Neuroprotective Effects of N-Acetyl Cysteine in an Acute Model of LPS Induced Neuroinflammation

By

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Master of Science
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(Neuroscience)

KOÇ
ÜNİVERSİTESİ

Date

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Koç University

Graduate School of Health Sciences

This is to certify that I have examined this copy of a master's thesis by

Judi Kesebi

and have found that it is complete and satisfactory in all respects,
and that any and all revisions required by the final
examining committee have been made.

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Prof./ Assoc. Prof / Assist. Prof. ABC (Advisor)

Date: _____

To my loving parents,

for your endless love and support

“This Study was conducted using the service and infrastructure of Koç University Research Center for Translational Medicine (KUTTAM)”

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Abstract:

Neuroinflammation has been a major focus for researchers in the fields of neurology and neuropathologies, as it had proven to play a key role in triggering pathogenesis of various neurodegenerative diseases like Alzheimer's disease, where inflammation, among many other interconnected factors inluding oxidative stress and mitochondrial dysfuction plays a crucial role in the pathological feedback loop contributing to disease progression. Therefore, there is an inevitable need for implicating variable models of neuroinflammation, and it remains promising to investigate potential therapeutics, those with a natural origin in particular. LPS induced neuroinflammation is a widely used model in animal research, and is able to mimic intrinsic neuroinflammatiory states seen in the human CNS by activating microglia and initiating the release of several proinflammatory cytokines. Numerous studies have pointed out the relevance of glial cells, like astrocytes and microglia to neuroinflammation, thus to possibly linked protein abnormalities like Tau fibrillary tangles and amyloid plaques. Moreover, recent works are focusing on the microglial activation derived perineuronal net disruptions in neurodegenerative and neuropsychiatric disorders. These disruptions might hold the answer to understanding the vulnerability of particular neuronal types to neuroinflammation.
In this project, we investigated the neuroprotective role of N-Acetylcystein, a well-known natural antioxidant, on an acute, 10 day model of centrally injected LPS rats. Animal Groups consisted of healthy controls, LPS-induced animals, NAC treated LPS-induced and a group treated with an additional prophylactic dose of NAC prior to model induction. We assesed the anti-inflammatory potential of NAC in terms of neuronal loss, perineuronal net degradation, glial cell activation, and protein aggregations, in addition to cognitive decline, using immunofluorescence stainings, ELISA and a behavioral test for working memory. Our results indicated that NAC was able to reduce LPS-induced $A\beta_{1-42}$ accumulations in the brain and spinal cord, as well as to halt neuronal loss and inflammatory astrocytosis in variable regions of the hippocampus. Moreover, NAC administration clearly protected PNN stuctures and alleviated PNN density loss associated with LPS-induced microglial activation in the prefrontal cortex. These results indicate the potential neuroprotective and anti-inflammatory impact of NAC on early neuroinflammation-associated CNS pathologies, and highlights the need

for more in-depth investigation regarding the neuro-protective role of NAC in models of inflammatory neurodegeneration.

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I am deeply indebted to my support system, my mother and father and my lovely sisters, none of what I have become would have been possible without you believing in me, your love and your continuous support.

Finally, to my loving husband, thank you for making this easier than it could have been, for your patience, your support, for giving me perspective on many levels. I love doing life with you.

Article I. Table of Contents

ABBREVIATIONS

CNS = Central Nervous System

AD = Alzheimer's Disease

NAC = N-Acetylcysteine

LPS = Lipopolysaccharide

PNN = Perineuronal Nets

IF = Immunofluorescnece

WFA = Wisteria floribunda agglutinin

Cy3 = Cyanine

MWM = Morris Water Maze

DAPI = 4′,6-diamidino-2-phenylindole

DPBS = Dulbecco's Phosphate Buffered Saline

ECM = Extracellular Matrix

i.p = Intraperitoneal

OCT = Optimal Cutting Temperature Compound

PFA = Paraformaldehyde

MAPK = Mitogen-Activated Protein Kinase

NF-κB = Nuclear factor kappa-light-chain-enhancer of activated B cells

DAMP = Damage-Associated Molecular Patterns

ROS = Reactive Oxygen Species

RNS = Reactive Nitrogen Species

GABA = γ-Aminobutyric acid

Chapter1: INTRODUCTION

1.1. Neuroinflammation in Neurodegenerative Disorders

Neuroinflammation is an essential biological process in which multiple cell types such as microglia, astrocytes, neutrophils, monocytes and neurons play a role. (Hey-Kyeong Jeong, 2013). With highly complex features, controlled neuroinflammation governs brain homeostasis in cases of brain damage resulting from insults either of an endogenous origin or exogenous pathogens; however, if inflammation persists to chronic states it may lead to serious pathologies (Wang & Qi, 2022). Therefore, models of neuroinflammation are recently playing an essential role in unraveling the pathways behind inflammatory driven CNS pathologies linked to several neurodegenerative diseases like Alzheimer's disease (Nazem et al., 2015), Parkinson's disease(Troncoso-Escudero et al., 2018) and muliple sclerosis (Bjelobaba et al., 2017). Such models are also of a highly practical significance in screening for potential inflammatory drugs. The pathological cascade driven by neuroinflammation includes CNS associated hallmarks like microglial activation, astrogliosis and release of pro-inflammatory cytokines, as well as recruitment of peripheral immune cells which leads to blood-brain barrier damage and subsequent neuronal loss (More et al., 2013).
Additionally, neuroinflammation is now considered as a significant associative to oxidative stress, one of the main drivers for several neurological disorders (Anwar, 2022).

1.2. Oxidative Stress and Its Link to Neuroinflammation

The brain is a highly susceptible organ to oxidative damage due to its high dependency on oxygen (utilizing about 20% of body's total oxygen usage). Moreover, the chemical nature of the brain is featured by a high content of lipid polyunsaturated fatty acids as well as high metal ion concentration, which indeed act as core inducers of oxidative stress (Anwar, 2022). The resulting imbalance between levels of ROS (reactive oxygen species) and antioxidants in the brain leads to serious brain tissue damage directly linked to the activation of TLR-4 (Toll-like receptor) inflammatory cascades (Sayre et al., 2001). Furthermore, an elevation in ROS was shown to activate disease associated

molecular patterns (DAMP), which in turn increases levels of NF-κB and MAPK in glial cells, essentially in microglia and astrcoytes, and exacerbates the inflammatory cascade (Anwar, 2022).

Importantly, such activation of microglial and astrocyte cells driven by DAMPs initiates several molecular events that facilitate abnormal protein accumulations like Aβ plaques and neurofibrillary TAU tangles, main hallmarks of Alzheimer's disease, leading eventually to apoptotic neuronal loss (Birecree et al., 1988) thus progressive cognitive decline and brain atrophy.

1.3. Oxidative Stress in Alzheimer's Disease

Recent clinical work has shown increased records of late onset AD among the elderly without previous predisposing factors or related genetic background (Anwar, 2022). Oxidative stress and accumulating reactive oxygen species have been a major focus being a potential cause for late onset AD especially the neuroinflammatory type. It has been suggested that increased oxidative stress levels that results from either extrinsic or intrinsic sources leads to homeostatic disruptions linked to oxidative stress/antioxidant balance, this leads to a further accumulation of ROS and RNS in addition to proinflammatory molecules. Resulting chronic inflammation interferes with natural gene expression leading to protein misfolding, then eventually amyloid beta deposition and leading to neuronal loss and cognitive decline.

Overproduction of ROS in the central nervous system was shown to be associated with clinical features of AD, such as amyloid beta deposition (Wang, 2010), mitochondrial dysfunction, as well as increased intracellular Ca+2 and associated excitotoxicity(Anwar, 2022). Furthermore, oxidative stress is shown to induce an overexpression of MAPK and NFkB pathways both in astrocytes and microglia via the activation of DAMPs. This leads to the release of nitric oxide and and other factors associated with Amyloid deposition and accumulation of Tau tangles (Anwar, 2022)

1.4. The Use of LPS in Models of Neuroinflammation

It is now known that in case of an insult, both innate and adaptive immune responses of the inflammatory type are seen in the CNS, through a cascade of cytokine and chemokine release and activation of microglia (Amor et al, 2010).

LPS injection in animals, either intracerebraly or via systemical way in variable doses, serves as a mimic for human AD by inducing the same key hallmarks, starting from oxidative stress (Sugaya K et al, 1998) and ending in cognitive decline (Lee et al, 2008). Lipopolysaccharide (LPS), is an endotoxin of a bacterial origin that is known to trigger inflammatory responses in rodents and thus has been used to induce models of neuroinflammation in a wide range of neurodegenerative disease studies (Batista CRA et al, 2019). For instance Lee and colleagues (2008) demonstrated that LPS induced neuroinflammation stimulates amyloidogenesis associated cognitive decline in mice. Moreover, LPS was shown to be able to activate glial cells and oxidative stress markers in rats (Sugaya K et al, 1998).

LPS causes the activation of TLR-4 found on the surface of microglia, and inhibiting the TLR-4 pathway prevents LPS induced neuroinflammation and asssociated cognitive decline (Zhao et al,2019).

Furthermore, a study (Koedel et al, 2007) showed that mice lacking the TLR2 and TRL4 receptors displayed lower cytokine release and thus less pro-inflammatory reactions upon traumatic brain injury.

It was further shown that advanced glycation end products (AGE) are accumulated in AD, and their receptors (RAGE) are over-expressed on the surface of neurons and astrocytes. Such increase in these receptors is seen in oxidative stress and neuroinflammatory processes as well. Which suggests a role of RAGE's in the propogation of disease pathology (Amor et al, 2010).

1.5. Defining Dementia

Dementia is a clinical condition manifested by a range of symptoms related to cognitive dysfunction. It is defined as impaired cognitive ability that interferes with one's routine activities and executive functions, which significantly affects their quality of life. Multiple cognitive domains are seen to be impaired in people with dementia, including memory, learning, spatial orientation, and mood (Arvanitakis et al, 2019).

Lately, with the increasing life span of the human population, dementia is rising as a major health problem with pronounced economic and social burden. It is now estimated that dementia sufferers are 47 million, with this number expected to reach 131 million by 2050 (Hebert et al, 2013).

While age stands as an important risk factor for dementia, it is also seen in younger population, and several other risk factors are highly attributed to early onset cognitive decline associated with dementia. These include genetics, hypertension, diabetes, diet, and poor exercise of social and physical activity (Pal et al, 2018) (Gupta et al, 2015) as well as head trauma (LoBue et al, 2019). Moreover, recent studies are focusing on the role that chronic neuroinflammation plays in increasing the risk of dementia, for instance Ahmad et al (2022) highlighted the link between persistent neuroinflammatory-induced microglial activation and dementia.

1.5.1 Alzheimer's Disease

Alzheimer's disease (AD) is the most common type of dementia accounting for about 75% of all dementia cases (Qiu, 2009). AD is a multifaceted neurodegenerative disease, characterized by progressive cognitive decline associated with pathological accumulation of misfolded proteins and a cascade of other pathological hallmarks leading to neuronal loss. Recently, AD has been imposing a huge burden on public health due to the increasing percentage of elderly population and the economic burden is huge as the cost of dementia in 2010 was reported to be approximately $157 billion (Hurd et al, 2013). The field of AD studies is therefore particularly focused on attempting to unravel the key pathological events associated with this disease, as well as discover potential treatments.

1.5.1.1 AD Hallmarks

Key underlying mechanisms of AD pathology are considered to be neuroinflammation, oxidative stress, and hippocampal neurodegeneration. This is because they are thought to link other factors contributing to disease progression such as mitochondrial dysfunction and protein aggregation. Thus, it is essential to study the causal link between these mechanisms and the basic hallmarks of AD, as well as those of other neurodegenerative diseases. Katafuchi et al, (2012) and Krstic et al, (2012) demonstrated this causal link by showing that both oxidative stress and neuroinflammation induce AD associated amyloid pathology, a key hallmark of AD, through glial activation. Moreover, oxidative stress is shown to induce an overexpression of MAPK and NFkB pathways both in astrocytes and microglia via the activation of DAMPs.

This leads to the release of nitric oxide and and other factors associated with Amyloid deposition and accumulation of Tau tangles (Anwar, 2022)

1.6. N-Acetylcysteine

N-acetylcysteine is a natural antioxidant of the thiol type with anti-inflammatory activity which acts through regulating the activation of NF-κB and HIF-1α, as well as directly scavenging reactive oxygen species (ROS) (Vida Mokhtari, 2017). The important role NAC plays as an antioxidant is through generating glutathione, one of the intrinsic antioxidants that have an essential role in redox regulations. Glutathione stands as an essential endogenous antioxidant, functioning in multiple roles associated with the regulation of immune responses (Shahripour et al, 2017) and regulating signal transduction through modulating oxidation-reduction reactions. During oxidative stress, NAC acts as a source of cysteine, one of the three amino acids that form the structure of glutathione and is the key limiting factor in GSH synthesis since it is the one less available intracellularly in normal conditions (Shahripour et al, 2017) (Aruoma, 1989). Various levels of evidence focus on the importance of NAC in restoring the body's antioxidant systems in chronic inflammation where there is an elevation in levels of ROS (Shahripour et al, 2017). For instance, Amin and colleagues (2008) indicated that NAC was able to reduce mitochondrial membrane depolarization. Similarly, in their review, Moreira et al, (2007) indicated the significant role of NAC in reducing oxidative and apoptotic markers resulting from mitochondrial damage in Alzheimer's disease patient fibroblasts. Furthermore, Fu Al et al (2006) showed that NAC administration was able to preserve learning and memory abilities in Aβ-induced AD mice models assessed by Morris water maze.

1.7. Perineuronal Nets

Perineuronal nets have recenly been driving the attention in the field of neuropathology, with recent literature focusing on their role in neurodegenerative and neuropsychiatric disorders. PNNs which are highly specialized extracellular matrix structures that enwrap particular neurons, preferentially fast spiking parvalbumin-expressing (PV+) GABAergic inhibitory interneurons of the hippocampus and cerebral cortex (Wen et al, 2018). They form an essential part of the tetrapartite synapse and contribute majorly to signal transduction (C. Reichelt, 2019). Moreover, interactions between neurons and their surrounding perineuronal nets form the essence of synaptic plasticity. For instance, immunostainings of the adult rat visual cortex showed that perineuronal net maturation overlaps with the closure of the critical period of plasticity, a time window of highly dynamic synapse formation, and neuronal interconnections (Pizzorusso T et al, 2002). Indeed, perineuronal nets play an essential role in aiding neuronal maturation and differentiation (Ana Jakovljevic, 2021). Therefore, researchers have particularly focused on these structures as a potential method in the treatment of several neurodegenerative and neuropsychiatric diseases, such as dementia (A.Suttkus et al, 2016) and schizophrenia (A. Mauney et al, 2013).

1.8. Perineuronal Net Disruptions in Neuroinflammatory States

One of the key downstream consequences of chronic neuroinflammation and oxidative stress is the disruption of extracellular matrix structures, including perineuronal nets, that normally surround and protect neurons from neurotoxins and damaging factors, such as reactive oxygen species. For instance, C.Reichelt (2020) indicated that chronic microglial activation, a main hallmark of AD pathology, is responsible for increasing the burden of neuronal damage, mainly by disrupting perineuronal net components; as chronically activated microglia respond to cell damage by activating TLR4 (Anwar, 2022) and releasing proinflammatory cytokines, as well as particular proteinases that cleave these components, stripping away the protective shield around neurons (C.Reichelt, 2020). Having such a major protective role against oxidative stress, microglial induced degradation of PNN components, as seen in AD, can render neurons vulnerable to damage, resulting in a feedback loop inducing microglial activation, PNN degradation, amyloid aggregation and neuronal death (D. Crapser J et al, 2020). Interestingly, it is not only microglial cells that engulf damaged perineuronal nets. D. Crapser (2020) showed that inclusions of aggrecan, a major component of the

perineuronal net structure, were found in amyloid plaques in mice models of AD. These findings raise important questions regarding the impact of neuroinflammation on PNN structures and how this phenomenon translates in terms of cognitive dysfunction seen in AD thus contributes to disease progression.

1.9. N-Acetyl Cysteine and Perineuronal net Disruptions

Given what was mentioned above, saving perineuronal nets from structural damage seen in multiple neurodegenerative diseases can serve as a key factor to halt neuronal loss. One way to achieve that is to target oxidative stress as a way to maintain balanced Redox regulations thus save ECM loss. In their research paper, Sharmin S (2021) showed that when administered postnatally, NAC was able to prevent PNN destruction in a transgenic mouse model of intrinsic defective PNN structures. Similarly, it was indicated that NAC was able to prevent delayed maturation of parvalbumin expressing interneurons and their surrounding PNNs in a mouse model of redox dysregulation (Cabungcal et al, 2013). In both works, NAC was administered early during the post-natal and pubertal period, respectively, which is known to be a highly flexible window for PNN maturation. Importantly, after puberty, PNN structures come to a mature state with reduction in their dynamic abilities, making it difficult to manipulate them pharmaceutically for the aim of neuroprotection against inflammatory damages associated with oxidative stress.

The aim behind this project was to build upon those works by investigating the neuro-protective impact of NAC by focusing on the basis of neuronal loss, astrocytic activation, amyloidosis and perineuronal net disruptions, along with behavioral assessment of the cognitive dysfunction in adult rats. Utilizing an acute model of neuroinflammation, we conducted a comprehensive analysis on LPS induced neuroinflammation that mimics AD pathology. Moreover, we wanted to test the neuroprotective efficacy of N-acetylcysteine (NAC) on LPS induced inflammatory processes and underlying pathology seen in our model. Our major focus in this study was to test whether our LPS model of neuroinflammation could activate microglia enough to disrupt the structure of perineuronal nets thus stripping away the protective shield around neurons against oxidative stress. More importantly, this work may well be

the first to investigate the antioxidant role NAC plays to save PNNs in an acute model of neuroinflammation in adult animals.

Chapter 2:

MATERIALS AND METHODS

2.1. Animals

In house bred 46 adult male Wistar rats, 8-9 weeks of age, weighing 187g in average were used for the expriment.
Animals were allowed free access to standard laboratory rat chow and water, they were housed in standard cages maintained in temperature (20°C – 26°C), humidity (30% – 60%) and lighting (12 hours light/dark cycle) controlled room.

All experiments and procedures were carried out in the light phase (between 9.00 am. and 17.00. pm) in accordance with the protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Koç University, and under compliance of the Animal Research Reporting In Vivo Experiments (ARRIVE) guidelines.

2.2. Study Groups and Experimental Design

Animals were randomly divided into 4 groups after assessing their motor function to exclude any abnormal motor deficits that might interfere with the results. Groups were then assigned as the following and shown in Table *1*:

G1, Healthy control group (n=7): Rats were housed at the same conditions as other groups and underwent the same MWM trainings and MWM test.
G2, LPS only group (n=7): Rats received intrahippocampal LPS injections without any treatment.
G3, LPS+NAC group (n=7): Rats received intrahippocampal LPS injections followed by 4 separate NAC doses (200 mg/kg per dose), one dose every other day along 10 days.
G4, LPS+Prophylaxis group (n=7): Animals received a prophylactic NAC dose prior to LPS administration, in addition to 4 treatment doses after model induction (200 mg/Kg per dose), one dose every other day along 10 days.

As demonstrated inFigure *1*, animals underwent habituation to the water maze where any potential intrinsic motor deficits were excluded. Following LPS administration, the model lasted for 10 days, during which animals underwent several MWM trainings, had regular weight measurements Figure *2* and (for groups G3 and G4) received intraperitoneal NAC injections (200 mg/kg) given every other day.
On the final day (10 days after model induction), MWM test was applied to assess the animals' retention of the platform, then they were sacrificed to collect tissues and samples.

Table 1 Experimental Groups.

Animal Group	Experimental Condition
G1	Healthy controls
G2	Rats received intra-hippocampal LPS injections (10 ul in total)
G3	Rats received intra-hippocampal LPS injections (10 ul in total) and received 4 seperate NAC i.p. injections (200mg/kg per dose) after model induction along 10 days.
G4	Rats received a (200mg/kg) single dose of NAC prior to model induction,

	underwent intra-hippocampal LPS injections (10 ul in total) and received 4 seperate NAC i.p. injections (200mg/kg per dose) after model induction along 10 days.

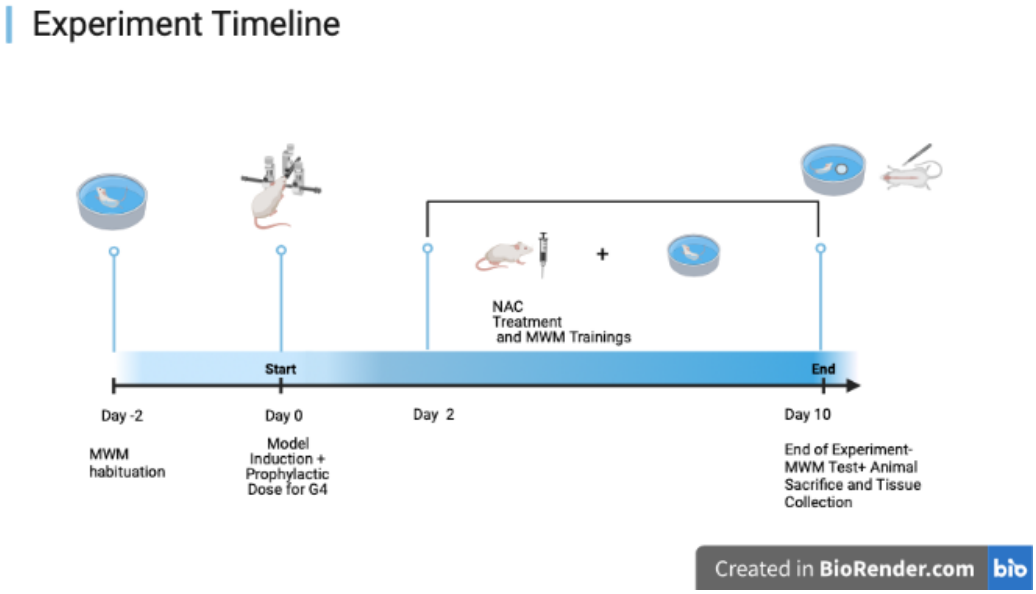

Figure 1 Experimental Timeline.

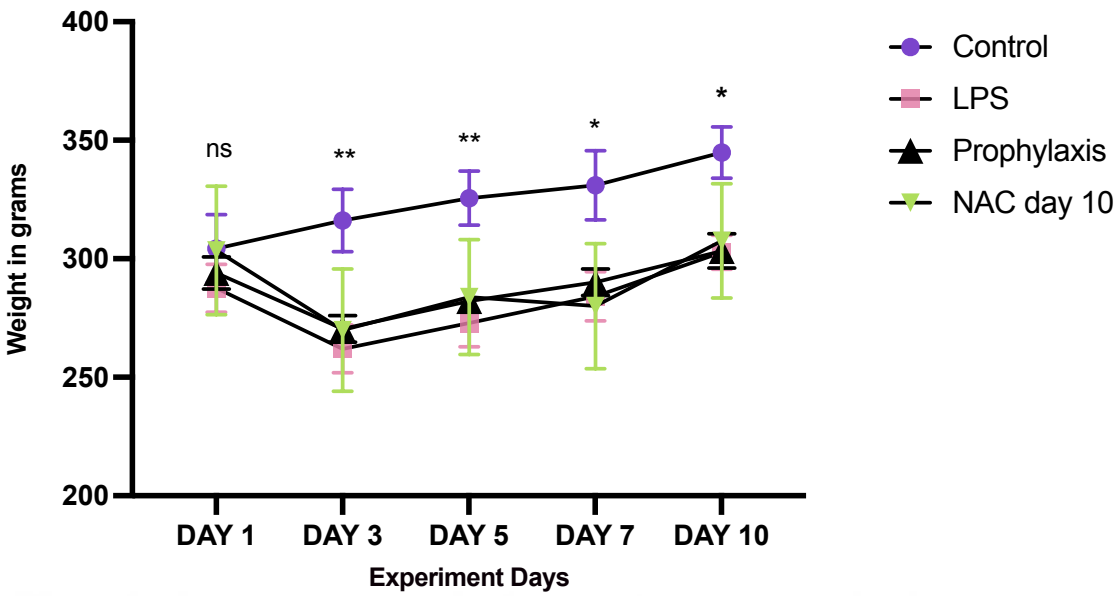

Figure 2 Weight change for all groups throughout the experiment.

2.3. *Surgery and Model Induction*

The model was induced by injecting LPS bilaterally into the rats' hippocampi, as LPS gram negative bacteria was proven to induce non genetically based neuroinflammatory type of AD (Catorce and Gevorkian, 2016). On the day of surgery, animals were anesthetized with ketamine/xylazine cocktail given intraperitoneally (0.1 mL/100g), then they were positioned on a rat specific stereotaxis apparatus (Stoelting, Wood Dale, IL, USA).

Lipopolysaccharide (LPS) purchased from Sigma, United States (Sigma-Aldrich, St. Louis,MO, L2630), was dissolved (1 mg/mL) in sterile PBS and 10 µL in total were given to both hippocampi, injections were done with a micro-syringe through midsagittal holes drilled into the neocortex using the following coordinates: 3.6 mm anterior, 2 mm medial and 3.6 mm posterior, 2 mm lateral from the bregma, both at a depth of 3.4 mm. Coordinates chosen were in accordance with previous literature (Espinosa et al., 2013).

Surgical incisions were cleaned and closed with surgical staples Figure *3* , then animals were placed on a warming pad to maintain their body temperature until they recovered from anaesthesia.

Surgical procedure was done in accordance with rules and regulations of Institutional Animal Care and Use Committee (IACUC) at Koç University.

Figure 3 Image of animals post-operation.

2.4. Behavioral Assessment

In order to assess hippocampal memory, Morris water maze test was performed (Morris et al, 1982). Animals were handled by a single experimenter and in the same room during the whole procedure.

The apparatus used was a circular pool Figure *4* (152 cm diameter and 38 cm height), filled with clean tap water, the water temperature was maintained throughout the experiment (26±1°).

Platform used was transparent, 17 cm in length, hidden 2 cm below water and 20 cm from the pool wall in a fixed quadrant (the southwest quadrant).

On the first day, all rats were allowed to swim freely for 1 minute to get habituated to the pool and to exclude any preexisting motor weaknesses.

On the following day, the platform was hidden in the middle of the southwest quadrant, rats were then released into the water and allowed to swim until they found the

platform; rats that fail to locate the plaform and escape within two minutes were gently guided to the platform and allowed to stand on it for 10 seconds, latency to the find the platform was then recorded as 120 seconds.

On each training day, two trials were applied for each animal; starting from a different quadrant chosen randomly for each trial. Animals had a total of 4 training days and a final test day (10 days after LPS injections) to assess their learning and memory, and escape latencies were recorded for each animal throughout the experiment.

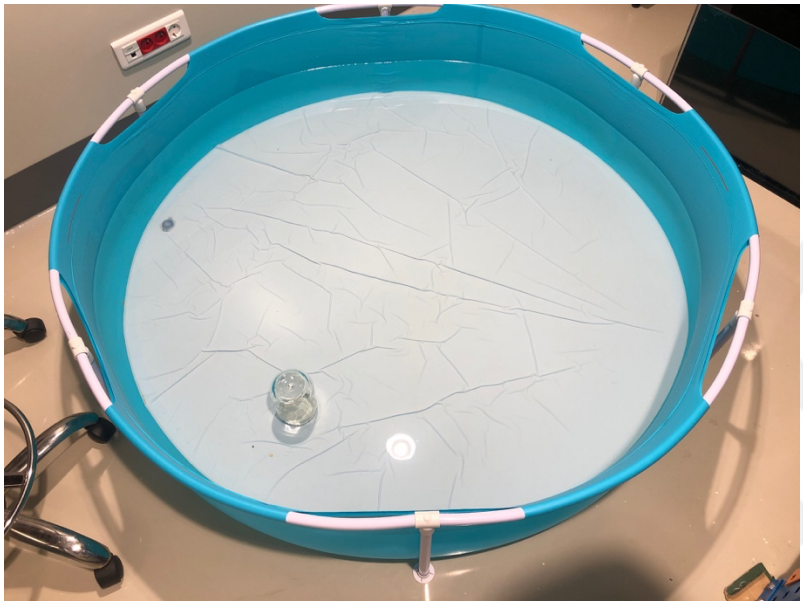

Figure 4 Apparatus used as MWM.

2.5. NAC administration

N-Acetyl Cysteine (NAC) was prepared from a pharmaceutically available mucolytic (ASIST, Bilimilac, Turkey) and dose was prepared with respect to NAC concentration. Each animal in G3 received one i.p. dose (200mg/kg) (Shahidi et al., 2017) of NAC starting from day 2 after surgery every other day until day 10. In addition to the same 4 doses, animals of the G4 group received one additional injection 4 hours prior to surgery and LPS induction to assess whether NAC has a prophylactic impact.

2.6. Animal Sacrifice and Tissue Collection

On the day of sacrifice, all animals were deeply anesthetized with ketamine/xylazine cocktail given intraperitoneally (0.1 mL/100g) combined with isoflurane gas.
Animals were divided randomly for immunohistochemical or protein analyses.
For histological studies, animals were transcardially perfused with 0,1M sterile phosphate buffer saline (PBS) followed by 4% PFA. Brains and spinal cords were removed and kept in 4% PFA overnight at +4°C.
For protein-based analyses, brains and spinal cords were removed without perfusion and homogenized with sterile PBS. Tissue homogenates were then kept at -80°C.
Cerebrospinal fluid samples were obtained and centrifuged at 15000g for 10 minutes at 4°C then stored at -80°C for subsequent analyses.

2.7. Immunofluorescence Staining of Frozen Sections

For histological analysis, collected brains and spinal cords were fixed with 4% PFA overnight at +4°C, then they were dehydrated with a sucrose gradient (10%, 20% and 30% respectively and 48 hours each). After that, frozen tissue blocks were prepared by embedding tissues in OCT (frozen tissue matrix) (OCT, Leica, #14020108926) and stored in -80°C Figure *5*.
Tissue blocks were later sliced with a cryostat microtome (Leica CM 1850, Leica Microsystems, Seoul, Korea) at a thickness of 10 µm. To proceed with immunofluorescence staining, first, slides were washed with distilled water to melt OCT surrounding tissue slices, then they were incubated in methanol for 5 minutes to allow permeabilization and washed with Dulbecco's phosphate-buffered saline (DPBS, Gibco, #14190-094).
To prevent non-specific reactions, tissue slices were blocked using a commercially available blocking solution (Super Block, Thermo Fisher Scientific™, PI37535).

Following the blocking step, tissue slices were incubated with primary antibodies (diluted in Super Block) at 37°C for 1.5 hours. Excess non-bound primary antibodies were then washed with DPBS.

Subsequently, tissues were incubated with suitable secondary antibodies to allow the visualization of target proteins, followed by thorough washing steps with DPBS again. Lastly, slides were mounted with 4 ', 6-diamidino-2-phenylindole (DAPI, Abcam, Ab104139).

Antibodies used for immunostaining are summarized in Table *2*.

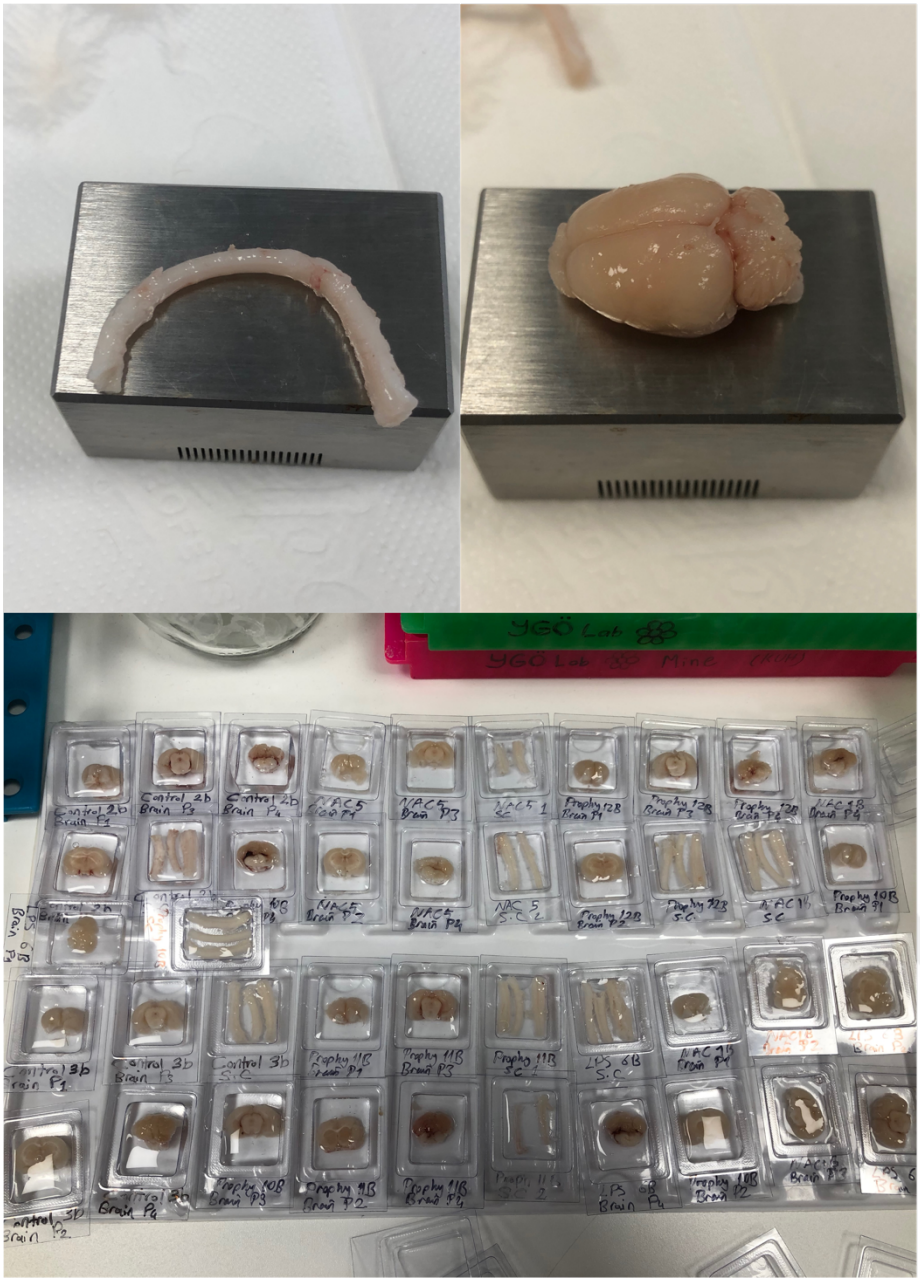

Figure 5 Tissue preparation for freezing.

Table 2 Antibodies used for IF staining.

Target	Primary Antibody (Catalog Number)	Dilution	Secondary Antibody	Dilution
Neurons	rabbit anti-NeuN (Abcam, Ab177487)	1/100	Goat-anti-rabbit Cy3	1/200
Astrocytes	rabbit anti-GFAP (Abcam, Ab7260)	1/100	Goat-anti-rabbit Cy3	1/200
Perineuronal Nets	WFA,Vector Labs, FL-1351-2)	1/500	Conjugated	

2.8. Image Analysis

Immunofluorescence stainings were observed using Leica DMI8 SP8 Confocal Scanning Microscope. Images from frozen sections were taken and exported as tiff files from the LAS X software (Leica, Wetzlar, Germany), then analyzed using ImageJ image processing program (NIH, MD, USA). For quantitative assessment of target markers, fluorescence intensities were compared between groups after collecting the images under the same applied microscopy settings, post-capture manipulations like contrast and background noise were also applied identically between groups. Differences between groups were analysed in matched areas and assessed for significance by One-way ANOVA or Kurskal Wallis tests using GraphPad PRISM (Version 9.2.0) (San Diego, USA).

2.9. ELISA

To assess inflammation induced pathology in terms of abnormal protein accumulation and in order to investigate the ability of NAC to reduce these hallmarks, commercially available ELISA kits (Elabscience, # E-EL-R3030 and # E-EL-R0943) were used to determine concentrations of Amyloid beta and Tau respectively in brain and spinal cord homogenates.

The protocol and sample preparation were applied in accordance with the manufacturer's instructions; target protein concentrations were determined compared with the standard curve.
ELISA results were compared between groups and differences were tested for significance using GraphPad PRISM (Version 9.2.0) (San Diego, USA).

2.10 Statistical Analysis

All results were expressed as mean ± SD unless otherwise mentioned. GraphPad PRISM (Version 9.2.0) (San Diego, USA) was used for statistical analyses. Appropriate statistical test was used upon Shapiro Wilk normality test, respective One-way ANOVA or Kruskal Wallis tests were used for group comparisons to analyse differences in matched areas. A p-value less than 0.05 was considered statistically significant.

Chapter 3:

RESULTS

3.1. ELISA Results:

3.1.1. A Single Intrahippocampal LPS Dose (10 μg) Increased Aβ Concentration in Brain and Spinal Cord Homogenates.

ELISA results were statistically analysed through GraphPad Prism using Kruskal Wallis test for over all comprison ($p=0.0026$ for brain samples and $p=0.004$ for spinal cord samples) and Mann-Whitney U test for pairwise comparisons (p values indicated in Figure *6*). Results indicated that $A\beta_{1–42}$ concentration was significantly increased in brain homogenates of G2 (LPS injected rats), as well as in spinal cord homogenates when compared to controls ($p<0.05$) Figure *6*.

3.1.2. N-Acetylcysteine Significantly Reduced Aβ1–42 Concentrations to Control Levels

In G3 (LPS+NAC) group, $A\beta_{1–42}$ was significantly reduced when compared to G2 (LPS only group) in brain and spinal cord sample homogenates ($p<0.05$). Interestingly, G4 (LPS+prophylaxis) group showed more reduction than G3 in $A\beta_{1–42}$ concentration when compared to LPS+NAC group and levels of $A\beta_{1–42}$ were shown to be closer to controls, however, difference between G3 and G4 did not reach statistical signficance ($p>0.05$) Figure *6*.

These results indicate the need for further research on the prophylactic impact of NAC against amyloid pathology in models of neuroinflammation.

3.1.3. No Pronounced Difference in pMAPT/pTAU Concentration Between Groups

Our ELISA results represented in Figure 7indicated no significant difference between groups in terms of pTAU concentration measured in brain and spinal cord homogenates ($p>0.05$)

These results indicate that a single LPS dose (10 µg in total) given intrahippocampally or the duration of the model was not enough to initiate AD like pathology in terms of protein aggregation for 10 days. More importantly, these results prove that amyolid aggregation precedes taupathy in this model of neuroinflammatory AD.

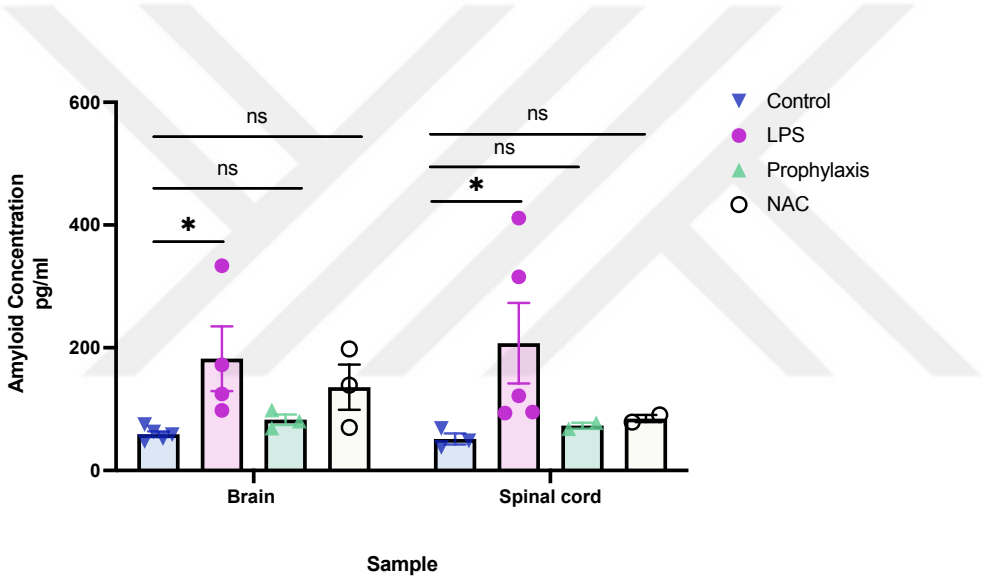

Figure 6 Aβ1–42 ELISA Results.

Kruskal Wallis results indicated an overall significant difference between groups ($p=0.0026$). Quantification of Aβ1–42 concentration in brain and spinal cord homogenates revealed a significant increase in Aβ1–42 levels in brain homogentaes in G2 compared to controls (Mann Whitney U results: $p=0.0079$). NAC clearly prevented the increase in Aβ1–42 concentration by keeping levels close to controls (Mann Whitney U results: G3 vs control $p=0.0714$, G4 vs control $p=0.0714$). Similar results were seen in spinal cord samples, where Kruskal Wallis results indicated an overall significant difference ($p= 0.0004$). Moreover, according to Mann Whitney U results, G2 homogenates displayed signficantly higher levels of Aβ1–42 than controls ($p=0.0357$).

No significant difference in Aβ1–42 concentrations between G3 and G4 for both brain and spinal cord homogenates (p= 0.400 and p=0.3333 respectively).

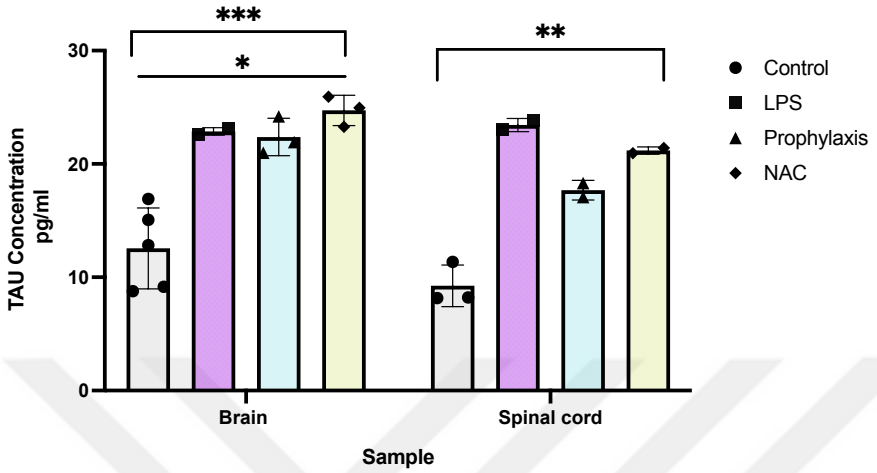

Figure 7 pTAU ELISA Results. Quantification of Tau concentration in brain and spinal cord homogenates revealed an increasing trend in LPS applied groups but it did not reach to significance (p=0.3333). Although prophylactic and non-prohylactic application of NAC show decerased trend it has no significance.

3.2. *Behavioral Results:* Administered Dose of LPS (10 μg) and/or Model Duration Did Not Cause Significant Memory Loss or Cognitive Dysfunction.

Our MWM results indicate that there was no significant difference in terms of platform retention latencies between groups (p>0.05) (Figure 7). There is however a general decrease in time taken by all groups when comparing first (corresponding to day3 after LPS induction) and last test days indicating a learning pattern in all animal groups.

This result can be explained by saying that neuronal loss (as indicate in 3.3) and amyloid aggregation as seen in abovementioned results, precedes cognitive decline in this type of model and duration of the model was not sufficient to observe potential memory loss.

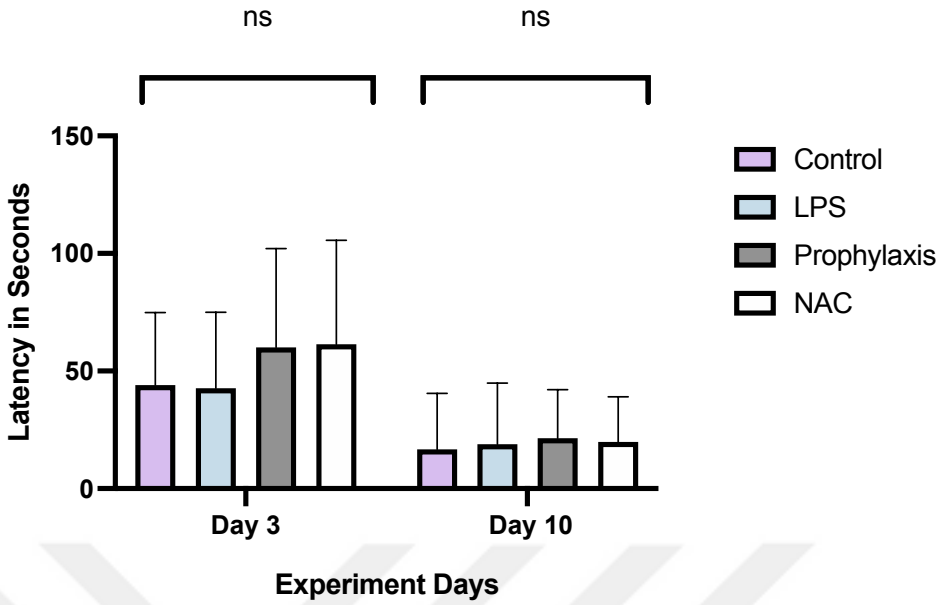

Figure 8 MWM Results. Latency in seconds taken by animals to reach the platform. Upon recording the time in seconds taken by animals of each group to reach the platform, results analysed using Kruskal Wallis test showed no signficant difference between groups, indicating no potential cognitive/memory loss indcued by LPS (p=0.9842 for Day3) and (p=0.2675).

3.3. NAC Prevented LPS Induced Neuronal Loss in Hippocampal Regions

To investigate whether the LPS dose administered, and duration of the model resulted in any significant neuronal loss, the number of NeuN positive cells was measured in different hippocampal regions (CA1, CA2, CA3 and DG) Figure *10* as well as in the parietal cortex using ImageJ software. Our results indicate that LPS induced a significant neuronal loss compared to control values ($p<0.05$). Moreover, Neuronal cell count measured in hippocampal regions of G4 (prophylactic NAC injection prior to model inudction) was significantly higher when compared with G2 ($p<0.05$) Figure *9*.

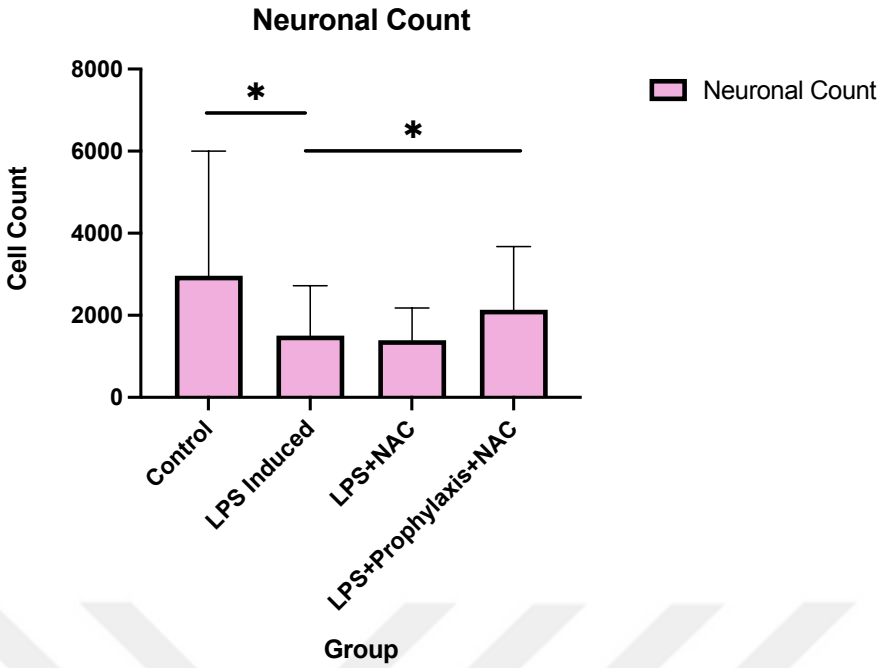

Figure 9 Neuronal Count in Hippocampal Regions. (Only significant differences are indicated). Neuron cellular count in hippocampal regions in LPS-induced group was significantly reduced when compared to controls (Mann Whitney U: p=0.0417). Prophylactic application of NAC in G4 clearly protected neuronal loss and kept neuronal counts significantly higher than LPS-induced animals (Mann Whitney U: p=0.0340) and close to controls (Mann Whitney U: p=0.8477).

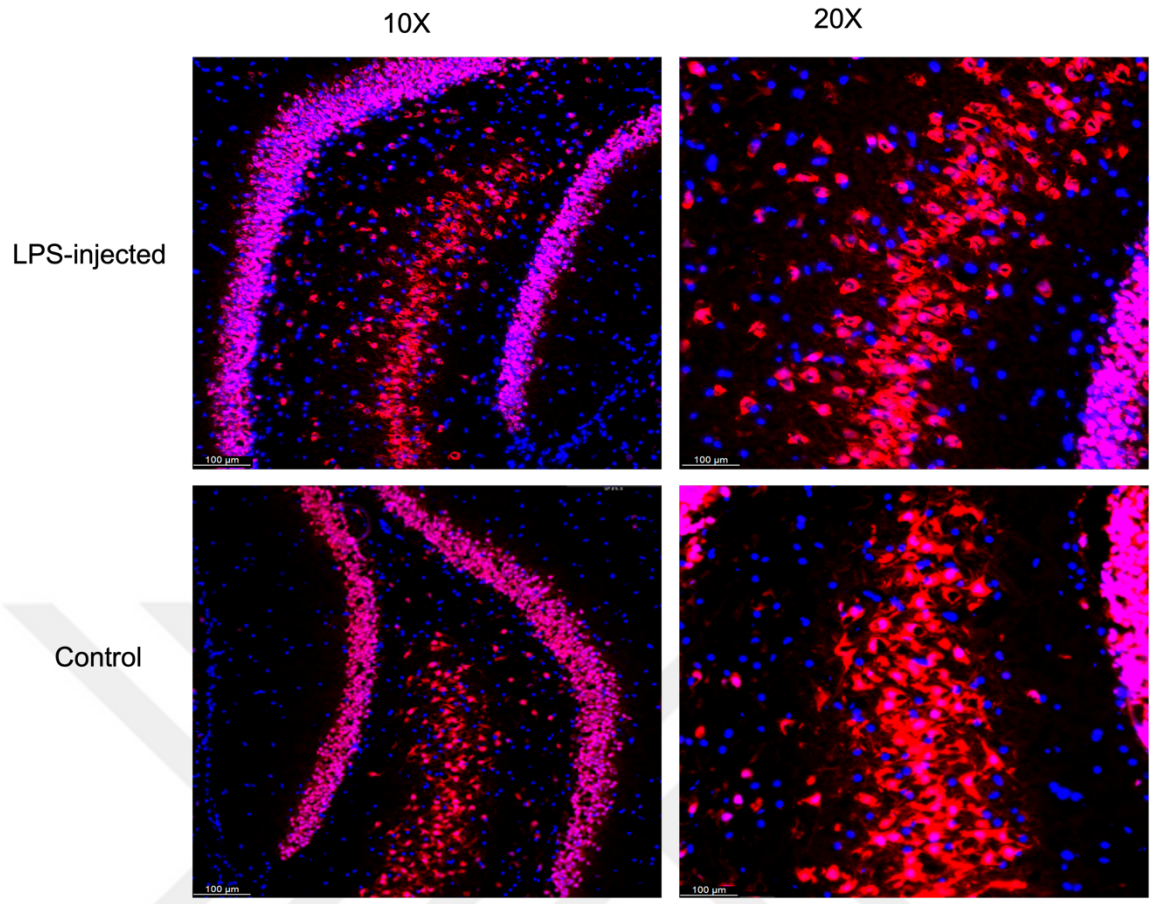

Figure 10 Representative images of brain hippocampal sections. Hippocampal sections stained for neurons by a special neuronal marker (NeuN in red) and DAPI (in blue). Upper two images are for stained sections of the LPS-induced group (G2) and lower two are from controls (G1), regions are shown in two different magnifications.

3.4. NAC Administration Reduced LPS Induced Astrogliosis

Upon measuring GFAP stained %area and integrated densities, results demonstrated a significant increase in astrocytic coverage corresponding to higher astrocyte count in hippocampal regions and parietal cortex in LPS induced animals compared to controls ($p<0.05$). Moreover, when compared to LPS induced animals, animals in both G3 and G4 showed a significant reduction in GFAP stained area and integrated density ($p<0.05$) Figure *11*.This indicates that NAC administration reversed LPS induced astrogliosis Figure *12* Figure *13*.

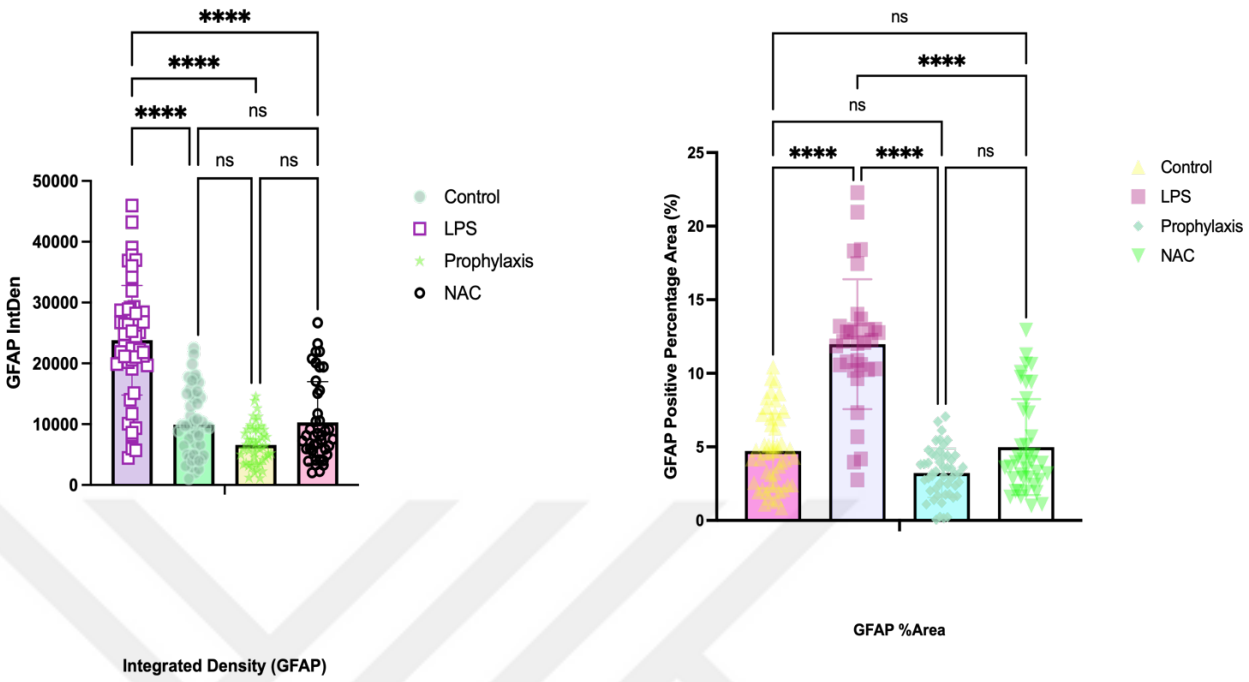

Figure 11 Analysis Results of GFAP Stainings Measures of GFAP integrated density (left) indicated an overall significant difference tested by Kruskal Wallis ($p<0.0001$). Mann Whitney U test results demonstrated a significant increase in GFAP intDen compared to controls ($p<0.0001$). NAC treated groups (G3 and G4) significantly reduced GFAP intDen when compared to G2 ($p<0.0001$ for both comparisons). Kruskal Wallis results for overall group comparisons for GFAP+ %area (right) indicated a significant difference between groups ($p>0.0001$). Mann-Whitney U results indicated an increase in GFAP stained %area in LPS group compared to controls ($p<0.0001$). G3 and G4 groups showed a significant reduction in GFAP expression when compared to LPS group ($p<0.0001$ for both).

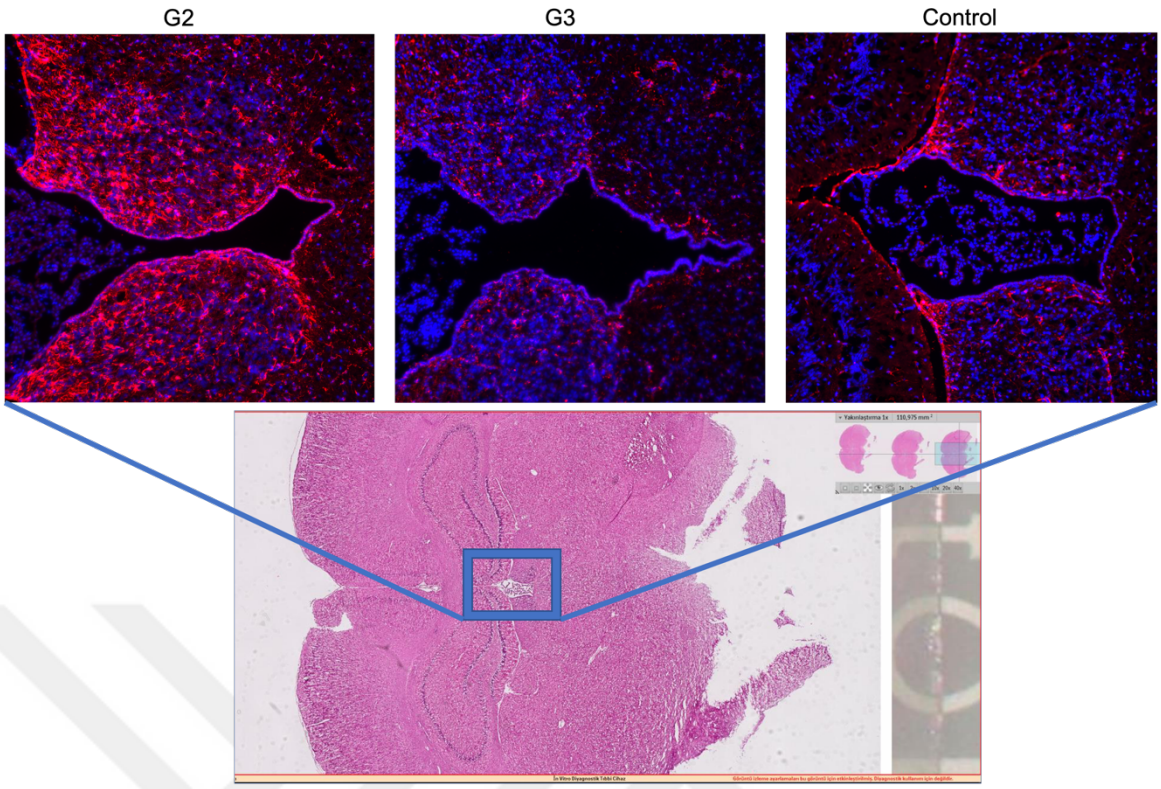

Figure 12 Representative images of GFAP stained brain sections. GFAP stained brain slices showing the third ventricle (Red: GFAP, Blue: DAPI). Animals in G2 group recieved a single intrahippocampal dose of LPS (10µg) in total to both hippocampi, there is a clear reactivity in astrocytes featured by increased GFAP expression. Animals in G2 group were treated with NAC (i.p. 200 mg/kg) for 10 days post LPS-injection. Compared to LPS-injected group, there is a clear reduction in GFAP reactivity in NAC treated brains.

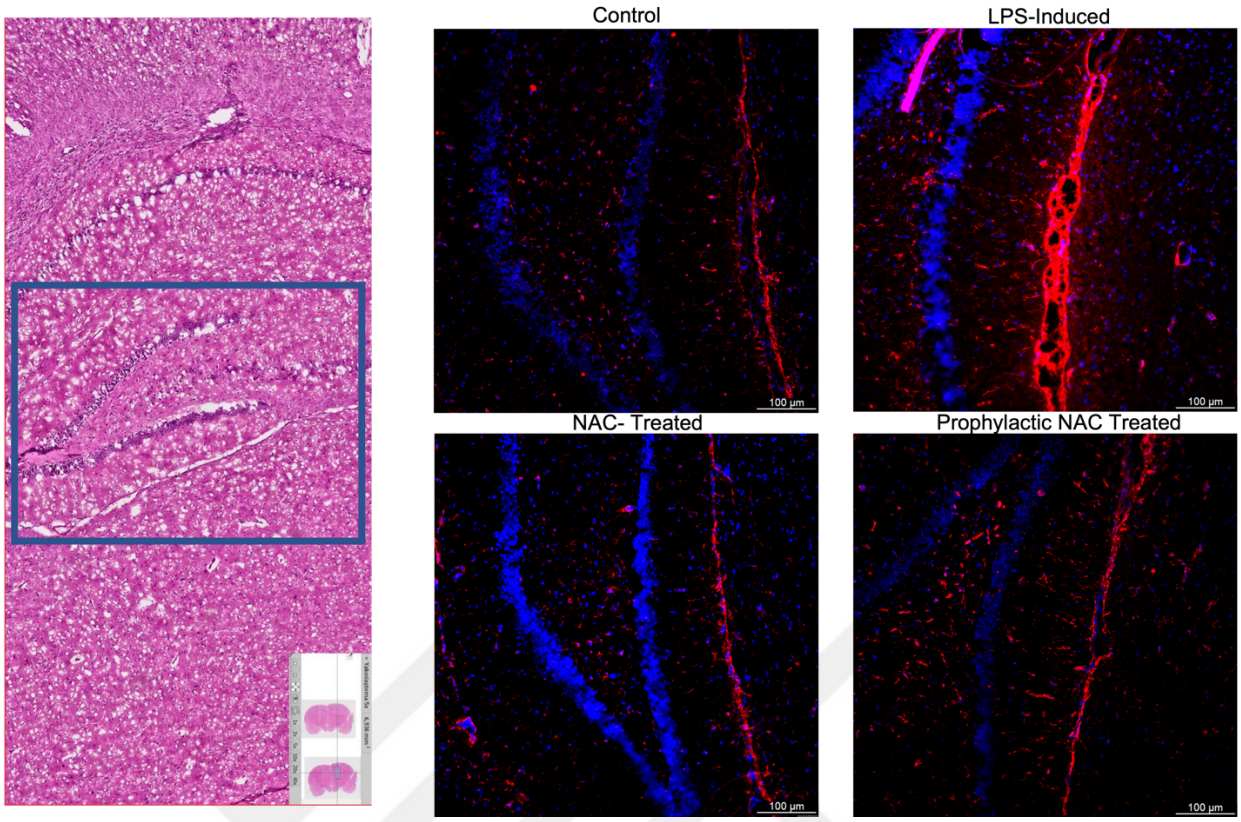

Figure 13 Representative images of GFAP stained hippampal regions (red: GFAP, Blue: DAPI). Animals the LPS group recieved a single intrahippocampal dose of LPS (10µg) in total to both hippocampi, there is a clear increase in GFAP expression compared to controls. Animals in NAC-treated group were i.p injected with NAC (200 mg/kg) for 10 days post LPS-injection. Prophyliactic-NAC treated animals recieved an additional NAC injection prior to model induction. Compared to LPS-injected group, GFAP reactivity in NAC treated and prophylactic NAC treated brains is significantly reduced.

3.5. NAC Reveresed PNN Loss Induced by LPS Injection in the Prefrontal Cortex

Staining of perineuronal nets via the lectin WFA showed a pronouced decrease in the number of PNN surrounded neurons in the prefrontal cortrex in LPS injected rats when compared to normal controls ($p<0.05$)Figure *15*. Results also demonstrate that in both LPS+NAC and LPS+Prophylaxis groups, the number of neurons

surrounded by WFA+ nets in the prefronal cortex was significantly higher than LPS treated animals ($p<0.05$) Figure *14*, Figure *16*.

This indicates a protective role of NAC against inflammatory LPS-induced perineuronal net loss in adult animals.

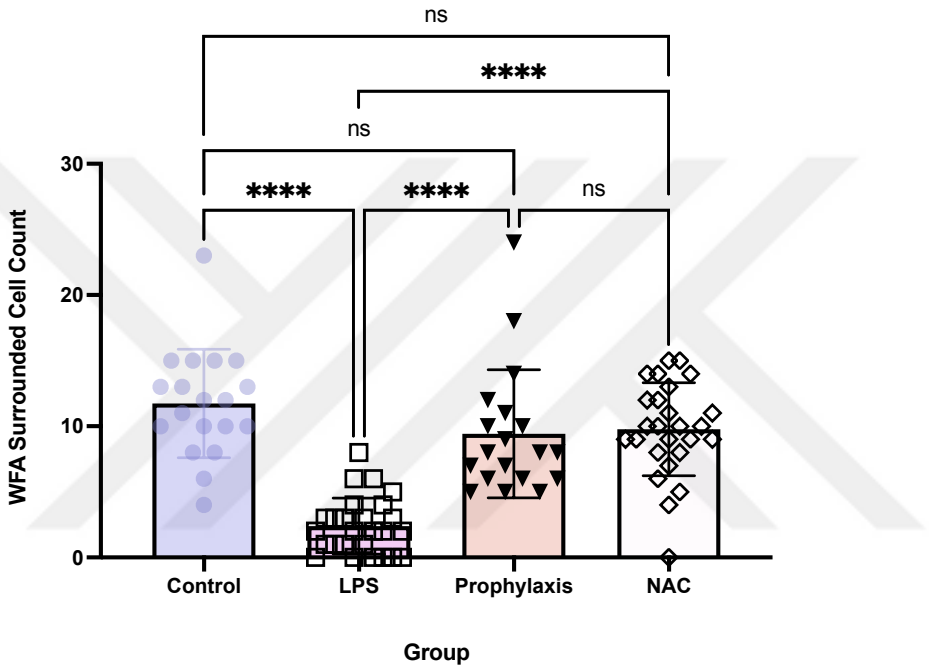

Figure 14 Quantification of PNN surrounded neurons in the PFC. Kruskal Wallis test indicated an overall significant difference between all groups ($p<0,0001$). Average count of WFA (PNN marker) surrounded neurons in the PFC of LPS-injected animals were significantly lower than controls ($p<0,0001$). Average number of WFA surrounded cells in NAC treated animals (G3) and prophylaxis group (G4) was significantly higher when compared to LPS-injected group ($p<0,0001$).

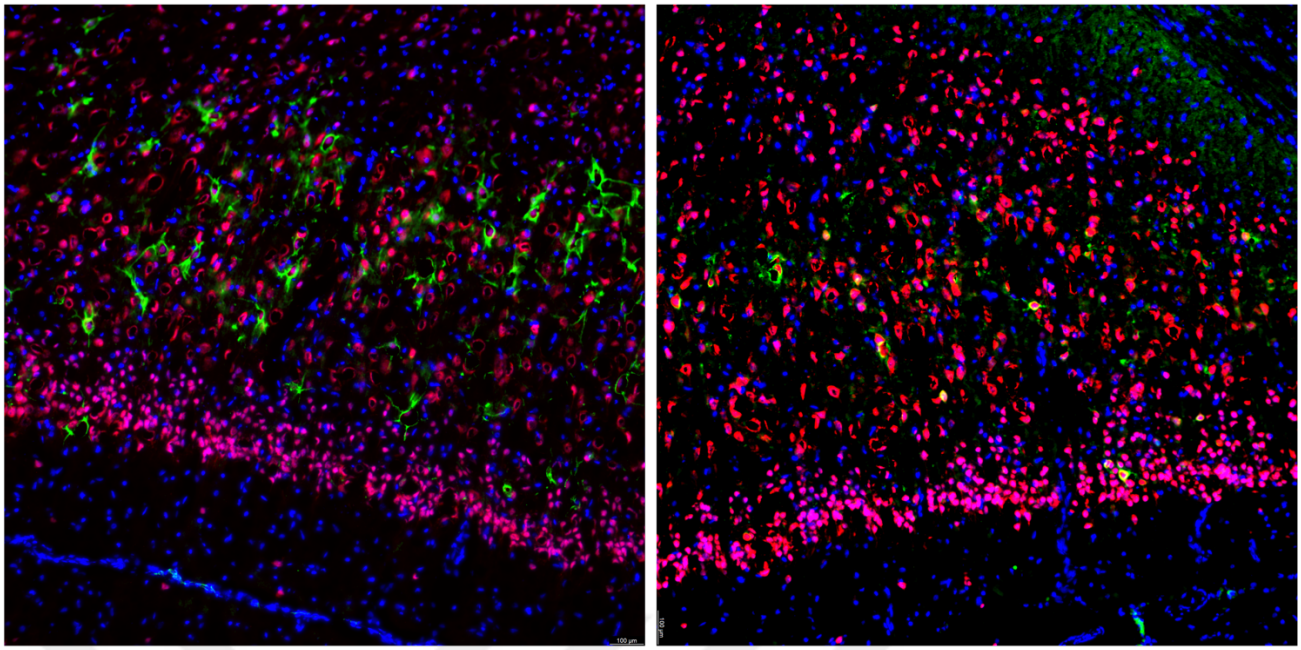

Figure 15 Loss of PNNs in LPS induced animals. On the left, IF stained brain slice of the PFC from a control animal (NeuN in red indicating neurons, WFA in green staining PNN structures and DAPI in blue). On the right: same staining applied on a brain slice of the PFC from an LPS-injected animal. Images depict a prominent loss of WFA+ neuronal surroundings in LPS-injected animals compared to controls.

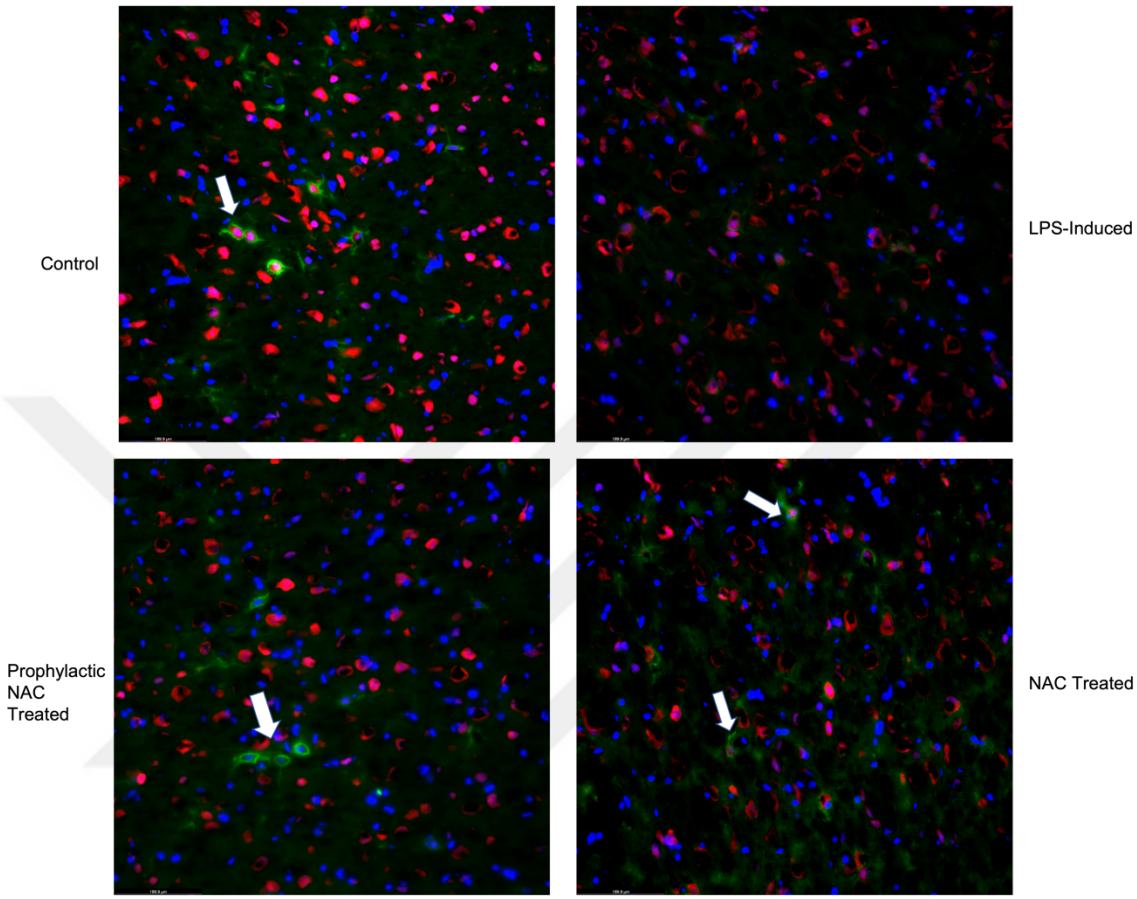

Figure 16 WFA stained PFCs in all groups. PNN surrounded neurons (white arrows). Red: NeuN, Blue: DAPI, Green: WFA. Staining against PNNs with WFA revealed a significant loss in PNN structures in regions of the PFC in LPS-induced animals. Stained brain slices from prophylactic NAC treated and NAC treated groups indicated that NAC provided some preservation of PNN structures against LPS.

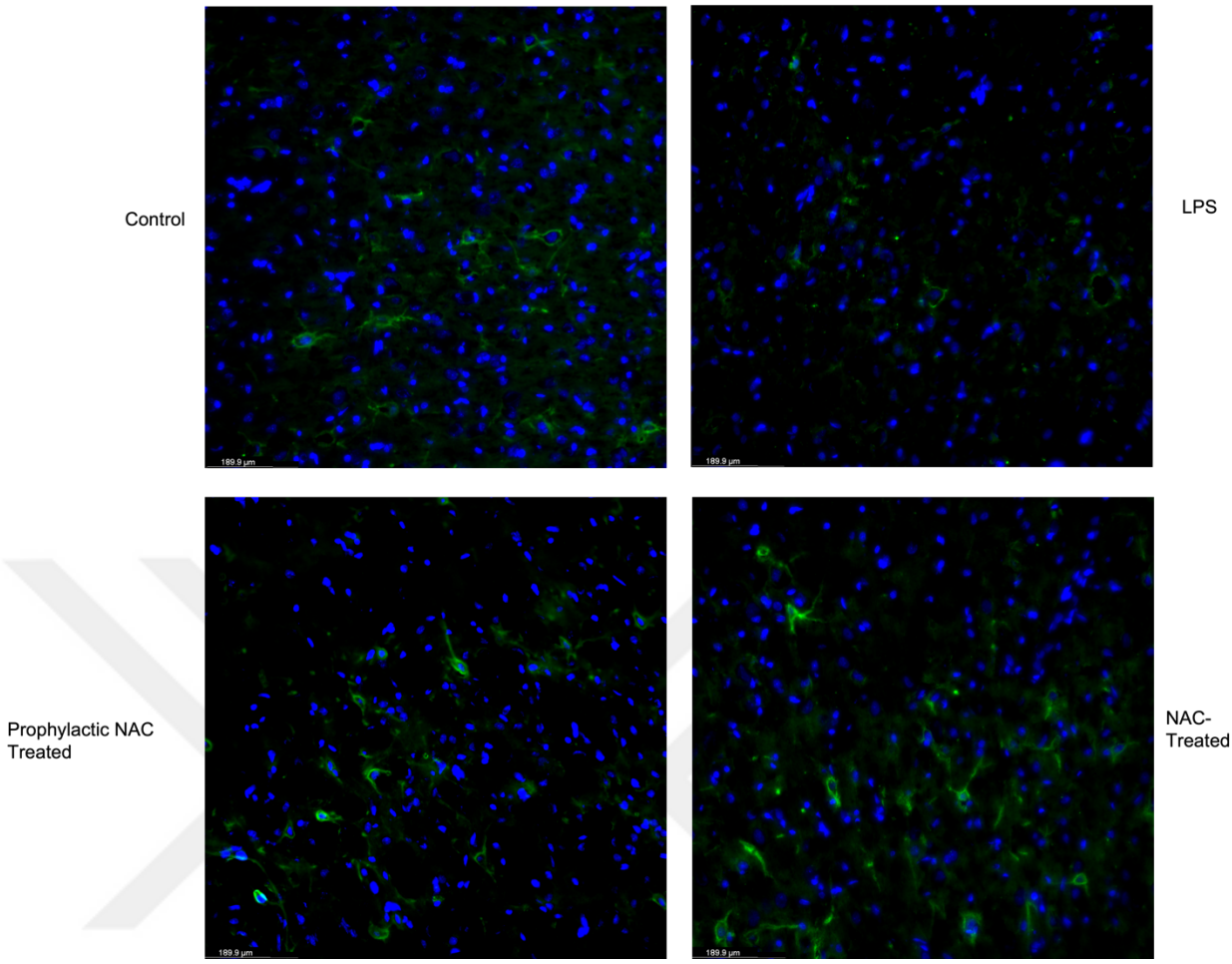

Figure 17 WFA stained PFCs in all groups. (Blue: DAPI, Green: WFA). Staining against PNNs with WFA revealed a significant loss in PNN structures in regions of the PFC in LPS-induced animals. Stained brain slices from prophylactic NAC treated and NAC treated groups indicated that NAC provided some preservation of PNN structures against LPS.

Chapter 4:

DISCUSSION

In this study, we aimed at demonstrating the neuroprotective role of NAC in an acute neuroinflammation model induced by intrahippocampal LPS in rats. Our goal was to assess the ability of NAC to attenuate LPS-induced components of neuroinflammation such as protein accumulation, neuronal loss and astrocytic activation. Moreover, we aimed at investigating whether LPS-induction in adult rat brain could lead to perineuronal net disruptions. It is important because it was long thought that these structures are almost impossible to reassemble after the closure of the critical period of plasticity, a structurally dynamic time window for perineuronal nets that allows the formation of new synapses. Upon the closure of the critical period, these specialized reticular structures provide a protective shield for neurons against environmental stressors like amyloid accumulations and oxidative stress components (Crasper et al, 2020), but it also limits the formation of new connections between neurons. Since our model was induced in adult rats, where perineuronal nets are shown to be fully mature and had lost their juvenile state-like plastic abilites, we wanted to assess the state of PNNs upon LPS-induced inflammation. Moreover, we wanted to investigate the ability of NAC to preserve PNN structures against inflammatory insult induced by LPS. Upon quantifying PNN surrounded neurons in the PFC of all groups, we found that intra-hippocampal LPS-injection caused a significant decrease in WFA+ neuronal surroundings, corresponding to loss in PNN structures. These results can be discussed with reference to findings presented by Crasper et al (2020), where they indicated that PNN loss in mouse models of AD is linked to microglial activation, and pharmacological depletion of microglia actually attenuated PNN loss. Moreover, their results demonstrate that degraded components of PNNs are found accumulated inside

dense core plaques (Crasper et al, 2020). Overall, rising evidence is clearly highlighting the importance of glial induced-ECM loss in models of neuroinflammation, as a bridge to undertsand neuronal vulnerability to oxidative damage and inflammatory insult. Furthermore, our results indicated that a single intra-hippocampal dose of LPS (10 μg) increased amyloid concentrations in brain and spinal cord homogenates when assessed with ELISA. These results align with previous literature demonstrating the impact of acute LPS-injection in inducing amyloidosis in rodents (Deng et al, 2014) (Batista CRA et al, 2019) (Wang et al, 2018) (Lee et al, 2008). However, Tau concentration measured by ELISA indicated a rising pattern detected in brain and spinal cord homogenates of LPS-injected animals but results did not reach significance. In their study, Wang et al (2018) showed that LPS given i.p at a dose of 10 mg/kg caused a signficant increase in phosphorylated Tau when measured by immunoblotting on brain homogenates of Sprague Dawley rats 24h post administration, their results also indicated significantly high levels of Aβ 7 to 9 days post LPS injection but no correlation was made between both results. Previous literature indicates a probable causative role of Aβ accumulation in the formation of Tau tangles (Götz et al, 2001) (Lewis et al, 2001). However, no definitive conslusion has been made regarding the progressive impact of LPS in such models, especially that animal type and age, as well as route and duration of administration, all contribute to highly heterogenous pathological phenotype (Batista et al, 2019) (Zakaria et al, 2017).

Assesed by Morris water maze 10 days after injection, animals in all experimental groups displayed a learning behaviour featured by reduced platform retention latency. However, when compared to controls, no significant difference in latency was seen in LPS-induced animals; which can be explained by the acute duration of our model (Zakaria et al, 2017) and that the resulting hippocampal neuronal loss seen in our LPS-induced group was not yet sufficient to cause memory dysfunction, whereas chronic-LPS injection was shown to cause spatial memory deficits (Wegrzyniak, 2018). These results align with findings indicated by (Bossu et al, 2019), where a single LPS injection in male Wistar rats caused no significant spatial memory loss tested by MWM 7 days post administration; our results also align with a study (Czerniawski, 2015) showing that acute LPS administration on rats does not affect MWM performance as it selectively disrupts other certain memory types. Different findings, however, where presented by Yamada et al, (1999), where a single intra-hippocampal LPS injection in

Wistar rats (5μg) resulted in spatial memory disturbance assessed by MWM 10 to 15 days post-administration. In a different study (Lee et al, 2008), rats injected intraperitoneally with (250 μg/kg) dose of LPS showed a worse performance in MWM test compared to controls.

In the present work, we demonstrated that LPS injected into the hippocampus caused significant neuronal loss evident by IF staining, these results align with previous literature (Yamada et al, 1999) (Lee et al, 2008). Astrocytes take up a percentage of about 1/3 of all cells in the CNS, occupying an important regulatory and immune function as well as providing trophic support to neurons. In cases of CNS insult, there is an upregulation in GFAP in astrocytes that indicates neurotoxic reactivity and transition to A1 phenotype and is mostly prominent close to injured brain regions (Giovannoni and Quintana, 2020). This phenotypic transition was shown to be associated with LPS-induced microglial activation (Liddelow et al, 2017). Thus, aligning with our expectations and previous literature (Fu *et al.* 2014) (Houdek et al, 2014), LPS-injection also produced significant astrogliosis indicated by elevated GFAP staining in hippocampal regions. Astrocytic activation in LPS-treated adult rat hippcampus is believed to play a role in clearing neuronal apoptotic debris (Cerbai et al, 2012). However, it is still not clear whether this reactivity is responsible for initiating the vicious cycle of neuronal damage or is solely a protective reaction to halt to progression of damage.

NAC is a natural compound with evident anti-oxidant and anti-inflammatory impact acting as a scavenger for ROS and regulating the release of critical inflammatory cytokines (Mokhtari et al, 2017). Having no recorded side effects (Tardiolo et al, 2018) it has attracted the interest of medical researchers for decades, and raised hope for its potential therapeutic applications in neurodegenerative and neuropsychiatric disorders. In our model, we used NAC by giving it intraperitoneally to LPS-injected rats. As we further wanted to test potential prophylaxis impact, we incorporated an additional experimental group in which animals recieved an i.p dose of NAC (200 mg/kg) prior to model induction as well as further treatment doses. Our results indicated that prophylactic NAC treatment prevented LPS-induced neuronal loss, however, direct treatment administration did not show significant neuronal protection when compared to LPS-injected group. These findings indicate a potential neuro-protective impact of prophylactic NAC use against acute neuroinflammatory cellular loss, and align with

previous evidence on its anti-apoptotic abilities through reducing damage associated NF-*k*B and TNF secretions (Shahripour et al, 2014) as well as in aging and neurodegenerative inflammation (Chandra et al, 2000). Another study conducted by Fu et al (2006), highlighted that pretreatment with NAC (i.p. 200 mg/kg) significantly preserved neuronal count as well as learning and memory in Aβ-treated mice. Additionally, NAC treatment (prophylactic and post-LPS injection) kept Aβ concentration in brain and spinal cord samples close to controls while it significantly elevated by LPS-induction; interestingly, in their study (Joy et al, 2019) showed that i.p. administration of NAC a week before and a week after ICV colchicine could not reduce amyloid pathology. On the other hand, NAC was able to reduce astrogliosis in their model which is what we also demonstrated in the present study; Both pre-treated and post-LPS injection NAC treated groups indicated a prominently significant reduction in brain astrogliosis when compared to LPS-injected groups.

Furthermore, our present work highlights the potential role of NAC in rescuing and maintaining PNN loss associated with inflammatory damage. Our results clearly indicate that NAC treated groups had more intact PNN strcutures and higher number of WFA+ stained perineuronal areas when compared to LPS-induced groups.

Indeed, these findings are important because PNNs serve as the intial protective barrier for neurons against inflammatory and oxidative stress insult. If this natural compound could preserve the integrity of PNN structural deformation, it could hold an important neuro-protective potential against acute inflammatory damage, and opens up a new path in the field of neurodegeneration research focusing on the role of ECM and neuronal vulnerability in certain pathological conditions.

Chapter 5

CONCLUSION

To conclude, as variable and heterogenous, models of neuroinflammation could bring us closer to deciphering important molecular pathways and help us understand the order of pathological processes involved in neurodegeneration. Also, understanding what happens in acute states holds promise for possible early intervention. Furthermore, neuron-extracellular matrix interactions play an essential role in several neurodegenerative diseases, thus, understanding the basic mechanisms underlying this phenomenon helps in guiding towards better and more efficient treatments.

PNNs have only recently attracted focus and we still lack a significant knowledge of their structures and ways of action in oxidative stress related pathology and synaptic plasticity.

This present study provides a step towards understanding the link between neurodegenration and perineuronal net disruptions, and highlights the potential of NAC in preventing early inflammatory insult.

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