Ward, Induction of type-1 diabetes mellitus in laboratory rats by use of alloxan: route of administration, pitfalls, and insulin treatment, Comp. Med. 54 (3) (2004) 252–257.
[31] Z.G. Li, W. Zhang, A.A.F. Sima, Alzheimer-like changes in rat models of spontaneous diabetes, Diabetes 56 (7) (2007) 1817–1824.
[32] NORMA Oficial Mexicana NOM-062-ZOO-1999. Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio. Diario Oficial de la Federación, 6 de diciembre de 1999.
[33] S. Lenzen, The mechanisms of alloxan- and streptozotocin-induced diabetes, Diabetologia 51 (2) (2008) 216–226.
[34] J.A. Ramos-Vara, Technical aspects of immunohistochemistry, Vet. Pathol. 42 (4) (2005) 405–426.
[35] A. Jiménez, M. Caba, C. Escobar, Food-entrained patterns in orexin cells reveal subregion differential activation, Brain Res. 1513 (2013) 41–50.
[36] G. Paxinos, C. Watson, The Rat Brain in Stereotaxic Coordinates, 4th ed., Academic Press, San Diego, 1998.
[37] D. Liu, K. Xie, X. Yang, J. Gu, L. Ge, X. Wang, et al., Resveratrol reverses the effects of chronic unpredictable mild stress on behavior, serum corticosterone levels and BDNF expression in rats, Behav. Brain Res. 264 (2014) 9–16.
[38] G. Griebel, R.J. Rodgers, G. Perrault, D.J. Sanger, Risk assessment behaviour: evaluation of utility in the study of 5-HT-related drugs in the rat elevated plus-maze test, Pharmacol. Biochem. Behav. 57 (4) (1997) 817–827.
[39] M. Radenković, M. Stojanović, M. Prostran, Experimental diabetes induced by alloxan and streptozotocin: the current state of the art, J. Pharmacol. Toxicol. Methods 78 (2016) 13–31.
[40] E. Castillo-Gómez, S. Coviello, M. Perez-Rando, Y. Curto, H. Carceller, A. Salvador, et al., Streptozotocin diabetic mice display depressive-like behavior and alterations in the structure, neurotransmission and plasticity of medial prefrontal cortex interneurons, Brain Res. Bull. 116 (2015) 45–56.
[41] A.N. Sharma, K.M. Elased, T.L. Garrett, J.B. Lucot, Neurobehavioral deficits in db/db diabetic mice, Physiol. Behav. 101 (3) (2010) 381–388.
[42] A. Dey, S. Hao, J.R. Erion, M. Wosiski-Kuhn, A.M. Stranahan, Glucocorticoid sensitization of microglia in a genetic mouse model of obesity and diabetes, J. Neuroimmunol. 269 (1-2) (2014) 20–27.
[43] A. Ernst, A.N. Sharma, K.M. Elased, P.C. Guest, H. Rahmoune, S. Bahn, Diabetic db/db mice exhibit central nervous system and peripheral molecular

alterations as seen in neurological disorders, Transl. Psychiatry 3 (5) (2013) e263.
[44] A. Belanger, N. Lavoie, F. Trudeau, G. Massicotte, S. Gagnon, Preserved LTP and water maze learning in hyperglycaemic-hyperinsulinemic ZDF rats, Physiol. Behav. 83 (3) (2004) 483–494.
[45] B.H. Allehyani, S.A. Elroby, S.G. Aziz, R.H. Hilal, Electronic structure of alloxan and its dimers: QM/QD simulations and quantum chemical topology analysis, J. Biomol. Struct. Dyn. 33 (10) (2015) 2121–2132.
[46] S. Lenzen, U. Panten, Alloxan: history and mechanism of action, Diabetologia 31 (6) (1988) 337–342.
[47] G.J. Biessels, K.P.J. Braun, R.A. de Graaf, P. van Eijsden, W.H. Gispen, K. Nicolay, Cerebral metabolism in streptozotocin-diabetic rats: an in vivo magnetic resonance spectroscopy study, Diabetologia 44 (3) (2001) 346–353.
[48] E.J. Akpan, J.E. Okokon, E. Offong, Antidiabetic and hypolipidemic activities of ethanolic leaf extract and fractions of *Melanthera scandens*, Asian Pac. J. Trop. Biomed. 2 (7) (2012) 523–527.
[49] V. Maithili, S.P. Dhanabal, S. Mahendran, R. Vadivelan, Antidiabetic activity of ethanolic extract of tubers of *Dioscorea alata* in alloxan induced diabetic rats, Indian J. Pharmacol. 43 (4) (2011) 455–459.
[50] O. Bashiru Shola, F. Olatunde Olugbenga, Hyperglycaemic environment: contribution to the anaemia associated with diabetes mellitus in rats experimentally induced with alloxan, Anemia 2015 (2015) 848921.
[51] G.J. Biessels, N.A. Cristino, G.J. Rutten, F.P. Hamers, D.W. Erkelens, W.H. Gispen, Neurophysiological changes in the central and peripheral nervous system of streptozotocin-diabetic rats: course of development and effects of insulin treatment, Brain 122 (Pt. 4) (1999) 757–768.
[52] H. Orskov, T. Steen Olsen, K. Nielsen, O.J. Rafaelsen, K. Lundbaek, Kidney lesions in rats with severe long-term alloxan diabetes: i Influence of age, alloxan damage, and insulin administration, Diabetologia 1 (3–4) (1966) 172–179.
[53] M. Pourghasem, E. Nasiri, H. Shafi, Early renal histological changes in alloxan-induced diabetic rats, Int. J. Mol. Cell Med. 3 (1) (2014) 11–15.
[54] N.D. Russell, M.E. Cooper, 50 years forward: mechanisms of hyperglycaemia-driven diabetic complications, Diabetologia 58 (8) (2015) 1708–1714.
[55] National Collaborating Centre for Chronic Conditions. Type 2 Diabetes: National Clinical Guideline for Management in Primary and Secondary Care (Update) [NICE Clinical Guidelines, No 66]. London: Royal College of Physicians.
[56] M. Steciuk, M. Kram, G.L. Kramer, F. Petty, Decrease in stress-induced c-Fos-like immunoreactivity in the lateral septal nucleus of learned helpless rats, Brain Res. 822 (1-2) (1999) 256–259.
[57] P. Averback, Structural lesions of the brain in young schizophrenics, Can. J. Neurol. Sci. 8 (1) (1981) 73–76.
[58] R. Brisch, H.G. Bernstein, H. Dobrowolny, D. Krell, R. Stauch, K. Trübner, et al., A morphometric analysis of the septal nuclei in schizophrenia and affective disorders: reduced neuronal density in the lateral septal nucleus in bipolar disorder, Eur. Arch. Psychiatry Clin. Neurosci. 261 (1) (2011) 47–58.
[59] American Psychiatric Association, Diagnostic and Statistical Manual of Mental Disorders, 4th ed., American Psychiatric Association, Washington DC, 1994.
[60] M. Davis, P.J. Whalen, The amygdala: vigilance and emotion, Mol. Psychiatry 6 (1) (2001) 13–34.
[61] Y.I. Sheline, M.H. Gado, J.L. Price, Amygdala core nuclei volumes are decreased in recurrent major depression, Neuroreport 9 (9) (1998) 2023–2028.
[62] J.D. Bremner, Structural changes in the brain in depression and relationship to symptom recurrence, CNS Spectr. 7 (2) (2002) (129-30, 135-9).
[63] I. Swart, J.M. Overton, T.A. Houpt, The effect of food deprivation and experimental diabetes on orexin and NPY mRNA levels, Peptides 22 (12) (2001) 2175–2179.
[64] Y. Oomura, H. Ooyama, M. Sugimori, T. Nakamura, Y. Yamada, Glucose inhibition of the glucose-sensitive neurone in the rat lateral hypothalamus, Nature 247 (5439) (1974) 284–286.
[65] J.C. Orsini, D.L. Armstrong, M.J. Wayner, Responses of lateral hypothalamic neurons recorded in vitro to moderate changes in glucose concentration, Brain Res. Bull. 29 (3–4) (1992) 503–505.
[66] T. Katafuchi, Y. Oomura, H. Yoshimatsu, Single neuron activity in the rat lateral hypothalamus during 2-deoxy-D-glucose induced and natural feeding behavior, Brain Res. 359 (1–2) (1985) 1–9.
[67] J.Y. Guo, H. Bian, Y. Yao, Chronic unpredictable mild stress induces parallel reductions of 15-PGDH in the hypothalamus and lungs in rats, Behav. Brain Res. 286 (2015) 278–284.
[68] S.M. Manschot, W.H. Gispen, L.J. Kappelle, G.J. Biessels, Nerve conduction velocity and evoked potential latencies in streptozotocin-diabetic rats: effects of treatment with an angiotensin converting enzyme inhibitor, Diab. Metab. Res. Rev. 19 (6) (2003) 469–477.
[69] A.P. Cruz, F. Frei, F.G. Graeff, Ethopharmacological analysis of rat behavior on the elevated plus-maze, Pharmacol. Biochem. Behav. 49 (1) (1994) 171–176.
[70] C. Estanislau, S. Morato, Behavior ontogeny in the elevated plus-maze: prenatal stress effects, Int. J. Dev. Neurosci. 24 (4) (2006) 255–262.
[71] A.M.A. Brands, J.M.L. Henselmans, E.H.F. de Haan, G.J. Biessels, [Diabetic encephalopathy: an underexposed complication of diabetes mellitus], Ned. Tijdschr. Geneeskd. 147 (1) (2003) 11–14.
[72] P.S. Koekkoek, G.E.H.M. Rutten, G.J. Biessels, Cognitive disorders in diabetic patients, Handb. Clin. Neurol. 126 (2014) 145–166.
[73] G.S. Mijnhout, P. Scheltens, M. Diamant, G.J. Biessels, A.M. Wessels, S. Simsek, et al., Diabetic encephalopathy: a concept in need of a definition, Diabetologia 49 (6) (2006) 1447–1448.
[74] E.O. Alvarez, J. Beauquis, Y. Revsin, A.M. Banzan, P. Roig, A.F. De Nicola, et al., Cognitive dysfunction and hippocampal changes in experimental type 1 diabetes, Behav. Brain Res. 198 (1) (2009) 224–230.
[75] M. Di Luca, L. Ruts, F. Gardoni, F. Cattabeni, G.J. Biessels, W.H. Gispen, NMDA receptor subunits are modified transcriptionally and post-translationally in the brain of streptozotocin-diabetic rats, Diabetologia 42 (6) (1999) 693–701.
[76] A. Kamal, G.J. Biessels, I.J. Urban, W.H. Gispen, Hippocampal synaptic plasticity in streptozotocin-diabetic rats: impairment of long-term potentiation and facilitation of long-term depression, Neuroscience 90 (3) (1999) 737–745.
[77] M. Popoviç, G.J. Biessels, R.L. Isaacson, W.H. Gispen, Learning and memory in streptozotocin-induced diabetic rats in a novel spatial/object discrimination task, Behav. Brain Res. 122 (2) (2001) 201–207.
[78] Y. Revsin, F. Saravia, P. Roig, A. Lima, E.R. de Kloet, F. Homo-Delarche, et al., Neuronal and astroglial alterations in the hippocampus of a mouse model for type 1 diabetes, Brain Res. 1038 (1) (2005) 22–31.