T.C.

DOKUZ EYLUL UNIVERSITY

IZMIR INTERNATIONAL

BIOMEDICINE AND GENOME

INSTITUTE

THE ROLE OF LONG NONCODING RNA NEAT1 IN REGULATION OF MICROGLIAL NLRP3 INFLAMMASOME ACTIVATION

BORA TAŞTAN

MOLECULAR BIOLOGY AND GENETICS

Ph.D. THESIS

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Supervisor: Prof. Dr. Şermin GENÇ

(This research is supported by TUBITAK 1001 Grant **219S939**)

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'The role of Long Noncoding RNA NEAT1 in regulation of microglial NLRP3 inflammasome activation'

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LIST OF ABBREVIATIONS

AD: Alzheimer's Disease

ALS: Amyotrophic Lateral Sclerosis

ASC: Apoptosis-associated Speck-like protein containing a CARD

ATP: Adenosine Triphosphate

BCA: Bicinchoninic Acid Assay

bp: Base pair

BSA: Bovine Serum Albumin

CNS: Central Nervous System

DAMPs: Danger-associated Molecular Patterns

ELISA: Enzyme-linked Immunosorbent Assay

FBS: Fetal Bovine Serum

FISH: Fluorescence in Situ Hybridization

FST: Forced Swimming Test

GSDMD: Gasdermin D

HD: Huntington's Disease

IF: Immunofluorescence

IL-18: Interleukin 18

IL-1β: Interleukin 1, beta

KO: Knock-out

LDH: Lactate Dehydrogenase Assay

lncRNAs: Long Noncoding RNAs

LPS: Lipopolysaccharide

miRNAs: MicroRNAs

mmu: *Mus Musculus*

MS: Multiple Sclerosis

NEAT1: Nuclear Paraspeckle Assembly Transcript 1

NLRP3: NLR Family Pyrin Domain Containing 3

ncRNAs: Noncoding RNAs

OFT: Open Field Test

PAMPs: Pathogen-associated Molecular Patterns

PD: Parkinson's Disease

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

RIP: RNA Immune Precipitation

RNA: Ribonucleic Acid

RPMI: Roswell Park Memorial Institute

RT: Room Temperature

SDS PAGE: Sodium Dodecyl-sulfate Polyacrylamide Gel Electrophoresis

siRNA: small interfering RNA

TST: Tail Suspension Test

WB: Western Blotting

WT: Wild type

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THE ROLE OF LONG NONCODING RNA NEAT1 IN REGULATION OF MICROGLIAL NLRP3 INFLAMMASOME ACTIVATION

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ABSTRACT

Microglia, resident macrophages, maintain homeostasis in the central nervous system and are able to respond to immune responses. Microglial activation may cause neuroinflammation, which is associated with various diseases observed in the CNS. Inflammasome activation is one of the causative mechanisms for inflammation in the CNS. Inflammasomes are evolutionarily conserved immune response mechanisms, and among them, the NLRP3 inflammasome complex, the most and best studied, has been associated with cardiovascular, autoimmune, and neurodegenerative diseases. NLRP3 inflammasome complex elicits immune responses to a wide range of molecular motifs than others, indicating the importance of the NLRP3 inflammasome complex. Therefore, regulation of NLRP3 inflammasome activation is of great importance.

Long non-coding RNAs, RNA molecules >200 bp, have been identified as key players in gene expression, transcriptional and post-transcriptional regulation, and epigenetic control. NEAT1 is a lncRNA located in paraspeckles and functions as a critical structural component. Evidence suggests that NEAT1 is associated with inflammation; In particular, it is thought to have a role in the regulation of NLRP3 inflammasome.

In our project, we aimed to determine the role of NEAT1 in the regulation of NLRP3 inflammasome activation. Therefore, we investigated whether lncRNA NEAT1 is involved in the regulation of microglial NLRP3 inflammasome activation utilizing *in vitro* and *in vivo* models. Since microglial NLRP3 inflammasome activation is at the center of diverse CNS diseases (neurodegenerative, neuropsychiatric, neuroimmune diseases), it is anticipated that the information obtained within the scope of the project will contribute to the treatment or prevention of these diseases.

Keywords: Microglia, NLRP3 Inflammasome, LncRNA, NEAT1

MİKROGLİAL NLRP3 İNFLAMAZOM AKTİVASYON REGÜLASYONUNDA UZUN-KODLAMA YAPMAYAN RNA NEAT1'İN ROLÜ

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ÖZET

Mikroglia, santral sinir sisteminde homeostazı sağlamakla görevli, immün yanıt verebilenmiyeloid kökenli hücrelerdir. Santral sinir sisteminde görülen birçok hastalığın patogenezinde, mikroglia aktivasyonuyla ortaya çıkan nöroenflamasyonun rolü bulunmaktadır. İnflamazom aktivasyonu, mikroglial aktivasyon ile ortaya çıkan inflamasyonun kaynaklarından birisidir. İnflamazomlar, doğal bağışıklık sisteminin bir parçası olup, evrimsel olarak korunmuş olan immün yanıt mekanizmalarından birisidir. İnflamazom komplekslerinin arasında, en çok çalışılmış olan NLRP3 inflamazom kompleksinin aktivasyonu, kardiyovasküler, otoimmün ve nörodejeneratif hastalıklar ile ilişkilendirilmiştir. Ayrıca, NLRP3 inflamazom kompleksinin, diğer inflamazom komplekslerinden daha fazla moleküler motife karşı immün yanıt oluşturması, NLRP3 inflamazom kompleksinin önemini göstermektedir. Bu sebeplerden dolayı, NLRP3 inflamazom aktivasyonunun regülasyonu da büyük önem taşımaktadır.

Uzun kodlama yapmayan RNA'lar (lncRNA'lar), epigenetik düzenleme yoluyla gen ekspresyonunun düzenlenmesi, transkripsiyonel düzenleme ve transkripsiyon sonrası düzenleme gibi çeşitli biyolojik işlevlerde yer aldığı bildirilen >200 baz çifti uzunluğunda kodlamayan RNA'lardır. Nuclear Enriched Abundant Transcript 1 (NEAT1), özellikle parabeneklerde bulunan ve temel bir yapısal belirleyici olarak işlev gören bir lncRNA'dır. Literatürdeki kanıtlar, NEAT1'in karmaşık bir şekilde inflamasyon ile ilişkili olduğunu öne sürmektedir; özellikle de NLRP3 inflamazomunun regülasyonunda rolü olabileceği düşünülmektedir.

Projemizde, mikroglial NLRP3 inflamazom aktivasyonunda, uzun kodlama-yapmayan RNA molekülü olan NEAT1'in, NLRP3 inflamazom aktivasyon regülasyondaki rolünün

belirlenmesi amaçlanmıştır. Bu doğrultuda, projemizde mikroglial NLRP3 inflamazom aktivasyon regülasyonunda lncRNA'lardan NEAT1'in yer alıp almadığının *in vitro* ve *in vivo* modeller ile araştırılmıştır. Mikroglial NLRP3 inflamazom aktivasyonunun, birçok santral sinir sistemi hastalığının (nörodejeneratif, nöropsikiyatrik, nöroimmün hastalıklar) merkezinde bulunmasından dolayı, proje kapsamında elde edilen bilgilerin bu hastalıkların tedavisinde veya önlenmesine katkı sağlayacağı öngörülmektedir.

Anahtar Sözcükler: Mikroglia, NLRP3 İnflamazom, LncRNA, NEAT1

1. INTRODUCTION & AIM

1.1. Aim of the study

Microglia, the primary immune cells in the central nervous system, act as the initial barrier against infections and injuries. When triggered by internal or external factors, microglia generate and release inflammatory cytokines and other signaling molecules. Prolonged overactivation of microglia and dysregulated neuroinflammation can result in neuronal cell death and worsen neuroinflammation, potentially contributing to the development of neurodegenerative diseases. The NLR Family Pyrin Domain Containing 3 (NLRP3) inflammasome complex, a crucial component of the innate immune system, is activated in response to "pathogen-associated molecular motifs" (PAMPs) such as microbial or viral infections, as well as "damage-associated molecular motifs" (DAMPs) like cellular damage and reactive oxygen species. This activation leads to the activation of Caspase-1, which subsequently triggers the cleavage and release of proinflammatory cytokines Interleukin -1β/Interleukin-18 (IL-1β/IL-18). Aberrant activation of the NLRP3 inflammasome has been linked to various conditions, including cancer, autoimmune diseases, cardiovascular diseases, Alzheimer's disease, and Parkinson's disease. Therefore, regulation of the NLRP3 inflammasome is of great importance.

Long non-coding RNAs (lncRNAs) are RNA molecules longer than 200 base pairs (bp) that do not encode proteins but play a role in various biological processes such as gene expression regulation, transcriptional control, and post-transcriptional regulation via epigenetic mechanisms. One specific lncRNA called Nuclear Enriched Abundant Transcript 1 (NEAT1) is predominantly present in paraspeckles, specialized structures within the nucleus, and serves as a crucial structural component. Existing evidence suggests that NEAT1 is strongly associated with inflammation, particularly in the context of regulating the NLRP3 inflammasome.

Thus, we aimed to unravel whether lncRNA NEAT1 is involved in the regulation of microglial NLRP3 inflammasome activation. This aim has brought the following objectives along;

1. Determination of NEAT1 expression change in LPS and ATP-induced NLRP3 inflammasome activation in N9 and HMC3 microglial cells *in vitro*

2. Determination of change in RNA-binding proteins and the paraspeckle structures inLPS and ATP-induced NLRP3 inflammasome activation *in vitro*

3. *In vitro* NEAT1 silencing with siRNA and investigation of inflammasome parameters

4. Investigation of NLRP3 inflammasome parameters in NEAT1 knock-out mice *in vivo*

5. Determination of the relationship between NEAT1 - miR-146a-5p

1.2. The hypothesis of the study

H1: lncRNA NEAT1 regulates microglial NLRP3 inflammasome activation.

H2: lncRNA NEAT1 exerts regulatory effects on microglial NLRP3 inflammasomeactivation through miR-146a-5p

2. BACKGROUND INFORMATION & LITERATURE

2.1. Microglia

Microglia, constituting approximately 10% of the total cells within the brain and spinal cord, are the macrophages that reside in the central nervous system (CNS). (Masuda, Sankowski, Staszewski, & Prinz, 2020). Microglia play essential roles in maintaining brain homeostasis, immunological surveillance, synaptic plasticity, and response to injury and illness. In the early 20th century, Pio del Rio-Hortega provided the initial characterization of microglial cells (Amanda Sierra, Paolicelli, & Kettenmann, 2019). Microglia continuously scan their microenvironment, stretching and retracting their processes to watch over synaptic and neuronal activity. By eliminating damaged synapses, they play a critical part in synaptic pruning throughout development (Borst, Dumas, & Prinz, 2021). Microglia also have an essential role in synaptic remodeling, supporting memory, learning, and plasticity in the adult brain.

The primary immune cells in the CNS are microglial cells, in addition to their other functions. When the brain is injured or infected, microglia become activated and give an immune response against the threat by releasing cytokines, chemokines, and reactive oxygen species (Olcum, Tastan, Kiser, Genc, & Genc, 2020). They promote tissue regeneration and remove potentially hazardous substances by phagocytosing pathogens, dead neurons, and cellular debris. However, neuroinflammation and neurodegenerative disorders can be exacerbated by deregulation of microglial activation. Chronic activation of microglia leading to excessive inflammation and the generation of neurotoxic substances is associated with the onset of various diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis, and amyotrophic lateral sclerosis (ALS). (Madore, Yin, Leibowitz, & Butovsky, 2020).

2.1.1. Discovery and origin of microglia

Microglia, distinguished by their distinct morphology and immune functions, have long been considered the CNS's resident immune cells. However, it was not known that

neuroglia is made up of astrocytes, oligodendrocytes, and microglia when Virchow introduced the idea of neuroglia in which he defined neuroglia as a sticky material that links neuroglia cells to neurons (Jäkel & Dimou, 2017). Pio Del Rio-Hortega was the first person to describe phagocytic and migrating cells in the CNS, and he proposed that they could have a mesodermal origin (Amanda Sierra et al., 2019). In a short period of time, he introduced the name "Microglia" and reclassified these cells as non-neuronal, non-astrocytic CNS components (Kabba et al., 2017). Over time, investigations have provided insights into the precise cellular lineage and developmental origins of microglia. Ginhoux et al. (2010) conducted studies employing fate mapping and ancestry tracing techniques, which revealed that microglia originate from primitive erythro-myeloid progenitors in the yolk sac. (Ginhoux et al., 2010).

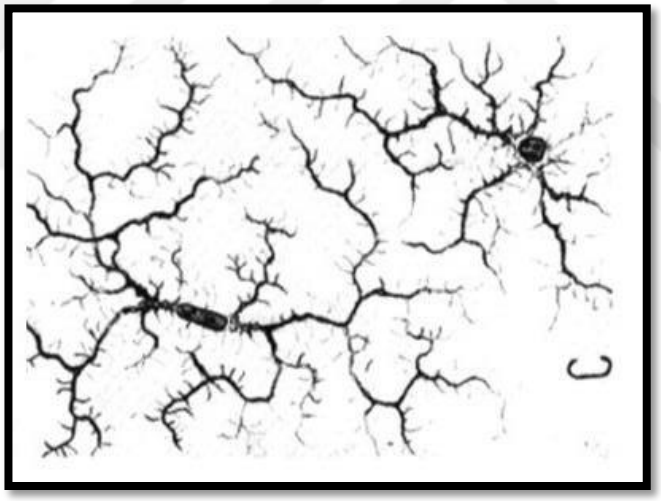

Figure 1. *Illustration of ramified microglia drawn by neuroscientist Pio del Rio-Hortega, who discovered microglial cells (Kettenmann, Hanisch, Noda, & Verkhratsky, 2011).*

These primitive progenitors leave the yolk sac and migrate to the developing brain. These cells colonize the neuroepithelium around E9.5 and undergo microglial differentiation. A recent study by Elmore *et al*. also contributed to unraveling the postnatal origin of microglial cells (Elmore et al., 2014). In the study, microglia in the

CNS of adult mice were eliminated by the use of CSF1R (important regulator myeloid cells) inhibitors. After 1 week, they discovered a subset of microglia in the adult brain derived from neural progenitor cells that express nestin. These neural progenitor-derived microglia were repopulated in the CNS.

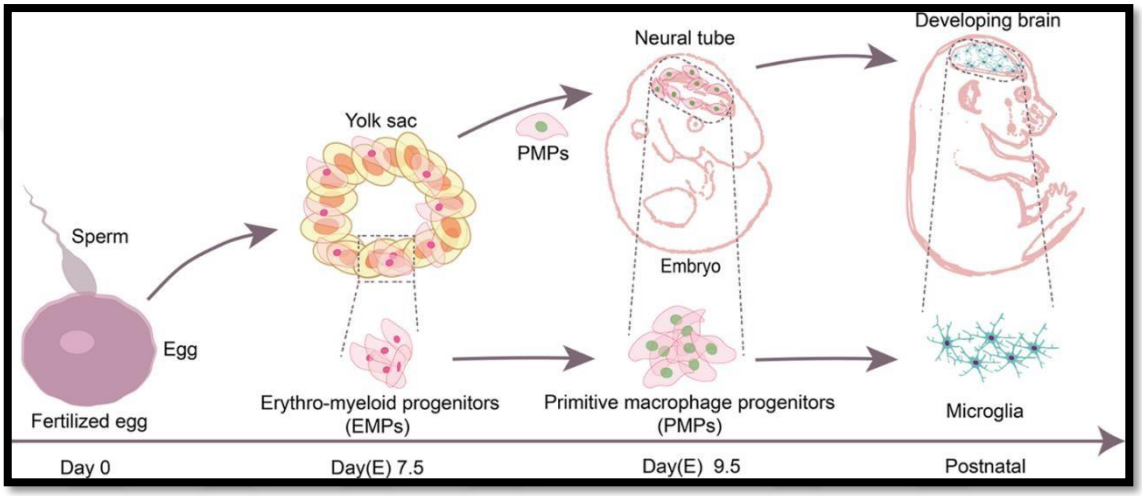

Figure 2. *The origination of microglia from the mesoderm cell lineage called erythro-myeloid progenitors in prenatal development (W. Zhang et al., 2023).*

2.1.2. Physiological Functions of Microglia

Microglial cells in the central nervous system (CNS) act as scavengers and perform vital functions in maintaining homeostasis. Microglial cells also retain other functions throughout the stages of development, adulthood, and aging. Research conducted by Tremblay et al. has demonstrated the essential role of microglia in the development of synapses, as well as in synaptic remodeling and plasticity in the developing brain (Tremblay, Lowery, & Majewska, 2010). They are responsible for carrying out functions such as maintaining homeostasis, promoting tissue healing, and conducting surveillance activities for neurons and tissues (Kettenmann et al., 2011; A. Sierra et al., 2010). They are also responsible for the elimination of damaged agents, apoptotic cells, and

misfolded proteins. According to Marin-Teva et al. (2011), it also plays an active role in initiating developmental apoptosis of Purkinje cells and the production of programmed death signals in neurons(Marin-Teva, Cuadros, Martin-Oliva, & Navascues, 2011). During the course of their work, microglial cells engage in a process known as phagocytosis. Two distinct kinds of receptors are connected to the phagocytosis processthat is carried out by microglial cells. These are Toll-like receptors (TLR), which permit the detection of pathogens that stimulate primary inflammation, and receptor-2 (TREM2) receptors produced in myeloid cells, which are critical for the anti-inflammatory response (Fu, Shen, Xu, Luo, & Tang, 2014). Toll-like receptors are responsible for the recognition of pathogens that stimulate primary inflammation. A pro-inflammatory response is produced when TLRs are stimulated and pathogens are ingested, but an anti- inflammatory response is produced when the apoptotic material is eliminated, and phosphatidylserine residues are identified (Piccio et al., 2007).

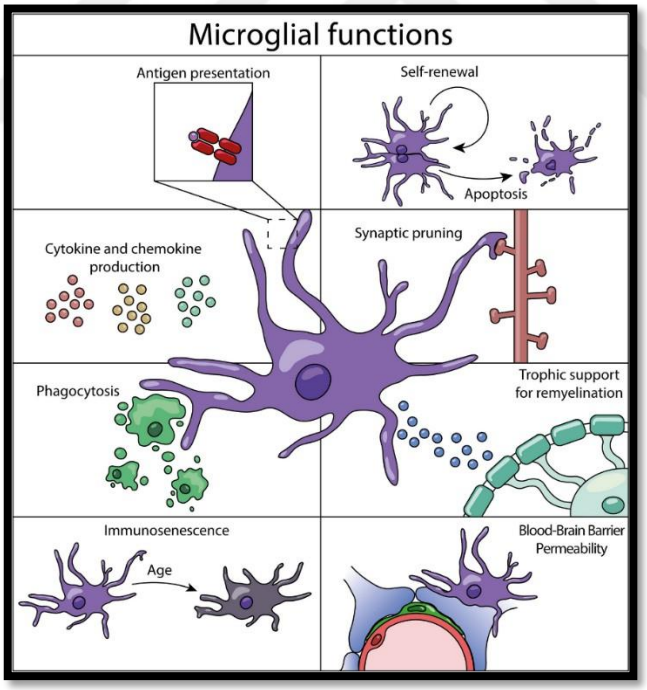

Figure 3. *The representation of physiological functions performed by microglia in the CNS (Amor et al., 2022).*

Research that was carried out on an animal model of multiple sclerosis (MS) demonstrated that TREM2 had a role that is protective. It has also been demonstrated that changes in TREM2 considerably increase the likelihood of developing PD, ALS, andAD (Cady et al., 2014; Pottier et al., 2013; Rayaprolu et al., 2013). Microglia are necessary for proper brain function; nevertheless, unregulated or overactive microglia can cause neurotoxicity. Microglia are essential for normal brain function. According to Takeuchi et al., the release of glutamate by activated microglia is a factor in the occurrence of extracellular autonomic neuronal injury (Takeuchi et al., 2011). According to Tremblay et al. (2010), activated microglia are capable of producing reactive oxygen species (ROS) via the enzyme nicotine adenine dinucleotide hydrophosphate (NADPH) oxidase and inducing poor blood-brain-barrier function (Tremblay et al., 2010). Several neurotrophic factors that are generated by activated microglial cells have an effect on the survival of neurons. These neurotrophic factors include IL-3 and IL-10, neurotrophin- 4/5, Nerve Growth Factor, TGF-, glia-derived neurotrophic factor, and fibroblast growth factor-2 (Nakajima, Tohyama, Maeda, Kohsaka, & Kurihara, 2007). microglial cells are responsible for controlling both synaptic function and synaptic development (Bilimoria & Stevens, 2015). The activity of neural precursor cell pools is controlled by microglial cells. In addition, microglial cells are the ones responsible for getting rid of extra synapses that are formed when neural precursor cells mature into neurons and generate more synapses than are required(Cunningham, Martinez-Cerdeno, & Noctor, 2013). Furthermore, microglial P2X4 receptors are essential for the development of pain hypersensitivity following neuron injury(Beggs, Trang, & Salter, 2012).

2.1.3. Microglia and neuroinflammation

Neuroinflammation is a natural immune response that develops to prevent viral/bacterial infections in the central nervous system, eliminate these pathogens, and eliminate cell debris or misfolded peptides/proteins (Yang & Zhou, 2019). Neuroinflammation occurs via the release of cytokines, chemokines, reactive oxygen/nitrogen products, and secondary messengers secreted from cells into the microenvironment. If

neuroinflammation, which plays an essential role in maintaining the health of the nervous system, cannot be eliminated and becomes chronic, this poses a threat to the central nervous system, primarily to neurons (Schain & Kreisl, 2017). Moreover, this chronic neuroinflammation causes the formation or progression of many diseases in the central nervous system. Microglia play a primary role in neuroinflammation (Wolf, Boddeke, & Kettenmann, 2017). Activated microglia activate multi-protein complexes called inflammasomes. During activation, the transcriptional profile of microglia changes, and inflammatory cytokines and chemokines are secreted.

2.2. Neuroinflammation and inflammasomes

A key role in both acute and chronic CNS disorders is played by neuroinflammation, the dynamic response of CNS to injury or infection. Inflammasomes, intracellular protein complexes that control the development and release of pro-inflammatory cytokines, are activated during neuroinflammation, which is a critical mechanism behind this condition (Voet, Srinivasan, Lamkanfi, & van Loo, 2019). In response to CNS injuries, inflammatory mediators are released, activating immune cells like microglia and astrocytes. Chronic neuroinflammation may cause tissue damage and contributes to the etiology of CNS-related disorders, including AD, PD, multiple sclerosis, and stroke, but acute neuroinflammation is a defensive reaction given against dangerous stimuli for its elimination (Onyango, Jauregui, Carna, Bennett, & Stokin, 2021). The cytosolic pattern recognition receptors (PRRs), such as NOD-like receptors (NLRs) and missing in melanoma 2 (AIM2)-like receptors, are part of multiprotein complexes called inflammasomes. They perform the role of molecular guardians by detecting pathogen- and danger-associated molecular patterns PAMPs and DAMPs. The maturation and release of pro-inflammatory cytokines like IL-1β and IL-18 are caused by the recruitment and cleavage of pro-caspase-1 by inflammasomes once they have been activated. Studies indicate that inflammasomes play a crucial role in controlling neuroinflammation (Olcum, Tastan, Kiser, et al., 2020). Amyloid-beta (Aβ) plaques, -synuclein aggregates, and mitochondrial dysfunction are a few examples of DAMPs that may cause inflammasome activation in the CNS. When inflammasomes are active, they help to

amplify the inflammatory response, which in turn causes the immune system to mobilize and become activated, the production of cytokines to increase, and damage to neurons.

Inflammasome activation that is abnormal and dysregulated neuroinflammation has been linked to a number of neurological diseases. The NLRP3 inflammasome is triggered by Aβ plaques in AD, IL-1β is released, and neurodegeneration is accelerated(Rajesh & Kanneganti, 2022). Similar to this, the death of dopaminergic neuronal cells in PD is a result of NLRP3 inflammasome activation by -synuclein aggregation. Inflammasomes also play a variety of functions in neuroinflammatory processes, as shown by the fact that they have been linked to stroke, multiple sclerosis, and traumatic brain injury.

2.3. NLRP3 inflammasome activation

The NLRP3 inflammasome complex may be activated by three different routes: the canonical, non-canonical, and alternative pathways. These pathways lead to the NLRP3 inflammasome complex's formation and the Caspase-1 enzyme's activation (Olcum, Tastan, Ercan, Eltutan, & Genc, 2020). The priming and activation steps comprise the first pathway, which is also known as the canonical pathway. Cells are prepared for an immunological response during the priming step because the abundance of the pro- inflammatory cytokines IL-1β and NLRP3 is insufficient for inflammasome activation. The priming process may be started by ligands for Toll-like receptors (TLRs), including lipopolysaccharide (LPS), cytokines, NOD1 and NOD2 receptors, and others. These stimuli cause Nuclear Factor Kappa B (NF-κB) to translocate into the nucleus and start the transcription of NLRP3 and pro-inflammatory cytokines pro-IL-1β (F. G. Bauernfeind et al., 2009). Recent studies have shown that NLRP3 undergoes post-translational modifications, including phosphorylation and ubiquitination, during the priming phase (Song et al., 2017). In the activation stage, NLRP3 recognizes danger signals or alterations in homeostasis, such as lysosomal degradation and mitochondrial dysfunction. For NLRP3's PYD domain to interact with the adaptor proteins Apoptosis- associated Speck-like protein containing a CARD (ASC), this causes NLRP3 to change conformation and oligomerize.

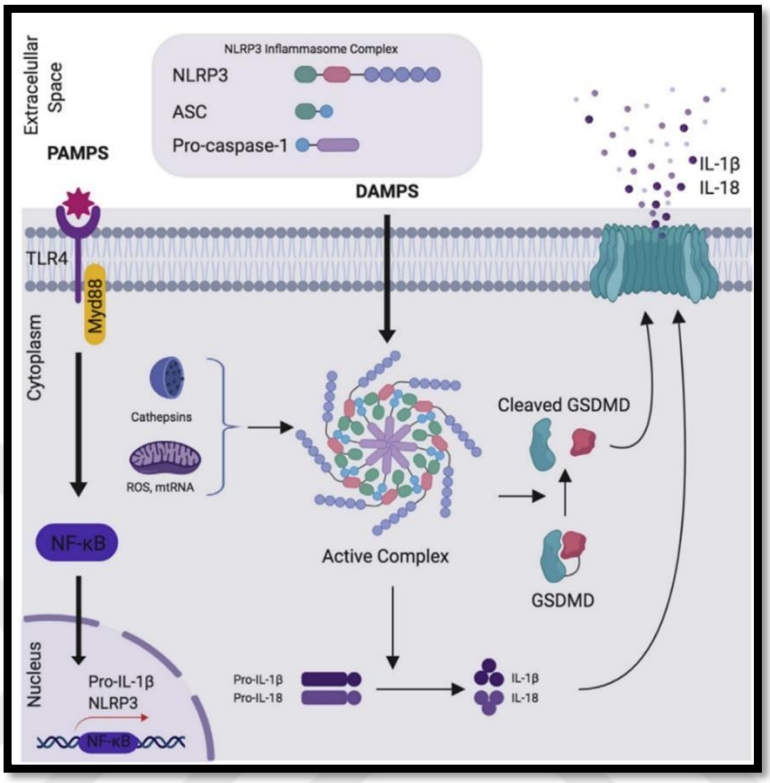

Figure 4. *Demonstration of canonical NLRP3 inflammasome activation in microglial cells (Olcum, Tastan, Kiser, et al., 2020).*

Through the CARD domain, the NLRP3-ASC complex interacts with effector protein pro-caspase-1. Pro-IL-1β and pro-IL-18 are then cleaved by activated caspase-1. The further cleavage of Gasdermin D (GSDMD) by caspase-1 also causes pyroptosis, an inflammatory form of programmed cell death. Since GSDMD cause the formation of pores on the cell membrane, the cellular integrity becomes broken, and pro- inflammatory cytokines are released, followed by pyroptotic cell death.

The NLRP3-ASC complex interacts with the effector protein pro-caspase-1 through the CARD domain. Activated caspase-1 then cleaves pro-IL-1β and pro-IL-18 (Mangan et al., 2018). GSDMD is further broken down by caspase-1, which results in pyroptosis, an inflammatory type of programmed cell death. Since GSDMD leads to the development of pores in the cell membrane, the integrity of the cell is compromised, which results in the release of pro-inflammatory cytokines and pyroptotic cell death (Liu et al., 2016).

In addition to the canonical pathway, the activation of the NLRP3 inflammasome can also be initiated through non-canonical and alternative pathways. Certain steps in the non-canonical pathway involve caspase-11, as well as its human counterparts, caspase- 4, and caspase-5 (Kayagaki et al., 2011; Vigano et al., 2015). The mechanism is initiated by LPS triggering TLRs, which subsequently activate NF-κB and induce the production of type I interferons, leading to the synthesis of caspase-4/5/11 through the JAK/STAT pathway (Schauvliege, Vanrobaeys, Schotte, & Beyaert, 2002). The cleavage of GSDMD by caspase-4/5/11 results in the canonical activation of the NLRP3 inflammasome. Additionally, type I interferons activate guanylate-binding proteins (GBPs), which assist in the autocatalytic activation of caspase-4/5/11 (Meunier et al., 2014). The NLRP3 inflammasome complex is canonically activated through the cleavage of GSDMD by caspase-4/5/11. Distinct from other pathways, the alternative activation route functions differently and is only found in human and porcine monocytes. It operates independently of pyroptotic cell death, ASC speck formation, or potassium efflux (Shi et al., 2015). Instead, the TLR4-TRIF-RIPK1-FADD axis induces the effector caspase-8. However, studies indicate that the outcome of this alternate route does not lead to pyroptotic cell death (Gaidt et al., 2016).

2.3.1. NLRP3 inflammasome activation in CNS Diseases

The involvement of the NLRP3 inflammasome in the pathophysiology of various CNS disorders has been extensively studied. Both acute and chronic CNS disorders, such as stroke, traumatic brain injury, and spinal cord injuries (Mortezaee, Khanlarkhani, Beyer, & Zendedel, 2018), AD (M. T. Heneka, R. M. McManus, & E. Latz, 2018), PD (Codolo et al., 2013), ALS (Meissner, Molawi, & Zychlinsky, 2010), multiple sclerosis (MS) (Gris et al., 2010), as well as neuropsychiatric conditions like depression and schizophrenia (Bhattacharya & Jones, 2018), have been the focus of numerous investigations regarding NLRP3 inflammasome activation.

In AD, there is an occurrence of hyperphosphorylation of tau protein within cytoplasmic neurofibrillary tangles, alongside the presence of abnormal deposition of amyloid plaques in the extracellular space. AD is the most commonly found progressive

neurodegenerative disorder affecting the central nervous system (CNS). Previous studies have demonstrated that the aggregation of β-amyloid peptides (Friker et al., 2020) and the hyperphosphorylation of tau (Stancu et al., 2019) can activate the NLRP3 inflammasome in microglia. Recent postmortem research has revealed that AD patients' frontal cortexes exhibited a higher expression of ASC, caspase-1, and IL-1β (L. Li et al.,2019). Similarly, in PD, α-synuclein deposits act as DAMPs that activate the microglial NLRP3 inflammasome. The clustering of α-synuclein leads to the generation of reactive oxygen species (ROS), increased cathepsin B activity, and the formation of the NLRP3 inflammasome complex. The microglial NLRP3 inflammasome has also been associated with PD pathogenesis through genetic factors and PD models induced by MPTP (Mouton-Liger et al., 2018; Panicker et al., 2019; Sarkar et al., 2017; Y. Zhou et al., 2016). In a neural cell model of PD, activation of the NLRP3 inflammasome using known inflammasome activators like nigericin, aluminum potassium sulfate crystals, bacterial LPS, and vitamin K3 (menadione) leads to the truncation and aggregation of α-synuclein by caspase-1 (W. Wang et al., 2016).

2.3.2. Regulation of NLRP3 inflammasome activation

Overactive or uncontrolled NLRP3 inflammasome complexes hold significance in the pathophysiology of a number of conditions, including infections, autoimmune diseases, and neurodegenerative diseases (Jo, Kim, Shin, & Sasakawa, 2016). Consequently, the many strategies used to control the evolutionarily conserved NLRP3 inflammasome complex closely. According to Song and Li (Song & Li, 2018), NLRP3 may be regulated at the transcriptional, post-transcriptional, and post-translational levels. Inflammasome activation is regulated post-translationally, and frequent changes to proteins include phosphorylation, methylation, acetylation, glycosylation, ubiquitination, SUMOylation, and S-nitrosylation. Ubiquitination-type alterations are crucial for controlling the NLRP3 inflammasome among these changes. Inflammasome activation may be triggered or suppressed by the NF-B-sensitive deubiquitinase A20 depending on cellular cues (Das, Chen, Hendriks, & Kool, 2018; Rothschild, McDaniel, Ringel-Scaia, & Allen, 2018; Yue et al., 2019). Another regulating mechanism for inflammasome activation is the

phosphorylation of NLRP3. Tyrosine-861 is phosphorylated by PTPN22 to activate NLRP3 (Spalinger et al., 2016), but serine-295 is phosphorylated to decrease ATPase function and stop the activation of the inflammasome (Mortimer, Moreau, MacDonald, & Chadee, 2016). However, it has been shown that JNK-1's phosphorylation of NLRP3 at serine-194 causes the development of the NLRP3 complex, functioning as a positive regulator of inflammasome activation (Song et al., 2017). By regulating the abundancy and stability of mRNAs encoding crucial proteins engaged in the preparation and/or activation phases, non-coding (ncRNA) RNA molecules, in particular microRNAs (miRNAs), play a critical role in the post-transcriptional control of NLRP3 inflammasome activity (Tezcan et al., 2019). It has been shown that a number of miRNAs have a role in controlling inflammasome activity. For example, miR-146a inhibits the NLRP3 inflammasome during the priming phase (Arulkumaran et al., 2018; Q. B. Zhang et al., 2018), whereas miR-223 inhibits NLRP3 expression by binding to the 3'UTR region of NLRP3 mRNA and is essential for innate immune responses because of its high expression in myeloid cells (G. Zhao et al., 2018).

2.4. Noncoding RNAs

The advancement in genomic science and technology altered the concept of RNA biology as genomic projects like ENCODE (E. P. Consortium, 2004), FANTOM(Kawai et al., 2001), and GTEx (G. T. Consortium, 2013) identified and rapidly expanded a new class of ncRNAs. These projects have indicated that the human genome is ubiquitously transcribed in RNAs, contrary to long-held assumptions. Those ncRNA molecules are characterized by RNA transcripts lacking protein-coding potential and play a fundamental role in the regulation of gene expression and protein biosynthesis (Ponting, Oliver, & Reik, 2009). ncRNAs could be classified based on their genomic location, functions, or size. Considering their functions, ncRNAs are grouped as constitutively expressed *housekeeping* ncRNAs and *regulatory* ncRNAs. Whilst miRNAs, PIWI- interacting RNAs (piRNAs), circular RNA (circRNA), and small interfering RNAs (siRNAs) constitute the small regulatory RNAs, ncRNAs longer than 200 nucleotides are called lncRNAs (Lodde et al., 2020).

2.4.1. lncRNAs

Another class of regulatory ncRNAs is lncRNAs, with a length of over 200 nucleotides (Mercer & Mattick, 2013). Since lncRNAs can be localized in both the nucleus and cytoplasm, the mechanism of lncRNA action depends on its localization (Cabili et al., 2015). lncRNAs modulate gene expression at transcription, translation, or post- transcriptional level (Kopp & Mendell, 2018) .lncRNAs acquired different mechanisms of action to modulate gene expression (**Fig.5**). LncRNAs can act as guides and scaffolds to ribonucleoprotein complexes modifying chromatin structures (Fang & Fullwood, 2016). Further, lncRNA can serve as a decoy or sponge-like structure for specific miRNAs (Sweta, Dudnakova, Sudheer, Baker, & Bhushan, 2019). Research demonstrated that lncRNAs play a fundamental role in a variety of biological processes, including metabolism (X. Sun & Wong, 2016), cell cycle (Kitagawa, Kitagawa, Kotake, Niida, & Ohhata, 2013), cell differentiation (Fatica & Bozzoni, 2014), immune response (Carpenter et al., 2013), and engage in diseases including cancers (Gupta et al., 2010), cardiovascular diseases (Uchida & Dimmeler, 2015), neurodegenerative diseases (Riva,Ratti, & Venturin, 2016). Only in humans, there are 173.112 lncRNA transcripts annotated so far (NONCODEV)(L. Zhao et al., 2021). The expression and abundancy of lncRNAs are tissue- and disease-specific, appointing those potential biomarkers and targets along with their involvement in the aforementioned biological processes/diseases. LncRNAs might regulate inflammasome activation during both the priming and activation steps. The most important mechanism of action of lncRNAs in the regulation of inflammasome is that they inhibit the activity of miRNAs. Recent studies have shown that LncRNAs FIRRE, LincRNA-Cox2, and SNHG participate in NLRP3 inflammasome regulation in microglia (B. Cao, T. Wang, Q. Qu, T. Kang, & Q. Yang, 2018; Hardeland, 2018; Zang, Zhou, Wang, Li, & Huang, 2018).

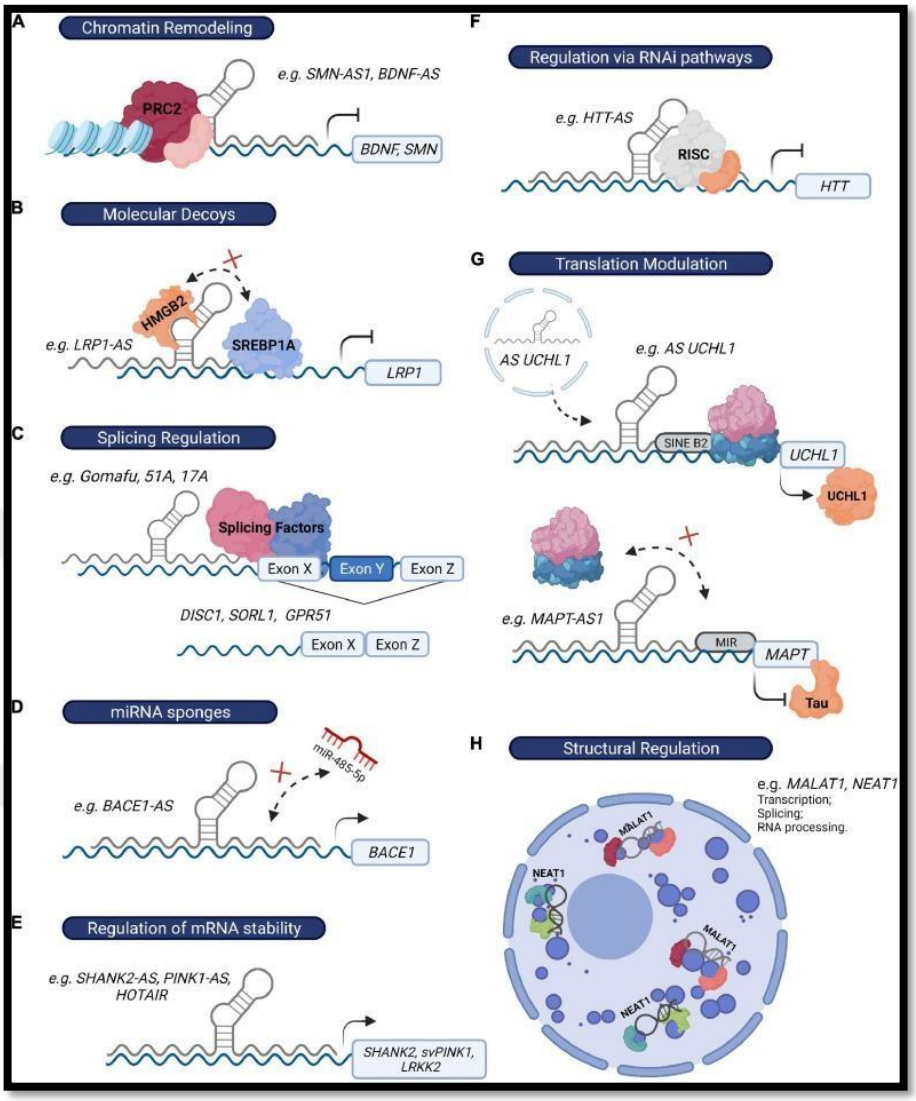

Figure 5. *Illustration of molecular mechanisms showing the routes in which lncRNAs may regulate gene expression (Policarpo, Sierksma, De Strooper, & d'Ydewalle, 2021).*

While FIRRE regulates the inflammasome by interacting with NF-KB, SNHG suppressesNLRP3 inflammasome activation via miR-7 (B. Cao et al., 2018; Zang et al., 2018). In addition, microarray performed in the retinal Ischemic/reperfusion model showed altered lncRNAs, of which lncRNA H19 contributed to microglial NLRP3 inflammasome and pyroptosis (Wan et al., 2020). However, since the expression of lncRNAs is tissue and disease-specific, the variation of microglial inflammasome activation is seen in the central nervous system.

2.4.2. NEAT1

Nuclear paraspeckle assembly transcript 1 (NEAT1) is one of the lncRNA molecules discovered in 2007. This lncRNA is expressed at the MEN type 1 locus on chromosome 11q13 having 2 isoforms, a short isoform (3.7 kb long) and a long isoform (23 kb long), which are expressed by alternative splicing. While NEAT1 is localized in the nucleus within the cell, it is also found in the cytoplasm, depending on its function (Dong et al., 2018). The long isoform of NEAT1 (NEAT1_2) is part of nucleo-protein complexes calledparaspeckles structures in the nucleus. The long isoform NEAT1_2 has three subdomains that interact with RNA-binding proteins such as NONO, SFPQ, etc. and form complex paraspeckle in the nucleus. The 5' and 3' ends of NEAT1 transcripts are located at the periphery, and the middle region of the transcripts is located in the center of the paraspeckles (**Fig.6**). These structures regulate gene expression by sponging mRNAs or miRNAs with adenosine-inosine conversion in the nucleus. Like other lncRNAs, NEAT1 forms complex structures with proteins. It also plays a regulatory role in pri-miRNA processing through these complexes (Prinz, Kapeller, Pichler, & Klec, 2019).

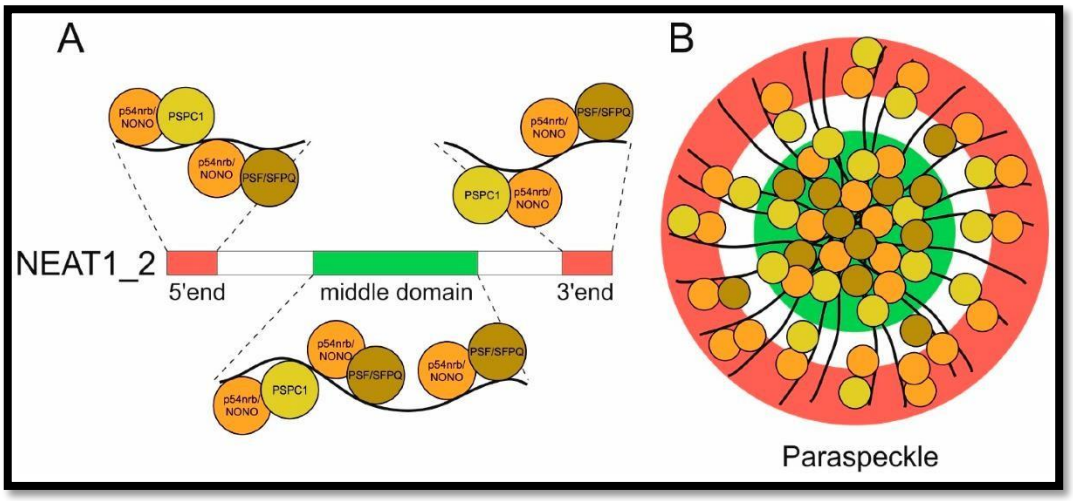

Figure 6. *Representation of NEAT1 domains and the subcellular structure called paraspeckle (Prinz et al., 2019).*

NEAT1 has been linked to a number of physiological processes, including the development of the breast, the formation of the corpus luteum, the development of embryonic stem cells, neuronal excitability, and immunological response. Additionally, research has shown that it has a role in the etiology of many illnesses, notably autoimmune disorders, and infections (Prinz et al., 2019). Increased expression of NEAT1 has been found in several neurodegenerative diseases, including ALS, AD, PD, frontotemporal dementia, epilepsy, and traumatic brain injury (An et al., 2019; An, Williams, & Shelkovnikova, 2018). These studies involved post-mortem human analysis, *in vivo* experiments, and *in vitro* studies. For instance, the development of paraspeckles in motor neurons and the buildup of at least eight RNA-binding proteins in this model have shed light on the involvement of NEAT1 in the pathogenesis of ALS (An et al., 2019; Nishimoto, Nakagawa, & Okano, 2021). Increased NEAT1 expression has been seen in post-mortem brain tissue from PD patients and animal models of the condition in the substantia nigra, according to studies. Suppression of NEAT1 expression has been shown to have a protective impact in PD animal models (Yan, Chen, Chen, & Chen, 2018). Similar to this, increased NEAT1 expression has been seen in several brain areas in investigations of AD and Huntington's disease in both humans and animals (Anet al., 2019; An et al., 2018).

In clinical studies, the increase in the level of NEAT1 in inflammatory diseases such as Sepsis and Lupus has suggested that NEAT1 may have a role in inflammation (Gast et al., 2019; S. Huang et al., 2017; F. Zhang et al., 2016). *In vitro,* studies have revealed that NEAT1 plays a vital role in the regulation of immune responses in monocyte/macrophage cells. For instance, it has been reported that NEAT1, which is increased by Ox-LDL, causes cytokine release from monocytes, and this effect is reversed by suppressing NEAT1 expression (Chen, Hui, Zhang, & Chang, 2019; L. Wang, Xia, Ke, & Zhang, 2019). It has been suggested that NEAT1 may be a promising drug target due to its effect on inflammation regulation(An et al., 2018). Furthermore, it has been shown that increased NEAT1 in oxygen-glucose deprivation and reoxygenation damage, in which microglial cells express NEAT1, cause cytokine release

(Han & Zhou, 2019). However, the current literature knowledge is limited regarding the role of NEAT1 in neuroinflammation. A study suggesting that NEAT1 has a regulatory role in NLRP3 inflammasome activation, it was found that NEAT1 overexpression in immortal bone marrow-derived macrophage cells caused an increase in inflammasome responses (P. Zhang, Cao, Zhou, Yang, & Wu, 2019). In this study, suppression of the expression of NEAT1 also led to a decrease in inflammasome responses, confirming the importance of NEAT1 in the regulation of inflammasome activation.

Similarly, it has been shown that NEAT1 plays a role in inflammasome activation in dendritic cells (M. Zhang et al., 2019). It is possible that NEAT1 plays a role in the activation of NLRP3 inflammasome in microglia cells, which are cognate cells in monocytes and macrophages. However, there is currently no data on whether NEAT1 participates in NLRP3 inflammasome regulation in microglial cells.

2.4.3. microRNAs

Compared to the other ncRNAs, miRNAs have been studied the most owing to their widespread regulatory roles in gene expression and earlier identification. miRNAs are evolutionarily conserved small ncRNAs with an average length of 22 nt. They function as gene silencers as they mainly bind to the 3' untranslated region (3'UTR) of target messenger RNAs (mRNAs), resulting in the inhibition of translation (O'Brien, Hayder, Zayed, & Peng, 2018). miRNAs regulate many biological processes varying from development, differentiation, and cell division to cell death (Tufekci, Meuwissen, & Genc, 2014). Furthermore, alterations in levels of miRNAs play roles in countless diseases, which makes them ideal biomarkers and even better therapeutic targets.

2.4.3.1. miR-146a-5p

miR-146a is a miRNA that regulates anti-inflammatory responses in a variety of immune cells and other cell types. It controls inflammatory responses in both the innate and acquired immune systems (Mortazavi-Jahromi, Aslani, & Mirshafiey, 2020). miR-146a is transcribed by RNA polymerase II from the primary transcript of the miR-146a gene on

human chromosome 5 (chromosome 5q33.3) and then processed by Drosha and Dicer to form the mature miRNA. According to the miRBase database, the mature -5p strand is the bioactive "guide strand," whereas the mature -3p strand is the "passenger strand" (Olivieri et al., 2021). In the innate immune system, the NF-κB transcription factor is highly correlated with miR-146a. Activating the NF-κB signaling pathway results in the expression of miR-146a, which acts as a negative regulator to inhibit the activity of the pathway. It is the first NF-κB-dependent miRNA discovered, upregulated in response to various immunological mediators. miR-146a inhibits important proteins in the conventional NF-κB pathway, such as Toll-like receptor 4 (TLR4), MyD88, IRAK1, TNF receptor-associated factor (TRAF6), and RelB (K. D. Taganov, M. P. Boldin, K.-J. Chang, & D. Baltimore, 2006). In the pathogenesis of neurological diseases, abnormal expression of miR-146a is commonly observed. In the CNS, it regulates cell cycle control, neuronal identity acquisition, neural cell differentiation, and immune responses (Aslani, Mortazavi-Jahromi, & Mirshafiey, 2021). A miR-146a deficiency has a significant impact on microglia, the primary source of miR-146a production in the CNS. In the absence of miR-146a, microglial activation is increased, and the expression of inflammatory cytokines is upregulated (Martin et al., 2020). During neurodegeneration, MiR-146a functions as a regulator to prevent excessive inflammation in the CNS. It modulates the activity of the NF-κB signaling pathway, thereby controlling the productionof neurotoxic inflammatory factors. The dysregulation of miR-146a results in the unrestrained production of inflammatory cytokines, which causes neuronal mortality and neurodegeneration. Additionally, it was shown that miR-146a can influence parameters associated to the NLRP3 inflammasome activation, including its downstream inflammatory components functioning in priming step (Q. B. Zhang et al., 2018). Thus, miR-146a functions as an anti-inflammatory regulator in various cell types and the immune system.

2.4.4. Relationship between NEAT1 and miR-146a-5p

The studies, mainly related to immunological responses in cancer and CNS disorders, revealed the possible relationship between lncRNA NEAT1 and miR-146a due to their

significant roles in inflammation (Olivieri et al., 2021; Yongli Pan et al., 2022). miR-146a has been identified as a target of NEAT1 by several studies. According to computational prediction methods and experimental confirmation, it was discovered that NEAT1 could interact with/ sponge miR-146a by specific binding sites.

Chanda *et al.* demonstrated that hsa-miR-146a-5p, along with hsa-miR-34a-5p, interacted with lncRNA NEAT1 in cell culture and transgenic model of AD. The silencing of NEAT1 resulted in the upregulation of the expression of both miRNAs in a unidirectional manner. The study indicated a complex regulatory network involving hsa- miR-146a-5p, hsa-miR-34a-5p, and NEAT1 (Chanda, Jana, & Mukhopadhyay, 2021). Another study by Ding and colleagues investigated the ceRNA networks in the hair follicle cycle (Ding et al., 2022). They identified several miRNA-lncRNA interactions, including Neat1-miR-30a/e-5p/miR-146a-5p-*N*otch1, which suggests their potential role in the development and homeostasis of hair follicles. A study in non-alcoholic fatty liver disease (NAFLD) models demonstrated that NEAT1 expression was upregulated, whereas the expression level of miR-146a-5p was decreased in the NAFLD models. They further confirmed that NEAT1 targeted miR-146a-5p and thus modulated lipid accumulation by activating the AMPK pathway. These findings were supported by knocking down NEAT1 and overexpressing miR-146a-5p in the NAFLD models. Lastly, the interaction between mir-146a-5p and NEAT1 has been studied in osteoarthritis (Ning, Zhu, Gao, & Deng, 2021). A study found increased expression of NEAT1 in osteoarthritis, which negatively correlated with miR-146a-3p expression. They further silenced NEAT1 resulting in elevated levels of miR-146a-3p and improved chondrocytes proliferation.

3. MATERIAL and METHOD

3.1. Type of the thesis study

This thesis is an experimental study consisting of *in vitro* and *in vivo* models.

3.2. Location and duration of the thesis study

All the experiments were carried out at Genc Laboratory in Izmir Biomedicine and Genome Center between July 2020 - May 2023 under the supervision of Prof. Dr. Şermin GENÇ.

3.3. Population, sampling, and experimental groups for the thesis study

In this thesis study, cell lines (N9, HMC3, RAW264.7) and mice (C57BL/6 Wild type and NEAT1 Knock Out (KO)) were used for *in vitro* and *in vivo* experiments. The study does not include any human specimens.

3.4. Materials used in the thesis study

The *in vitro* studies utilized N9 murine microglia, HMC3 human microglia, and RAW264.7 murine macrophage cell lines. The N9 microglial cell line (Righi et al., 1989) was kindly provided by Dr. Paola Ricciardi-Castagnoli (Toscana Life Science- Italy). On the other hand, the commercial HMC3 human microglia (cat#: CRL-3304) and RAW264.7 murine macrophage cell line (cat#: TIB-71) were purchased from ATCC.

In the *in vivo* part, Wild type C57BL/6 mice and NEAT1 KO mice were used. Wild-type C57BL/6 mice were supplied by Izmir Biomedicine and Genome Center Vivarium. NEAT1 KO mice on the C57BL/6 background were created (Nakagawa, Naganuma, Shioi, & Hirose, 2011) and kindly provided by Dr. Shinichi NAKAGAWA in October 2021. Following ethical and animal care guidelines, the mice were housed and handled in Izmir Biomedicine and Genome Center Vivarium.

3.5. Variables of the Study

This thesis study consists of *in vitro* and *in vivo* experimental setups. For *in vitro* experiments, LPS and Adenosine triphosphate (ATP) model is independent variables. On the other hand, the biological parameters, such as protein/mRNA levels of NLRP3 inflammasome markers or cell death rates measured with different assays, are dependent variables. For *in vivo* experiments, while wild-type (C57BL/6) mice and NEAT1 KO mice are independent variables, protein/mRNA levels or clinical/behavioral phenotypes are dependent variables.

3.6. Tools for Data Collection

3.6.1.1. Reagents, Devices, Tools

3.6.1.1.1. Antibodies

Table 1. *The complete list of antibodies used in this thesis study.*

Antibody	Host Species	Catalog Number	Application	Dilution	Blocking	Company
Anti-SFPQ	Rabbit	PA5-29948	WB	1:4000	5% Milk	ThermoFisher
Anti-SFPQ	Rabbit	PA5-29948	RIP	1:200	-	ThermoFisher
Anti-NONO	Rabbit	NB100-1556	WB	1:2000	5% Milk	Novus Biologicals
Anti-NONO	Rabbit	NB100-1556	RIP	1:200	-	Novus Biologicals
Anti-IL-1β	Rabbit	Ab9722	WB	1:1000	5% Milk	Abcam
Anti-Caspase-1	Mouse	AG-20B-0042-C100	WB	1:1000	5% Milk	Adipogen
Anti-	Mouse	AG-20B-	WB	1:1000	5% Milk	Adipogen

NLRP3		0014-C100				
Anti-GSDMD	Rabbit	Ab209845	WB	1:1000	5% Milk	Abcam
Anti-β-actin	Rabbit	Ab8227	WB	1:3000	5% Milk	Abcam
Anti-Rabbit	Goat	7074	WB	1:2000	5% Milk	Cell Signaling
Anti-Mouse	Horse	7076	WB	1:2000	5% Milk	Cell Signaling
DAPI	-	62248	IF	1:1000	-	ThermoFisher
Hoechst	-	H1399	IF	1:1000	-	ThermoFisher
NEAT1_1	SMF-3009-1		FISH	125 nM	-	Stellaris
NEAT1_2	SMF-3010-1		FISH	125 nM	-	Stellaris

3.6.1.1.2. Chemicals, Reagents, And Commercial Kits

Table 2. *The complete list of items used in this thesis study.*

Chemicals/Reagents		
Items	**Vendors**	**Catalog Number**
Ultrapure LPS (LPS from *E. coli* 0111: B4)	InvivoGen	tlrl-3pelps
Adenosine 5′-triphosphate disodium salt hydrate (ATP)	Sigma-Aldrich	A6419
LPS (LPS from *E. coli* 055: B5)	Sigma-Aldrich	L2880
FBS	Gibco	10500064
Phosphate buffer saline	Gibco	10010015
Glutamax	Gibco	35050061
RPMI 1640	Gibco	21875034
DMEM	Gibco	11965092
MEM	Biowest	B.L0416-500
Penicillin-streptomycin	PAN-Biotech	P06-07100
Trypsin-EDTA (0.25%)	PAN-Biotech	P10-019100

Trypan Blue	Sigma-Aldrich	T8154-100ML
DMSO	AppliChem	A3672
Methanol	Merck	106008
Ethanol	Merck	108543
Isopropanol	Merck	109634
APS	Sigma-Aldrich	A3678
Bovine serum albumin	BioShop	ALB001.500
Skimmed milk powder	BioShop	SKI400.500
Tween-20	BioShop	TWN510
TEMED	Sigma-Aldrich	T9281
Nitrocellulose Membrane	Macherey-Nagel	741260
SDS	BioShop	SDS003.500
Tris	BioShop	TRS003.1
Triton X-100	BioShop	TRX777
Glycine	BioShop	GLN002
NaCl	BioShop	SOD001.1
Agarose	BioShop	AGA002
Gel Loading Dye	NEB	B7024S
100 bp DNA Ladder	NEB	N3231S
SafeView Classic	ABM	G108
Trizol Reagent	ThermoFisher Scientific	15596026
Paraformaldehyde	BioShop	PAR070
HALT Protease Inhibitor	ThermoFisher Scientific	78440
RIPA Buffer	ThermoFisher Scientific	89901
Prestained Protein Ladder	ThermoFisher Scientific	26620
Stripping Solution	ThermoFisher Scientific	46430
SuperSignal West Pico PLUS Chemiluminescent Substrate	ThermoFisher Scientific	34580
Lincode NEAT1 Smartpool siRNA	Horizon Discovery	R-160022-00-0020

Lincode Non-targeting Control Pool	Horizon Discovery	D-001320-10-20
5x siRNA Buffer	Horizon Discovery	B-002000-UB-100
DharmaFECT Transfection Reagent	Horizon Discovery	T-2001-03
Syn-mmu-miR-146a-5p miScript miRNA Mimic	Qiagen	219600
miScript Negative Control	Qiagen	1027272
HiPerFect Transfection Reagent	Qiagen	1029975
Commercial Kits		
GoTaq qPCR Master Mix	Promega	A6001
RevertAid First Strand cDNA Synthesis Kit	ThermoFisher Scientific	K1621
miRneasy Mini Kit	Qiagen	217004
Pierce BCA Protein Assay Kit	ThermoFisher Scientific	23225
Mouse IL-1β ELISA Kit	Invitrogen	88-7013-88
Xpert Fast Hotstart Mastermix (2X) with dye	Grisp	GE45.5001
Magna RIP Kit	Merck	17-700

3.6.1.1.3. Devices

Table 3. *The complete list of devices used in this thesis study*

Items	Vendors	Catalog Number
Biological safety cabinet	ThermoFisher Scientific	-
Pipette – Single Channel	Gilson	PIPETMAN G starter kit (P20G, P200G, P1000G)
Pipette – Multi Channel	Eppendorf	3125000028
Multiskan™ FC Microplate	ThermoScientific	51119000

Photometer		
Real-Time PCR	Applied Biosystem	ABI Fast 7500
Nanodrop 2000	ThermoScientific	ND-2000
Confocal Microscope	Zeiss	LSM 880
SimpliAmp™ Thermal Cycler	Applied Biosystems	A24811
My Spin™ 6 Mini Centrifuge	ThermoScientific	75004061
Centrifuge	Eppendorf	5810R
pH Meter	Hanna Instruments	HI2002-02
Centrifuge MicroCL 17R	ThermoScientific	75002499
LP Vortex Mixer	ThermoScientific	88880017
Cimarec Stirring Hot Plate	ThermoScientific	SP88857105
Mini PROTEAN® Tetra Cell and Mini Trans-Blot ® Module	Bio-Rad	1658029
PowerPac™ Basic Power Supply	Bio-Rad	1645050
Digital Heating Shaking Drybath	ThermoScientific	88880028
CO_2 incubator	MEMMERT	INCOmed 246
Ice machine	HOSHIZAKI	FM-300AKE-N

3.6.2. Methods

3.6.2.1. Cell Culture

3.6.2.1.1. N9 Murine Microglia Cell Line

The N9 microglial cells (**Fig.7**) were primarily isolated from the mouse brain andimmortalized with over-expression of the *v-myc* transcription factor (Righi et al., 1989). It

has been demonstrated that N9 cells feature typical characteristics of primary microglia and can generate an immune response against inflammatory stimuli (Weinstein, Swarts, Bishop, Hanisch, & Moller, 2008).To maintain N9 cells, RPMI 1640 cell culture media supplemented with 10% Fetal Bovine Serum (FBS) (ThermoScientific, USA), 2 mM L- Glutamine (ThermoScientific, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin (ThermoScientific, USA) was used.

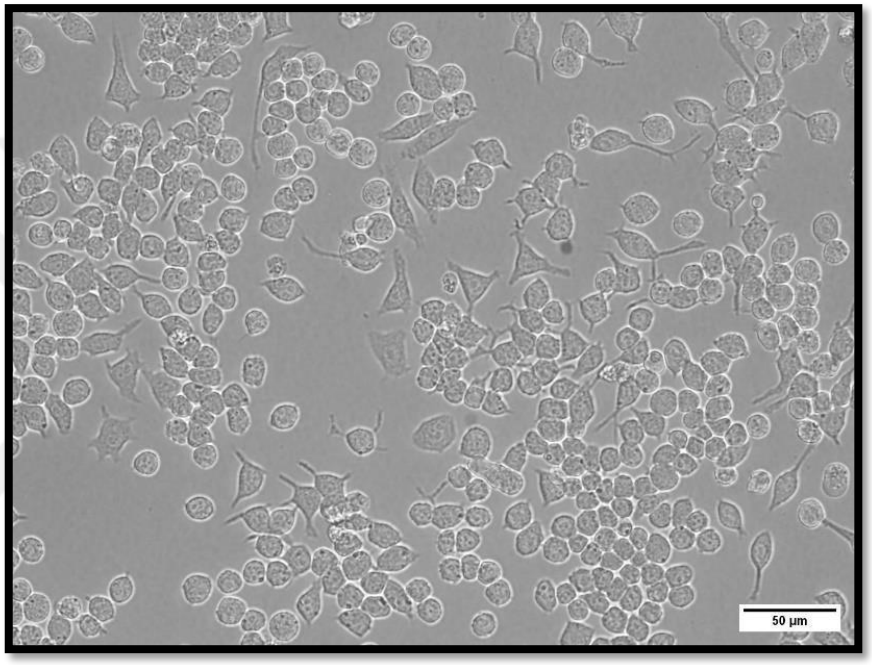

Figure 7. *N9 murine microglia cell line.*

Cells were grown on 75 cm^2 culture flasks and passaged 3 times a week. First, the media was discarded for passage, and the cells were washed with 1x Phosphate Buffered Saline (PBS) (ThermoScientific, USA). Then, the cells were detached with 4 ml of 0.25% (w/v) Trypsin-EDTA (PAN-Biotech, Germany) solution. 6 ml of RPMI (ThermoScientific, USA) with 10% FBS (ThermoScientific, USA) was added to the flask to stop the enzymatic reaction. The mixture was taken into a 15-ml falcon and centrifuged at 1.500 rpm to collect the cells. Next, the media were removed, and the cells were suspended in 2 ml of media. Finally, the cells were counted with the help of the Thoma slide (Marienfeld, Germany), and $1x10^6$ cells were seeded into a new culture

flask containing 10 ml of fresh medium. All the procedures were carried out in a Class II B2 biosafety cabinet, and the cells were cultured in an incubator at 37°C with 5% CO_2 and 80% humidity.

3.6.2.1.2. HMC3 Human Microglia Cell Line

The human microglial clone 3 (HMC3) –cell line (**Fig.8**) was purchased from the American Type Culture Collection (ATCC) (Manassas, VA). To culture HMC3 cells, Eagle's Minimum Essential Medium (EMEM) medium supplemented with10% FBS (ThermoScientific, USA), 2 mM L-Glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (PAN-Biotech, Germany) was used. The cells were cultured and kept in an incubator at 37°C with 5% CO_2 and 80% humidity. The cells were passaged once they reached 80-90 % confluency under the Class II B2 biosafety cabinet. Briefly, the medium was discarded, and the cells were washed with 1x PBS once. Then, 0.25% (w/v) Trypsin-EDTA (PAN-Biotech, Germany) solution was used to detach cells, and the detached cells were collected in a 15-ml falcon tube by centrifugation at 1.500 rpm. The pellet was suspended in EMEM complete medium. Depending on the experiment, the suspended cells were seeded into 75 cm2 culture flasks to maintain culture or well- plates.

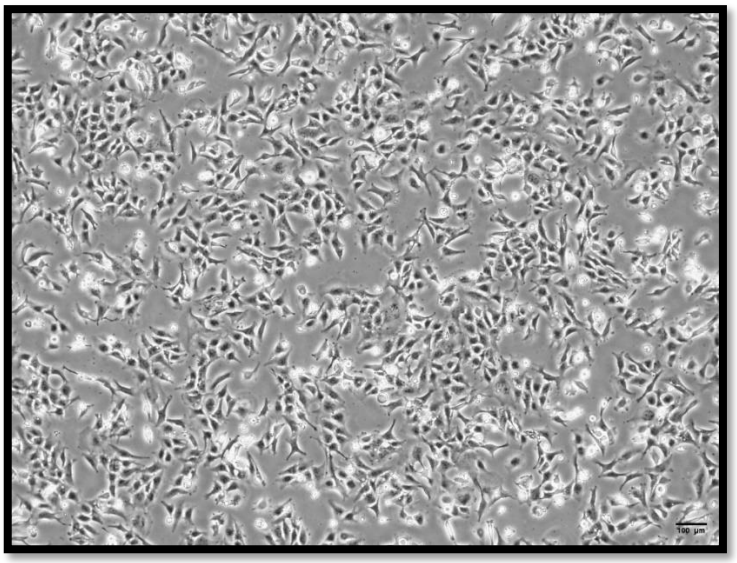

Figure 8. *HMC3 human microglia cell line.*

3.6.2.1.3. RAW 264.7 Murine Macrophage Cell Line

RAW264.7 murine macrophage cell line (**Fig.9**) was purchased from ATCC (Manassas, VA). This cell line was used as a positive control for NEAT1 expression. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) containing 10% FBS, 2 mM L-Glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Once the cells reached 80-90% confluency, the cells were passaged by detaching cells with 0.25% (w/v) Trypsin-EDTA solution. Then, the cells were collected by centrifugation at
1.500 rpm and resuspended in complete DMEM. To induce NEAT1 expression, RAW264.7 cells were seeded in a 6-well plate with $3x10^5$ concentration and further treated with LPS (LPS from E. coli 055: B5) for 24 hours. At the end of the 24 hours, treated cells were scraped and lysed with Trizol, and subsequent RNA isolation was carried out. The method for RNA isolation is given in the corresponding section.

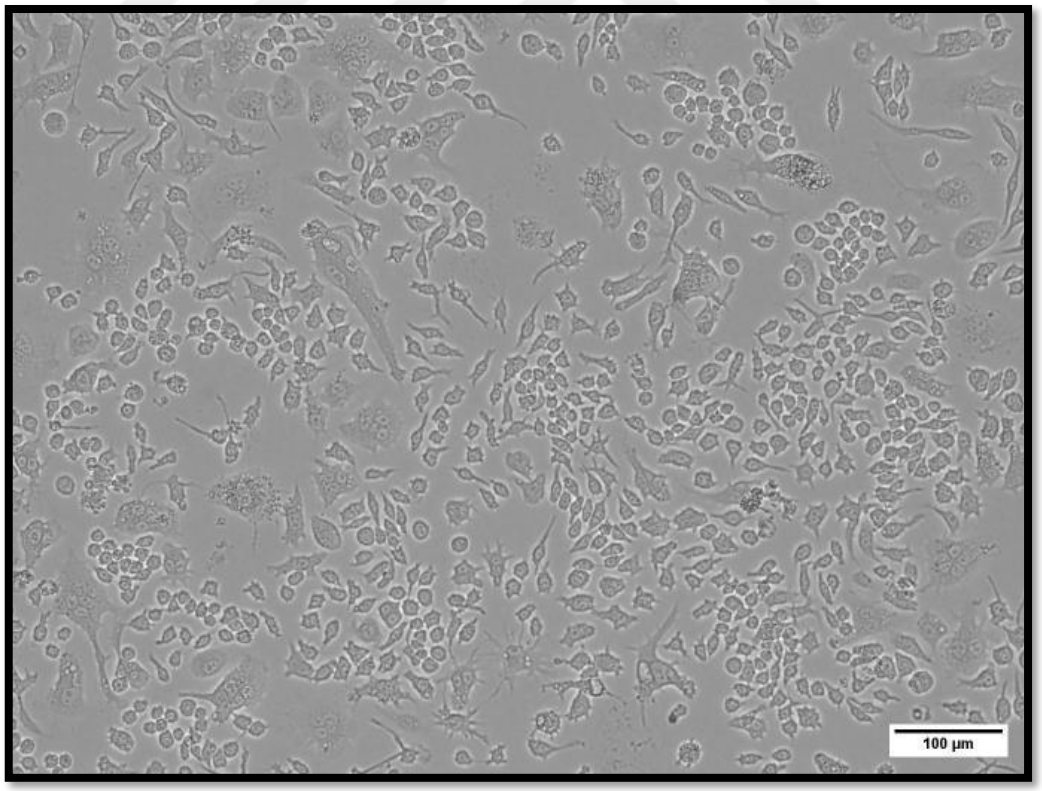

Figure 9. *RAW 264.7 murine macrophage cell line.*

3.6.2.1.4. NLRP3 Inflammasome Activation in N9 and HMC3 Microglial Cell Line

To induce microglial NLRP3 inflammasome activation, LPS and ATP model was used (Arioz et al., 2019). As the canonical NLRP3 inflammasome activation requires 2-signal; priming and activation, LPS primes cells for NLRP3 inflammasome activation, initiating transcription of NLRP3 and IL-1β while ATP induces assembly and activation of inflammasome complex (Y. Pan et al., 2016).

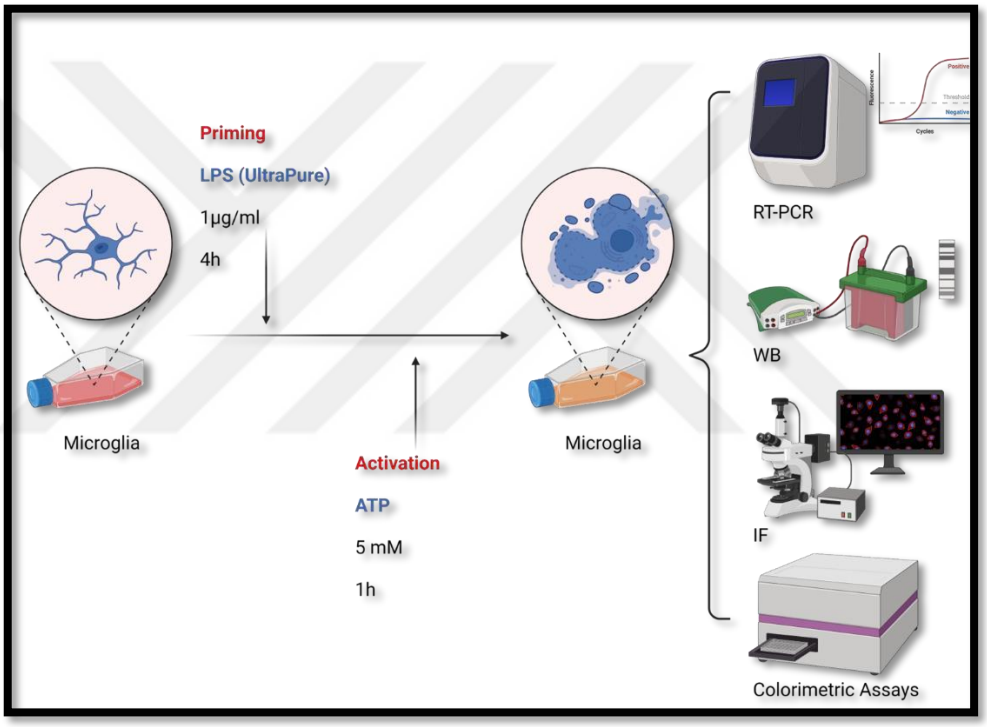

Figure 10. *The illustration of the experimental setup to induce microglial NLRP3 inflammasome activation in the thesis study.*

In the experiments performed on N9 murine microglia, the cells were primed with LPS (1µg/ml) for 4 hours and then incubated with ATP (5 mM) for 1 hour (**Fig.10**). As NLRP3 inflammasome activation causes cell swelling due to the formation of the GSDMD pore on the membrane, the cells were swollen and took an egg-like shape, as shown in **Fig.11**. LPS and ATP model to induce NLRP3 inflammasome activation is also performed in HMC3 human microglial cell line. Different treatment times and concentration schemes of LPS were implemented. The results demonstrated that

NLRP3 inflammasome is not activated in HMC3 cells upon LPS and ATP treatment. Furthermore, secretion of IL-1β, a hallmark of NLRP3 inflammasome activation, could not be induced in HMC3 cells **Figure 12**. When the literature was reviewed, we found that the cytokine response of this cell line was relatively low. It was also reported that TNF-α expression, which increases most upon immune activation, was not seen in the immune response (Dello Russo et al., 2018). Although some publications have reported that HMC3 cells express low levels of IL-1β in immune activation, we could not confirm this finding in our experiments (Y. J. Wang, Downey, Choi, Shoup, & Elmaleh, 2021). Thus, the experiments were continued with N9 microglial cell line.

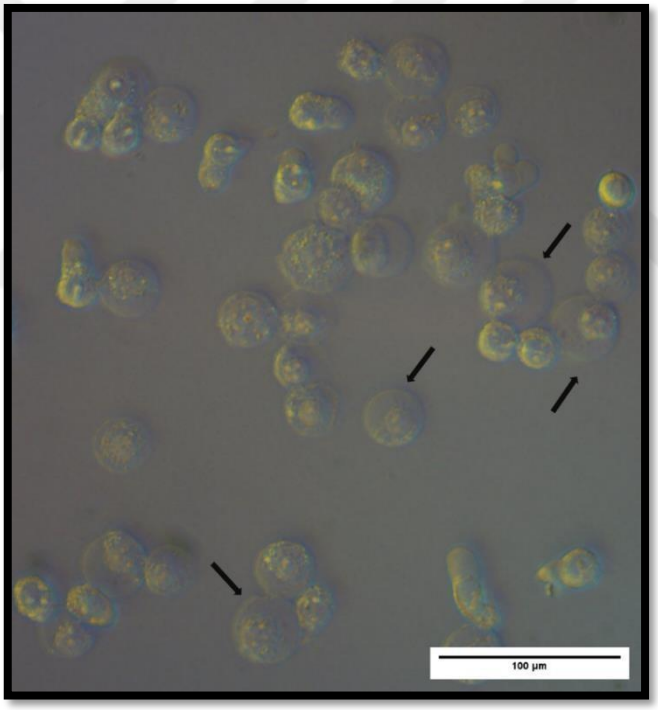

Figure 11. *The demonstration of the egg-like shape of N9 microglia after LPS and ATP-induced NLRP3 inflammasome activation.*

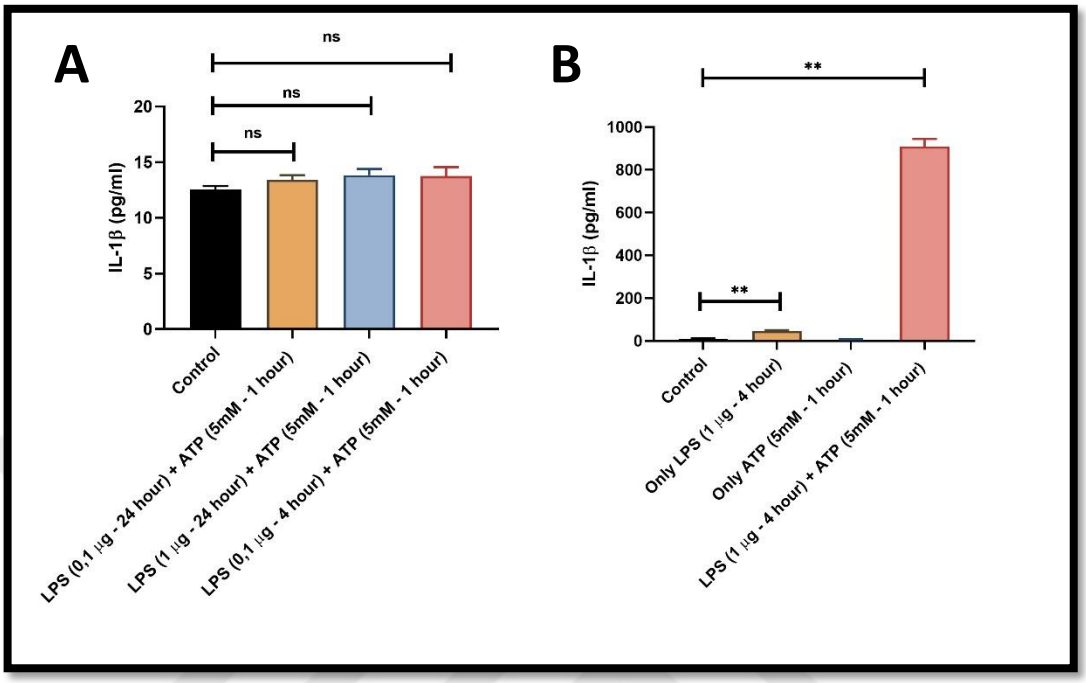

Figure 12. *LPS and ATP induced NLRP3 inflammasome activation in N9 murine microglia but not in HMC3 human microglia. The cells were primed with UP-LPS (1 µg/ml) for 4 hours and followed by ATP (5 mM) for 1 hour.* ***A****) The protein levels of secreted IL-1β evaluated with ELISA in the HMC3 Cell line.* ***B****) The protein levels of secreted IL-1β evaluated with ELISA in the N9 cell line. Data are presented as mean ± S.E.M, n = 5. **p < 0.01, ns: not significant.*

3.6.2.1.5. Animal Maintenance and Experimental Setup

For *in vivo* experiments, Wild type (WT) C57BL/6 mice and NEAT1 KO on the C57BL/6 background were used. NEAT1 KO mice were generated and kindly provided by Dr. Shinichi Nakagawa (Faculty of Pharmaceutical Sciences, Hokkaido University, Hokkaido, Japan). Briefly, NEAT1 KO mice have LacZ insertion prior to the downstream region of the transcription start site of the Neat1 gene (Nakagawa et al., 2011). The mice sent by Dr. Nakagawa reached Izmir Biomedicine and Genome Center – Vivarium on

03.12.21 (**Fig.13**), and they were kept in quarantine for 1 week. At the end of the quarantine period, mice were taken to standard animal housing rooms where they were fed *ad libitum* feed and water under controlled conditions (22±2°C; 12 h light/dark

periods). Mice were mated with wild-type C57BL/6 mice for use in the experiment (**Fig.14**). Izmir Biomedicine and Genome Center HADYEK guidelines were followed in animal care and *in vivo* experiments.



Figure 13. *The arrival of NEAT1 KO mice in October 2021. The mice were quarantined for a week and then habituated in the vivarium.*



Figure 14. *The newborn mice (F1) from the breeding between NEAT1 Heterozygous Mice and WT mice.*

3.6.2.1.6. Tissue Collection, DNA Isolation, Genotyping, and Gel Electrophoresis

To genotype mice, the tissue from mouse tail tips (~2 mm) was collected for genotyping. DNA isolation was done using a tail buffer (**Table 4**). The collected mouse tails were incubated overnight in the water bath at 55°C in 200 µl of tail buffer containing 2 µl proteinase K (20 mg/ml). The following day, the samples were boiled for 5 minutes and centrifuged for 5 minutes at 1500 rpm speed, 4°C. The supernatant was transferred to new Eppendorf tubes and stored at -20°C until needed.

Table 4. *The recipe of Tail Buffer used in DNA isolation from mouse tail*

Components	Final Concentration	Weight/Volume
Tris-Cl pH8.0	10 mM	
KCl	50 mM	
Gelatin		50 mg

Autoclave		
NP40		2,25 ml
Tween-20		2,25 ml
Total Volume		**500 ml**

For the NEAT1 genotyping, PCR was carried out using Xpert Fast Hotstart Mastermix (2X) with dye (Grisp, Portugal) (**Table 6**) and appropriate primers for WT and NEAT1 KOmice (**Table 5**). The conditions were followed as the manufacturer provided (**Table 7**). Two different PCR reactions (WT allele and KO allele) were performed for each mouse. Finally, the genotype was identified using the results of both reactions.

Table 5. *The list of primers used in genotyping of NEAT1 KO mice*

Gene	Primers	Sequence	Product Size
NEAT1	NEAT1 WT Forward	5'-CTAGTGGTGGGGAGGCAGT-3'	WT Allele: 210 bp KO Allele: 315 bp
	NEAT1 WT Reverse	5'-AGCAGGGATAGCCTGGTCTT-3'	
	LacZ 5 Reverse	5'-GCCATTCAGGCTGCGCAACTG-3'	

Table 6. *The preparation of the PCR reaction mix*

Components	Volume	Final Concentration
Xpert Fast Hotstart Mastermix (2X) with dye	12.5 µl	1X
Forward primer (5 pmol/ µl)	2 µl	0.4 µM
Reverse primer (5 pmol/ µl)	2 µl	0.4 µM
Template DNA	1.5 µl	1-250 ng
Nuclease-free Water	7 µl	

Final Volume	25 µl	

Table 7. *The cycling program of Xpert Fast Hotstart PCR mix*

Steps	Cycles	Temperature (°C)	Time
Enzyme Activation	1	95	3 Minutes
Denaturation	40	95	15 Seconds
Annealing		58	15 Seconds
Extension		72	2 Seconds
Final Extension	1	72	3 Minutes

The PCR products were analyzed by gel electrophoresis. For the gel electrophoresis analysis, 3% (w/v) agarose gel containing SafeView Classic (ABM, Canada) was cast. Agarose was dissolved in 1X TAE buffer by heating a microwave. After casting the gel, it was placed into a tank filled with 1X TAE buffer. The PCR products were loaded into gels after being mixed with 6X gel loading dye.

Furthermore, the first and last well of the gel was loaded with a 100 bp ladder to visualize the gel after the electrophoresis process. The gel was run at 90V for 1 hour and was visualized by the Chemidoc imaging system (Biorad, USA). The sample gel image for WT and NEAT1 KO mice is given in **Fig.15** below.

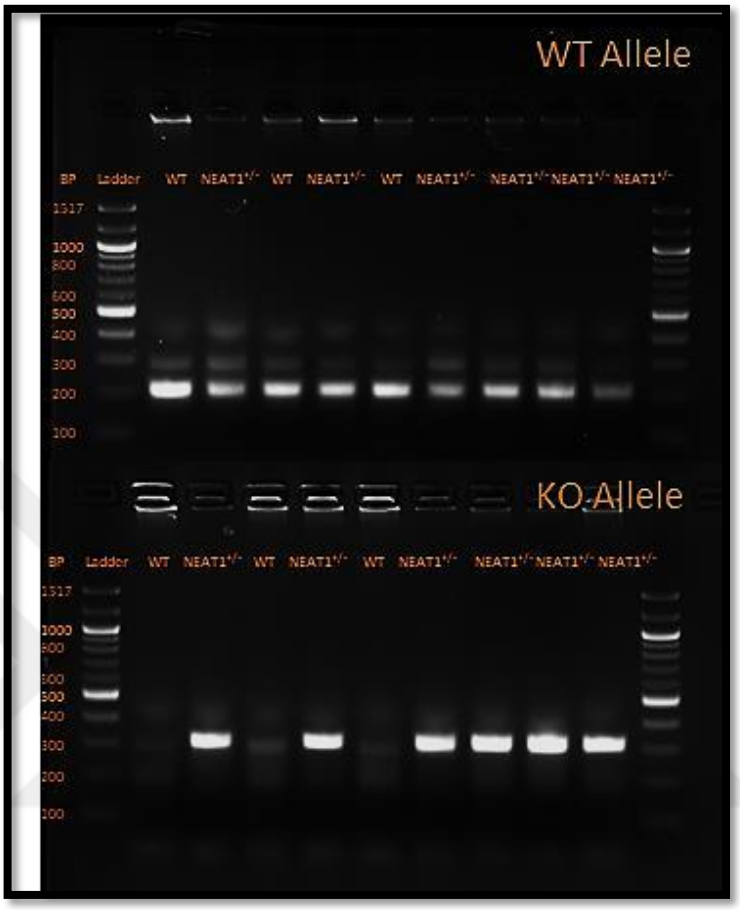

Figure 15. *The agarose gel image of PCR products of WT and NEAT1 KO alleles. The length of the amplicons are as follows: WT allele: 210bp; NEAT1 KO: 315bp*

3.6.2.1.7. LPS Injection

In the experimental setup, mice (**Table 8**) were injected intraperitoneally with 5 mg/kg LPS (Lipopolysaccharide from *E. coli* 055: B5) dissolved in sterile PBS to establish the NLRP3 inflammasome activation model (Tastan et al., 2021). Then, 24 hours after the LPS injection, the mice were subjected to behavioral tests to evaluate the effects of NEAT1 on sickness behaviors; they then were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and perfused with ice-cold PBS using a peristaltic pump to eliminate the blood in the brain vessels and eventually were sacrificed.

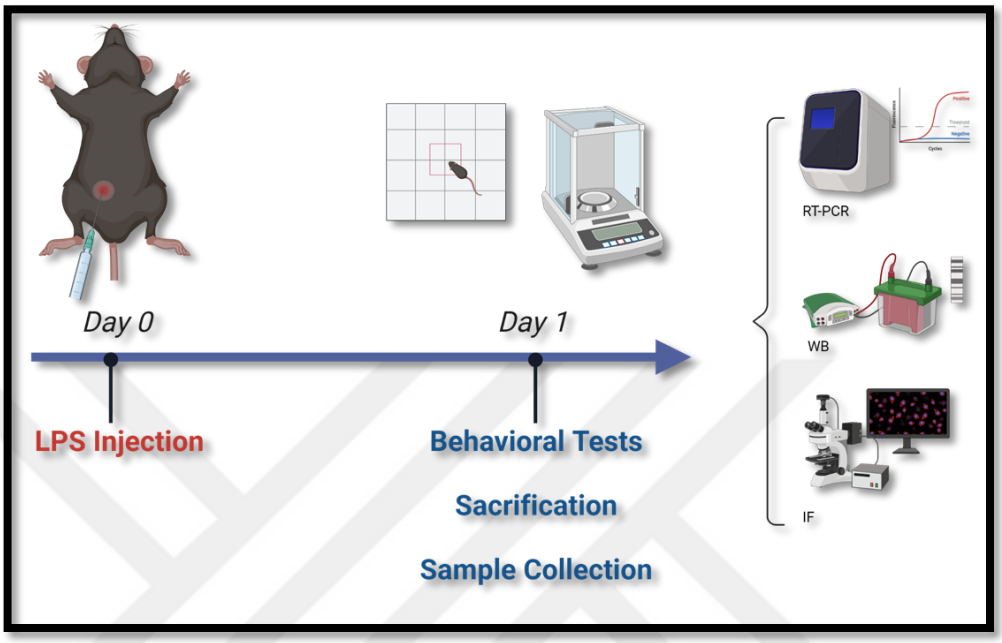

Figure 16. *The illustration of in vivo experimental setup showing the LPS-induced sickness model and the analysis to assess sickness behaviors and molecular changes.*

After sacrification, the brains of animals were divided into two hemispheres for use in molecular biological and immunohistopathological examinations, one of the hemispheres was quickly frozen in liquid nitrogen and stored in a -80°C refrigerator; the other hemisphere was incubated overnight in 4% paraformaldehyde solution for use in immunohistopathological examinations, then dehydrated in 30% sucrose solution and embedded in Tissue-Tek OCT compound to be ready for immunohistopathological examinations (**Fig.16**).

Table 8. *The experimental groups formed in the in vivo study*

Group 1 WT: Control (n=7):	Animals were injected with PBS intraperitoneally and sacrificed 24 hours after the LPS injection.
Group 2 WT: LPS (n=7):	Animals were injected intraperitoneally with 5 mg/kg LPS and sacrificed 24 hours after LPS injection.

Group 3 NEAT1 KO: Control (n=7):	Animals were injected with PBS intraperitoneally and sacrificed 24 hours after the LPS injection.
Group 4 NEAT1 KO: LPS (n=7):	Animals were injected intraperitoneally with 5 mg/kg LPS and sacrificed 24 hours after the LPS injection.

3.6.2.1.8. Behavioral Analysis

3.6.2.1.8.1. Clinical Scoring and Weighing

In order to evaluate sickness behavior and its outcomes, mice used in *in vivo* experiments were weighed prior to LPS injection. Weight measurement was repeated 24 hours after LPS injection, and the change in weight of the animals was calculated. In addition to weight change, clinical scoring was performed 24 hours after LPS injection, and the effects of disease caused by LPS were evaluated. Accordingly, mice were scored for symptoms of lethargy (decreased mobility), huddling (curled body posture), and ptosis (droopy eyelids), and each symptom was recorded as one point (one symptom = 1 point; two symptoms = 2 points, three symptoms = 3 points)(Gandhi, Hayley, Gibb, Merali, & Anisman, 2007).

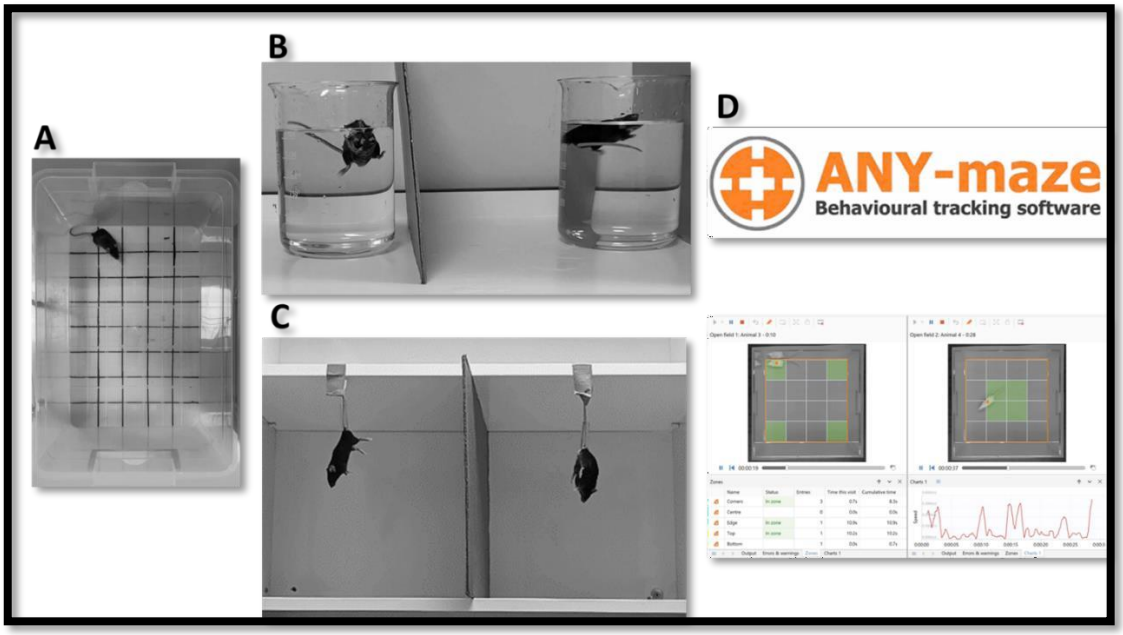

Figure 17. *The behavioral tests used in the assessment of sickness behaviors in mice.* ***A)*** *Open-Field test,* ***B)*** *Forced-swim test,* ***C)*** *Tail-suspension test, and* ***D)*** *the software used in the analysis of the recorded videos.*

3.6.2.1.8.2. Open-field Test

For the open field test (OFT) (**Fig.17A**), mice were placed in 30 x 30 x 45 cm boxes divided into equal squares, and their movements were recorded for 6 minutes. Boxes were thoroughly cleaned and dried between mice with 30% ethanol (Beckman et al., 2015). Videos recorded during testing were analyzed using Any-maze (Stoult Company) software.

3.6.2.1.8.3. Tail Suspension Test

For the tail suspension test (TST) (**Fig.17C**), mice were suspended from a 30 × 30 × 30 cm arm in a white enclosed area where they could not see each other. The movements of mice were recorded for 6 minutes. Complete immobility during the test was considered to be passively suspended for 5 seconds or longer (Bambico et al., 2010; Beckman et al., 2015). Videos recorded during testing were analyzed using Any-maze (Stoult Company) software.

3.6.2.1.8.4. Forced Swim Test

For the forced swimming test (FST) (**Fig.17B),** 65 cm (h) x 30 cm (w) beakers were filled to 45cm high with 24°C water, and mice were forced to swim in this water for 6 minutes. To prepare the mice for the test, they were allowed to acclimate to the beaker for 15 minutes the day before. The water was changed after each test, and the mice were dried in a warm environment. During testing, complete immobility was considered having made the necessary movements to keep their heads above water for 5 seconds or longer (Porsolt, Bertin, & Jalfre, 1977). Videos recorded during testing were analyzed using Any-maze (Stoult Company) software.

3.6.2.1.9. Lactate Dehydrogenase and Cell Viability Assay

To evaluate cell death and cell viability, lactate dehydrogenase assay (LDH) and cell viability assay [Cell Counting Kit-8 (CCK-8)] were used, respectively. LDH assay is based on releasing LDH enzyme into the supernatant when the plasma membrane is damaged. The amount of LDH released from the dead cells is determined via colorimetric measurements by spectrophotometer. To perform LDH assay, first, N9 microglia were plated to a 96-well plate with a concentration of $1X10^4$ cells/well in 200 µl volume for each group. The plate was incubated overnight. The next day, the medium to be used in the experiment was prepared. The colorless RPMI 1640 medium does not contain FBS and contains L-Glutamine, Penicillin, and Streptomycin used during the experiment. After LPS and ATP model was implemented on cells, they were taken from the incubator, and 50 µl of supernatant was collected from the wells. These supernatants were subjected to Cytotoxicity Detection Kit LDH (Roche, Germany) assay mixture and incubated for 15 minutes at room temperature. Lastly, the plates were read at a wavelength of 490 nm and a reference wavelength of 600 nm. Percent cell death will be calculated using the following formula: % Cytotoxicity = (Found absorbance - Control absorbance) / (Maximum absorbance - Control absorbance) X100.

CCK-8 was used to assess cell viability. CCK-8 is based on converting WST-8 tetrazolium monosodium salt into water-soluble formazan dye by cellular dehydrogenases to an orange formazan product soluble in a tissue culture medium. In order to perform the CCK-8 assay, 15 µl of CCK-8 solution per well was added onto theplate, which contains 150 µl supernatant (because 50 µl of supernatant was used for LDH assay). The well plate was incubated at 37 ℃ for 2-4 hours more. The color change was read at 450 nm with the multi-plate reader Varioskan (ThermoScientific, USA) and at 630 nm as a reference. Cell viability was calculated using the following formula: % Cell Viability = (Sample value absorbance/Control absorbance) *100

3.6.2.1.10. Propidium Iodide (PI) Staining

In order to visualize and analyze pyroptotic cell death in N9 microglia, PI staining was performed. First, N9 microglia were seeded into a 48-well plate with a density of 3x104 cells/well, and the cells were incubated overnight at 37 °C. The next day, LPS and ATP model was implemented on them to induce NLRP3 inflammasome activation. In the last 15 minutes of ATP treatments, 50 μg/ml of PI stain was added per well and incubated for 15 minutes. Images were acquired using a fluorescent microscope (Olympus IX-71, Japan). ImageJ software (National Institutes of Health, USA) was used to count the number of PI-positive and PI-negative cells. The percentages of PI-positive cells were calculated by the ratio of PI-positive cells to the total number of cells.

3.6.2.1.11. ELISA

The pro-inflammatory cytokines IL-1β and IL-18 levels were determined with commercially available Enzyme-linked Immunosorbent Assay (ELISA) kits (Invitrogen, USA). The protocols were followed according to the manufacturer's instructions. On day zero, N9 cells ($50x10^3$ cells/well, 96-well plate) were plated based on the experimental setup. On the same day, a 96-well plate (Nunc, USA) was coated against capture antibody (IL-1β or IL-18) and left for overnight incubation. On the second day, after LPSand ATP model was implemented on N9 cells ($50x10^3$ cells/well, 96-well plate), the plate was centrifuged briefly, and the supernatant was collected. Along with that, coated ELISA plates were washed and blocked. After the blocking step, standards and collected supernatants were put into wells, and the plate was sealed and incubated overnight at +4°C. The next day, the following steps were implemented respectively; aspiration of the wells, washing, the addition of detection antibody, incubation for 1 hour at RT, aspiration, washing, the addition of Streptavidin-HRP solution, and incubation for
30 minutes, aspiration, washing and addition of TMB/substrate solution. After the substrate solution was added and incubated at room temperature, the enzymatic reaction was stopped, and the plate was read at absorbance 450 nm and 570 nm with a spectrophotometer (ThermoScientific, USA). The protein concentrations were calculated using a standard curve plotted from standards.

3.6.2.1.12. RT-qPCR

3.6.2.1.12.1. RNA Isolation

RNA isolation was performed with Trizol Reagent (ThermoFisher, USA) following the manufacturer's protocol with slight modification. Briefly, the cells were scraped and lysedwith a Trizol reagent after experiments. Next, 1 ml of Trizol reagent was added to 1 X 10^6 cells, and the lysate was heated at 55° C for 10 minutes and agitated at 1000 rpm speed. This step is primarily performed for the experiments in which NEAT1 expression is detected (Chujo et al., 2017). After this step, the samples were incubated for 5 minutes for the nucleoprotein complex to dissociate completely. Then, 0,2 ml of chloroform was added to the samples, and the tubes were shaken thoroughly. After 3 minutes of incubation, the samples were centrifuged at 12,000 × g for 15 minutes at

+4°C resulting in 3-separated layers in the tubes. The top colorless layer was carefully collected into a new tube to isolate the total RNA. Next, 0,5 ml of isopropanol was addedto the new tube containing the collected top layer. The mixture was incubated for 10 minutes at +4°C and then centrifuged at 12,000 × g for 10 minutes at 4°C. The total RNA precipitated as a pellet on the bottom of the tube. Thus, the supernatant was discarded, and the pellet was washed with 1 mL of 75% ethanol. Next, the tube was vigorously vortexed and centrifuged for 5 minutes at 7500 × g at 4°C. In the end, the supernatant was removed carefully, and the pellet was air-dried for 10 minutes. Finally, total RNA was solubilized in 30 µl of RNase-free water. The concentration and quality of RNA samples were checked by a Nanodrop spectrophotometer by checking 260/280 and 260/230. If the samples passed through quality check, samples were used for cDNAsynthesis and further downstream application.

3.6.2.1.12.2. cDNA Synthesis

After RNA isolation, cDNA was synthesized with the RevertAid First Strand cDNASynthesis Kit (ThermoFisher, USA). During synthesis, a reaction was prepared 1µg of the total RNA sample. The RevertAid First Strand cDNA synthesis kit consists of 2-step. The first and second step was prepared as shown in

Table 9 and **Table 10,** respectively. The reactions were set up as shown in **Table 11**. When the second reaction was complete, the cDNA samples were taken from the thermal cycler and put to -20ºC for later use.

Table 9. *The preparation of the step-1 reaction mix*

Components	Volume	Final Concentration
Template RNA	X	1 µg
Random Hexamer Primer	1 µl	
Nuclease-Free Water	(11-x) µl	

Final Volume	12 µl	

Table 10. *The preparation of the step-2 reaction mix*

Components	Volume	Final Concentration
Reaction Buffer (5X)	4 µl	1X
RiboLock RNase Inhibitor (20 U/µL)	1 µl	1U
10 mM dNTP Mix	2 µl	1 mM
RevertAid M-MuLV RT (200 U/µL)	1 µl	10 U

Final Volume	20 µl	

Table 11. *The cycling program of RevertAid First Strand cDNA Synthesis Kit*

Steps	Cycles	Temperature (°C)	Time
Step-1			
Incubation	1	65	5 Minutes
Step-2			
Activation	1	25	5 Minutes
Synthesis	1	42	60 Minutes
Termination	1	70	5 Minutes

3.6.2.1.12.3. RT-qPCR

After cDNA synthesis, RT-qPCR analysis was performed to examine differential gene expression between the biological groups. For the mRNA expression of NLRP3 inflammasome markers and the lncRNA NEAT1, GoTaq qPCR (Promega, USA) SYBR green mix was used in the RT-qPCR. First, the qPCR master mix included in the kit was thawed on ice. After thawing, the reaction was set up as described in **Table 12**. The complete list of the primers used in RT-qPCR analysis is given in **Table 14**. Briefly, 10 µlof 2X Master mix SYBR green, 1 µl forward and 1 µl reverse primer, and 1 µg cDNA were used for the reactions. The mix was completed to 20 µl with nuclease-free water depending on the cDNA volume. ABI Fast 7500 instrument was used for the RT-qPCR reaction. The reaction was carried out in 40 cycles of 95ºC for 10 minutes, 60ºC for 10 seconds, and 72ºC for 30 seconds (**Table 13**). After that, the quality control of the PCR reaction was done by melting curve analysis (**Fig.18**). For the calculation of relative gene expression, the housekeeping gene GAPDH was used for normalization. Then, the changes in the gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method.

Table 12. *The preparation of the reaction mix.*

Components	Volume	Final Concentration
qPCR Master Mix (2X)	10 µl	1X
Forward Primer	1 µl	
Reverse Primer	1 µl	
Nuclease-Free Water	(8-x) µl	
cDNA	x µl	1 µg

Final Volume	20 µl	

Table 13. *The cycling program of GoTaq® qPCR master mix*

Steps	Cycles	Temperature (°C)	Time
Hot Start Polymerase activation	1	95	2 Minutes
Denaturation	40	95	15 Seconds
Annealing & Extension		60	1 Minute

Table 14. *The list of primers used for RT-qPCR.*

Gene		Primers	Product
Ms_NEAT1_1	**F**	TGGTCTCACTCTTCCCTACTC	141 bp
	R	CCACCTTTACCAAAATGCCG	
Ms_NEAT1_2	**F**	CATGTGTTATGGTGGGGCCT	287 bp
	R	GCAGAGGGAATAGGTACGGC	
IL-1β	**F**	TTCTTTTCCTTCATCTTTGAAGAAG	365 bp

	R	TCCATCTTCTTCTTTGGGTATTGTT	
IL-18	**F**	CTTTGGAAGCCTGCTATAATCC	363 bp
	R	GGTCAAGAGGAAGTGATTTGGA	
NLRP3	**F**	TGCCTGTTCTTCCAGACTGGTGA	143 bp
	R	CACAGCACCCTCATGCCCGG	
GAPDH	**F**	ACCACAGTCCATGCCATCAC	452 bp
	R	TCCACCACCCTGTTGCTGTA	

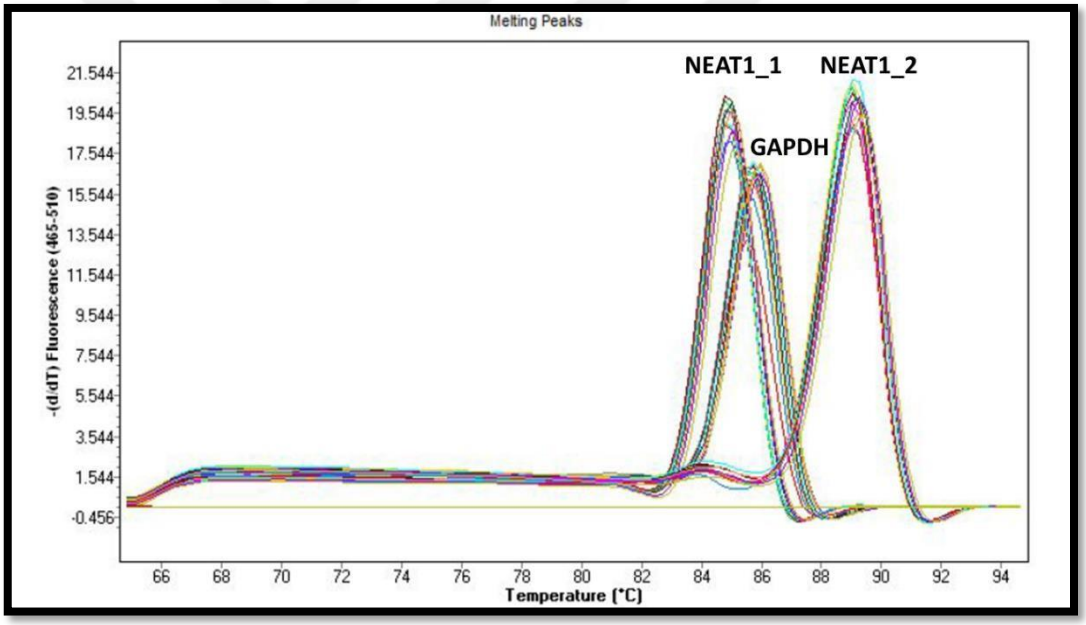

Figure 18. *The melting curve analysis of designed primers for NEAT1_1 and NEAT1_2.*

3.6.2.1.13. Western Blot

3.6.2.1.13.1. Protein Isolation

At the end of the experiments, cells were scraped off and collected in a 15-ml falcon. Then the cells were centrifuged at 1500 RPM for 5 minutes at RT, and after washing with 1X PBS, they were again centrifuged. Next, the pellet was lysed with ice-cold RIPA

buffer (ThermoFisher, USA) supplemented with HALT protease inhibitor (ThermoFisher, USA). Next, the samples were left on ice for 40 minutes. During this incubation, they were vortexed every 5 minutes to increase the lysis yield. Finally, the samples were centrifuged at 12.000 g at 4°C for 15 minutes, and the supernatant containing total protein was transferred into a new tube and stored at -80°C for further experiments.

3.6.2.1.13.2. Determination of Protein Concentration

The Pierce Bicinchoninic Acid Assay (BCA) protein assay kit was used to determine the concentration of protein samples. Firstly, BSA standards were prepared in different concentrations ranging from 1000 µg/ml to 100 µg/ml. Furthermore, samples were diluted accordingly. Then, both standards and samples were put into a 96-well plate (25 µl/ sample) as duplicates. BCA solution consisting of reagent A (200 µl/sample) and B (4 µl/sample) were put onto samples, and the plate was incubated at 37° C for 30 minutes. The plate was read via spectrophotometer at 562 nm wavelength. After plotting the standard curve using BSA standards, the concentration of the samples was determined, and the values were used in further experiments.

3.6.2.1.13.3. SDS-Page and Transfer of the gel

An SDS page was carried out to visualize and quantify the protein of interest. To do that,the first SDS-Page gels were prepared. As SDS-page gels consist of two different gels, Resolving and Stacking, their preparations differ. Resolving gel was cast based on the size of the protein of interest. **Table 15** indicates recipes for resolving gels ranging from 6% - 15% acrylamide content. After the resolving gel was poured and polymerized, stacking gel was poured onto resolving gel using the recipe in **Table 16,** and a 10- or 15-well comb, depending on the sample number, was placed onto the stacking gel. SDS-page were run using Mini PROTEAN® Tetra Cell equipment (Bio-Rad, USA). The samples, containing 50 µg of total protein in 1X Laemmli buffer and boiled at 95° C for 5minutes, were run in 1X running buffer (in **Table 14**) at constant mA (12 mA for each gel in the tank) until the loading dye put into the first well of each gel was completely separated which takes typically 1,5-2 hours).

After SDS-Page was run, the gels were transferred into the PVDF membrane (Millipore, USA) using a mini–Trans-Blot module (Bio-Rad, USA). The transfer was carried out in 1X Towbin transfer buffer at 375 mA for 2 hours.

Table 15. *The recipe and preparations of buffers used in SDS-Page and Western Blotting.*

Buffers & Solutions	**Recipe**
1,5 M Tris Solution, pH 8,8 – 0,1 L	• 18.15 g Tris - Dissolved in 90 mL distilled water - pH adjusted to 8,8 with HCl - Completed up to 0,1 L
1 M Tris Solution, pH 6,8 – 0,1 L	• 12.10 g Tris - Dissolved in 90 mL distilled water - pH adjusted to 6,8 with HCl - Completed up to 0,1 L
30% Acrylamide/Bis Solution (29:1) – 0,1 L	• 29 g Acrylamide • 1 g Bis-acrylamide - Dissolved in 600 ml distilled water - Completed up to 0,1 L at 37° C
10X SDS Running Buffer – 1L	• 10g SDS • 30.3g Tris Base • 144.1g Glycine -Dissolved in 800 ml distilled water -Completed up to 1 L
10X Towbin Transfer Buffer – 0,8 L	• 30g Tris Base • 144g Glycine • 20 ml SDS (%10) - Dissolved in 600 ml distilled water - Completed up to 800 ml

	- 200 ml of methanol was added to 800 ml of 1X Towbin Buffer.
5X Laemmli Buffer – 10 ml	• 1 g SDS • 2.5 ml 1M Tris, pH 6.8 • 5 mg Bromophenol Blue • 4.45 ml H2O • 3 ml Glycerol • 50µl 2M DTT
10X TBS – 1 L	• 24 g Tris base • 88 g NaCl - Dissolved in 900 mL distilled water - pH adjusted to 7.6 with HCl - Completed up to 1 L
1X TBS-T (0,05% Tween 20) – 1 L	• 100 mL 10x TBS • 900 mL distilled water • 0,5 mL Tween 20

Table 16. *The recipe for resolving gel based on its acrylamide concentrations*

Components	**6%**	**8%**	**10%**	**12%**	**15%**
Distilled Water	5,3 ml	4,6 ml	4,0 ml	3,3 ml	2,3 ml
30% Acrylamide/Bis Solution	2,0 ml	2,7 ml	3,3 ml	4,0 ml	5 ml
1,5 M Tris Solution, pH 8,8	2,5 ml	2,5 ml	2,5 ml	2,5 ml	2,5 ml
10% SDS	0,1 ml	0,1 ml	0,1 ml	0,1 ml	0,1 ml

10% APS	0,1 ml	0,1 ml	0,1 ml	0,1 ml	0,1 ml
TEMED	0,008 ml	0,006 ml	0,004 ml	0,004 ml	0,004 ml

Table 17. *The recipe for 5% Stacking Gel*

Components	**5%**
Distilled Water	6.8 ml
30% Acrylamide/Bis Solution	1.7 ml
1,0 M Tris Solution, pH 6,8	1.25 ml
10% SDS	0.1 ml
10% APS	0.1 ml
TEMED	0.01 ml

3.6.2.1.13.4. Immunoblotting and Stripping of the membranes

After the transfer step, membranes were blocked with milk or BSA, whose concentration depends on the antibody of protein of interest, for 1 hour at RT. Then, membranes were incubated with primary antibodies against the protein of interest overnight at 4°C. The next day, membranes were washed with TBS-T and incubated with secondary antibodies at RT for 1 hour. Lastly, membranes were washed 3 times with TBS-T for 15 minutes. As the immunoblotting step was completed, the membranes were soaked into till imaging step. The list of antibodies used in the immunoblotting step and the blocking agent/concentration is given in **Table 1**.

Occasionally, stripping of the membranes was required as different proteins of interest were analyzed. In this case, the membranes were incubated with stripping buffer (ThermoFisher, USA) for 40 minutes at RT. Next, membranes were washed with TBS-T twice for 15 minutes to eliminate the stripping buffer as much as possible. Then, the immunoblotting process was started from the beginning.

3.6.2.1.13.5. Imaging and Analysis

The Chemidoc imaging system (Bio-Rad, USA) was utilized for the imaging of the membranes. To do that, Chemiluminescent Substrate (ThermoFisher, USA) was put onto membranes and incubated for a short period. Then they were imaged, and the images were analyzed via Image Studio software. In the analysis, the expression of cellular/nuclear proteins of interest was normalized with β-actin, and the expression of secreted proteins (cytokines) was given as arbitrary units.

3.6.2.1.14. RNA immunoprecipitation (RIP) assay

In order to validate the interaction between RNA-binding proteins NONO/SFPQ and NEAT1, an RNA immunoprecipitation (RIP) assay was performed. Briefly, N9 microglia were seeded into a 150 mm cell culture dish to work with a high number of cells. The next day, after the cells were treated with LPS and ATP, the cells were collected using cell scrapers and washed with cold PBS. Subsequently, cells were lysed using 100 µl of RIP Lysis Buffer containing protease and RNase inhibitors. The cell lysates were centrifuged at 14,000 rpm for 10 minutes at 4°C. The collected supernatants were incubated with NONO or SFPQ antibody or control IgG. The immunoprecipitation process involved the use of preactivated magnetic beads, which were prepared according to the instructions provided with the kit. The RNA binding protein-RNA complexes were immunoprecipitated by incubating the samples with the prepared magnetic beads at 4°C overnight with agitation. After the immunoprecipitation, the beads were washed using an ice-cold RIP Wash Buffer from the Magna RIP kit. To digest the RNA binding proteins, the beads were treated with proteinase K at 55°C for 30 minutes with shaking. The purified RNA was then isolated using Trizol reagent, and quantification of RIP assay was performed with qRT-PCR to determine NEAT1 expression level.

3.6.2.1.15. FISH & IF

To detect and observe lncRNA NEAT1, Fluorescence in Situ Hybridization (FISH) and IF staining were performed. The NEAT1 probes were obtained commercially (Stellaris,

BioSearch Technologies, USA). The commercial kit provided all the necessary buffers for the FISH protocol. Probes were used in the experiments to detect both isoforms (long and short) of NEAT1. First, N9 cells were grown on a cell culture dish with a concentration of $1x10^6$. After LPS and ATP model was implemented, cells were detached from the cell culture dish with trypsin/EDTA (0,25%). The cells were collected and precipitated onto slides with a Cytospin centrifuge (Hettich, USA).

Then, the slides were washed with 1 mL of 1X PBS. The cells were incubated with a 1 mL addition of fixation buffer and allowed to sit at room temperature for 10 minutes. The cells were then rinsed twice in 1 mL of 1X PBS. Next, the cells were suspended in 1 mL of 70% ethanol and kept between +4 °C for an hour to make them permeable. After that, the hybridization buffer containing probes was prepared by mixing 1 µL of the probe stock with 100 µL of hybridization buffer yielding with 125 nM working probe solution. The 70% ethanol was removed from the slides containing precipitated N9 microglia. After that, 1 mL of Wash Buffer A was added, and the slides were incubated for 5 minutes at room temperature. A mixture of 100 µL of the hybridization buffer containing the probe and the primary antibodies was applied to the slides on the parafilm. The droplet containing the hybridization buffer, probe, and primary antibody was gently placed on the slides. The slides were incubated in the dark at 37 °C overnight in the humidified chamber to prevent evaporation of the working solution. The following day, the slides were soaked with 1 mL of Wash Buffer A and a properly diluted secondary antibody. The slides were further incubated for 30 minutes at 37 °C in the dark.

Lastly, 1 mL of DAPI nuclear stain (5 ng/mL DAPI) and adequately diluted secondary antibody in the Wash Buffer A were added into slides and kept in the dark for another 30 minutes. Next, the wash buffer A containing DAPI was removed, and 1 mL of Wash Buffer B was added and incubated for 5 minutes at room temperature. A little drop of Vectashield Mounting Medium was added to the slides at the end of the staining procedure. Then the coverglass was then mounted onto the slide with the cells facing down. Around the coverglass, any extra anti-fade solution was carefully wiped away. After being dried enough, coverglass edges were sealed using nail polish. Samples

were visualized with Zeiss LSM880 confocal microscope. Images were analyzed with ImageJ software (National Institutes of Health, USA) (Schindelin et al., 2012).

3.6.2.1.16. siRNA Transfection

To test the effect of NEAT1 on microglial NLRP3 inflammasome activation, cells were transfected with siRNA specific to the lncRNA NEAT1 (Lincode Mouse Neat1 siRNA, Horizon Discovery, USA) to silence NEAT1. Additionally, a non-targeting siRNA pool (Lincode Non-targeting Control Pool, Horizon Discovery, USA) was also used to eliminate the effect of the transfection process. First, N9 microglia were seeded in dishes/plates depending on the experiment to carry out and cultured overnight in the RPMI media, excluding antibiotics. The following day, 5 µM siRNA solution was prepared in siRNA buffer. siRNAs were diluted with DharmaFECT transfection reagent according to the manufacturer's instructions. Next, media containing siRNA and media containing transfection reagents were mixed. Then, to mediate forming complexes between the transfection reagent and siRNA, the mixture was set still for 20 minutes at room temperature. After the siRNA mixture was ready, the culture medium was removed from the plates/dishes, and the siRNA-transfection Reagent mixture was slowly added dropwise at 200 µl per well. Finally, the cells were incubated in the incubator for 48 hours, and the experiments (LPS and ATP model) were carried out afterward.

3.6.2.1.17. miRNA mimic Transfection

In order to reveal the relation between lncRNA NEAT1 and miR-146a-5p and how miR- 146a-5p affects microglial NLRP3 inflammasome activation, transfection of miR-146a-5p mimics was carried out using commercially available tools and instructions. While miScript Inhibitor Negative Control was used as the negative control, Syn-mmu-miR- 146a-5p miScript miRNA mimic was used as the mimic. First, cells were seeded in a cellculture flask with an antibiotic-free medium. The next day, cells were transfected with 20 nM mimic or negative control by HiPerFect Transfection Reagent. The cells were then cultured for 48 with miR-146 and mimic miR-146 to complete the transfection process. Afterward, the media was changed when this process was complete, and the cells were

treated with LPS for 4 hours and ATP for 1 hour to induce NLRP3 inflammasome activation. Before any experimental analysis, transfection efficiency was checked by measuring mmu-mir146a-5p expression by RT-qPCR.

3.6.2.1.18. Data Analysis

3.7. Research plan and timeline

The thesis study has followed the timeline given in **Fig.19**.

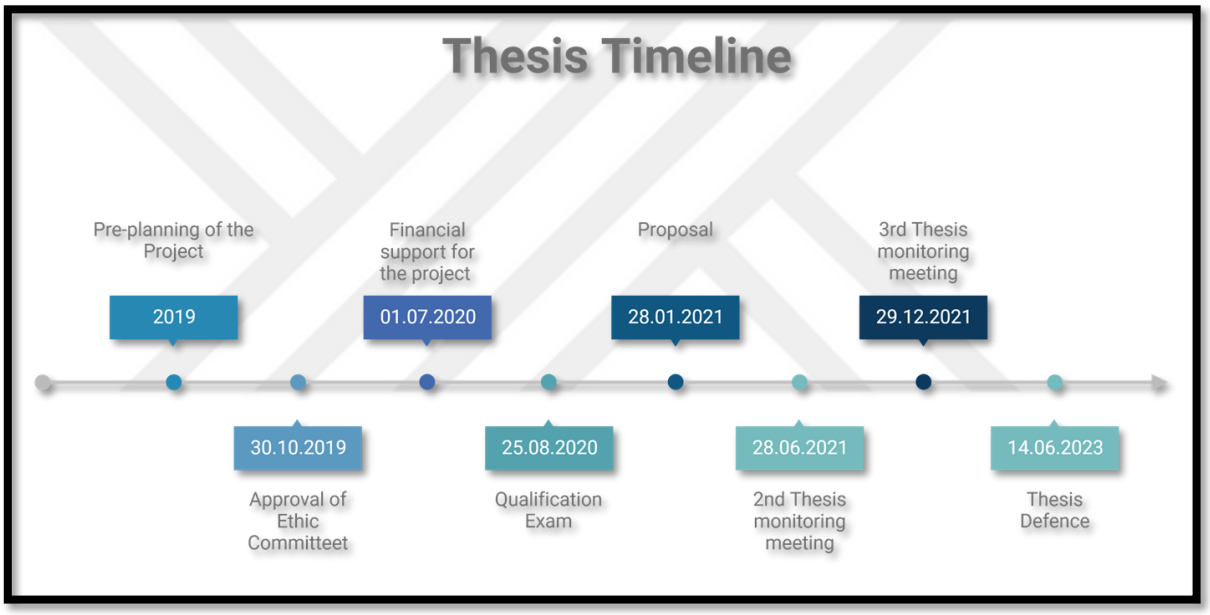

Figure 19. *The schematic representation of the research plan and important milestones of this thesis study*

3.8. Data analysis

All statistical analyses were performed with GraphPad Prism 9.0 (GraphPad Software Inc., CA, USA). The collected data were initially examined with the Shapiro-Wilk test to determine their normality. For non-parametric data, the Mann-Whitney U-test was used, while the student's t-test was used for parametric data. Then, multiple normally distributed groups were compared using one-way ANOVA with Bonferroni multiple comparison corrections. The Dunn's multiple comparison Kruskal-Wallis test was used

to compare three or more non-normally distributed groups. All information was presented as the mean, standard error of the mean. In all experiments, a p-value less than 0.05 (p0.05) was considered statistically significant and represented in the graphicsas *p 0.05, **p 0.01, and ***p 0.001.

3.9. Limitations of the research

Although *in vitro* experiments were carried out on microglial cell lines from different species, the results were not confirmed in primary microglia. Therefore, this could be counted as the only experimental limitation of this thesis study. Furthermore, to gain more insight into the lncRNA NEAT1-ceRNA network, NGS sequencing could be performed with the NLRP3 inflammasome activation model. However, the budget allocated for this thesis study was insufficient for this kind of elaborate approach.

3.10. Ethics committee approval

This thesis study has been approved by Izmir International Biomedicine and Genome Institute Local Ethics Committee for Animal Experiments (IBG-AELEC) with protocol number 33/2019. All the experiments and the animal care were strictly performed according to the ethical guideline. The certificate of committee's approval is given in the Appendix.

4. RESULTS

4.1. NEAT1 KO mice had lower body weight compared to Wild-type C57BL/6 mice

Behavioral assessment of wild-type mice and NEAT1 KO mice were performed 24 hoursafter the generation of the disease model by LPS injection (**Fig. 20A**). Before the behavioral tests, the weights of the mice used in the *in vivo* experiment were measured (**Fig. 20B**). As seen in results, NEAT1 KO mice were found to be significantly slimmer/ smaller than wild-type mice.

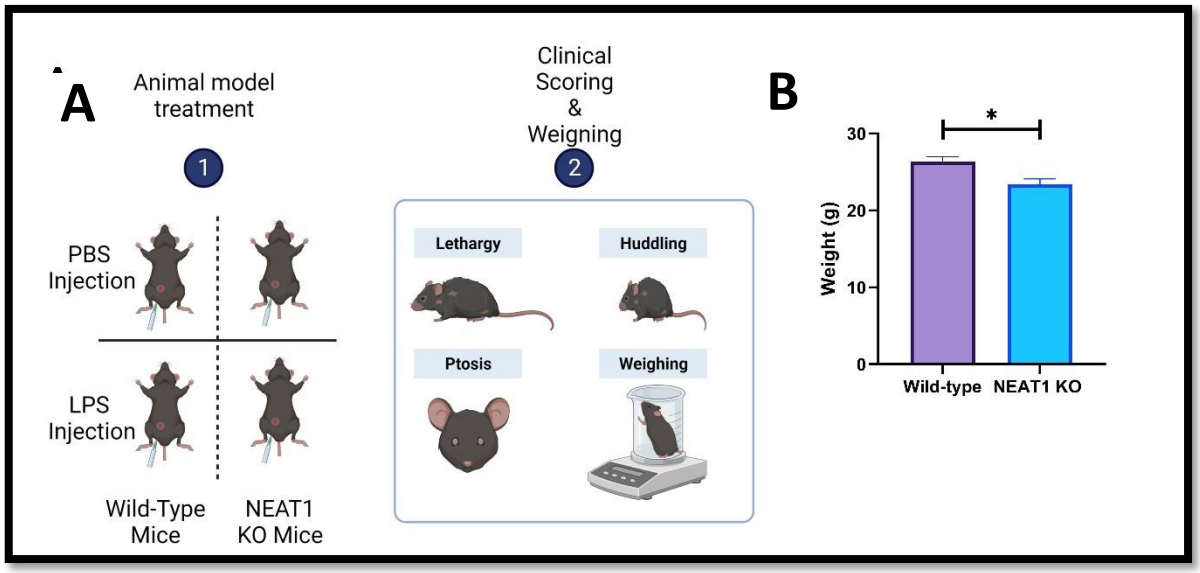

Figure 20. *Representation of intergroup weight change and clinical score in the disease model created by LPS injection (5mg/kg).* ***A)*** *The schematic representation of the experimental setup and clinical assessment.* ***B)*** *Weight difference between wild-type mice and NEAT1 Knock-Out mice. Data are given as mean ± S.E.M. n=7, *p <0.05*

4.2. Lower body weight change and mild sickness-related phenotype were observed in NEAT1 KO mice after LPS injection.

The weights of the mice were measured before and after the LPS injection allowing us to determine how LPS affected body weight in both wild-type mice and NEAT1 KO mice. Results indicated that LPS injection caused higher weight loss in wild-type mice.

However, NEAT1 KO mice displayed less body weight change than wild-type mice (**Fig. 21A**).

Furthermore, mice were clinically scored to determine the effect of LPS injection in NEAT1 KO mice (**Fig. 20A**). To do that, three different parameters (lethargy, ptosis, and huddling) were used for clinical evaluation of the mice. In line with previous results, LPS-injected NEAT1 mice had improved clinical scores compared to LPS-injected wild-type mice. These demonstrated that NEAT1 has a regulatory role in systemic immune response upon LPS administration causing milder disease phenotype.

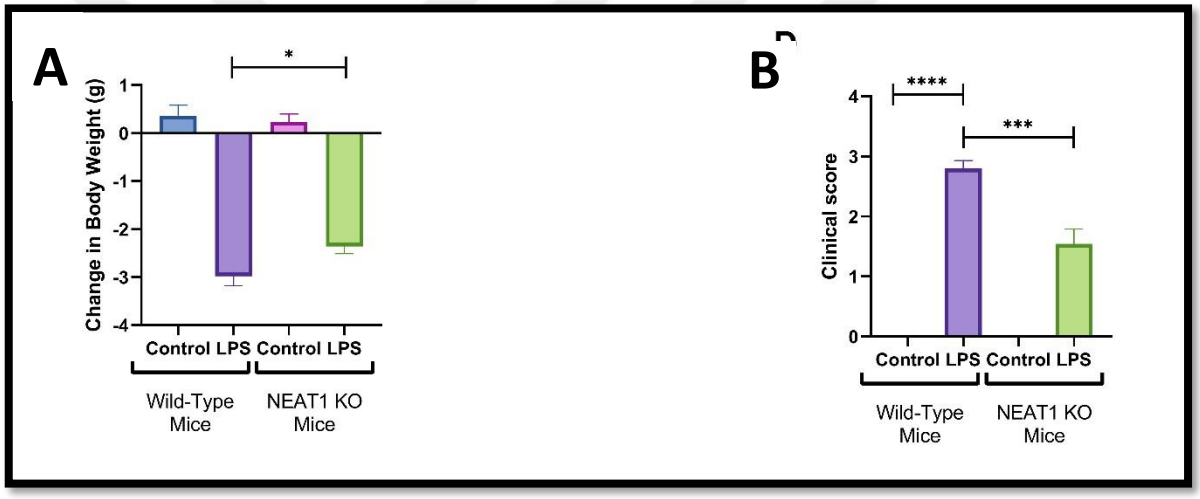

Figure 21. *Comparison of change in the body weight and clinical score in the disease model created by LPS injection (5mg/kg).* ***A)*** *Alteration in the body weight B**)** Clinical assessment of wild-type and NEAT1 KO mice. Data are given as mean ± S.E.M. n=7, *p <0.05, ***p <0.001, ****p<0.0001*

4.3. LPS-injected NEAT1 KO mice displayed milder sickness behaviors compared to wild-type mice.

After examination of the change in the body weight among groups and the clinical scoring of mice, behavioral tests were carried out to evaluate sickness behaviors in the

disease model generated by LPS injection on both NEAT1 KO and wild-type mice. We further aimed to demonstrate the effect of NEAT1 on these behaviors.

In OFT, the first behavioral test performed, several parameters were analyzed, including the number of lines crossed by mice, total immobility, average speed, and the zones mice wandered. Based on the OFT results, NEAT1 KO mice performed better in the OFT test compared to wild-type mice upon LPS injection (**Fig. 22**). The number of lines crossed, which decreased with LPS injection in wild-type mice, increased in NEAT1 KO mice injected with LPS (Fig. **22A**). NEAT1 KO mice displayed better score in terms of the line crossed. Also, the total immobility time, which increases with LPS injection, was lower in LPS-injected NEAT1 KO mice compared to LPS-injected wild-type mice (Fig. **22B**). The further analysis indicated that the average speed of control NEAT1 mice was significantly higher than the rest of the other group. These findings support the observation that NEAT1 KO mice were hyperactive during the OFT test. This hyperactivity led to the escape of control NEAT1 KO mice occasionally by jumping out of the OFT box (Fig. **22C**).

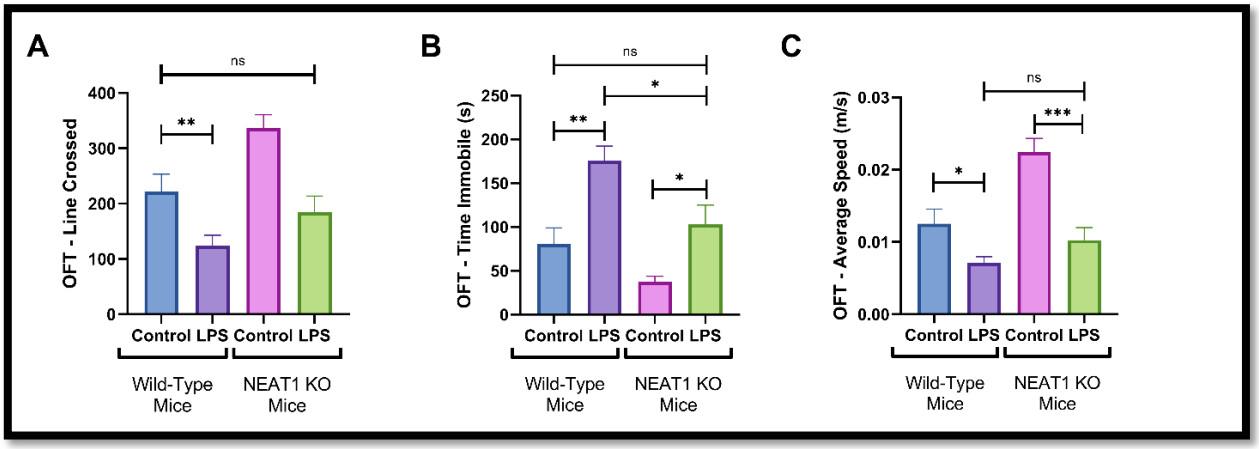

Figure 22. *The behavioral assessment of sickness-behavior by OFT* ***A)*** *Comparison of the line crossed by mice in the OFT* ***B)*** *the total time of immobility* ***C)*** *Average speed of mice measured during OFT. Data are given as mean ± S.E.M. n=7, *p <0.05, **p <0.01, ***p<0.001, ns: not significant*

While there was no difference between the groups in the amount of distance traveled (**Fig. 23A**), their preferences for which areas of the OFT box they wandered were altered. LPS-injected wild-type mice tended to spend time in corners, whereas LPS- injected NEAT1 KO mice wandered the box more evenly, including the center and peripheral areas (**Fig. 23B-C**).

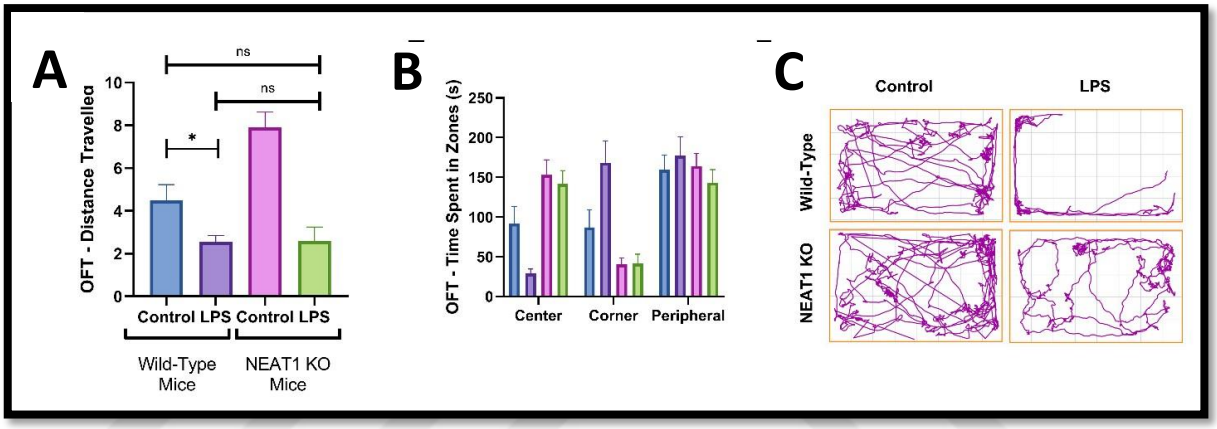

Figure 23. *The behavioral assessment of sickness-behavior by OFT* ***A)*** *Comparison of distance traveled by wild-type and NEAT1 KO mice in the OFT* ***B)*** *The time mice spent in the region of the OFT box* ***C)*** *The demonstration of OFT-tracking system record showing the zones of OFT box. Data are given as mean ± S.E.M. n=7, *p <0.05, ns: not significant*

Another test used to assess sickness behavior in mice is the TST. In the TST, the parameters of total immobility and latency to the first immobility episode of mice were investigated. In the test, in which more than 5 seconds of immobility was considered immobilized, wild-type mice injected with LPS were immobilized longer than wild-type control mice. In contrast, there was no significant change between control and LPS- injected NEAT1 KO mice (**Fig. 24A**). LPS-injected wild-type mice were immobilized

earlier than wild-type control mice. However, this situation was reversed in LPS-injected NEAT1 KO mice compared to wild-type mice (**Fig. 24B**).

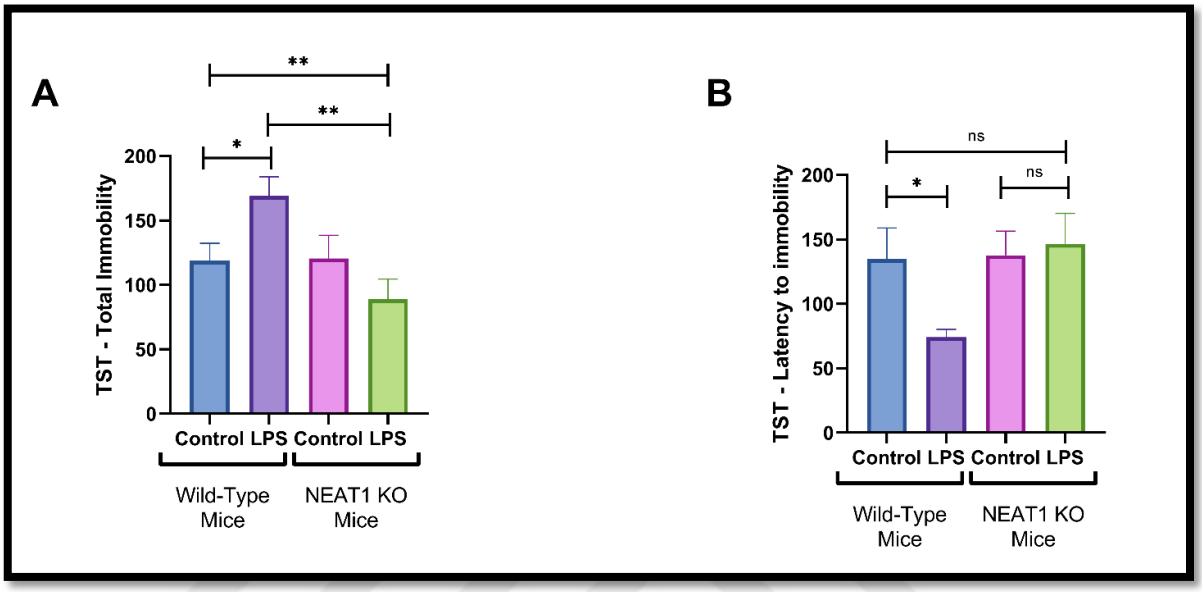

Figure 24. *The behavioral assessment of sickness-behavior by TST* ***A)*** *Comparison of the total time of immobility of mice in the TST* ***B)*** *The latency of the immobility during TST. Data are given as mean ± S.E.M. n=7, *p <0.05, **p <0.01, ns: not significant*

Finally, the FST was performed to evaluate sickness behaviors *in vivo*. As in the TST test, the total immobility of the mice and the latency to the first immobilization parameters were analyzed in this test. In the test, in which more than 5 seconds of immobility was considered immobilization, the total time in which LPS-injected NEAT1 KO mice were less immobilized compared to the wild-type mice (**Fig. 25A**). Again, LPS- injected wild-type mice gave up earlier than its counterpart LPS-injected NEAT1 KO mice (**Fig. 25B**). Thus, NEAT1 KO mice reversed adverse effects of LPS-injection and displayed improved behaviors compared to wild-type mice.

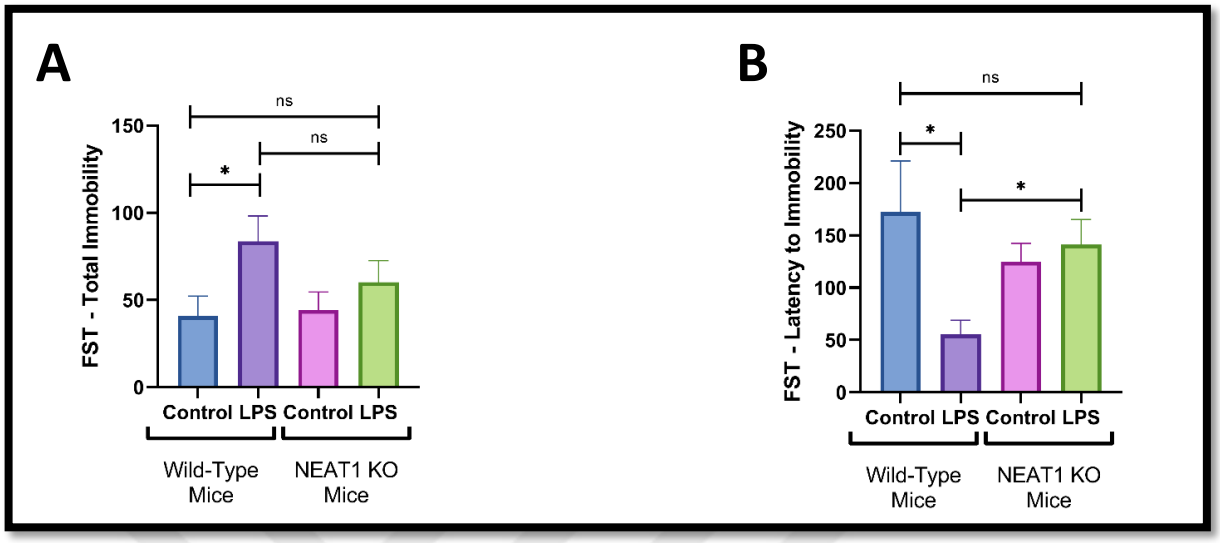

Figure 25. *The behavioral assessment of sickness-behavior by FST* ***A)*** *Comparison of the total time of immobility of mice in the TST* ***B)*** *The latency of the immobility during TST. Data are given as mean ± S.E.M. n=7, *p <0.05, ns: not significant*

4.4. Systemic LPS injection increased the mRNA expression of NLRP3 inflammasome markers in both wild-type and NEAT1 KO mice.

After the disease model was generated and behavioral tests were carried out on wild- type and NEAT1 KO mice, the animals were sacrificed, and the effect of NEAT1 on *in vivo* activation of NLRP3 inflammasome was studied.

First, the change in NLRP3 inflammasome activation markers was examined by the RT- qPCR method (**Fig. 26**). The changes in the mRNA levels of the pro-inflammatory cytokines IL-1β (**Fig. 26A**) and IL-18 (**Fig. 26C**), and NLRP3 mRNA level (**Fig. 26B**) were determined. Results indicated that IL-1β mRNA level is upregulated by LPS injection in wild-type mice, and this was not reversed in NEAT1 KO mice. On the other hand, IL-18 mRNA levels remain the same among the groups. Lastly, NLRP3 levels were significantly elevated in LPS-injected wilt-type and NEAT1 KO mice. These pieces of evidence indicate that lncRNA NEAT1 does not alter mRNA levels of NLRP3 inflammasome activation markers.

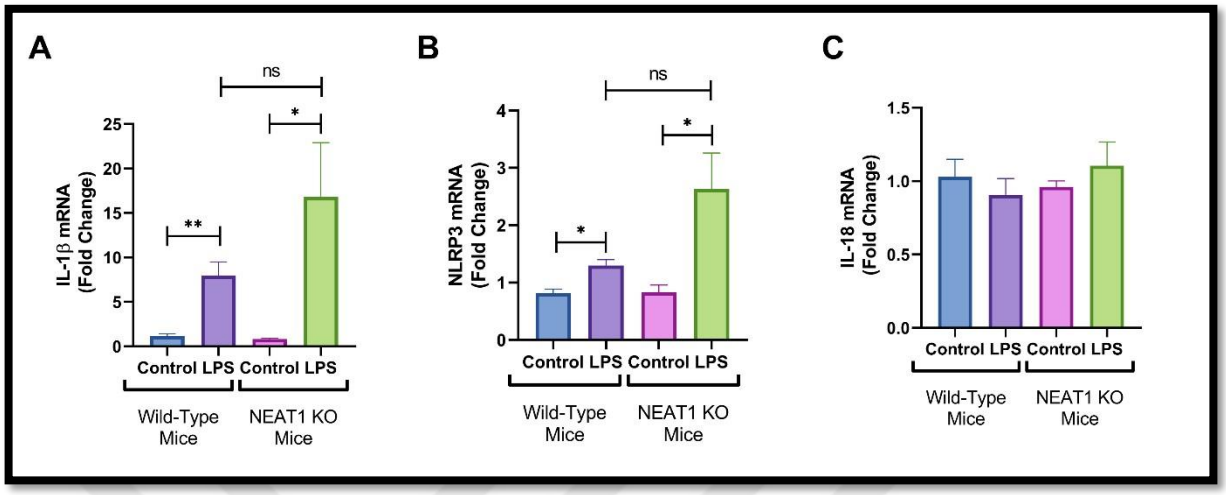

Figure 26. *Demonstration of the altered mRNA levels of NLRP3 inflammasome activation markers,* ***A)*** *IL-1β,* ***B)*** *IL-18, and* ***C)*** *NLRP3, in the disease model created by LPS injection (5mg/kg). Data are given as mean ± S.E.M. n=7, *p <0.05, **p <0.01, ns: not significant*

4.5. Knocking out NEAT1 reversed the upregulation of protein levels of NLRP3 inflammasome markers observed in wild-type mice.

After mRNA levels were examined by the RT-qPCR method, the change in protein levels of NLRP3 inflammasome activation markers was evaluated by the Western blot method. For this purpose, NLRP3, pro-inflammatory cytokine IL-1β (mature and cleaved forms), pro-Caspase-1(p-45), and active Caspase-1(p20) protein levels were examined (**Fig. 28**). The results showed that the protein expression levels of inflammasome markers NLRP3(**Fig. 28A**), Pro-IL-1β (**Fig. 28B**), cleaved IL-1β (**Fig. 28C**), and active Caspase-1 (**Fig. 28D**) were significantly increased in LPS-injected wild-type mice indicating activation of NLRP3 inflammasome activation. These protein expression levels were increased by LPS injection. These increases in protein levels were reversed in LPS- injected NEAT1 KO mice. Knocking out NEAT1 alleviated NLRP3 inflammasome activation in LPS-injected NEAT1 KO mice. In addition to these results, there was no significant change in pro-Caspase-1 levels among the groups.

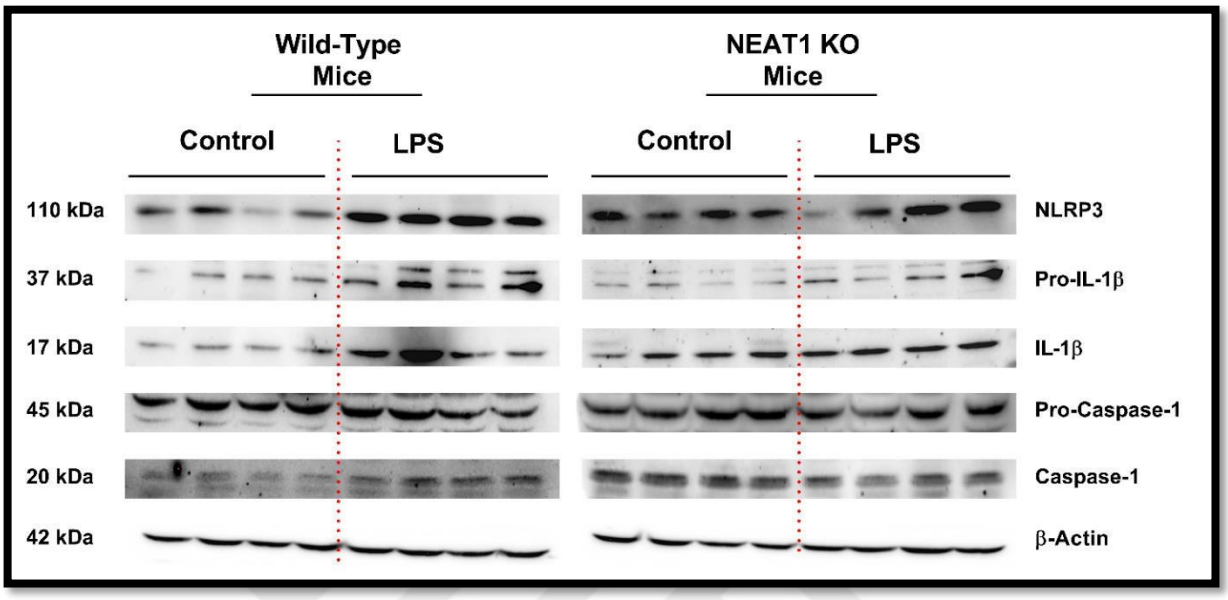

Figure 27. *Western blot result showing the change in protein levels of IL-1β, NLRP3, and Caspase-1, markers of NLRP3 inflammasome activation, in the disease model created by LPS injection (5mg/kg).*

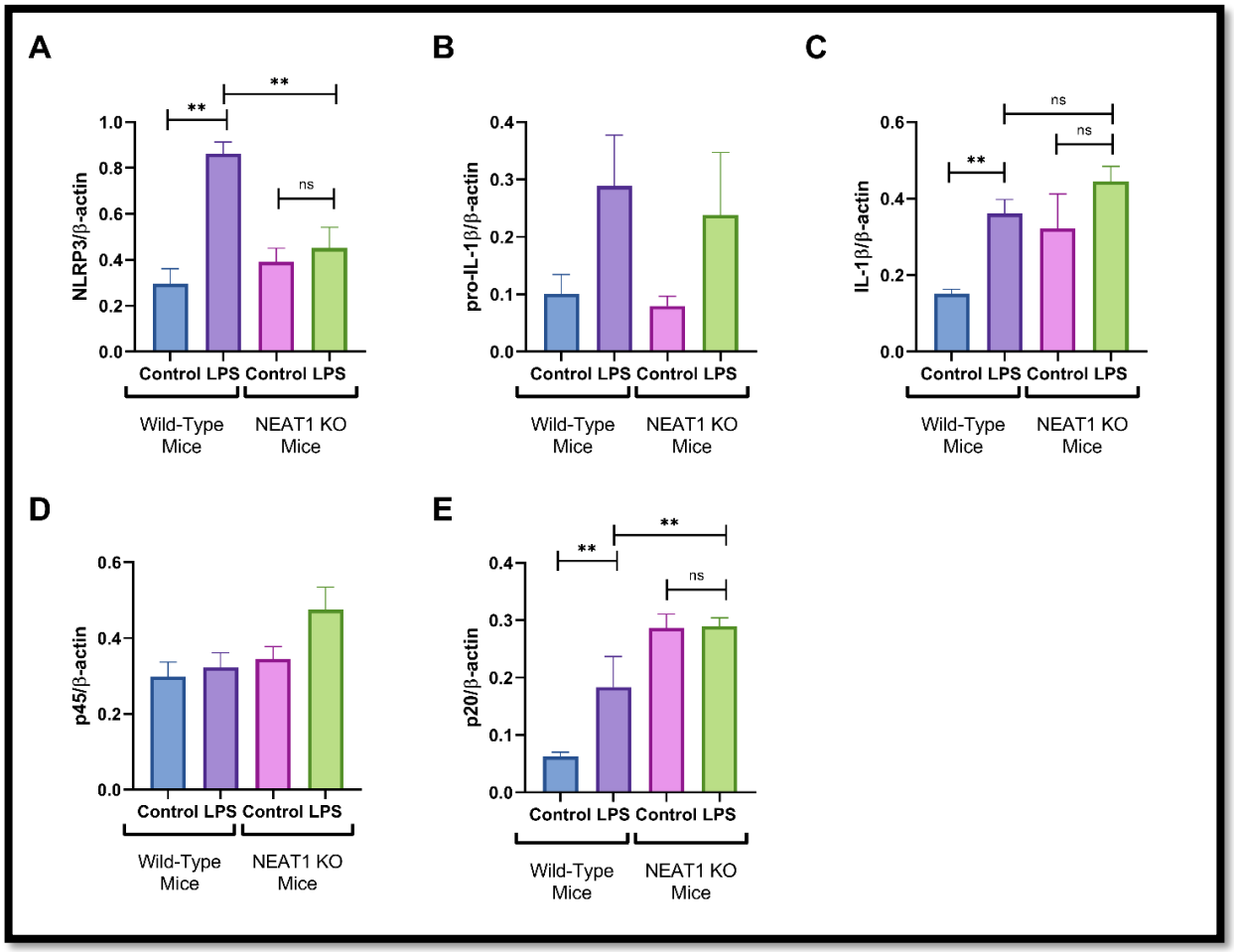

Figure 28. *The analysis of protein levels* ***A)*** *NLRP3,* ***B)*** *Pro-IL-1β,* ***C)*** *cleaved IL-1β,* ***D)*** *Pro-Caspase-1(p45), and* ***E)*** *activated Caspase-1 (p20) by Western blot. Data are given as mean ± S.E.M. n = 5, **p < 0.01, ns= not statistically significant.*

We further studied the effect of NEAT1 on protein levels of GSDMD and Traf6 in the disease model (**Fig. 29A**). Since Traf6 is the target for mmu-miR-146a whose expression is increased in LPS-injected NEAT1 KO mice, reduced protein level of Traf6 was expected in NEAT1 KO mice. The result demonstrated that Traf6 protein levels were reduced in NEAT1 KO mice after LPS injection compared to LPS-injected wild-typemice (**Fig. 29B**). The GSDMD levels were also similar to active Caspase-1 level as

GSDMD is cleaved by active Caspase-1 to form pore on the cell membrane eventually inducing pyroptotic cell death (**Fig. 29C**).

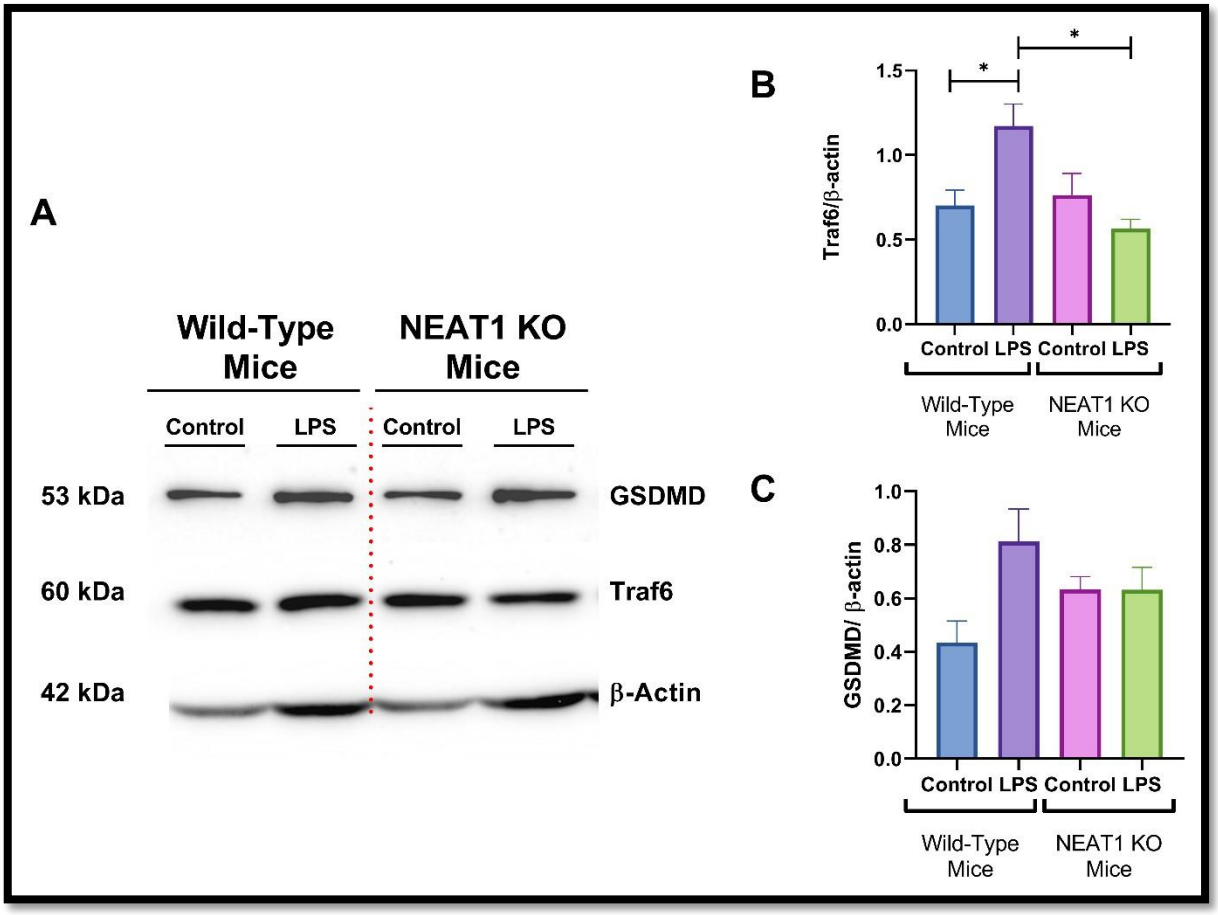

Figure 29. *The demonstration of **A)** western blot and the analysis of corresponding proteins **A)** Gasdermin D, and **C)** Traf6. Data are given as mean ± S.E.M. n = 5, **p < 0.01, ns= not statistically significant.*

4.6. LPS and ATP model activated NLRP3 inflammasome activation in the N9 cell line

To induce NLRP3 inflammasome activation, LPS, and ATP model is utilized. First, N9 microglia were treated with 1 μg/ml LPS for 4 hours to mimic the priming step of NLRP3

inflammasome activation. Then, after 4 hours of incubation, the cells were treated with 5mM ATP, which induces the formation of the NLRP3 inflammasome complex, subsequently causing activation of the NLRP3 inflammasome complex. Thus, we first check the change at mRNA levels of IL-β (**Fig. 30A**) and NLRP3 (**Fig. 30B**). The RT- qPCR results showed that LPS and ATP model successfully led to the upregulation of mRNA levels of these NLRP3 inflammasome markers. The experimental setup includes only LPS and the only ATP groups, along with the RAW264.7 macrophage group, which was only treated with LPS for 24 hours. As can be seen in the figure below, there was a significant increase in IL-β and NLRP3 mRNA levels even in only LPS-treated cells.

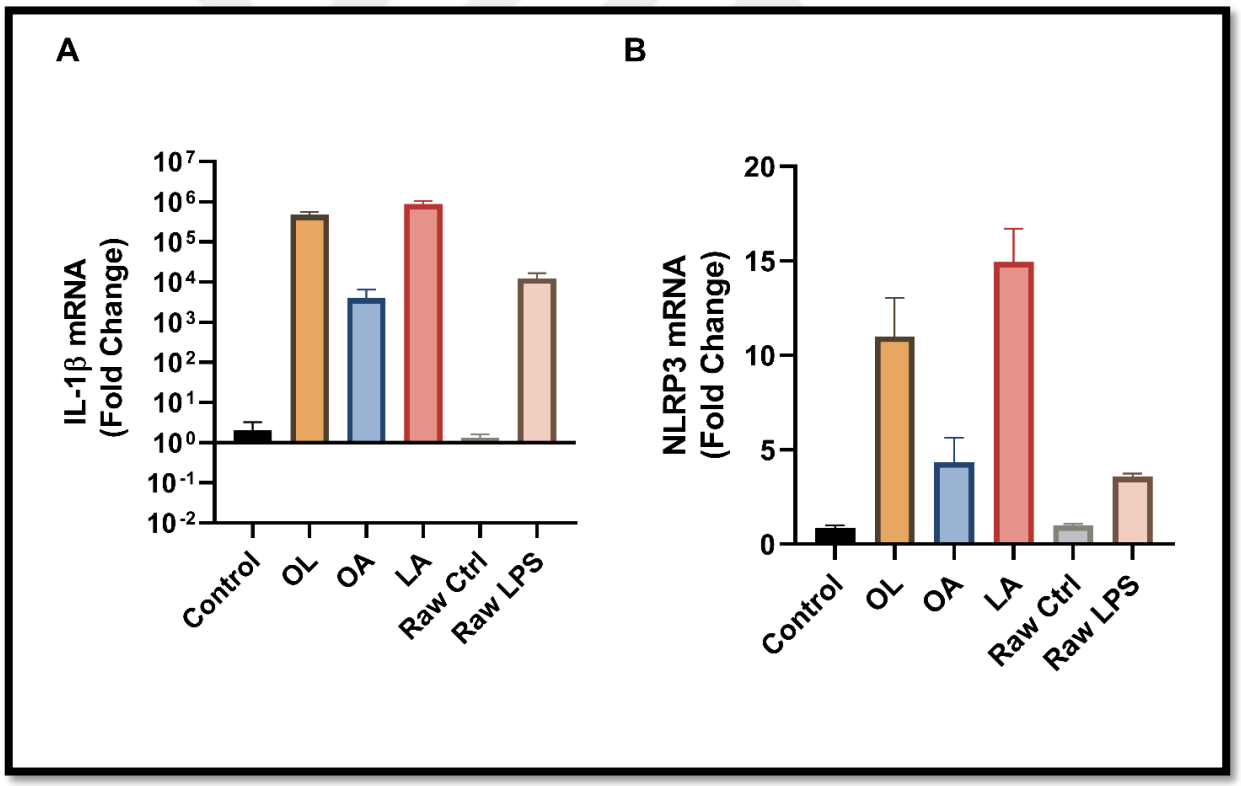

Figure 30. *RT-qPCR results showing altered mRNA levels of NLRP3 inflammasome activation markers,* ***A****) IL-1β,* ***B****) NLRP3, in LPS (1 µg, 4 h) and ATP (5 mM, 1 h) treated microglial cells. Data are given as mean ± S.E.M. **p < 0.01, ***p < 0.01ns= not statistically significant,* ***CT****: Control,* ***OL****: Only LPS,* ***OA****: Only ATP,* ***LA****: LPS, and ATP.*

Next, we performed western blot and ELISA test to examine NLRP3 inflammasome markers at protein levels in LPS and ATP-treated N9 microglia (**Fig.31**). According to theresults, the level of NLRP3, the sensor protein of the inflammasome complex, was significantly increased in NLRP3 inflammasome activation (**Fig.31A-B**). NLRP3 protein level was significantly higher than any other groups. Furthermore, pro-IL-1β (**Fig.31A- C**), expressed by NF-kB activation, was increased with NLRP3 inflammasome activation. Although Pro-IL-1β levels were lower in the LA group compared with the OL group, it is the result of Caspase-1 activity that cleaves Pro-IL-1β (p37) into mature IL-1β to be secreted. The ELISA results confirmed these findings as secreted IL-1β is significantly higher than the OL group (**Fig.31E**). Additionally, Pro-Caspase-1 levels were not changed among groups (**Fig.31D**) which could be explained by the fact that NLRP3 inflammasome activation altered Caspase-1 cleavage/activity rather than its abundancy (Tastan et al., 2021). Overall, LPS and ATP model is sufficient to mimic the NLRP3 inflammasome activation model in N9 microglia, offering a rapid and robust model.

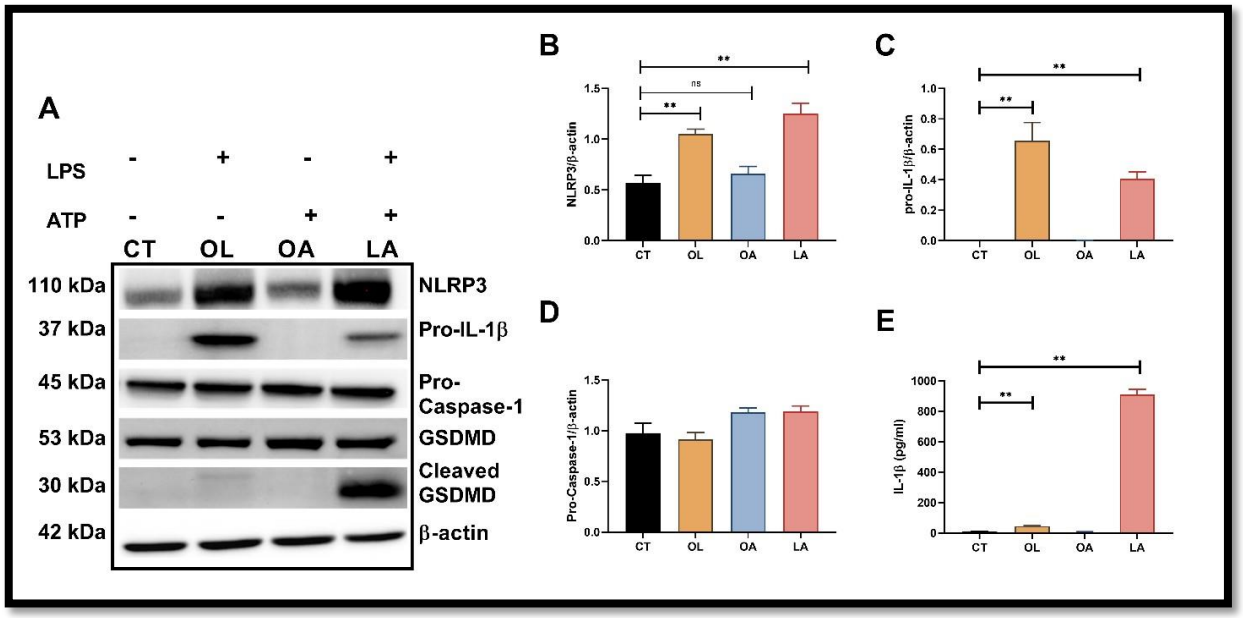

Figure 31. *The western blot results show protein levels of NLRP3 inflammasome activation markers* ***B)*** *NLRP3,* ***C)*** *Pro-IL-1β,* ***D)*** *Pro-Caspase-1(p45), and* ***E)*** *ELISA result showing cleaved IL-1β levels in LPS (1 µg, 4 h) and ATP (5 mM, 1 h) treated microglial cells. Data are given as mean ± S.E.M. **p < 0.01, ns= not statistically significant,* ***CT****: Control,* ***OL****: Only LPS,* ***OA****: Only ATP,* ***LA****: LPS, and ATP.*

4.7. LPS and ATP-induced NLRP3 inflammasome activation model induced pyroptotic cell death in N9 microglial cells

Another hallmark of NLRP3 inflammasome activation is Caspase-1-dependent pyroptotic cell death. The pyroptotic cell death was examined by Western blot and PI staining. To do that, GSDMD protein levels were checked (**Fig.32A**). As the GSDMD protein is cleaved by Caspase-1 and then N-terminals of cleaved GSDMD form a pore on the cell membrane leading to swelling of the cell, and eventual cell death called pyroptosis. Accordingly, the results showed no significant change among groups for uncleaved GSDMD levels (**Fig.32B**). However, the protein levels of cleaved GSDMD levels differ among groups. As indicated in **Fig.32C**, the activation of NLRP3 inflammasome activation by LPS and ATP treatment caused significant increases in the cleavage of GSDMD in the LA group compared to the rest of the groups. There were also significant increases in the OL group, which could be interpreted as non-canonical activation of NLRP3 inflammasome complex by only LPS treatment (He et al., 2015). The pyroptotic cell death was also examined by PI staining in LPS, and ATP-treated N9 cells (**Fig.32D**). Microglial cells were stained with PI dye after treatment with LPS and ATP. Cells stained red in the LPS + ATP group indicate that cells underwent pyroptotic cell death. Therefore, the result demonstrated that LPS and ATP model induces pyroptotic cell death in N9 microglia.

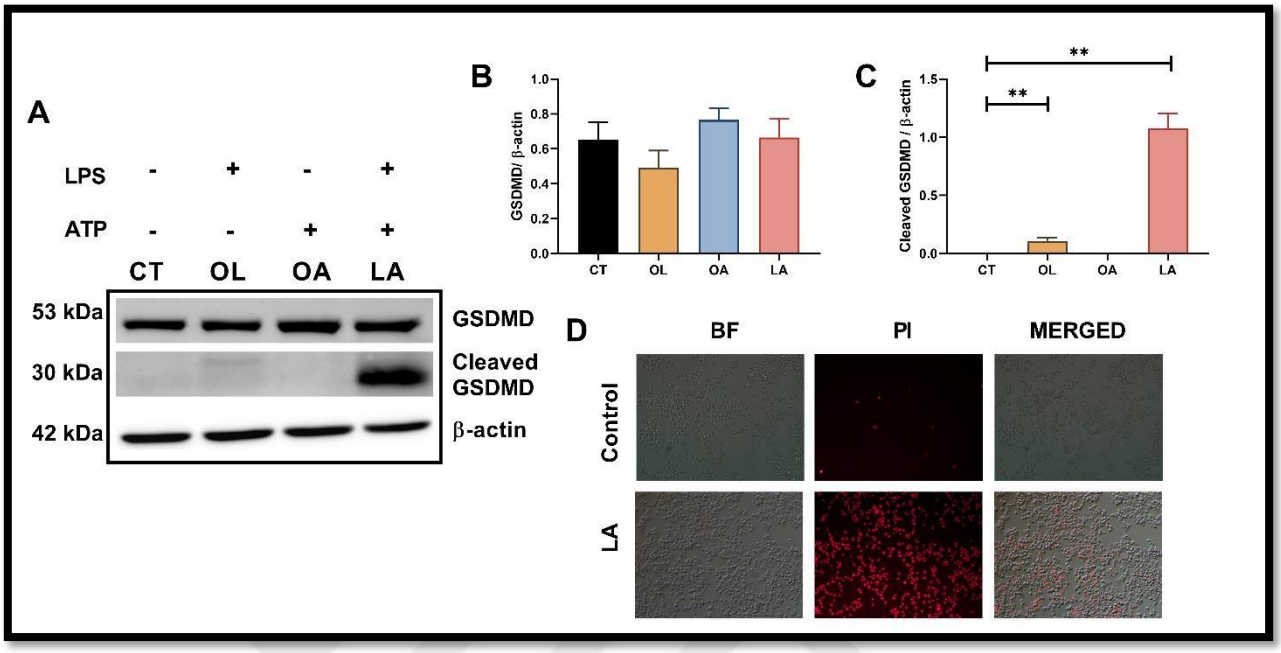

Figure 32. *Results demonstrating pyroptotic cell death by **A**) western blot of **B-C**) Gasdermin D and **D**) PI staining in microglial NLRP3 inflammasome activation induced by LPS (1 µg, 4 h) and ATP (5 mM, 1 h). Data are given as mean ± S.E.M. **p < 0.01, ns= not statistically significant, **CT**: Control, **OL**: Only LPS, **OA**: Only ATP, **LA**: LPS, and ATP.*

4.8. NEAT1 expression was downregulated in LPS and ATP-induced microglial NLRP3 inflammasome activation

NEAT1, our lncRNA of interest, has two isoforms, and these variants contribute to the formation of the paraspeckle structure, primarily found in the nucleus. These variants interact with RNA-binding proteins (**Fig.33A**), and the paraspeckle structure arises. RT- qPCR was performed to determine the changes in NEAT1 expression in NLRP3 inflammasome activation in microglial cells. In the experimental setup, N9 cells were primed with LPS and ATP to induce NLRP3 inflammasome activation. Further, RAW

264.7 cells were treated with LPS for 24 hours as a positive control in terms of NEAT1 expression (Both NEAT1_2 and NEAT1_1 isoforms), as it is shown that NEAT1

expression is upregulated when RAW264.7 cells are treated with LPS for 24 hours (D. Xia et al., 2020). As RT-qPCR results showed, both long isoform NEAT1_2 (**Fig.33B**) and short isoform NEAT1_1 (**Fig.33C**) were downregulated after activation of NLRP3 inflammasome activation. Besides these isoform-specific NEAT1 primers, a common primer for NEAT1 was also used for RT-qPCR analysis (**Fig.33D**). Further, total NEAT1 expression was decreased compared to the control group. Besides the comparison of the control group and LA group, the other groups in the experimental setup (OL, OA) had lower NEAT1 expression than the control group.

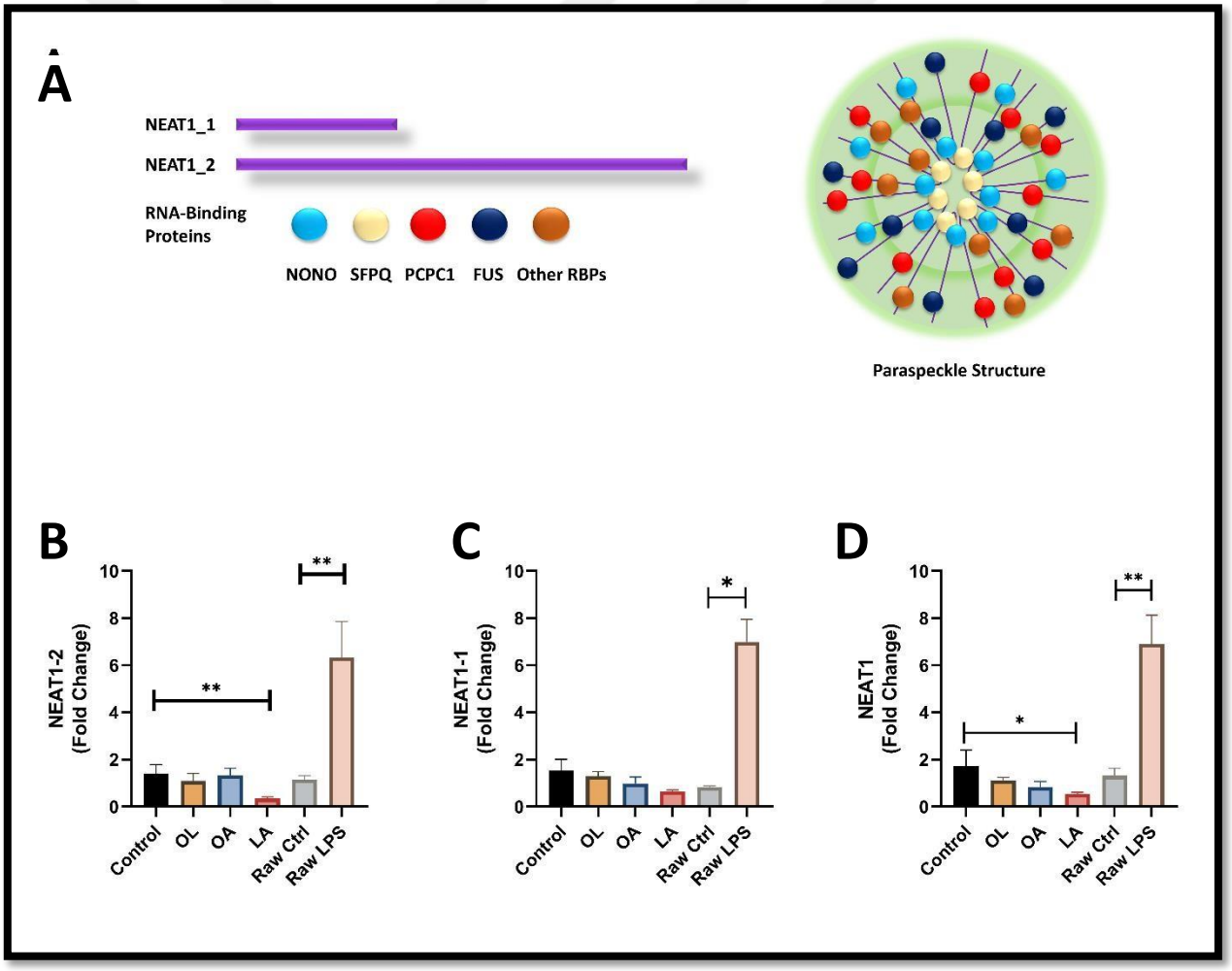

Figure 33. A) *LncRNA NEAT1 and the paraspeckle structure. The short and long isoforms of lncRNA NEAT1 and the RNA-binding proteins such as SFPQ and NONO form a subcellular structure called paraspeckle. The figure shows RNA levels of* ***B)*** *long isoform NEAT1_2,* ***C)*** *short isoform NEAT1_1, and* ***D)*** *NEAT1 upon microglial NLRP3 inflammasome activation induced by LPS (1 µg, 4 h) and ATP (5 mM, 1 h). Data are given as mean ± S.E.M. *p < 0.05, **p < 0.01, ns= not statistically significant,* ***CT****: Control,* ***OL****: Only LPS,* ***OA****: Only ATP,* ***LA****: LPS, and ATP.*

4.9. Microglial NLRP3 inflammasome activation did not alter protein expression levels of paraspeckle proteins.

After determining the changes in NEAT1 expression in NLRP3 inflammasome activation in N9 microglial cells, the expression changes in the protein levels involved in the formation of the paraspeckle structure were determined. Accordingly, two RNA-binding proteins, SFPQ and NONO, were examined by Western Blot analysis (**Fig. 34A**). Results showed that no changes were found in the levels of NONO and SFPQ proteins, which are RNA-binding proteins that participate in the paraspeckle structure and form a nucleoprotein complex with NEAT1 isoforms.

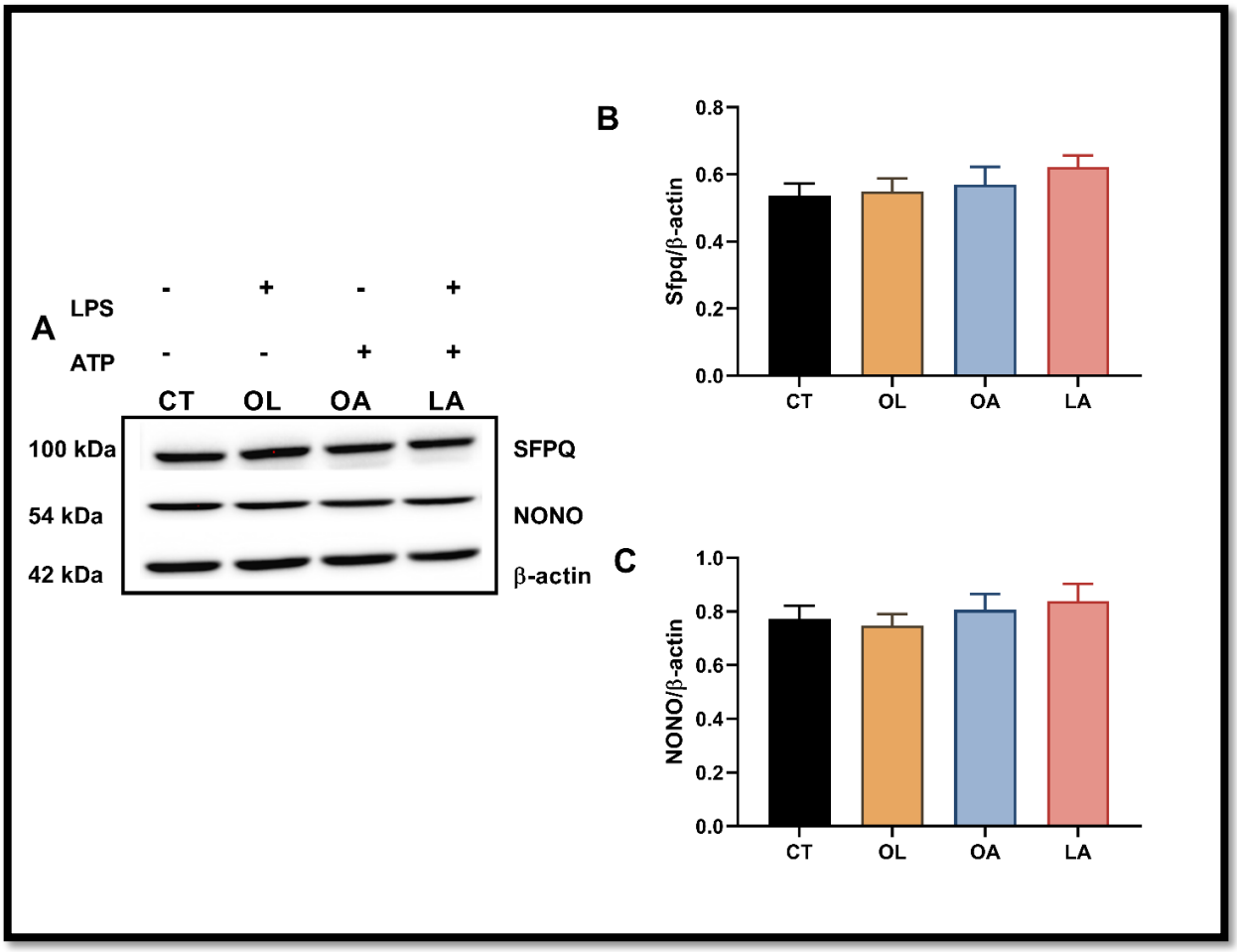

Figure 34. *The western blot analysis of RNA-binding proteins* ***B)*** *SFPQ and* ***C)*** *NONO in microglial NLRP3 inflammasome activation induced by LPS (1 µg, 4 h) and ATP (5 mM, 1 h). Data are given as mean ± S.E.M. *p < 0.05, **p < 0.01, ns= not statistically significant,* ***CT****: Control,* ***OL****: Only LPS,* ***OA****: Only ATP,* ***LA****: LPS, and ATP.*

4.10. Microglial NLRP3 inflammasome activation decreased the number of paraspeckle structures.

In order to visualize the paraspeckle structure, FISH staining was performed. As we demonstrated a decrease in the RNA level of NEAT1 and its isoforms and the protein level of RNA-binding proteins NONO and SFPQ, the localization and abundancy of NEAT1 and paraspeckles were determined via FISH staining in which two different

probes (for long and short isoforms) were used. As seen in **Fig.35** and **Fig.36**, the abundancy of NEAT1 was higher in the control group. The speckles were located in the nucleus. However, NLRP3 inflammasome activation by LPS and ATP treatment reducedNEAT1 abundancy and the number of speckles located in the nucleus. Accordingly, relative fluorescence intensity was lower in the LA group compared to the control group in both FISH staining.

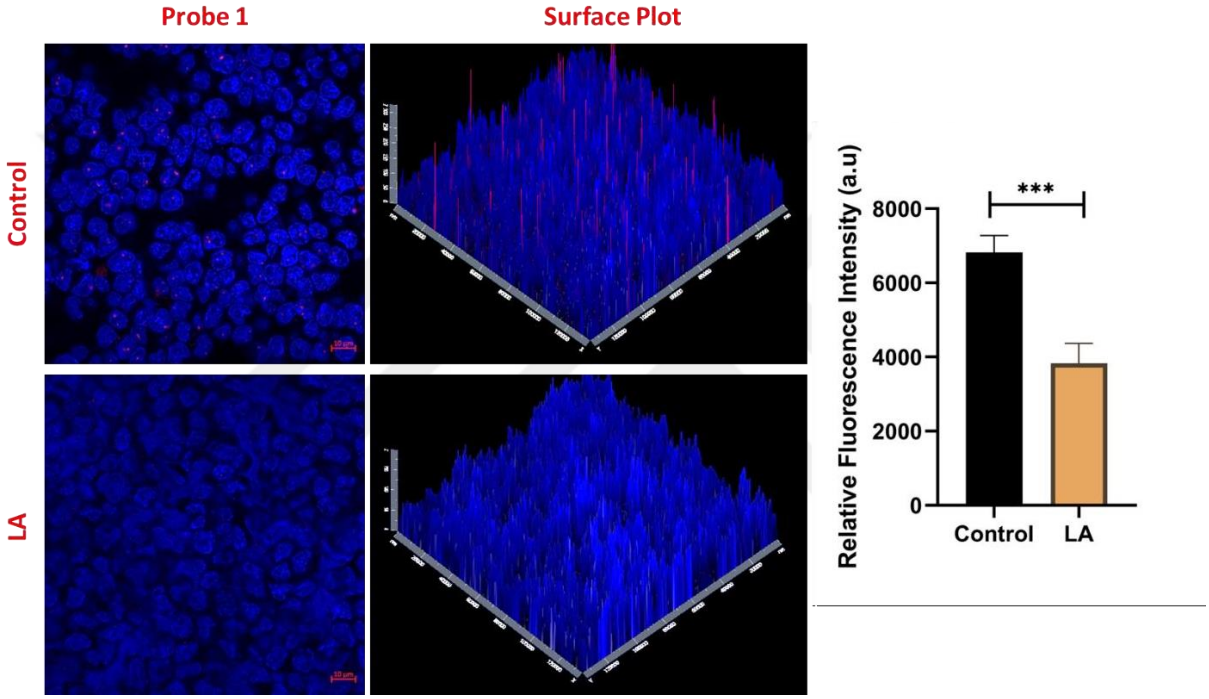

Figure 35. *FISH staining using NEAT1 probe (Red staining)* ***B)*** *Relative fluorescence intensity of NEAT1 in microglial NLRP3 inflammasome activation induced by LPS (1 µg, 4 h) and ATP (5 mM, 1 h). Data are given as mean ± S.E.M. ***p < 0.001, ns= not statistically significant,* ***LA****: LPS, and ATP.*

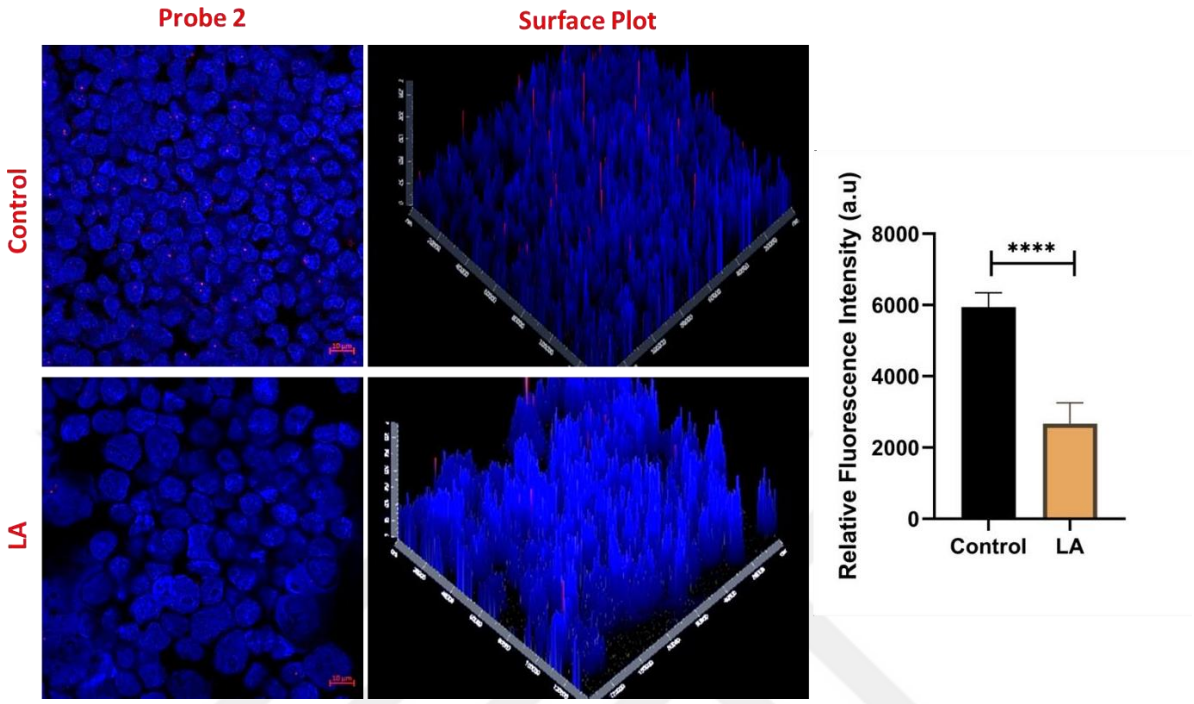

Figure 36. *FISH staining using NEAT1 probe (Red staining)* ***B)*** *Relative fluorescence intensity of NEAT1 in microglial NLRP3 inflammasome activation induced by LPS (1 µg, 4 h) and ATP (5 mM, 1 h). Data are given as mean ± S.E.M. ***p < 0.001, ns= not statistically significant,* ***LA****: LPS, and ATP.*

4.11. Knocking down NEAT1 by NEAT1-specific siRNA did not alter NLRP3 inflammasome activation markers at mRNA levels.

To determine the effect of the lncRNA RNA NEAT1 on microglial NLRP3 inflammasome activation and inflammasome parameters, N9 microglia cells were transfected with NEAT1-specific siRNA to knock down lncRNA NEAT1. As seen in the figure (**Fig.37**), there was a significant reduction in both isoforms of NEAT1 in N9 microglial cells transfected with siRNA. This result confirmed the efficiency of the transfection process using NEAT1-siRNA.

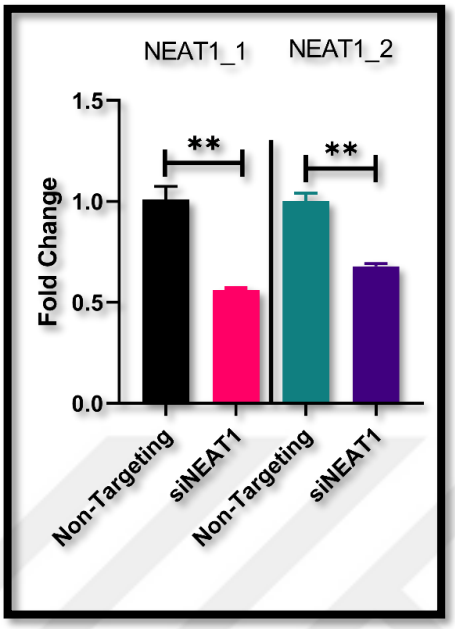

Figure 37. *The RT-qPCR results showing siNEAT1 transfection efficiency for both isoforms, long NEAT1_2 and short NEAT1_1, in N9 microglia*

After confirming the efficiency of siRNA transfection, RT-PCR was performed to determine the effect of lncRNA NEAT1 on mRNA levels of inflammasome markers, IL- 1β (**Fig.38A**), IL-18 (**Fig.38B**) and NLRP3 (**Fig.38C**). According to the RT-qPCR results, the expression levels of these markers were upregulated in LPS and ATP-treated N9 microglial cells. On the other hand, similar effects were seen in the siNEAT1-transfected groups. Thus, no changes were detected in the mRNA levels of proinflammatory IL-1β, IL-18, and NLRP3 among groups.

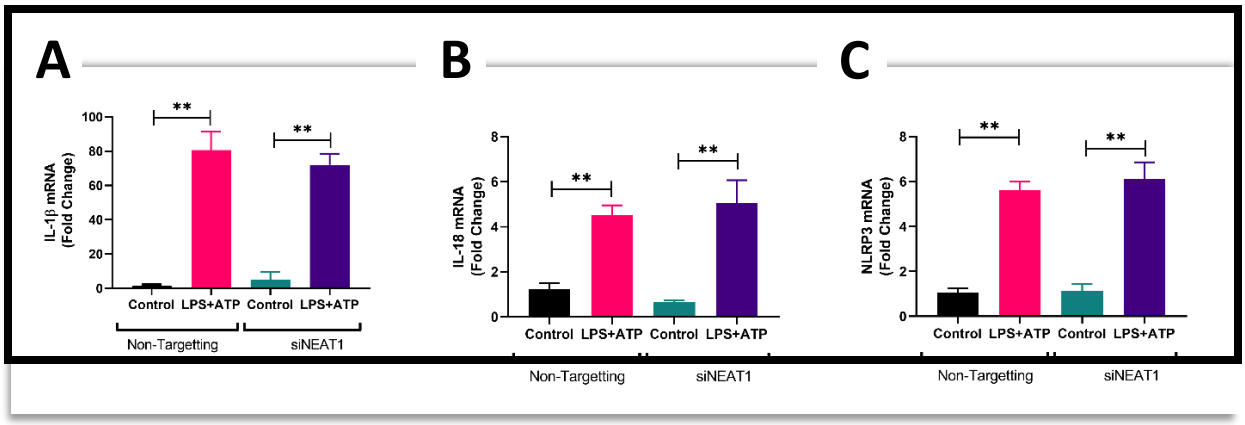

Figure 38. *Comparison of mRNA levels of NLRP3 inflammasome activation markers,* ***A)*** *IL-1β,* ***B)*** *IL-18, and* ***C)*** *NLRP3, between control and siNEAT1 transfected microglial cells upon microglial NLRP3 inflammasome activation induced by LPS (1 µg, 4 h) and ATP (5 mM, 1 h). Data are given as mean ± S.E.M. **p < 0.01*

4.12. Knocking down of NEAT1 by NEAT1-specific siRNA reduced secreted IL-1β levels in LPS and ATP-treated cells.

The effects of NEAT1 on microglial NLRP3 inflammasome activation were assessed by Western blot in N9 microglial cells transfected with siNEAT1. Accordingly, NLRP3, pro- inflammatory cytokine IL-1β (mature form), pro-Caspase-1(p-45), and active Caspase- 1(p20) protein levels were examined (**Fig.39**). Further, the cleaved form of IL-1β was analyzed by ELISA test (**Fig.41C**).

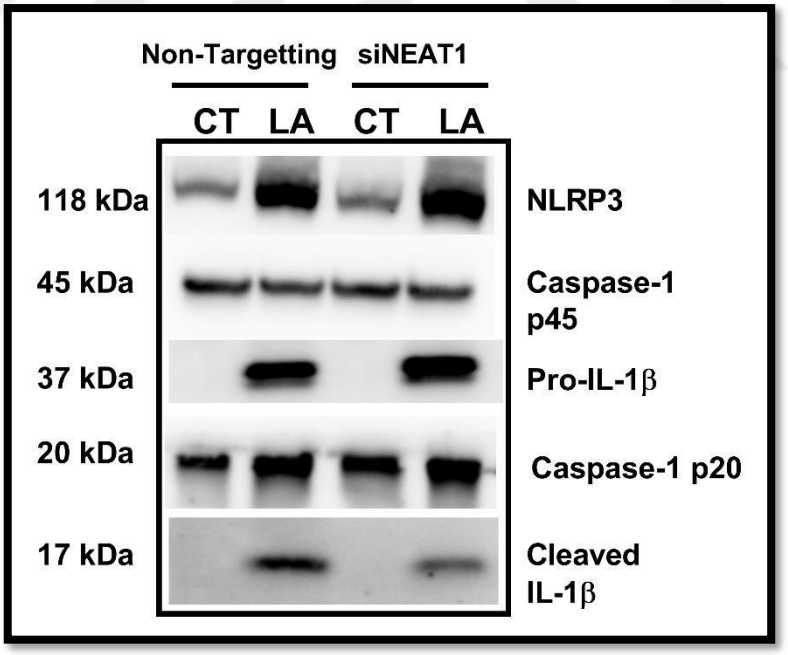

Figure 39. *The protein levels of NLRP3 inflammasome activation markers are shown by western blot results.*

The NLRP3 expression levels were increased by LPS and ATP treatment in the group transfected with non-targeting siRNA. Similarly, there was a significant rise in NLRP3 protein levels in the group transfected with siNEAT1. Thus, knocking down NEAT1 did not affect the NLRP3 expression pattern (**Fig.40A**). The results also showed that while the change in expression levels of pro-Caspase-1 between the non-targeting and siNEAT1 transfected LA group is similar (**Fig.40B**), the knocking down NEAT1 in N9 microglia reversed the effect of LPS and ATP which significantly increased cleaved Caspase-1 level in nontargeting LA group (**Fig.40C**).

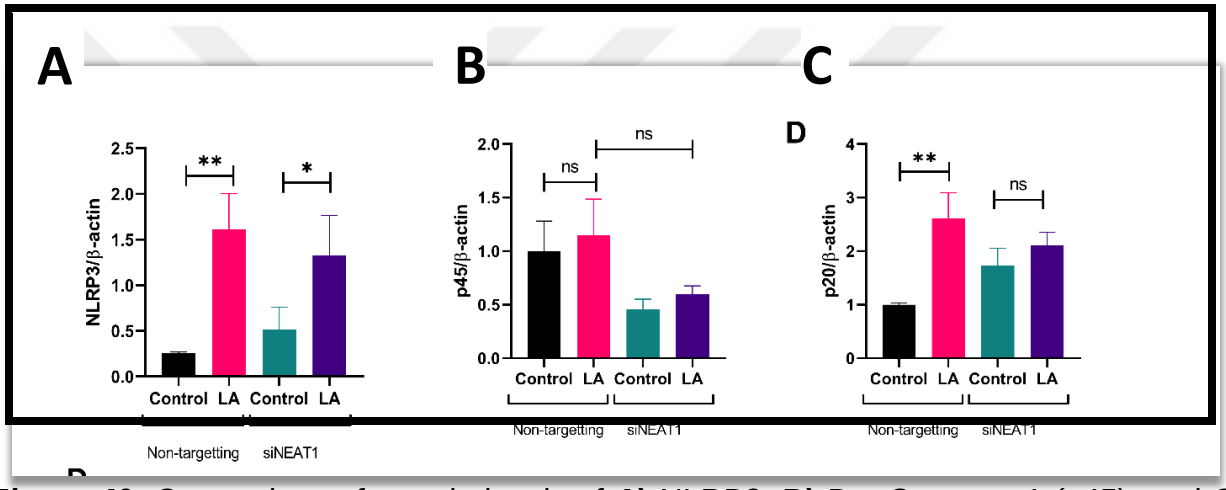

Figure 40. *Comparison of protein levels of **A)** NLRP3, **B)** Pro-Caspase-1 (p45), and **C)** Cleaved Caspase-1 (p20) between control and siNEAT1-transfected microglial cells upon microglial NLRP3 inflammasome activation induced by LPS (1 µg, 4 h) and ATP (5 mM, 1 h). Data are given as mean ± S.E.M. *p < 0.05, **p < 0.01, ns: not significant*

The western blotting results further demonstrated that Pro-IL-1β levels were increased in both LA groups, and knocking down NEAT1 did not reverse this process (**Fig.41A**). On the other hand, there was a significant decrease in cleaved IL-1β level in microglial cells induced by LPS and ATP in the LA group transfected with siNEAT1 (**Fig.41B**) which wasalso corroborated with the ELISA result (**Fig.41C**). Overall, evidence revealed that NEAT1 play the activity of Caspase-1 and subsequent cleavage of Pro-IL-1Beta into mature and released IL-1β levels.

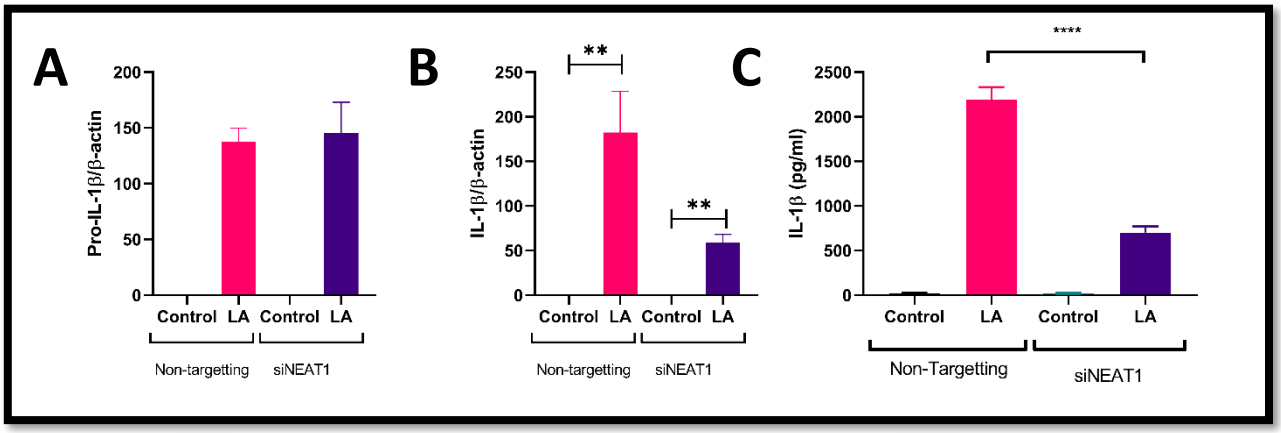

Figure 41. *Protein levels of* ***A)*** *Pro-IL-1β,* ***B)*** *Cleaved IL-1β by Western blot analysis, and* ***C****) Cleaved IL-1β by ELISA test in control and siNEAT1-transfected microglial cells upon microglial NLRP3 inflammasome activation induced by LPS (1 μg, 4 h) and ATP (5 mM, 1 h). Data are given as mean ± S.E.M. **p < 0.01, ****p < 0.001, ns: not significant*

4.13. Pyroptotic cell death was reversed by the knocking down of NEAT1 by NEAT1-specific siRNA in LPS and ATP-treated cells.

After examining the changes in protein levels, the change in cell death was examined bythe LDH release test and PI staining. The LDH test revealed that LDH release resulting from the pyroptotic cell death decreased to the control group level. Transfection with siNEAT1 reversed the increased level of LDH release (**Fig.42A**). In parallel with this result, cell viability assay also demonstrated that knocking down of NEAT1 significantly improved cell viability compared to the LA group transfected with non-targeting siRNA (**Fig.42B**). Lastly, we showed the effects of NEAT1 on pyroptotic cell death by PI staining on N9 microglia (**Fig.42D**). LPS and ATP treatment caused pyroptotic cell death resulted with an increased number of PI-positive cells in LA group transfected with non- targeting siRNA. However, transfection with siNEAT1 reversed this incident and decreased PI-positive cell number in the LA group (**Fig.42C**).

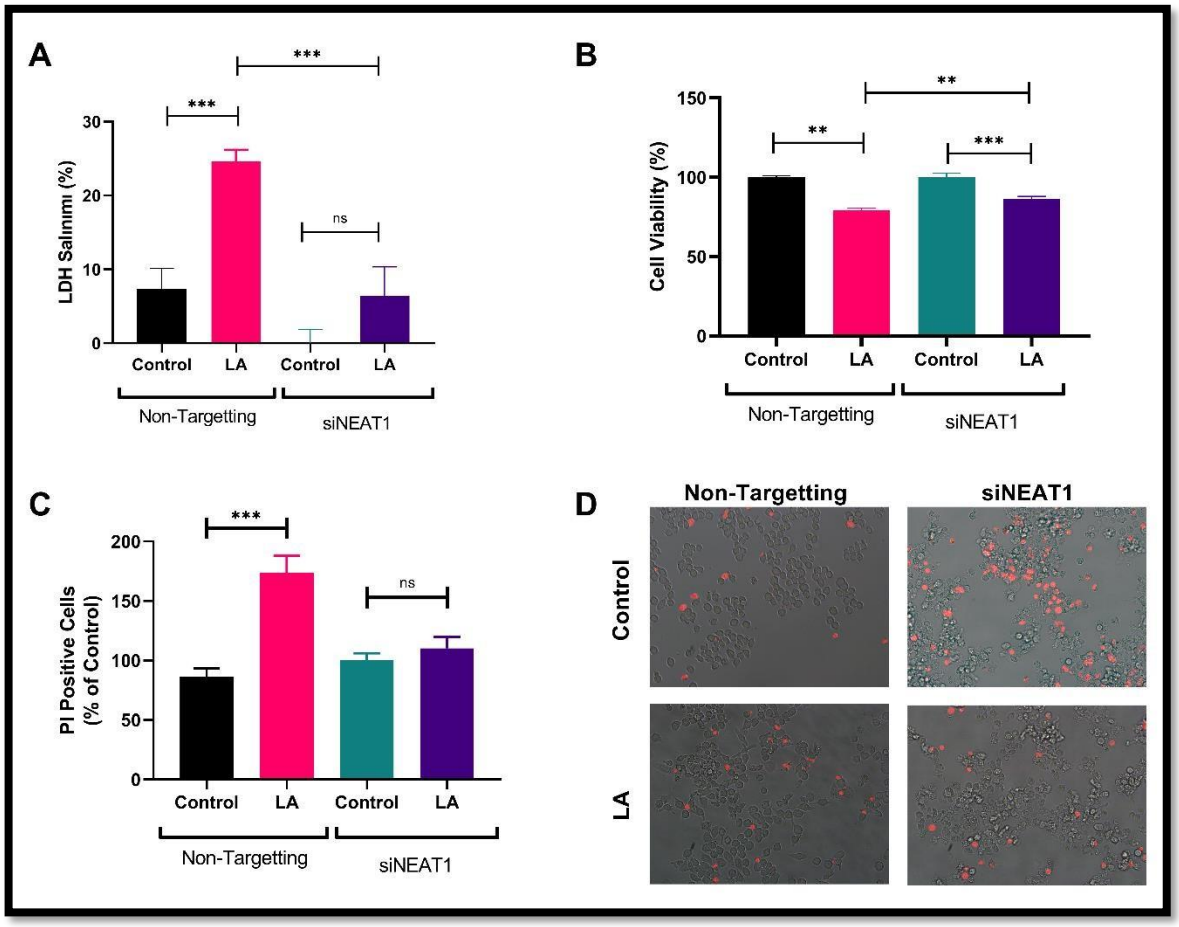

Figure 42. *Results demonstrating pyroptotic cell death by* ***A)*** *LDH Release,* ***B)*** *viability test, and* ***C-D)*** *PI staining in control and siNEAT1 transfected microglial cells upon microglial NLRP3 inflammasome activation induced by LPS (1 μg, 4 h) and ATP (5 mM, 1 h). Data are given as mean ± S.E.M. **p < 0.01, ***p < 0.001, ns= not statistically significant,* ***LA:*** *LPS and ATP.*

4.14. Examination of the relation between lncRNA NEAT1 and mmu-miR-146a-5p

Our second hypothesis was that NEAT1 regulates microglial NLRP3 inflammasome activation through miRNA mmu-miR-146a-5p, one of the targets of lncRNA NEAT1. By sponging miR-146a-5p, lncRNA NEAT1 might have a regulatory role in the NF-κB signaling pathway, an essential signaling pathway for the priming step of NLRP3 inflammasome activation. To test our second hypothesis, first, the relationship between

NEAT1 and mmu-miR-146a-5p was examined. RNAhybrid database (Kruger & Rehmsmeier, 2006) revealed the complementary domain between NEAT1 and mmu- miR-146a-5p (**Fig.43A**). Furthermore, the miRWALK database offering search for targets of miRNA of interest in three different databases showed that Traf6 is the only target gene for mmu-miR-146a-5p which all three databases pointed out (Sticht, De La Torre, Parveen, & Gretz, 2018) (**Fig.43B**). Hence, we examined the effects of miR- 146a-5p on microglial NLRP3 inflammasome activation to show whether NEAT1 regulate NLRP3 inflammasome activation via miR-146a.

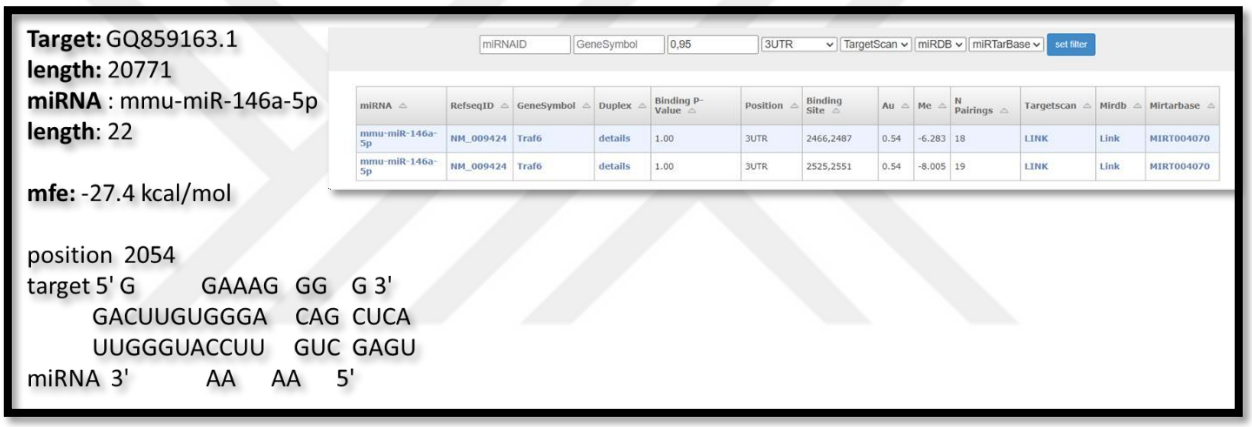

Figure 43. *The representation of the relationship between lncRNA NEAT1 and miRNA miR-146a-5p* ***A)*** *complementary site between lncRNA NEAT1 and miRNA miR-146a-5p.* ***B)*** *The TRAF6 target of miR-146a-5p is selected based on the comparison of 3 different databases (TargetScan, miRDB, and miRTarBase).*

4.15. mmu-miR-146a-5p levels were increased in both *in vitro* and *in vivo* models of the NLRP3 inflammasome activation model

After the first step, the expression of mmu-miR-146a-5p *in vitro* N9 microglia NLRP3 inflammasome model and *in vivo* LPS model was investigated by RT-qPCR method (**Fig.44**). First, NLRP3 inflammasome activation was induced by LPS and ATP in N9 microglia, and the change in mmu-miR-146a-5p expression was examined. Accordingly,

the expression of mmu-miR-146a-5p was upregulated *in vitro* NLRP3 inflammasome model (**Fig.44A**). Comparably, levels of mmu-miR-146a-5p were measured in wild-type and NEAT1 KO mice in which the LPS injection induced NLRP3 inflammasome activation. While the level of mmu-miR-146a-5p was significantly increased in wild-type mice injected with LPS compared to the control group, this increase was not observed inLPS-injected NEAT1 KO mice.

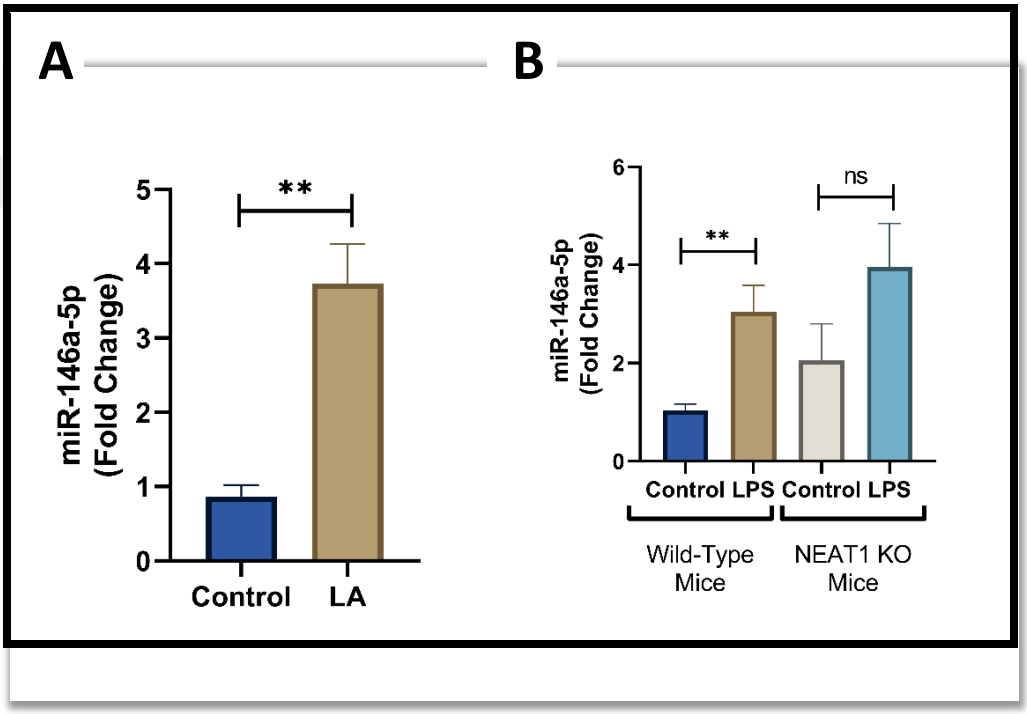

Figure 44. *The levels of miR-146a-5p in both* ***A)*** *in vitro LPS and ATP model and* ***B)*** *in vivo LPS-injected NLRP3 inflammasome activation model. In vitro NLRP3 inflammasome was activated by LPS (1 µg, 4 h) and ATP (5 mM, 1 h) treatment. In vivo NLRP3 inflammasome activation was induced by LPS injection (5mg/kg). Data are given as mean ± S.E.M. **p < 0.01, ****p < 0.001, ns: not significant.* ***LA:*** *LPS and ATP*

4.16. Transfecting N9 microglial cells with miR-146a-5p mimic did not alter NLRP3 inflammasome activation markers at mRNA levels.

To determine the effect of mmu-miR-146a-5p on NLRP3 inflammasome activation, N9 cells were transfected with the mmu-miR-146a-5p mimic after the levels of mmu-miR- 146a-5p were determined by RT-qPCR. Prior to further experiments, the efficiency of transfection was confirmed by RT-qPCR (**Fig.45A**). Being transfected using the mmu- miR-146a-5p mimic increased the level of mmu-miR-146a-5p on average 500-fold. After the transfection efficiency was determined, the change in NLRP3 inflammasome activation markers was first examined by the RT-PCR method (**Fig.45B-D**). The results demonstrated that the expression of levels of the proinflammatory cytokines IL-1β (**Fig.45B**), IL-18 (**Fig.45C**), and NLRP3 (**Fig.45D**) upregulated LPS and ATP-treated N9 microglia in the non-targeting group. Similarly, IL-1β, IL-18, and NLRP3 mRNA levels were elevated in the LA group transfected with the mmu-miR-146a-5p mimic suggesting that miR-146a-5p did not alter mRNA expression profiles in N9 microglia.

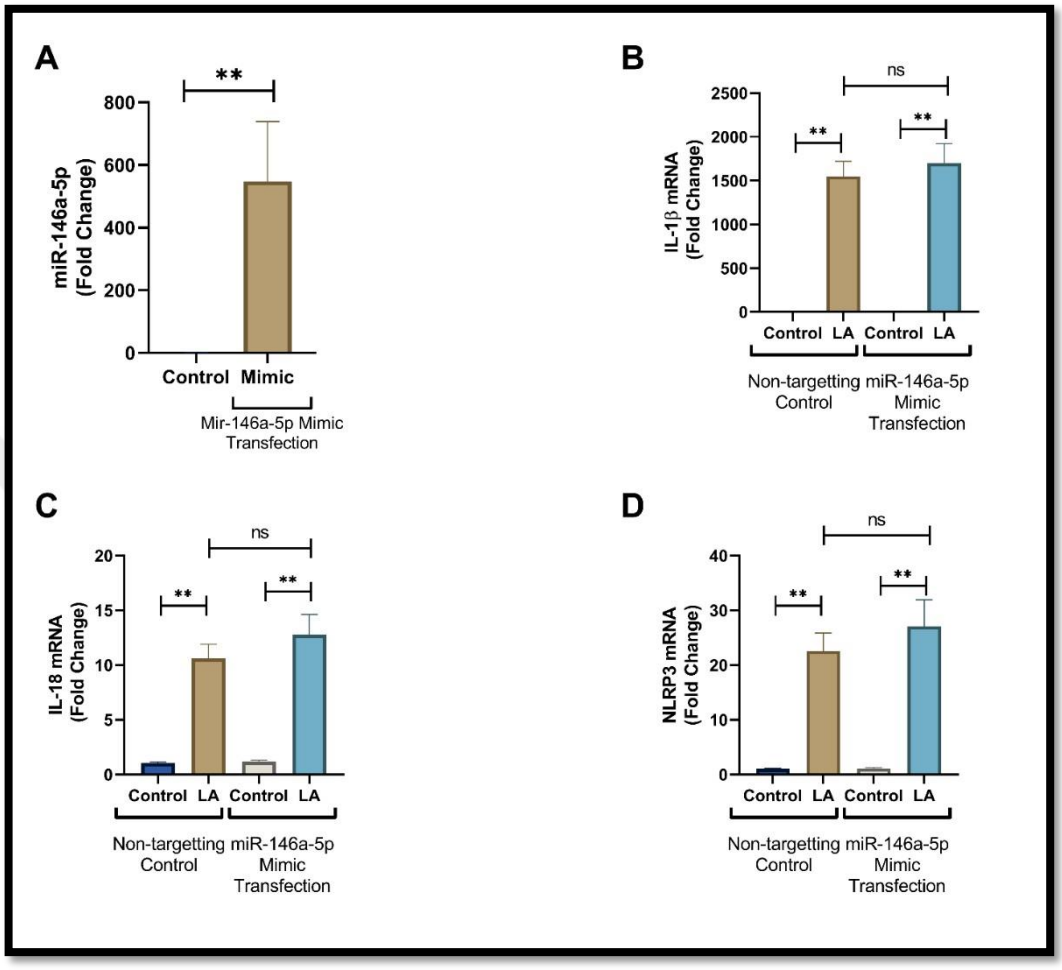

Figure 45. *RT-qPCR results showing altered **A)** miR-146a-5p, **B)** IL-1β, **C)** IL-18, and **D)** NLRP3 in miR-146a-5p mimic-transfected microglial cells upon microglial NLRP3 inflammasome activation induced by LPS (1 µg, 4 h) and ATP (5 mM, 1 h). Data are given as mean ± S.E.M. **p < 0.0, ns: not significant.* ***LA:*** *LPS and ATP*

4.17. miR-146a-5p mimic reduced protein expression of NLRP3 inflammasome activation markers and its target Traf6

In order to uncover how mmu-miR-146a-5p affect Traf6 and NLRP3 inflammasome markers at protein levels, western blot analysis was performed (**Fig.46A**). When mimic- transfected LA, and its counterpart group was compared, the level of Traf6 protein was significantly reduced in the mimic-transfected LA group (**Fig.46B**). Results also showed

that miR-146a-5p mimic reduced NLRP3 inflammasome activation by decreasing NLRP3 inflammasome markers, NLRP3 (**Fig.46C**) and the pro-IL-1β (**Fig.46D**).

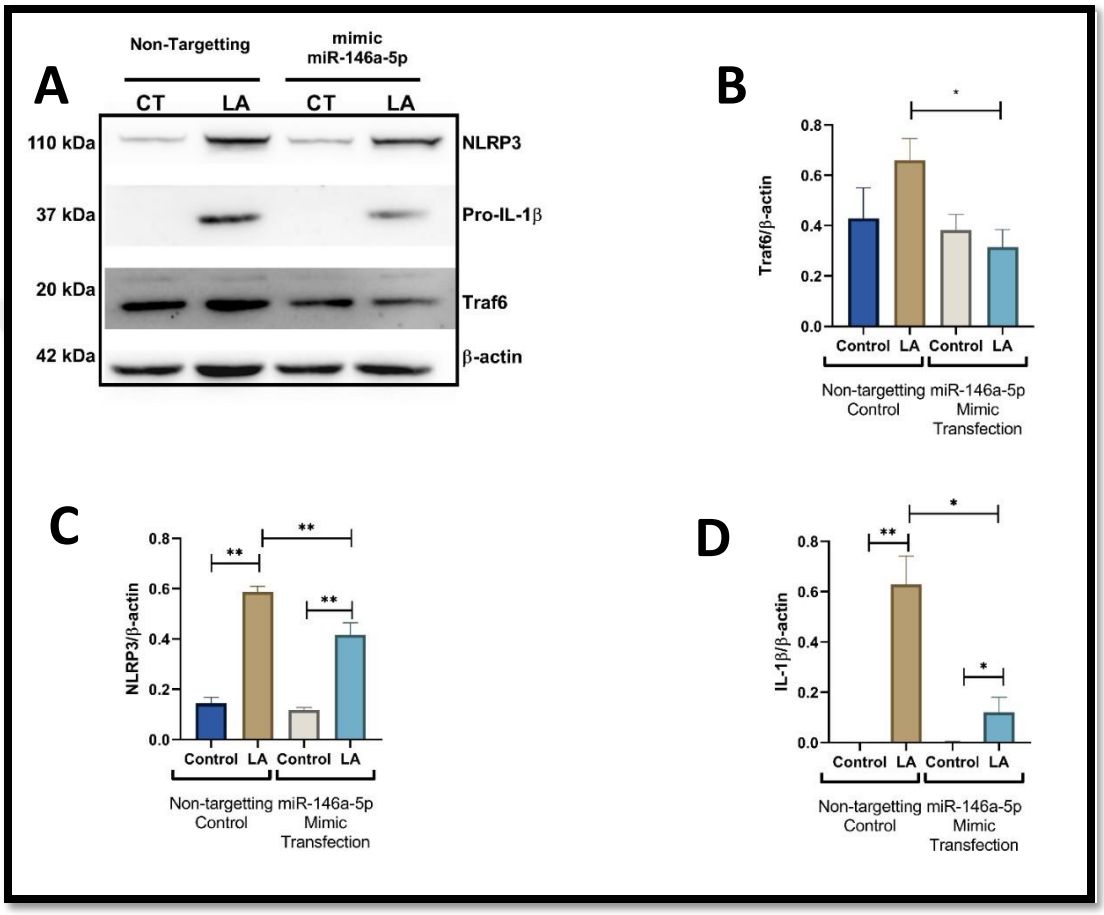

Figure 46. *Western blot results showing altered* ***B)*** *Traf6,* ***C)*** *NLRP3,* ***D)*** *pro-IL-1β levels in miR-146a-5p mimic-transfected microglial cells upon microglial NLRP3 inflammasome activation induced by LPS (1 μg, 4 h) and ATP (5 mM, 1 h). Data are given as mean ± S.E.M. *p < 0.05, **p < 0.01, ns: not significant.* ***LA:*** *LPS and ATP*

4.18. miR-146a-5p mimic alleviated pyroptotic cell death

Lastly, the change in cell death was examined by the LDH test release test (Fig. 31). It was observed that LDH release was increased in N9 microglia treated with LPS and ATP, while LDH release was significantly reduced in the LA group transfected with mmu-

miR-146a-5p mimic. This indicated that miR-146a-5p ameliorated pyroptotic cell death resulting from NLRP3 inflammasome activation.

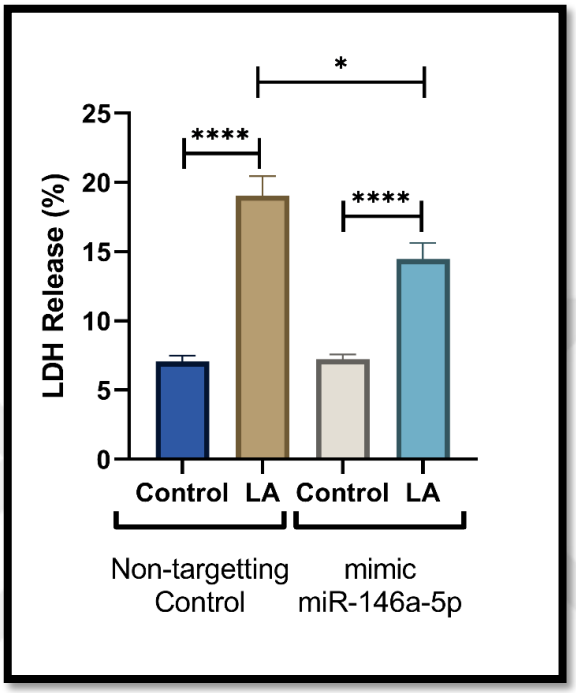

Figure 47. *The assessment of pyroptotic cell death by LDH Assay, Comparison of control and miR-146a-5p mimic-transfected microglial cells upon microglial NLRP3 inflammasome activation induced by LPS (1 µg, 4 h) and ATP (5 mM, 1 h). Data are given as mean ± S.E.M. *p < 0.05, ****p < 0.0001.* ***LA:*** *LPS and ATP*

5. DISCUSSION

The present thesis study examines the role of lncRNA NEAT1 in regulation of microglial NLRP3 inflammasome activation. First, *in vivo*, research demonstrated a substantial role for NEAT1 in mediating the immunological response to systemic LPS administration. This was corroborated by NEAT1 KO animals having a milder clinical phenotype and scoring better behaviorally than wild-type mice that had received LPS injections. Our *in vitro* research using the N9 microglial cell line clearly demonstrated that the lncRNA NEAT1 controls NLRP3 inflammasome activation by inhibiting Caspase-1 activation and consequent IL-1 production. Additionally, it was shown that these effects were followed by a significant reduction in the rate of pyroptotic cell death, leading to increased cell viability. Results obtained *in vitro* further suggested that miR-146a-5p may mediate these NEAT1-induced effects on microglial NLRP3 inflammasome activation.

Multicomponent protein complexes called inflammasomes are found in the cytoplasm and control how the immune system reacts to various pathogen and damage signals. These signals, such as pathogen-associated molecular motifs and damage-associated molecular motifs, cause the inflammasome complexes to become activated, at which point Caspase-1, an enzyme linked to the inflammasome, begins to function (Michael T. Heneka, Róisín M. McManus, & Eicke Latz, 2018). Proinflammatory cytokines IL-1β andIL-18, as well as the pore-forming protein GSDMD, are proteolytically cleaved by activated Caspase-1, which might result in pyroptotic cell death. The removal of pathogens and injured cells depends on adequate/appropriate inflammasome activation,while excessive or dysregulated inflammasome complex activation is linked to a wide range of illnesses, including cancer, neurological, cardiovascular, and autoimmune disorders (Tastan, Arioz, & Genc, 2022).

In vitro, NLRP3 inflammasome activation was induced using the LPS and ATP model. Accordingly, 4 hours of LPS (1 g/ml) incubation followed by 1 hour of ATP (5 mM) incubation led to N9 microglia cells to activate the NLRP3 inflammasome. In this case, ATP activates the NLRP3 inflammasome complex, whereas LPS acts as the priming

signal necessary for classical NLRP3 inflammasome activation (Arioz et al., 2019; Tastan et al., 2021). The active NLRP3 inflammasome complex in N9 microglia was confirmed by our findings regarding NLRP3 inflammasome markers and pyroptotic cell death. In order to activate the NLRP3 inflammasome *in vivo*, LPS was injected into mice,and a disease model was created. Studies have shown that the LPS-induced sickness model, which is accompanied by disease behaviors, involves activation of the NLRP3 inflammasome. According to research done on rats, LPS decreased locomotion and motility while increasing the production of pro-inflammatory cytokines such as IL-1β and IFN-gamma, which are signs of sickness (Mazuco, Biondi, Silva, Bernardi, & Kirsten, 2019). Studies in which mice were given injections of LPS also showed a rise in other pro-inflammatory cytokines, including TNF-α and IL-6 (Dantzer, 2009). In our study, molecular biological tests carried out in mice given LPS injections revealed that NLRP3 inflammasome activation occurred. This demonstrates that the pathophysiology of the LPS-induced sickness model involves NLRP3 inflammasome activation, and it suggests that inhibiting NLRP3 inflammasome activation may be a possible treatment approach for inflammatory disorders.

In order to prevent excessive tissue injury or disruption of homeostasis, the NLRP3 inflammasome complex, along with other innate immune system components, must be tightly regulated. Several neurodegenerative diseases, such as AD and PD (Hulse & Bhaskar, 2022), cardiovascular diseases, such as atherosclerosis (Tanase et al., 2023), and metabolic/inflammatory disorders, such as type 2 diabetes, gout, and rheumatoid arthritis (Kong, Sun, Li, & Wang, 2021), have been linked to overactivated or unregulated NLRP3 inflammasome activation as etiological factors. Regulated activationof the NLRP3 inflammasome prevents aberrant inflammation and tissue injury. The activation of the NLRP3 inflammasome complex can be regulated on multiple levels. Post-transcriptional modifications are an essential control mechanism (J. Xia, Jiang, Dong, Liao, & Zhou, 2023). For instance, phosphorylation of the NLRP3 receptor at specific positions can increase or decrease NLRP3 activation.

In addition, studies have demonstrated that the ubiquitination, deubiquitination, and SUMOylation of NLRP3 and other inflammasome components modulate the formation and activity of the inflammasome complex (Nanda, Vollmer, & Perez-Oliva, 2022). ncRNAs, including miRNAs and lncRNAs, play a crucial role in additional regulatory mechanisms. Acting as gene expression regulators and targeting distinct NLRP3 inflammasome signaling pathway components, these ncRNAs regulate the upstream or downstream of the NLRP3 inflammasome signaling pathway. According to studies, miRNAs such as miR-223 and miR-155 inhibit the activation of the NLRP3 inflammasome by targeting the NLRP3 and NF-κB pathways. Overexpressing miR-223 (F. Bauernfeind et al., 2012) or inhibiting miR-155 (Luo et al., 2018) decreases inflammasome activation and IL-1β release, according to studies. The regulatory function of lncRNAs on the activation of the NLRP3 inflammasome has also been investigated in a variety of diseases. In terms of NLRP3 inflammasome activation, studies have demonstrated the regulatory role of MALAT1 in multiple sclerosis and PD (Cai et al., 2020), SNHG1 (Bingqing Cao, Tao Wang, Qiumin Qu, Tao Kang, & Qian Yang, 2018), HOTAIR (Q. Zhang et al., 2020), GAS5 (Xu, Zhang, Geng, Liu, & Zhang, 2020) and HOTTIP (P. Sun et al., 2022) in PD and SNHG14 (Duan et al., 2021) in AD pathogenesis. Understanding the complex network of noncoding RNAs involved in the regulation of NLRP3 inflammasome activation could lead to the development of novel treatment strategies for inflammasome-associated pathologies.

A subset of lncRNAs is engaged in innate immunity; although they are becoming acknowledged as critical regulators in several biological processes (Heward & Lindsay, 2014), lncRNAs are intensively investigated as an essential factor in a variety of disorders. lncRNAs have been linked to cell proliferation, apoptosis, inflammation, differentiation, and cancer development (Carrasco-Leon et al., 2021; Y. Huang et al., 2019; C. C. Zhang & Niu, 2019). NEAT1 is a lncRNA that is characterized by two isoforms: NEAT1_2 (long isoform - 23 kb) and NEAT1_1 (short isoform 3.7 kb). According to research, NEAT1 forms paraspeckle structures with RNA-binding proteins like SFPQ and NONO, in which the ratio of these isoforms varies dynamically depending

on cell type. However, it has been proven that the NEAT1_2 isoform is required for the formation of paraspeckle structures (Sasaki, Ideue, Sano, Mituyama, & Hirose, 2009). When the biological function of NEAT1 was studied, it was discovered that it is importantin hypoxia (Choudhry et al., 2015), viral infection (Imamura et al., 2014), and human embryonic stem cell differentiation (Standaert et al., 2014). Furthermore, it has been observed that NEAT1 expression is dynamically altered in a variety of cancer diseases. Furthermore, NEAT1 has been shown to have a role in cancer etiology by acting as an oncogene (Yu, Li, Zheng, Chan, & Wu, 2017).

Considering our thesis study, the significance of NEAT1 and the role it plays in the central nervous system become apparent. Research conducted on the central nervous system suggests that NEAT1 may have a dual function in the degenerative processes that occur in the CNS. Nishimoto et al. provided evidence that NEAT1 offers some degree of protection against ALS via TDP-43 and FUS/TLS (Nishimoto et al., 2013). However, it has been suggested that NEAT1 could have a dual role in the etiology of disorders, including AD, PD, and Huntington's disease (K. Li & Wang, 2023). This would mean that it might either protect or damage the patient. In individuals with ALS, there was an increase in the expression of the NEAT1_2 isoform as well as the occurrence of the paraspeckle structure in the motor neurons of the spinal cord (Nishimoto et al., 2013). In another research, it was shown that inhibiting the expression of NEAT1 in rats that were subjected to a chronic constriction injury model led to an increase in the levels of NEAT1 as well as a reduction in the levels of inflammatory cytokines, such as IL-6, IL-1β, and TNF-α (L. X. Xia, Ke, & Lu, 2018).

There has been little research on the impact of NEAT1 on the control of NLRP3 inflammasome activation. NEAT1-induced inflammasome activation in macrophages hasbeen shown by Zhang et al. (P. Zhang et al., 2019). NEAT1 facilitated the formation and activation of the NLRP3 inflammasome complex, as well as the NLRC4 and AIM2 complexes. NEAT1 subsequently bonded with active Caspase-1 and stabilized it, leading to enhanced interleukin-1 production and pyroptotic cell death. They also observed that when the NLRP3 inflammasome was activated, NEAT1 translocated into

the cytoplasm, indicating that NEAT1 possibly controls inflammasome/caspase-1 activity (P. Zhang et al., 2019). Previous research has shown that Neat1 expression is firmly controlled by a range of signals, including those that activate the tumor suppressor p53 (Adriaens et al., 2016). Our research addressed the involvement of the lncRNA NEAT1 in the activation of microglial NLRP3 inflammasomes. NEAT1 suppression decreased microglial NLRP3 inflammasome activation by lowering active-Caspase-1 and IL-1β production, as well as pyroptotic cell death. Furthermore, NEAT1 altered the immunological response to systemic LPS administration. NEAT1 knockout mice performed better in behavioral tests evaluating illness behaviors and had a milder disease phenotype.

miRNAs are ncRNAs that control the expression of several gene targets and have been conserved throughout evolution. Studies have shown that miRNAs regulate the activation of the NLRP3 inflammasome. Numerous inflammatory disorders and conditions have been linked to the dysregulation of NLRP3 by miRNAs such as miR- 223, miR-142, miR-7, miR-20a, and miR-20b-5p (Feng et al., 2021). By being targetedby miRNAs like miR-146a, miR-124, miR-155, and miR-23a, the inflammasome complex/components and signaling pathways (NF-kB, TXNIP, and FOXO3) found in the upstream of NLRP3 inflammasome activation, may modulate NLRP3 inflammasome activation (Zamani, Oskuee, Atkin, Navashenaq, & Sahebkar, 2020). LncRNAs have also been shown to control miRNAs. NEAT1 has been shown to have the potential to behave as a ceRNA for miRNAs, inhibiting miRNA functions with its paraspecklesstructure and thus controlling the expression of miRNA endogenous targets (Prinz et al., 2019; H. Zhou et al., 2022). For instance, miR-124-3p, miR-125a, miR-146b, let-7e, miR-449b, and miR-181d have been discovered as direct target genes of NEAT1 (M. Zhang et al., 2022).

Based on this knowledge, it was proposed that NEAT1 may modify the activation of the NLRP3 inflammasome by sponging miRNAs. In order to support this theory, RNA-RNA interaction databases were examined, and the results revealed that NEAT1 targeted miR-146a-5p, which may be involved in the control of NLRP3 inflammasome activation

(Sasaki et al., 2009). It has been shown that miR-146a-5p may adversely influence the NF-kB pathway and related genes found upstream of the activation of the NLRP3 inflammasome (K. D. Taganov, M. P. Boldin, K. J. Chang, & D. Baltimore, 2006). In our work, we first used bioinformatic methods to show that miR-146a-5p and NEAT1 can bind to each other, and we then found that miR-146a-5p levels were elevated when the NLRP3 inflammasome was activated in microglia and when LPS was injected into an *in vivo* disease model. Then, using miR-146a-5p mimics, we looked into the regulatory function of miR-146a-5p in NLRP3 inflammasome activation. The results showed that miR-146a-5p affects the activation of the microglial NLRP3 inflammasome. NLRP3inflammasome activity was reversed by miR-146a-5p after pyroptotic cell death. This supports the findings from our *in vivo* NEAT1 siRNA and *in vivo* NEAT1 KO mouse experiments that NEAT1 sponges miR-146a-5p and controls the NLRP3 inflammasome.

6. CONCLUSION & FUTURE DIRECTIONS

This thesis study investigated the regulatory role of lncRNA RNA NEAT1 on microglial NLRP3 inflammasome activation. With this regard, *in vitro* LPS and ATP-induced NLRP3 inflammasome activation model and *in vivo* LPS-induced NLRP3 inflammasome activation model were utilized. First, we determined that NEAT1 expression was downregulated in microglial NLRP3 inflammasome activation. Later, it was shown that the nucleoprotein structure called paraspeckles by lncRNA NEAT1 and RNA-binding proteins were reduced by the activation of NLRP3 inflammasome. Transfection experiments with NEAT1-specific siRNA showed that although NEAT1 did not change the mRNA levels, it affected the protein levels of IL-1β, an active Caspase-1 protein. As a result of these changes, pyroptotic cell death also decreased in a direct proportion. *In vivo,* experiments using the NEAT1 KO mouse have shown that NEAT1 modulates LPS- induced disease behavior. However, in our *in vivo* LPS model, NEAT1 reduced the LPS- induced disease behavior. This showed that NEAT1 reduced neuroinflammation in the brain.

In conclusion, the role of NEAT1 in the regulation of microglial NLRP3 inflammasome activation was shed light on and supported our initial hypothesis. On the other hand, it has not been determined exactly by which miRNA and/or signaling pathways NEAT1 exert this effect. Therefore, in subsequent studies, how NEAT1 achieves this effect will be studied using more inclusive techniques (transcriptomics and proteomics).

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8. APPENDIX

8.1. ARBIS



BORA TAŞTAN

Kişisel Bilgiler

İletişim Bilgileri

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Telefon	(
E-posta	b
İnternet Sayfası	v ŗrams/groups/genc-lab

Öğrenim Bilgileri

02 Şubat 2018 - Şu Anda (4 yıl 10 ay)
Doktora, Doktora, DOKUZ EYLÜL ÜNİVERSİTESİ, TÜRKİYE
İZMİR ULUSLARARASI BİYOTIP VE GENOM ENSTİTÜSÜ, MOLEKÜLER BİYOLOJİ VE GENETİK (DR) (İNGİLİZCE)
Diploma Numarası: -
Ağırlıklı Genel Not Ortalaması: 3.64 / 4.0

31 Ağustos 2016 - 09 Ocak 2018 (1 yıl 5 ay)
Yüksek Lisans, Tezli Program, DOKUZ EYLÜL ÜNİVERSİTESİ, TÜRKİYE
SAĞLIK BİLİMLERİ ENSTİTÜSÜ, TEMEL SİNİR BİLİMLER (YL) (TEZLİ)
Diploma Numarası: 201800270
Ağırlıklı Genel Not Ortalaması: 3.91 / 4.0

01 Eylül 2010 - 01 Temmuz 2016 (5 yıl 11 ay)
Lisans, Anadal/Normal Öğretim, İZMİR YÜKSEK TEKNOLOJİ ENSTİTÜSÜ, TÜRKİYE
FEN FAKÜLTESİ, MOLEKÜLER BİYOLOJİ VE GENETİK PR. (İNGİLİZCE)
Diploma Numarası: 3226
Ağırlıklı Genel Not Ortalaması: 2.68 / 4.0

Yabancı Dil Bilgileri

İNGİLİZCE (Okuma: İyi, Yazma: İyi, Konuşma: İyi)

Bilimsel Teknolojik Faaliyet Alanları

Bilimsel Teknolojik Faaliyet Alanı Bilgileri

Sağlık Bilimleri -- Tıp -- Dahili Tıp Bilimleri -- Nöroloji

Temel Bilimler -- Yaşam Bilimleri -- Nörobiyoloji -- Diğer

Anahtar Kelimeler

Mikroglia
Nöroenflamasyon
Eksozomlar
Sinirbilim
Glia

Ar-Ge Yetkinlik

Kitaplar

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Projeler	TEMEL ARAŞTIRMA, BURSİYER, Mikroglial NLRP3 Inflamazom Aktivasyon Regülasyonunda Uzun-Kodlama Yapmayan RNA NEAT1'in Rolü, Yürütülen Kuruluş: İZMİR BİYOTIP VE GENOM MERKEZİ (Devam ediyor) . DİĞER, BURSİYER, Alzheimer Hastalığının Tanısında ve Yayılımında Nöron Kökenli Ekzozomlar, Yürütülen Kuruluş: İZMİR BİYOTIP VE GENOM MERKEZİ, Destek Alınan Kuruluş: TÜBİTAK BAŞKANLIK (Yurt İçi) , 01 Haziran 2018, 01 Haziran 2020.
Ödüller	Diğer, Uluslararası, EMBO Short Term Fellowship, Araştırma Ödülü, Ödül Alınan Kurum: EMBO (Sistemde kayıtlı olmayan kuruluş), İSPANYA, 22 Mayıs 2020. Diğer, Ulusal, BİDEB 2211A Bursu, Burs, Ödül Alınan Kurum: TÜBİTAK BAŞKANLIK, TÜRKİYE, 01 Ekim 2019. Diğer, Ulusal, 100/2000 Doktora Bursu, Doktora Bursu, Ödül Alınan Kurum: YÖK (Sistemde kayıtlı olmayan kuruluş), TÜRKİYE, 01 Mart 2018.

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Proje Bilgileri	217S584, Alzheimer Hastalığının Tanısında ve Yayılımında Nöron Kökenli Ekzozomlar, 1001 - Araştırma, Burslu, Sonuçlandı, ARDEB, SBAG - Sağlık Bilimleri Araştırma Destek Grubu, Projeye Katılma/Ayrılma Tarihleri: 23.07.2018 - 01.06.2019, Proje Başlangıç/Bitiş Tarihleri: 01.06.2018 - 01.06.2020. 219S939, Mikroglial NLRP3 Inflamazom Aktivasyon Regülasyonunda Uzun-Kodlama Yapmayan RNA NEAT1'in Rolü, 1001 - Araştırma, BİDEB Burslusu, Yürürlükte, ARDEB, SBAG - Sağlık Bilimleri Araştırma Destek Grubu, Projeye Katılma/Ayrılma Tarihleri: 04.08.2020 - 01.12.2022, Proje Başlangıç/Bitiş Tarihleri: 01.07.2020 - 01.12.2022.
BİDEB Destekleri	BORA TAŞTAN, Etkinlik Destekleri ve Eğitim Bursları Müdürlüğü, 2250-Lisansüstü Bursları Performans Programı, Destekleniyor, 2022 - 1. BORA TAŞTAN, Yarışma Programları Müdürlüğü, 2204-A Lise Öğrencileri Araştırma Projeleri Yarışması, Başvurusu İnceleniyor, 2010 - 1. BORA TAŞTAN, Etkinlik Destekleri ve Eğitim Bursları Müdürlüğü, 2211-A Genel Yurt İçi Doktora Burs Programı, Destekleniyor, 2019 - 2, 01.06.2019 - 31.05.2023.

Panelistlik/İzleyicilik/Raporörlük Sayısı

Hakemlik/Panelistlik/Dış Danışmanlık Sayısı	ARDEB/BİDEB 0	TEYDEB 0	Toplam 0

8.2. Publication List

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ORIGINAL PAPER

Melatonin Alters the miRNA Transcriptome of Inflammasome Activation in Murine Microglial Cells

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Abstract
Systemic inflammation can have devastating effects on the central nervous system via its resident immune cells, the microglia. One of the primary mediators of this inflammation is inflammasomes, multiprotein complexes that trigger a release of inflammatory proteins when activated. Melatonin, a hormone with anti-inflammatory effects, is an attractive candidate for suppressing such inflammation. In this study, we have investigated how melatonin alters the microRNA (miRNA) transcriptome of microglial cells. For that purpose, we have performed RNA sequencing on a lipopolysaccharide and adenosine triphosphate (LPS + ATP) induced NLR family pyrin domain containing 3 (NLRP3) inflammasome activation model in the N9 mouse microglial cell line, with and without melatonin pre-treatment. We have identified 136 differentially expressed miRNAs in cells exposed to LPS + ATP compared to controls and 10 differentially expressed miRNAs in melatonin pre-treated cells compared to the inflammasome group. We have identified miR-155-3p as a miRNA that is upregulated with inflammasome activation and downregulated with melatonin treatment. We further confirmed this pattern of miR-155-3p expression in the brains of mice injected intraperitoneally with LPS. Moreover, an overexpression study with miRNA-155-3p mimic supported the idea that the protective effects of melatonin in NLRP3 inflammasome activation are partly associated with miRNA-155-3p inhibition.

Keywords Melatonin · Microglia · NLRP3 inflammasome · microRNA · LPS · ATP

Abbreviations

ATP	Adenosine triphosphate
ASC	Apoptosis-associated speck-like protein containing a caspase activation and recruitment domain
CNS	Central nervous system
Ctrl	Control
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSDMD	Gasdermin D
IL	Interleukin
IP	Intraperitoneal
KEGG	Kyoto encyclopedia of genes and genomes
LA	LPS and ATP
LPS	Lipopolysaccharide
miRNA	MicroRNA
MLA	Melatonin and LPS and ATP
MT	Melatonin
NLRP3	NLR family pyrin domain containing protein 3
PBS	Phosphate-buffered saline
qPCR	Quantitative real-time PCR
TLR	Toll-like receptor

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Introduction

Microglia are the primary immune cells in the central nervous system (CNS), responsible for or auxiliary to the inflammatory processes that protect, regulate, and occasionally damage the neuronal milieu [1]. Inflammation in the brain involves a significant degree of inflammasome

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Pros and cons of NRF2 activation as adjunctive therapy in rheumatoid arthritis

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Fibroblast-like synoviocytes

ABSTRACT

Rheumatoid arthritis (RA) is an autoimmune disease with an important inflammatory component accompanied by deregulated redox-dependent signaling pathways that are feeding back into inflammation. In this context, we bring into focus the transcription factor NRF2, a master redox regulator that exerts exquisite antioxidant and anti-inflammatory effects. The review does not intend to be exhaustive, but to point out arguments sustaining the rationale for applying an NRF2-directed co-treatment in RA as well as its potential limitations. The involvement of NRF2 in RA is emphasized through an analysis of publicly available transcriptomic data on NRF2 target genes and the findings from NRF2-knockout mice. The impact of NRF2 on concurrent pathologic mechanisms in RA is explained by its crosstalk with major redox-sensitive inflammatory and cell death-related pathways, in the context of the increased survival of pathologic cells in RA. The proposed adjunctive therapy targeted to NRF2 is further sustained by the existence of promising NRF2 activators that are in various stages of drug development. The interference of NRF2 with conventional anti-rheumatic therapies is discussed, including the cytoprotective effects of NRF2 for alleviating drug toxicity. From another perspective, the review presents how NRF2 activation would be decreasing the efficacy of synthetic anti-rheumatic drugs by increasing drug efflux. Future perspectives regarding pharmacologic NRF2 activation in RA are finally proposed.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune joint disease with a marked inflammatory component that affects cartilages and bones, leading to a progressive decline in physical function and quality of life as well as cumulative comorbid risk, putting therefore a huge burden on patients, healthcare systems and society. As reviewed by Smolen et al. [1], RA has an elusive autoimmune component involving autoantibodies like rheumatoid factors against the Fc portion of IgG as well as anti-citrullinated protein antibodies (ACPAs) against many citrullinated proteins such as fibrinogen, α-enolase, vimentin, collagen type II and fibronectin [2]. ACPAs, which appear before disease onset and correlate well with disease progression, are used as diagnostic tool early in the course of the disease [3]. In addition to autoantibodies, there is a genetic risk related to HLA-DRB1 polymorphisms that are strongly implicated in self-peptide binding and antigen presentation to autoreactive T cells [4].

Unlike other autoimmune diseases, the triggering autoantigen in RA still remains a mystery. Instead, a wealth of evidence has been brought for the self-sustaining inflammatory processes that drive RA progression. Pro-inflammatory cytokines such as TNFα, IL-1β, IL-6 and IL-17 have a key role in regulating the immunological 'prodrome' that precedes clinically manifested arthritis, underlines synovitis and perpetuates

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Alteration of miRNAs in Small Neuron-Derived Extracellular Vesicles of Alzheimer's Disease Patients and the Effect of Extracellular Vesicles on Microglial Immune Responses

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Abstract

Alzheimer's disease (AD) is one of the most severe neurodegenerative diseases observed in the elderly population. Although the hallmarks of AD have been identified, the methods for its definitive diagnosis and treatment are still lacking. Extracellular vesicles (EVs) have become a promising source for biomarkers since the identification of their content. EVs are released from multiple cell types and, when released from neurons, they pass from the brain to the blood with their cargo molecules. Hence, neuron-specific EV-resident microRNAs (miRNAs) are promising biomarkers for diagnosis of AD. This study aimed to identify altered miRNA content in small neuron-derived extracellular vesicles (sNDEVs) isolated from AD patients and healthy individuals. Furthermore, we examined the role of sNDEV-resident miRNAs in neuron-glia cellular interaction to understand their role in AD propagation. We identified 10 differentially expressed miRNAs in the sNDEVs of patients via next-generation sequencing and validated the most dysregulated miRNA, let-7e, with qRT-PCR. Let-7e was significantly increased in the sNDEVs of AD patients compared with those of healthy controls in a larger cohort. First, we evaluated the diagnostic utility of let-7e via ROC curve analysis, which revealed an AUC value of 0.9214. We found that IL-6 gene expression was increased in human microglia after treatment with sNDEVs of AD patients with a high amount of let-7e. Our study suggests that sNDEV-resident let-7e is a potential biomarker for AD diagnosis, and that AD patient-derived sNDEVs induce a neuroinflammatory response in microglia.

Keywords Alzheimer's disease · Extracellular vesicle · miRNA · Cellular interaction · Let-7e

Abbreviations

AD	Alzheimer's disease
Aβ	Amyloid beta
APP	Amyloid precursor protein
AUC	Area under the curve
BACE1	Beta-secretase 1
cDNA	Complementary DNA
CNS	Central nervous system
CSF	Cerebrospinal fluid
EV	Extracellular vesicle
GO	Gene ontology
HC	Healthy control
IL	Interleukin
KEGG	Kyoto Encyclopedia of Genes and Genomes
L1CAM	Neural cell adhesion protein
miRNA	MicroRNA
MISEV	Minimal information for studies of extracellular vesicles
MMSE	Mini-Mental State Exam
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
sNDEV	Small neuron-derived extracellular vesicle
NGS	Next-generation sequencing
NTA	Nanoparticle tracking analysis
PET	Positron emission tomography

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REVIEW
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Targeting NLRP3 Inflammasome With Nrf2 Inducers in Central Nervous System Disorders

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The NLRP3 inflammasome is an intracellular multiprotein complex that plays an essential role in the innate immune system by identifying and eliminating a plethora of endogenous and exogenous threats to the host. Upon activation of the NLRP3 complex, pro-inflammatory cytokines are processed and released. Furthermore, activation of the NLRP3 inflammasome complex can induce pyroptotic cell death, thereby propagating the inflammatory response. The aberrant activity and detrimental effects of NLRP3 inflammasome activation have been associated with cardiovascular, neurodegenerative, metabolic, and inflammatory diseases. Therefore, clinical strategies targeting the inhibition of the self-propelled NLRP3 inflammasome activation are required. The transcription factor Nrf2 regulates cellular stress response, controlling the redox equilibrium, metabolic programming, and inflammation. The Nrf2 pathway participates in anti-oxidative, cytoprotective, and anti-inflammatory activities. This prominent regulator, through pharmacologic activation, could provide a therapeutic strategy for the diseases to the etiology and pathogenesis of which NLRP3 inflammasome contributes. In this review, current knowledge on NLRP3 inflammasome activation and Nrf2 pathways is presented; the relationship between NLRP3 inflammasome signaling and Nrf2 pathway, as well as the pre/clinical use of Nrf2 activators against NLRP3 inflammasome activation in disorders of the central nervous system, are thoroughly described. Cumulative evidence points out therapeutic use of Nrf2 activators against NLRP3 inflammasome activation or diseases that NLRP3 inflammasome contributes to would be advantageous to prevent inflammatory conditions; however, the side effects of these molecules should be kept in mind before applying them to clinical practice.

Keywords: NLRP3 inflammasome, Nrf2, inflammation, central nervous system, dimethyl fumarate, sulforaphane

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Abbreviations: AD, Alzheimer's Disease; ALS, Amyotrophic Lateral Sclerosis; ARE, Antioxidant Response Element; ATP, Adenosine Triphosphate; ASC, Apoptosis-associated Speck-like Protein containing a CARD; CBP, CREB-binding protein; CNS, Central Nervous System; DAMPs, Danger-Associated Molecular Patterns; DMF, Dimethyl Fumarate; GSDMD, Gasdermin D; HAMPs, Homeostasis-Altering Molecular Processes; IL, Interleukin; KEAP1, Kelch-like ECH associated protein; LncRNA, Long Non-coding RNA; LPS, Lipopolysaccharide; miRNA, MicroRNA; MS, Multiple Sclerosis; NF-κB, Nuclear Factor Kappa B; NLRP3, NLR Family Pyrin Domain Containing Protein 3; Nrf2, Nuclear Factor Erythroid 2-related Factor 2; PAMPs, Pathogen-associated molecular patterns; PD, Parkinson's Disease; PPI, Protein-Protein Inhibitor; PRR, Pattern Recognition Receptors; ROS, Reactive Oxygen Species; RRMS, Relapsing-Remitting Multiple Sclerosis; SFN, Sulforaphane; TLR, Toll-like Receptor; TXNIP, Thioredoxin-interacting protein.

Chapter 14

Role of Exosomal MicroRNAs in Cell-to-Cell Communication

Bora Tastan, Emre Tarakcioglu, Yelda Birinci, Yongsoo Park, and Sermin Genc

Abstract

Exosomes, a type of extracellular vesicle, are small vesicles (30–100 nm) secreted into extracellular space from almost all types of cells. Exosomes mediate cell-to-cell communication carrying various biologically active molecules including microRNAs. Studies have shown that exosomal microRNAs play fundamental roles in healthy and pathological conditions such as immunity, cancer, and inflammation. In this chapter, we introduce the current knowledge on exosome biogenesis, techniques used in exosome research, and exosomal miRNA and their functions in biological and pathological processes.

Key words Exosome, MicroRNA, Cancer, Immune System, Inflammation, Central Nervous System, Cardiovascular System

1 The Extracellular Vesicles

The term "extracellular vesicle" is used to define cell-derived and membrane-surrounded structures, which are released into extracellular space from multiple cell types ranging from prokaryotes to eukaryotes [1, 2]. The size of extracellular vesicles (EVs) ranges from 30 to 10,000 nm [3]. These structures were first described in 1946 as "pro-coagulant platelet-derived particles" [4]. After that, in 1967, Peter Wolf identified microvesicles as "Platelets dust" [5]. Secretion of EVs occurs in healthy and diseased conditions including injury, cellular stress, or changes in homeostasis [6, 7]. EVs are composed of a lipid bilayer membrane, which protects the EVs and their cargo from protease and nuclease activity [6]. The EVs are differ in their size/shape and origin that EVs are further divided into mainly two sub-classes: exosomes and microvesicles [6, 8]. The exosome derives from the endosome while microvesicles form through shedding from plasma membranes [2]. Also apoptotic bodies, a type of EV are released after apoptosis [1]. Evidently, the EVs are found almost in all bodily fluids including blood, urine, saliva, cerebrospinal fluid, milk, semen [6]. EVs

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Dimethyl Fumarate Alleviates NLRP3 Inflammasome Activation in Microglia and Sickness Behavior in LPS-Challenged Mice

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NLRP3 inflammasome activation contributes to several pathogenic conditions, including lipopolysaccharide (LPS)-induced sickness behavior characterized by reduced mobility and depressive behaviors. Dimethyl fumarate (DMF) is an immunomodulatory and anti-oxidative molecule commonly used for the symptomatic treatment of multiple sclerosis and psoriasis. In this study, we investigated the potential use of DMF against microglial NLRP3 inflammasome activation both *in vitro* and *in vivo*. For *in vitro* studies, LPS- and ATP-stimulated N9 microglial cells were used to induce NLRP3 inflammasome activation. DMF's effects on inflammasome markers, pyroptotic cell death, ROS formation, and Nrf2/NF-κB pathways were assessed. For *in vivo* studies, 12–14 weeks-old male BALB/c mice were treated with LPS, DMF + LPS and ML385 + DMF + LPS. Behavioral tests including open field, forced swim test, and tail suspension test were carried out to see changes in lipopolysaccharide-induced sickness behavior. Furthermore, NLRP3 and Caspase-1 expression in isolated microglia were determined by immunostaining. Here we demonstrated that DMF ameliorated LPS and ATP-induced NLRP3 inflammasome activation by reducing IL-1β, IL-18, caspase-1, and NLRP3 levels, reactive oxygen species formation and damage, and inhibiting pyroptotic cell death in N9 murine microglia *via* Nrf2/NF-κB pathways. DMF also improved LPS-induced sickness behavior in male mice and decreased caspase-1/NLRP3 levels *via* Nrf2 activation. Additionally, we showed that DMF pretreatment decreased miR-146a and miR-155 both *in vivo* and *in vitro*. Our results proved the effectiveness of DMF on the amelioration of microglial NLRP3 inflammasome activation. We anticipate that this study will provide the foundation consideration for further studies aiming to suppress NLRP3 inflammasome activation associated with in many diseases and a better understanding of its underlying mechanisms.

Keywords: dimethyl fumarate (DMF), microglia, NLRP3 inflammasome, lipopolysaccharide (LPS), sickness behaviors, pyroptosis

antioxidants

MDPI

Article

Ethyl Pyruvate Attenuates Microglial NLRP3 Inflammasome Activation via Inhibition of HMGB1/NF-κB/miR-223 Signaling

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Abstract: Ethyl pyruvate is a molecule with anti-inflammatory and pro-metabolic effects. Ethyl pyruvate has been shown to ameliorate the clinical and pathological findings of neurodegenerative diseases such as Alzheimer's and Parkinson's Diseases in rodents. Its anti-inflammatory and neuroprotective effects are widely investigated in animal and cellular models. Our study aimed to investigate the mechanism of the impact of Ethyl pyruvate on NLRP3 inflammasome activation in the N9 microglial cell line. Our results indicated that ethyl pyruvate significantly suppressed LPS and ATP-induced NLRP3 inflammasome activation, decreased active caspase-1 level, secretion of IL-1β and IL-18 cytokines, and reduced the level of pyroptotic cell death resulting from inflammasome activation. Furthermore, ethyl pyruvate reduced the formation of total and mitochondrial ROS and suppressed inflammasome-induced HMGB1 upregulation and nuclear NF-κB translocation and reversed the inflammasome activation-induced miRNA expression profile for miR-223 in N9 cells. Our study suggests that ethyl pyruvate effectively suppresses the NLRP3 inflammasome activation in microglial cells regulation by miR-223 and NF-κB/HMGB1 axis.

Keywords: ethyl pyruvate; microglia; NLRP3 inflammasome; microRNA; HMGB1; NF-κB

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1. Introduction

Microglial cells are the primary innate immune component of the central nervous system (CNS), which make up 10–15% of the brain [1]. They are mainly involved in maintaining tissue homeostasis by constantly scavenging the environment for possible threats and initiating the proper inflammatory responses, including inflammasomes. Microglial cells are the primary source of inflammasomes in the central nervous system [2]. Microglial inflammasome activation can be triggered by lipopolysaccharide (LPS), alpha-synuclein, prion peptide, and ATP, which then causes the secretion of pro-inflammatory cytokines and reactive oxygen species [3].

Cytoplasmic pattern recognition receptors (PRRs) are the first to encounter and recognize foreign bodies and pathogens as they enter the cells [4]. NOD-like receptor (NLR) family of PRRs can recognize damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) to activate the appropriate inflammatory response [5]. NLRP3 inflammasome complex is one of the inflammatory responsive complexes of the NLR family, which is activated via ATP and leads to pro-inflammatory cytokine release and pyroptotic cell death [6]. The proteolytic multiprotein complex structure of the NLRP3 inflammasome complex induces the caspase-1 enzyme to get activated

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Sulforaphane inhibits NLRP3 inflammasome activation in microglia through Nrf2-mediated miRNA alteration

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ABSTRACT

The NLRP3 inflammasome is a multiprotein complex that activates caspase-1 and triggers the release of the proinflammatory cytokines IL-1β and IL-18 in response to diverse signals. Although inflammasome activation plays critical roles against various pathogens in host defense, overactivation of inflammasome contributes to the pathogenesis of inflammatory diseases, including acute CNS injuries and chronic neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. In the current study, we demonstrated that Sulforaphane (SFN), a dietary natural product, inhibits NLRP3 inflammasome mediated IL-1β and IL-18 secretion and pyroptosis in murine microglial cells. SFN decreased the secretion of IL-1β and IL-18, and their mRNA levels in LPS primed microglia triggered by ATP. SFN suppressed the overexpression of cleaved caspase-1 and NLRP3 protein expressions as measured by caspase activity assay and western blot, respectively. SFN also prevented caspase-1 dependent pyroptotic cell death in microglia. Our data indicate that SFN suppresses NLRP3 inflammasome via the inhibition of NF-κB nuclear translocation and Nrf2 mediated miRNAs expression modulation in murine microglia.

1. Introduction

Inflammasomes are multiprotein complexes composed of NLRs, apoptosis-associated speck-like protein (ASC), and pro-caspase-1. They are crucial to innate immunity and have roles in the protection of the host against the pathogen [1]. Inflammasomes are also essential in sterile inflammation following tissue injury. NLRP3 is an extensively studied and well-characterized type of inflammasome in innate immune responses and inflammatory diseases, like gout, arthritis, Type 2 Diabetes, and Alzheimer's disease [2]. As a result of activation, NLRP3 recruits ASC and caspase-1 (known as interleukin-1 converting enzyme, ICE) to form complexes mediating maturation and secretion of potent proinflammatory cytokines, such as Interleukin-1β (IL-1 β) and Interleukin -18 (IL-18). The release of proinflammatory cytokines amplifies the inflammatory response [3].

Sulforaphane (SFN) is one of the isothiocyanates commonly found in cruciferous vegetables. The immunomodulatory properties of SFN have led the extensive research on its use against inflammation in numerous disease models. SFN also exhibits anti-inflammatory effects in the brain [4]. In rodent microglia, SFN suppresses pro-inflammatory cytokines secretion in LPS-induced inflammation [5]. These effects of SFN are closely related to the inhibition of pro-inflammatory transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1(AP-1) [5,6]. Several other intracellular

Abbreviations: ASC, Apoptosis-associated Speck-like Protein; ATP, Adenosine Triphosphate; CNS, Central Nervous System; DAMPs, Danger-associated Molecular Patterns; ELISA, Enzyme-linked Immunosorbent Assay; FBS, Fetal Bovine Serum; Gclc, Glutamate-cysteine Ligase Catalytic Subunit; Gstp1, Glutathione S-transferase P; HO-1, Heme Oxygenase-1; IL, Interleukin; LPS, Lipopolysaccharide; miRNA, microRNA; NF-κB, Nuclear Factor kappa B; NLRP3, NLR Family Pyrin Domain Containing 3; Nqo1, NAD (P)H Quinone Oxidoreductase 1; Nrf2, Nuclear Factor (erythroid-derived 2)-related Factor 2; PAMPs, Pathogen-associated Molecular Patterns; PRR, Pattern Recognition Receptor; ROS, Reactive Oxygen Species; SFN, Sulforaphane; TLR, Toll-like Receptor.

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Review

Inhibitory effects of phytochemicals on NLRP3 inflammasome activation: A review

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ABSTRACT

Background: The NLRP3 inflammasome formation and following cytokine secretion is a crucial step in innate immune responses. Internal and external factors may trigger inflammasome activation and result in inflammatory cytokine secretion. Inflammasome formation and activity play critical roles in several disease pathologies such as cardiovascular, metabolic, renal, digestive, and CNS diseases. Underlying pathways are not yet clear, but phytochemicals as alternative therapies have been extensively used for suppression of inflammatory responses.

Purpose: In this review, we aimed to summarize *in vivo* and *in vitro* effects on NLRP3 inflammasome activation of selected phytochemicals.

Method: Three phytochemicals; Sulforaphane, Curcumin, and Resveratrol were selected, and studies were reviewed to clarify their intracellular signaling mechanism in NLRP3 inflammasome activity. PubMed and Scopus databases are used for the search. For sulforaphane, 8 articles, for curcumin, 25 articles, and for resveratrol, 41 articles were included in the review.

Conclusion: *In vitro* and *in vivo* studies pointed out that the selected phytochemicals have inhibitory properties on NLRP3 inflammasome activity. However, neither the mechanism is clear, nor the study designs and doses are standardized.

Introduction

Nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain containing 3 (NLRP3) inflammasome is a multi-protein complex that contributes to innate immune responses. NLRP3 complex is composed of NLRP3 sensor protein, the apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) adaptor protein, and caspase-1 protease (Fig. 1) (de Zoete et al., 2014). NLRP3 inflammasome activity is primarily seen in macrophages and monocytes, but it is not exclusive to those cells. NLRP3 inflammasome complex has been shown in various cell types from neurons to mesenchymal stromal cells (Shao et al., 2015). Besides being a defense mechanism, NLRP3 inflammasome overactivation is related to several types of diseases such as cardiovascular, metabolic, renal, digestive, and central nervous system (CNS) diseases (Chen et al., 2017; Kammoun et al., 2018; Martinez et al., 2018; Nasoohi et al., 2018; Sharma et al., 2018; Wu et al., 2018; Yuan et al., 2018). Therefore, NLRP3 inflammasome activation is one of the most attractive therapeutic targets for drug studies.

Phytochemicals are defined as a substance synthesized in a medicinal plant and extracted for therapeutic purposes (Katz and Baltz, 2016). Plants have been used since ancient times for their

Abbreviations: AMPK, AMP-activated protein kinase; ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; ATP, adenosine triphosphate; CNS, central nervous system; ER, endoplasmic reticulum; I/R, ischemia/reperfusion; IL, interleukin-; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; NLRP3, nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing 3; Nrf2, nuclear factor erythroid 2-related factor; ROS, reactive oxygen species; RSV, resveratrol; SFN, sulforaphane; SIRT1, sirtuin-1; STAT-1, signal transducer and activator of transcription 1; TXNIP, thioredoxin interacting protein

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Microglial NLRP3 inflammasome activation in multiple sclerosis

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Melatonin Attenuates LPS-Induced Acute Depressive-Like Behaviors and Microglial NLRP3 Inflammasome Activation Through the SIRT1/Nrf2 Pathway

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Inflammation is a crucial component of various stress-induced responses that contributes to the pathogenesis of major depressive disorder (MDD). Depressive-like behavior (DLB) is characterized by decreased mobility and depressive behavior that occurs in systemic infection induced by Lipopolysaccharide (LPS) in experimental animals and is considered as a model of exacerbation of MDD. We assessed the effects of melatonin on behavioral changes and inflammatory cytokine expression in hippocampus of mice in LPS-induced DLB, as well as its effects on NLR Family Pyrin Domain Containing 3 (NLRP3) inflammasome activation, oxidative stress and pyroptotic cell death in murine microglia *in vitro*. Intraperitoneal 5 mg/kg dose of LPS was used to mimic depressive-like behaviors and melatonin was given at a dose of 500 mg/kg for 4 times with 6 h intervals, starting at 2 h before LPS administration. Behavioral assessment was carried out at 24 h post-LPS injection by tail suspension and forced swimming tests. Additionally, hippocampal cytokine and NLRP3 protein levels were estimated. Melatonin increased mobility time of LPS-induced DLB mice and suppressed NLRP3 expression and interleukin-1β (IL-1β) cleavage in the hippocampus. Immunofluorescence staining of hippocampal tissue showed that NLRP3 is mainly expressed in ionized calcium-binding adapter molecule 1 (Iba1) -positive microglia. Our results show that melatonin prevents LPS and Adenosine triphosphate (ATP) induced NLRP3 inflammasome activation in murine microglia *in vitro*, evidenced by inhibition of NLRP3 expression, Apoptosis-associated speck-like protein containing a CARD (ASC) speck formation, caspase-1 cleavage and interleukin-1β (IL-1β) maturation and secretion. Additionally, melatonin inhibits pyroptosis, production of mitochondrial and cytosolic reactive oxygen species (ROS) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling. The beneficial effects of melatonin on NLRP3 inflammasome activation were associated with nuclear factor erythroid 2–related factor 2 (Nrf2) and Silent information regulator 2 homolog 1 (SIRT1) activation, which were reversed by Nrf2 siRNA and SIRT1 inhibitor treatment.

Keywords: melatonin, NLRP3 inflammasome, microglia, lipopolysaccharide, depressive-like behaviors, Nrf2, SIRT1

8.3. Ethical Approval

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Bilgilerinizi ve gereğini rica ederiz.

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