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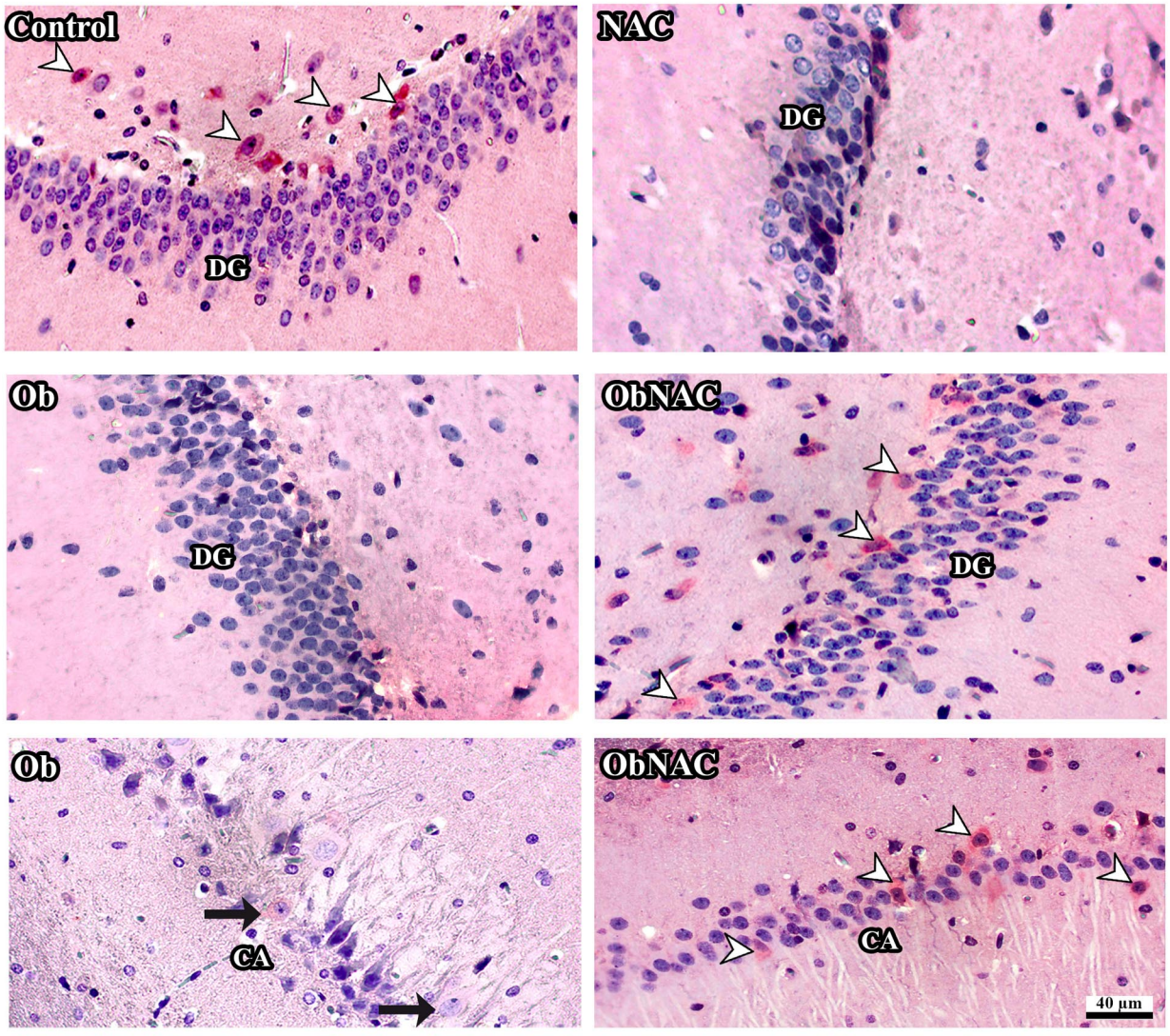

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AIM AND SCOPES
Journal of Cellular Neuroscience and Oxidative Stress is an online journal that publishes original research articles, reviews and short reviews on the molecular basis of biophysical, physiological and pharmacological processes that regulate cellular function, and the control or alteration of these processes by the action of receptors, neurotransmitters, second messengers, cation, anions, drugs or disease.

Areas of particular interest are four topics. They are;

A- Ion Channels (Na^+- K^+ Channels, Cl^- channels, Ca^{2+} channels, ADP-Ribose and metabolism of NAD^+, Patch-Clamp applications)

B- Oxidative Stress (Antioxidant vitamins, antioxidant enzymes, metabolism of nitric oxide, oxidative stress, biophysics, biochemistry and physiology of free oxygen radicals)

C- Interaction Between Oxidative Stress and Ion Channels in Neuroscience
(Effects of the oxidative stress on the activation of the voltage sensitive cation channels, effect of ADP-Ribose and NAD^+ on activation of the cation channels which are sensitive to voltage, effect of the oxidative stress on activation of the TRP channels in neurodegenerative diseases such Parkinson's and Alzheimer's diseases)

D- Gene and Oxidative Stress
(Gene abnormalities. Interaction between gene and free radicals. Gene anomalies and iron. Role of radiation and cancer on gene polymorphism)

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Keywords
Ion channels, cell biochemistry, biophysics, calcium signaling, cellular function, cellular physiology, metabolism, apoptosis, lipid peroxidation, nitric oxide, ageing, antioxidants, neuropathy, traumatic brain injury, pain, spinal cord injury, Alzheimer's Disease, Parkinson's Disease.

RESEARCH ARTICLE

J Cell Neurosci Oxid Stress 2023;15(2): 1137-1146

The protective effect of N-acetylcysteine on hippocampal ferroptosis in an experimental obesity model

Kıymet Kubra TUFEKCI[1*] and Musa TATAR[2]

[1]Department of Histology and Embryology, Faculty of Medicine, Kastamonu University, Kastamonu, Turkiye
[2]Department of Histology and Embryology, Faculty of Veterinary Medicine, Kastamonu University, Kastamonu, Turkiye

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***Address for correspondence:**
Dr. Kıymet Kübra TÜFEKCİ
Department of Histology and Embryology,
Faculty of Medicine, Kastamonu University,
Kastamonu, Turkiye
Email: kkyurt@kastamonu.edu.tr; kubrayurt55@gmail.com
ORCID: 0000-0002-4722-3813

List of Abbreviations;

***CA**, cornu ammonis; **DG**, dentate gyrus; **GPX4**, Glutathione peroxidase 4; **GSH**, glutathione; **H-score,** histoscore; **NAC**, N-acetylcysteine; **Ob,** Obesity; **ObNAC,** Obesity and N-acetylcysteine; **PBS**, phosphate-buffered saline; **RCD**, regulated cell death; **ROS**, reactive oxygen species; **SLC7A11**, cystine transporter solute carrier family 7- member 11 or Xc^-system; **TBI,** traumatic brain injury; **WAT**, white adipose tissue*

Abstract

Ferroptosis is a non-apoptotic form of cell death closely associated with a metabolic pathway involving components including iron overload, imbalanced glutathione metabolism, oxidative stress, and lipid peroxidation damage. A close association exists between obesity and these imbalances. This study was intended to investigate the effect of hippocampal ferroptosis in an obesity model and the potential role of N-acetylcysteine (NAC) against this. A high-fat (60%) dietary pattern was used over 15 weeks to establish an obesity model. NAC was administered to the NAC and Obese+NAC (ObNAC) groups by oral gavage at 150 mg/kg for three weeks. Glutathione peroxidase 4 (GPX4) and the cystine transporter solute carrier family 7- member 11 (SLC7A11) expression levels were investigated immunohistochemically to detect ferroptosis in hippocampal tissues. GPX4 and SLC7A11 H-scores in the hippocampus sections from the obese group were significantly lower than those in the control, NAC, and ObNAC groups ($p \leq 0.01$). However, NAC treatment significantly attenuated hippocampal ferroptosis by maintaining both GPX4 and SLC7A11 expression levels in the ObNAC group ($p \leq 0.01$). These findings imply that ferroptosis may play an essential role in the hippocampus, an area of the brain of crucial importance to memory, learning, and emotional reactions, in obese individuals. In addition, NAC, a promoter of GSH biosynthesis, may attenuate hippocampal ferroptosis in obesity by inhibiting the downregulation of GPX4 and SLC7A11 expression signaling.

Keywords: Brain, Ferroptosis, GPX4, Hippocampus, N-acetylcysteine, SLC7A11

Introduction

Obesity is a severe metabolic disorder impacting on adipose tissue mass, distribution, or function, thus increasing morbidity and mortality. Genetic, environmental, physiological, behavioral, and societal variables can lead to the condition. Numerous factors influence energy intake and use, essential factors in obesity. Eating patterns are controlled by the central nervous system's hunger-satiety circuit (He et al., 2023).

Ferroptosis refers to a form of regulated cell death (RCD) resulting from oxidative stress in the cellular microenvironment, which can be regulated by glutathione peroxidase 4 (GPX4). The event is triggered by significant lipid peroxidation caused by an overload of iron and reactive oxygen species (ROS) (Dixon et al., 2012). Ferroptosis causes several morphological changes, including shrinkage of mitochondria, decreases in or the disappearance of cristae, and increased membrane density (Li et al., 2021). Numerous disorders, particularly nervous system diseases, are affected by ferroptosis at their onset and during their development (He et al., 2023). When investigating the pathogenesis of various diseases, it is therefore important to accurately define the mechanisms involved and to target and evaluate the ferroptotic death pathway in novel therapeutic applications and disease prevention strategies (Thilaganathan, 2017).

The antioxidant defense enzyme GPX4 is also the principal regulatory factor involved in ferroptosis. It is therefore also essential for cell survival, and GPX4 deficiency can result in cell death via ferroptosis. As an important central regulatory factor, it therefore performs a critical function in mitochondrial oxidative stress and ferroptosis (Li et al., 2022a). Iron chelators, iron intake inhibitors, and lipophilic antioxidants can also prevent ferroptosis (Chen et al., 2021; Yu et al., 2021).

Ferroptosis is strongly correlated with various disease mechanisms, including oxidative stress, inflammatory response, and autophagy. It has also been found to play an important role in the development of obesity (Wu et al., 2018; Sun et al., 2020; Zhou et al., 2020; Zhang et al., 2022). Obesity is a consequence of the excessive intake of nutrients and the subsequent accumulation of fat in white adipose tissue. A correlation has been shown between inadequate iron consumption and overweight or obesity among children and adolescents. Understanding the regulatory mechanisms involved in ferroptosis is therefore important to the prevention and treatment of obesity. Excess iron-dependent ROS production, a decrease in glutathione (GSH) levels, and GPX4 inactivation occur during ferroptosis (Geng et al., 2021).

The inactivation of the cellular antioxidant system, including suppression of the cystine/glutamate antiporter system (SLC7A11 or system Xc-) and GPX4, diminished iron homeostasis, and lipid peroxidation are currently thought to represent the primary cause of cell death in ferroptosis. Decreased antioxidant capacity and intracellular lipid ROS accumulation thus lead to oxidative cell death as a result (Li et al., 2020; Xu et al., 2021).

N-acetylcysteine (NAC) is a mucolytic agent that contains a thiol group and is the subject of increasing research due to its potential anti-inflammatory and antioxidant effects. In addition, NAC is on the list of essential medicines maintained by the World Health Organization, and its well-established safe history makes it an appealing candidate for treating a diverse range of conditions. Interestingly, a growing body of experimental data confirms the advantages of NAC therapy in managing obesity-related problems (Dludla et al., 2019). NAC exhibits both direct and indirect antioxidant properties by serving as a precursor of reduced GSH. The thiol group in NAC can activate the body's endogenous defenses against oxidative damage by replenishing the thiol group in the GSH system and restoring the intracellular GSH pool (Li et al., 2022b). NAC can prevent the development of ferroptosis by enhancing the accumulation of cysteine, providing biologically available cysteine. Reducing ROS production is crucially important in preventing the incidence of ferroptosis (Wang et al., 2021).

In the light of the foregoing discussion, this study investigated the immunohistochemical expression of the ferroptosis indicators GPX4 and SLC7A11 in the hippocampus of rats exposed to obesity. The potential protective effects of NAC in the relationship between obesity and ferroptosis in brain tissue were also examined.

Materials and Methods

Animals and Experimental Study Design

Sixteen female Wistar albino rats (weight 250±300 g, age 12 weeks-old) were randomly divided into four groups: Control, NAC, Obesity (Ob), and Obesity+NAC

(ObNAC). The control group received a standard diet (10% kcal) for 15 weeks. The NAC group received a standard diet (10% kcal) for 15 weeks and 150 mg/kg NAC (Sigma-Aldrich, Merck, Germany) via the intragastric route for three weeks (Hart et al., 2004). The obese group was fed a high-fat diet (60% kcal) (Arden Research and Experiment Company, Ankara, Turkiye) for obesity induction, while the ObNAC group received a high-fat diet (60% kcal) for three weeks along with 150 mg/kg NAC. The animals were housed under conventional conditions in a 12-hour light:dark cycle at an ambient temperature of 24±1°C. At the end of the experiment, cervical dislocation was performed under xylazine-ketamine (intraperitoneal, 10 mg/kg-50 mg/kg) anesthesia. The dissected brain tissues were fixed for 48 hours in 10% neutral buffered formaldehyde and subsequently embedded in paraffin blocks following routine tissue procedures. The experimental procedures were approved by the Kastamonu University Animal Experiments Local Ethics Committee with approval number 2023/33.

Immunohistochemical analysis

Sections 4 µm in thickness sections were placed onto poly-lysine slides and stained as described in the Mouse and Rabbit Specific HRP/AEC IHC Detection (Ab93705) kit procedure for immunohistochemical examination. Accordingly, the sections were dehydrated, incubated in 3% hydrogen peroxide (Cat No.108597, Merck) solution for 10 minutes in the dark to block endogenous peroxidase activity, and washed with phosphate-buffered saline (PBS) solution for a further 10 minutes. The sections were then placed into citrate buffer for antigen retrieval and boiled in an microwave oven for 10 minutes. Protein block solution was subsequently dripped onto the cooled sections. These were then left at room temperature for 10 minutes and finally washed with PBS. The sections were incubated overnight at +4°C with primary antibody (**Table 1**). Following primary antibody incubation, sections were again washed with PBS, and Biotinylated Goat Anti-Polyvalent was applied. Streptavidin peroxidase enzyme was applied to the sections washed with PBS for 10 minutes. Sections treated with AEC chromogen were washed with distilled water, counterstained with Gill's hematoxylin, covered, and examined under light microscopy. PBS was employed instead of primary antibody after protein blocking in the negative control group. The prepared sections were photographed under a light microscope (Zeiss, Axiolab) with a camera attachment. Staining intensities were evaluated as (-); no staining, (-/+); partial staining, (+); weak staining, (++); moderate staining, (+++); strong staining (Tatar et al., 2023; Tufekci et al., 2023).

Table 1: *Antibodies used as the primary antibodies in immunohistochemical staining*

Primary antibodies	IHC Dilution	Code	Company
GPX4 Monoclonal antibody	1/1000	67763-1-Ig	Proteintech Group
SLC7A11 Polyclonal antibody	1/200	26864-1-AP	Proteintech Group

Semi-quantitative analysis

The immunoreactivities of GPX4 and SLC7A11 primary antibodies in the hippocampus were scored using the modified Histoscore (H-score), a semi-quantitative staining intensity and percentage assessment. Ten randomly selected regions in brain tissue sections from each rat were graded semi-quantitatively using an objective magnification of 40X. The median H-score value was used to classify each region's expression level as either low or high (Numata et al., 2013; Tatar et al., 2023).

Statistical analysis

SPSS version 21.0 (IBM SPSS Statistics, IBM Corporation, Chicago, IL, USA) software for MAC was used for the statistical analyses. The Shapiro-Wilks test was used to determine whether the data were normally distributed. Group comparisons were conducted using Kruskal Wallis testing. Interquartile range [Me (Q25-Q75)] and p values <0.05 were adopted as statistically significant values for the study data, which were expressed as median values.

RESULTS

GPX4 Immunohistochemistry staining results

Evaluation of anti-GPX4 positive staining in the dentate gyrus (DG) and cornu ammonis (CA) regions of the hippocampus sections from the control group that some of the cells in the granular and subgranular layer of the DG were strongly anti-GPX4 positive. Strongly positively anti-GPX4 stained cells and their cellular extensions were also

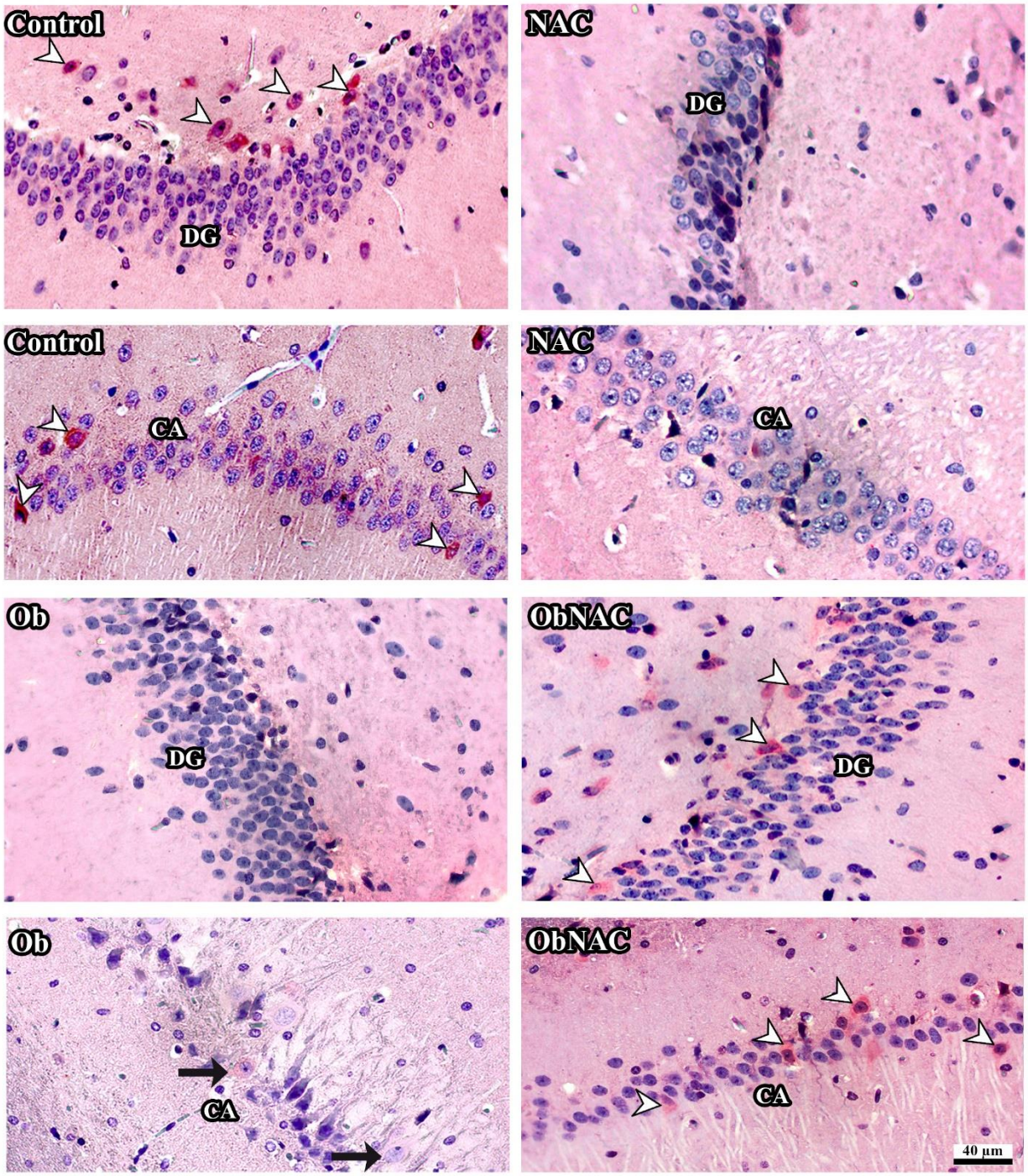

Figure 1. *Anti-GPX4 (+) stained cells (arrowhead) in the dentate gyrus (DG) and cornu ammonis (CA) regions of the hippocampus from all groups. In the control group, strong positive staining was detected in the DG subgranular layer and in some pyramidal neurons in the CA region. While moderate immunoreactivity was observed in the NAC group, no activity was observed in the granular cells of the DG in the Ob group, and weak immunoreactivity was detected in the CA region (black arrow). In the ObNAC group, strong positive staining was observed in both the DG and pyramidal neurons in the CA region. Counterstaining was performed with Gill's hematoxylin. Scale bar: 40 µm for all panels.*

frequently observed in pyramidal neurons in the CA.

NAC group sections exhibited moderate positivity in DG granular cells and some cells of the polymorphic layer. Pyramidal neurons in the CA region were similarly moderately anti-GPX4 positive.

Examination of the DG sections from the Ob group revealed no positive staining in granular, subgranular, or polymorphic layer cells, while a few pyramidal cells in the CA region were weakly positive.

Examination of the sections from the ObNAC group revealed strong anti-GPX4 positive staining in the granular layer and intense staining in the polymorphic layer of the DG. Strong staining was also detected in pyramidal cells in the CA region (**Figure 1, Table 2**).

Analysis of the GPX4 immunoreactivity H-scores in the hippocampus sections from the four groups revealed a decrease in the Ob group compared to the Control, NAC, and ObNAC groups ($p \leq 0.01$). However, the ObNAC group H-score was not statistically different from that in the Control group ($p > 0.05$). The NAC group H-score was lower than in the Control and ObNAC groups, but higher in the Ob group ($p \leq 0.01$, **Table 2**).

Table 2: *Semi-quantitative analysis of GPX4 immunostaining in hippocampal sub-regions from all the study groups (data presented as median and the interquartile range)*

Groups	DG	CA1	CA2	CA3	GPX4 positivity score
Control	+++	+++	+++	+++	225 (210-240) [b,c]
NAC	++	++	+	+	140 (120-150) [a,c,d]
Ob	-	-/+	-	-/+	50 (0-70) [a, b, d]
ObNAC	+++	+++	+++	++	240 (225-255) [b, c]

(-, no staining; -/+, partial; +, weak; ++, moderate; +++, strong positive staining). [a]$p \leq 0.01$; versus the control group, [b]$p \leq 0.01$; versus the NAC group, [c]$p = \leq 0.01$; versus the Ob group, [d]$p \leq 0.01$; versus the ObNAC group, Kruskal–Wallis/Tamhane T2 test, (n=4).

SLC7A11 Immunohistochemistry staining results

Immunohistochemical SLC7A11 antibody staining in hippocampus sections from the control group revealed strong SLC7A11 immunoreactivity in cells in the DG granular layer and in pyramidal neurons in the CA regions.

In the NAC group sections, positive cells were observed in the DG subgranular layer, and moderate positively stained cells were frequently observed in the CA region.

Weak anti-SLC7A11 immunoreactivity was observed in a small number of cells in the DG in hippocampus sections from the Ob group, while no anti-SLC7A11 staining was observed in cells in the CA region.

Hippocampus sections from the ObNAC group exhibited strong anti-SLC7A11 positive staining in the cells in the polymorphic layer, especially in the subgranular zone of the DG. A strong reaction was also observed in pyramidal neurons in the CA region (**Figure 2, Table 3**).

Statistical analysis of SLC7A11 immunoreactivity in the hippocampus sections from all the study groups revealed that the Ob group H-score was significantly lower compared to those of the Control, NAC, and ObNAC groups ($p \leq 0.01$). However, the ObNAC group H-score was not statistically different to those in the Control and NAC groups ($p > 0.05$, **Table 3**).

Table 3: *Semi-quantitative analysis of SLC7A11 immunostaining in hippocampal sub-regions from all the study groups (data presented as median and the interquartile range)*

Groups	DG	CA1	CA2	CA3	SLC7A11 positivity score
Control	++/+++	+++	++	++	180 (160-210) [b]
NAC	++/+	++	+	+	160 (140-160) [b,d]
Ob	-/+	-	-	-	10 (0-30) [a,b,d]
ObNAC	+++	+++	++	++	210 (200-240) [b,c]

(-, no staining; -/+, partial; +, weak; ++, moderate; +++, strong positive staining). [a]$p \leq 0.01$; versus the control group, [b]$p \leq 0.01$; versus the NAC group, [c]$p = \leq 0.01$; versus the Ob group, [d]$p \leq 0.01$; versus the ObNAC group, Kruskal–Wallis/Tamhane T2 test, (n=4).

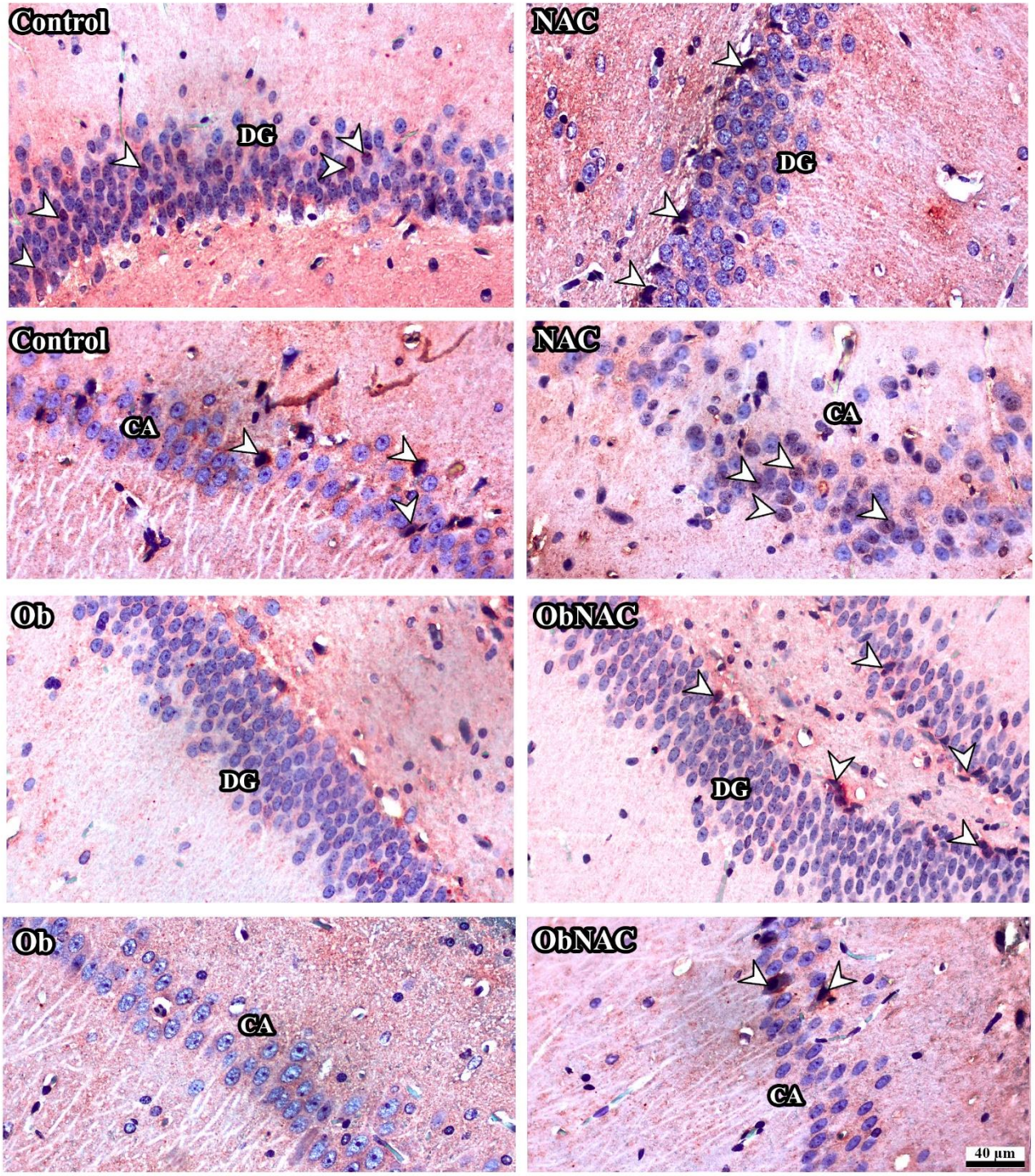

Figure 2. *Anti-SLC7A11 positive stained cells (arrowhead) in the dentate gyrus (DG) and cornu ammonis (CA) regions of the hippocampus from all groups. Strongly positive stained cells can be seen in the granular layer of the DG and CA regions from the Control group. In the cross-sectional images from the NAC group SLC7A11 was moderately stained in the DG and the pyramidal neurons in the CA region. No anti-SLC7A11 activity was found in the DG and CA regions of the hippocampus sections from the Ob group. In the ObNAC group, intense positive staining was found in the subgranular layers of the DG and pyramidal layers of the CA. Counterstaining of the sections was performed with Gill's hematoxylin. Scale bar: 40 µm for all panels.*

Discussion

Studies conducted in recent years are increasingly clarifying the connection between obesity and ferroptosis from various different perspectives. The present study found a significant depletion of GPX4 and SLC7A11 immunoreactivity in Ob group hippocampus sections. In addition, NAC treatment against obesity prevented a decrease in both significant indicators of ferroptosis. Obesity is a metabolic disease characterized by iron metabolism disorders, GSH deficiency, and oxidative stress (Gonzalez-Dominguez et al., 2020). The brain possesses relatively limited antioxidant factors but is rich in iron, which can react with endogenous H_2O_2 and promote lipid peroxidation, making the organ vulnerable to ferroptosis (Zhang et al., 2022).

According to some research, ferroptosis may play a role in the degenerative processes of a variety of neurological diseases, such as stroke, Alzheimer's disease, Parkinson's disease, and traumatic brain injury (TBI) (Hambright et al., 2017; Alim et al., 2019; Rui et al., 2021). Studies have also reported that obesity reduces the proliferation of neural progenitor cells and hippocampal neurogenesis by elevating lipid peroxidation and lowering brain-derived growth factor levels (Tozuka et al., 2009; Park et al., 2010). Neuronal membranes with high levels of PUFA-PL are especially vulnerable to peroxidation (Cobley et al., 2018). Due to its antioxidant capacity, GPX4 is capable of using GSH as a cofactor to detoxify intracellular lipid and cholesterol hydroperoxide product accumulation (Zhang et al., 2022). Loss of GPX4 activity has been identified as a specific feature of ferroptosis (Wang et al., 2021).

Obesity-related excessive ROS generation can lower GPX4 expression levels and exacerbate lipid oxidative damage. GPX4-deficient animals have been found to exhibit noticeable cognitive impairment and hippocampal neuronal degeneration on the basis of in vivo tests (Chen et al., 2015). GPX4 deficiency has been detected in the ipsilateral hippocampus following TBI (Fang et al., 2023). Similarly, ferroptosis has been linked to a number of brain injury disorders, including intracerebral hemorrhage (ICH) and subarachnoid hemorrhage (Alim et al., 2019; Chen et al., 2020). Hambright et al. (2017) detected severe behavioral alterations and neurodegeneration in the hippocampus of mice with GPX4 ablation in forebrain neurons (Hambright et al., 2017). Guan et al. (2019) found that Carvacrol protected hippocampal neurons against ischemia and reperfusion injury in gerbils by increasing GPX4 expression and inhibiting ferroptosis (Guan et al., 2019). Ferroptosis agonists have also been reported to drastically reduce GPX4 expression in cardiomyocytes, causing an imbalance in iron metabolism and lipid peroxidation in these cells (Liu et al., 2019).

Studies have reported that a high-fat, high-sucrose diet resulted in reduced GPX4 expression and activity in mice, which was connected to greater GPX4 levels in white adipose tissue (Schriever et al., 2017). However, a study involving a mouse model of ICH reported, as a frustrated adaptive response, significantly greater GPX4 mRNA in the striatum six hours following collagenase injection (Alim et al., 2019). On the other hand, research keeps demonstrating that GPX4 deletion may exacerbate neuroinflammation and lead to oxidative damage to membranes. Another study reported that adult mice with conditional knockdown of neuronal GPX4 caused by Tamoxifen treatment exhibited paralytic phenotypes linked to motor neuron degeneration with ferroptosis-like characteristics (Chen et al., 2015). Reducing the oxidative stress caused by ferroptosis occurring in the hypothalamus and nervous system is therefore a crucial factor in treating obesity (Zhang et al., 2022). Fang et al. (2023) demonstrated the neuroprotective role of GPX4 against ferroptosis in hippocampal neurons in a TBI model in vitro and in vivo using the adeno-associated virus-mediated GPX4 overexpression method.

We also found a significant decrease in SLC7A11 expression levels as a ferroptosis marker in hippocampal sections from the Ob group. Down-regulation of SLC7A11 is also known to reduce intracellular cysteine levels, thus suppressing GSH biosynthesis. GPX4 activity is thus inhibited, ROS generation increases, and the integrity of the mitochondrial structure is compromised as a result (Gout et al., 2001). Changes in GSH status are common in many neurodegenerative diseases, and this finding from the present study emphasizes the importance of cysteine homeostasis in obesity. Consistent with our findings, altered concentrations of ferroptosis indicators such as GPX4, SLC7A11, and GSH have been observed in tissues from rats exposed to spinal cord injury (Yao et al., 2019). However, the structural features of mitochondria could not be examined in the present study. Electron microscopic studies examining the morphological features of ferroptosis are therefore now needed.

A cysteine donor, NAC has been shown to inhibit ferroptosis in vitro and to serve as a precursor to GSH (Yang et al., 2014). The antioxidant NAC is also known to modify the function of the Xc- system and has recently attracted attention as a potential treatment for obesity-related neuronal damage and behavioral disorders (Tufekci et al., 2023). NAC may also enhance central nervous system function by increasing glutamatergic neurotransmission by activating the Xc-transporter (Baker et al., 2002). The present study also tested NAC as a potential neuroprotective agent against the adverse effects of obesity. It was observed to maintain GPX4/SLC7A11 levels in the ObNAC group hippocampus, similarly to the control group.

A previous study concluded that NAC mitigated ferroptosis by activating the SIRT3-SOD2/GPX4 pathway in diabetic nephropathy and preserving mitochondrial redox homeostasis (Li et al., 2022b). NAC has been found to directly maintain mitochondrial redox steady state via the SITR3-SOD2 pathway (Li et al., 2022b). In the present study, it was found to activate GPX4/SLC7A11 expression in the ObNAC group through a similar mechanism. Additionally, NAC has been reported to reduce neuronal ferroptosis and to enhance behavior in mice after ICH by neutralizing nuclear arachidonate 5-lipoxygenase-derived harmful lipid species, which are essential for ferroptosis and involved in cellular lipid production (Karuppagounder et al., 2018). Since GPX4 is a ferroptosis-preventing factor, NAC can reverse ferroptosis by reducing lipid peroxidation damage in the hippocampus, due to its ability to protect GPX4 levels. Additionally, SLC7A11, the Xc-system's catalytic component, can be upregulated to increase intracellular cysteine levels, which produces GSH and increases GPX4 activity while reducing ferroptosis (Gong et al., 2019).

In conclusion, this study found that obesity reduces GPX4/SLC7A11 expression in the hippocampus, and that NAC exhibits a protective role against hippocampal ferroptosis. NAC may exert these effects by attenuating mitochondrial dysfunction and lowering ROS levels, although further research is needed to confirm the molecular mechanisms involved.

According to recent studies, oxidative stress, lipid peroxidation, iron metabolism, and other as yet unidentified pathways represent potential causes of ferroptosis. This is regulated in a variety of ways and contributes to a range of illnesses. The immunohistochemical results of the present study showed a decrease in ferroptosis markers in the hippocampus in the obesity model. In addition, this study identified the potential nature of NAC as an important protective agent against obesity-induced ferroptosis. The principal limitation of this research is that changes associated with oxidative stress, lipid peroxidation, and iron metabolism were not investigated. Further studies investigating possible underlying mechanisms are now needed.

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Author contribution

Conceptualization, methodology: K.K.T., Experimental procedures: K.K.T, M.T. All authors wrote, read and approved the final version of the manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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