Molecular Neurobiology (2023) 60:1499–1514
https://doi.org/10.1007/s12035-022-03158-x

A Maternal High-Fat Diet Causes Anxiety-Related Behaviors by Altering Neuropeptide Y1 Receptor and Hippocampal Volumes in Rat Offspring: the Potential Effect of N-Acetylcysteine

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Received: 21 September 2022 / Accepted: 1 December 2022 / Published online: 11 December 2022

Abstract

The children of obese mothers are known to have a high risk of obesity and metabolic disease and are prone to developing cognitive deficits, although the underlying mechanism is not yet fully understood. This study investigated the relationship between neuropeptide Y1 receptor (NPY1R) and anxiety-like behaviors in the hippocampi of male rat offspring exposed to maternal obesity and the potential neuroprotective effects of N-acetylcysteine (NAC). A maternal obesity model was created using a high-fat (60% k/cal) diet. NAC (150 mg/kg) was administered by intragastric gavage for 25 days in both the NAC and obesity + NAC (ObNAC) groups. All male rat offspring were subjected to behavioral testing on postnatal day 28, the end of the experiment. Stereological analysis was performed on hippocampal sections, while NPY1R expression was determined using immunohistochemical methods. Stereological data indicated significant decreases in the total volume of the hippocampus and CA1 and dentate gyrus (DG) regions in the obese (Ob) group ($p < 0.01$). Decreased NPY1R expression was observed in the Ob group hippocampus ($p < 0.01$). At behavioral assessments, the Ob group rats exhibited increased anxiety and less social interaction, although the ObNAC group rats exhibited stronger responses than the Ob group ($p < 0.01$). The study results show that NAC attenuated anxiety-like behaviors and NPY1R expression and also protected hippocampal volume against maternal obesity. The findings indicate that a decrease in NPY1R-positive neurons in the hippocampus of male rats due to maternal conditions may be associated with increased levels of anxiety and a lower hippocampal volume. Additionally, although there is no direct evidence, maintenance of NPY1R expression by NAC may be critical for regulating maternal obesity-induced anxiety-related behaviors and hippocampal structure.

Keywords High-fat diet · Anxiety · Hippocampus · N-acetylcysteine · Stereology

Introduction

A significant increase has been observed in the global prevalence of obesity over the last 10 years. Research has associated a high maternal pre-pregnancy body mass index (BMI) with developmental delay, anxiety behaviors, autism spectrum disorders, attention deficit/hyperactivity disorder, and cognitive disorders in children [1]. However, the mechanisms underlying these relationships are still not fully understood. Obesity during pregnancy has also been linked to phenomena such as systemic and placental inflammation, dysregulation of metabolic and neuro-endocrine signals, increased oxidative stress, and antioxidant deficiencies in both rodent and human studies. Placental inflammation has been reported to cause fetal neuronal inflammation in obese rodent pups, in turn giving rise to changes in fetal neurogenesis, myelination, and synaptic plasticity in organs such as the hippocampus and hypothalamus [2].

Animal models have also suggested that maternal obesity causes impairment in cognitive functions. Memory, learning, and visual and motor functions all play a key role in cognitive functions [3]. Rodent studies have reported impaired performance among offspring of obese individuals in the Morris water maze and Barnes maze, tests that

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assess visuospatial orientation, memory, and learning and that are associated with new object recognition [4].

The hippocampus plays an important role in regulating emotions, behaviors, motivation, and memory functions. However, it is also susceptible to the deleterious impacts of maternal obesity. Epidemiological studies have revealed dysfunction and an increased risk of development of autism-like behavioral disorders in the children of overweight and obese women [5–7]. However, the mechanisms involved in the relationship between cognitive dysfunction and maternal obesity are still unknown, and experimental studies are needed on this subject.

One previous study associated maternal obesity with a decreased hippocampal volume in children [8]. Although the underlying mechanisms involved in this are not yet fully understood, animal studies have shown that exposure to fetal obesity results in impairment of neurogenesis in the dentate gyrus (DG) and compromise of hippocampal learning [8, 9]. The hippocampus is particularly important in terms of memory, and its development is highly susceptible to the intrauterine metabolic environment. Both human and animal studies have revealed that a diet regimen containing increased fat-cholesterol plays an important role in behavioral abnormalities associated with social behavior, aggression, and brain plasticity [10, 11]. Another animal study reported disruption of the blood–brain barrier and a decrease in brain-derived neurotrophic factor (BDNF) levels, in turn impacting on the hippocampus and memory performance, after 10 days of administration of a Western-style diet [12]. Similarly, smaller hippocampal volumes were observed in obese humans compared to normal weight individuals in another study [13]. The hippocampus, with its important role in regulating emotions, behaviors, motivation, and memory functions, is therefore susceptible to the adverse effects of obesity.

Neuropeptide Y (NPY) plays a modulatory role in energy homeostasis, nutrition, sexual behavior, learning and memory, cognition, and neuroplasticity [14]. The NPY system functions as a communication medium between the hypothalamus and adipose tissue. Energy intake is linked to a positive energy balance through increases in storage and reduced energy consumption. Compromise of the NPY system has been linked to diseases including obesity, type II diabetes, and metabolic syndrome [15]. NPY has been shown to interact with a family of G protein-coupled receptors in the brain, including Y1 (Y1R), Y2, and Y5 receptors [16]. Numerous studies have shown that NPY affects metabolism and feeding behavior, particularly through activation of Y1R [16–18]. The majority of these studies have shown that the *NPY1R* is a critical component and plays an important role in the regulation of anxiety-related behavior.

In that context, the present study investigated the effect of exposure to an obese environment in fetal life on *NPY1R* and anxiety-related behaviors in the male rat offspring brain.

N-Acetylcysteine (NAC) is a thiol compound responsible for stimulating glutathione-S-transferase activity. It also behaves as a free radical scavenger and antioxidant by normalizing the intracellular glutathione (GSH) pool. It has been widely employed in the treatment of chronic obstructive pulmonary disease [19], and there is increasing interest in its potential beneficial effects on metabolic disorders, as well as in its antioxidant and anti-inflammatory activities. Studies have shown low GSH levels in obese individuals [20]. Glutaminergic dysregulation in the brain is also seen in obesity. GSH serves as a reservoir of neuronal glutamate. Synaptic excitability is affected by the equilibrium of the glutamate–glutamine cycle between glial cells and glutaminergic neurons [21], and obesity is characterized by GSH deficiency. If insufficient GSH is available, heavy metals and toxins are stored in adipose tissue. The nervous system is one component of the important risk group due to its fatty structure. GSH can be increased by providing the body with raw materials and cofactors. NAC is an essential amino acid required for the formation of GSH. It converts glutamate to GSH and then releases glutamate into the extracellular space, thereby reducing glutamatergic neurotransmission at the synapses [22]. NAC may therefore represent an important raw material capable of use in order to increase the level of GSH in tissue and thus prevent the deleterious cognitive effects of obesity. NAC supplementation has also been shown to suppress hyperglycemia and hyperinsulinemia caused by a fructose and high-sucrose diet and to improve peripheral insulin sensitivity [23].

Strong associations have been reported between maternal obesity and an increased incidence of anxiety-like behavior [24, 25]. The present study investigated the relationship between behavioral disorders and limbic *NPYR1* expression in rats exposed to obesity in the prenatal period. It also evaluated the potential protective effects of NAC against cognitive dysfunctions closely related to anxiety-like behavior caused by maternal obesity.

Material and Methods

Sixteen female Wistar albino rats (age 25 days) were randomly divided into four groups of four animals each. These received the diets listed below. At 12 weeks of age, the animals were allowed to mate with male breeding rats in separate cages for 1 day under appropriate conditions. Vaginal smear samples were taken the following day. Those rats whose smear samples contained sperm were considered to be on day 0 of pregnancy. The experimental animals were allowed ad libitum access to water and feed under

conventional conditions at a 24 ± 1 °C ambient temperature for 12 h a day during the study. The experimental animals were provided by and cared for at the Adıyaman University Experimental Animals Research Center, Turkey, following receipt of Adıyaman University Experimental Animals Ethical Committee approval (No. 2021/001 dated 25.02.2021).

The experimental procedure was performed on 16 pregnant Wistar albino rats, while analyses were performed on their male offspring ($n = 8$ for each group, 32 in total). The animals were sacrificed at the end of the postnatal 28th day.

Groups

Control Group (Cont, n = 8)

The four female rats in this group received a standard diet (10% k/cal) and were mated at the age of 12 weeks. A control group was formed with their male offspring ($n = 8$), and these were subjected to analyses.

N-Acetylcysteine Group (NAC, n = 8)

The four female rats in this group were given a standard diet (10% k/cal) and mated at the age of 12 weeks. NAC (Sigma-Aldrich, Merck, Germany) was administered via intragastric gavage from the beginning of pregnancy until postnatal day 25 (150 mg/kg) [26]. The NAC group was formed with male offspring ($n = 8$) born to the pregnant rats in this group and was subjected to analyses.

Obesity Group (Ob, n = 8)

The four female rats in this group received a high-fat (60% k/cal) diet and were also mated at 12 weeks. An obesity group was formed with male offspring ($n = 8$) born to the pregnant rats in this group, and these male offspring were subjected to analyses. The induction of obesity is explained in detail below [27, 28].

Obesity + N-Acetylcysteine Group (ObNAC, n = 8)

The four female rats in this group received a high-fat (60% k/cal) diet and were also mated at 12 weeks of age. NAC (Sigma-Aldrich, Merck, Germany) (150 mg/kg) was administered via intragastric gavage from the beginning of pregnancy until postnatal day 25 [26]. The obesity + NAC (ObNAC) group was established with male offspring ($n = 8$) born to the pregnant rats in this group and subjected to analyses.

The rats in the control group received a commercial rat diet (7–10% fat, 68–70% carbohydrates, 18–20% protein, 1–2% vitamins and minerals; 210 kcal 100 g/day). The obesity model was created using feed with 60% kcal fat (Arden Research and Experiment Company, Ankara, Turkey). The dietary compounds are shown in Additional file 1. While the animals were fed a standard or high-fat diet, their water needs were met ad libitum from tap water. All rats were weighed initially and once weekly throughout the experiment. BMI calculations were performed in both groups following 9-week follow-up to establish whether or not the animals were obese. During BMI calculation, height (length) was defined as the distance between the tip of the nose and the beginning of the tail. Weight and length parameters were calculated with the BMI formula, and animals with BMI greater than 5 kg/m^2 were considered obese [27, 28].

Behavioral Tests

Male pups were subjected to the open field maze test, elevated plus maze test, and social interaction test to measure stress, anxiety, and social behavior. The rats were transferred to the test room 1 h before the start of the behavioral tests, and the test boxes were cleaned with 70% alcohol after each rat. The tests were performed between 9:00 and 15:00 on the 28th postnatal day.

Open Field Maze Test

The open field maze test is used to determine the animal's anxiety-related emotions and locomotor activity. Anxiety behavior is triggered by removing the animal from its own environment and leaving it alone in an unfamiliar one. The time the animal is left in the open during the test is usually 5 min. During this time, the animal's movements in the horizontal (transition from one frame to another) and vertical (rearing on its hind limbs) planes, grooming behaviors, and number of defecations are determined. Locomotor activity is directly proportional to the number of line crossings and environmental exploration behavior and the number of rearings on the hind extremities. Grooming and numbers of defecations are regarded as indicators of autonomous functions. The apparatus used for the open field test consists of 49 equal squares 100 cm × 100 cm × 30 cm in size.

The Elevated Plus Maze Test

The elevated plus maze test was used to determine behavioral changes related to anxiety (Kumar et al., 2015). The maze consists of two closed and two open arms. The labyrinth is kept 50 cm above the ground. Wooden ledges (0.5 × 0.5 cm) were added along the edges of the open arms to prevent the rats falling out of the apparatus. At the start of the test, the rats were placed in the center of the maze facing an open

arm. They were then permitted to explore their new environment for 5 min. The number of times they entered the open arms and the total time spent in these arms were adopted as the "anxiety index." A low index indicates high anxiety, and the total number of arms (open + closed) is used as a measure of locomotion. Behavioral tests were performed with appropriate apparatus specially created for each test.

Social Interaction Test

Social interaction behavior in rats was evaluated with a three-compartment social behavior apparatus (70 cm wide and 30 cm long and divided into three equal chambers) [29].

For the social interaction test, in phase 1 (the habituation phase), a rat was placed in the central chamber with the doors open. They were then permitted to explore the other two side chambers for 5 min. In the social preference stage, an age- and sex-matched stranger rat (familiarized with the wire cage in the apparatus 24 h before testing) was placed beneath a small wire cage with a radius of 5.5 cm in the left or right chamber. Another side chamber wireframe was left empty during the socializing phase. The room where the stranger animal is located is known as the "stranger chamber" and the side with the empty wireframe as the "empty chamber." In stage 2, the social innovation stage, a new, stranger rat was placed in the wire cup that had been left empty in the previous session. The time spent in all three chambers by the test animal was observed over 10 min. Sociability is expressed in terms of the "sociability index," defined as the proportion of time spent on the foreign side to that spent on the empty side. The "social preference index" was calculated as the proportion of time spent on the novel side to that spent on the familiar side [30].

Routine Histological Procedures

After the completion of the behavioral tests, the experimental animals were sacrificed by cervical dislocation under ketamine (50 mg/kg)/xylazine (10 mg/kg) anesthesia. The brain tissues were removed for stereological, histopathological, and immunohistochemical examination.

Tissue Processing

Brain tissues were dissected and fixed in 10% paraformaldehyde for 1 week. Tissues were washed in running water for 1 day and then dehydrated by being passed through gradually increasing alcohol series. After clearing in xylene, the tissues were embedded in paraffin blocks. Sections of the prepared tissue blocks were taken for stereological analysis (5 µm) and for immunohistochemical analysis (4 µm) in accordance with the systematic random sampling rule (1/50) for volumetric analysis in the hippocampus. Sampling rates were determined by means of a pilot study. Sections were stained with cresyl violet for stereological analysis [31].

Stereological Analysis

CA and DG volumes in the hippocampus were estimated using the Cavalieri volume estimation method, one of the unbiased stereological counting methods. Reference volume estimation using this method relies on multiplying the total section area (S_{ai}) of the region of interest in the sections by the section thickness (t). A point counting grid is employed to determine the section area of the parameter of interest in the section. The point counting grid consists of equidistant systematic cross-shaped points. The center of each cross is considered to represent a point on the grid, while each point represents a unit area between the four points ((a)). If the point counting grid is placed on a section image, its points will be superimposed on the searching area; the total number of points superimposed on the areas in the section images will be shown as (ΣPi). When the total number of points (ΣPi) superimpose with the section is multiplied by ((a)), the total area in that sections is estimated using the formula [32, 33]:

$$V_{ref} = \Sigma S_{ai} \bullet t$$

$$S_{ai} = P_{(a)}$$

where V_{ref} is the total volume of the structure of interest, ΣP_i is the total number of points intersecting with the cross-section projections, t is the average section thickness, and $P_{(a)}$ is the area represented by a point. Field images were taken of tissue sections with a 4 × objective lens (Zeiss Axiolab 5, Jena, Germany) with the sampling interval (1/50) determined in the pilot study. The area represented by a point on the dotted area measurement ruler was determined by means of a pilot study and then ImageJ software (Java image processing program, NIH, USA). The volume was calculated on ImageJ.

Immunohistochemical Analysis

Serial Sections 4 µm in thickness taken from the paraffin blocks prepared from brain tissues were placed on polylysine slides and stained according to the Mouse and Rabbit Specific HRP/DAB IHC Detection Kit-Micro polymer (ab236466) kit procedure. Following dehydration, the antigen retrieval procedure was initiated with Proteinase K for 15 min. In order to block endogenous peroxidase activity, the slides were incubated in a 3% hydrogen peroxide (Cat no: 108597, Merck, Darmstadt, Germany) solution for 20 min in the dark. Following incubation, the sections were washed once again with PBS solution for 15 min, and then

the protein block solution was dropped onto the hippocampal sections, left at room temperature for 10 min, and washed with PBS. Anti-*NPY1R* (Cat no: bs-1070R, Bioss, USA, 1:200) was used as the primary antibody, and sections were incubated for 1.30 h. Following primary antibody incubation, the sections were washed with PBS for 15 min, and the mouse detection reagent solution was dropped onto the sections for 10 min to show the reaction. The sections were incubated and washed with goat anti-rabbit HRP-conjugate for 15 min. The DAB chromogen was used to display the antigen–antibody complex. The sections were counterstained with Mayer's hematoxylin, covered with occlusive medium, and examined under a microscope. After protein blocking in the negative control, PBS was used instead of the primary antibody, and the tissues were incubated for 1 h at room temperature. The sections were examined under a light microscope and photographed (Zeiss Axiolab 5, Jena, Germany). The intensity of immunohistochemical staining was scored as mild (+, 1), moderate (++, 2), or severe (+++, 3) [34].

Statistical Analysis

Analysis of numerical data was performed on SPSS software (SPSS version 21.0; SPSS Inc., Chicago, IL, USA). The data were expressed as mean ± standard deviation. The Shapiro–Wilk test was applied to assess normality of distribution. Normally distributed data were compared between groups using one-way ANOVA and the Tukey test. The Kruskal–Wallis and Tamhane tests were applied in the comparison of multiple groups not exhibiting normal distribution. $p < 0.05$ and $p < 0.01$, as appropriate, were regarded as statistically significant.

Results

Open Field Test Results

Number of Inner Squares Entered

In the open field test, the control and NAC group rats entered a similar number of inner squares during the observation period. The number of times the Ob group rats entered the inner squares was lower than in the control and NAC group ($p < 0.01$). However, NAC-treated obese (ObNAC) animals entered more inner squares than the Ob group rats ($p < 0.01$). No significant differences were observed between the control, NAC, and ObNAC groups ($p > 0.05$) (Fig. 1A).

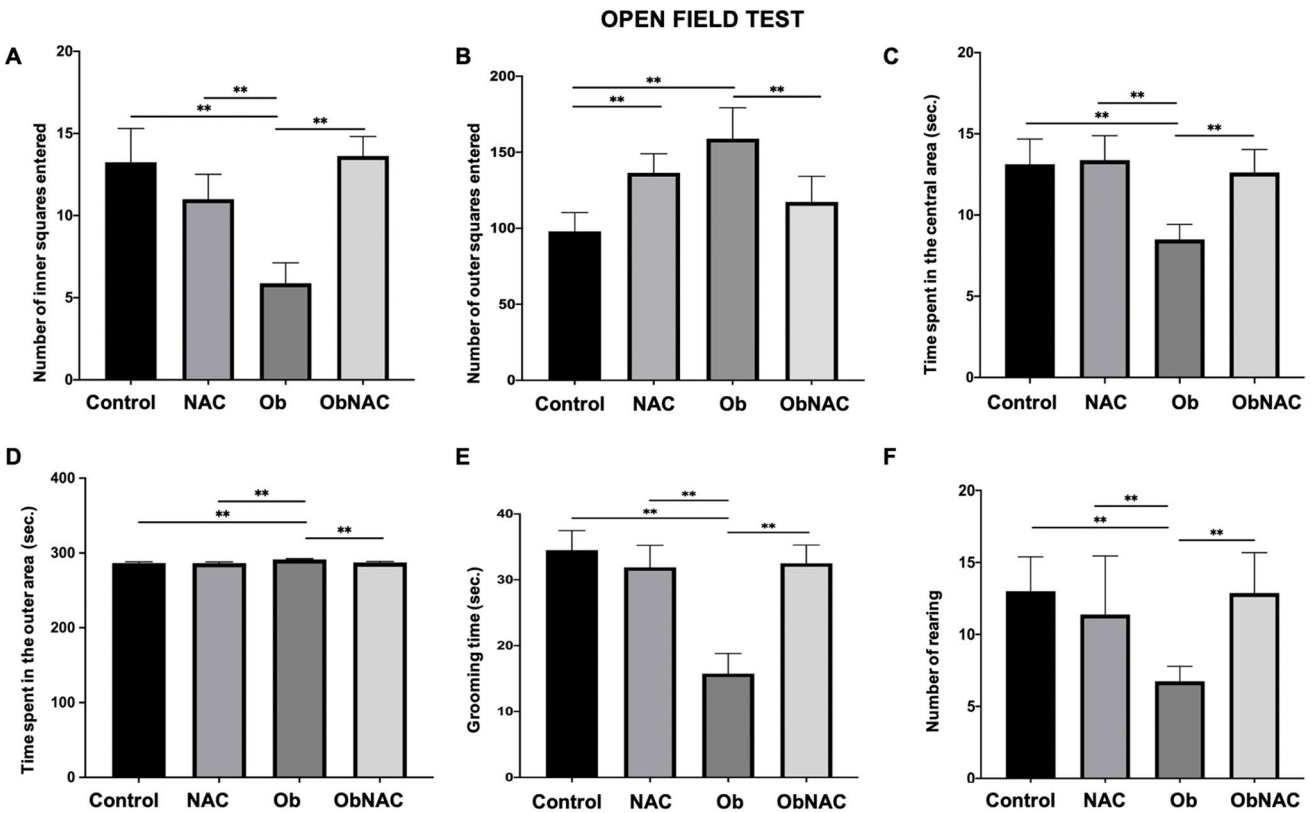

Fig. 1 Open field test results from male offspring. Data expressed as mean ± SD were determined for each animal over a 5-min session

Number of Outer Squares Entered

Statistical analysis showed that Ob and NAC groups rats discovered significantly more outer squares than the control group animals ($p < 0.01$). In addition, NAC treatment in obesity reduced locomotion on the outer squares in the ObNAC group ($p < 0.01$) (Fig. 1B).

Time Spent in the Central Area

Rats exposed to maternal obesity spent less time in the central area than the control and NAC groups ($p < 0.01$). The time spent in the center increased in the ObNAC group compared to the Ob group ($p < 0.01$). No significant differences were observed between the control, NAC, and ObNAC groups ($p > 0.05$) (Fig. 1C).

Time Spent in the Outer Area

The time spent in the outer area was greater in the Ob group than in the control and NAC groups ($p < 0.01$). There was no difference between the ObNAC, control, and NAC groups ($p > 0.05$) (Fig. 1D).

Grooming Time

The duration of grooming was significantly affected by maternal obesity. Statistical analysis showed that the Ob group rats groomed less than the control and NAC groups in the open field ($p < 0.01$). NAC treatment prevented an obesity-induced decrease in grooming in the ObNAC group ($p < 0.01$) (Fig. 1E).

Number of Rearings

The number of rearings was significantly lower in the Ob group compared with the control and NAC groups ($p < 0.01$). In the ObNAC group, NAC treatment prevented an obesity-induced decrease in the number of rearings ($p < 0.01$) (Fig. 1F).

Number of Fecal Boli

No difference was determine between any of the groups in terms of fecal boli numbers ($p > 0.05$) (not shown in figures).

The Elevated Plus Maze Test

Anxiety Index

Control animals spent more time in the open arm and open arm entries in the elevated plus maze compared to the closed arm, indicating normal behavior. Statistical analyses revealed a significant decrease in the amount of time spent in open arms and open arm entries in the Ob group compared to the control group ($p < 0.01$), a phenomenon indicative of the induction of anxiety in these animals. Furthermore, NAC treatment in obese rats (the ObNAC group) exhibited a protective effect against obesity-induced anxiety ($p < 0.01$) (Fig. 2A–C).

Social Interaction Test

Social Preference and Social Preference Index

During the sociability test on the three-chamber apparatus, the Ob group spent less time spent in the stranger chamber but more time in the empty chamber than the other groups (Fig. 3A). This indicates that the Ob group animals had a lower sociability index than the other groups. While the ObNAC group improved the obesity-induced decrease in the time spent in the empty room, the time spent in the stranger room was close to that of the control group. This suggests that the ObNAC group animals had a higher sociability index than the Ob group ($p < 0.01$) (Fig. 3B).

In the social preference index, Ob group animals spent a greater period of time in the familiar compartment than the other groups but a shorter time in the new compartment ($p < 0.01$) (Fig. 3C). This indicates a lower social preference in animals exposed to obesity in prenatal life (Fig. 3D).

The ObNAC group spent significantly less time in the familiar chamber, while a correction was observed of the obesity-induced decrease in time spent in the new chamber ($p < 0.01$) (Fig. 3C). Ob group animals also exhibited a decreased social preference index compared with the control animals ($p < 0.01$) (Fig. 3D).

NAC administration in maternal obesity thus corrected social preference as well as impairment in socialization in offspring.

Stereological Results

Hippocampus Volume

Total hippocampal and hippocampal subregion volumes were analyzed using Cavalieri's method.

Statistical analyses revealed significant differences in total hippocampus volumes between the control and Ob groups ($p < 0.01$). The total volume of the hippocampus was significantly higher in the NAC and ObNAC groups than in the Ob group ($p < 0.01$). These results indicate that maternal obesity significantly reduced the hippocampal volume, while NAC treatment in obesity significantly reversed that decrease (Table 1).

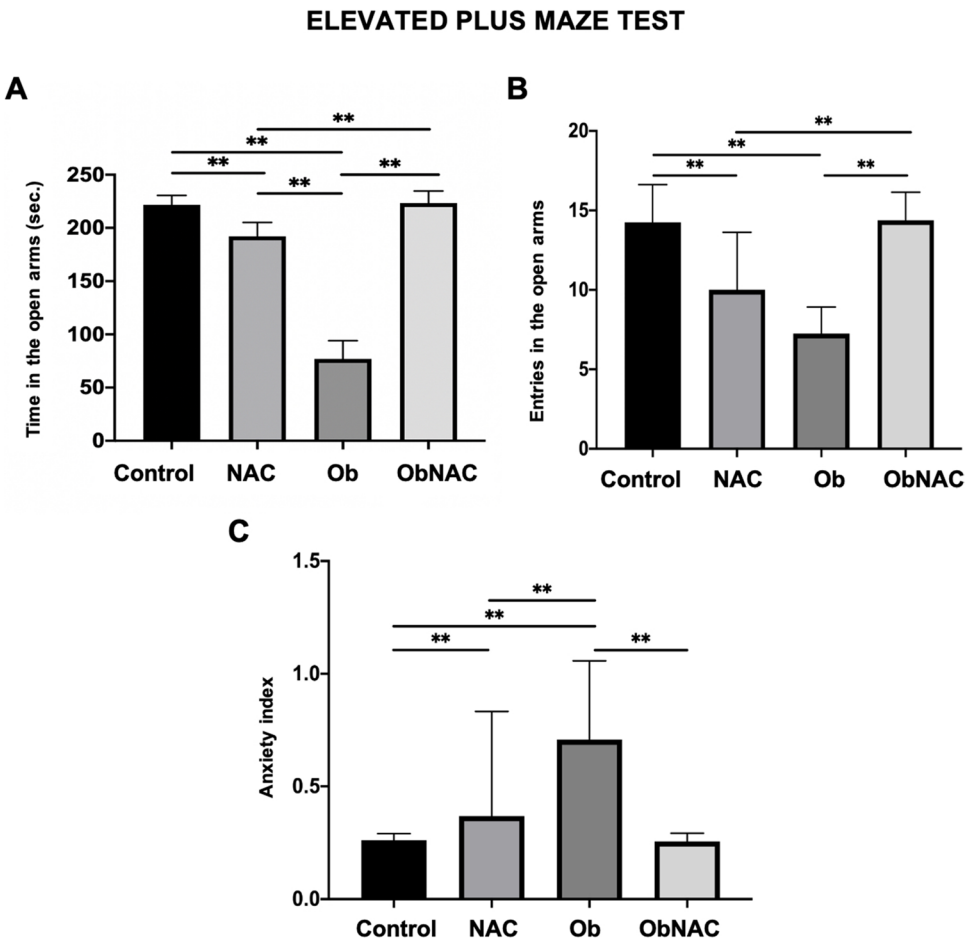

Fig. 2 Elevated plus maze test results from male offspring. Data expressed as mean ± SD were determined for each animal over a 5-min session

Hippocampal Subregions

The mean volume of the CA1 region was significantly lower in the Ob group than in the control group ($p < 0.01$). Treatment with NAC in the Ob group significantly attenuated the obesity-induced volume reduction in the CA1 region. However, no significant differences were observed between the groups in the CA2 or CA3 regions.

Moreover, a significantly lower volume was determined in the DG of the Ob group compared to the control group ($p < 0.001$). The DG volume was significantly higher in the ObNAC group than in the Ob group ($p < 0.01$). No differences were observed in the ObNAC group and the NAC or control groups ($p > 0.05$) (Table 1).

Histopathological Results

Light microscopic images of the CA1 region of the hippocampus revealed a large number of dark-stained pyramidal neurons with indistinct cell borders in sections from the Ob group compared to the other groups. These were interpreted as apoptotic neurons that had lost their function. An irregular neuron arrangement and the intracellular spaces between the cells were particularly noteworthy in the CA1 region (Fig. 4A). The nuclear borders of the pyramidal neurons were prominent in the CA1 region in the control, NAC, and ObNAC groups, with one or more prominent nucleoli in the euchromatic nucleus and an intact cellular layer. In the control group, the neurons in the CA2 region were tightly packed, the intercellular space was normal, and the cells exhibited prominent nuclei and nucleoli. However, the presence of dark-stained neurons in the CA2 region was noteworthy in the Ob group. The cell borders were indistinct, and there were spaces between the cells. The cell structures were normal in the NAC group. Notably, the structures of the neurons in the CA2 region were preserved in the ObNAC group, there were spaces between the cells, and some cells are dark-stained (Fig. 4A). The presence of a large number of dark-stained cells in the CA3 region of the Ob group was particularly notable. However, other cells appeared normal. Similarly, cells in the control, NAC, and ObNAC groups exhibited normal structures. Examination of the DG area of the hippocampus revealed that the granular neurons in the Ob group were dark-stained, with wide spaces between the cells. Dark-stained cells

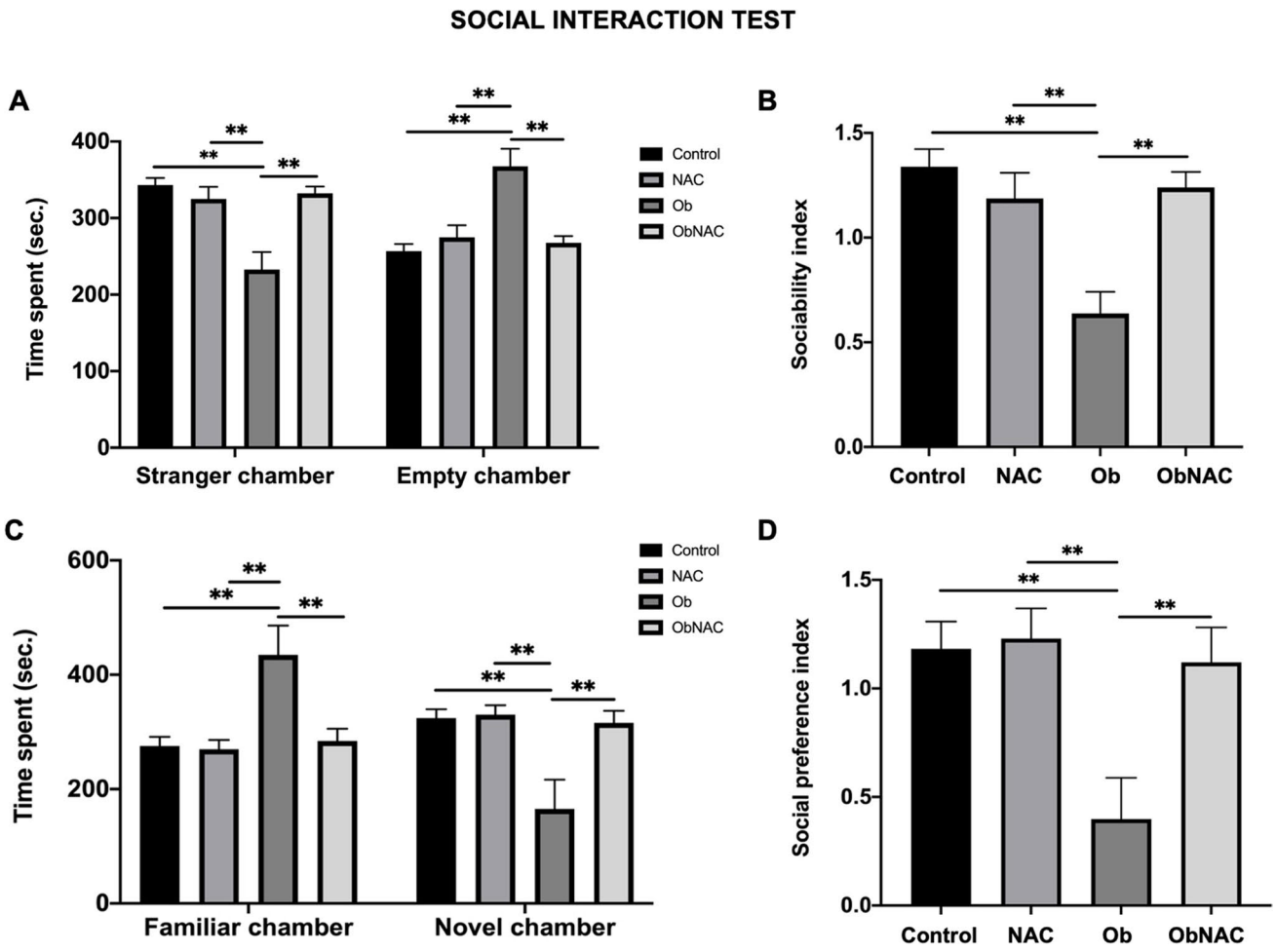

Fig. 3 Social interaction test results from male offspring. Data expressed as mean ± SD were determined for each animal over a 10-min session

Table 1 Statistical expression of the total volumes of the cornu ammonis (CA1, CA2 and CA3), dentate gyrus (DG), and hippocampus between the control and study groups

Volume (mean ± SD) mm^3					
Groups	CA1	CA2	CA3	DG	Total
Control	12.69 ± 2.00^{c}	8.01 ± 2.51	12.98 ± 1.14	27.63 ± 3.60^{c}	61.33 ± 5.93^{c}
NAC	12.44 ± 2.06^{c}	5.48 ± 2.51	11.06 ± 1.50	26.57 ± 3.80^{c}	55.56 ± 6.45^{c}
Ob	$8.86 \pm 1.20^{a,b,d}$	5.48 ± 1.74	11.72 ± 1.697	$18.98 \pm 1.74^{a,b,d}$	$45.05 \pm 3.85^{a,b,d}$
ObNAC	12.61 ± 2.09^{c}	7.17 ± 2.81	12.88 ± 1.130	27.42 ± 3.34^{c}	60.08 ± 5.00^{c}

[a]Different vs. control ($p < 0.01$). [b]Different vs. NAC ($p < 0.01$). [c]Different vs. Ob ($p < 0.01$). [d]Different vs. ObNAC ($p < 0.01$).

are interpreted as cells that have lost their function. The granular cells in the control, NAC, and ObNAC groups had spherical nuclei, and their cell borders were clearly defined. In addition, the granular cells in these groups were tightly packed, and the intercellular spaces were normal (Fig. 4B). These pathological changes were also confirmed by stereological volume analysis (Fig. 4C–F).

Immunohistochemical Results

Pyramidal neurons in the CA1 region from the control, NAC, and ObNAC groups exhibited strong anti-*NPY1R* (+) staining, while neurons in the Ob group were weakly stained. Nuclei shrinkage and dark staining were observed in most of the cells in the Ob group that did not exhibit a sufficient

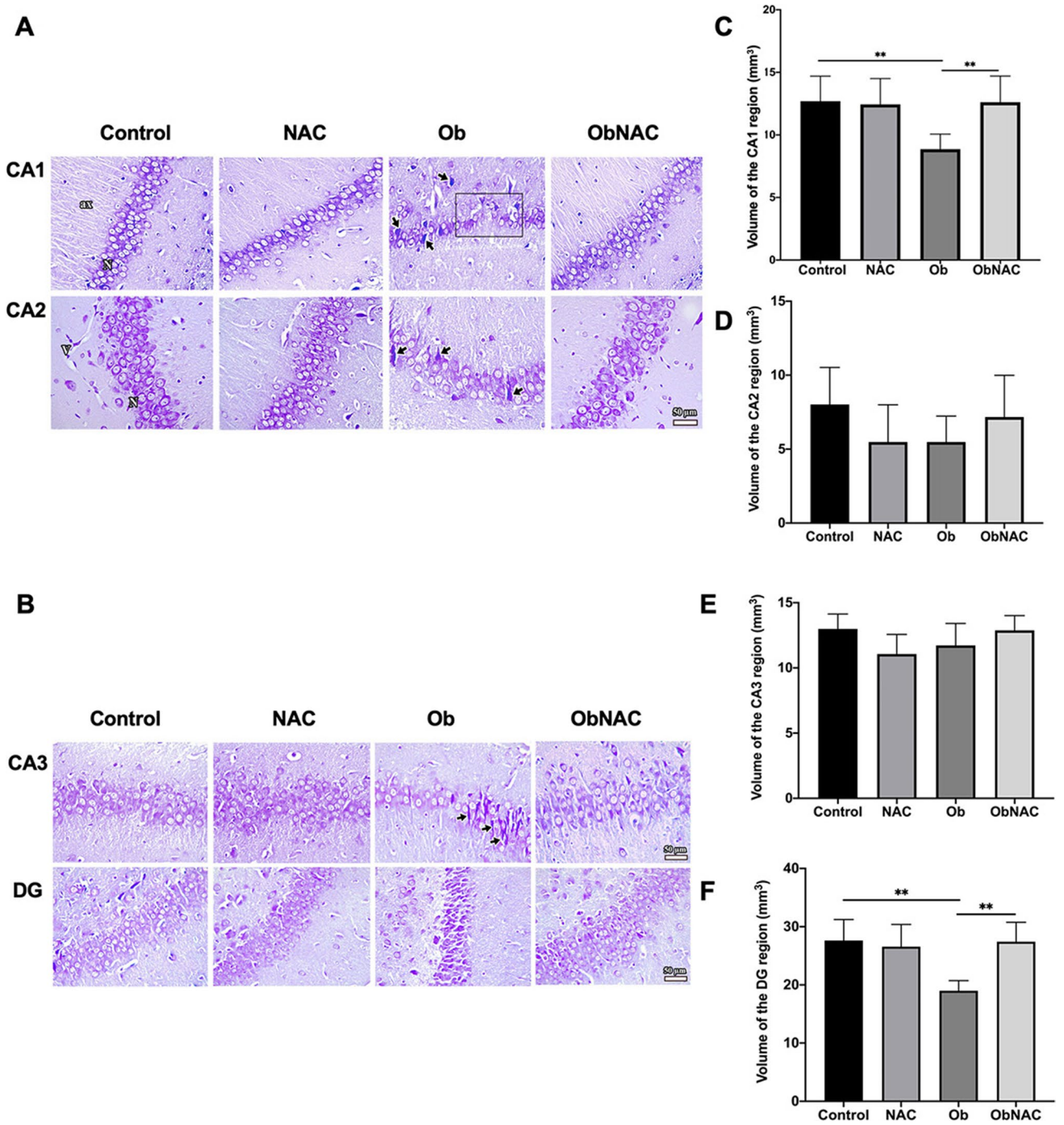

Fig. 4 **A**, **B** Cresyl violet staining in the cornu ammonis (CA) and DG regions of the hippocampus. Control, NAC, and ObNAC groups: normal histological structure of pyramidal and granular neurons. Ob group: degenerative changes in pyramidal cells (black arrows), spaces between granular cells (rectangle). Bar, 50 µm. **C**–**F** The volumes of the CA1, CA2, and CA3 region and DG in the control, NAC, Ob, and ObNAC groups (mean ± SD). The asterisk indicates $p < 0.01$; ax, axon; V, vessel; N, neuron

positive reaction. The pyramidal neurons in the CA2 region exhibited strong anti-NPY1R (+) staining in general and did not differ between any groups (Fig. 5A). Similarly, strong anti-NPY1R (+) staining of pyramidal neurons was observed in the CA3 region of hippocampus sections in all, while a few cells were darkly stained. There was no difference between the groups in terms of anti-NPY1R (+) staining in the CA3 region. Anti-NPY1R (+) granular neurons were widely located in the DG region in the control, NAC, and ObNAC groups. anti-NPY1R (+) staining in the

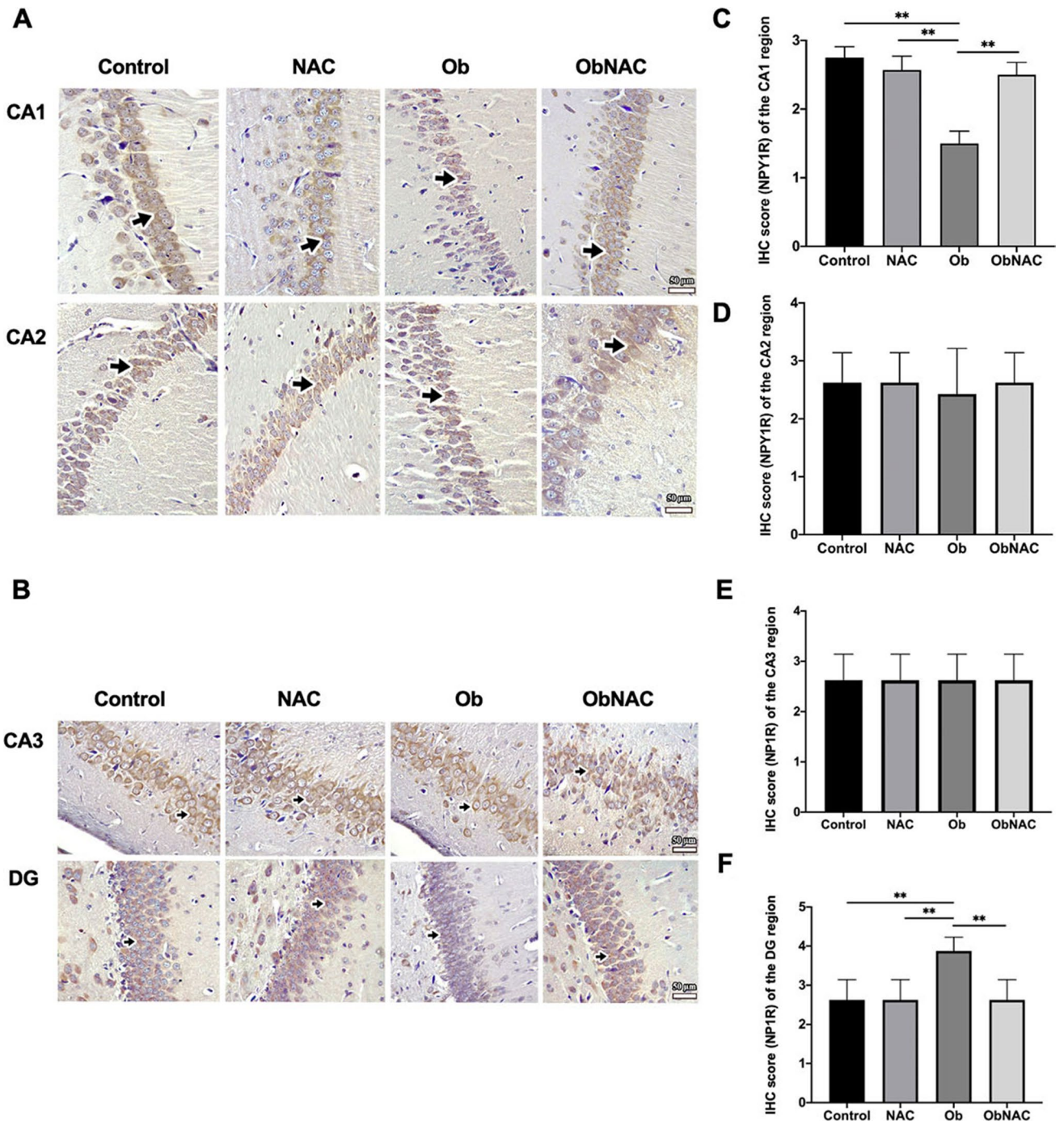

Fig. 5 **A**, **B** Immunohistochemical staining of the cornu ammonis (CA) and DG regions of the hippocampus. Control, NAC, and ObNAC groups: pyramidal neurons exhibited strong anti-NPY1R (+) staining (Arrow). Ob group: weak anti-NPY1R (+) staining in pyramidal neurons (Arrow). Bar, 50 μm. **C**–**F** IHC scores of anti-NPY1R (+) cells of the CA and DG regions of the hippocampus in all groups

DG region was especially intense in the subgranular region. Neurons in the DG region of the Ob group exhibited low-intensity anti-NPY1R (+) staining (Fig. 5B).

Statistical analysis of semiquantitative scoring revealed that Ob group animals exhibited significantly lower anti-NPY1R (+) cells in the CA1 region of the hippocampus compared to the control group ($p < 0.01$). No difference was found in anti-NPY1R (+) cells in the CA1 region in the ObNAC group compared to the control group ($p > 0.05$), indicating that NAC treatment protected NPY1R expression

levels in the ObNAC group (Fig. 5C). No significant differences were observed between any groups in terms of anti-NPY1R (+) cells in the CA2 and CA3 subregions ($p > 0.05$) (Fig. 5D, E). The IHC score of anti-NPY1R (+) cells in the DG region was significantly lower in the Ob group compared to the control group ($p < 0.01$). However, stronger anti-NPY1R (+) granular cells were commonly found in the ObNAC group DG region, and their IHC score was no different to that of the control group ($p > 0.05$) (Fig. 5F).

Discussion

This study examined the effects of high-fat diet exposure in the prenatal and lactational periods on behavioral changes, hippocampal NPY1R expression, and hippocampal volumes in male rat offspring. The principal findings were that male offspring born to mothers fed a high-fat diet exhibited more anxiety-like behavior, fewer hippocampal NPY1R-positive cells in the CA1 and DG regions, and lower total hippocampal volumes than the controls.

Previous studies have observed an association between maternal obesity and offspring neurobehavior [24, 35, 36]. Maternal obesity or excessive caloric nutrition in the early developmental stages (pre-gestational, gestational, and lactational) has been shown to cause a nutrition programming effect in offspring behavior [37]. Another study showed that prenatal exposure to maternal obesity led to an alteration in anxiety and stress coping behaviors in elderly mice [38]. Even minor changes in maternal nutritional status can cause significant changes in the fetal environment. The mechanistic basis for this is unclear, suggesting the possibility of a link between these environmental changes and altered expression of essential genes responsible involved in the tissue remodeling response and the risk of subsequent disease. Nutritional factors can also trigger these processes by compromising placental functions, such as the control of maternal–fetal endocrine changes or epigenetic regulation of gene expression [39]. A recent study demonstrated gender-specific effects of maternal obesity on the metabolic health of offspring, with male offspring seeming to be more powerfully affected by maternal obesity than females [40].

The results of the behavioral test in this study suggest that a high-fat diet during the prenatal and lactational periods reduced locomotor activity and sociability index values while increasing the anxiety index values in male offspring. We investigated the effect of a maternal high-fat diet on sociability and social memory using the three-chamber assay. Ob group rats spent less time in the stranger and novel chambers, followed by an increased amount time in the empty and familiar chambers, compared to the control animals, suggesting a lower social preference in the members of the Ob group. Similar behavioral abnormalities have been observed in previous studies in mice fed high-fat diets, female offspring from dams given a high-fat diet exhibiting reduced sociability compared to controls [41]. That study suggested that increased maternal IL-6 may play a role in reducing sociability in offspring born to high-fat diet mothers. Another study reported that a high-fat diet group exhibited decreased locomotor activity and social recognition, but not sociability, in the three-chamber sociability and social novelty test [42]. These partly inconsistent findings may be attributable to differences in the protocol designs adopted for the behavioral testing.

In the open field test, the Ob group entered fewer inner squares and spent less time spent in the central area, with a shorter grooming time. This may be a sign of increased anxiety. Consistent with these results, Kang et al. reported that a maternal high-fat diet during pregnancy resulted in increased anxiety among female offspring in the open field test [41].

In the elevated plus maze test, the Ob group spent less time in the open arms and exhibited a lower number of entries into the open arms. An increase was also detected in the anxiety index of the Ob group. Likewise, a previous study suggesting that maternal high-fat diet consumption during crucial stages of fetal development may increase the risk of abnormal behavior related to anxiety in adulthood reported a decreased tendency to time spent in the elevated plus maze test open arms and increased anxiety-like behaviors in the high-fat diet group [36].

Although studies have employed different behavioral measures, preclinical studies have frequently endeavored to link effective changes to dysfunctional hypothalamic–pituitary–adrenal (HPA) axis activity, changes in neuronal plasticity, and increased neuroinflammation [43]. Two studies showed that maternal obesity compromises the activity of the HPA axis feedback system in the hypothalamus, a phenomenon characterized by a heightened corticotropin-releasing factor transcription levels and ACTH responses [44] and alterations in transcription levels of the glucocorticoid receptor in rat offspring [38]. Other rat studies have focused on the hippocampus and amygdala, key areas involved in the regulation of emotional states, and have reported high protein levels and mRNA expression of proinflammatory markers, including IL6, TNFα, NFkB, and MCP-1, in the offspring of obese mothers [45–47].

Limbic neuropeptide Y1R, implicated in both emotional behavior and regulation of energy homeostasis [48], may be suggested as another mechanism in the light of the present study. Limbic NPY1R expression is regulated by the early maternal environment and is furthermore implicated in the regulation of anxiety, stress reactions, circadian rhythm, and cognition, this receptor being widely distributed in the central nervous system [48].

Studies involving emotionality, stress, and anxiety have confirmed the role of NPY1R, which is closely linked to

appetite stimulation [49]. NPY1R deficiency has been reported to result in increased anxiety and related behaviors [50]. The anxiolytic effect of NPY is predominantly mediated by its receptor NPY1 [50].

Studies have shown that NPY is capable of modulating noradrenergic and serotonergic systems and of impacting on cortical and limbic functions through Y1 receptors [51]. Studies involving genetic animal models of depression have reported alteration of NPY-like immunoreactivity and NPY Y1-type receptor binding sites in different cortical regions and in the hippocampal DG [52, 53]. The hippocampus is one of the regions of the brain where NPY1 receptors are concentrated in the central nervous system [54]. Specifically, NPY has been reported to exhibit anxiolytic effects in the dentate gyrus and the CA1 region of the hippocampus, and researchers have suggested that the Y1 receptor participates in the effect of NPY on memory [55, 56]. In the present study, we observed decreased NPY1R-positive cells in the CA1 and DG regions of the hippocampus in addition to increased anxiety and decreased sociability in the Ob group. Decreased NPY1R together with behavioral impairments and volumetric changes highlight the heightened effect of NPY on hippocampal learning–memory and neurogenesis-related receptors, emphasizing the role of NPY as an important regulator in obesity.

In the light of the link between hippocampal NPY and memory processing, researchers have speculated that memory problems in depressed humans may be associated with low hippocampal levels of NPY and Y1 mRNA [56]. Flinders sensitive line rats, fawn-hooded rats, and bulbectomized rats, regarded as depression models, have all been observed to exhibit low NPY levels in the hippocampus together with extreme immobility in the Porsolt swim test [57, 58]. Studies of rats with overexpression of hippocampal NPY have demonstrated impairment of spatial learning associated with reduced NPY-Y1 binding in transgenic rats [59].

The association between obesity, diet, and anxiety is a complex one. NPY1R plays a significant role in the regulation of the HPA axis [60]. However, previous studies have strongly suggested the involvement of HPA axis activity in obesity [61, 62]. Bertocchi et al. (2011) demonstrated that conditional inactivation of the NPY1R gene in forebrain excitatory neurons of adolescent mice resulted in greater peripheral corticosterone and corticotropin-releasing hormone (CRH) immunoreactive cell bodies in the hypothalamic paraventricular nucleus than in control offspring. This suggests that inactivation of limbic NPY1 receptors induces activation of the HPA axis [48]. The HPA axis produces a stress response manifesting with the release of glucocorticoids (either cortisol in humans or corticosterone in rodents) that indirectly affects hippocampal sensitivity with the onset of the stressor. Moreover, researchers have hypothesized that high glucocorticoid levels may be associated with damage and volume reduction in the hippocampus [63–65].

The stereological results of the present study suggest that the decrease in the volume of the hippocampal CA1 and dentate gyrus regions may be due to an increase in glucocorticoid levels. The decrease in limbic NPY1 receptors in these hippocampal regions also supports this hypothesis. Further studies investigating the relationships between glucocorticoid levels and NPY1 receptors are now needed.

Ventral CA1 contains enriched levels of anxiety cells activated in anxiogenic settings and essential for avoidance behavior in mice. Animal studies have demonstrated that obesity and diabetes inhibit cell proliferation [66–69], neuronal differentiation [67, 70], and survival [71] of newborn cells in the hippocampal DG. In the present study, the granular cell volume in the Ob group decreased in the dentate gyrus, and NPY1-positive cells in this region also decreased. Although there are studies proving that a high-fat diet compromises hippocampal neurogenesis in rats, the role of NPY1 receptors in hippocampal subregions as an underlying mechanism has not been previously investigated. The present study is thus the first to suggest that disrupted NPY1 receptors may represent the underlying mechanism involved in the decreased hippocampal volume in case of exposure to maternal obesity.

However, we found no differences between the CA2 and CA3 regions. This may be due to the CA2-3 regions being relatively small, without clear and precise boundaries, to their being investigated separately by some researchers but together in the present study, or to the staining intensity in these regions being higher than that in the CA1 region [72]. Based on these data, considering that NPY modulates both anxiety and depression through the same receptor, type-Y1, it seems plausible to speculate that changes in total hippocampal NPY1R content may impact on a number of behavioral patterns. NPY1R causing reduced locomotor activity may account for the reduced dentate neurogenesis [73].

Furthermore, we hypothesized that NAC may constitute a potential protective agent against obesity-related alterations. NAC is emerging as a useful agent in the treatment of the most common psychiatric disorders, such as depression and anxiety. In addition to being an antioxidant and a precursor to glutathione, it exhibits benefits such as modulating glutamatergic, neurotropic, and inflammatory pathways [74]. NAC has been found to reduce anxiety behaviors measured using open field, light/dark, hole-board, social interaction, and stress-induced hyperthermia models in mice and the light/dark test in zebrafish [75, 76]. We therefore tested the effect of NAC treatment on behavioral changes induce by a maternal high-fat diet. NAC administration to obese mothers (the ObNAC group) attenuated behavioral parameters. Compared with the Ob group, the ObNAC group rats exhibited

a higher sociality index in the sociability test, a lower anxiety index in the elevated plus maze test, more spent time in the inner area and central area, and exhibited a longer grooming time in the open field test. The effects of NAC treatment on behavioral changes have also been reported in previous studies. Vukovic et al. reported that the anxiogenic effect induced by cisplatin was significantly attenuated in the NAC-treated group in open field and elevated plus maze. Those researchers evaluated hippocampal slices in their investigation of the underlying mechanism involved in the protective effect of NAC and showed that NAC supplementation was beneficial by exhibiting antioxidant and antiapoptotic effects [77]. Another study of male BALB/c mice reported that NAC exhibited antidepressant and anxiolytic effects, reversing anxiety and depressive-like behaviors triggered by noise exposure. Those researchers attributed the mechanism underlying the anxiolytic effect of NAC to its antioxidant activities in the prefrontal cortex and hippocampus [74]. Similarly, another recent study showed that NAC suppresses doxorubicin and cyclophosphamide-induced anxiety-like behavior and cognitive impairment by lowering levels of oxidative stress in the hippocampus [78]. Our stereological results also supported the idea that NAC treatment exhibited a protective role in terms of hippocampal volume in the ObNAC group. The role of NAC in protecting the hippocampus volume can also be attributed to its antioxidant properties, although further studies are now needed to determine the specific mechanisms involved. IHC analysis showed that NAC protected NPY1R expression in the hippocampal CA1 and DG subregions and total hippocampus in the ObNAC group. Y1 receptor activation has been found to increase cell survival by modulating intracellular calcium levels as well as immediate activity in retinal ganglion cells [79]. The proliferative effects of NPY on neuronal precursor cells derived from the postnatal rat olfactory epithelium have been demonstrated via NPY1R [80].

Researchers have also shown that NPY is capable of stimulating the proliferation of neuronal precursor cells in the hippocampal subgranular region [81], retinal glial (Muller) cells [82], endothelial cells [83], and neuronal precursor cells in the subventricular region [84].

Previous studies have reported that NAC treatment inhibits fat accumulation in obesity and reduces the expression of proteins associated with obesity, such as monoamine oxidase A, heat shock protein 70, aminoacylase-1, and transketolase [85]. NAC can also prevent brain oxidative stress and neuroinflammation through stimulation of glutathione synthesis (via γ-glutamylcysteine ligase) [86]. One previous study reported that memory impairment and metabolic changes induced by high-fat diet in the hippocampus of female mice were completely ameliorated by NAC treatment and that NAC caused glutathione accumulation in the hippocampus, thus providing an enhanced antioxidant defense [87].

Conclusion

The findings of the present study indicate that limbic NPY1R is affected by maternal obese conditions. Moreover, NAC-mediated protection of NPY1R preserved cell survival in the hippocampus and improved behavioral parameters. Further studies are now needed to identify the underlying mechanisms.

The limitations of the study are as follows:

- Data for the components of energy metabolism were not collected, and plasma NPY levels were not measured.
- Only males were used in this study, females being employed in a different, but parallel study.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12035-022-03158-x.

Author Contribution All authors contributed to designing, writing, and editing the manuscript. Kıymet Kübra Tüfekci and Elfide Gizem Bakırhan performed the experiments and behavior tests analyzed as well as interpreted the data and contributed to writing the paper. Funda Terzi wrote and revised the paper. Kıymet Kübra Tüfekci contributed to experimental design, supervised the data writing, and revised the whole manuscript.

Funding This study was supported by a grant from the Scientific Research Foundation of Kastamonu University (Kıymet Kübra Tüfekci; KUBAP01/2021–03).

Data Availability Data are available on request from the authors. The data that support the findings of this study are available from the corresponding author, upon request.

Declarations

Ethics Approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Adıyaman University (Dated 25.02.2021/No. 2021/001).

Consent to Participate Not applicable.

Consent for Publication All authors read and approved the final manuscript.

Competing Interest The authors declare no competing interests.

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