Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

TRPM2 may be involved in high fructose corn syrup-induced anxiety-like behavior in adult male rats

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ARTICLE INFO

Original paper

Article history:
Received: August 5, 2022
Accepted: March 13, 2023
Published: March 31, 2023

Keywords:

Anxiety, depression, high fructose corn syrup, oxidative stress, chronic immobilization stress, TRPM2

ABSTRACT

Excessive high fructose corn syrup (HFCS) consumption is known to cause oxidative stress, which induces transient receptor potential melastatin type 2 (TRPM2) channel gating. Oxidative stress-induced TRPM2 gating is suggested to play an important role in neurons, indicating a role for the TRPM2 channel in a variety of neuropsychiatric disorders including depression and anxiety. We investigated the effects of HFCS and chronic immobilization stress (CIS) on TRPM2 channel immunoreactivity, anxiety, and depressive-like behaviors in adult male rats. The male rats (n=8/group) were divided into 4 groups: Control, 20% HFCS (F20), 40% HFCS (F40), and stress. The control group received tap water, and F20 and F40 groups were exposed to 20% and 40% HFCS respectively for 14 consecutive days. Rats in the stress group were subjected to immobilization stress for 3 or 6 hours daily in the first and second weeks to induce CIS. Then, light/dark test, open field test (OFT), and tail suspension test (TST) were performed, respectively. In the light/dark test, the time spent in the dark chamber significantly increased in all groups vs the control group ($P<0.01$). In support of this result, the time spent in the light chamber significantly decreased in all groups vs the control group ($P<0.01$). Besides, CIS significantly increased depressive-like behavior in the stress group vs the control group ($P<0.05$). In serum hormone levels, corticosterone (CORT) levels significantly increased in the F40 and stress groups vs the control group ($P<0.01$). TRPM2 immunoreactivity significantly increased in the hippocampus, prefrontal cortex (PFC), nucleus accumbens (NaC), and amygdala regions by HFCS and CIS treatments. For the first time the present study showed that increased immunoreactivity of the TRPM2 cation channels may be linked to the anxiety-like behavior induced by HFCS.

Doi: http://dx.doi.org/10.14715/cmb/2023.69.3.3

Introduction

Fructose consumption has increased significantly over the last two centuries, mainly due to the intensive consumption of high fructose corn syrup (HFCS) in the food and beverage industry (1). HFCS accounts for 40% of all caloric sweeteners currently consumed (2-4). Various forms of HFCS contain 30%, 40%, and 55% fructose (F30, F42, F55), and the most commonly used is F55 (5). Epidemiological evidence suggests that excessive sugar consumption including fructose has several significant negative effects on human health (6). In recent years, attention has been focused on the detrimental effects of fructose consumption on the brain. Prolonged high fructose consumption has been shown to reduce hippocampal neurogenesis and cause altered mitochondrial activity in the hippocampus. Oxidative stress was suggested to be involved as a central part of fructose-induced damage to the brain (1). Moreover, present evidence links oxidative stress to many psychiatric disorders, including anxiety and depression, though the mechanism and pathway implicated are not clear yet (7). Oxidative stress is formed because of an imbalance between oxidant-antioxidant systems, which can be due to the increased free radical formation and attenuated activity of antioxidants (8).

Currently, findings regarding the modulation of intracellular ion channels by oxidative stress are constantly emerging (9). One of these channels has been suggested to be the transient receptor potential melastatin type 2 (TRPM2) channel. The TRPM2 cation channel can be gated by reactive oxygen species (ROS) from oxidative stress (10). Functional TRPM2 channels are found in many neuronal populations, including the hippocampus and cortex, and TRPM2 is one of the main factors in cell death in response to oxidative stress in neurons (11, 12). In connection with this situation, at this point, as the most

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established channel serving as a cellular redox potential sensor, TRPM2 may be likely to be associated with anxiety and depression since oxidative stress was reported as one of the major factors in the pathogenesis of anxiety and depression (13). Consistent with this hypothesis, one study showed that deletion of TRPM2 (*Trpm2*$^{-/-}$) decreases depressive-like behavior in mice, suggesting it may have a key role as a target therapeutic ion channel in this behavior (14). On the other hand, another similar study reported that TRPM2 knockout mice (*Trpm2*$^{-/-}$) were suggested to have increased anxiety-like behavior (15). In this way, the reason for this opposite effect in the pathology of both diseases has not been revealed yet.

Immobilization stress is one of the classical techniques to induce physiological and pathological stress by changing antioxidant enzyme activities (8, 16). Immobilization stress is known as a suitable and reliable model to mimic psychological stress (17). The hypothalamus–pituitary–adrenal (HPA) axis is well-known to be the main neuroendocrine system that is activated as a stress response, culminating in the release of glucocorticoids (GCs) from the adrenal gland; cortisol in humans, and corticosterone (CORT) in rodents. CORT levels in the blood are a good predictor of stress in rodents (13). Corticosteroids are known to be critical hormones in the regulation of the stress response (18).

The hippocampus and prefrontal cortex (PFC) are involved in the regulation of corticosteroid release and mediate emotional behaviors as well. The PFC connects the hippocampus and amygdala and projects to the nucleus accumbens (NaC) (19). At this circuitry, the amygdala is well-known to be implicated in emotional processes, and corticosteroids can decrease the sensitivity of the amygdala indicating corticosteroids may play a crucial role in the balance of homeostasis by normalizing/desensitizing brain processing after stress exposure (20). Lastly, NaC is known to regulate the control of the stress cascade by changing the dopamine transporter and enhancing dopamine metabolism (19). Attenuated connectivity and plasticity in the PFC, hippocampus, and amygdala were suggested as the origin of the pathology of anxiety and depression (21). To the best of our knowledge, there is almost no study investigating the effects of HFCS-feeding and immobilization stress on anxiety and depressive-like behaviors and their histopathological connection with the TRPM2 cation channel in PFC, hippocampus, amygdala, and NaC.

In conclusion, in light of these findings, we aimed to elucidate the effects of HFCS and chronic immobilization stress (CIS) on anxiety and depressive-like behaviors, serum oxidative stress markers, and the immunoreactivity of the TRPM2 cation channel in PFC, hippocampus, amygdala, and NaC in adult male rats.

Materials and Methods

Chemicals

HFCS (F55: 55% fructose and 42% glucose) was prepared from fructose and glucose. Fructose and glucose used in experiments were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Merck Co. (Darmstadt, Germany), respectively. The concentration of HFCS was determined based on a study investigating the sugar content of sweetened beverages (22).

Animals and Treatments

Thirty-two (10-12 weeks old) male Sprague-Dawley rats, weighing 200-250 g, obtained from the University of Firat Experimental Research Unit (FUDAM) were used. This study was conducted at FUDAM in accordance with the guidelines for the ethical use of laboratory animals that were approved by the Firat University Ethic's Committee of Experimental Animals Research as of 17.05.2017 with the number 139 (Elazig, Turkey). The rats were individually housed in standard cages (42x32x16 cm) with a 12:12 hr light-dark cycle with constant temperature (21±1°C), and humidity (55±5%). Pelleted food was provided *ad libitum* to all groups. Rats were randomly separated into four groups, with 8 rats per group. The animal groups were as follows: Control, F20, F40, and stress. The control group received tap water for 2 weeks. The F20 group received 20% (w/v) HFCS, and F40 group received 40% (w/v) HFCS dissolved in tap water daily for 2 weeks. The stress group received tap water for 2 weeks and animals were exposed to CIS every day between 08:00 a.m. and 2:00 p.m. in the first week for 3 hours and in the second week for 6 hours (23).

Behavioral Procedures

To investigate the behavioral effects of HFCS and to compare this effect with the animals treated with CIS, light/dark test, open field tests (OFT), and tail suspension tests (TST) were performed on the 10th, 12th, and 14th days of the study in all groups, respectively. In order to prevent the effects between the behavior tests, the one-day break was given, while the light and dark test and OFT were performed to assess the anxiety-like behavior, and TST was performed to test the depressive-like behavior. All behavioral tests were recorded by video-tape recording unit and scored by the trained and blinded observer to experimental design in all-male rats.

TST

The TST is a highly preferred paradigm for determining the measurement of depressive-like behavior. This test was performed by Steru *et al.*(1985) to test and investigate the efficacy of antidepressant drugs (24). In this test method, it is tried to determine the immobility time against active movement by hanging from the tail in rodents. In our study, each rat was placed in the apparatus (height 50 cm, metal rod) by the 20 cm length band from the tail, and the duration of struggling movements and immobility was recorded for 6 min (25). In normal conditions, the rats attempt to escape from this stressful situation and climb the metal rod. After a while, hopelessness occurs about the situation of the rats, and the rat leaves its struggling motion and remains immobilized. The moment it stands still is interpreted as hopelessness. The immobility time is then determined (26, 27). This period is considered a depressive-like behavior indicator (28).

OFT

The open-field test is a behavioral test that evaluates locomotor activity and anxiety-induced behaviors (29, 30). In this procedure, rodents are used, and it is utilized in an environment previously unknown to the rodent, in a system surrounded by walls the height to prevent the animal from escaping (31). Currently, the OFT is one of the popular procedures used to evaluate animal psychology (32).

In the OFT, the number of frames (horizontal mobility), grooming movements, and the frequency of rearing (vertical mobility) are evaluated by placing the experimental animal at the center of the apparatus for 2 to 20 min (usually 5 min) (29). The increase in locomotor movements, the time spent in the central region, and vertical mobility are interpreted as an anxiolytic-like effect, while the opposite is defined as an anxiogenic effect (29). The wider area, larger than its cage leads to avoidance behavior, allowing the evaluation of locomotor and investigative behavior (33). In our study, the apparatus used in the OFT had a square shape and was illuminated by a light source suspended at a height of 1 m through a bulb of 60 w. The apparatus was an 80 cm wide square apparatus surrounded by plexiglass white walls consisting of a total of 25 squares in 9 central and 16 in the periphery. The animals were left individually, in the square of the center and then the video was recorded immediately. In the recording performed for 10 min, two basic parameters were evaluated: 1. The total number of passed lines (horizontal activity), 2. The number of rearing (vertical activity). The total number of lines passed by the animal is one of the parameters that determine locomotor activity. Locomotor activity is used to express the position change. A new environment is discovered with the rearing where the animal is standing on the lower extremities, raising the upper extremities, or resting on the wall. After each animal, the OFT apparatus was disinfected by cleaning with 70% alcohol.

Light/Dark Test

This test was first used by Cawley and Goodwin in 1980 to evaluate investigative behaviors, which are indicative of the anxiolytic effects of some drugs in rodents (34). These investigators used a device consisting of two parts, one-third of which was dark, and two-thirds of which were light. In this test, the places considered to be safe by the animal are small dark chambers and the avoided areas are large, illuminated chambers. The time spent by animals in each chamber and the number of passes between the two chambers were evaluated (35). The light/dark test is based on the innate aversion of rodents to brightly illuminated areas and the spontaneous exploratory behavior of rodents in response to mild stressors, that is, novel environments and light (36). The increases in the time spent in the light chamber and the number of passes to the light chamber are called anxiolytic behaviors, while the decrease in these parameters is an indicator of anxiety-like behaviors (37). In our study, as a light/dark test device, a device form consisting of two equal-area sections made of transparent plexiglass material and one side black was used (38). An open section was formed between the two compartments that allowed the passage of animals. In the dark chamber, there was a cover made of black plexiglass material, and a bulb was used on the light chamber lighting (39). Then, the rats were placed in the middle of the light chamber, facing away from the dark, and it was left to explore the apparatus for 5 min (40). In this process, the number of passes from the light chamber to the dark chamber and the time spent in both chambers were recorded by camera. After each test, light and dark chambers were cleaned with 70% alcohol (41).

Surgery

Before starting surgical procedures, animals were decapitated using a guillotine. Following decapitation, the heads of the animals were removed, bone tissues were cut along the two parietal-temporal suture lines in the occipitofrontal direction, and the brain tissue was quickly removed by blunt dissection. In addition, after decapitation, 4-5 ml of blood was collected in cooled test tubes from each animal, then centrifuged for 5 min at 4000 rpm, and finally stored at −20°C until Enzyme-Linked Immunosorbent Assay (ELISA) and auto-analyzer assays were performed.

Hormone Measurements

Serum CORT levels were studied according to the kit user manual using the rat CORT ELISA kit (Wuhan Fine Biotech Co., Ltd., China). Absorbances were performed by ELX 800 ELISA reader (BioTek Instruments, Winooski, VT, USA) spectrophotometrically at 450 nm. Test results were multiplied by 10 because of 1:10 dilution; ng/ml. Serum total antioxidant status (TAS) and total oxidant status (TOS) levels were determined by automated colorimetric methods developed by Erel (42, 43) and measured using commercial kits (Rel Assay Diagnostics, Gaziantep, Turkey) in an Advia 2400 auto-analyzer (Siemens Healthcare Diagnostics Inc., Tarrytown, USA). The intensity of the colored complex formed by ferric ions in an acidic medium was measured spectrophotometrically, and the results were expressed as μmol hydrogen peroxide (H_2O_2) Equiv./L. The measurement calibration was performed with Trolox, a water-soluble vitamin E analog, and the results were reported as mmol Trolox Equiv./L.

Immunohistochemistry

The deparaffinized tissues were passed through the graded alcohol series and boiled in the microwave oven (750W) for 7+5 min in the pH: 6 in citrate buffer solution for antigen retrieval. After boiling, the tissues were allowed to cool at room temperature for about 20 min and washed 3 times with PBS (Phosphate Buffered Saline, P4417, Sigma-Aldrich, USA) for a total of 15 min. It was then incubated with a hydrogen peroxide block solution for 5 min to prevent endogenous peroxidase activity (Hydrogen Peroxide Block, TA-125-HP, Lab Vision Corporation, USA). Ultra V Block (TA-125-UB, Lab Vision Corporation, USA) solution was applied to the tissues for 5 min, which were washed with PBS for 3x5 min. It was then incubated at room temperature in a humid medium of 60 min with diluted primary antibody (Rabbit Anti-TRPM2 antibody, Abcam, Cambridge, UK) at a rate of 1/200. Tissues were washed 3x5 min with PBS after primary antibody application and then incubated at room temperature in a humid medium for 30 min with secondary antibodies (biotinylated Goat Anti-Poliyvalent Anti-mouse/rabbit IgG), (TP-125-BN, Lab Vision Corporation, USA). After secondary antibody application, the tissues were washed with PBS for 3x5 min and incubated with Streptavidin Peroxidase (TS-125-HR, Lab Vision Corporation, USA) for 30 min at room temperature in a humid medium. After secondary antibody treatment, tissues were washed for 3x5 min with PBS and then 3-amino-9-ethyl carbazole (AEC) Substrate +AEC Chromogen (AEC Substrate, TA-015, and HAS, AEC Chromogen, TA-002-HAC, Lab Vision Corporation, USA) solution were added to tissues, and then the image signal was taken in the light microscope and then was simultaneously washed with PBS. Rabbit IgG was

used for negative control. The tissues were contrasted with Mayer's hematoxylin. It was then passed through PBS and distilled water, and the appropriate closure solution (Large Volume Vision Mount, TA-125-UG, Lab Vision Corporation, USA) was used. Prepared preparations were examined and photographed in a Leica DM500 microscope (Leica DFC295). Histoscore was established based on the prevalence (0.1: <25%, 0.4: 26-50%, 0.6: 51-75%, 0.9: 76-100%) and severity (0: none, +0.5: very small, +1: less, + 2: medium, +3: severe) of immunoreactivity in staining.

Statistics

All data are presented as mean±standard error of the mean (S.E.M). The statistical difference between values from the study groups was analyzed using one-way ANOVA followed by Tukey's posthoc test by OriginPro 8 SR1 (OriginLab Co, Northampton, MA, USA). A p-value <0.05 was considered statistically significant.

Results

Effects of F20, F40, and CIS on Depression and Anxiety-like Behavior

TST

TST results are shown in Table 1. In the TST, the immobility time, which is a marker of depression, significantly increased ($F_{(3,28)}$=2.909, P=0.05) in the stress group vs control group (P<0.05). Also, the mobility time significantly decreased ($F_{(3,28)}$=2.941, P=0.05) in the stress group vs the control group (P<0.05). In the F20 and F40 groups, the immobility time increased vs the control group, but this was not significant (Table 1).

Light/Dark Test

Light/Dark Test results are shown in Table 2. As a marker of anxiety, the time spent in the dark chamber significantly increased ($F_{(3,28)}$=46.560, P<0.001) in F20, F40, and stress groups vs the control group (P<0.01), (Table 2). Also, the time spent in the light chamber significantly decreased ($F_{(3,28)}$=46.560, P<0.001) in F20, F40, and stress groups vs the control group (P<0.01). Hence, it can be said that both HFCS and CIS can increase anxiety-like behavior in male rats. We also examined the transition number to darkness, and it did not change ($F_{(3,28)}$=2.807, P=0.058) between the groups (Table 2).

OFT

OFT results are shown in Table 3. In the OFT, which is an indirect measure of locomotor activity, the number of lines passed in the stress group significantly decreased ($F_{(3,28)}$= 17.096, P<0.001) vs the control group (P<0.01; Table 3). The number of lines passed in the F20 and F40 groups decreased vs the control group, but this was not significant. The number of rearing significantly increased ($F_{(3,28)}$=19.022, P<0.001) in F20 and F40 groups vs control and stress groups (P<0.01). There was no significant difference in the number of rearing in the stress group vs the control group.

Effects of F20, F40, and CIS on CORT, TAS, and TOS Levels

The serum levels of CORT, TAS, and TOS are shown in Figure 1. TAS levels were not significantly changed in F20, F40, and stress groups ($F_{(3,28)}$=1.636, P=0.213) vs the control group (Figure 1A). However, TOS levels were significantly higher ($F_{(3,28)}$=7.779, P<0.01) in the stress group vs the control group (P<0.01; Figure 1B). TOS levels also increased in F20 and F40 groups vs the control group, but this was not significant. However, the TOS levels in the F20 group significantly decreased vs the stress group (P<0.05; Figure 1B). The CORT levels significantly in-

Table 1. Effects of F20, F40, and CIS on the mobility and immobility time in the TST in the male rats.

Groups	Mobility Time (s)	Immobility Time (s)
Control	223.32 ± 10.81	136.68 ± 10.81
F20	198.65 ± 22.53	161.35 ± 22.53
F40	195.01 ± 10.02	164.99 ± 10.02
Stress	164.50 ± 8.19^{*a}	195.50 ± 8.19^{*a}

a: vs control group, *P<0.05, n=8 for each group.

Table 2. Effects of F20, F40, and CIS on the parameters of the light/dark test in the male rats.

Groups	Time spent in the light chamber (s)	Time spent in the dark chamber (s)	Transition Number to Darkness
Control	187.63 ± 12.99	112.37 ± 12.99	34.21±4.68
F20	73.69 ± 5.18^{**a}	226.31 ± 5.18^{**a}	22.37 ±2.94
F40	64.76 ± 5.57^{**a}	235.24 ± 5.57^{**a}	21.90 ± 3.17
Stress	66.65 ± 8.94^{**a}	233.35 ± 8.94^{**a}	21.31 ± 3.72

a: vs control group, **P<0.01, n=8 for each group.

Table 3. Effects of F20, F40, and CIS on the parameters of the OFT in the male rats.

Groups	Number of lines passed	Number of rearing
Control	194.40 ± 8.68	19.37 ± 2.32
F20	184.50 ± 6.52^{**b}	35.50 ± 1.49^{**a,**b}
F40	175.75 ± 6.13^{**b}	37.66 ± 1.61^{**a,**b}
Stress	127.83 ± 6.95^{**a}	23.37 ± 2.58

a: vs control group **P<0.01, b: vs stress group **P<0.01, n=8 for each group.

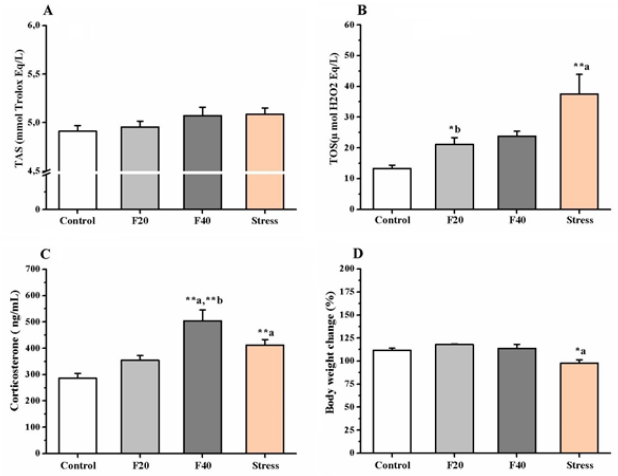

Figure 1. Effects of F20, F40, and CIS on **(A)** TAS levels and **(B)** TOS levels, and **(C)** CORT levels, and **(D)** body weights on the 14th day in male rats. a: vs control group **P<0.01, b: vs stress group *P<0.05, n=8 for each group.

creased in F40 and stress groups ($F_{(3,28)}$=14.043, P<0.001) vs the control group (P<0.01; Figure 1C). Also, CORT levels significantly increased in the F40 group vs the F20 group (P<0.01; Figure 1C).

Effects of F20, F40, and CIS on Body Weight

The body weights of all groups on the 14th day are shown in Figure 1D. The HFCS did not change body weight in the F20 or F40 group vs the control group. However, CIS significantly decreased body weight ($F_{(3,28)}$=8.426, P<0.001) on the 14th day in the stress group vs the control group (P<0.05).

Effects of F20, F40, and CIS on TRPM2 Immunoreactivity in the Amygdala, NaC, Hippocampus, and PFC

The results of the immunohistochemical staining for TRPM2 immunoreactivity in four brain areas are shown in Table 4.

In the evaluation of immunohistochemical staining for TRPM2 immunoreactivity under light microscopy, TRPM2 immunoreactivity in the amygdala significantly increased ($F_{(3,28)}$=18.770, P<0.001) in F40 and stress groups vs control group (P<0.01). On the other hand, it significantly decreased in the F20 group vs the stress group (P<0.01, Figure 2). Similarly, it also significantly increased in NaC ($F_{(3,28)}$=19.633, P<0.001) in F40 and stress groups vs control group while it significantly decreased in the F20 group vs stress group (P<0.01, Figure 3). On the other hand, TRPM2 immunoreactivity significantly increased in the hippocampus ($F_{(3,28)}$=18.558, P<0.001) in F20, F40, and stress groups vs the control group (P<0.01, Figure 4). Correlatively, it also significantly increased in PFC ($F_{(3,28)}$=8.062, P<0.01) in F20, F40, and stress groups vs the control group (P<0.01, Figure 5).

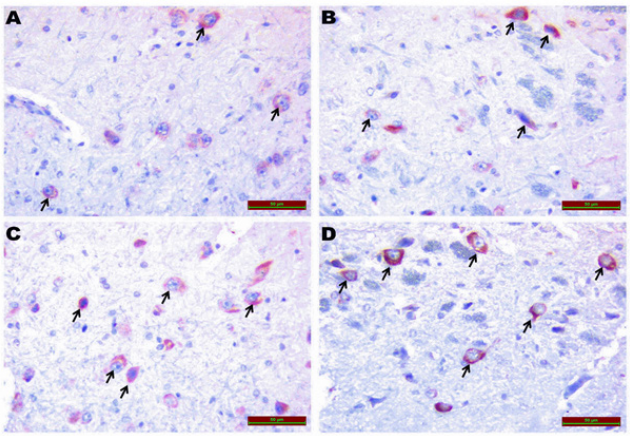

Figure 2. Immunohistochemical illustration of TRPM2 immunoreactivity in amygdala **(A)** Control group, **(B)** F20 group, **(C)** F40 group, and **(D)** Stress group. 50 µm, 40 x.

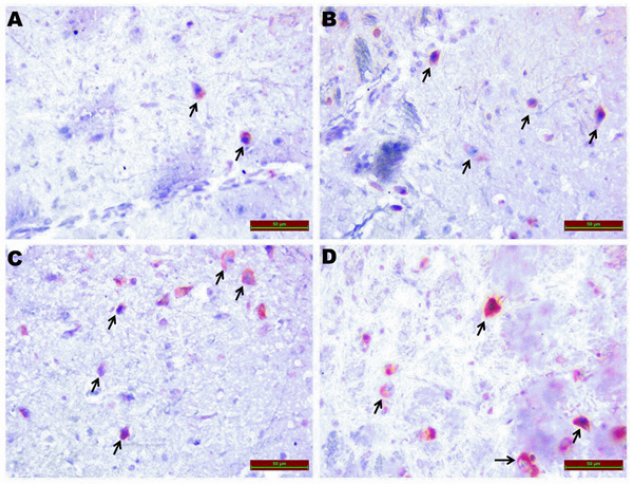

Figure 3. Immunohistochemical illustration of TRPM2 immunoreactivity in NaC **(A)** Control group, **(B)** F20 group, **(C)** F40 group, and **(D)** Stress group. 50 µm, 40x.

Discussion

In this study, it was shown for the first time that HFCS consumption caused anxiety-like behavior with CIS-like effects by parallel increased TRPM2 channel immunoreactivity in adult male rats.

Hormone Levels and Body Weights

Fructose is known to induce CORT secretion from the adrenal gland and it was reported that the overload of fructose during 9 weeks significantly increased plasma CORT levels in rats (44). It is well-established that as a response to this stress stimulus, the paraventricular nucleus secretes vasopressin which stimulates the anterior hypophysis for

Table 4. Effects of F20, F40, and CIS on the histological scores of TRPM2 immunoreactivity in the amygdala, NaC, hippocampus, and PFC areas of the male rat.

Groups	Amygdala	NaC	Hippocampus	PFC
Control	0.43 ± 0.09	0.50 ± 0.16	0.35 ± 0.16	0.45 ± 0.19
F20	0.63 ± 0.18^{**b}	0.52 ± 0.15^{**b}	1.33 ± 0.51^{**a}	1.80 ± 0.73^{**a}
F40	1.40 ± 0.69^{**a}	1.70 ± 0.61^{**a}	1.75 ± 0.55^{**a}	1.60 ± 0.86^{**a}
Stress	1.55 ± 0.63^{**a}	1.73 ± 0.81^{**a}	1.53 ± 0.91^{**a}	1.63 ± 0.90^{**a}

a: vs control group **: P<0.01, b: vs stress **: P<0.01, n=8 for each group.

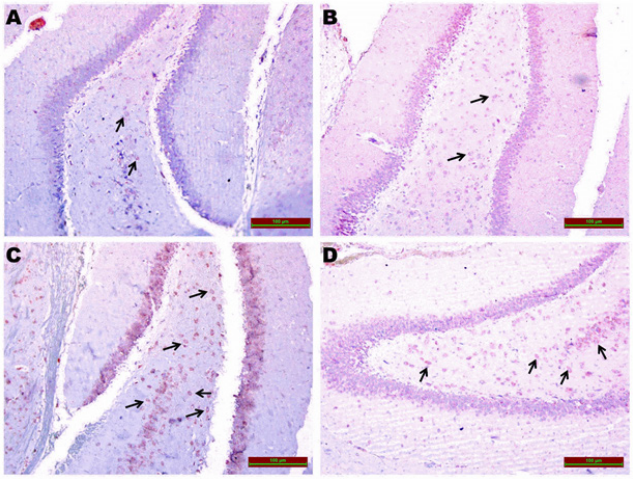

Figure 4. Immunohistochemical illustration of TRPM2 immunoreactivity in the hippocampus **(A)** Control group, **(B)** F20 group, **(C)** F40 group, and **(D)** Stress group. 100 µm, 20x.

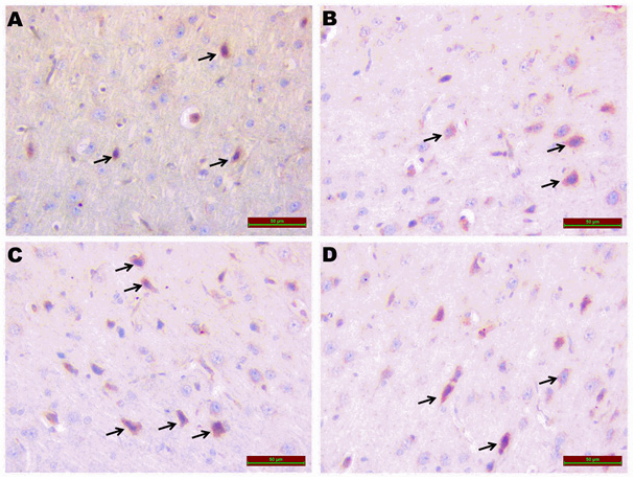

Figure 5. Immunohistochemical illustration of TRPM2 immunoreactivity in PFC **(A)** Control group, **(B)** F20 group, **(C)** F40 group, and **(D)** Stress group. 50 µm, 40x.

secreting the corticotrophin-releasing hormone and adrenocorticotropic hormone, and culminating the GCs to secrete CORT in rodents (45, 46). The immobilization stress is known to end the increase of CORT in rats for long years (47). In accordance with the literature, in our study, there was an increase in the CORT levels in the stress group vs the control group Similarly, F40 also increased serum CORT levels vs the control group for the first time in the present study. 11β-hydroxysteroid dehydrogenase type 1 isoenzyme (11β-HSD-1) is known to catalyze the turnover of cortisol from cortisone in men and CORT from 11-dehydrocorticosterone in rodents. Cortisone and 11-dehydrocorticosterone are inactive corticosteroids that are activated by 11β-HSD-1 to their active forms (48). In our study, HFCS might have led to an activation of the HPA axis and increased the serum CORT levels by increasing 11β-HSD-1, and there is no exact mechanism of action to explain these findings.

The nutrition which consists of much fructose leads to excessive ROS occurrence and a simultaneous decrease in the antioxidant defense mechanism, and therefore it accelerates oxidative stress (49). ROS production is the main parameter of oxidative stress, and as a result of the increase in ROS production, the degree of oxidative damage also increases (50). In one study, fructose at the 60% rate was given to male rats for 10 weeks, and it was observed that there was a significant decrease in TAS levels in the fructose group in which there was a significant increase in TOS levels (51). All in all, in our study, there was no change in TAS levels of F20, F40, and stress groups vs the control group, but there was a significant increase in TOS levels in only the stress group vs the control group. Several hypotheses can be made as to why HFCS did not change TAS and TOS levels, but both parameters might not have changed depending on HFCS concentrations and the duration of treatment.

Stress was suggested to alter body weight in animal models, and the CIS model is known to be most commonly used in various models since it can imitate robust physical and psychological stress. The CIS model can be used as an animal model in anorexia nervosa (52). Hence, several studies reported that CIS decreased body weight and body weight gain in rodents (52-54). A decrease in body weight gain may depend on various physiological factors and a decrease in body weight gain was suggested to be associated with anorexia as well (55). Jeong *et al.* (2013) revealed that CIS decreased body weight by initially modulating major food intake-related genes and then later modifying other genes implicated in energy metabolism. More importantly, these genetic alterations were reported to be mediated, at least in part, by CORT (52). Consistent with the literature, in the present study, serum CORT levels significantly increased in the stress group, and parallel to this finding, body weight also decreased in the same group vs the control group. Body weight might have attenuated depending on changes in orexigenic and anorexigenic peptides gene expressions in the brain.

Anxiety and Depressive-like Behaviors

Light/dark test and OFT are generally used to determine anxiety-like behavior in experimental animals (56). In the light/dark test, time spent in the light chamber is evaluated as the anxiolytic behavior while the time spent in the dark chamber is evaluated as the anxiogenic behavior (57). In another test used to determine anxiety-like behavior, OFT, the rearing behavior, and horizontal and vertical moves are used as the parameters of OFT. A decrease in the number of lines passed and the increase in the number of rearing in this behavioral test are described as anxiogenic behavior (29). Previous studies showed that anxiogenic findings increased when the OFT was performed on rats exposed to stress (58).

In our study, the light and dark test and OFT were used to determine anxiety-like behavior in adult male rats. According to the light and dark test results, the time spent in the dark chamber significantly increased in F20, F40, and stress groups vs the control group. Compatible with these findings, the time spent in the light chamber significantly decreased in F20, F40, and stress groups vs the control groups. On the other hand, the transition number to darkness did not change in any group vs the control groups. Overall, it can be said that HFCS and CIS elevated the levels of anxiety-related parameters in the light/dark test. For the first time, the present study revealed that HFCS caused anxiety-like behaviors in adult male rats.

According to the OFT results, the number of lines passed significantly decreased in only the stress group vs control group while the number of rearing significantly increased in F20 and F40 groups vs the control group. The

number of lines passed and the number of rearing are typically used as markers of locomotor activity, but these are also parameters of exploration and anxiety The increase of such behaviors refers to a rise in locomotion and exploration and indicates a lower level of anxiety (59). Hence, CIS caused anxiety-like behavior by decreasing the number of lines passed. Interestingly, on the other hand, HFCS caused anxiolytic effects by increasing the number of rearing. Although we have considered that locomotor response to novelty is one of the parts of the anxiety-like behavior, the number of rearing in the OFT was also suggested to be relevant with activity-like levels more than the anxiety-like levels (60). Hence, only the light/dark test was able to reveal the anxiety-like effect of HCFS.

Some tests have been used in experimental animals in order to determine depression. TST and forced swimming test are used to determine depressive-like behavior (61). The duration of immobility time in the TST is accepted as a depression sign (28). In one study conducted with the rats exposed to the immobilization stress for 21 days, it was observed that the immobility time increased vs the control group in TST (62). Also, two other studies that were conducted on rodents showed that CIS increased immobility time in TST (63, 64). Compatible with the literature, in our study, the immobility time in F20, F40, and stress groups increased while this increase was at a significant level for the stress group. Hence, CIS can be said to cause depressive-like behavior in adult male rats. On the other hand, HFCS did not change mobility or immobility time in TST, and as mentioned earlier above, this situation may likely depend on HFCS concentration and the length of treatment. It needs to be elucidated by much more detailed molecular and cellular mechanisms of action in future studies.

TRPM2 Immunoreactivity

TRPM2 is a nonselective cation channel and that becomes active in oxidative stress conditions. It also leads to an increase in intracellular free calcium concentration and cell death (65). Although the brain and neurons generally consume oxygen and multi-unsaturated fatty acids, which can be oxidized, they have a weak antioxidant system. Therefore, they may become the target of ROS (66). Also, the brain and neural system are quite sensitive to stress, and the complex pathologies cause some important problems, which lead to neuron death, such as increased oxidative damage, mitochondrial dysfunction, inflammation, and defects in protein clearance (67).

TRPM2 plays a role in neural development and has a preventing role in neuritis development. It was shown that the overexpression of TRPM2 decreased axonal growth while the knockdown of TRPM2 increased. In the same study, it was also revealed that neurons of *Trpm2*$^{-/-}$ rats had longer neuritis and more dendrite vs the control group, and TRPM2 was suggested to mediate the axonal growth in the lysophosphatidic acid-induced depression (68).

In the present study, TRPM2 immunoreactivity was shown to be increased in four brain areas in F20, F40, and stress groups vs the control group. However, the increase in the F20 group was not significant in both amygdala and NaC areas vs the control group. On the other hand, TRPM2 immunoreactivity in both stress and F40 groups significantly increased in four brain areas vs the control group. In the PFC, amygdala, hippocampus, and NaC, stressors cause a change in the extracellular concentration of different neurotransmitters, and this change is related to the behavioral activation and modulation to cope with stressors (69, 70). The hippocampus which is the key region in response to stress is the first brain region identified as the target of GCs. Stress and stress hormones produce both adaptive and non-adaptive effects on this brain region during life (71). Besides the hippocampus, the amygdala and PFC which are related to emotions and cognitive functions are exposed to exposed to reshape structurally by changing the behavioral and physiologic responses originating from stress (71). These brain areas are the target of stress and stress hormones and the acute and chronic effects of stressful experiences determine how these areas will respond. It was reported in the autopsy studies of chronic depressive individuals of the amygdala, PFC, and hippocampus that there was a decrease in the glial cell number (71). Also, it was observed in positron emission tomography and functional magnetic resonance studies that the volumes of these three brain structures decreased in individuals who had repeated depression (71). Also, CIS led to dendritic spine density in the medial prefrontal cortex (Mpfc) and enhanced dendritic arborization in the basolateral amygdala (BLA) (72, 73). Also, in concern with the amygdala, the CIS which lasts longer than 21 days, was shown to disrupt the hippocampal-dependent cognitive function increasing the amygdala-dependent fear and fear conditioning (74). In our study, we showed that the CIS increased TRPM2 immunoreactivity in these brain areas. The major award pathway of the brain constitutes the dopaminergic projections projecting to NaC from the ventral tegmental area and the BLA, Mpfc, and their dense connections (69). NaC, BLA, and Mpfc are defined as mediators in the response to the award (75). Since the Mpfc and BLA arrange the responses to stress, are related to the conditioning to the apparent emotional stimulus, and may arrange the HPA and autonomous neural system responses against both conditional and unconditional award exposure. In our study, there was an increase in TRPM2 immunoreactivity in NaC, amygdala, and PFC areas in stress and HFCS groups vs the control group, and this is a sign of disruption in the stress-award pathway. Altogether, in the present study, TRPM2 immunoreactivity might have increased depending on behavioral changes, including anxiety-like and depressive-like behaviors in all treatment groups or vice versa. Hence, these findings indicate that there may be a close cross-talk between TRPM2, and depression and anxiety-like behaviors in male rats.

In conclusion, here we showed for the first time that HFCS and CIS may lead to anxiety-like behavior by modulation of TRPM2 immunoreactivity in the amygdala, NaC, hippocampus, and PFC in male rats. Besides, we revealed for the first time, CIS may also cause depressive-like behavior by changing the same cation channel immunoreactivity indicating TRPM2 can be implicated in depressive-like behavior as well. Further research is needed to determine whether or not TRPM2 plays a part in the development of anxiety and depression-like symptoms in response to HFCS and CIS.

Fundings

This research was funded by Firat University Scientific Research Projects Unit (Project No: TF.17.29).

Data availability statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement
I.S., E.K., G.K., M.O., and H.K. designed and supervised the study. A.Y., N.U.E., Z.E. and F.T. conducted all animal experiments. O.B., F.G.B., T.K. and N.K. conducted all hormone measurements. I.S., F.T. and G.Z. contributed to the data analysis. G.K. and I.S. wrote the manuscript, and all authors approved the final manuscript.

Conflict of Interest
The authors declare that they have no conflict of interest.

Abbreviation List
BLA: Basolateral amygdala
CIS: Chronic immobilization stress
CORT: Corticosterone
F20: 20% HFCS
F40: 40% HFCS
GCs: Glucocorticoids
HFCS: High fructose corn syrup
H_2O_2: Hydrogen peroxide
HPA: Hypothalamus–pituitary–adrenal axis
Mpfc: Medial prefrontal cortex
NaC: Nucleus accumbens
OFT: Open field test
PFC: Prefrontal cortex
ROS: Reactive oxygen species
TRPM2: Transient receptor potential melastatin type 2
TST: Tail suspension test
TAS: Total antioxidant status
TOS: Total oxidant status
11β-HSD-1: 11β-hydroxysteroid dehydrogenase type 1 isoenzyme

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