2023

YÜKSEK LİSANS TEZİ

Aida Nurul BAROKAH

T.C.

ANKARA YILDIRIM BEYAZIT ÜNİVERSİTESİ

SAĞLIK BİLİMLERİ ENSTİTÜSÜ

INVESTIGATION of mRNA EXPRESSION LEVELS of AMPA RECEPTOR GENES in MICE EXPOSED to MATERNAL STRESS

YÜKSEK LİSANS TEZİ

Aida Nurul BAROKAH

TRANSLASYONEL TIP PROGRAMI

Ankara, 2023

T.C.

ANKARA YILDIRIM BEYAZIT ÜNİVERSİTESİ

SAĞLIK BİLİMLERİ ENSTİTÜSÜ

INVESTIGATION of mRNA EXPRESSION LEVELS of AMPA RECEPTOR GENES in MICE EXPOSED to MATERNAL STRESS

YÜKSEK LİSANS TEZİ

Aida Nurul BAROKAH

TRANSLASYONEL TIP PROGRAMI

This study was supported by Scientific Research Projects Unit at Ankara Yıldırım Beyazıt University. (Project Number: 2473)

Ankara, 2023

I dedicate this thesis to my parents, Siti Muslimah, A.Md., and Gunawan Suprianto, S.Ag., M.Ag., who have always been my inspiration;
To my son, Albirru Rafatul Izza, my trusted discussion partner; and
To my husband, Humam Ari Wayhudin, my eternal supporter.

ACKNOWLEDGMENTS

I would first like to express my sincere gratitude to Asst. Prof. Keziban KORKMAZ BAYRAM for her kind support and supervision throughout my Master's study. I greatly appreciate her for giving me a valuable opportunity and experience to improve myself academically and personally. I am the luckiest student that has grown with her 'lab venture.'

I am grateful to Asst. Prof. Faruk MERT for being my second supervisor and supporting me throughout my academic journey. I am especially thankful for his help and understanding, and for allowing me to learn about translational research areas within his field.

I would like to thank Assoc. Prof. Ramazan KARSLIOĞLU and Asst. Prof. Mustafa GÜNGÖRMÜŞ for their support during my thesis research in Ankara Yıldırım Beyazıt University's Central Research Laboratory, Application and Research Centre (Merkez Araştırma Laboratuvarı Uygulama ve Araştırma Merkezi).

I would like to express my gratitude to Dr. Arslan BAYRAM for providing me with invaluable guidance during the statistical analysis for my thesis writing. His weekly discussion section throughout my Master's program was also very thoughtful and greatly appreciated.

I would like to thank Merve Hilal DÖNMEZ for her assistance in conducting my experiments, her unwavering support, and our valuable friendship. Additionally, I am thankful to the members of Korkmaz Bayram's lab, Şevval Nebahat Işıktan and İhsan Kıvanç Gürsoy, for their meaningful friendship and support.

I would like to thank Ankara Yıldırım Beyazıt Scientific Research Projects Coordination Unit (BAP) for their financial assistance through grant number 2473 during my studies and for the completion of my thesis.

Last but not least, I would be thankful to my family in Indonesia for their patience and support during my studies. A special thank you to my husband, Humam Ari Wayhudin, for his unwavering support, understanding, and care for our child, Albirru Rafatul İzza, throughout my academic journey. I would like to express my appreciation to my brother, Hafidz Amarulloh, for his assistance during my thesis writing.

TABLE OF CONTENTS

ÖZET

Maternal Strese Maruz Kalmış Farelerde AMPA Reseptör Genlerinin mRNA İfade Düzeylerinin Araştırılması

Neonatal dönemde maruz kalınan stres, yaşam boyu devam eden antisosyal davranışlar ve kişilik bozuklukları gibi psikopatolojik sorunlara yatkınlık oluşturabilmektedir. Amino-3-hydroxy-5-methyl-4-isoxazole propionic asit (AMPA) reseptörleri glutamat ile bağlanarak aktifleşmektedir. Tahmin algoritmalarına göre yüksek skor ile miR-124-3p'nin hedefleri arasında yer alan *Gria2-4*'e ve *Gria1* (hedef olmayan) genleri AMPA reseptörlerinin alt birimlerini kodlamaktadır. miR-124-3p ifade düzeyinin, maternal stresli farelerin plazma ve hipokampusunda arttığını tespit ettiğimiz çalışmadan ilham alarak maternal stres varlığında *Gria1-4* genlerinin mRNA düzeylerini hipokampus ve kan örneklerinde araştırmayı amaçladık.

Çalışma, kontrol, maternal ayrılık (MS) ve maternal ayrılık ve öngörülemeyen maternal stres (MSUS) gruplarından oluşmaktadır. Postnatal (PND) 1-14 günlerinde MS grubu öngörülemeyen maternal ayrılmaya maruz bırakılırken, MSUS grubuna öngörülemeyen maternal ayrılma ve maternal stres uygulanmıştır (kısıtlama ve zorla yüzme). PND35'de sakrifiye edilen farelerin hipokampus ve kan örneklerinden Fenol-Kloroform tekniği ile total RNA izole edilerek QuantiTect Reverse Transcription Kit ile cDNA sentezlenmiştir. QuantNova SYBR Green PCR Kit kullanılarak *Gria1-4* genlerinin mRNA ifade düzeyleri qPCR tekniği ile ölçülmüştür. Elde edilen veriler GraphPad Prism'de One-Way ANOVA testleri kullanılarak analiz edilmiştir.

Hipokampusta sırasıyla *Gria2* ve *Gria3* mRNA ifade düzeylerinin kontrole kıyasla hem dişi hem erkeklerde MS (sırasıyla p=0.0071, p=0.0001) ve MSUS (sırasıyla p<0.0001, p<0.0001) gruplarında azaldığı, *Gria4* mRNA ifadesinin ise dişilerde MS ve MSUS gruplarında azaldığı MSUS erkeklerde (p=0.0094) ise arttığı tespit edilmiştir. Ancak kontrole kıyasla *Gria1* mRNA ifade düzeyi dişilerde MS (p=0.0021) ve MSUS grubunda azaldığı, *Gria1* ve *Gria4* mRNA ifade düzeyinin MS

ve MSUS gruplarının erkeklerinde ise arttığı tespit edilmiştir. Kan örneklerinde kontrole kıyasla dişilerde *Gria2* mRNA ifade düzeyi değişmezken *Gria3* MS ve MSUS grubunda artmıştır ayrıca *Gria2-3* MS erkeklerde de artmıştır. Kontrole kıyasla hem erkek hem dişilerde *Gria1* mRNA ifade düzeyinin MSUS grubunda artarken (p<0.0001), *Gria4*'ün ise azaldığı (p=0.0161) tespit edilmiştir.

Öngörülen şekilde, miR-124-3p ifade düzeyi artışı ile olası hedeflerinin hipokampusda mRNA ifade düzeylerinin azaldığı, ancak bu profilin kanda sergilenmediği gözlenmiştir. Ancak ilginç şekilde, annelerine stres uygulanan yavruların kanında anlamlı şekilde mRNA ifade düzeyinin *Gria4* için azalırken *Gria1* için arttığı tespit edilmiştir. Bu çalışma, neonatal dönemde maruz kalınan maternal stresin kanda saptanabilmesi açısından *Gria1* ve *Gria4* genlerinin birer biyobelirteç olma potansiyelini ortaya koyması sebebiyle orijinaldir ve gelecek çalışmalar için umut vadedicidir.

Anahtar Kelimeler: AMPA reseptörü, uyarıcı amino asit reseptörü, gen ekspresyonu, glutamat reseptörü, hipokampus**.**

ABSTRACT

Investigation of mRNA Expression Levels of AMPA Receptor Genes in Mice Exposed to Maternal Stress

Early-life stress may predispose to psychopathological problems such as antisocial behaviours and personality disorders that continue throughout life. Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors are activated by binding with glutamate. According to prediction algorithms, miR-124-3p encodes subunits of *Gria2-4* and *Gria1* (non-target) AMPA receptors among the targets of miR-124-3p with high scores. We investigated *Gria1-4* gene expression in the hippocampus and blood of mice with maternal stress, inspired by our previous research showing raised miR-124-3p levels in these same mice.

This study examined control, maternal separation (MS), and maternal separation with unpredictable maternal stress (MSUS). The MS and MSUS groups experienced unpredictable separation from their dams during postnatal days 1-14. The researchers analysed RNA from the hippocampus and blood samples of mice sacrificed on PND35 using qPCR and One-Way ANOVA tests in GraphPad Prism.

It was found that *Gria2* and *Gria3* mRNA expression levels in the hippocampus decreased in the MS ($p=0.0071$, $p=0.0001$, respectively) and MSUS ($p<0.0001$, $p<0.0001$, respectively) groups in both females and males compared to the control. In contrast, *Gria4* mRNA expression decreased in the MS and MSUS groups in females and increased in MSUS males ($p=0.0094$). However, in females, *Gria1* mRNA expression level decreased in the MS ($p=0.0021$) and MSUS groups, while in males of the MS and MSUS groups, *Gria1* and *Gria4* mRNA expression levels increased compared to controls. In blood samples, *Gria2* mRNA expression level did not change in females, *Gria3* increased in MS and MSUS groups, and *Gria2-3* MS increased in males. *Gria1* mRNA expression level was increased in the MSUS group ($p<0.0001$), while *Gria4* was decreased ($p=0.0161$) in both genders compared to the control.

The study found that miR-124-3p increases in the hippocampus and its targets decrease, but no changes were seen in the blood. The mRNA levels of Gria4 and Gria1 in the blood were significantly different in the litter of stressed mothers, and these genes could serve as potential biomarkers for detecting maternal stress during the neonatal period.

Keywords: AMPA receptor, excitatory amino acid receptor, gene expression, glutamate receptor, hippocampus.

LIST OF SYMBOLS AND ABBREVIATIONS

MS : Maternal separation/Unpredictable maternal separation

MSUS : Maternal separation/Unpredictable maternal separation combined with unpredictable maternal stress

AMPA : α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMPAR : α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

RNA : Ribonucleic acid

DNA : Deoxyribonucleic acid

mRNA : Messenger ribonucleic acid

miRNA : MicroRNA

Gria1 : Glutamate Ionotropic Receptor AMPA Type Subunit 1

Gria2 : Glutamate Ionotropic Receptor AMPA Type Subunit 2

Gria3 : Glutamate Ionotropic Receptor AMPA Type Subunit 3

Gria4 : Glutamate Ionotropic Receptor AMPA Type Subunit 4

Gapdh : Glyceraldehyde-3-phosphate dehydrogenase

Ca^{2+} : Calcium ion

Na^{+} : Sodium ion

K^{+} : Potassium ion

GABA : Gamma-aminobutyric acid

TCA : Tricarboxylic acid

NMDA : N-methyl-D-aspartate

PSD : Postsynaptic density

CA : Cornu ammonis

CA1 : Cornu ammonis 1

CA2 : Cornu ammonis 2

CA3 : Cornu ammonis 3

CA4 : Cornu ammonis 4

PTSD : Post-traumatic stress disorder

HPA : Hypothalamo-pituitary-adrenocortical

CRH : Corticotropin releasing hormone

PVN : Paraventricular nucleus

ACTH : Adrenocorticotrophic hormone

GR : Glucocorticoid receptors

RISC : RNA-induced silencing complex

UTR : Untranslated Region

ELS : Early-life stress

FST : Forced swim test

PND : Postnatal Day

NFW : Nuclease-free water

cDNA : Complementary DNA

qPCR : Quantitative Polymerase Chain Reaction

PCR : Polymerase Chain Reaction

gDNA : Genomic DNA

RT : Reverse transcriptase

TBE : Tris-Borate-EDTA

Ct : Cycle threshold

ANOVA : Analysis of variance

IS : Immobilization stress

HFSCs : Hair follicle stem cells

Gas6 : Growth Arrest Specific 6

PTSD : Posttraumatic stress disorder

REST : Repressor element 1 silencing transcription factor

NRSF : Neuron-Restrictive Silencing Factor

RCOR1 : REST corepressor 1

cfNAs : Cell-free nucleic acids

BBB : Blood brain barrier

CNS : Central nervous system

SZ : Schizophrenia

BP : Bipolar Disorder

PFC : Prefrontal cortex

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

Various circumstances, such as natural disasters, can profoundly impact individuals, causing significant physical and psychological consequences [1], [2]. These events often lead to the separation of families, a critical concern recognized by the United Nations Children's Fund (UNICEF) in various crises, including natural disasters [3]. In such situations, maternal separation (MS) commonly occurs, where mothers and their children can be separated in various circumstances. MS refers to the stress experienced by mothers during the postnatal period [4], which can be a significant early-life stressful event. It arises when the continuity of the mother-child relationship is disrupted after it has been established [5]. This issue is further highlighted by recent research indicating that maternal separation was prevalent during the COVID-19 pandemic, with numerous babies born to mothers with COVID-19 separated after birth [6]. This separation resulted in lower breastfeeding rates and limited skin-to-skin contact [6], underscoring the impact of family separation on essential aspects of early infant care.

Early-life postnatal stress is essential for developing depression later in life [7]. However, despite recognizing the detrimental effects of early-life postnatal stress, the specific molecular mechanisms underlying its impact on litter development remain unclear. One area of interest in understanding these mechanisms is the expression level of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor genes in the hippocampus, a brain region crucial for learning, memory, and stress response. The AMPA receptor plays a vital role in mediating excitatory neurotransmission and synaptic plasticity [7], making it a potential candidate for studying the effects of early-life stress. Studies in rodents has focused on studying the effects of early postnatal period stress, such as MS, better to understand its impact on neuronal alterations in offspring [7]–[17]. Furthermore, to look also the physiological stress effect this study model often combined with forced swim test and restraint stress [8], [10].

This study aims to examine the mRNA expression levels of AMPA receptor subunits in both the hippocampus and blood to understand better the molecular changes associated with early-life stress. Specifically, the aims of this study on AMPA receptor mRNA expression in the hippocampus in a maternal stress mice model (**Figure 3.1.**) are as follows:

A. To investigate the impact of maternal stress on the mRNA expression levels of *Gria1, Gria2, Gria3* and *Gria4*, which are AMPA receptor subunits, in the hippocampus of mice.
B. To examine any alterations or changes in the expression patterns of *Gria1-4* genes in response to maternal stress.
C. To assess the potential correlation between maternal stress and the expression levels of *Gria1-4* genes, providing insights into the molecular mechanisms involved in the hippocampus.
D. Include blood samples to explore systemic changes and potential peripheral biomarkers related to the stress response.
E. To explore the implications of altered *Gria1-4* mRNA expression on synaptic plasticity, neuronal excitability, and neurotransmission in the hippocampus, which are crucial for learning, memory, and stress response.
F. To better understand the specific role of *Gria1-4* genes in the hippocampus under conditions of maternal stress and their potential implications for litter development.

The findings from this research will contribute to our understanding of the molecular changes associated with maternal stress and their potential impact on hippocampal function and neuronal processes.

2. GENERAL OVERVIEW

2.1. Stress and Its Health Implications

Stress is generally defined as any condition that disrupts an organism's homeostasis, the physiological or psychological equilibrium [18]. It encompasses the biological and psychological changes influenced by environmental factors, which can increase the risk of illness. Stress can be categorized as acute or chronic and manifest as physiological or psychological disturbances [19].

Acute stress typically occurs in sudden situations like confrontations or emergencies [19], [20], whereas chronic stress is a prolonged state of enduring a heavy burden without relief. As a result, chronic stress can lead to various health problems, including insomnia, digestive system disorders [21], anxiety, depression [22], cardiovascular diseases, neurodegenerative diseases [23] and even cancer [19].

To describe the cumulative impact of chronic stress on the body, Bruce McEwen and Stellar introduced the concept of "allostatic load." Allostatic load refers to the wear and tear that builds up in the body when a person experiences repeated or chronic stress [24]. A psychological stressor can activate the hypothalamo-pituitary-adrenocortical (HPA) axis. The HPA stress response is primarily triggered by neural mechanisms, specifically the release of corticotrophin releasing hormone (CRH) from paraventricular nucleus (PVN) neurons in the hypothalamus. Stressors cause the release of CRH into the hypophysial portal vessels, which transport the peptide to the anterior pituitary, allowing access to corticotrophs. Stimulated corticotrophs then release adrenocorticotrophic hormone (ACTH) into the systemic circulation, promoting the synthesis and secretion of glucocorticoids (such as cortisol in humans or corticosterone in rodents) by the adrenal cortex. Glucocorticoids are subsequently released into the systemic circulation and can bind to specific receptors in almost every organ system, including the brain [25].

Glucocorticoids, the principal end-products of the HPA axis, act on multiple organ systems, including the brain, to maintain homeostatic balance [26]. Although they are beneficial for short-term survival, prolonged exposure to glucocorticoids can

lead to various issues with metabolism, immune function, and psychological well-being. Therefore, it is crucial to tightly regulate the secretion of glucocorticoids to prevent these negative effects [26].

The hippocampus is rich in glucocorticoid receptors (GR) and mineralocorticoid receptors, allowing it to sense various levels of glucocorticoids circulating in the body. As a result, the hippocampus is strategically positioned to regulate the negative feedback inhibition of the stress axis based on the levels of glucocorticoids induced by stress [26]. Glucocorticoids play a regulatory role in the trafficking of AMPA receptors, vital for synaptic transmission and plasticity. These stress hormones can rapidly and persistently modulate the function of AMPA receptors, thus influencing synaptic strength and plasticity. Maintaining precise control over the abundance of AMPA receptors on the postsynaptic membrane is crucial for facilitating changes in synaptic strength and plasticity [27].

2.2. Early-Life Postnatal Maternal Stress

Postnatal maternal stress is a type of stress that occurs after childbirth and can have negative effects on both the mother and the child [28]. In addition, postnatal maternal stress is associated with impairments in litter's neurodevelopment [29]. The development of social skills in humans, primates, and rodents heavily relies on the care provided by the mother and the establishment of a bond between the mother and offspring during the early stages of life [30]. Extensive research conducted on animal models has consistently shown that early-life stressors, such as maternal separation, have detrimental effects on behaviour in adulthood. These effects include impaired stress responses and increased behavioural despair, indicating maladaptive behavioural patterns [8], [10].

2.3. Heritability of Negative Social Behaviour and Transgenerational Epigenetic Inheritance

Clinical studies on humans have indicated a significant heritability rate for negative social behaviours associated with stress [31]. The causes of depression are not yet confirmed, but it is commonly accepted that genetic and environmental factors contribute to its development. Furthermore, emerging evidence suggests that an individual's personal life experiences and the experiences inherited from their ancestors can impact their health and the likelihood of healthy aging [19], [32]. However, further experimental research is needed to support this hypothesis more robustly. The occurrence of certain traits in both parents and their litter, which does not follow the traditional inheritance pattern described by Mendel, is known as a transgenerational epigenetic inheritance [31]. This form of inheritance relates to changes in inherited traits that do not alter the DNA sequence. DNA methylation, histone modification, and small non-coding RNAs can affect gene regulation [33]. One important group of these non-coding RNAs is microRNAs (miRNAs), which bind to specific regions of messenger RNA (mRNA) and prevent their translation into proteins [34]. It is well-known that changes in the levels of miRNAs can significantly affect behaviour and neurodevelopment [35].

2.4. mRNA Expression

The Central Dogma of molecular biology describes the flow of genetic information from DNA to RNA to protein. At the core of this process is mRNA synthesis, where messenger RNA (mRNA) molecules are produced to carry genetic instructions from the DNA in the nucleus to the ribosomes in the cytosol. mRNA is chemically similar to DNA and is synthesized as a single strand [46]. Gene expression refers to converting genetic information from genes into proteins through the intermediate step of mRNA. The term "Gene expression" is often used interchangeably with mRNA measurements [47].

mRNA expression involves a complex series of coordinated events regulated at multiple levels. These levels include transcription, where genes are transcribed into

RNA molecules through processes such as initiation, elongation, and termination. Following transcription, there is posttranscriptional regulation involves RNA movement, splicing to remove introns, and ensuring RNA stability. Once the RNA is prepared, translation occurs, where proteins are synthesized using the RNA as a template. This process includes initiation, elongation, and termination of translation. Finally, there is posttranslational regulation, which encompasses modifications and processes that impact protein stability, localization, and functionality. These regulatory mechanisms control mRNA expression finely, allowing cells to respond to signals and adapt to their surroundings [48].

This study mainly investigates the expression of genes encoding AMPA receptor channel proteins (*Gria1, Gria2, Gria3* and *Gria4*). The widely used housekeeping gene *Glyceraldehyde-3-Phosphate Dehydrogenase* (*Gapdh*) was employed to ensure accurate comparisons and normalization of mRNA expression data. *Gapdh* is a reference gene commonly used in mRNA expression studies, enabling reliable assessment and interpretation of the expression levels of the AMPA receptor channel protein-coding genes of interest [49].

2.5. MicroRNAs and Their Biogenesis

MicroRNAs (miRNAs) are small non-coding RNA molecules, typically composed of 18-25 nucleotides, that play a crucial role in regulating gene expression. They achieve this by binding to the 3' Untranslated Region (3'UTR) of messenger RNAs (mRNAs). This binding interaction hinders the translation of the mRNA into proteins, effectively inhibiting protein synthesis [34].

The biogenesis of miRNAs begins with the transcription of specific miRNA genes by RNA polymerase II, producing long primary transcripts known as pri-miRNAs. These pri-miRNAs possess characteristic structures, including a 5' cap and a 3' poly-A tail [36]. Pri-miRNA is an elongated form of RNA that exists as a double-stranded structure. This long dsRNA undergoes cleavage by an enzyme called Drosha, which belongs to the RNase III family. As a result of this cleavage, shorter hairpin-shaped dsRNA molecules known as pre-miRNA are formed. These pre-miRNA

molecules are typically around 60 nucleotides in length [37]. Drosha, in conjunction with other proteins like Pasha (DGCR8), interacts to form a protein complex. This complex facilitates the transportation of pre-miRNA from the nucleus to the cytoplasm, aided by a nuclear transport molecule called exportin 5 [38].

In the cytoplasm, another RNase III enzyme called Dicer recognizes and cleaves the pre-miRNAs, generating approximately 22 nucleotide-long RNA duplexes known as mature miRNA-miRNA* duplexes. The mature miRNA's dsRNA structure is transformed into a single-stranded mature miRNA upon its loading into the Argonaute (AGO) protein, achieved by the expulsion of the single miRNA* strand [39], [40]. The interaction between miRNA and AGO leads to the formation of the functional RNA-induced silencing complex (RISC), which plays a crucial role in regulating gene expression by directly binding to mRNA targets. **Figure 2.1.** illustrates the step-by-step process of miRNA synthesis, beginning with its transcription in the nucleus and concluding with its functionalization in the cytoplasm [41].

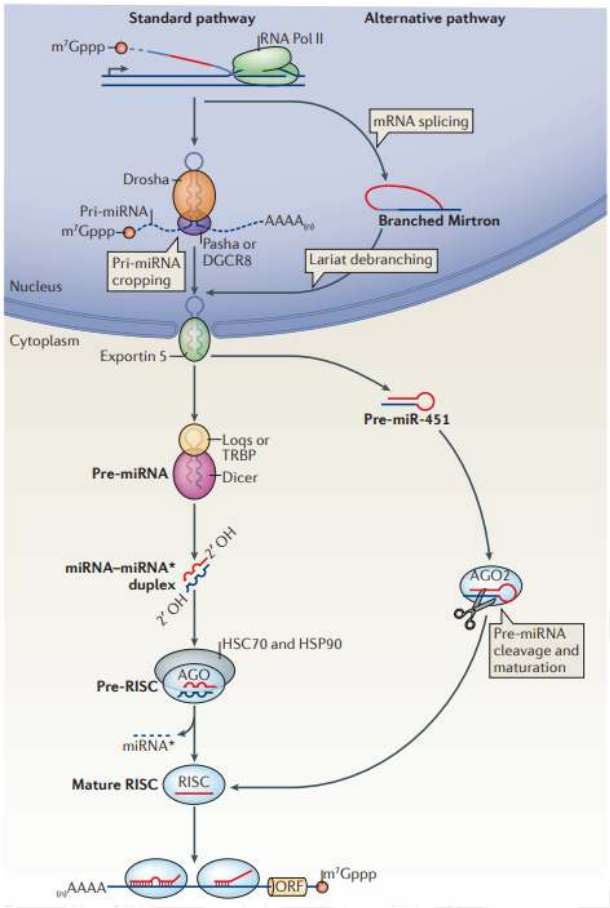

Figure 2.1. miRNA biogenesis pathway. *Copyright permission for the figure is provided in the Appendix Section.*

The interaction between miRNAs and their target mRNAs triggers the destabilization of the mRNA molecules. This destabilization occurs through processes such as mRNA cleavage and inhibition of translation, ultimately leading to a reduction in the protein production from the target mRNA [42]. In the context of animals, the interaction between miRNA and the 3'-UTR of mRNA targets primarily occurs in the vicinity of the miRNA's 5' terminus, known as the "seed" sequence. This specific sequence, typically 6-8 nucleotides long, is conserved across different species and plays a crucial role in determining the range of miRNA targets. Understanding the significance of the miRNA seed sequence provides insights into how a single miRNA

can regulate the expression of multiple mRNA targets and how a single mRNA can be controlled by multiple miRNAs. [43]–[45].

In this study, we investigate the mRNA expression of *Gria1*, *Gria2*, *Gria3*, and *Gria4* in relation to miR-124-3p. The data for miR-124-3p was obtained from our unpublished preliminary study. Based on miRDB, miR-124-3p targets *Gria2*, *Gria3*, and *Gria4*, and therefore, we aimed to explore the correlation between their expression levels and miR-124-3p. We also examined the expression levels of the *Gria1* gene, which belongs to the same gene family but is not targeted by miR-124-3p, in animal models subjected to stress conditions [50]. The AMPA receptors, known for their crucial involvement in glutamate ionotropic transmembrane receptor signalling, have emerged as prominent candidates for further investigation in light of their potential association with miR-124-3p.

miR-124-3p is a vital microRNA for neuronal development and immune responses, constituting the largest proportion (25-48%) of brain microRNA [51]. Studies have shown that miR-124 is closely related to depression [52]. Research demonstrates that the expression of microRNA-124 (miR-124) in the hippocampus has a significant impact on resilience or susceptibility to depression-like behaviours induced by chronic stress. Modulating miR-124 pathways in the hippocampus could potentially have antidepressant effects [53].

In the STRING database, **Figure 2.2.** demonstrates the close correlation among the *Gria1, Gria2, Gria3* and *Gria4* genes, which encode the AMPA receptors. Within this group, three specific genes, namely *Gria2*, *Gria3*, and *Gria4*, have been identified as direct targets of miR-124-3p. However, although *Gria1* is not listed among the target genes of miR-124-3p, it has been included in the study due to its association with the other genes.

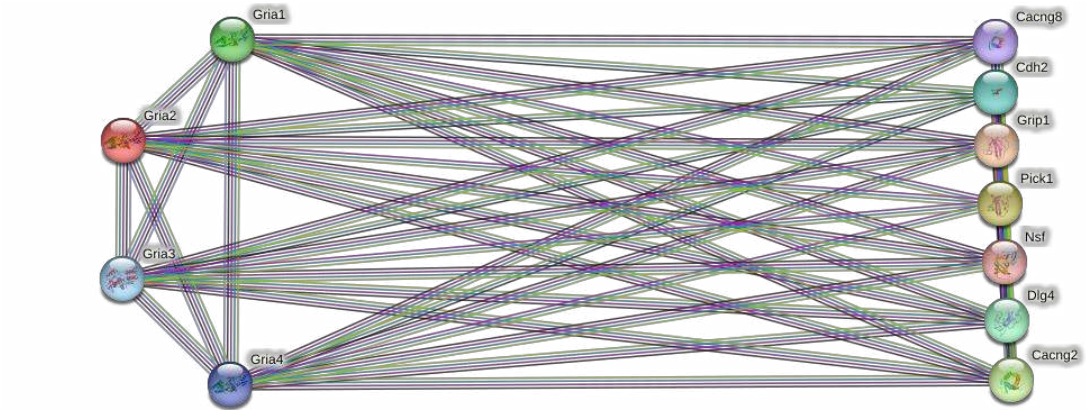

Figure 2.2. STRING output reflecting the connection between *Gria1*, *Gria2*, *Gria3*, and *Gria4*.

The target gene and corresponding scores of miR-124-3p, pertaining to the AMPA receptor genes, are provided in **Table 2.1**. AMPA receptor genes are targeted by miR-124-3p, and their respective scores according to the miRDB prediction algorithm are provided in **Table 2.1**. The scoring of target transcripts ranges between 100 and 50, depending on the sequence of the core region of miR-124-3p.

Table. 2.1. Target transcripts and scores of miR-124-3p for *Gria1*, *Gria2*, *Gria3* and *Gria4*.

miRNA	Hedef Transkriptler	Skor
miR-124-3p	*Gria2*	92
	Gria3	93
	Gria4	91

In **Figure 2.3.** the sequence complementarity between mouse miR-124-3p and *Gria2*, *Gria3* was demonstrated. Although sequence data for miR-124-3p and *Gria4* were limited in TargetScan v8.0, miRDB analysis indicated the targeting of *Gria4* by miR-124-3p. Both miRDB and TargetScan analyses confirmed that *Gria1* is not predicted to be targeted by miR-124-3p.

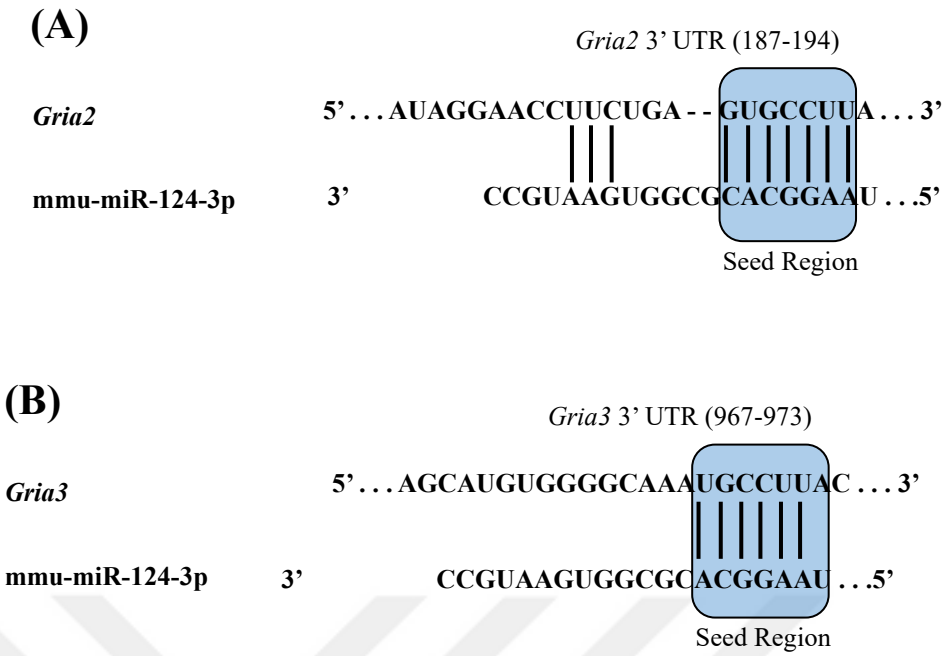

Figure 2.3. Graphical representation of sequence complementarity between miR-124-3p and *Gria2* (A), *Gria3* (B) seed region and binding site of *Gria2* (A), Gria3 (B) 3' UTR retrieved from TargetScan v8.0.

2.6. Hippocampus

The hippocampus is a bilateral structure located within each brain's temporal lobe. It occupies the upper and inner regions of the temporal lobes, positioned on the top of the parahippocampal gyrus (**Figure 2.4.**). Each hippocampus is composed of two interconnected folds of gray matter. One of these folds is referred to as the cornu ammonis (CA) or "Ammon's horn," located in the hippocampus's upper and outer region. This region is also known as the hippocampus proper. The other fold is called the dentate gyrus, and it is located in the lower and inner part of the hippocampus [54].

When observed in a transverse (coronal) section, the CA region can be subdivided into four distinct regions: CA1, CA2, CA3, and CA4 [54]. The CA region of the hippocampus is primarily composed of pyramidal cells. The CA region predominantly consists of pyramidal cells, which are excitatory neurons that utilize glutamate as a neurotransmitter [55]. Most (90%) of neurons in CA1 are pyramidal

cells, serving as glutamatergic projection neurons, while the remaining 10% are interneurons. Conversely, the dentate gyrus primarily comprises granular neurons [55].

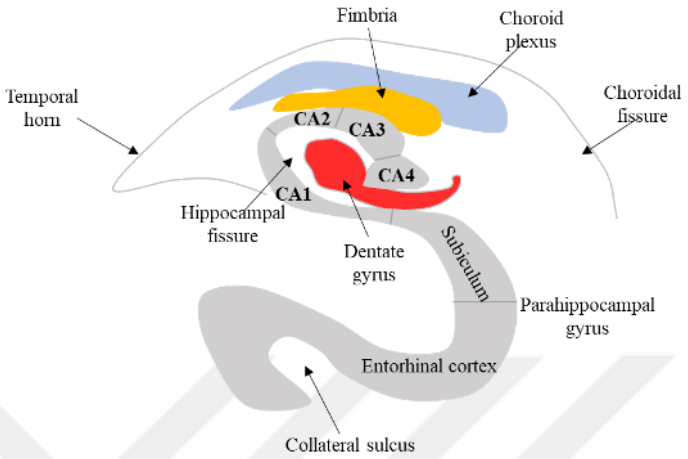

Figure 2.4. Hippocampal coronal section [54].

Figure 2.5 illustrates a schematic representation of the mouse hippocampus, specifically focusing on a coronal slice and providing a cross-sectional view to highlight its internal organization.

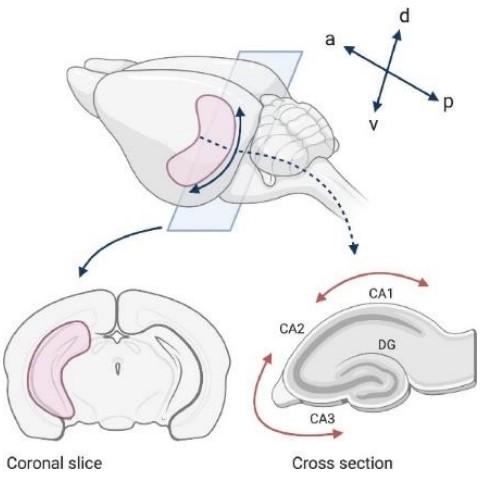

Figure 2.5. Schematic of mouse hippocampus [56].

Studies have found that the hippocampus, which plays a critical role in memory formation [54][57], is influenced by various pathways due to uncontrolled stress [58]. Stress can disrupt memory tasks, alter synaptic plasticity and neuronal firing patterns, and induce changes in the structure of the hippocampus. These stress-induced changes in the hippocampus have been associated with stress-related disorders like post-traumatic stress disorder (PTSD) [58]. Therefore, understanding these changes' mechanisms is crucial for developing effective treatments for stress-related disorders.

2.7. Synaptic Transmission

Neurons establish communication between each other through specialized connections known as synapses. These synapses are junctions where one neuron transmits a message to a target neuron or another cell. Most synapses function through chemical signalling, utilizing specific chemical messengers to relay information. However, there are also electrical synapses where ions flow directly between cells to facilitate communication. Within the synapse, when an action potential is generated in the presynaptic neuron (the sender), it triggers the transmission of a signal to the postsynaptic neuron (the receiver). This signal influences the postsynaptic neuron, increasing or decreasing its likelihood of generating an action potential of its own [59].

The brain contains various types of synapses, which can be broadly classified into two categories: electrical synapses and chemical synapses. Electrical synapses facilitate the direct and passive flow of electrical current between neurons. This flow occurs through specialized membrane channels called gap junctions, which directly connect the two cells. In contrast, chemical synapses enable communication between cells by releasing neurotransmitters. Neurotransmitters, which are chemical agents released by the presynaptic neurons, induce secondary current flow in postsynaptic neurons by binding to specific receptor molecules and activating them [60].

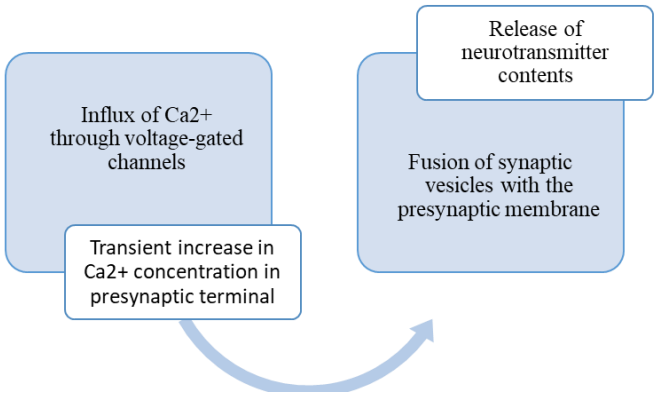

Figure 2.6. Secretion of neurotransmitters.

Neurotransmitter release as presented at **Figure 2.6.**, is initiated by the entry of calcium ions (Ca^{2+}) through voltage-gated channels, leading to a transient increase in Ca^{2+} concentration within the presynaptic terminal. This elevated Ca^{2+} level triggers the fusion of synaptic vesicles, which store neurotransmitters, with the presynaptic plasma membrane. Consequently, the neurotransmitters are released into the space between the pre- and postsynaptic cells. These chemicals subsequently attach to chemical receptors located in the dendrites of the receiving (post-synaptic) neuron. This interaction alters the cell membrane's permeability to specific ions, opening specialized gates or channels. As a result, a large influx of charged particles, including calcium, sodium, potassium, and chloride ions, enters the neuron. The precise mechanism by which Ca^{2+} induces exocytosis has yet to be fully understood. However, it is believed that specific proteins on the surface of the synaptic vesicle and throughout the presynaptic terminal play a crucial role in mediating this process [60] [61].

2.7.1. Neurotransmitters

Neurotransmission is the process by which signalling molecules called neurotransmitters are released by the axon terminal of a neuron (the presynaptic neuron), and bind to and react with the receptors on the dendrites of another neuron (the postsynaptic neuron) a short distance away [22]. Neurotransmission in the nervous

system begins at the presynaptic terminals, where synaptic vesicles merge with the plasma membrane and release chemical transmitters or neurotransmitters through exocytosis [61] (**Figure 2.6.**). Neurotransmitters play a vital role in human neural function. While the exact number of neurotransmitters in humans remains uncertain, over 100 neuroactive peptides have been identified [62]. A substance is typically classified as a neurotransmitter if it is produced within a neuron, present in the presynaptic terminal, released to exert an influence on the postsynaptic cell, can mimic its effects when externally applied to the postsynaptic cell, and has a specific mechanism for terminating its action [62]. Neurotransmitters can be categorized as small molecules and much larger neuropeptides [63]. Neurotransmitters can be classified based on their effects on the target cell. If a neurotransmitter stimulates the target cell and elicits a response, it is categorized as an excitatory neurotransmitter.

This type of neurotransmitter acts within an excitatory synapse to enhance neuronal activity. On the other hand, if a neurotransmitter inhibits the target cell and reduces its activity, it is classified as an inhibitory neurotransmitter. Inhibitory neurotransmitters function within inhibitory synapses to dampen neuronal activity and maintain balance in the neural circuitry [64]. Excitatory neurotransmitters facilitate depolarization of postsynaptic cells, triggering action potentials. Inhibitory neurotransmitters, on the other hand, induce hyperpolarization of target cells, moving them further away from the action potential threshold and suppressing their activity[60]. Excitatory neurotransmitters include acetylcholine, epinephrine, norepinephrine, histamine, glutamate, and dopamine. Inhibitory neurotransmitters include Gamma-aminobutyric acid (GABA), serotonin, and dopamine. Neurotransmitters like acetylcholine, norepinephrine, serotonin, and dopamine can exhibit either excitatory or inhibitory effects, depending on the receptors they interact with. This diversity poses challenges in categorizing neurotransmitters accurately [63].

Glutamate is the most prominent excitatory neurotransmitter in the mammalian central nervous system [65], [66]. It plays a vital role in regulating the overall excitability of the central nervous system, as well as in processes related to learning and memory. Glutamate facilitates the transmission of signals between nerve cells by stimulating their firing. As a nonessential amino acid, glutamate is synthesized locally in neurons from local precursors and cannot cross the blood-brain barrier [65].

Glutamine, derived from glial cells, is the primary precursor for glutamate in synaptic terminals. Within presynaptic terminals, glutamine undergoes metabolic conversion to glutamate through the action of the mitochondrial enzyme glutaminase. Additionally, glutamate can be synthesized through the transamination of 2-oxoglutarate, an intermediate of the tricarboxylic acid (TCA) cycle. Consequently, neurons can utilize a portion of their metabolized glucose to synthesize glutamate [65]. While glutamate is essential for normal brain function, abnormal levels or activity of glutamate, often associated with chronic stress, can contribute to the development of epilepsy, cognitive impairments, and mood disorders [67].

2.8. AMPA Receptors

Glutamate receptors can be divided into two categories based on their pharmacological and physiological properties: metabotropic and ionotropic receptors. The ionotropic subtype includes AMPA, N-methyl-D-aspartate (NMDA), and kainate receptors, which are named after specific agonists that activate them [68]. Ionotropic glutamate receptors are non-selective cation channels that allow the passage of Na^+ and K^+ ions, and in certain cases, Ca^{2+} ions as well [68]. Recent research has demonstrated that AMPA receptors lacking the GluA2 subunit, encoded by the *Gria2* gene, become permeable to calcium ions, leading to significant implications for neuronal function and synaptic plasticity [69].

Information transfer from one neuron to another through chemical signals is fundamental to the communication between neurons in the brain [59]. AMPA receptors are one of the most important ligand-gated or ionotropic glutamate receptors that mediate fast excitatory neurotransmission in the central nervous system. AMPA-type glutamate receptors (AMPAR) mediate a majority of excitatory synaptic transmission in the brain [70], [71]. AMPA receptors, which facilitate rapid excitatory synaptic transmission and are essential for synaptic plasticity and the formation of new memories [36]. AMPARs are composed of four subunits (GluA1-4) encoded by separate genes, which can be diversified through alternative splicing and mRNA editing [72]. AMPARs are tetrameric complexes assembled from four identical (homomeric) or similar (heteromeric) subunits [71]. Different subunit compositions

confer distinct biophysical and molecular properties to AMPARs. For instance, GluA2-containing receptors in principal neurons have a linear current-voltage relationship and are impermeable to Ca^{2+} due to Q/R editing of the GluA2 subunit [73]. In contrast, GluA2-lacking receptors in non-principal cells show double-rectification, higher conductance, and Ca^{2+} permeability [74], [75]. Both the number and the sub-unit compositions of AMPARs vary amongst different brain regions/neuronal cell types [74] and change during development, synaptic plasticity, and pathological states such as epilepsy [71].

AMPA receptors composed of subunits (GluA1, GluA2, GluA3, GluA4) with a modular domain arrangement, beginning with the relatively long amino-terminal domain (ATD), shorter intracellular C-terminal region, the ligand- or agonist-binding domain (LBD), three hydrophobic pore-forming transmembrane domains (TMD) and intramembrane loop between TMD1 and TMD2 [76], [77] (**Figure 2.7.** and **Figure 2.8.**). Although AMPA receptors are widely distributed throughout the brain, the central channel structure of these receptors is formed by only four genes. There may not be much variation in their molecular composition across different brain regions. However, recent proteomic studies indicate that AMPA receptors may exist as complex assemblies with additional subunits, which have proven difficult to isolate [78]. Most AMPARs are synthesized in the neuronal cell body and reach their synaptic targets after a complicated journey involving multiple sorting and transportation steps along different cytoskeleton structures and through various membrane compartments [79]. The trafficking, localization, kinetics and pharmacology of AMPA receptors are tuned by an ensemble of auxiliary protein subunits, which are integral membrane proteins [80]. The trafficking of AMPA receptors to synapses is regulated by subunit-specific mechanisms [81].

AMPARs on the cell membrane are not determined solely by mRNA expression. Specific subunits and splice variants preferentially assemble in the endoplasmic reticulum. For example, GluA3 subunits form homo-tetramers alone but form obligate heteromers with other subunits. Understanding the structural elements that influence AMPAR assembly is crucial to unravel the mechanisms and regulation of this process [71].

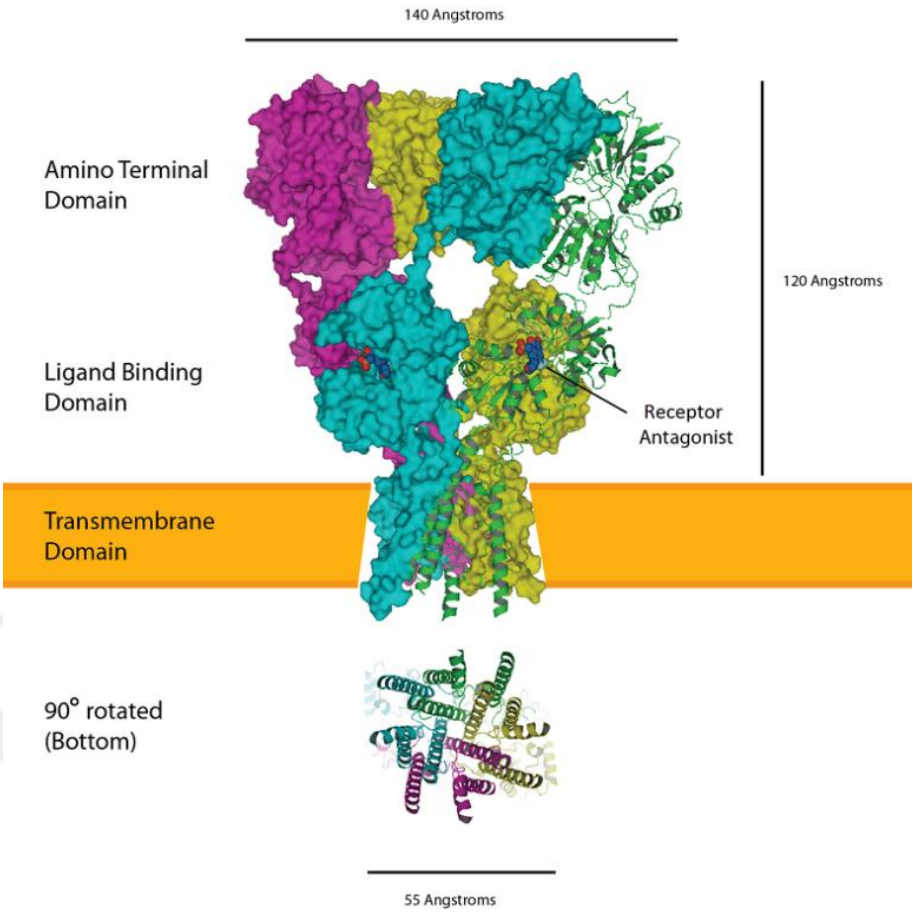

Figure 2.7. The structure of AMPA receptors.

Figure 2.8 provides an overview of the structural domains of AMPA receptors (AMPARs), highlighting their key regions and functional elements.

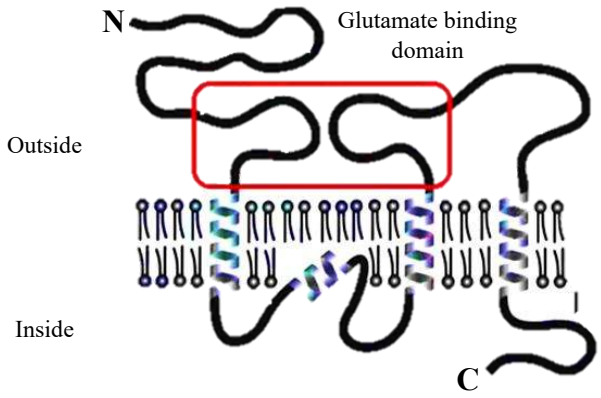

Figure 2.8. The structure of AMPAR domains.

Furthermore, recent findings on hippocampal AMPARs [78] have revealed:

- AMPA receptors are widely distributed in the brain, but formed by only four genes encoding their central channel structure.
- Proteomic studies suggest that AMPA receptors may be complex structures with additional subunits.
- AMPA receptors that have recently been assembled are inserted into the cell membrane and can be enlisted in the postsynaptic density (PSD), specialized region within the postsynaptic neuron where various proteins and signalling molecules accumulate to facilitate synaptic transmission and communication between neurons.
- Scaffold proteins like PSD-95 regulate the recruitment of AMPA receptors based on synaptic activity, allowing for changes in synaptic strength through the addition or removal of receptors.
- Hippocampal AMPA receptors are of particular interest due to their involvement in synaptic plasticity. They are decorated with a diverse group of over 30 auxiliary proteins, potentially giving them region-specific characteristics in different brain regions.

2.9. miR-124-3p-AMPA Receptors Interaction Networks

The miR-124-AMPAR pathway, which involves the regulation of AMPA receptors by microRNA-124 (miR-124), has been identified as a neurobiological pathway linking schizophrenia and bipolar disorder. These two mental disorders exhibit significant shared genetic risks and display similar clinical manifestations. However, the specific neurobiological mechanisms that connect these genetic risks to the observed behavioral changes in both conditions were previously unknown.

In a study by Namkung et al. [85], a microRNA–AMPA receptor pathway has been revealed, providing insights into the underlying connection between schizophrenia and bipolar disorder. This pathway suggests that microRNA-124 (miR-124) regulates the expression of AMPA receptors, which play a crucial role in neuronal signalling and synaptic plasticity. Dysregulation of this pathway could contribute to

the shared features and overlapping symptoms observed in schizophrenia and bipolar disorder [85].

Notably, schizophrenia is a complex mental disorder characterized by distinct changes in the hippocampus, which can be observed in both early and advanced stages of the disease. These hippocampal deviations, often accompanied by increased neuronal activity, underscore the significant role of the hippocampal formation in the manifestation of schizophrenia [82]. In addition to schizophrenia, bipolar disorder (formerly manic-depressive illness or manic depression) is another mental illness that causes unusual shifts in a person's mood, energy, activity levels, and concentration. Both conditions share the common risk factor of early life stress (ELS) [83], [84].

2.10. Blood

This study examined the mRNA expression levels of *Gria1, Gria2, Gria3* and *Gria4* genes in the blood to identify potential biomarkers. Peripheral blood may be the most feasible tissue source in clinical assessment of differences in mRNA expression between diseases and drug treatments due to accessibility. Analyzing mRNA expression in peripheral blood poses a persistent challenge. Blood is a complex biological system that encompasses various cell types at different developmental stages. Moreover, blood is known for its high variability in mRNA expression analysis compared to other tissue types. Therefore, the success of a blood microarray study relies heavily on careful selection of cell isolation methods and preparation techniques [86].

2.11. Animal Models in Early-Life Postnatal Maternal Stress Research

BALB/c mice are albino and have pink eyes with white skin hair. They are an inbred strain and are among the five most commonly used inbred strains in biomedical research [87]. The BALB/c mouse strain's susceptibility to stress makes it a valuable model for studying the health implications of chronic stress [88]. In this study, we employed an animal model using BALB/c mice.

To provide a comprehensive understanding of the research findings and emphasize key aspects, **Figure 2.9.** presents a general overview of the study.

Figure 2.9. Overview of the study methodology.

2.11.1. Forced Swim Test and Restraint Stress

The maternal stress procedure in this study incorporated several models, including maternal separation, maternal forced swim test, and maternal restraint stress. Restraint stress is a technique employed to induce physiological responses in animals by limiting their mobility. One notable aspect of restraint stress is that it primarily acts as a psychological stressor, where the physical discomfort resulting from restriction is intentionally secondary to the cognitive evaluation of the organism's inability to move freely. The popularity of this stress-inducing method is due to its relatively low cost and technical requirements. Additionally, restraint stress allows for parametric adjustments, such as altering the duration number of restraint session, number of restraint sessions, intervals between sessions, and manipulating food intake or deprivation before restraint. Furthermore, various methodologies have been developed to cater to the specific needs of researchers from diverse disciplines, including neuroscience [89].

The Forced Swim Test (FST) is a widely used experimental paradigm specifically designed to assess parameters related to depression rather than exercise performance [90]. Porsolt and colleagues developed the FST to measure the effects of antidepressant compounds in rats and mice. In the test, the rodent is placed in an inescapable water container, typically cylindrical. After attempting to escape for a certain period, the rodent eventually adopts a posture of immobility [91].

3. MATERIALS AND METHODS

3.1. Materials and Equipment

3.1.1. Equipment

In this scientific experiment, we employed a comprehensive set of specialized equipment, meticulously selected to meet the precise requirements of our study. This included the utilization of the following instruments:

1. Ultrasonic Tissue Homogenizer (Bandelin SONOPLUS ultrasonic homogenizer HD 2070, Berlin, Germany)
2. Rotor-Gene Q (Qiagen, Germany)
3. Refrigerated Microfuge 20R Centrifuge (Beckman Coulter, United States)
4. T100 Thermal Cycler PCR (Bio-Rad, California, United States)
5. Vortex Mixer (SciLogex, United States)
6. Mini Centrifuge (Isolab, Germany)
7. Cold Block (Qiagen, Germany)
8. Micropipettes (Gilson, France)
9. NanoDrop 2000c (Thermo Fisher Scientific, Massachusetts, United States)
10. -20 °C Freezer (Uğur, Türkiye)
11. -80 °C Freezer (Sanyo, Japan)
12. Autoclave (Nüve, Türkiye)
13. Analytical Balance
14. PowerPac™ Basic Electrophoresis Power Supply (Bio-Rad, California, United States)
15. Owl™ EasyCast™ B2 Mini Gel Electrophoresis Systems (Thermo Fisher Scientific, Massachusetts, United States)
16. Vilber Gel Imaging System (Vilber Lourmat, France)

3.1.2. Materials and Reagents

In this scientific experiment, we also employed a diverse selection of specialized materials and reagents, carefully chosen to fulfill the specific requirements of our study. These encompassed a variety of essential components, such as:

1. Eppendorf tubes 1.5 mL (Eppendorf AG, Germany)
2. PCR tubes 0.2 mL
3. Falcon tubes 50 mL
4. 1 mL insulin syringe
5. Micropipette tips with filter (10-100-1000 µL)
6. Chloroform (Merck, Darmstadt, Germany)
7. Ethanol (Merck, Darmstadt, Germany)
8. Isopropanol (Merck, Darmstadt, Germany)
9. Ethylenediamine tetraacetic acid
10. Nuclease-free water (Lonza, Switzerland)
11. SeaKem® LE Agarose (Lonza, Switzerland)
12. QIAzol Lysis Reagent (Qiagen, Germany)
13. QuantiTect Reverse Transcription Kit (Qiagen, Germany)
14. QuantiNova SYBR Green PCR Kit (Qiagen, Germany)
15. TBE Buffer (Tris-Borate-EDTA) (Thermo Fisher Scientific, Massachusetts, United States)
16. DNA Gel Loading Dye (6X) (Thermo Fisher Scientific, Massachusetts, United States)
17. 50 bp DNA Ladder (BioLabs, United States)
18. Ethidium bromide (Merck, Germany)
19. Sodium hydroxide

3.2. Methods

The molecular analysis, involving RNA isolation, RNA measurement, cDNA synthesis, qPCR amplification, and agarose gel electrophoresis, was conducted at Ankara Yıldırım Beyazıt University's Central Research Laboratory, Application and Research Centre (Merkez Araştırma Laboratuvarı Uygulama ve Araştırma Merkezi). We followed standard laboratory protocols and used validated methods to ensure precise and dependable results.

3.2.1. Mice Welfare and Housing Conditions

In this study, we utilized BALB/c strain mice as the experimental model. These mice were housed at the Ankara University Faculty of Medicine Laboratory for Experimental Animals and Research under appropriate conditions. The housing facilities provided for the mice used in this study met the criteria of a minimum cage area of 180 cm^2 and a minimum cage height of 12 cm, ensuring their needs were adequately met from birth onwards. The mice were maintained in an environment with a 20-40 °C temperature range and relative humidity of 45-70%, considered the most suitable living conditions for their well-being. (Ethical committee approval number: 2023-5-34).

3.2.2. Maternal Stress Procedure

The study commenced with 8-week-old female BALB/c strain mice of the control group (6 females), unpredictable maternal separation (MS) group (6 females), and unpredictable maternal separation combined with unpredictable maternal stress (MSUS) group (6 females) (**Figure 3.1.**).

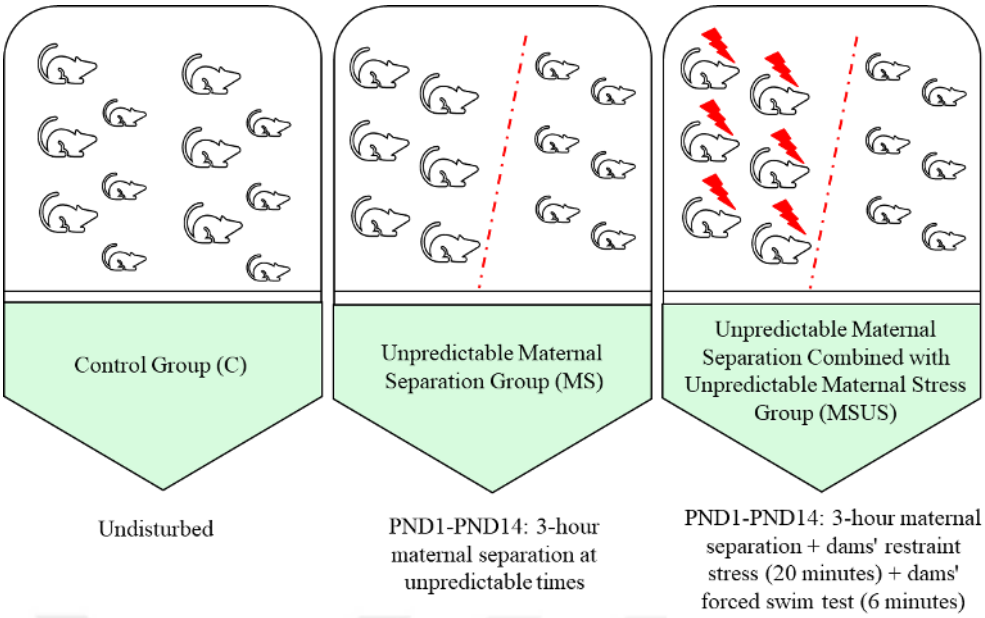

Figure 3.1. Categorization of groups in the study.

Each female mouse was individually housed in a cage and mated with a male mouse. After a few days of mating, the male mice were removed from the cages, leaving the female mice alone throughout the gestation period. The control, MS and MSUS groups and their respective litter were weighed every five days from postnatal day 1 (PND1) to PND35. Cage cleaning was performed once a week for all groups. The control group experienced minimal disturbance, except for cage cleaning and weighing. The MS and MSUS group mice underwent unpredictable maternal separation during early-life postnatal period started from PND1 until PND14 [8], [10]. Feed and water restrictions were not imposed on the mothers during this period. Mice litter continued to consume milk from their mother even during maternal stress.

The maternal separation stress procedure including separated MS and MSUS group dams from the litter and placed the dams in a clean new cage for 3 hours every experiment day at random times. During the 3-hour maternal separation period, specific stress tests were conducted at different intervals.

In the last 20 minutes of separation, the dams in the MSUS group underwent a restraint stress test, which involved being placed inside a Falcon tube (**Figure 3.2.**). A small hole was created for easier breathing by cutting a section of the narrower end of

the Falcon tube used for the MSUS dams. The tubes were then securely held in a holder, ensuring stability and reducing any potential disturbances that could impact the experimental procedure.

The following day, during the last 6 minutes of the 3-hour separation period, the dams from the MSUS group underwent the forced swim test (**Figure 3.3.**). The dams were placed in a beaker filled with cold water (18 °C). The water level in the beaker was approximately three-fourths of its capacity. The dams were forced to swim in the cold water for 6 minutes, adding stressor to their experience.

The pattern continued, with the dams experiencing the restraint stress test for 20 minutes on one day and the forced swim test for 6 minutes the following day during the last portion of the 3-hour separation period.

This daily cycle of stress tests, with restraint stress followed by a forced swim test, was repeated throughout the maternal separation period to induce unpredictable stress in the dams from the MSUS group.

After the sex determination of the litter at PND30, hippocampus and blood samples were collected from a total of 72 litter at PND35, with 12 female and 12 male litter selected from each group. The collected litter samples were then subjected to a mRNA expression study.

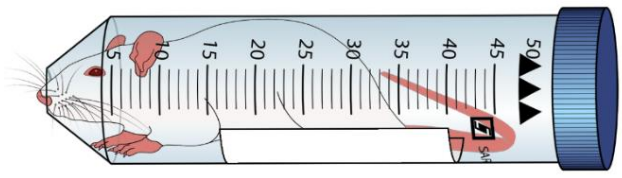

Figure 3.2. Restraint stress test.

Figure 3.3. Forced swim test.

3.2.3. Weight Monitoring

Weight monitoring of dams and litter was performed from postnatal day 1 (PND1) until PND35. Individual weight monitoring for the litter began at PND10. To assign numerical identifiers to the litter, their nails were trimmed accordingly. Specifically, the nail of the fifth digit on the left hind paw was trimmed for the first litter, the nail of the first digit on the right hind paw for the sixth litter, and so on, following a systematic pattern.

3.2.4. Sample Collection from Litter

Collection of Blood Samples

1. At PND35 the mice were euthanized by cervical dislocation following ethical guidelines.
2. Blood samples were collected from mice using the cardiac puncture method. The chest area was gently palpated with the thumb and index finger to identify where the heartbeat could be felt. An aseptic technique was employed, and a 1 mL insulin syringe (previously washed with 0.5 M liquid EDTA) was carefully inserted into the identified region.
3. Approximately 1 mL of blood was collected directly from the heart and promptly transferred into a 500 mL QIAzol Lysis Reagent (Qiagen, Germany).

4. The collected blood sample was vortexed and stored at -80 °C until RNA isolation.

Collection of Hippocampus and Homogenization

1. After blood collection, the entire brain was carefully removed from the head and placed on an ice pack wrapped in aluminium foil.
2. The cerebellum was dissected initially.
3. Subsequently, the brain hemispheres were gently lifted towards the forebrain to remove the hippocampus using fine forceps.
4. To minimize tissue cross-contamination between mice, surgical instruments were cleaned with 75% ethanol during each transition.
5. The obtained hippocampus tissue was placed in an Eppendorf tube containing 500 mL of QIAzol Lysis Reagent (Qiagen, Germany).
6. The tissue was disintegrated using an ultrasonic tissue homogenizer (Bandelin SONOPLUS ultrasonic homogenizer HD 2070, Berlin, Germany)..
7. To ensure proper sample processing, the homogenizer was thoroughly cleaned between each sample by sequentially passing 0.5M Sodium Hydroxide (NaOH), 75% ethanol (twice), autoclaved water, and nuclease-free water through the instrument.
8. The homogenized samples were then stored at -80°C until RNA isolation.

3.2.5. RNA Isolation

The RNA isolation process was conducted using the phenol-chloroform method with QIAzol lysis reagent (Qiagen, Germany), following the provided protocol.

Sample Homogenization and Phase Separation

1. The samples were gradually thawed from -80 ºC to room temperature for RNA isolation.

2. Both the hippocampus and blood samples were vortexed for 15 seconds. Then, 100 µL chloroform was added and vortexed for 15 seconds to ensure thorough mixing of the solution.
3. The samples were incubated at room temperature for 15 minutes.
4. The samples were centrifuged at 12,000 x *g* for 15 minutes at +4 ºC. After centrifugation, a distinct yellowish-whitish interphase band appeared. This band separated the upper aqueous phase, which contains the total RNA, from the lower organic phase.
5. The upper aqueous phase was carefully transferred to a new 1.5 mL Eppendorf tube, and other phases were carefully avoided during the transfer.

RNA Precipitation

6. Isopropanol (500 µL) was added to the aqueous phase. The tube was subjected to repeated up-down movements every 3 minutes during 10-minute incubation at -20 °C.
7. The samples were centrifuged at 12,000 x *g* for 10 minutes at +4 ºC. A white pellet, representing the precipitated RNA, was formed after centrifugation. The size and visibility of the pellet depended on the concentration of RNA. The supernatant was then discarded.

RNA Washes

8. One millilitre (1 mL) of 75% ethanol was added to the pellet, followed by brief vortexing to dislodge the pellet.
9. The tubes were centrifuged at 7,500 x *g* for 5 minutes at +4 ºC. The supernatant was discarded.
10. Steps 8 and 9 were repeated, followed by discarding the supernatant. The tubes were left open at room temperature for 5 minutes to air dry.

RNA Solubilization

11. The pellet of the hippocampus and blood samples were resuspended by adding 50 µL of nuclease-free water (NFW) and placed on a cold block.
12. The purity and quantity of total RNA were measured using NanoDrop 2000c (Thermo Fisher Scientific, Massachusetts, United States).

3.2.6. Nucleic Acid Measurement

The RNA purity and quantity was measured using the NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Massachusetts, United States), according to the manufacturer's protocol as described below:

1. The measuring equipment, including the micropipette and the area around the machine installation, was sterilized using 75% ethanol.
2. The NanoDrop 2000c Spectrophotometer software on the PC connected to the machine was opened.
3. The Nucleic Acid application was selected from the main menu. The wavelength verification window appeared, and it was ensured that the arm was down before clicking OK.
4. The RNA type of the sample to be measured was selected from the "Type" drop-down list.
5. The ng/µL concentration units were chosen from the drop-down list next to the color-coded concentration box.
6. A wavelength of 340 nm was used for bichromate normalization, and the baseline correction box was selected.
7. The "Add to report" checkbox was selected before the measurement to save the sample data to a workbook.
8. The bottom pedestal was cleaned with HCl and distilled water. A volume of 1.5 µL of the nuclease-free water was pipetted onto the bottom pedestal as the blanking solution. The arm was lowered, and the "Blank" button was clicked followed by the measurement. The blank solution typically refers to the buffer in which the molecule of interest is suspended or dissolved.

9. The measurement report was saved to the designated folder. Following the blank measurement, the bottom pedestal was wiped clean using lint-free tissue.
10. The sample ID was entered for the first sample. Prior to pipetting the sample into the bottom pedestal, the bottom of the sample tube was tapped gently to ensure proper mixing. Then, 1.5 µL of the sample was pipetted onto the bottom pedestal, the arm was lowered, and the measurement was initiated. This process was repeated for all the samples. The upper and lower pedestals were wiped using a dry laboratory wipe between each sample. A blank measurement was performed every 30 minutes during the measurement process. After completing the measurements, the pedestal was wiped with distilled water.
11. The RNA concentration of the hippocampus and blood samples was found to be above 1 pg and below 1 µg, indicating that they are suitable for cDNA synthesis.
12. The 260/280 ratio is typically measured to be 2.0 or higher, while the 260/230 ratio is usually in the range of 1.8-2.2. The 260/280 ratio compares the absorbance at 260 nm (associated with nucleic acid absorption) and 280 nm (related to protein absorption). A ratio of approximately 2.0 indicates pure RNA, whereas a lower ratio suggests the presence of protein or other contaminants. The 260/230 ratio compares the absorbance at 260 nm (associated with nucleic acid absorption) and 230 nm (related to contaminant absorption), providing additional information on RNA purity. Higher 260/230 ratios indicate cleaner RNA samples.

3.2.7. Reverse Transcription

The reverse transcription reaction was performed using the Complementary DNA (cDNA) QuantiTect Reverse Transcription Kit (Qiagen, Germany) according to the manufacturer's protocol. A total of 1 µg of total RNA was used for each sample. The reaction was carried out in a thermocycler (Bio-Rad, California, United States) with the program provided in **Table 3.3.** The step-by-step details are presented below:

1. All reactions were set up on a cold block to minimize the risk of RNA degradation.

2. Template RNA was thawed on the cold block. The 7x gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, 5x Quantiscript RT Buffer, RT Primer Mix and RNase-free water were thawed at room temperature (15-25 °C).
3. Each solution was mixed by gently flicking the tubes and briefly centrifuged using a mini centrifuge to collect any residual liquid from the sides of the tubes. The solutions were then stored on the cold block.

Genomic DNA Elimination Reaction

4. Two microliters of 7x gDNA Wipeout Buffer was added to the 0.2 µL PCR tube, followed by addition of 5 µL of template RNA and 7 µL of RNase-free water. The total volume of this reaction was 14 µL (**Table 3.1.**)
5. The tubes were incubated for 2 minutes at 42 °C using the T100 Thermal Cycler PCR (Bio-Rad, California, United States). Then placed immediately on the cold block.

Table 3.1. Genomic DNA elimination reaction components.

Component	Volume/reaction
gDNA Wipeout Buffer, 7x	2 µL
Template RNA	5 µL
RNase-free water	7 µL
Total volume	14 µL

Reverse-transcription Reaction

6. One microlitre of Quantiscript Reverse Transcriptase was added, followed by adding 4 µL of 5x Quantiscript RT Buffer. Then, 1 µL of RT Primer Mix was added (**Table 3.2.**).

Table 3.2. Reverse-transcription reaction components.

Component	Volume/reaction
Quantiscript Reverse Transcriptase	1 µL
Quantiscript RT Buffer, 5x	4 µL
RT Primer Mix	1 µL
gDNA Elimination reaction (Table 1.1.)	14 µL
Total volume	20 µL

7. The tubes were incubated for 15 minutes at 42 °C using T100 Thermal Cycler PCR (Bio-Rad, California, United States).
8. Afterward, they were incubated for 3 minutes at 95 °C to inactivate Quantiscript Reverse Transcriptase.
9. After the reaction was completed, the tubes were stored on a cold block then diluted with nuclease-free water. Any cDNA that was not immediately used in real-time PCR was stored undiluted at -20 °C.
10. The cDNA samples were diluted to a 1:20 ratio and used for further qRT-PCR experiments.

Table 3.3. Thermocycler program for reverse transcription reaction.

Reaction	Temperature	Time
Genomic DNA Elimination Reaction	42 °C	2 minutes
Cold block (outside thermocycler)		-
Reverse-transcription Reaction	42 °C	15 minutes
	95 °C	3 minutes
	4 °C	∞

3.2.8. qRT-PCR for mRNA Quantification

qRT-PCR was performed using Rotor-Gene Q (Qiagen, Germany) and the QuantiNova SYBR Green PCR Kit (Qiagen, Germany). The master mix was prepared according to the specifications provided in **Table 3.4.,** then it was distributed to each tube and placed into Rotor-Gene Q (Qiagen, Germany) and run using program described in **Table 3.5.** The gene-specific primer pairs listed in **Table 3.6.** were used for amplification. The primer pairs used in the qRT-PCR experiments were carefully designed using primer designing software and multiple molecular databases.

To confirm the specificity of the primers, gel electrophoresis was performed as described in section **3.2.8.** (**Agarose Gel Electrophoresis**). The presence of expected product bands at the appropriate positions validated the accuracy of the primers. This result is shown in **Figure 3.4.**

Table 3.4. Master mix composition used for qRT-PCR reaction.

Component	Volume/reaction
2x QuantiNova SYBR Green PCR Master Mix	10 µL
Forward and Reverse Primer Mix (10 µM)	1 µL
Nuclease-free water	5 µL

First, 17 µL of the master mix was added to each tube, and then 4 µL cDNA was added on top of the master mix. The program details of thermocycler are provided in **Table 1.5.** Two reactions were conducted for each sample. *Gapdh* was used as the housekeeping gene.

Table 3.5. Thermocycler program used for qRT-PCR.

Parameters			**Value**					
Run			Three Steps with Melt					
Sample Volume			20 µL					
Thermal cycling profile	Step	Hold	Cycling					Melt
			45 cycles					
			Denaturation	Annealing			Extension	
				Hippocampus		Blood		
				Gapdh	*Gria1-4*	*Gapdh*	*Gria1-4*	
	Time	10 min	15 s	30 s	30 s	30 s	30 s	45 s	90 s (First step) 5 s (next steps)
	Temp (°C)	95	95	60	55	55	55	72	Ramp from 65 °C to 95 °C

Table 3.6. Sequences of forward and reverse primers used for qRT-PCR reaction.

Gene Symbol	Gene ID	Forward Primer	Reverse Primer	Product Length (base pairs)
Gapdh	14433	CTCTCTGCTCCTCCCTGTTC	TACGGCCAAATCCGTTCACA	105 bp
Gria1	14799	CTCCTCATACACAGCCAACC	TTTAGACCTCCTGAAGAACTCCT	151 bp
Gria2	14800	GGATCCTCATTAAGAAATGCGGT	CCCGACAAGGATGTAGAATACTC	189 bp
Gria3	53623	CGATGAAAGTTGGTGGAAATCTG	GCACTGGTCTTGTCCTTACTC	199 bp
Gria4	14802	ATGGAGGAGCAAATGTCACTG	AGAGCAGAAGTGTACTTTGGAG	139 bp

3.2.9. Agarose Gel Electrophoresis

1. A 2% agarose gel was prepared using 1x TBE (Tris-Borate-EDTA) buffer and 2g of agarose powder.
2. To prepare the gel, 2g of agarose powder was measured and added to the desired volume of 1x TBE buffer in a flask.
3. The mixture was gently swirled to ensure the agarose powder was fully dispersed in the buffer.
4. The flask containing the mixture was heated in a microwave, with intermittent stirring, until the agarose completely dissolved.
5. Once the agarose solution cooled down to approximately 60°C, it was carefully poured into the gel casting tray, ensuring no bubbles were trapped.
6. The gel was allowed to solidify at room temperature for about 20-30 minutes.
7. After solidification, the comb was gently removed, and the gel was carefully placed into the electrophoresis tank filled with 1x TBE buffer.
8. The samples, along with the DNA ladder, mixed with 6x DNA Loading dye, were loaded into the wells.
9. The gel was then subjected to electrophoresis at a constant voltage of 120 V.
10. Electrophoresis was terminated once the DNA bands migrated close to the end of the gel.
11. Following electrophoresis, the gel was stained by incubating it in ethidium bromide solution for 15 minutes.
12. The stained gel was visualized under UV light using a UV imaging device to observe and document the DNA bands. The visualized bands are presented in **Figure 3.4.**

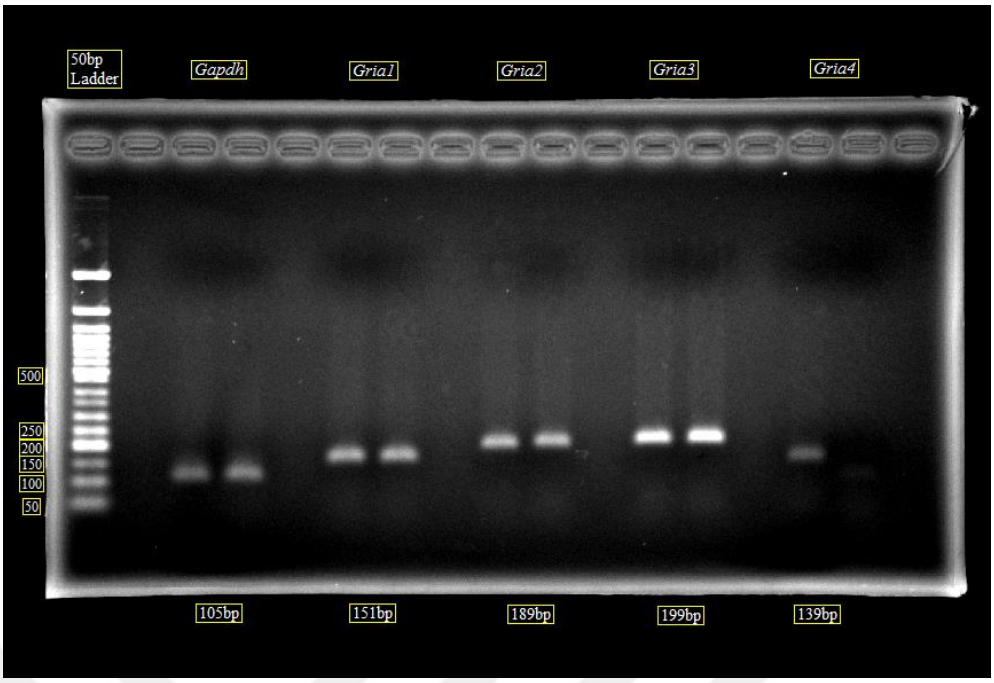

Figure 3.4. The visualization of the bands on the gel.

3.2.10. Dataset Analysis

The Cycle Threshold (Ct) values of the control and stress group were transferred to an Excel format. The average Ct values of the control group were calculated and used as a reference calibrator for data normalization. The data was analysed using the delta-delta Ct ($2^{-\Delta\Delta Ct}$) method (**Figure 3.5.**).

$$2^{-\Delta\Delta Ct} = 2\text{^-}\ ((Ct_{target} - Ct_{ref}) - (Avg.\ Control\ Group\ Ct_{target} - Avg.\ Control\ Group\ Ct_{ref}))$$

Figure 3.5. Equation for delta-delta Ct ($2^{-\Delta\Delta Ct}$) method.

3.2.11. Statistical Analysis and Bioinformatic Analysis

The statistical analysis of the data was performed with GraphPad Prism 9.1.0 (GraphPad Software, California, United States). The distribution of the data was assessed through histogram, q-q plot, and Shapiro-Wilk test. Outliers were identified and excluded based on the approach described by David C. Hoaglin and Boris Iglewicz [92]. Independent t-test and Mann-Whitney U tests were used to compare data between two groups. For more comparisons involving three or more groups, ANOVA and/or Kruskal-Wallis H tests were used to determine the significance of the difference in accordance with distribution of the data, and Tukey HSD and/or Dunn's test were used as post-hoc analysis. The relationship between the data was evaluated by Pearson or Spearman correlation. Chi-square test was used to analyse two independent categorical criteria. Summary statistics provided include count (n), percentage (%), mean, and standard deviation values. Statistical significance was determined at a level of $p < 0.05$. Graphs were generated using GraphPad Prism 9.1.0 software (GraphPad Software, California, United States).

In our study, we specifically mentioned miR-124-3p in relation to the expression of *Gria2*, *Gria3*, and *Gria4* genes. To explore this relationship, we utilized the miRDB and miRBase databases. These databases served as valuable resources for analysing and identifying the specific genes that are targeted and regulated by miRNAs.

4. RESULTS

This study involved female BALB/c mice that were divided into three groups: the control group (n=6), the MS (unpredictable maternal separation) group (n=6), and the MSUS (maternal separation combined with unpredictable maternal stress) group (n=6). Mice were then mated with male mice to initiate pregnancy. After the mice became pregnant and gave birth, the stress procedures were initiated. The unpredictable maternal separation stress was directly exposed to the MS and MSUS group dams and their corresponding litter. However, the unpredictable maternal stress was specifically applied to the MSUS dams and did not directly expose the litter. For the mRNA expression study, only the litter (n=72) were included, with an equal representation of 12 male and 12 female litter per group.

4.1. Phenotypic Differences in Mice Subjected to Maternal Separation Stress

In comparison to the control group, both the MS and MSUS groups of mice showed developmental delays. The following observations were made regarding phenotypic differences in mice exposed to maternal separation stress compared to the control group.

Hair Development

The stress group showed noticeable differences in hair development compared to the control group (**Figure 4.1. A**). The hair structure appeared sparser, and the colour was paler in the stress group mice (**Figure 4.1. B**).

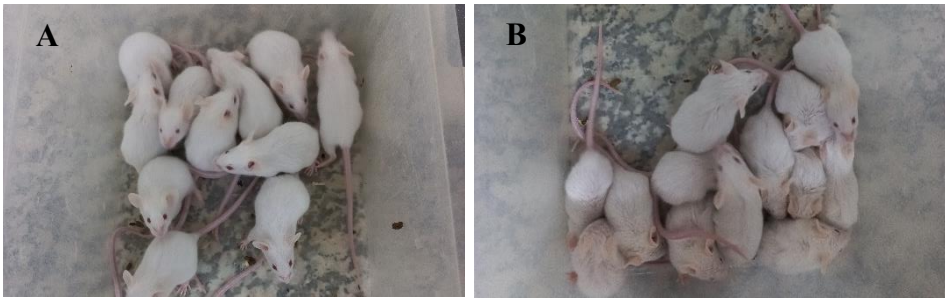

Figure 4.1. A) Control group mice litter B) Stress group mice litter.

Skull Development

Upon sacrifice of the mice on day 35, distinct variations were observed in the skulls of the different groups. The skulls of the control group litter exhibited greater rigidity when cut with scissors, indicating more advanced ossification. In contrast, the MS and MSUS group litter's skulls displayed more elasticity when cut, suggesting incomplete ossification.

4.2. Weight Monitoring Analysis

The weights of the mice litter were measured at specific time points, including postnatal day 1 (PND1), PND5, PND10, PND15, PND20, PND25, PND30, and PND35. To assess the influence of the treatment group and weighing period on the mice litter' weight, a two-way analysis of variance (ANOVA) was conducted. The resulting means and standard deviations of the mice litter' weight can be found in **Table 4.1.** below.

Table 4.1. Descriptive statistics for mice litter weight monitoring result.

Group	Mean	Standard deviations
Control Female	9.574	6.466
MS Female	7.546	4.957
MSUS Female	6.019	3.965
Control Male	9.484	6.828
MS Male	7.566	5.213
MSUS Female	6.348	4.287

Female Litter Weight Result

A two-way ANOVA revealed that there was a statistically significant interaction between the effects of treatment group and weighing period on female litter' weight ($F(14, 264) = 8.391$, $p < 0.0001$) (**Figure 4.2.**). Simple main effects analysis showed that both treatment groups and weighing period did have a statistically significant effect on the weight of the mice litter ($p < 0.0001$, $p < 0.0001$, respectively).

To evaluate the significance of weight differences, post hoc analysis using Tukey's HSD was conducted among the control, MS, and MSUS female litter. The results are presented in the **Table 4.2.**

Table 4.2. Summary of post hoc analysis results for weight differences among different female groups at different time points

Time Point (PND)	Comparison Groups	*p*-value	Weight Change	Comparison Result
1	Control Female vs MS Female	0.9679		No significant difference
	Control Female vs MSUS Female	0.8448		No significant difference
	MS Female vs MSUS Female	0.9484		No significant difference
5	Control Female vs MS Female	0.7434		No significant difference
	Control Female vs MSUS Female	0.3467		No significant difference
	MS Female vs MSUS Female	0.7883		No significant difference
10	Control Female vs MS Female	0.1376		No significant difference
	Control Female vs MSUS Female	0.0026 (**)	↓	MSUS Female < Control Female
	MS Female vs MSUS Female	0.3194		No significant difference
15	Control Female vs MS Female	0.0504		No significant difference
	Control Female vs MSUS Female	<0.0001 (****)	↓	MSUS Female < Control Female

Table 4.2. (Continued)

	MS Female vs MSUS Female	0.0747		No significant difference
20	Control Female vs MS Female	0.0062 (**)	↓	MS Female < Control Female
	Control Female vs MSUS Female	<0.0001 (****)	↓	MSUS Female < Control Female
	MS Female vs MSUS Female	0.0591		No significant difference
25	Control Female vs MS Female	<0.0001 (****)	↓	MS Female < Control Female
	Control Female vs MSUS Female	<0.0001 (****)	↓	MSUS Female < Control Female
	MS Female vs MSUS Female	0.0010 (***)	↓	MSUS Female < MS Female
30	Control Female vs MS Female	<0.0001 (****)	↓	MS Female < Control Female
	Control Female vs MSUS Female	<0.0001 (****)	↓	MSUS Female < Control Female
	MS Female vs MSUS Female	<0.0001 (****)	↓	MSUS Female < MS Female
35	Control Female vs MS Female	<0.0001 (****)	↓	MS Female < Control Female
	Control Female vs MSUS Female	<0.0001 (****)	↓	MSUS Female < Control Female
	MS Female vs MSUS Female	<0.0001 (****)	↓	MSUS Female < MS Female

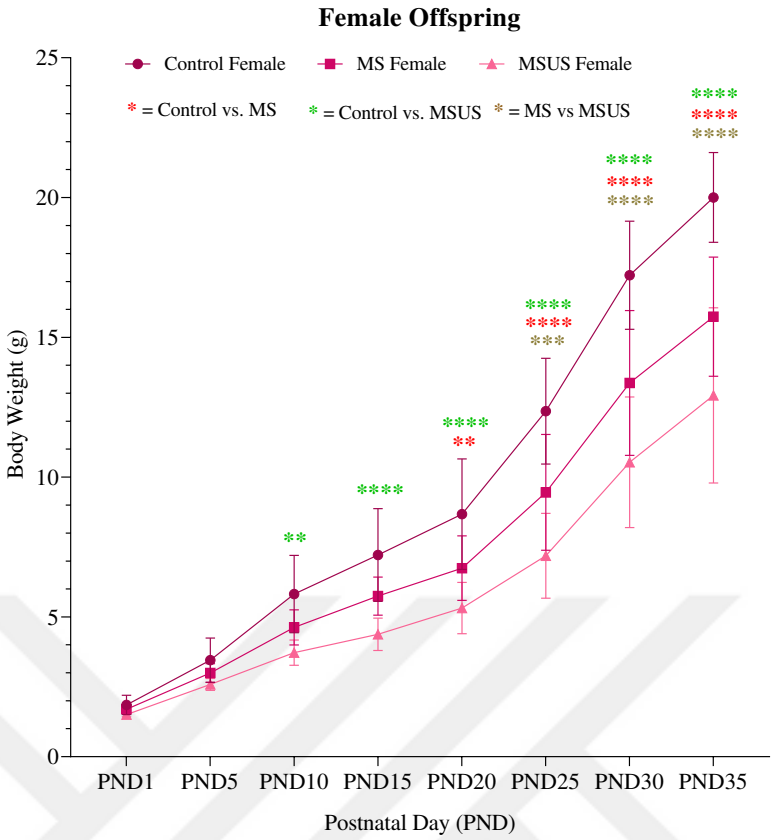

Figure 4.2. Effect of early-life postnatal maternal stress on the body weight of female litter.

Male Litter Weight Result

A two-way ANOVA revealed that there was a statistically significant interaction between the effects of treatment group and weighing period on male litter' weight ($F(14, 264) = 10.70$, $p < 0.0001$) (**Figure 4.3**). Simple main effects analysis showed that both treatment groups and weighing period did have a statistically significant effect on the weight of the mice litter ($p < 0.0001$, $p < 0.0001$, respectively).

To evaluate the significance of weight differences, post hoc analysis using Tukey's HSD was conducted among the control, MS, and MSUS male litter. The results are presented in the **Table 4.3**.

Table 4.3. Summary of post hoc analysis results for weight differences among different male groups at different time points.

Time Point (PND)	Comparison Groups	*p*-value	Weight Change	Comparison Result
1	Control Male vs MS Male	0.9983		No significant difference
	Control Male vs MSUS Male	0.9224		No significant difference
	MS Male vs MSUS Male	0.9427		No significant difference
5	Control Male vs MS Male	0.8445		No significant difference
	Control Male vs MSUS Male	0.3970		No significant difference
	MS Male vs MSUS Male	0.7369		No significant difference
10	Control Male vs MS Male	0.1686		No significant difference
	Control Male vs MSUS Male	0.0129 (*)	↓	MSUS Male < Control Male
	MS Male vs MSUS Male	0.7369		No significant difference
15	Control Male vs MS Male	0.0641		No significant difference
	Control Male vs MSUS Male	0.0003 (***)	↓	MSUS Male < Control Male
	MS Male vs MSUS Male	0.2151		No significant difference
20	Control Male vs MS Male	0.0098 (**)	↓	MS Male < Control Male
	Control Male vs MSUS Male	<0.0001 (****)	↓	MSUS Male < Control Male
	MS Male vs MSUS Male	0.2775		No significant difference
25	Control Male vs MS Male	<0.0001 (****)	↓	MS Male < Control Male
	Control Male vs MSUS Male	<0.0001 (****)	↓	MSUS Male < Control Male
	MS Male vs MSUS Male	0.0078 (**)	↓	MSUS Male < MS Male
30	Control Male vs MS Male	<0.0001 (****)	↓	MS Male < Control Male
	Control Male vs MSUS Male	<0.0001 (****)	↓	MSUS Male < Control Male

Table 4.3. (Continued)

	MS Male vs MSUS Male	0.0001 (***)	↓	MSUS Male < MS Male
35	Control Male vs MS Male	<0.0001 (****)	↓	MS Male < Control Male
	Control Male vs MSUS Male	<0.0001 (****)	↓	MSUS Male < Control Male
	MS Male vs MSUS Male	<0.0001 (****)	↓	MSUS Male < MS Male

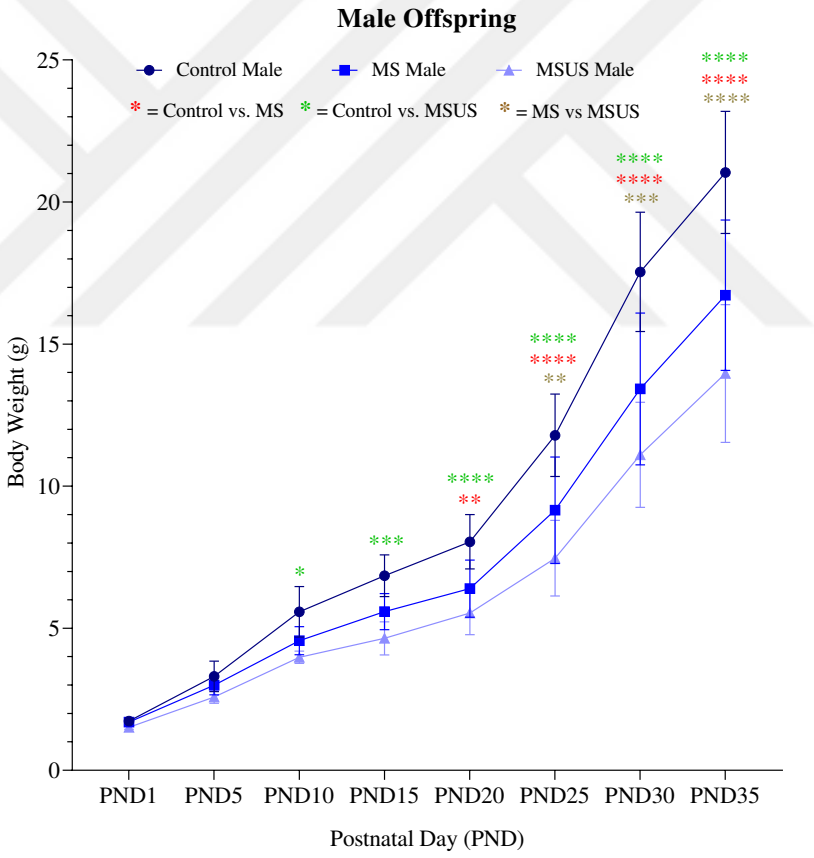

Figure 4.3. Effect of early-life postnatal maternal stress on the body weight of male litter.

4.3. mRNA Expression Analysis Results

4.3.1. mRNA Expression Analysis Results of Hippocampal Samples

Regarding the results of the mRNA expression analysis, the values were normalized to housekeeping gene *Gapdh*. A one-way ANOVA was conducted to compare *Gria1* mRNA expression levels among three groups: Control female, MS female and MSUS female groups. The ANOVA revealed a significant main effect of group on mRNA expression levels ($F(2,33) = 7.024$, $p = 0.0029$, **). Tukey's multiple comparison test showed that the MS female group had significantly lower mRNA expression levels of the *Gria1* gene compared to the Control female group ($p = 0.0021$, **), while there were no significant differences between the other groups (**Figure 4.4. A**).

Similarly, a one-way ANOVA was conducted to assess the differences in *Gria1* mRNA expression levels among three groups: Control male, MS male and MSUS male groups. The ANOVA summary revealed that there was no significant main effect of group on mRNA expression levels ($F(2,33) = 1.723$, $p = 0.1941$). Tukey's multiple comparison test was used to further investigate the differences between the groups, and it was found that there were no significant differences between any of the groups (**Figure 4.4. B)**.

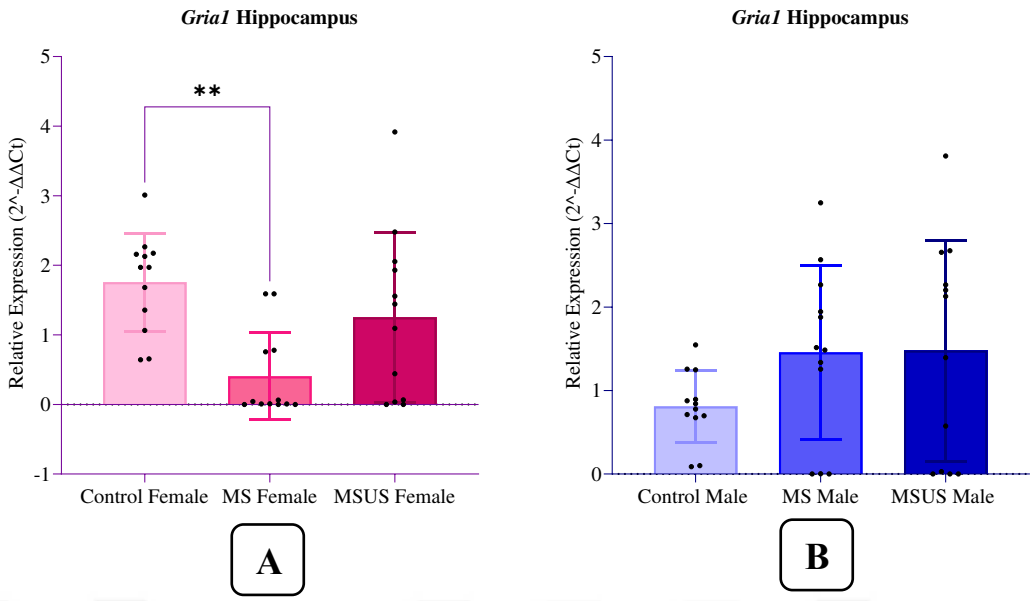

Figure 4.4. Bar Graph showing the quantification of *Gria1* expression levels in the female (A) and male (B) hippocampus after exposure to maternal stress compared to non-stressed mice

Table 4.4. Details of *Gria1* mRNA expression in the female hippocampus; sample size and normalized expression information.

Group	Number of Litter	Minimum-Maximum (Mean ± S.D)
MS	12	0.00 - 1.59 (0.40 ± 0.62)
MSUS	12	0.00 - 3.92 (1.25 ±1.23)
Control	12	0.64 - 3.01 (1.76 ± 0.71)

Table 4.5. Details of *Gria1* mRNA expression in the male hippocampus; sample size and normalized expression information.

Group	Number of Litter	Minimum-Maximum (Mean ± S.D)
MS	12	0.00 - 3.25 (1.46 ± 1.04)
MSUS	12	0.00 - 3.81 (1.48 ± 1.32)
Control	12	0.09 - 1.55 (0.81 ± 0.43)

In this study, we also investigated the mRNA expression result of the *Gria2* gene. The Kruskal-Wallis test was used to compare the median values of three groups. The H statistic was 19.37, p <0.0001, indicating that there was a significant difference in *Gria2* mRNA expression levels between at least two groups. Post-hoc analysis using Dunn's multiple comparison test revealed that the MSUS female group had significantly lower median values than Control female group ($p < 0.0001$), but there was no significant difference between the other groups (**Figure 4.5. A**).

Furthermore, a one-way ANOVA was performed to compare *Gria2* mRNA expression levels among three groups: Control male, MS male and MSUS male groups. The ANOVA revealed a significant main effect of group on mRNA expression levels ($F(2, 28) = 16.77$, $p < 0.0001$, ****). Tukey's multiple comparison test showed that the MS male and MSUS male group had significantly lower mRNA expression levels of the *Gria2* gene compared to the Control male group ($p = 0.0001$, ***, p <0.0001, ****, respectively) (**Figure 4.5. B**).

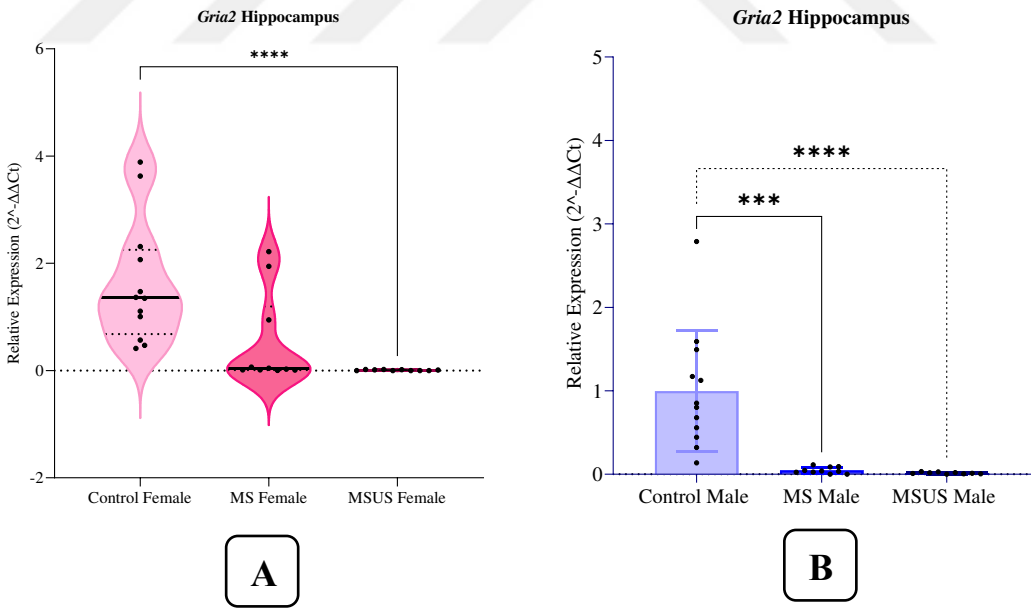

Figure 4.5. Violin plot showing the quantification of *Gria2* expression levels in the female hippocampus (A), and a bar graph depicting the *Gria2* expression levels in the male hippocampus (B) after exposure to maternal stress, compared to non-stressed mice.

Table 4.6. Details of *Gria2* mRNA expression in the female hippocampus; sample size and normalized expression information.

Group	Number of Litter	Minimum-Maximum (Median)
MS	10	0.00 - 2.22 (0.04)
MSUS	10	0.00 - 0.02 (0.00)
Control	12	0.41 - 3.89 (1.36)

Table 4.7. Details of *Gria2* mRNA expression in the male hippocampus; sample size and normalized expression information.

Group	Number of Litter	Minimum-Maximum (Mean ± S.D)
MS	10	0.00 - 0.11 (0.05 ± 0.04)
MSUS	9	0.00 - 0.03 (0.01 ± 0.01)
Control	12	0.14 - 2.79 (0.10 ± 0.72)

In addition, a Mann-Whitney test was performed to compare *Gria2* mRNA expression levels between the Control female and MS female groups. There was a significant difference in *Gria2* mRNA expression levels between the Control female (mdn =1.356, 0.6793 – 2.252) and MS Female (mdn = 0.0354, 0.01163 – 1.196) groups (U = 20, p = 0.0071, **) (**Figure 4.6.**).

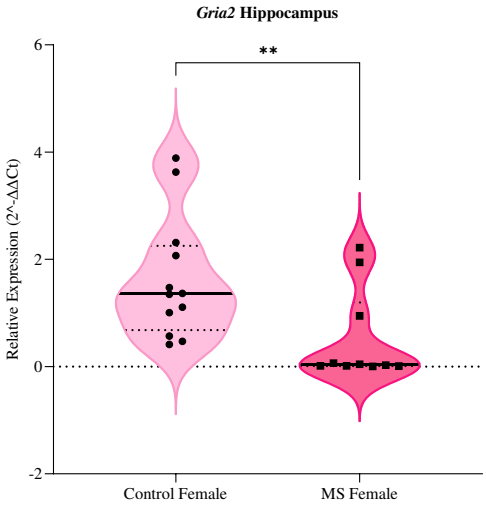

Figure 4.6. Violin plot showing the quantification of *Gria2* expression levels in the female hippocampus after exposure to maternal stress compared to non-stressed female mice.

Continuing our investigation, we further examined the mRNA expression level of the *Gria3* gene. We conducted a one-way ANOVA to compare *Gria3* mRNA expression levels among three groups: Control female, MS female and MSUS female groups. The ANOVA revealed a significant main effect of group on mRNA expression levels ($F(2,32) = 6.425$, $p = 0.0045$, **). Tukey's multiple comparison test showed that the MSUS female group had significantly lower mRNA expression levels of the *Gria3* gene compared to the Control female group ($p = 0.0033$, **), while there were no significant differences between the other groups (**Figure 4.7. A)**.

Similarly, in the male group, a one-way ANOVA was conducted to compare *Gria3* mRNA expression levels among three groups: Control male, MS male and MSUS male groups. The ANOVA revealed a significant main effect of group on mRNA expression levels ($F(2, 33) = 6.244$, $p = 0.0050$, **). Tukey's multiple comparison test showed that the MS male and MSUS male group had significantly lower mRNA expression levels of the *Gria3* gene compared to the Control male group (P = 0.0087, **, $p = 0.0166$, *, respectively) (**Figure 4.7. B**).

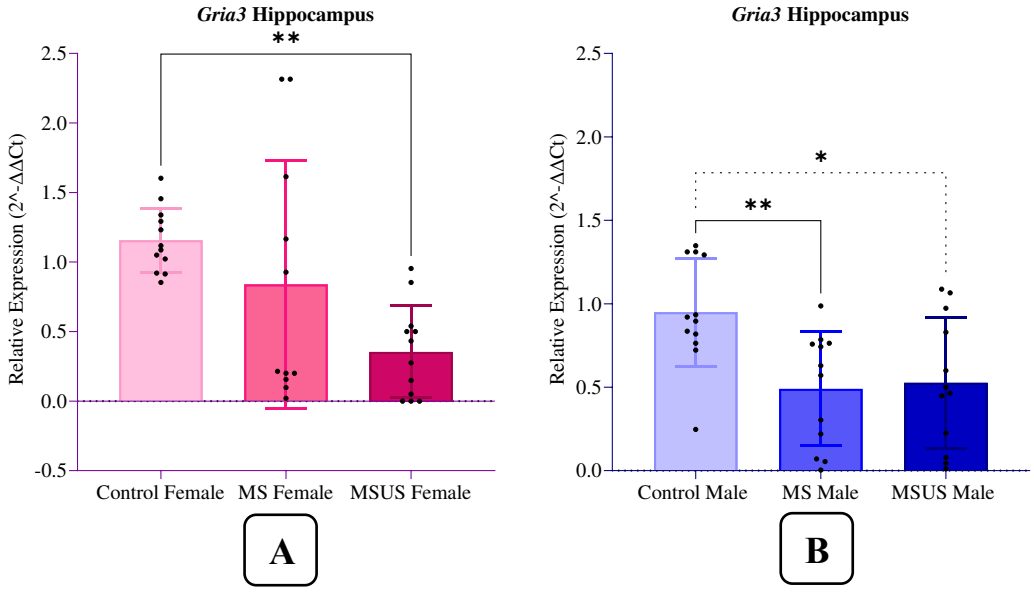

Figure 4.7. Bar Graph showing the quantification of *Gria3* expression levels in the female (A) and male (B) hippocampus after exposure to maternal stress compared to non-stressed mice.

Table 4.8. Details of *Gria3* mRNA expression in the female hippocampus; sample size and normalized expression information.

Group	Number of Litter	Minimum-Maximum (Mean ± S.D)
MS	11	0.02 - 2.32 (0.84 ± 0.89)
MSUS	12	0.00 - 0.95 (0.35 ± 0.33)
Control	12	0.85 - 1.60 (1.16 ± 0.23)

Table 4.9. Details of *Gria3* mRNA expression in the male hippocampus; sample size and normalized expression information.

Group	Number of Litter	Minimum-Maximum (Mean ± S.D)
MS	12	0.00 - 0.99 (0.49 ± 0.34)
MSUS	12	0.01 - 1.09 (0.53 ± 0.39)
Control	12	0.25 - 1.35 (0.95 ± 0.32)

Next, we examined the mRNA expression levels of the *Gria4* gene. For the female groups, the Kruskal-Wallis test was used to compare the median values among the three groups: Control female, MS female and MSUS female. The H statistic was 0.4604, $p = 0.7944$, indicating that there was no significant difference in *Gria4* mRNA expression levels between at least two groups. Post-hoc analysis using Dunn's multiple comparison test revealed that there was no significant difference between groups (**Figure 4.8. A)**.

On the other hand, for male groups, the Kruskal-Wallis test was used to compare the median values of three male groups. The H statistic was 9.376, $p = 0.0092$, indicating that there was a significant difference in *Gria4* mRNA expression levels between at least two groups. Post-hoc analysis using Dunn's multiple comparison test revealed that the MSUS male group had significantly higher median values than Control male group ($p = 0.0094$), but there was no significant difference between the other groups (**Figure 4.8. B**).

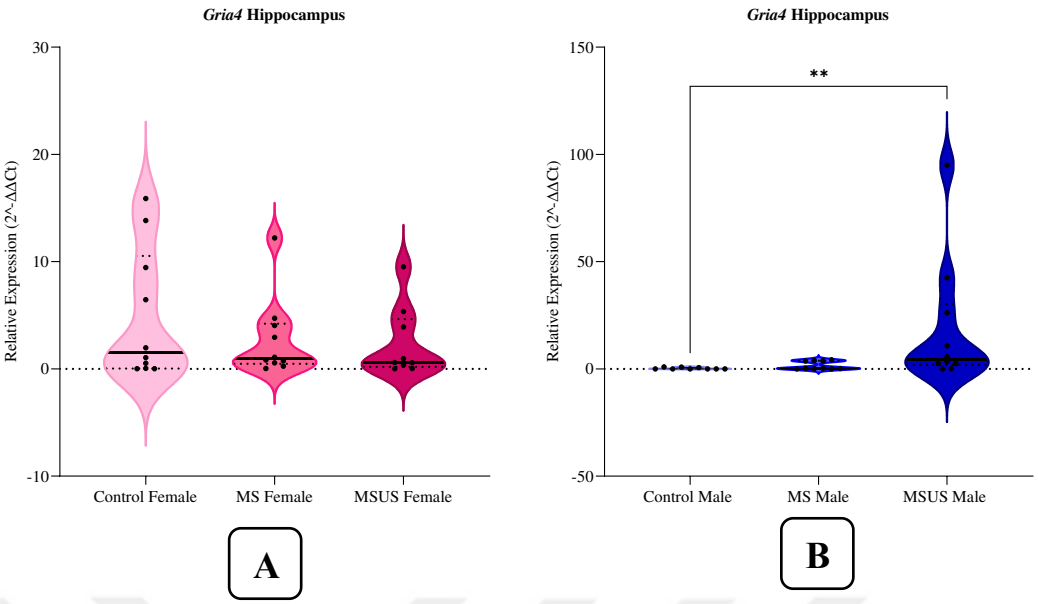

Figure 4.8. Violin plots showing the quantification of Gria4 expression levels in the female (A) and male (B) hippocampus after exposure to maternal stress compared to non-stressed mice.

Table 4.10. Details of *Gria4* mRNA expression in the female hippocampus; sample size and normalized expression information.

Group	Number of Litter	Minimum-Maximum (Median)
MS	10	0.03 - 12.21 (0.95)
MSUS	9	0.01 - 9.51 (0.60)
Control	10	0.01 - 15.88 (1.51)

Table 4.11. Details of *Gria4* mRNA expression in the male hippocampus; sample size and normalized expression information.

Group	Number of Litter	Minimum-Maximum (Median)
MS	10	0.02 - 4.20 (0.30)
MSUS	10	0.02 - 94.98 (4.53)
Control	9	0.01 - 0.89 (0.03)

4.3.2. mRNA Expression Analysis Results of Blood Samples

For the female groups, the Kruskal-Wallis test was used to compare the median values of three female groups. The H statistic was 8.976, $p = 0.0112$, indicating that there was a significant difference in *Gria1* mRNA expression levels between at least two groups. Post-hoc analysis using Dunn's multiple comparison test revealed that the MSUS female group had significantly higher median values than Control female group ($p < 0.0001$), but there was no significant difference between the other groups (**Figure 4.9. A)**.

Similarly, for the male groups, the Kruskal-Wallis test was used to compare the median values of three male groups. The H statistic was 3.716, $p = 0.1560$, indicating that there was no significant difference in *Gria1* mRNA expression levels between at least two groups. Post-hoc analysis using Dunn's multiple comparison test revealed that there was no significant difference between groups (**Figure 4.9. B**).

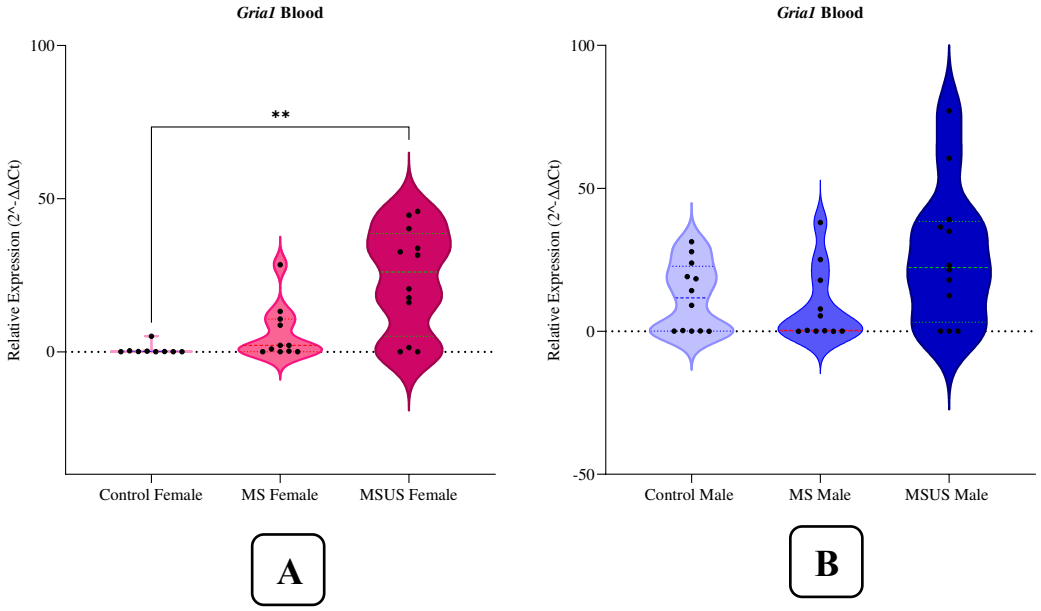

Figure 4.9. Violin plots showing the quantification of *Gria1* expression levels in the female (A) and male (B) blood after exposure to maternal stress compared to non-stressed mice.

Table 4.12. Details of *Gria1* mRNA expression in the female blood; sample size and normalized expression information.

Group	Number of Litter	Minimum-Maximum (Median)
MS	11	0.04 - 28.43 (2.10)
MSUS	12	0.02 - 45.86 (26.03)
Control	9	0.04 - 5.10 (0.07)

Table 4.13. Details of *Gria1* mRNA expression in the male blood; sample size and normalized expression information.

Group	Number of Litter	Minimum-Maximum (Median)
MS	11	0.03 - 38.03 (0.30)
MSUS	12	0.04 - 77.13 (22.32)
Control	12	0.03 - 31.32 (11.67)

We investigated the *Gria2* mRNA expression in blood samples. The Kruskal-Wallis test was used to compare the median values of three female groups. The H statistic was 5.706, $p = 0.0577$, indicating that there was no significant difference in *Gria2* mRNA expression levels between at least two groups. Post-hoc analysis using Dunn's multiple comparison test revealed that there was no significant difference between groups (**Figure 4.10. A**).

Similarly, for the male groups, the Kruskal-Wallis test was used to compare the median values of three groups. The H statistic was 5.311, $p = 0.0703$, indicating that there was no significant difference in *Gria2* mRNA expression levels between at least two groups. Post-hoc analysis using Dunn's multiple comparison test revealed that there was no significant difference between groups (**Figure 4.10. B**).

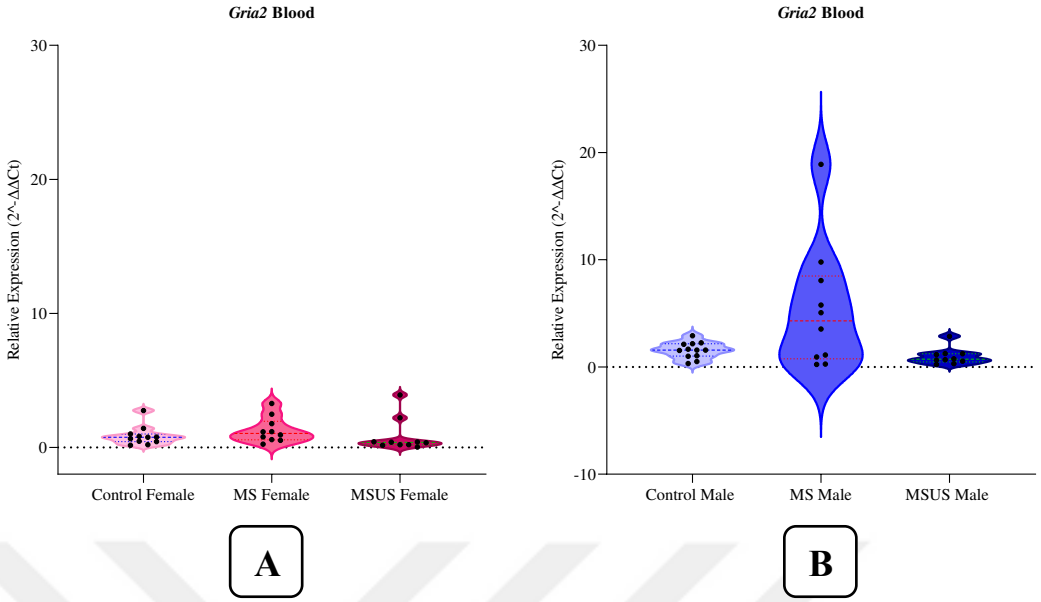

Figure 4.10. Violin plots showing the quantification of *Gria2* expression levels in the female (A) and male (B) blood after exposure to maternal stress compared to non-stressed mice.

Table 4.14. Details of *Gria2* mRNA expression in the female blood; sample size and normalized expression information.

Group	Number of Litter	Minimum-Maximum (Median)
MS	10	0.24 - 3.27 (1.05)
MSUS	10	0.03 - 3.92 (0.38)
Control	11	0.16 - 2.75 (0.76)

Table 4.15. Details of *Gria2* mRNA expression in the male blood; sample size and normalized expression information.

Group	Number of Litter	Minimum-Maximum (Median)
MS	10	0.24 - 18.91 (4.30)
MSUS	10	0.23 - 2.87 (0.72)
Control	12	0.32 - 2.91 (1.58)

Next, the *Gria3* mRNA expression levels in blood samples were investigated. For the female groups, the Kruskal-Wallis test was used to compare the median values of three groups. The H statistic was 2.583, $p = 0.2748$, indicating that there was no significant difference in *Gria3* mRNA expression levels between at least two groups. Post-hoc analysis using Dunn's multiple comparison test revealed that there was no significant difference between groups (**Figure 4.11. A**).

Similarly, for male groups, the Kruskal-Wallis test was used to compare the median values of three groups. The H statistic was 3.758, $p = 0.1527$, indicating that there was no significant difference in *Gria3* mRNA expression levels between at least two groups. Post-hoc analysis using Dunn's multiple comparison test revealed that there was no significant difference between groups (**Figure 4.11. B**).

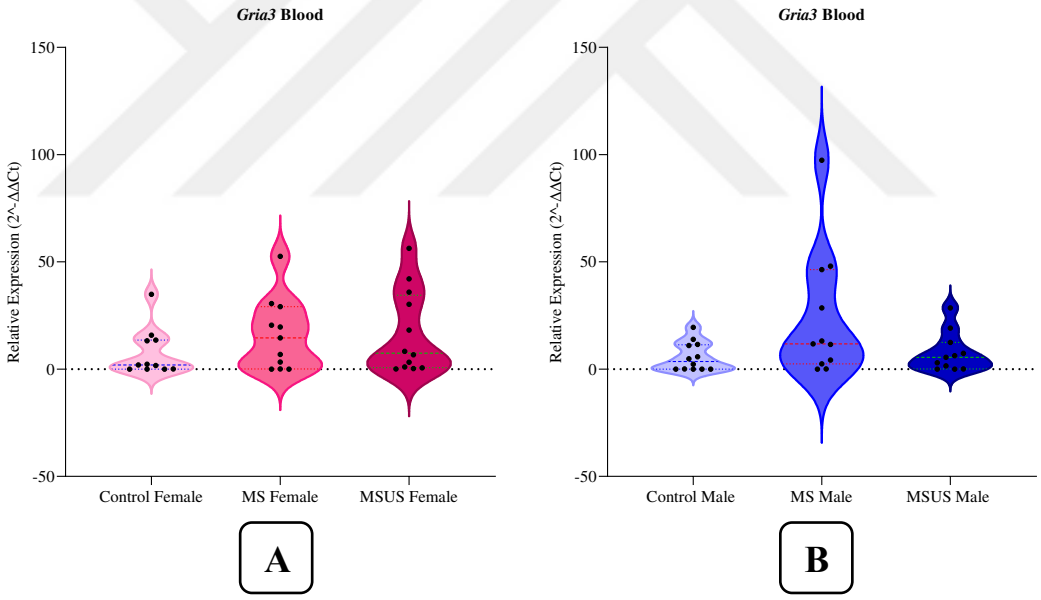

Figure 4.11. Violin plots showing the quantification of *Gria3* expression levels in the female (A) and male (B) blood after exposure to maternal stress compared to non-stressed mice.

Table 4.16. Details of *Gria3* mRNA expression in the female blood; sample size and normalized expression information.

Group	Number of Litter	Minimum-Maximum (Median)
MS	11	0.01 - 56.33 (14.58)
MSUS	12	0.05 - 56.33 (7.48)
Control	11	0.01 - 34.92 (1.97)

Table 4.17. Details of *Gria3* mRNA expression in the male blood; sample size and normalized expression information.

Group	Number of Litter	Minimum-Maximum (Median)
MS	11	0.03 - 97.40 (11.76)
MSUS	11	0.02 - 28.56 (5.52)
Control	12	0.02 - 19.51 (3.56)

Lastly, we focused our analysis on the mRNA expression levels of the *Gria4* gene in the blood samples. For the female groups, a one-way ANOVA was conducted to compare *Gria4* mRNA expression levels among three groups: Control female, MS female and MSUS female groups. The ANOVA revealed a significant main effect of group on mRNA expression levels ($F(2, 28) = 4.309$, $p = 0.0234$, *). Tukey's multiple comparison test showed that the MSUS female group had significantly lower mRNA expression levels of the *Gria4* gene compared to the MS female group ($p = 0.0176$, *), while there were no significant differences between the other groups (**Figure 4.12. A)**.

Similarly, for the male groups, a one-way ANOVA was conducted to compare *Gria4* mRNA expression levels among three groups: Control male, MS male and MSUS male groups. The ANOVA revealed a significant main effect of group on mRNA expression levels ($F(2, 30) = 4.372$, $p = 0.0216$, *). Tukey's multiple comparison test showed that the MSUS male group had significantly lower mRNA expression levels of the *Gria4* gene compared to the Control male group ($p = 0.0161$, *), while there were no significant differences between the other groups (**Figure 4. 12. B)**.

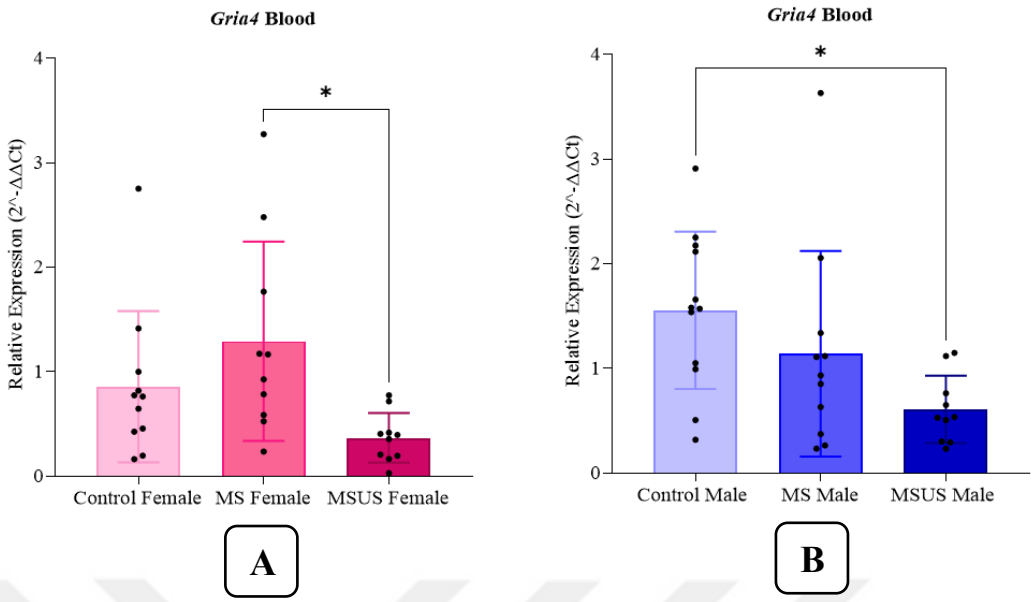

Figure 4.12. Bar Graph showing the quantification of *Gria4* expression levels in the female (A) and male (B) blood after exposure to maternal stress compared to non-stressed mice.

Table 4.18. Details of *Gria4* mRNA expression in the female blood; sample size and normalized expression information.

Group	Number of Litter	Minimum-Maximum (Mean ± S.D)
MS	10	0.24 - 3.27 (1.29 ± 0.95)
MSUS	10	0.03 - 0.77 (0.37 ± 0.24)
Control	11	0.16 - 2.75 (0.86 ± 0.72)

Table 4.19. Details of *Gria4* mRNA expression in the male blood; sample size and normalized expression information.

Group	Number of Litter	Minimum-Maximum (Mean ± S.D)
MS	11	0.24 - 3.63 (1.14 ± 0.98)
MSUS	10	0.23 - 1.15 (0.61 ± 0.32)
Control	12	0.32 - 2.91 (1.56 ± 0.75)

4.3.3. Expression Correlation Analysis Results

The Spearman correlation test was performed for every mRNA expression between the hippocampus and blood samples. The results of these tests are presented in **Figures 4.13**, **4.14**, **4.15**, and **4.16**. Correlation strength for each mRNA expression is interpreted according to **Table 4.20**.

Table 4.20. Interpretation table of Spearman Rank-Order Correlation Coefficients[93].

Spearman ρ	Correlation
0.90 to 1.00 (-0.90 to -1.00)	Very high positive (negative) correlation
0.70 to 0.90 (−0.70 to −0.90)	High positive (negative) correlation
0.50 to 0.70 (−0.50 to −0.70)	Moderate positive (negative) correlation
0.30 to 0.50 (−0.30 to −0.50)	Low positive (negative) correlation
0.00 to 0.30 (0.00 to −0.30)	Negligible correlation

Correlation of *Gria1-4* in Hippocampus and Blood

Spearman's rank correlation was computed to assess the relationship between hippocampus *Gria1* expression and blood *Gria1* expression. There was no correlation between the two variables both in female and male group (r = -0.2986, p = 0.0970; r = -0.1791, p = 0.3032, respectively) (**Figure 4.13.**).

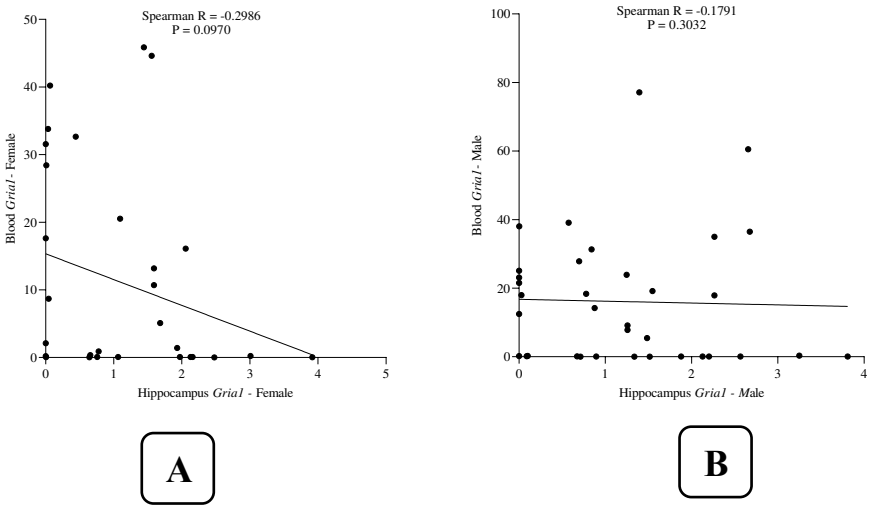

Figure 4.13. Expression correlation between hippocampus *Gria1* and blood *Gria1* both in (A) female groups and (B) male groups.

Spearman's rank correlation was computed to assess the relationship between hippocampus *Gria2* expression and blood *Gria2* expression. There was no correlation between the two variables both in female and male group (r = 0.1581, p = 0.4126; r = 0.0744, p = 0.7066, respectively) (**Figure 4.14.**).

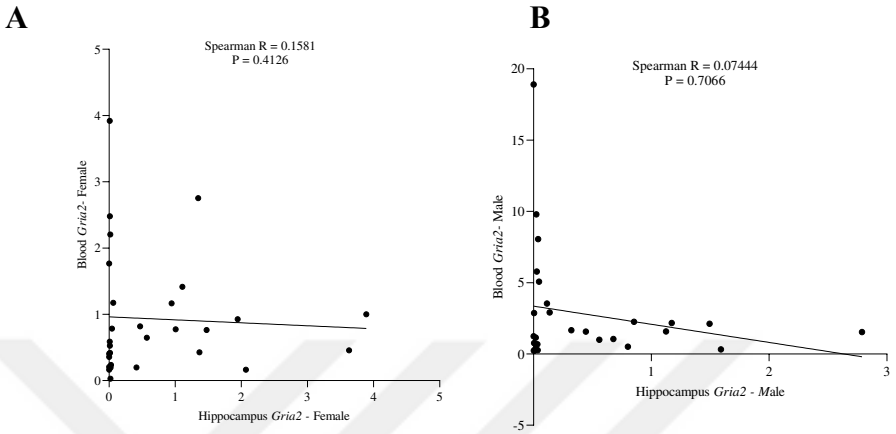

Figure 4.14. Expression correlation between hippocampus *Gria2* and blood *Gria2* both in (A) female groups and (B) male groups.

Spearman's rank correlation was computed to assess the relationship between hippocampus *Gria3* expression and blood *Gria3* expression. There was no correlation between the two variables both in female and male group (r = 0.0033, p = 0.9853; r = -0.1796, p = 0.3095, respectively) (**Figure 4.15.**).

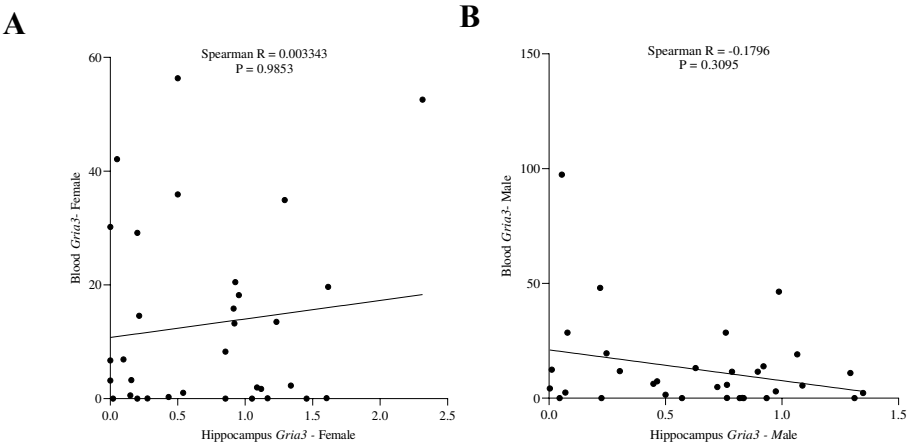

Figure 4.15. Expression correlation between hippocampus *Gria3* and blood Gria3 both in (A) female groups and (B) male groups.

Spearman's rank correlation was computed to assess the relationship between hippocampus *Gria4* expression and blood *Gria4* expression. There was no correlation between the two variables both in female and male group (r = -0.1210, p = 0.5558; r = -0.3466, p = 0.0766, respectively) (**Figure 4.16.**).

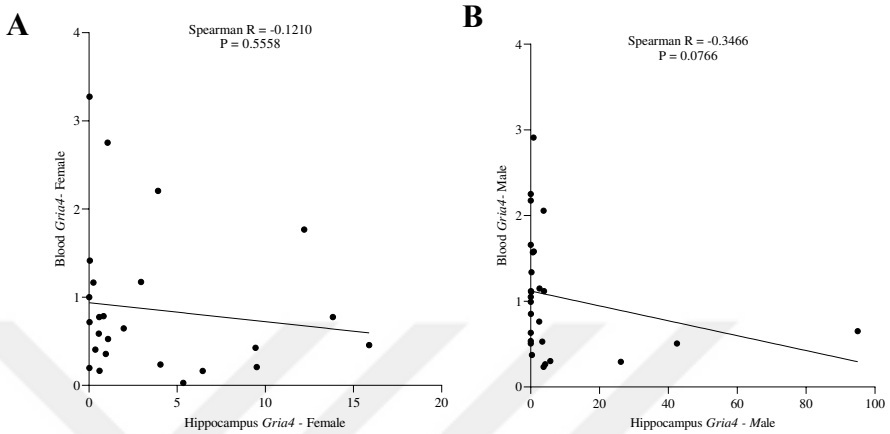

Figure 4.16. Expression correlation between hippocampus *Gria4* and blood *Gria4* both in (A) female groups and (B) male groups.

Correlation of Hippocampus *Gria1-4* expression and Hippocampus miR-124-3p expression (Unpublished Data)

A correlation analysis was conducted to examine potential regulatory relationships between hippocampus *Gria1*, *Gria2*, *Gria3*, and *Gria4* expression levels and hippocampus miR-124-3p expression levels. The expression levels of *Gria1-4* mRNA and miR-124-3p were analysed in order to identify any possible connections, although the miRNA data is not included in this thesis.

This analysis aimed to provide insights into potential regulatory interactions, although the primary focus of this study remains on *Gria1-4* mRNA expression. Due to this focus, the miRNA data will not be shown or discussed extensively. However, the correlation analysis lays the groundwork for future investigations into the intricate regulatory networks involving *Gria1-4* genes and miR-124-3p.

Spearman's rank correlation was computed to assess the relationship between hippocampus *Gria1* expression and hippocampus miR-124-3p expression. The results of these tests are presented in **Figures 4.17**, **4.18**, **4.19**, and **4.20**. Correlation strength for each mRNA expression is interpreted according to **Table 4.20**. There was no correlation between the two variables both in female and male group (r = -0.2410, p = 0.1568; r = 0.1599, p = 0.3589, respectively) (**Figure 4.17.**).

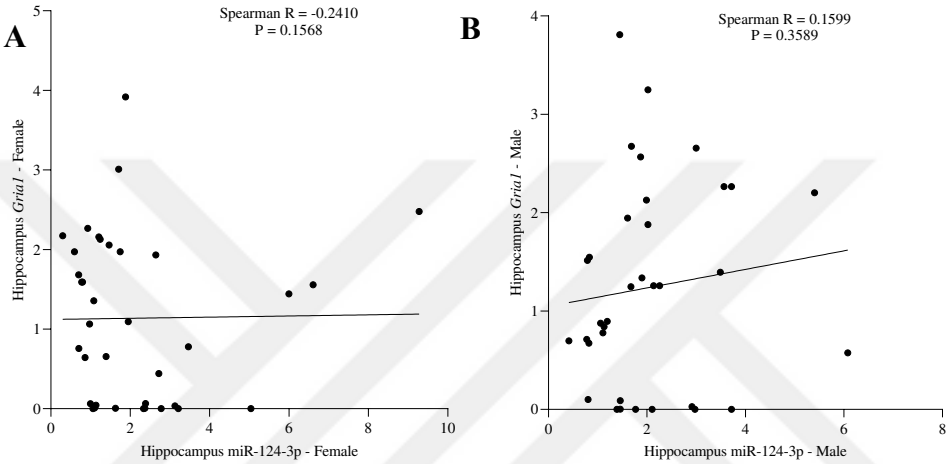

Figure 4.17. Expression correlation between hippocampus *Gria1* and hippocampus miR-124-3p both in (A) female groups and (B) male groups

Spearman's rank correlation was computed to assess the relationship between hippocampus *Gria2* expression and hippocampus miR-124-3p expression. There was a moderate negative correlation between the two variables both in female and male group (r = -0.5952, p = 0.0003; r = -0.6365, p = 0.0002, respectively) (**Figure 4.18.**).

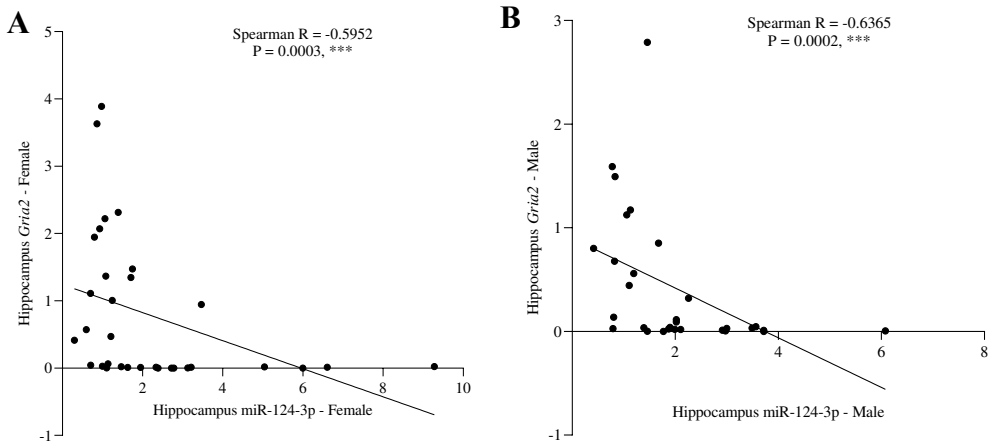

Figure 4.18. Expression correlation between hippocampus *Gria2* and hippocampus miR-124-3p both in (A) female groups and (B) male groups.

Spearman's rank correlation was computed to assess the relationship between hippocampus *Gria3* expression and hippocampus miR-124-3p expression. There was a low or moderate negative correlation between the two variables both in female and male group (r = -0.4901, p = 0.0028; r = -0.3014, p = 0.0785, respectively) (**Figure 4.19.**).

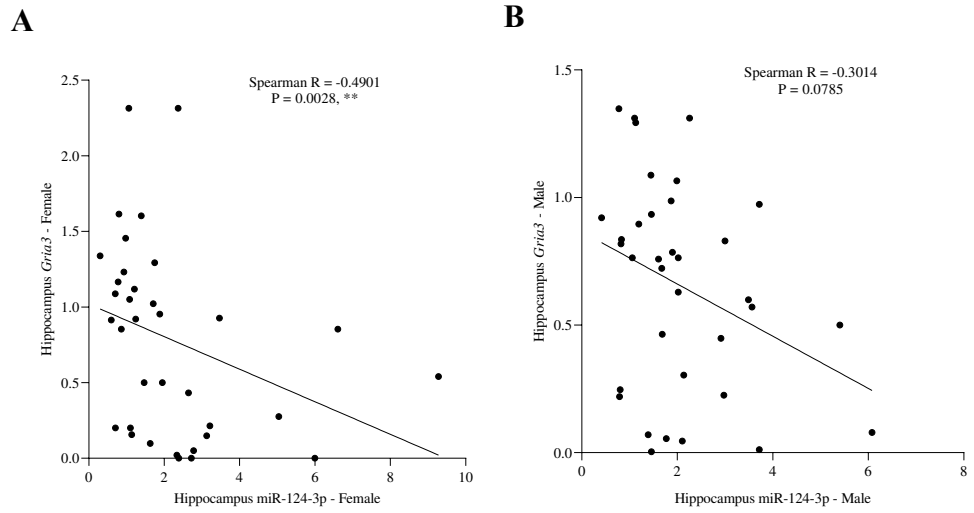

Figure 4.19. Expression correlation between hippocampus *Gria3* and hippocampus miR-124-3p both in (A) female groups and (B) male groups.

Spearman's rank correlation was computed to assess the relationship between hippocampus *Gria4* expression and hippocampus miR-124-3p expression. There was no correlation between the two variables both in female and male group (r = -0.1099, p = 0.5705; r = 0.1489, p = 0.4496, respectively) (**Figure 4.20.**).

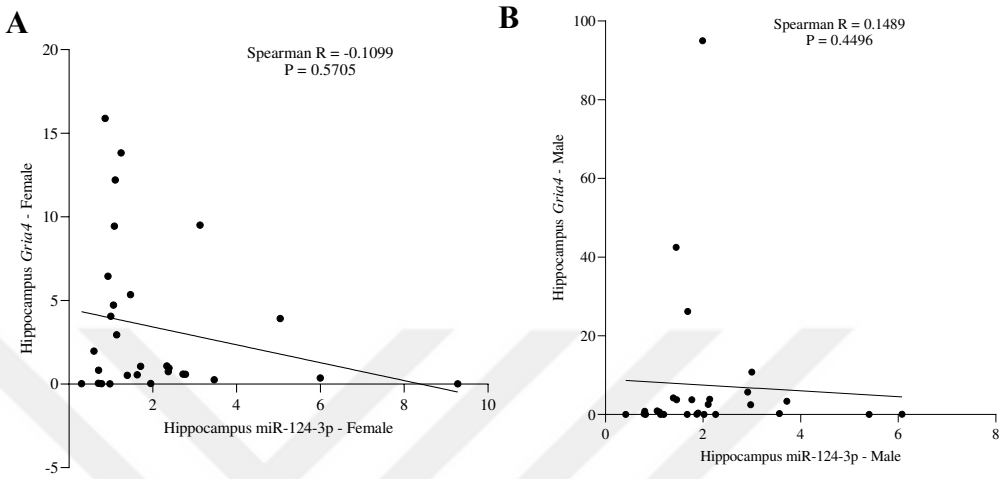

Figure 4.20. Expression correlation between hippocampus *Gria4* and hippocampus miR-124-3p both in (A) female groups and (B) male groups.

Correlation Analysis Among *Gria1, Gria2, Gria3* and *Gria4* in Hippocampus

Spearman's rank correlation was computed to assess the relationship among *Gria1-4* in hippocampus of female groups. There was a low positive correlation between the *Gria1* and *Gria2* (r = 0.4003, p = 0.0232), as well as between *Gria1* and *Gria3* (r = 0.4033, p = 0.0163). In addition, there was a high positive correlation between *Gria2* and *Gria3* (r = 0.8753, $p < 0.0001$). However, there was no correlation between *Gria1* and *Gria4, Gria2* and *Gria4, Gria3* and *Gria4* (r = -2193, p = 0.2530; r = 0.1087, p = 0.5893; r = -0.1366, p = 0.4881, respectively) (**Figure 4.21.**).

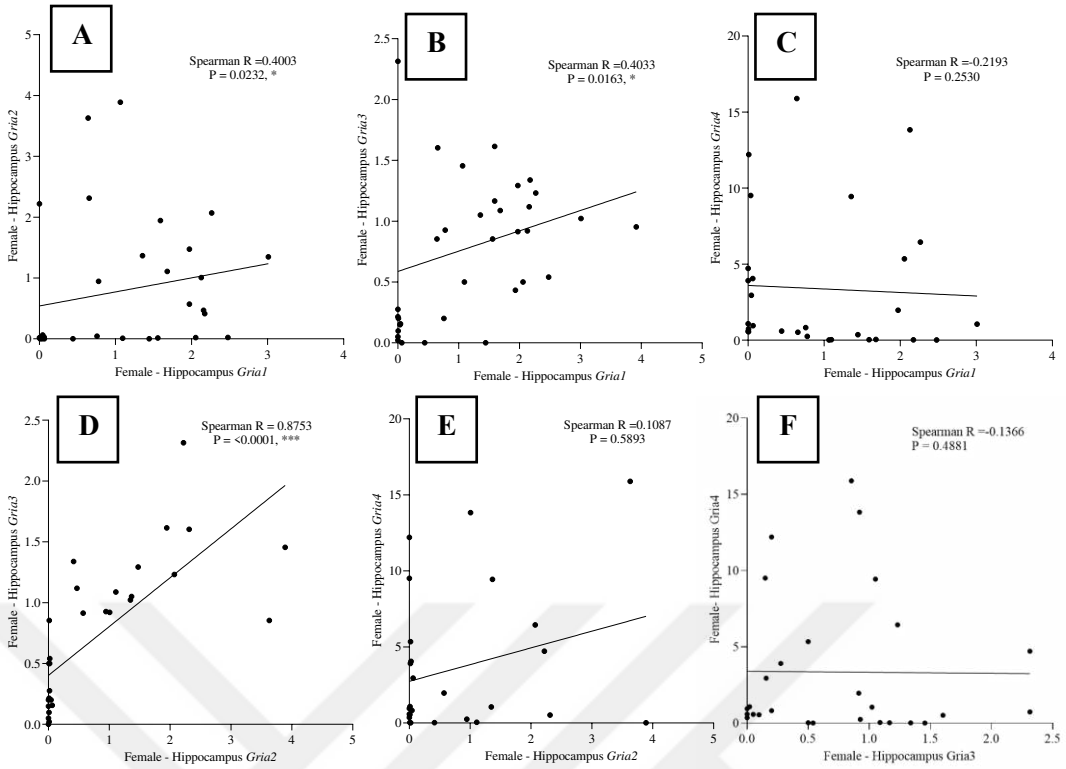

Figure 4.21. Expression correlation in female hippocampus between *Gria1* and *Gria2* (A), *Gria1* and *Gria3* (B), *Gria1* and *Gria4* (C), *Gria2* and *Gria3* (D), *Gria2* and *Gria4* (E), *Gria3* and *Gria4* (F).

Spearman's rank correlation was computed to assess the relationship among *Gria1-4* in hippocampus of male groups. There was a low positive correlation between the *Gria1* and *Gria3* (r = 0.4315, p = 0.0086). In addition, there was a moderate positive correlation between *Gria2* and *Gria3* (r = 0.6195, p = 0.0002). On the other hand, there was a moderate negative correlation between *Gria2* and *Gria4* (r = -0.6324, p = 0.0007). However, there were no correlations between *Gria1* and *Gria2, Gria1* and *Gria4, Gria3* and *Gria4* (r = 0.1964, p = 0.2897; r = -0.0333, p = 0.8637; r = -0.3040, p = 0.1089, respectively) (**Figure 4.22**).

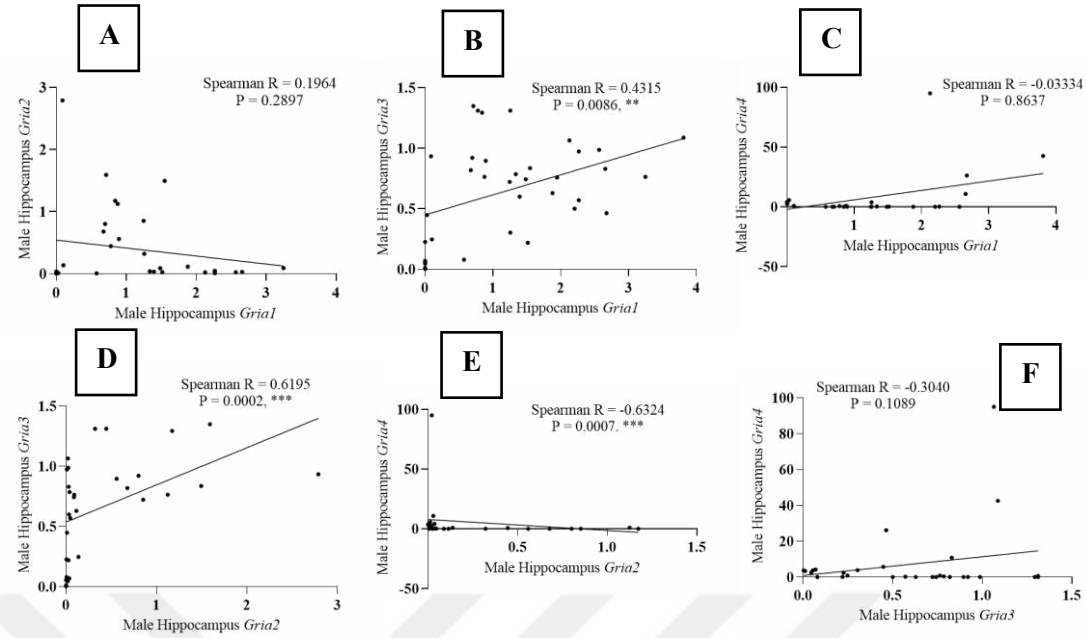

Figure 4.22. Expression correlation in male hippocampus between *Gria1* and *Gria2* (A), *Gria1* and *Gria3* (B), *Gria1* and *Gria4* (C), *Gria2* and *Gria3* (D), *Gria2* and *Gria4* (E), *Gria3* and *Gria4* (F).

Correlation Analysis Between Blood *Gria1, Gria2, Gria3* and *Gria4* and Plasma miR-124-3p (Unpublished Data)

A correlation analysis was conducted to examine potential regulatory relationships between blood *Gria1*, *Gria2*, *Gria3*, and *Gria4* expression levels and plasma miR-124-3p expression levels. The expression levels of *Gria1-4* mRNA and miR-124-3p were analysed in order to identify any possible connections, although the miRNA data is not included in this thesis. This analysis aimed to provide insights into potential regulatory interactions, although the primary focus of this study remains on *Gria1-4* mRNA expression. Due to this focus, the miRNA data will not be shown or discussed extensively. However, the correlation analysis lays the groundwork for future investigations into the intricate regulatory networks involving *Gria1-4* genes and miR-124-3p.

Spearman's rank correlation was computed to assess the relationship between blood *Gria1-4* and plasma miR-124-3p in female groups. There was a moderate positive correlation between the *Gria1* expression in blood and miR-124-3p expression in plasma (r = 0.5077, p = 0.0081). However, no significant correlations were observed between blood *Gria2* and plasma miR-124-3p, *Gria3* and plasma miR-124-3p, and *Gria4* and plasma miR-124-3p (r = 0.0161, p = 0.9389; r = 0.0312, p = 0.8748; r = -0.0701, p = 0.7337). (**Figure 4.23**).

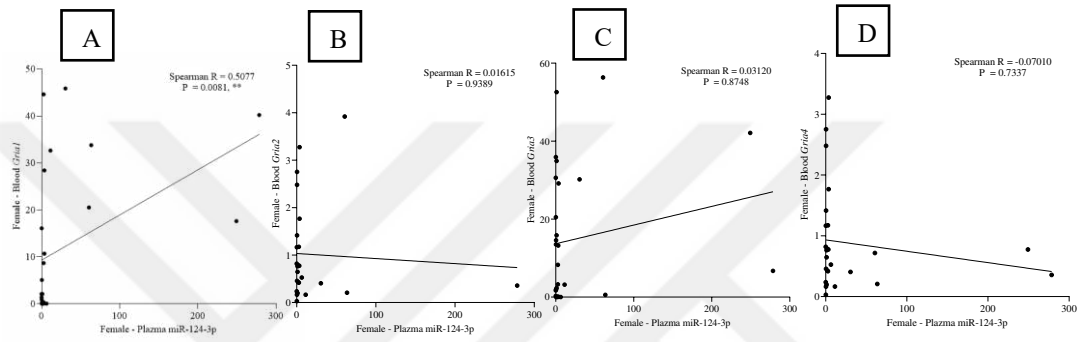

Figure 4.23. Expression correlation between blood *Gria1* and plasma miR-124-3p (A), *Gria2* and plasma miR-124-3p (B), *Gria3* and plasma miR-124-3p (C), *Gria4* and plasma miR-124-3p in female groups (D).

Spearman's rank correlation was computed to assess the relationship between blood *Gria1-4* and plasma miR-124-3p in male groups. There was a low negative correlation between the *Gria4* expression in blood and miR-124-3p expression in plasma (r = -0.4370, p = 0.0157). However, no significant correlations were observed between blood *Gria1* and plasma miR-124-3p, *Gria2* and plasma miR-124-3p, and *Gria3* and plasma miR-124-3p (r = -0.1927, p = 0.2989; r = -0.3276, p = 0.0828; r = -0.0254, p = 0.8921). (**Figure 4.24**).

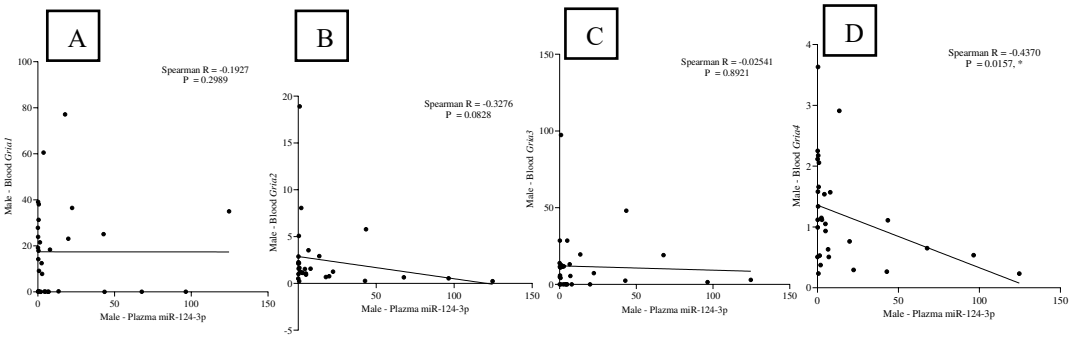

Figure 4.24. Expression correlation between blood *Gria1* and plasma miR-124-3p (A), *Gria2* and plasma miR-124-3p (B), *Gria3* and plasma miR-124-3p (C), *Gria4* and plasma miR-124-3p in male groups (D).

The expression values of the *Gria1, Gria2, Gria3* and *Gria4* in hippocampus were compared between the MS and MSUS groups and the Control group. The results, depicted in heatmap, showed that the expression of *Gria1, Gria2, Gria3* and *Gria4* genes was consistently decreased in both the Female MS and Female MSUS groups when compared to the Female Control group (**Figure 4.25**).

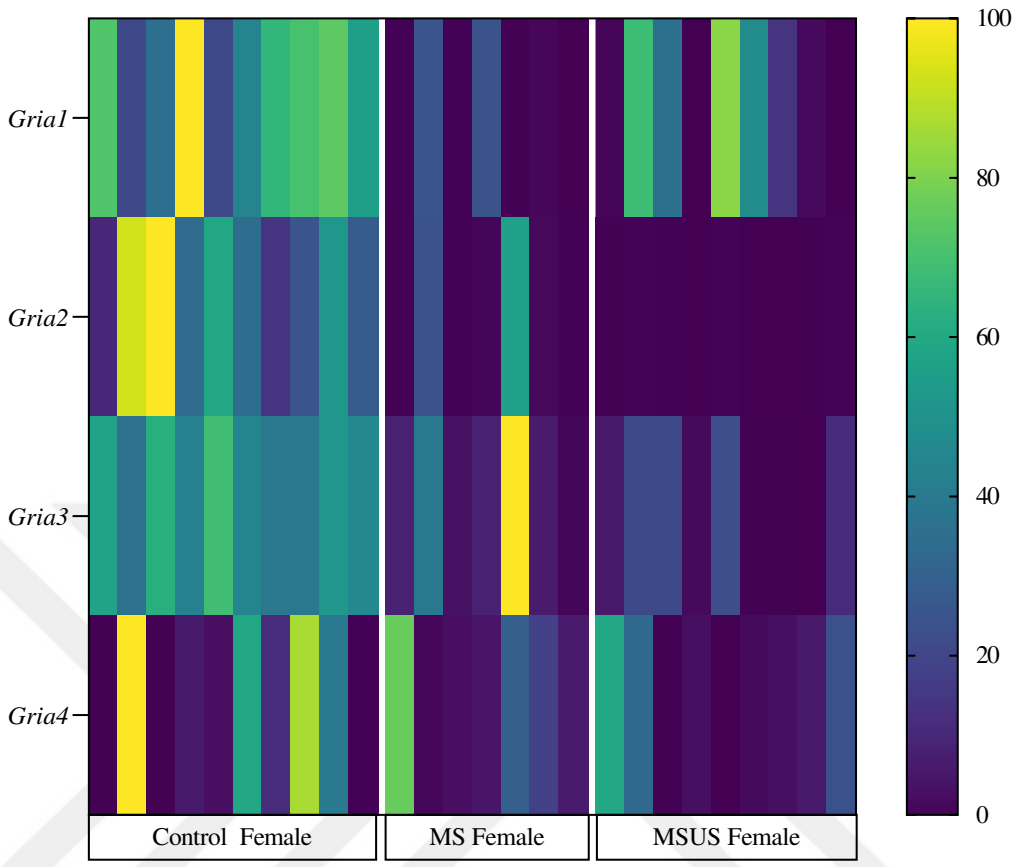

Figure 4.25. Differential mRNA expression heatmap in female MS (n=7) and female MSUS (n=9) groups compared to female control group (n=10).

Gria1 exhibited increased expression in both male MS and male MSUS groups, while *Gria2* showed decreased expression in both groups. Similarly, *Gria3* demonstrated decreased expression in both the male MS and male MSUS groups. In contrast, *Gria4* slightly increased expression in both groups. (**Figure 4.26**).

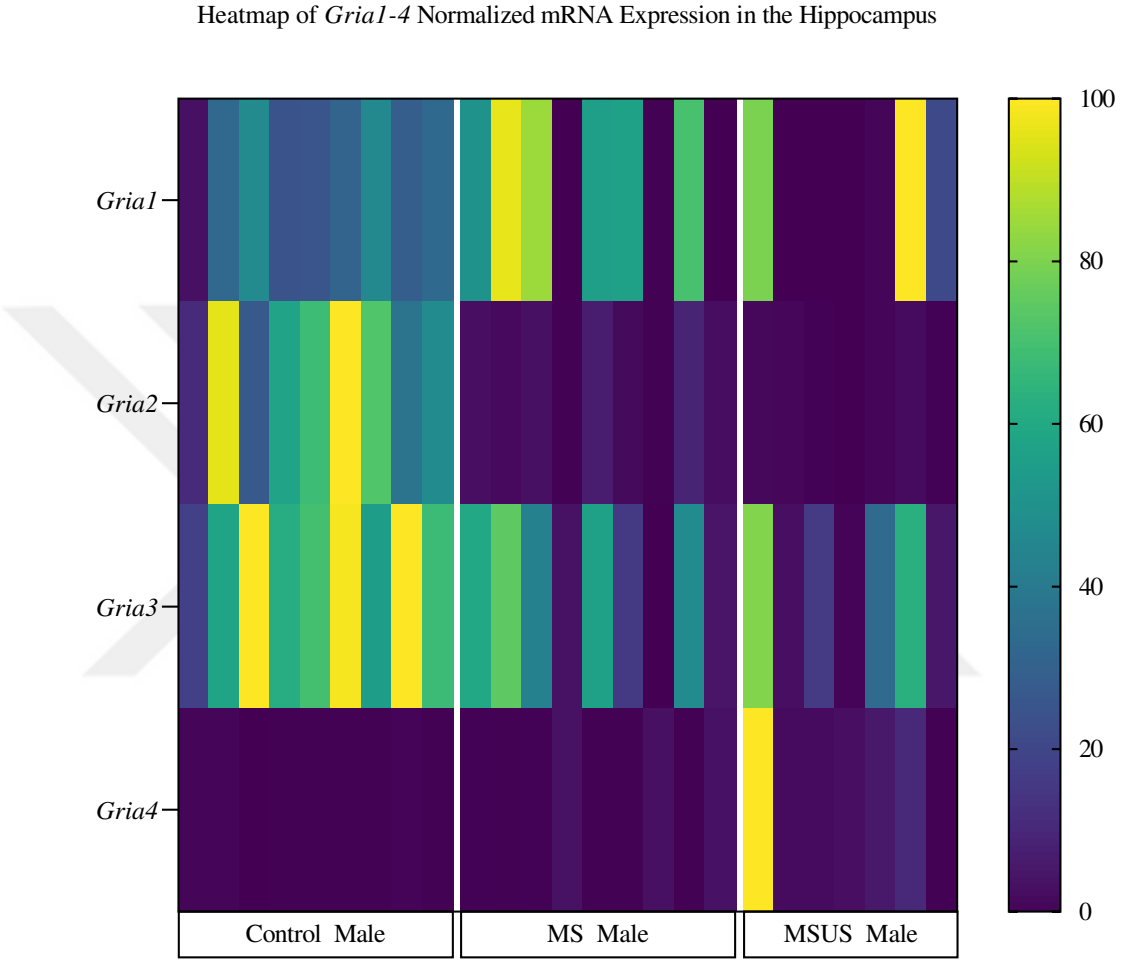

Figure 4.26. Differential mRNA expression heatmap in male MS (n=9) and male MSUS (n=7) groups compared to female control group (n=9).

The expression values of the *Gria1, Gria2, Gria3* and *Gria4* genes in blood were compared between the MS and MSUS groups and the Control group. The results showed that the expression of *Gria1, Gria2, Gria3* and *Gria4* genes was consistently increased in the Female MS group when compared to the Female Control group (**Figure 4.23**). In the Female MSUS group, *Gria1* expression was increased compared to the Female Control group. However, in *Gria2*, despite one sample with a high value,

the overall trend showed decreased expression in the Female MSUS group. On the other hand, *Gria3* exhibited increased expression in the Female MSUS group, while *Gria4* showed decreased expression compared to the Female Control group (**Figure 4.27**).

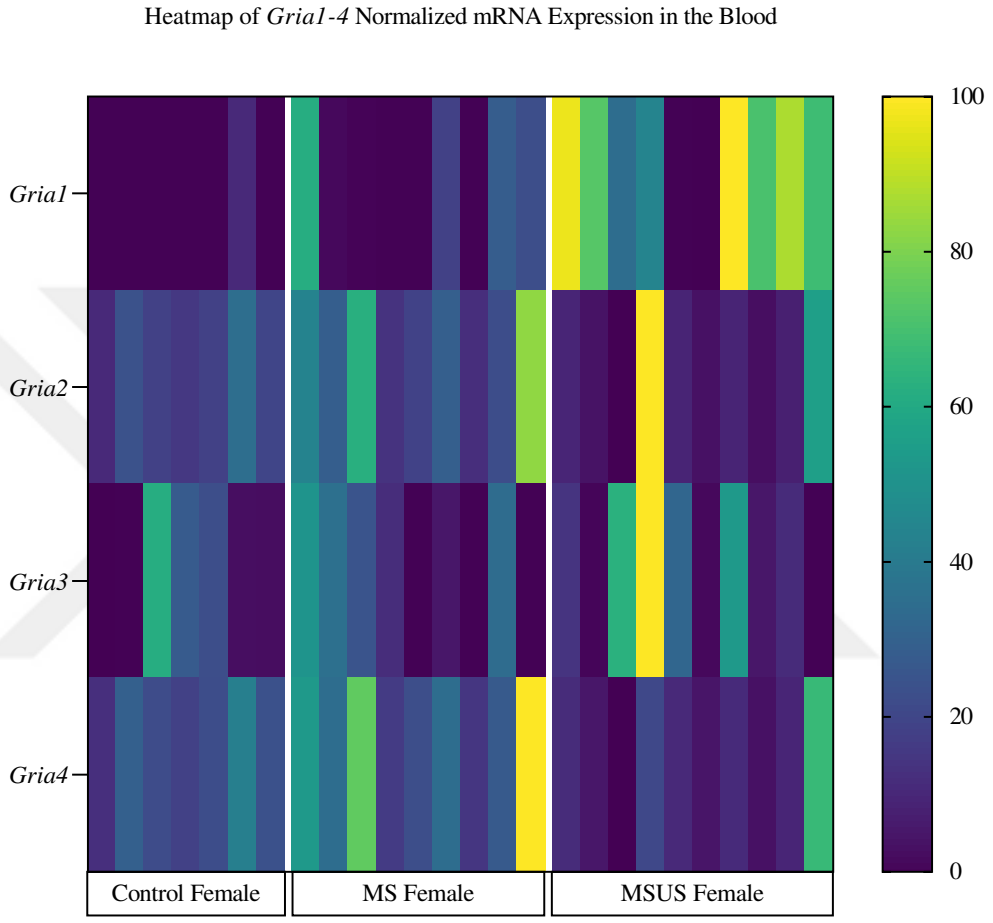

Figure 4.27. Differential mRNA expression heatmap in female MS (n=9) and female MSUS (n=10) groups compared to female control group (n=7).

In the male MS group, *Gria1* slightly decreased, while in the male MSUS group, it increased. *Gria2* increased in the male MS group and slightly decreased in the male MSUS group. *Gria3* increased in the male MS group and showed a slight increase in the male MSUS group. Both the male MS and male MSUS groups exhibited a decreased expression of *Gria4* compared to the male Control group (**Figure 4.28.**).

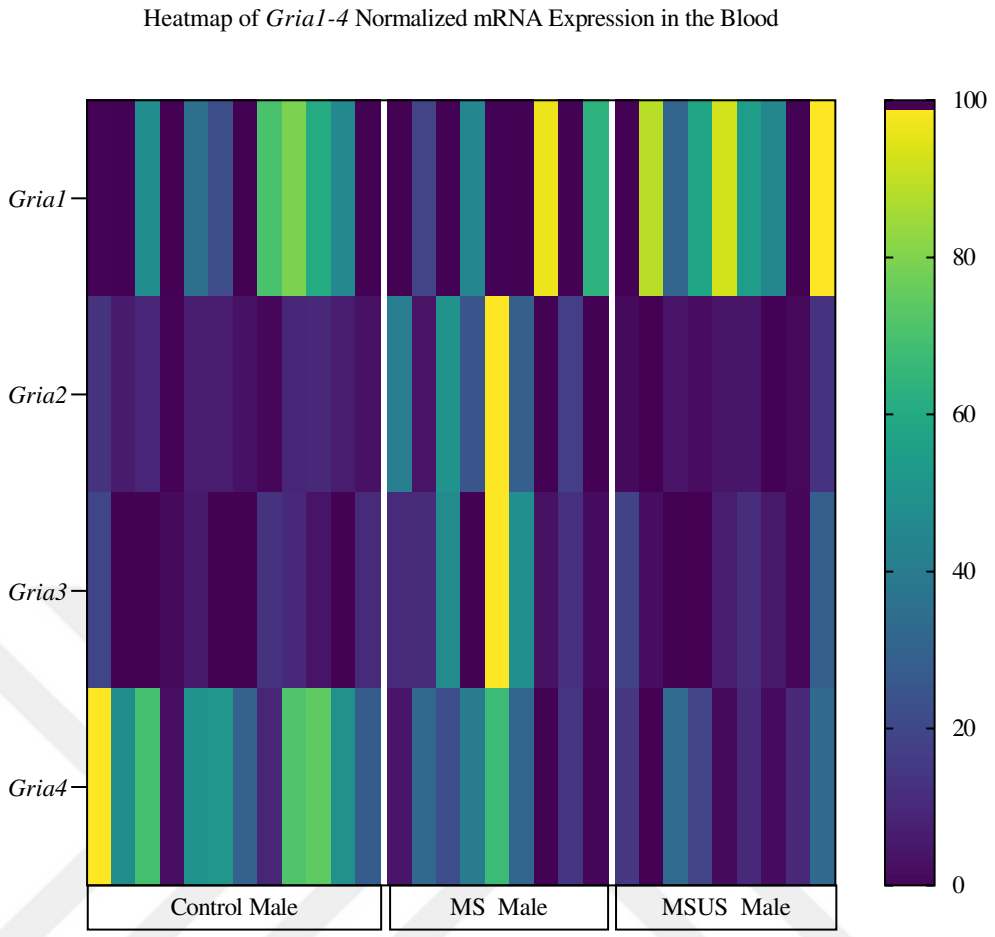

Figure 4.28. Differential mRNA expression heatmap in male MS (n=9) and male MSUS (n=9) groups compared to female control group (n=12).

5. DISCUSSION

Childhood trauma can have long-term consequences, including an increased risk of psychiatric disorders like depression and anxiety in adulthood. Both genetic and epigenetic factors may influence these effects. Furthermore, maternal stress and separation during the neonatal period can also impact various physiological systems. In our previous study, we investigated the maternal stress mouse model under the guidance of my supervisor, Dr. Keziban Korkmaz Bayram, and discovered higher levels of miR-124-3p in both plasma and hippocampus samples. However, it's important to note that these findings are currently unpublished.

Moreover, while animal models are limited in fully replicating human physiology's complexity, they serve as valuable tools for studying biological systems and evaluating potential treatments. Animal models allow researchers to create experimental conditions that would be unethical to impose on humans.

This study involved a detailed comparison of the weights of animals from three distinct groups (**Figure 3.1.**) to assess the efficacy of the stress model employed. The litter weights were measured every five days from birth (postnatal day one or PND1) until PND35. This allowed us to monitor their weight throughout the entire maternal stress procedure, which lasted from PND1 to PND14, as well as the period after the stress procedure until the day of euthanasia (PND15-PND35). During the first 16 days of their lives (PND1-PND16), the litter relied solely on their mother's milk for nutrition [94]. However, starting from PND16, they began to develop self-feeding behaviour [95]. This transition from milk to solid food consumption usually occurs during the weaning period, typically between PND17 and PND22 [94]. Based on the findings, it can be inferred that the maternal stress procedures in this study were implemented during the lactation phase of the litter when they relied on their mother's milk for sustenance. The main objective of the weight monitoring was to assess the effects of early-life maternal stress on the weight of mouse litter. It is noteworthy that the MS dams were subjected to unpredictable maternal separation, whereas the MSUS dams experienced both unpredictable maternal separation and additional stressors such as the forced swim test and restraint stress. However, the litter themselves solely

experienced maternal separation and were not directly exposed to the forced swim test or restraint stress.

The weight monitoring results were presented in **Figure 4.2.** for females and **Figure 4.3.** for males. Female and male litter weight data were presented separately, following confirmation that the graphs displayed similar trends when plotted together. Each data point on the graphs represents the average body weight of the respective groups on each weighing day. The findings from both female and male litter indicated that, from postnatal day 1 (PND1) to PND35, all groups (control, maternal stress (MS), and maternal stress with unpredictable stressors (MSUS) experienced weight gain, albeit to varying extents. The control group exhibited the highest weight gain, followed by the MS group, while the MSUS group displayed the most minor weight gain, indicating their relatively leaner status. At PND10, the MSUS group started showing a significant difference in weight compared to the control group.

Furthermore, significant weight loss in the MS group began from PND20 onwards. Both female and male MSUS groups demonstrated significant weight loss from PND10 until PND35. Both female and male MS groups demonstrated significant weight loss from PND20 until PND35, which became increasingly significant as time progressed. The significant weight loss of both female and male MSUS groups compared to MS groups was established from PND 25 until PND35. The study model has demonstrated remarkable success, highlighting its efficacy and reliability.

The weight loss observed in the MSUS group cannot be solely attributed to the deprivation of maternal milk, as both the MS and MSUS litter were directly exposed to maternal separation. It is important to note that the MSUS group dams experienced unpredictable maternal stress, which may have indirectly affected the litter. Therefore, factors related to the MSUS experimental conditions likely play a role in reducing body weight in the litter.

Does body weight loss due to mother's milk deprivation? If so, the weight loss should decrease starting from the litter self-feeding behaviour day. Nevertheless, the results showed that weight loss was more significant even litter started self-feeding with solid foods.

It is pertinent to question whether the body weight loss results from the deprivation of the mother's milk. If that were the case, the weight loss would be expected to decrease once the litter transition to self-feeding behaviour. However, the results indicate that the weight loss remains significant even after the litter has started consuming solid foods, indicating that factors beyond maternal milk deprivation contribute to the observed weight reduction.

In a previous study [96] examining the impact of maternal separation on rats, no significant differences in body weight were found among the groups from postnatal day 2 (PND2) to PND15. Notably, this study specifically focused on the maternal separation period and included ten rats in each group. We found no significant differences in body weight among the maternal separation groups until PND15 in the previous study and our study. This indicates that maternal separation may not have a statistically significant impact on body weight until PND15. However, our study observed an exciting pattern within the MS group. While there were no significant weight differences at PND15, we noticed a significant weight loss trend starting from PND20 in the MS group. Strikingly, this weight loss became statistically significant at later time points. These findings suggest that the effect of maternal separation on body weight may take some time to manifest and become noticeable.

A related study on rats found no significant difference in body weight within the maternal separation (MS) group when the separation procedure was consistently conducted from 09:30 to 12:30 daily, starting from PND2 to PND14 [97]. However, in our study, we observed a significant difference in body weight between the control group and the MS group. The significant body weight difference observed between the control and MS group in our study can be attributed to the unpredictable timing of the maternal separation procedure, and each day, the separation periods started at different times, making it inconsistent for the litter to experience maternal separation. As a result, the litter in the MS group did not have a consistent separation schedule, which likely contributed to the observed weight difference compared to the control group.

Another study conducted on a mice model demonstrated that both the unpredictable maternal separation (MS) and unpredictable maternal separation with

unpredictable stressors (MSUS) groups exhibited normal body weight despite the early stress treatment compared to the control group. This finding contrasts our study, where significant weight loss was observed in the MS and MSUS groups [10]. The difference in weight outcomes between our study and the previous one could be due to differences in the experimental protocols and the timing of stress exposure. Our study experimented with the stress model during the daylight period, in contrast to the previous study, which used the dark cycle of mice. Cortisol levels in humans tend to be low during the night and rise in the morning, which is the diurnal rhythm of cortisol [98]. As mice are nocturnal animals [99] and primarily active at night, the highest percentage of free corticosterone (unbound and available for use) is observed during the dark phase of the light/dark cycle. Conversely, during the light phase of the cycle, the lowest values of free corticosterone were reached [100]. By implementing our stress procedure, we can potentially disrupt our animal models' natural diurnal rhythm of corticosterone. This disruption may result in variations in the physiological stress response and have a notable impact on the body weight of the mice. Our suggestion is supported by the findings of a previous study on maternal separation (MS) conducted during the light cycle of mice. The study's findings indicated that the MS group had lower body weight than the control group by week 4 [101]. Furthermore, another study involving maternal separation for a period of 21 days and exposure to chronic unpredictable mild stress in BALB/c mice also demonstrated a significant reduction in the average weight of the offspring[102]. These results align with our own findings, despite differences in the duration of stress exposure.

Studies on restraint stress, such as immobilization stress (IS) (for the current study is restraint stress) on the mouse dams in the early postnatal period, showed that IS during lactation resulted in low body weight of male mouse litter caused by malnutrition [103]. Additionally, restraint stress experienced by lactating mice has been found to reduce maternal care provided to their litter. This decrease in maternal care has been linked to a subsequent decrease in the body weight of the litter [104].

Although the specific effect of the maternal forced swim test on litter body weight was not individually examined, it is believed that the combined impact of restraint stress and the forced swim test in MSUS dams contribute to weight decrease

in litter. This belief is based on the observed changes in maternal care behaviour following the test, which may influence the litter's weight.

In addition to the weight-related results that demonstrated the effectiveness of the animal model in this study, the presence of phenotypic differences (**Figure 4.1.**) in mice further confirms the validity and success of the model.

Chronic stress triggers a cascade of events that disrupts tissue homeostasis, particularly in the context of hair growth regulation. In mice, the stress hormone corticosterone, similar to cortisol in humans, plays a vital role in regulating hair follicle stem cells (HFSCs) and hair growth. When subjected to chronic stress, the increased levels of corticosterone prolong the quiescent state of HFSCs, leading to an extended resting phase of the hair follicles [105].

The mechanism involves corticosterone acting on the dermal papillae, a structure within the hair follicle, suppressing the expression of a gene called *Gas6*. *Gas6* encodes a secreted factor known as growth arrest-specific 6. By reducing *Gas6* expression, chronic stress inhibits the activation of HFSCs and hinders hair growth [105].

However, the inhibitory effects of chronic stress on hair growth can be overcome. By restoring the expression of Growth Arrest Specific 6 (*Gas6)*, the suppression caused by stress-induced corticosterone levels can be counteracted, allowing for the activation of HFSCs and promoting hair growth despite the ongoing stress [105] (**Figure 5.1.**).

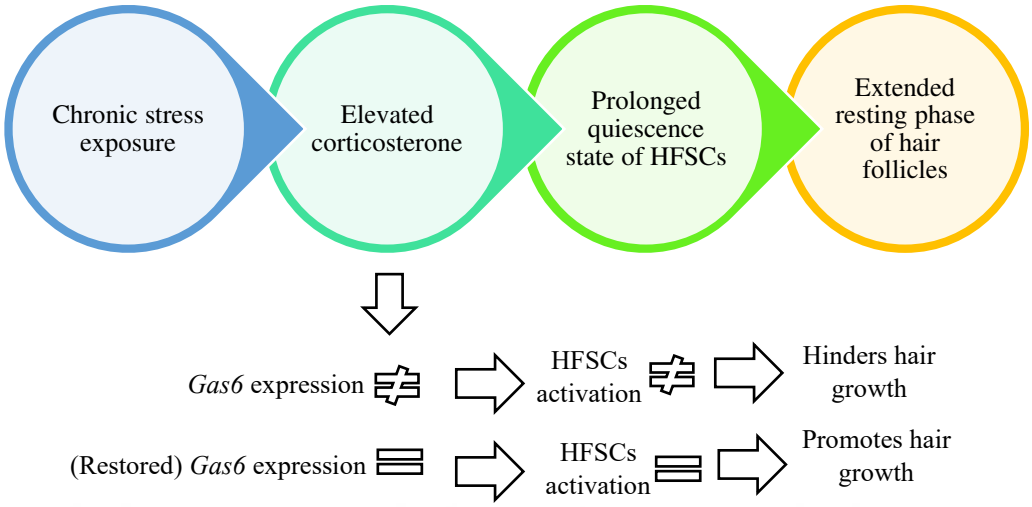

Figure 5.1. Mechanisms of chronic stress-induced hair growth disruption [105].

Based on the evidence presented, the observed decline in hair growth and changes in hair phenotypes are associated with the upregulation of corticosterone in response to chronic stress. This suggests that corticosterone has a negative impact on the normal hair growth cycle, potentially impeding the activation of hair follicle stem cells.

Furthermore, upon the sacrifice of the mice on day 35, we observed notable differences in skull development across the various groups. The skulls of the control group litter demonstrated higher rigidity when cut with scissors. Conversely, the MS and MSUS group's skulls exhibited more elasticity upon cutting. Previous research has highlighted the disruptive effects of early-life postnatal stress on fetal bone formation, leading to a range of skeletal anomalies. Notably, the nature of the stress experienced, and the developmental phase in which it occurs can influence the diversity and severity of these bone abnormalities [106].

Karin Wuertz-Kozak et al. (2020) found that early-life stress affects bone metabolism in mice. They observed that in MSUS mice, there was a clear indication of a shift toward a catabolic environment. This was characterized by a decrease in the expression of neurogenic and osteogenic factors, including *NGF, NPYR1, VIPR1*, and

TACR1. Additionally, they noted an increase in innervation density within the bone and elevated levels of CTX-I in the serum. Interestingly, despite these changes, no observable alterations were found in the bone microarchitecture of these relatively young mice, who were not subjected to additional life stresses during the study [107].

In order to address the substantial impact of sex on mRNA expression patterns and networks [108], separate plots were generated for the female and male groups. It has been well-documented that male and female rodents exhibit differential responses to acute and chronic stress regarding their endocrine and neurobiological reactions. Therefore, considering the sex differences is crucial in understanding the findings of this study.

There is a clear association between early life stress and various psychiatric disorders such as depression, posttraumatic stress disorder (PTSD), and bipolar disorder. Clinical studies have identified early life stress as a significant risk factor for increased susceptibility to these disorders. Recent experimental evidence suggests that glutamatergic mechanisms may play a central role in developing these disorders [109].

In our initial investigation that served as the basis for this study, the miR-124-3p expression level was significantly increased in the hippocampus in males of the MS and MSUS groups relative to the control group. We also observed increased miR-124-3p in specific samples of females of the MS and MSUS groups compared to the control group. Expanding on these findings., this study focused on investigating the mRNA expression levels of miR-124-3p and AMPA receptor subunits genes, including *Gria2*, *Gria3*, *Gria4* (targets), and *Gria1* (non-target). These target genes were specifically chosen based on their strong scores in prediction algorithms, indicating their significant involvement in the regulatory network of miR-124-3p. Correlation analyses explored potential variations in response to stress conditions attributed to individual epigenetic differences. When microRNAs (miRNAs) attach to mRNA transcripts, they prevent them from being translated into proteins. As a result, changes in gene expression can be detected by analyzing protein levels. However, intriguingly, our study revealed an increase in mRNA transcripts without a corresponding elevation in protein levels. Despite the conventional expectation of a relationship at the protein level based on theoretical knowledge, we observed that the correlation between miR-

124-3p and *Gria1*-4 expression could also be observed at the mRNA level through these correlation analyses. The subsequent sections provide a more comprehensive elucidation of these results:

The *GRIA1* gene encodes the GluA1 subunit of AMPA receptors, which are ligand-gated ion channels that act as excitatory receptors for the neurotransmitter L-glutamate (Glu) [110]. GluA1-containing AMPARs play a crucial role in brain function and can form either homomeric receptors composed of identical subunits or heteromeric receptors when combined with GluA2-4 subunits [110]. In the synapses of principal hippocampal neurons, most AMPA receptors are heteromers of GluA1 and GluA2 (80%), while the remaining 20% are assembled from GluA2 and GluA3 [111]. Interestingly, we found that there was a decrease in *Gria1* expression in the hippocampus of mice female subjected to stress (**Figure 4.4. A**). Furthermore, correlation analyses revealed a significant low positive correlation between the decrease in *Gria1* expression and the decrease in *Gria2* expression within the female hippocampus (**Figure 4.21. A**). Moreover, we also observed a similar correlation between the decrease in *Gria1* and *Gria3* expression both in females and males. This indicates that as *Gria1* expression decreases, there is a tendency for *Gria2* and *Gria3* expression to decrease as well. However, it is important to note that the relationship between their decreases is not very strong. While there is a general trend of decrease in both variables, the magnitude of the decrease is not substantial, suggesting a weak positive correlation between these factors (**Figure 4.21. B, 4.22. B**).

The expression of hippocampal *Gria1* depends on the duration of stress exposure, with short-term stress increasing and long-term stress decreasing *Gria1* expression [109]. The alteration in AMPA receptor expression observed in patients with depression and after stress induction in rodents may result in changes in synaptic plasticity. Conversely, changes in synaptic plasticity may underlie the observed differences in AMPA receptor expression [109].

Our results demonstrate a significant decrease in *Gria1* expression in the hippocampus of mice female subjected to maternal separation stress (MS female) compared to the control group (**Figure 4.4. A**). One possible explanation for this decrease is the activity of the repressor element 1 silencing transcription factor

(REST). REST, also known as Neuron-Restrictive Silencing Factor (NRSF), has been reported as a neuroprotective molecule in certain neurological disorders [112]. REST regulates glutamate receptors and immediate early genes, thereby influencing synaptic plasticity [113]. Moreover, in the maternal separation stress paradigm, NRSF is upregulated [114]. Maternal separation has also been found to increase the mRNA and protein levels of REST4 in the mouse prefrontal cortex [115]. Decreased levels of REST/NRSF in the nucleus can induce the expression of death-related genes, leading to neuronal death. Under physiological stress conditions, overexpression of REST/NRSF activates neuronal survival mechanisms in the brain [112]. REST plays a role in maintaining synaptic plasticity by affecting *GRIA1* expression, as evidenced by a study conducted on primary mouse hippocampal neurons, which showed that REST knockdown increased *GRIA1* mRNA expression. At the same time, REST downregulation decreased *GRIA1* mRNA expression [113].

Although *Gria1* is not predicted to be a target gene of miR-124-3p, according to miRDB, miR-124-3p does target the REST corepressor 1 (*RCOR1*) with a score of 86. A study on embryonic brain tissue [116] identified a repressor/corepressor complex consisting of REST corepressors RCOR1 and RCOR2, and eliminating RCOR1 from this complex induced neuro-proliferation while causing overexpression of REST. In the case of both MS females and MSUS females (our preliminary study), the high expression of miR-124-3p may be related to a protective mechanism against alterations in neurodevelopment. The current findings of our research demonstrate a decrease in both MS females and MSUS females *Gria1*, which may be attributed to the high level of REST. Additionally, a study by Jaya Visvanathan et al. suggested that miR-124 may play a role in promoting or facilitating neural development by counteracting the actions of the REST/SCP1 pathway, which inhibits or suppresses neural development [117]. Another study on mice found a significant loss of neurons in the dentate gyrus in mice subjected to maternal separation compared to control mice [118].

In contrast to MS females, the expression level of *Gria1* in MSUS females did not significantly decrease. This could be attributed to certain samples within the MSUS group that exhibited high expression of *Gria1* (**Figure 4.4. A**). MS females and MSUS females experienced maternal separation during the early-life postnatal period. The only difference between them is the additional maternal stress experienced by the

MSUS female dams. While the MS female dams were not exposed to forced swim tests and restraint stress, the MSUS female mothers were exposed to these stressors. We suggest that the level of miR-124-3p expression influences the expression of *Gria1* in MSUS females. At a certain point, miR-124-3p may suppress RCOR1, but beyond that point, hippocampal miR-124-3p may enhance resilience to chronic stress in mice [119].

Sex differences in stress-related disorders can manifest at various levels, as demonstrated by previous research [120]. We investigated the effects of early-life postnatal maternal stress on mRNA expression levels of AMPA receptor subunits, focusing on *Gria1*, in the hippocampus of male mice litter. Previous studies have also examined *Gria1* expression in the hippocampus and frontal cortex and reported no significant changes in *Gria1* mRNA expression in male mice litter exposed to maternal separation [121]. These findings align with our study, which observed no significant changes in *Gria1* expression in the hippocampus of male mice litter exposed to maternal separation (**Figure 4.4. B**).

Interestingly, considering the RCOR1 pathway suggests that another factor must be present in MS male litter that contributes to their resilience against unpredictable maternal separation stress. In our previous study, we observed significant differences in the expression levels of miR-124-3p in maternally stressed male litter. Specifically, MS males exhibited higher levels of miR-124-3p than the control, and MSUS males showed the highest expression levels. It suggests that increased miR-124-3p may be protective against stress in MS male litter. Supporting our findings, the study conducted by S. Katsouli et al. (2014) on Wistar rats, which involved daily 15-minute maternal separation, aligns with our results. They discovered that in the dorsal hippocampus, *GluA1* (*Gria1*) mRNA levels increased in males subjected to maternal separation, while females exposed to the same stress exhibited decreased GluA1 mRNA levels. This study further highlights the sexually dimorphic effects of maternal separation on AMPA receptor subunits, namely GluA1 and GluA2, within the limbic system [122]. Our analysis of correlation did not find a noteworthy relationship between miR-124-3p and *Gria1*, which is a subunit of AMPAR. This indicates that miR-124-3p might not have a direct impact on *Gria1*, as predicted by miRDB.

However, it is important to consider the possibility of an indirect interaction between miR-124-3p and *Gria1*.

Our research aimed to identify potential biomarkers by examining the levels of *Gria1* mRNA expression in the blood. (**Figure 4.9.**) Our investigation yielded novel findings. We observed a significant increase in *Gria1* expression in the blood samples of MSUS females compared to control females (**Figure 4.9. A**), and a similar pattern was observed in MSUS males compared to control males, although their difference was not statistically significant (**Figure 4.9. B**). However, there were no significant differences between MS female and MS male groups compared to their control groups. Furthermore, we found that there was no significant correlation between *Gria1* expression in the hippocampus and *Gria1* expression in blood (**Figure 4.13**).

We also studied the expression of miR-124-3p in plasma samples from litter exposed to early-life maternal stress (Unpublished data). Our analysis revealed a moderate positive correlation between miR-124-3p expression in plasma and *Gria1* expression in female blood (**Figure 4.23. A**). This significant result suggests that increased *Gria1* expression is associated with increased plasma miR-124-3p levels in response to early-life maternal stress. Additionally, we observed a negative correlation between plasma miR-124-3p and *Gria4* in male blood samples (**Figure 4.24. D**). This suggests that increased miR-124-3p levels in male plasma may suppress *Gria4* expression in male blood, leading to decreased *Gria4* expression in the blood.

Examining gene expression in peripheral blood presents an ongoing challenge, despite the advantages of its minimally invasive collection using standard clinical procedures[86]. This method isolates cell-free nucleic acids (cfNAs) with diagnostic and screening potential [123]. Considering these benefits, our study also investigated mRNA expression in blood. Analyzing mRNA expression in blood holds promise for various clinical drug development and disease diagnosis applications.

It is worth noting that the ligand of AMPAR, glutamate, is a nonessential amino acid that cannot cross the blood-brain barrier (BBB) and instead must be synthesized within neurons from local precursors [65]. The BBB is a highly selective semipermeable border separating circulating blood from the brain and extracellular

fluid in the central nervous system (CNS). Its role is to limit the entry of water-soluble molecules into brain tissue [124]. The presence of the BBB likely contributes to the non-significant correlations observed between AMPAR expression in the hippocampus and blood samples (**Figure 4.13; 4.14; 4.15; 4.16**).

Detecting cell-free mRNA (cf-mRNA) poses challenges due to its fragmented nature and relatively low abundance [123]. The existing literature needs to provide more information about the presence of *Gria1*, *Gria2*, *Gria3*, and *Gria4* protein-coding RNA in blood.

The mRNA expression profiles of *Gria2*, *Gria3*, and *Gria4* in the hippocampus and blood are discussed collectively in this study, as it aims to elucidate the AMPAR-miR-124-3p pathway. The pathway involving AMPAR and miR-124-3p connects the genetic risks and behavioural changes in schizophrenia and bipolar disorder. AMPA receptors are tetrameric glutamate-gated ion channels that play a crucial role in fast excitatory neurotransmission in the brain. These receptors exist in two subtypes: calcium-impermeable (CI-) and calcium-permeable (CP-) [69]. The *Gria2* gene encodes the GluA2 subunit of AMPA receptors, determining their calcium permeability. AMPA receptors lacking the GluA2 subunit are calcium-permeable (CP) and contribute to calcium influx in plasticity and disease processes [125]. Therefore, the downregulation of *Gria2* by miR-124-3p provides a potential mechanism for the altered synaptic transmission and behavioural deficits observed in these disorders [126].

Studies have revealed that abnormal trafficking of AMPA receptors plays a role in the dysregulated glutamate neurotransmission observed in schizophrenia [127]. They observed that miR-124 is upregulated in biopsied neuronal cells obtained from patients with schizophrenia (SZ) and bipolar disorder (BP). According to the miRDB prediction algorithm, it has been discovered that miR-124-3p targets *Gria2*, *Gria3*, and *Gria4* with scores of 92, 91, and 93, respectively. The miR-124-3p-AMPAR pathway has emerged as a common neurobiological mediator connecting the shared polygenic risks and behavioural changes observed in schizophrenia and bipolar disorder [85]. This pathway may contribute translating genetic vulnerabilities into the clinical manifestations of these psychotic disorders.

In individuals with schizophrenia (SZ) and bipolar disorder (BP), a significant downregulation of *GRIA2* was confirmed in both human olfactory neuronal cells and postmortem prefrontal cortex (PFC) samples [128]. AMPARs can exhibit calcium permeability in two situations: when the *GRIA2* subunit is absent or when they contain the unedited *GRIA2*(Q) subunit [129]. The upregulation of miR-124-3p leads to the downregulation of Gria2, which subsequently enhances the abundance of calcium-permeable AMPARs (CP-AMPARs) [128].

In a mouse model, upregulation of miR-124-3p in the medial prefrontal cortex resulted in elevated *GRIA2*-lacking calcium-permeable AMPARs, disrupting excitatory synaptic transmission mediated by AMPARs. Overexpression of miR-124-3p in excitatory neurons of the mouse mPFC causes behavioural and synaptic deficits. These molecular changes were associated with deficits observed in the shared behavioural dimensions of schizophrenia and bipolar disorder [128]. The upregulation of miR-124-3p in neuronal cells and the prefrontal cortex of individuals with schizophrenia and bipolar disorder indicates its potential role in developing these disorders. The shared polygenic risks observed in both conditions further support the notion that miR-124-3p is a common mechanism underlying schizophrenia and bipolar disorder. Additionally, a previously unpublished study provided further support by demonstrating that early-life maternal separation induced miR-124-3p expression in the hippocampus of both female and male mouse litter. This finding is relevant, considering that early-life stress has been implicated in the developing of long-term psychiatric disorders such as schizophrenia and bipolar disorder [84][83].

In our recent study, we madae an exciting discovery in female mice exposed to stress. We found that miR-124-3p levels were increased, indicating higher expression, while the expression of AMPAR subunits (*Gria2* (**Figure 4.5. A**), *Gria3* (**Figure 4.7. A**), and *Gria4* (**Figure 4.8. A**)) in female mice, as well as *Gria2* (**Figure 4.5. B**) and *Gria3* (**Figure 4.7. B**) in male mice, showed a decrease in the stress group compared to the control group. These findings align with previous research that showed lower GluA2 (*Gria2*) mRNA levels in the ventral hippocampus and amygdaloid complex of female litter exposed to maternal separation [122]. Another study also reported reduced *Gria2* mRNA in the dorsal hippocampus, *Gria3* in various hippocampal

regions, and *Gria4* in the ventral hippocampus of male and female rats subjected to maternal stress [122]. We noticed an intriguing pattern with *Gria2* that supports the mRNA expression results from correlation analyses. The levels of *Gria2* mRNA were significantly reduced in both female and male stress groups (MS, MSUS), showing a strong inverse relationship with miR-124-3p (**Figure 4.18.**). In other words, when the miR-124-3p expression was higher, the levels of *Gria2* were lower. A similar inverse correlation was observed between miR-124-3p and *Gria3* mRNA expression in both female and male groups, suggesting that increased miR-124-3p expression led to a decrease in *Gria3* levels (**Figure 4.19.**). Furthermore, our findings revealed a high positive correlation between *Gria2* and *Gria3* expression in the female hippocampus (**4.21. D**). In the male groups, a moderate positive correlation was observed between *Gria2* and *Gria3* expression. These results indicate that when there is a decrease in *Gria2* expression, there is also a tendency for *Gria3* expression to decrease, with a stronger correlation in females compared to males. Additionally, we observed a moderate inverse correlation between *Gria2* and *Gria4* in mmale's hippocampus (**Figure 4.22**. **E**), suggesting that the decrease in *Gria2* tends to be associated with an increase in *Gria4* expression.

Interestingly, we did not find a significant correlation between *Gria4* and miR-124-3p in the hippocampus (**Figure 4.20. A**). Although the correlation was not statistically significant in the female groups, there was a trend towards an inverse correlation between miR-124-3p expression and *Gria4* mRNA expression. This suggests that higher levels of miR-124-3p may be associated with decreased expression of *Gria4* in females. However, we did not observe an inverse correlation between miR-124-3p and *Gria4* in the male subjects (**Figure 4.20 B**). These findings indicate a discrepancy between the expression patterns of miR-124-3p and *Gria4* in male and female subjects.

To gain further insights into these observations, we can refer to a previous study [130] that investigated a rodent model of depressive symptoms associated with major depressive disorder. This study revealed that although the binding site for miR-124-3p on the *Gria4* 3' UTR region showed less conservation across mammalian species than *Gria3*, there was still notable transcriptional repression of *Gria4*. However, our study did not find a significant inverse correlation between miR-124-3p and *Gria4* in male

subjects, despite this miRNA targeting *Gria4*. This discrepancy suggests that factors other than direct miRNA regulation may influence the expression pattern of *Gria4* in males. Further investigation is needed to explore the specific binding site on the *Gria4* 3' UTR region or other regulatory mechanisms that may contribute to the observed differences in miR-124-3p-*Gria4* correlation between male and female subjects.

This observation further strengthens the concept of an AMPAR-miR-124-3p regulatory pathway and underscores the specific impact of early-life maternal stress on modulating this relationship within the hippocampus of litter.

To gain a comprehensive understanding of the relationship between the expression patterns of AMPAR and miR-124-3p in our findings and their relevance to the shared changes observed in schizophrenia (SZ) and bipolar disorder (BP), further research is warranted.

We examined the mRNA expression levels of *Gria2* and *Gria3* genes in the blood to identify potential biomarkers (**Figure 4.10.; 4.11.**). However, we did not obtain any significant results that could be attributed to stress-related factors. Regarding *Gria2*, its expression was found to be increased but not significant in both the female and male MS groups. However, *Gria3* did not show significant changes in expression. We did not observe significant correlations between these gene expression levels in the hippocampus and blood (**Figure 4.14; 4.15.**).

Regarding *Gria4* in MSUS males, we observed a distinct pattern compared to the other *Gria* subunits. The control group exhibited decreased *Gria4* expression (**Figure 4.8. B**), whereas MSUS males showed a significant increase in *Gria4* expression. The expression of the GluA4 AMPAR subunit in hippocampal pyramidal neurons is transient, occurring during the early postnatal period when glutamatergic synaptic connections in the hippocampus undergo activity-dependent refinement. After this transient phase, the expression of the GluA4 subunit may decrease or be downregulated as synaptic connectivity becomes more stable [131]. The differential expression of *Gria4* in the male and female hippocampus may be attributed to the factors mentioned earlier.

We examined the mRNA expression levels of *Gria4* genes in the blood to identify potential biomarkers. Interestingly, we observed a significant decrease in *Gria4* expression in the MSUS male group compared to the control group (**Figure 4.12. B**). Significant changes in *Gria4* expression were observed in the MSUS female compared to the MS female groups (**Figure 4.12. A**). Similar to other genes. The correlation between *Gria4* expression in the hippocampus and blood also did not exhibit a significant correlation (**Figure 4.16.**). This lack of correlation suggests that the expression levels of *Gria1*, *Gria2*, *Gria3*, and *Gria4* may be regulated independently in the hippocampus compared to the blood. These findings highlight the complexity of gene regulation mechanisms in different tissues and emphasize the need for further research to elucidate the specific factors that govern the expression patterns of these genes in the hippocampus and blood. Although there is limited literature on their presence in blood, our study provides valuable insights into their potential involvement in stress-related mechanisms. Further research is necessary to fully understand the functional significance of these expression patterns concerning maternal stress and its impact on litter.

In summary, the weight data obtained from the Control, MS, and MSUS groups showed significant differences (**Figure 4.2.** and **Figure 4.3.**). Notably, the MS litters showed lower weight gain compared to the control group, while the MSUS litters exhibited even more pronounced weight reduction, irrespective of gender. This animal model study explored the impact of maternal stress and separation on litter mice's weight, physiology, and psychology. Stress in mice causes the release of cortisol, which is regulated by the HPA axis. Elevated cortisol, specifically corticosterone, can lead to muscle atrophy. To investigate this further, a stress model was created, and it was found that stressed mice had reduced weight compared to the control group. These findings indicate the successful establishment of a maternal stress mouse model.

Additionally, notable phenotypic differences in hair structure (**Figure 4.1.**) and decreased skull ossification were observed in the stressed groups, further confirming the success of the stressed mouse model implementation. Furthermore, our findings revealed that stress-induced elevation of miR-124-3p expression in the hippocampus was associated with decreased expression of specific AMPA receptors. The qPCR results from this study support this hypothesis in the case of *Gria2*, *Gria3*, and *Gria4*

in the MS and MSUS groups of female litters and *Gria2* and *Gria3* in male litters. However, this hypothesis was not supported in the case of *Gria1* in both female and male litters, likely because it is not a target of miR-124-3p. Additionally, other factors may influence the lack of support for this hypothesis in the MS and MSUS groups of male litters for *Gria4*. Although qPCR primer design was based on the transcript translated into protein, current knowledge suggests that miRNAs affect the expression of mRNAs at the protein level. Therefore, this hypothesis may be supported by the results obtained from techniques such as western blotting, where protein expression levels can be determined. It is recommended to add techniques such as western blot, immunohistochemistry.

6. CONCLUSION AND FUTURE PERSPECTIVES

Our stress model [8], [10] successfully induced stress in the litter, and its effectiveness was further enhanced by incorporating variability and unpredictability in the timing of stress exposure. This approach allowed us to capture a range of responses and yielded more significant results.

Early-life postnatal maternal stress had a profound impact on AMPA receptor subunit gene mRNA expression in both the hippocampus and blood of the litter. These alterations in mRNA expression may be regulated by epigenetic factors, such as miR-124-3p, and contribute to the behavioural deficits shared between schizophrenia (SZ) and bipolar disorder (BP) [85].

The findings in the hippocampus regarding AMPA receptor subunits, supported by our previous study on miR-124-3p, shed light on the AMPA receptor-miR-124-3p regulatory pathway. This novel pathway may play a crucial role in mediating the effects of early-life postnatal stress on the hippocampus and behaviour.

Interestingly, the transcriptome analysis of AMPA receptors in blood did not exhibit a correlation with the mRNA expression patterns in the hippocampus. However, this finding contributes to the existing literature and suggests that further research is needed to explore the utility of blood biomarkers in detecting early-life postnatal changes.

Our study revealed that early-life postnatal stress resulted in altered development in the litter, including significant decreases in body weight during the period from one week to three weeks after the maternal stress period. Additionally, we observed changes in hair characteristics in the litter, further highlighting the long-term impact of early-life stress.

These alterations in the litter may be transmitted from mothers to *litter* through two factors: maternal milk and maternal care behaviours. Epigenetic regulation,

possibly mediated by miR-124-3p transmission through maternal milk, may contribute to the long-term effects observed.

In future studies, it would be beneficial to measure corticosterone levels during the maternal stress period and post-maternal stress period to better understand the physiological response to stress. Additionally, incorporating experimental groups with specific stressors, such as a group exposed to forced swim tests only or a group subjected to restraint stress only, could help elucidate additional regulatory factors, including the role of thermoregulation.

Furthermore, investigating protein expression levels and employing specific procedures to detect potential disease-related changes in the *Gria2* calcium-permeable subunit may offer valuable insights into the underlying mechanisms.

Moreover, our research suggests that *Gria1* and *Gria4* may be useful biomarkers for identifying early-life stress in litters. However, further studies are necessary to confirm their effectiveness in accurately detecting and assessing the impact of such stress. It would be beneficial to investigate the mRNA expression levels of *Gria1* and *Gria4* across generations in the maternal stress mouse model to determine if these transcripts indeed serve as biomarkers for detecting early-life maternal stress.

Overall, our research sheds light on the lasting effects of stress during early life and offers paths for future studies to delve into biomarkers, epigenetic regulation, and the molecular pathways that are involved in disorders related to stress.

7. BIBLIOGRAPHY

[1] A. F. Waters ve A. L. Copeland, "Behavioral Health After a Natural Disaster", içinde *The Intersection of Trauma and Disaster Behavioral Health*, 2021, ss. 285–295.

[2] R. Zhang, Y. Zhang, ve Z. Dai, "Impact of Natural Disasters on Mental Health: A Cross-Sectional Study Based on the 2014 China Family Panel Survey", *Int. J. Environ. Res. Public Health*, c. 19, sayı 5, 2022, doi: 10.3390/ijerph19052511.

[3] "UNICEF". https://www.unicef.org/protection/family-separation-during-crisis.

[4] K. L. K. Tamashiro ve R. R. Sakai, "Early postnatal and adolescent stress", içinde *Encyclopedia of Behavioral Neuroscience*, 2010.

[5] F. Delavari, V. Sheibani, S. Esmaeili-Mahani, ve N. Nakhaee, "Maternal Separation and the Risk of Drug Abuse in Later Life.", *Addict. Heal.*, c. 8, sayı 2, ss. 107–114, 2016.

[6] G. Dowse *vd.*, "Born into an isolating world: family-centred care for babies born to mothers with COVID-19", *eClinicalMedicine*, c. 56, s. 101822, 2023, doi: 10.1016/j.eclinm.2022.101822.

[7] Y. Bian *vd.*, "Prolonged Maternal Separation Induces the Depression-Like Behavior Susceptibility to Chronic Unpredictable Mild Stress Exposure in Mice", *Biomed Res. Int.*, c. 2021, 2021, doi: 10.1155/2021/6681397.

[8] K. Gapp *vd.*, "Early life stress in fathers improves behavioural flexibility in their offspring", *Nat. Commun.*, c. 5, 2014, doi: 10.1038/ncomms6466.

[9] M. I. Zalosnik, A. Pollano, V. Trujillo, M. M. Suárez, ve P. E. Durando, "Effect of maternal separation and chronic stress on hippocampal-dependent memory in young adult rats: evidence for the match-mismatch hypothesis", *Stress*, c. 17, sayı 5, ss. 445–450, 2014, doi: 10.3109/10253890.2014.936005.

[10] I. C. Weiss, T. B. Franklin, S. Vizi, ve I. M. Mansuy, "Inheritable effect of

unpredictable maternal separation on behavioral responses in mice", *Front. Behav. Neurosci.*, c. 5, sayı FEBRUARY, ss. 1–12, 2011, doi: 10.3389/fnbeh.2011.00003.

[11] Y. Yang, Z. Zhong, B. Wang, Y. Wang, ve W. Ding, "Activation of D1R signaling in the medial prefrontal cortex rescues maternal separation-induced behavioral deficits through restoration of excitatory neurotransmission", *Behav. Brain Res.*, c. 441, s. 114287, Mar. 2023, doi: 10.1016/J.BBR.2023.114287.

[12] Z. Jiang *vd.*, "H3K9me2 regulation of BDNF expression in the hippocampus and medial prefrontal cortex is involved in the depressive-like phenotype induced by maternal separation in male rats", *Psychopharmacology (Berl).*, c. 238, sayı 10, ss. 2801–2813, Eki. 2021, doi: 10.1007/S00213-021-05896-7.

[13] J. Huang, C. Shen, R. Ye, Y. Shi, ve W. Li, "The Effect of Early Maternal Separation Combined With Adolescent Chronic Unpredictable Mild Stress on Behavior and Synaptic Plasticity in Adult Female Rats", *Front. Psychiatry*, c. 12, sayı March, ss. 1–11, 2021, doi: 10.3389/fpsyt.2021.539299.

[14] L. Zhou *vd.*, "Long-term maternal separation potentiates depressive-like behaviours and neuroinflammation in adult male C57/BL6J mice", *Pharmacol. Biochem. Behav.*, c. 196, Eyl. 2020, doi: 10.1016/J.PBB.2020.172953.

[15] K. Gapp *vd.*, "Potential of environmental enrichment to prevent transgenerational effects of paternal trauma", *Neuropsychopharmacology*, c. 41, sayı 11, ss. 2749–2758, 2016, doi: 10.1038/npp.2016.87.

[16] M. Asadullah, S. N. Khan, H. M. Safdar, R. A. Aslam, ve I. Shaukat, "Sustainability and Development of Aquaponics System: a Review", *Earth Sci. Pakistan*, c. 4, sayı 2, ss. 78–80, 2020, doi: 10.26480/esp.02.2020.78.80.

[17] C. Shu, L. Xiao, J. Tang, G. Wang, X. Zhang, ve X. Wang, "Blunted behavioral and molecular responses to chronic mild stress in adult rats with experience of infancy maternal separation", *Tohoku J. Exp. Med.*, c. 235, sayı 2, ss. 18–87, Oca. 2015, doi: 10.1620/TJEM.235.81.

[18] J. J. Kim ve D. M. Diamond, "The stressed hippocampus, synaptic plasticity

and lost memories", *Nat. Rev. Neurosci. 2002 36*, c. 3, sayı 6, ss. 453–462, 2002, doi: 10.1038/nrn849.

[19] S. Dai *vd.*, "Chronic Stress Promotes Cancer Development", *Front. Oncol.*, c. 10, sayı August, ss. 1–10, 2020, doi: 10.3389/fonc.2020.01492.

[20] Y. M. Ulrich-Lai ve J. P. Herman, "Neural regulation of endocrine and autonomic stress responses", *Nat. Rev. Neurosci.*, c. 10, sayı 6, ss. 397–409, 2009, doi: 10.1038/nrn2647.

[21] C. Alonso *vd.*, "Maladaptive Intestinal Epithelial Responses to Life Stress May Predispose Healthy Women to Gut Mucosal Inflammation", *Gastroenterology*, c. 135, sayı 1, 2008, doi: 10.1053/j.gastro.2008.03.036.

[22] C. Menard *vd.*, "Social stress induces neurovascular pathology promoting depression", *Nat. Neurosci.*, c. 20, sayı 12, ss. 1752–1760, Ara. 2017, doi: 10.1038/s41593-017-0010-3.

[23] H. Song *vd.*, "Association of Stress-Related Disorders with Subsequent Neurodegenerative Diseases", *JAMA Neurol.*, c. 77, sayı 6, ss. 700–709, Haz. 2020, doi: 10.1001/jamaneurol.2020.0117.

[24] B. S. McEwen ve T. Seeman, "Protective and damaging effects of mediators of stress. Elaborating and testing the concepts of allostasis and allostatic load", *Ann. N. Y. Acad. Sci.*, c. 896, ss. 30–47, 1999, doi: 10.1111/J.1749-6632.1999.TB08103.X.

[25] J. P. Herman *vd.*, "Regulation of the hypothalamic-pituitary-adrenocortical stress response", c. 6, sayı 2, ss. 603–621, 2016, doi: 10.1002/cphy.c150015.Regulation.

[26] R. Jankord ve J. P. Herman, "LIMBIC REGULATION OF HYPOTHALAMO-PITUITARYADRENOCORTICAL FUNCTION DURING ACUTE AND CHRONIC STRESS", ss. 64–73, 2009, doi: 10.1196/annals.1410.012.LIMBIC.

[27] H. J. Krugers, C. C. Hoogenraad, ve L. Groc, "Stress hormones and AMPA receptor trafficking in synaptic plasticity and memory", *Nat. Rev. Neurosci.*, c. 11, sayı 10, ss. 675–681, 2010, doi: 10.1038/nrn2913.

[28] Y. Lin *vd.*, "Effects of prenatal and postnatal maternal emotional stress on toddlers' cognitive and temperamental development", *J. Affect. Disord.*, c. 207, ss. 9–17, Oca. 2017, doi: 10.1016/J.JAD.2016.09.010.

[29] K. Koutra, L. Chatzi, M. Bagkeris, M. Vassilaki, P. Bitsios, ve M. Kogevinas, "Antenatal and postnatal maternal mental health as determinants of infant neurodevelopment at 18 months of age in a mother-child cohort (Rhea Study) in Crete, Greece", *Soc. Psychiatry Psychiatr. Epidemiol.*, c. 48, sayı 8, ss. 1335–1345, 2013, doi: 10.1007/s00127-012-0636-0.

[30] T. B. Franklin, N. Linder, H. Russig, B. Thöny, ve I. M. Mansuy, "Influence of early stress on social abilities and serotonergic functions across generations in mice", *PLoS One*, c. 6, sayı 7, ss. 1–7, 2011, doi: 10.1371/journal.pone.0021842.

[31] G. Van Steenwyk, M. Roszkowski, F. Manuella, T. B. Franklin, ve I. M. Mansuy, "Transgenerational inheritance of behavioral and metabolic effects of paternal exposure to traumatic stress in early postnatal life: Evidence in the 4th generation", *Environ. Epigenetics*, c. 4, sayı 2, ss. 1–8, 2018, doi: 10.1093/eep/dvy023.

[32] M. Ambeskovic, T. J. Roseboom, ve G. A. S. Metz, "Transgenerational effects of early environmental insults on aging and disease incidence", *Neuroscience and Biobehavioral Reviews*, c. 117. Elsevier Ltd, ss. 297–316, Eki. 2020, doi: 10.1016/j.neubiorev.2017.08.002.

[33] S. Mahurkar-Joshi ve L. Chang, "Epigenetic Mechanisms in Irritable Bowel Syndrome", *Front. Psychiatry*, c. 11, sayı August, ss. 1–15, 2020, doi: 10.3389/fpsyt.2020.00805.

[34] P. J. Dexheimer ve L. Cochella, "MicroRNAs: From Mechanism to Organism", *Front. Cell Dev. Biol.*, c. 8, sayı June, ss. 1–18, 2020, doi: 10.3389/fcell.2020.00409.

[35] K. K. Bayram *vd.*, "Gene expression of mouse hippocampal stem cells grown in a galactose-derived molecular gel compared to in vivo and neurospheres", *Processes*, c. 9, sayı 4, 2021, doi: 10.3390/pr9040716.

[36] Y. Lee *vd.*, "MicroRNA genes are transcribed by RNA polymerase II", *EMBO*

J., c. 23, sayı 20, ss. 4051–4060, 2004, doi: 10.1038/sj.emboj.7600385.

[37] Y. Lee *vd.*, "The nuclear RNase III Drosha initiates microRNA processing", *Nature*, c. 425, sayı 6956, ss. 415–419, Eyl. 2003, doi: 10.1038/NATURE01957.

[38] E. Lund, S. Güttinger, A. Calado, J. E. Dahlberg, ve U. Kutay, "Nuclear export of microRNA precursors", *Science*, c. 303, sayı 5654, ss. 95–98, Oca. 2004, doi: 10.1126/SCIENCE.1090599.

[39] H. Zhang, F. A. Kolb, L. Jaskiewicz, E. Westhof, ve W. Filipowicz, "Single processing center models for human Dicer and bacterial RNase III", *Cell*, c. 118, sayı 1, ss. 57–68, Tem. 2004, doi: 10.1016/j.cell.2004.06.017.

[40] S. Cheloufi, C. O. Dos Santos, M. M. W. Chong, ve G. J. Hannon, "A dicer-independent miRNA biogenesis pathway that requires Ago catalysis", *Nat. 2010 4657298*, c. 465, sayı 7298, ss. 584–589, Nis. 2010, doi: 10.1038/nature09092.

[41] S. L. Ameres ve P. D. Zamore, "Diversifying microRNA sequence and function", *Nat. Rev. Mol. Cell Biol.*, c. 14, sayı 8, ss. 475–488, 2013, doi: 10.1038/nrm3611.

[42] A. Lujambio ve S. W. Lowe, "The microcosmos of cancer", *Nat. 2012 4827385*, c. 482, sayı 7385, ss. 347–355, Şub. 2012, doi: 10.1038/nature10888.

[43] Y. Cai, X. Yu, S. Hu, ve J. Yu, "A brief review on the mechanisms of miRNA regulation", *Genomics. Proteomics Bioinformatics*, c. 7, sayı 4, ss. 147–154, Ara. 2009, doi: 10.1016/S1672-0229(08)60044-3.

[44] M. Mutlu *vd.*, "miR-564 acts as a dual inhibitor of PI3K and MAPK signaling networks and inhibits proliferation and invasion in breast cancer", *Sci. Reports 2016 61*, c. 6, sayı 1, ss. 1–14, Eyl. 2016, doi: 10.1038/srep32541.

[45] H. Seitz, "Redefining MicroRNA Targets", *Curr. Biol.*, c. 19, sayı 10, ss. 870–873, 2009, doi: 10.1016/j.cub.2009.03.059.

[46] C. D. Berdanier ve H. C. Freake, "Nutrient-gene interactions | Molecular aspects", *Encycl. Hum. Nutr.*, ss. 269–276, 2005, doi: 10.1016/B0-12-226694-

3/02291-2.

[47] C. Buccitelli ve M. Selbach, “mRNAs, proteins and the emerging principles of gene expression control”, *Nat. Rev. Genet. 2020 2110*, c. 21, sayı 10, ss. 630–644, Tem. 2020, doi: 10.1038/s41576-020-0258-4.

[48] K. G. Le Roch ve E. A. Winzeler, “Genome-Wide Analysis of Gene Expression”, *Encycl. Biol. Chem.*, ss. 175–180, 2004, doi: 10.1016/B0-12-443710-9/00264-7.

[49] K. Sikand, J. Singh, J. S. Ebron, ve G. C. Shukla, “Housekeeping Gene Selection Advisory: Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and β-Actin Are Targets of miR-644a”, *PLoS One*, c. 7, sayı 10, Eki. 2012, doi: 10.1371/JOURNAL.PONE.0047510.

[50] F. Freudenberg, “Quantitative analysis of Gria1, Gria2, Dlg1 and Dlg4 expression levels in hippocampus following forced swim stress in mice”, *Sci. Rep.*, c. 9, sayı 1, ss. 1–8, 2019, doi: 10.1038/s41598-019-50689-w.

[51] N. Vuokila *vd.*, “Chronic Regulation of miR-124-3p in the Perilesional Cortex after Experimental and Human TBI”, *Int. J. Mol. Sci. 2020, Vol. 21, Page 2418*, c. 21, sayı 7, s. 2418, Mar. 2020, doi: 10.3390/IJMS21072418.

[52] C. Miao ve J. Chang, “The important roles of microRNAs in depression: new research progress and future prospects”, *J. Mol. Med.*, c. 99, sayı 5, ss. 619–636, 2021, doi: 10.1007/s00109-021-02052-8.

[53] F. Higuchi *vd.*, “Hippocampal MicroRNA-124 Enhances Chronic Stress Resilience in Mice”, *J. Neurosci.*, c. 36, sayı 27, s. 7253, Tem. 2016, doi: 10.1523/JNEUROSCI.0319-16.2016.

[54] K. Hammoud, N. Spittler, K. Buch, R. Hakimelahi, J. Nguyen, ve M. Lanfranchi, “Camping Out in the Hippocampus: Imaging Spectrum of Etiologies That Affect the Hippocampi and Tips for Honing the Differential Diagnosis”, *Neurographics*, c. 9, sayı 1, ss. 37–54, 2019, doi: 10.3174/ng.1800032.

[55] P. Chauhan, K. Jethwa, A. Rathawa, G. Chauhan, ve S. Mehra, “The Anatomy of the Hippocampus”, *Cereb. Ischemia*, sayı November, ss. 17–30, 2021, doi:

10.36255/exonpublications.cerebralischemia.2021.hippocampus.

[56] B. Gunnarsdóttir, V. Zerbi, ve C. Kelly, “Multimodal gradient mapping of rodent hippocampus”, *Neuroimage*, c. 253, sayı March, 2022, doi: 10.1016/j.neuroimage.2022.119082.

[57] V. Costa, S. Lugert, ve R. Jagasia, *Role of adult hippocampal neurogenesis in cognition in physiology and disease: pharmacological targets and biomarkers*, c. 228. 2015.

[58] E. J. Kim, B. Pellman, ve J. J. Kim, “Stress effects on the hippocampus: A critical review”, *Learn. Mem.*, c. 22, sayı 9, ss. 411–416, 2015, doi: 10.1101/lm.037291.114.

[59] D. M. Lovinger, “Communication Networks in the Brain: Neurons, Receptors, Neurotransmitters, and Alcohol”, *Alcohol Res. Heal.*, c. 31, sayı 3, s. 196, 2008.

[60] P. Dale, A. GJ, ve F. D, *Neuroscience. 2nd edition*. 2001.

[61] A. E. Pereda, “Electrical synapses and their functional interactions with chemical synapses”, *Nat. Rev. Neurosci.*, c. 15, sayı 4, ss. 250–263, 2014, doi: 10.1038/nrn3708.

[62] J. Cuevas, “Neurotransmitters and Their Life Cycle”, *Ref. Modul. Biomed. Sci.*, Oca. 2019, doi: 10.1016/B978-0-12-801238-3.11318-2.

[63] R. Knapp ve H. I. Yamamura, “Neurotransmitter Receptors”, içinde *Encyclopedia of the Neurological Sciences*, 2003.

[64] D. Kim ve J. S. Lee, “Neurotransmitter-Induced Excitatory and Inhibitory Functions in Artificial Synapses”, *Adv. Funct. Mater.*, c. 32, sayı 21, s. 2200497, May. 2022, doi: 10.1002/ADFM.202200497.

[65] D. Purves *vd.*, “Glutamate”, 2001.

[66] Y. Zhou ve N. C. Danbolt, “Glutamate as a neurotransmitter in the healthy brain”, *J. Neural Transm.*, c. 121, sayı 8, ss. 799–817, 2014, doi: 10.1007/s00702-014-1180-8.

[67] M. M. Pal, “Glutamate: The Master Neurotransmitter and Its Implications in

Chronic Stress and Mood Disorders", *Front. Hum. Neurosci.*, c. 15, sayı October, ss. 1–4, 2021, doi: 10.3389/fnhum.2021.722323.

[68] D. Purves *vd.*, "Glutamate Receptors", 2001.

[69] S. G. Cull-Candy ve M. Farrant, "Ca2+-permeable AMPA receptors and their auxiliary subunits in synaptic plasticity and disease", *J. Physiol.*, c. 599, sayı 10, ss. 2655–2671, 2021, doi: 10.1113/JP279029.

[70] D. Jane, "AMPA glutamate receptor", *xPharm Compr. Pharmacol. Ref.*, ss. 1–19, 2007, doi: 10.1016/B978-008055232-3.60374-5.

[71] Q. Gan, C. L. Salussolia, ve L. P. Wollmuth, "Assembly of AMPA receptors: Mechanisms and regulation", *J. Physiol.*, c. 593, sayı 1, ss. 39–48, 2015, doi: 10.1113/jphysiol.2014.273755.

[72] S. F. Traynelis *vd.*, "Glutamate receptor ion channels: Structure, regulation, and function", *Pharmacol. Rev.*, c. 62, sayı 3, ss. 405–496, 2010, doi: 10.1124/pr.109.002451.

[73] J. T. R. Isaac, M. Ashby, ve C. J. McBain, "The role of the GluR2 subunit in AMPA receptor function and synaptic plasticity", *Neuron*, c. 54, sayı 6, ss. 859–871, Haz. 2007, doi: 10.1016/J.NEURON.2007.06.001.

[74] S. Cull-Candy, L. Kelly, ve M. Farrant, "Regulation of Ca2+-permeable AMPA receptors: synaptic plasticity and beyond", *Curr. Opin. Neurobiol.*, c. 16, sayı 3, ss. 288–297, Haz. 2006, doi: 10.1016/J.CONB.2006.05.012.

[75] J. R. P. Geiger *vd.*, "Relative abundance of subunit mRNAs determines gating and Ca2+ permeability of AMPA receptors in principal neurons and interneurons in rat CNS", *Neuron*, c. 15, sayı 1, ss. 193–204, 1995, doi: 10.1016/0896-6273(95)90076-4.

[76] J. Kumar ve M. L. Mayer, "Functional Insights from Glutamate Receptor Ion Channel Structures", *Annu. Rev. Physiol.*, c. 75, s. 313, Şub. 2013, doi: 10.1146/ANNUREV-PHYSIOL-030212-183711.

[77] F. Conti ve R. J. Weinberg, "Shaping excitation at glutamatergic synapses", *Trends Neurosci.*, c. 22, sayı 10, ss. 451–458, 1999, doi: 10.1016/S0166-2236(99)01445-9.

[78] A. J. R. Plested, “Glutamate receptor complexes open up and reveal their molecular dialect”, *News views Struct.*, c. 594, ss. 338–339, 2021.

[79] M. J. Kennedy ve M. D. Ehlers, “Organelles and Trafficking Machinery for Postsynaptic Plasticity”, *Annu. Rev. Neurosci.*, c. 29, s. 325, 2006, doi: 10.1146/ANNUREV.NEURO.29.051605.112808.

[80] J. Yu, P. Rao, S. Clark, J. Mitra, T. Ha, ve E. Gouaux, “Hippocampal AMPA receptor assemblies and mechanism of allosteric inhibition”, *Nature*, c. 594, sayı 7863, ss. 448–453, 2021, doi: 10.1038/s41586-021-03540-0.

[81] J. G. Hanley, “Subunit-specific trafficking mechanisms regulating the synaptic expression of Ca2+-permeable AMPA receptors”, *Semin. Cell Dev. Biol.*, c. 27, ss. 14–22, 2014, doi: 10.1016/j.semcdb.2013.12.002.

[82] D. Wegrzyn, G. Juckel, ve A. Faissner, “Structural and Functional Deviations of the Hippocampus in Schizophrenia and Schizophrenia Animal Models”, *Int. J. Mol. Sci.*, c. 23, sayı 10, 2022, doi: 10.3390/ijms23105482.

[83] S. Bahari-Javan *vd.*, “HDAC1 links early life stress to schizophrenialike phenotypes”, *Proc. Natl. Acad. Sci. U. S. A.*, c. 114, sayı 23, ss. E4686–E4694, 2017, doi: 10.1073/pnas.1613842114.

[84] L. Fregna, F. Seghi, M. Locatelli, ve C. Colombo, “The impact of early-life stress in the development and course of bipolar disorder: Mechanisms and implications”, *Neurobiol. Bipolar Disord. Road to Nov. Ther.*, ss. 303–318, Oca. 2021, doi: 10.1016/B978-0-12-819182-8.00027-2.

[85] H. Namkung *vd.*, “The miR-124-AMPAR pathway connects polygenic risks with behavioral changes shared between schizophrenia and bipolar disorder”, *Neuron*, c. 111, sayı 2, ss. 220-235.e9, Oca. 2023, doi: 10.1016/J.NEURON.2022.10.031.

[86] H. Fan ve P. Hegde, “The Transcriptome in Blood: Challenges and Solutions for Robust Expression Profiling”, *Curr. Mol. Med.*, c. 5, sayı 1, ss. 3–10, 2005, doi: 10.2174/1566524053152861.

[87] H. Nakamura, “BALB/c Mouse”, *Brenner’s Encycl. Genet. Second Ed.*, ss. 290–292, Şub. 2013, doi: 10.1016/B978-0-12-374984-0.00133-9.

[88] H. Okamura *vd.*, "Long-Term Effects of Repeated Social Defeat Stress on Brain Activity during Social Interaction in BALB/c Mice", *eNeuro*, c. 9, sayı 3, May. 2022, doi: 10.1523/ENEURO.0068-22.2022.

[89] R. J. Servatius, G. Salameh, K. M. Coyle, ve W. P. Paré, "Restraint Stress", *Encycl. Stress*, ss. 389–390, 2007, doi: 10.1016/B978-012373947-6.00333-0.

[90] R. C. Noland, *Exercise and Regulation of Lipid Metabolism*, 1. baskı, c. 135. Elsevier Inc., 2015.

[91] M. PORSOLT, R., LE PICHON, M. & JALFRE, "Depression: a new animal model sensitive to antidepressant treatments.", *Nature*, 1977.

[92] D. C. Hoaglin ve B. Iglewicz, "Fine-tuning some resistant rules for outlier labeling", *J. Am. Stat. Assoc.*, c. 82, sayı 400, ss. 1147–1149, 1987, doi: 10.1080/01621459.1987.10478551.

[93] M. M. Mukaka, "Statistics corner: A guide to appropriate use of correlation coefficient in medical research", *Malawi Med. J.*, c. 24, sayı 3, ss. 69–71, 2012.

[94] B. König ve H. Markl, "Maternal care in house mice", *Behav. Ecol. Sociobiol.*, c. 20, sayı 1, ss. 1–9, 1987, doi: 10.1007/bf00292161.

[95] T. Londei, M. Calci, ve V. G. Leone, "First experiences with solid food by mouse pups: Effects of age and the presence of the mother", *Bolletino di Zool.*, c. 55, sayı 1–4, ss. 155–159, 1988, doi: 10.1080/11250008809386612.

[96] F. C. Vilela, J. S. Vieira, A. Giusti-Paiva, ve M. L. da Silva, "Experiencing early life maternal separation increases pain sensitivity in adult offspring", *Int. J. Dev. Neurosci.*, c. 62, sayı 2600, ss. 8–14, 2017, doi: 10.1016/j.ijdevneu.2017.07.003.

[97] H. Toda *vd.*, "Maternal separation enhances conditioned fear and decreases the mRNA levels of the neurotensin receptor 1 gene with hypermethylation of this gene in the rat amygdala", *PLoS One*, c. 9, sayı 5, 2014, doi: 10.1371/journal.pone.0097421.

[98] E. K. Adam, M. E. Quinn, R. Tavernier, M. T. McQuillan, K. A. Dahlke, ve K. E. Gilbert, "Diurnal cortisol slopes and mental and physical health

outcomes: A systematic review and meta-analysis”, *Psychoneuroendocrinology*, c. 83, ss. 25–41, Eyl. 2017, doi: 10.1016/J.PSYNEUEN.2017.05.018.

[99] S. Steinlechner, “Biological Rhythms of the Mouse”, *Lab. Mouse*, ss. 383–407, 2012, doi: 10.1016/B978-0-12-382008-2.00017-9.

[100] J. D’Agostino, G. F. Vaeth, ve S. J. Henning, “Diurnal rhythm of total and free concentrations of serum corticosterone in the rat”, *Acta Endocrinol. (Copenh).*, c. 100, sayı 1, ss. 85–90, 1982, doi: 10.1530/acta.0.1000085.

[101] H. M. Savignac, T. G. Dinan, ve J. F. Cryan, “Resistance to early-life stress in mice: Effects of genetic background and stress duration”, *Front. Behav. Neurosci.*, sayı APRIL, 2011, doi: 10.3389/fnbeh.2011.00013.

[102] Y. Zhang *vd.*, “Comparison of the chronic unpredictable mild stress and the maternal separation in mice postpartum depression modeling”, *Biochem. Biophys. Res. Commun.*, c. 632, ss. 24–31, Ara. 2022, doi: 10.1016/J.BBRC.2022.09.063.

[103] P. Gao *vd.*, “Maternal stress affects postnatal growth and the pituitary expression of prolactin in mouse offspring”, *J. Neurosci. Res.*, c. 89, sayı 3, ss. 329–340, 2011, doi: 10.1002/jnr.22550.

[104] T. Nishigawa, S. Nagamachi, ve H. Ikeda, “Restraint stress in lactating mice alters the levels of sulfur-containing amino acids in milk”, ss. 503–509, 2018, doi: 10.1292/jvms.17-0661.

[105] S. Choi *vd.*, *Corticosterone inhibits GAS6 to govern hair follicle stem-cell quiescence*, c. 592, sayı 7854. Springer US, 2021.

[106] X. G. Pappalardo *vd.*, “Early Life Stress (ELS) Effects on Fetal and Adult Bone Development”, *Children*, c. 10, sayı 1, 2023, doi: 10.3390/children10010102.

[107] K. Wuertz-Kozak *vd.*, “Effects of early life stress on bone homeostasis in mice and humans”, *Int. J. Mol. Sci.*, c. 21, sayı 18, ss. 1–25, 2020, doi: 10.3390/ijms21186634.

[108] R. J. G. Hartman, M. Mokry, G. Pasterkamp, ve H. M. den Ruijter, “Sex-

dependent gene co-expression in the human body", *Sci. Rep.*, c. 11, sayı 1, ss. 1–10, 2021, doi: 10.1038/s41598-021-98059-9.

[109] F. Freudenberg, T. Celikel, ve A. Reif, "The role of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in depression: central mediators of pathophysiology and antidepressant activity?", *Neurosci. Biobehav. Rev.*, c. 52, ss. 193–206, May. 2015, doi: 10.1016/J.NEUBIOREV.2015.03.005.

[110] V. Ismail *vd.*, "Identification and functional evaluation of GRIA1 missense and truncation variants in individuals with ID: An emerging neurodevelopmental syndrome", *Am. J. Hum. Genet.*, c. 109, sayı 7, ss. 1217–1241, 2022, doi: 10.1016/j.ajhg.2022.05.009.

[111] W. Lu *vd.*, "Subunit composition of synaptic AMPA receptors revealed by a single-cell genetic approach", *Neuron*, c. 62, sayı 2, ss. 254–268, Nis. 2009, doi: 10.1016/J.NEURON.2009.02.027.

[112] T. Soga, S. Nakajima, M. Kawaguchi, ve I. S. Parhar, "Repressor element 1 silencing transcription factor /neuron-restrictive silencing factor (REST/NRSF) in social stress and depression", *Prog. Neuro-Psychopharmacology Biol. Psychiatry*, c. 104, s. 110053, Oca. 2021, doi: 10.1016/J.PNPBP.2020.110053.

[113] C. Xu *vd.*, "Repressor element-1 silencing transcription factor regulates glutamate receptors and immediate early genes to affect synaptic plasticity", *Aging (Albany NY)*, c. 13, sayı 11, s. 15569, Haz. 2021, doi: 10.18632/AGING.203118.

[114] V. Kreouzis, G.-L. Chen, ve G. M. Miller, "Perturbations of Neuron-Restrictive Silencing Factor Modulate Corticotropin-Releasing Hormone Gene Expression in the Human Cell Line BeWo", *Complex Psychiatry*, c. 4, sayı 2, ss. 100–110, 2018, doi: 10.1159/000492635.

[115] S. Uchida *vd.*, "Early life stress enhances behavioral vulnerability to stress through the activation of REST4-mediated gene transcription in the medial prefrontal cortex of rodents", *J. Neurosci.*, c. 30, sayı 45, ss. 15007–15018, 2010, doi: 10.1523/JNEUROSCI.1436-10.2010.

[116] C. E. Monaghan *vd.*, "REST corepressors RCOR1 and RCOR2 and the repressor INSM1 regulate the proliferation- differentiation balance in the developing brain", *Proc. Natl. Acad. Sci. U. S. A.*, c. 114, sayı 3, ss. E406–E415, 2017, doi: 10.1073/pnas.1620230114.

[117] J. Visvanathan, S. Lee, B. Lee, J. W. Lee, ve S. K. Lee, "The microRNA miR-124 antagonizes the anti-neural REST/SCP1 pathway during embryonic CNS development", *Genes Dev.*, c. 21, sayı 7, s. 744, Nis. 2007, doi: 10.1101/GAD.1519107.

[118] K. Fabricius, G. Wörtwein, ve B. Pakkenberg, "The impact of maternal separation on adult mouse behaviour and on the total neuron number in the mouse hippocampus", *Brain Struct. Funct.*, c. 212, sayı 5, ss. 403–416, 2008, doi: 10.1007/s00429-007-0169-6.

[119] F. Higuchi *vd.*, "Hippocampal microRNA-124 enhances chronic stress resilience in mice", *J. Neurosci.*, c. 36, sayı 27, ss. 7253–7267, 2016, doi: 10.1523/JNEUROSCI.0319-16.2016.

[120] E. Brivio, J. P. Lopez, ve A. Chen, "Sex differences: Transcriptional signatures of stress exposure in male and female brains", *Genes, Brain Behav.*, c. 19, sayı 3, s. e12643, Mar. 2020, doi: 10.1111/GBB.12643.

[121] V. V. Reshetnikov, A. A. Lepeshko, Y. A. Ryabushkina, A. A. Studenikina, T. I. Merkulova, ve N. P. Bondar, "The Long-Term Effects of Early Postnatal Stress on Cognitive Abilities and Expression of Genes of the Glutamatergic System in Mice", *Neurochem. J.*, c. 12, sayı 2, ss. 142–151, 2018, doi: 10.1134/s1819712418020095.

[122] S. Katsouli, A. Stamatakis, P. Giompres, E. D. Kouvelas, F. Stylianopoulou, ve A. Mitsacos, "Sexually dimorphic long-term effects of an early life experience on AMPA receptor subunit expression in rat brain", *Neuroscience*, c. 257, ss. 49–64, 2014, doi: 10.1016/j.neuroscience.2013.10.073.

[123] O. Pös, O. Biró, T. Szemes, ve B. Nagy, "Circulating cell-free nucleic acids: Characteristics and applications", *Eur. J. Hum. Genet.*, c. 26, sayı 7, ss. 937–945, 2018, doi: 10.1038/s41431-018-0132-4.

[124] W. G. Mayhan ve S. P. Didion, "Glutamate-Induced Disruption of the Blood-

Brain Barrier in Rats", *Stroke*, c. 27, sayı 5, ss. 965–970, 1996, doi: 10.1161/01.STR.27.5.965.

[125] I. D. Coombs, C. Bats, C. A. Sexton, D. Studniarczyk, S. G. Cull-Candy, ve M. Farrant, "Enhanced functional detection of synaptic calcium-permeable AMPA receptors using intracellular NASPM.", *Elife*, c. 12, Nis. 2023, doi: 10.7554/ELIFE.66765.

[126] J. Rogers, "A shared pathway connects schizophrenia and bipolar disorder", *Nat. Rev. Neurosci.*, c. 24, sayı 1, s. 2, 2023, doi: 10.1038/s41583-022-00662-w.

[127] J. Tucholski, M. S. Simmons, A. L. Pinner, V. Haroutunian, R. E. McCullumsmith, ve J. H. Meador-Woodruff, "Abnormal N-linked glycosylation of cortical AMPA receptor subunits in schizophrenia", *Schizophr. Res.*, c. 146, sayı 1–3, ss. 177–183, 2013, doi: 10.1016/j.schres.2013.01.031.

[128] H. Namkung *vd.*, "The miR-124-AMPAR pathway connects polygenic risks with behavioral changes shared between schizophrenia and bipolar disorder", *Neuron*, c. 111, sayı 2, ss. 220-235.e9, 2023, doi: 10.1016/j.neuron.2022.10.031.

[129] J. M. Henley ve K. A. Wilkinson, "Synaptic AMPA receptor composition in development, plasticity and disease", *Nat. Rev. Neurosci. 2016 176*, c. 17, sayı 6, ss. 337–350, Nis. 2016, doi: 10.1038/nrn.2016.37.

[130] B. Roy, M. Dunbar, R. C. Shelton, ve Y. Dwivedi, "Identification of MicroRNA-124-3p as a Putative Epigenetic Signature of Major Depressive Disorder", *Neuropsychopharmacology*, c. 42, sayı 4, ss. 864–875, 2017, doi: 10.1038/npp.2016.175.

[131] N. V. Luchkina *vd.*, "Molecular mechanisms controlling synaptic recruitment of GluA4 subunit-containing AMPA-receptors critical for functional maturation of CA1 glutamatergic synapses", *Neuropharmacology*, c. 112, ss. 46–56, Oca. 2017, doi: 10.1016/J.NEUROPHARM.2016.04.049.

8. APPENDIX

APPENDIX-1. Ethical statement for animal model studies

APPENDIX-2. Copyright permission

SPRINGER NATURE LICENSE
TERMS AND CONDITIONS

Jun 10, 2023

This Agreement between Ankara Yıldırım Beyazıt University -- Aida Nurul Barokah ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.

License Number	5565300407340
License date	Jun 10, 2023
Licensed Content Publisher	Springer Nature
Licensed Content Publication	Nature Reviews Molecular Cell Biology
Licensed Content Title	Diversifying microRNA sequence and function
Licensed Content Author	Stefan L. Ameres et al
Licensed Content Date	Jun 26, 2013
Type of Use	Thesis/Dissertation
Requestor type	academic/university or research institute
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Would you like a high resolution image with your order?	no
Will you be translating?	no

Circulation/distribution	500 - 999
Author of this Springer Nature content	no
Title	INVESTIGATION of mRNA EXPRESSION LEVELS of AMPA RECEPTOR GENES in MICE EXPOSED TO MATERNAL STRESS
Institution name	Ankara Yıldırım Beyazıt University
Expected presentation date	Jun 2023
Portions	Figure 1: MicroRNA biogenesis.
Requestor Location	Ankara Yıldırım Beyazıt University Ayvalı Mah. 150. Sok. B-Blok -2. Kat Etlik-Keçiören/ Ankara Ankara, Keçiören 06010 Turkey Attn: Mrs. Aida Nurul Barokah
Total	0.00 USD

Terms and Conditions

Springer Nature Customer Service Centre GmbH Terms and Conditions

The following terms and conditions ("Terms and Conditions") together with the terms specified in your [RightsLink] constitute the License ("License") between you as Licensee and Springer Nature Customer Service Centre GmbH as Licensor. By clicking 'accept' and completing the transaction for your use of the material ("Licensed Material"), you confirm your acceptance of and obligation to be bound by these Terms and Conditions.

1. Grant and Scope of License

1. 1. The Licensor grants you a personal, non-exclusive, non-transferable, non-sublicensable, revocable, world-wide License to reproduce, distribute, communicate to the public, make available, broadcast, electronically transmit or create derivative works using the Licensed Material for the purpose(s) specified in your RightsLink Licence Details only. Licenses are granted for the specific use requested in the order and for no other use, subject to these Terms and Conditions. You acknowledge and agree that the rights granted to you under this License do not include the right to modify, edit, translate, include in collective works, or create derivative works of the Licensed Material in whole or in part unless expressly stated in your RightsLink Licence Details. You may use the Licensed Material only as permitted under this Agreement and will not reproduce, distribute, display, perform, or otherwise use or

exploit any Licensed Material in any way, in whole or in part, except as expressly permitted by this License.

1. 2. You may only use the Licensed Content in the manner and to the extent permitted by these Terms and Conditions, by your RightsLink Licence Details and by any applicable laws.

1. 3. A separate license may be required for any additional use of the Licensed Material, e.g. where a license has been purchased for print use only, separate permission must be obtained for electronic re-use. Similarly, a License is only valid in the language selected and does not apply for editions in other languages unless additional translation rights have been granted separately in the License.

1. 4. Any content within the Licensed Material that is owned by third parties is expressly excluded from the License.

1. 5. Rights for additional reuses such as custom editions, computer/mobile applications, film or TV reuses and/or any other derivative rights requests require additional permission and may be subject to an additional fee. Please apply to journalpermissions@springernature.com or bookpermissions@springernature.com for these rights.

2. Reservation of Rights

Licensor reserves all rights not expressly granted to you under this License. You acknowledge and agree that nothing in this License limits or restricts Licensor's rights in or use of the Licensed Material in any way. Neither this License, nor any act, omission, or statement by Licensor or you, conveys any ownership right to you in any Licensed Material, or to any element or portion thereof. As between Licensor and you, Licensor owns and retains all right, title, and interest in and to the Licensed Material subject to the license granted in Section 1.1. Your permission to use the Licensed Material is expressly conditioned on you not impairing Licensor's or the applicable copyright owner's rights in the Licensed Material in any way.

3. Restrictions on use

3. 1. Minor editing privileges are allowed for adaptations for stylistic purposes or formatting purposes provided such alterations do not alter the original meaning or intention of the Licensed Material and the new figure(s) are still accurate and representative of the Licensed Material. Any other changes including but not limited to, cropping, adapting, and/or omitting material that affect the meaning, intention or moral rights of the author(s) are strictly prohibited.

3. 2. You must not use any Licensed Material as part of any design or trademark.

3. 3. Licensed Material may be used in Open Access Publications (OAP), but any such reuse must include a clear acknowledgment of this permission visible at the same time as the figures/tables/illustration or abstract and which must indicate that the Licensed Material is not part of the governing OA license but has been reproduced with permission. This may be indicated according to any standard referencing system but must include at a minimum 'Book/Journal title, Author, Journal Name (if applicable), Volume (if applicable), Publisher, Year, reproduced with permission from SNCSC'.

4. STM Permission Guidelines

4. 1. An alternative scope of license may apply to signatories of the STM Permissions Guidelines ("STM PG") as amended from time to time and made available at

https://www.stm-assoc.org/intellectual-property/permissions/permissions-guidelines/.

4. 2. For content reuse requests that qualify for permission under the STM PG, and which may be updated from time to time, the STM PG supersede the terms and conditions contained in this License.

4. 3. If a License has been granted under the STM PG, but the STM PG no longer apply at the time of publication, further permission must be sought from the Rightsholder. Contact journalpermissions@springernature.com or bookpermissions@springernature.com for these rights.

5. Duration of License

5. 1. Unless otherwise indicated on your License, a License is valid from the date of purchase ("License Date") until the end of the relevant period in the below table:

Reuse in a medical communications project	Reuse up to distribution or time period indicated in License
Reuse in a dissertation/thesis	Lifetime of thesis
Reuse in a journal/magazine	Lifetime of journal/magazine
Reuse in a book/textbook	Lifetime of edition
Reuse on a website	1 year unless otherwise specified in the License
Reuse in a presentation/slide kit/poster	Lifetime of presentation/slide kit/poster. Note: publication whether electronic or in print of presentation/slide kit/poster may require further permission.
Reuse in conference proceedings	Lifetime of conference proceedings
Reuse in an annual report	Lifetime of annual report
Reuse in training/CME materials	Reuse up to distribution or time period indicated in License
Reuse in newsmedia	Lifetime of newsmedia
Reuse in coursepack/classroom materials	Reuse up to distribution and/or time period indicated in license

6. Acknowledgement

6. 1. The Licensor's permission must be acknowledged next to the Licensed Material in print. In electronic form, this acknowledgement must be visible at the same time as the figures/tables/illustrations or abstract and must be hyperlinked to the journal/book's homepage.

6. 2. Acknowledgement may be provided according to any standard referencing system and at a minimum should include "Author, Article/Book Title, Journal name/Book imprint, volume, page number, year, Springer Nature".

7. Reuse in a dissertation or thesis

7. 1. Where 'reuse in a dissertation/thesis' has been selected, the following terms apply: Print rights of the Version of Record are provided for; electronic rights for use only on institutional repository as defined by the Sherpa guideline (www.sherpa.ac.uk/romeo/) and only up to what is required by the awarding institution.

7. 2. For theses published under an ISBN or ISSN, separate permission is required. Please contact journalpermissions@springernature.com or bookpermissions@springernature.com for these rights.

7. 3. Authors must properly cite the published manuscript in their thesis according to current citation standards and include the following acknowledgement: '*Reproduced with permission from Springer Nature*'.

8. License Fee

You must pay the fee set forth in the License Agreement (the "License Fees"). All amounts payable by you under this License are exclusive of any sales, use, withholding, value added or similar taxes, government fees or levies or other assessments. Collection and/or remittance of such taxes to the relevant tax authority shall be the responsibility of the party who has the legal obligation to do so.

9. Warranty

9. 1. The Licensor warrants that it has, to the best of its knowledge, the rights to license reuse of the Licensed Material. **You are solely responsible for ensuring that the material you wish to license is original to the Licensor and does not carry the copyright of another entity or third party (as credited in the published version).** If the credit line on any part of the Licensed Material indicates that it was reprinted or adapted with permission from another source, then you should seek additional permission from that source to reuse the material.

9. 2. EXCEPT FOR THE EXPRESS WARRANTY STATED HEREIN AND TO THE EXTENT PERMITTED BY APPLICABLE LAW, LICENSOR PROVIDES THE LICENSED MATERIAL "AS IS" AND MAKES NO OTHER REPRESENTATION OR WARRANTY. LICENSOR EXPRESSLY DISCLAIMS ANY LIABILITY FOR ANY CLAIM ARISING FROM OR OUT OF THE CONTENT, INCLUDING BUT NOT LIMITED TO ANY ERRORS, INACCURACIES, OMISSIONS, OR DEFECTS CONTAINED THEREIN, AND ANY IMPLIED OR EXPRESS WARRANTY AS TO MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. IN NO EVENT SHALL LICENSOR BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, PUNITIVE, OR EXEMPLARY DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, VIEWING OR USE OF THE LICENSED MATERIAL REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION APPLIES NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

10. Termination and Cancellation

10. 1. The License and all rights granted hereunder will continue until the end of the applicable period shown in Clause 5.1 above. Thereafter, this license will be terminated and all rights granted hereunder will cease.

10. 2. Licensor reserves the right to terminate the License in the event that payment is not received in full or if you breach the terms of this License.