

INVESTIGATING BIOLOGICAL ROLE OF MIRNAS IN TAY-SACHS DISEASE MOUSE CELLS

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ABSTRACT

INVESTIGATING BIOLOGICAL ROLE OF MIRNAS IN TAY-SACHS DISEASE MOUSE CELLS

Tay-Sachs disease is a lysosomal storage disorder arising from the deficiency of β -N-acetylhexosaminidase A enzyme due to a mutated *HEXA* gene. Lysosomal accumulation of GM2 ganglioside and impaired autophagic flux were reported previously in a mouse model of Tay-Sachs disease, *Hexa*^{-/-}*Neu3*^{-/-}. Although several treatment strategies have been investigated, there is no curative treatment for Tay-Sachs disease. ML-SA1 is a lysosomal calcium channel agonist, and its ability to induce autophagy and exocytosis has been reported in various diseases. miRNAs are noncoding small RNAs that regulate gene expression post-transcriptionally. Their dysregulated expression was studied for several lysosomal storage disorders. However, there is no study about comprehensive miRNA profiling and ML-SA1 treatment on Tay-Sachs disease. Here, it was aimed to determine in-depth miRNA profiles of *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts and neuroglia in untreated and ML-SA1 treated conditions to identify specific miRNAs associated with disease pathology and to evaluate the effect of ML-SA1 on lysosomal exocytosis and impaired autophagy. Accordingly, next-generation sequencing alongside evaluations of enzymatic activity, protein, and gene expression of autophagy and exocytosis markers were performed. As a result, ML-SA1 increased lysosomal exocytosis and induced autophagy. However, autophagic clearance could not be observed. In untreated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts and neuroglia mmu-miR-672-5p and mmu-miR-5624-3p were significantly upregulated, while mmu-miR-6940-3p was significantly downregulated compared to *WT* controls. Upon ML-SA1 treatment, mmu-miR-375-3p was significantly upregulated in both cell types, whereas mmu-miR-7091-5p was significantly downregulated compared to *WT* cells, with mmu-miR-672-5p remaining unaffected. Overall, these findings suggest that lysosomal calcium modulation distinctly influences miRNA regulation under disease conditions.

ÖZET

TAY-SACHS HASTALIĞI FARE HÜCRELERİNDE MİRNA'LARIN BİYOLOJİK ROLÜNÜN ARAŞTIRILMASI

Tay-Sachs hastalığı, *HEXA* geminin mutasyonu ve β -N-asetilheksosaminidaz A enzim eksikliğine bağlı bir lizozomal depo hastalığıdır. GM2 ganglioizidinin lizozomal birikimi ve bozulmuş otofajik akış daha önce Tay-Sachs hastalığı fare modeli olan *Hexa-/-Neu3-/-*'de bildirilmiştir. Birkaç tedavi stratejisi araştırılmış olsa da Tay-Sachs hastalığı için küratif bir tedavi yoktur. ML-SA1 bir lizozomal kalsiyum kanalı agonistidir ve otofaji ve ekzositozu indüklemeye yeteneği çeşitli hastalıklarda bildirilmiştir. miRNA'lar, gen ekspresyonunu transkripsiyon sonrası düzenleyen kodlamayan küçük RNA'lardır. miRNA'ların düzensiz ekspresyonları birkaç lizozomal depo hastalığı için incelenmiştir. Ancak, Tay-Sachs hastalığında kapsamlı miRNA profillendirmesi ve ML-SA1 tedavisi hakkında bir çalışma yoktur. Bu çalışmada ML-SA1 ile tedavi edilmiş ve edilmemiş koşullarda *Hexa-/-Neu3-/-* fibroblastları ve nöroglialarının kapsamlı miRNA profillerini belirleyerek hastalık patolojisiyle ilişkili spesifik miRNA'ları tanımlamak ve ML-SA1'in lizozomal ekzositoz ve bozulmuş otofaji üzerindeki etkisini değerlendirmek amaçlanmıştır. Buna göre, yeni nesil dizileme ile birlikte enzim aktivite analizi, otofaji ve ekzositoz belirteçlerinin protein ve gen ekspresyon analizleri gerçekleştirilmiştir. Sonuç olarak, ML-SA1 lizozomal ekzositozu artırmış ve otofajiyi indüklemiştir. Ancak otofajik temizlenme gözlemlenmemiştir. ML-SA1 uygulanmamış *Hexa-/-Neu3-/-* fibroblastlar ve nöroglialarda mmu-miR-672-5p ve mmu-miR-5624-3p, *WT* kontrollerine kıyasla önemli ölçüde yukarı regüle edilirken, mmu-miR-6940-3p önemli ölçüde aşağı regüle edilmiştir. ML-SA1 tedavisi üzerine, *WT* hücrelerine kıyasla mmu-miR-375-3p her iki hücre tipinde de önemli ölçüde yukarı regüle edilirken mmu-miR-7091-5p önemli ölçüde aşağı regüle edilmiştir ve mmu-miR-672-5p'nin yukarı regülasyonu etkilenmemiştir. Bu bulgular lizozomal kalsiyum modülasyonunun hastalık koşulları altında miRNA regülasyonunu belirgin şekilde etkilediğini göstermektedir.



Dedicated to my beloved family...

TABLE OF CONTENTS

LIST OF FIGURES	ix
LIST OF TABLES.....	xiv
CHAPTER 1. INTRODUCTION	1
1.1 Lysosomal Storage Disorders (LSDs).....	1
1.2 Gangliosides	2
1.2.1 GM2 Gangliosidosis	3
1.2.2 Tay-Sachs Disease.....	5
1.2.3 Mouse Model of Tay-Sachs Disease	6
1.3 Lysosomes.....	7
1.4 Lysosomal Exocytosis.....	9
1.4.1 Lysosomal Exocytosis in LSDs.....	11
1.5 Autophagy	12
1.5.1 Autophagy in LSDs	14
1.6 Effect of ML-SA1 on Lysosomal Exocytosis and Autophagy.....	15
1.7 microRNAs (miRNAs).....	16
1.7.1 miRNAs in LSDs.....	19
1.8 The Aim of the Study	20
CHAPTER 2. MATERIALS & METHODS	21
2.1 Mouse Breeding	21
2.2 Mouse Genotyping	21
2.2.1 Isolation of DNA	21
2.2.2 Polymerase Chain Reaction (PCR) for Genotyping.....	22
2.3 Generation of Immortal Cell Lines	23
2.3.1 Generation of Primary Neuroglia Cell Lines.....	23
2.3.2 Generation of Primary Fibroblast Cell Lines	25
2.3.3 immortalization of Primary Cell Lines.....	25
2.4 MTT Assay.....	26
2.5 ML-SA1 Treatment	26
2.6 Enzyme Activity Assays	27
2.6.1 Sample Preparation.....	27

2.6.2 β -hexosaminidase B (Hexb) Assay	28
2.6.3 β -hexosaminidase A (Hexa) Assay	29
2.7 Real-Time Polymerase Chain Reaction (RT-PCR).....	31
2.7.1 Total RNA Isolation	31
2.7.2 cDNA Conversion	32
2.7.3 RT-PCR	32
2.8 Western Blot Analysis.....	33
2.8.1 Protein Isolation.....	34
2.8.2 Bradford Assay	34
2.8.3 SDS-PAGE	35
2.9 Immunocytochemical (ICC) Staining	36
2.10 microRNA (miRNA) Profiling	37
2.10.1 Sample Preparation for miRNA Sequencing.....	37
2.10.2 miRNA Isolation and Sequencing	37
2.10.3 Raw Data Analysis	38
CHAPTER 3. RESULTS.....	40
3.1 Mouse Genotyping	40
3.2 MTT Assay.....	41
3.3 Enzyme Activity Assays	42
3.4 RT-PCR.....	44
3.5 Western Blot.....	46
3.6 Immunocytochemical (ICC) Staining	49
3.7 miRNA Profiling	57
CHAPTER 4. DISCUSSION.....	85
CHAPTER 5. CONCLUSION	101
REFERENCES	103

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
Figure 1.1. Biosynthesis pathways of ganglio-series gangliosides.....	3
Figure 1.2. Schematic explanation of GM2 gangliosidoses.....	4
Figure 1.3. A pathway illustration representing the role of Neuraminidase 3 (Neu3) as the main contributor to the metabolic bypass mechanism in the context of the <i>Hexa</i> ^{-/-} mouse model.....	7
Figure 1.4. The illustration of the lysosome structure.....	8
Figure 1.5. The representative illustration of lysosomal exocytosis.....	10
Figure 1.6. Schematic representation of macroautophagy.....	14
Figure 1.7. Canonical pathway of miRNA biogenesis.....	18
Figure 3.1. The images of agarose gel electrophoresis after PCR of <i>Hexa</i> (A), and <i>Neu3</i> (B) genes. The wild-type allele of the <i>Hexa</i> gene is 420 bp and the mutant allele is 210 bp	40
Figure 3.2. Comparative representation of the viability percentages of 5-month-old <i>WT</i> neuroglia (A), <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} neuroglia (B), <i>WT</i> fibroblast (C), <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} fibroblast.....	41
Figure 3.3. Specific activity of extracellular Hexb (A), intracellular Hexb (B), and intracellular Hexa (C) in 5-month-old <i>WT</i> and <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} neuroglia under the untreated.....	42
Figure 3.4. Specific activity of extracellular Hexb (A), intracellular Hexb (B), and intracellular Hexa (C) in 5-month-old <i>WT</i> and <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} fibroblasts under the untreated.....	43
Figure 3.5. Relative expression levels of autophagy-related genes <i>Atg7</i> (A), <i>p62</i> (B), <i>Beclin1</i> (C), and <i>Lamp1</i> (D) in 5-month-old <i>WT</i> and <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} neuroglia cells under.....	44
Figure 3.6. Relative expression levels of autophagy-related genes <i>Atg7</i> (A), <i>p62</i> (B), <i>Beclin1</i> (C), and <i>Lamp1</i> (D) in 5-month-old <i>WT</i> and <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} fibroblast cells.....	45

<u>Figure</u>		<u>Page</u>
Figure 3.7.	Representative Western Blot results for autophagy markers Beclin1 (A), p62 (C), and LC3 (E) in 5-month-old <i>WT</i> and <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} neuroglia cells under.....	47
Figure 3.8.	Representative Western Blot results for autophagy markers Beclin1 (A), p62 (C), and LC3 (E) in 5-month-old <i>WT</i> and <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} fibroblast cells under.....	48
Figure 3.9.	Immunostaining of p62 protein in 5-month-old <i>WT</i> and <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} neuroglia (A) and fibroblast (C) cells under the untreated (none) and ML-SA1-treated	50
Figure 3.10.	Immunostaining of LC3 protein in 5-month-old <i>WT</i> and <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} neuroglia (A) and fibroblast (C) cells under the untreated (none) and ML-SA1-treated.....	51
Figure 3.11.	Immunostaining of surface Lamp1 protein in 5-month-old <i>WT</i> and <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} neuroglia (A) and fibroblast (C) cells under the untreated (none) and ML-SA1.....	52
Figure 3.12.	Immunostaining of total Lamp1 protein in 5-month-old <i>WT</i> and <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} neuroglia (A) and fibroblast (C) cells under the untreated (none) and ML-SA1.....	53
Figure 3.13.	Immunostaining of LC3 and Lamp1 protein in 5-month-old <i>WT</i> and <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} neuroglia cells under the untreated (none) and ML-SA1-treated conditions.....	55
Figure 3.14.	Immunostaining of LC3 and Lamp1 protein in 5-month-old <i>WT</i> and <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} fibroblast cells under the untreated (none) and ML-SA1-treated conditions.....	56
Figure 3.15.	Heatmap of significantly upregulated (A) and downregulated (B) miRNAs in ML-SA1 treated <i>WT</i> fibroblasts compared to untreated <i>WT</i> fibroblasts.....	57
Figure 3.16.	Dot plot of KEGG enrichment analysis of the significantly up and down-regulated miRNAs in ML-SA1 treated <i>WT</i> fibroblasts compared to untreated <i>WT</i> fibroblasts.....	58

<u>Figure</u>	<u>Page</u>
Figure 3.17. Dot plot of Reactome enrichment analysis of the significantly up and down-regulated miRNAs in ML-SA1 treated <i>WT</i> fibroblasts compared to untreated <i>WT</i> fibroblasts.....	59
Figure 3.18. Heatmap of significantly upregulated (A) and downregulated (B) miRNAs in untreated <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} fibroblasts compared to untreated <i>WT</i> fibroblasts.....	60
Figure 3.19. Dot plot of KEGG enrichment analysis of the significantly up and down-regulated miRNAs in untreated <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} fibroblasts compared to untreated <i>WT</i> fibroblasts.....	61
Figure 3.20. Dot plot of Reactome enrichment analysis of the significantly up and down-regulated miRNAs in untreated <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} fibroblasts compared to untreated <i>WT</i> fibroblasts.....	62
Figure 3.21. Heatmap of significantly upregulated (A) and downregulated (B) miRNAs in ML-SA1 treated <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} fibroblasts compared to untreated <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} fibroblasts.....	63
Figure 3.22. Dot plot of KEGG enrichment analysis of the significantly up and down-regulated miRNAs in ML-SA1 treated <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} fibroblasts compared to untreated <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-}	64
Figure 3.23. Dot plot of Reactome enrichment analysis of the significantly up and down-regulated miRNAs in ML-SA1 treated <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} fibroblasts compared to untreated <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-}	65
Figure 3.24. Heatmap of significantly upregulated (A) and downregulated (B) miRNAs in ML-SA1 treated <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} fibroblasts compared to ML-SA1 treated <i>WT</i> fibroblasts.....	66
Figure 3.25. Dot plot of KEGG enrichment analysis of the significantly up and down-regulated miRNAs in ML-SA1 treated <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} fibroblasts compared to ML-SA1.....	67
Figure 3.26. Dot plot of Reactome enrichment analysis of the significantly up and down-regulated miRNAs in ML-SA1 treated <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} fibroblasts compared to ML-SA1.....	68

<u>Figure</u>	<u>Page</u>
Figure 3.27. Heatmap of significantly upregulated (A) and downregulated (B) miRNAs in ML-SA1 treated <i>WT</i> neuroglia compared to untreated (none) <i>WT</i> neuroglia.....	69
Figure 3.28. Dot plot of KEGG enrichment analysis of the significantly up and down-regulated miRNAs in ML-SA1 treated <i>WT</i> neuroglia compared to untreated <i>WT</i>	70
Figure 3.29. Dot plot of Reactome enrichment analysis of the significantly up and down-regulated miRNAs in ML-SA1 treated <i>WT</i> neuroglia compared to untreated <i>WT</i>	71
Figure 3.30. Heatmap of significantly upregulated (A) and downregulated (B) miRNAs in untreated (none) <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} neuroglia compared to untreated (none) <i>WT</i>	72
Figure 3.31. Dot plot of KEGG enrichment analysis of the significantly up and down-regulated miRNAs in untreated <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} neuroglia compared to untreated.....	73
Figure 3.32. Dot plot of Reactome enrichment analysis of the significantly up and down-regulated miRNAs in untreated <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} neuroglia compared to untreated.....	74
Figure 3.33. Heatmap of significantly upregulated (A) and downregulated (B) miRNAs in ML-SA1 treated <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} neuroglia compared to untreated (none) <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-}	75
Figure 3.34. Dot plot of KEGG enrichment analysis of the significantly up and down-regulated miRNAs in ML-SA1 treated <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} neuroglia compared to untreated.....	76
Figure 3.35. Dot plot of Reactome enrichment analysis of the significantly up and down-regulated miRNAs in ML-SA1 treated <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} neuroglia compared to untreated.....	77
Figure 3.36. Heatmap of significantly upregulated (A) and downregulated (B) miRNAs in ML-SA1 treated <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} neuroglia compared to ML-SA1 treated <i>WT</i> neuroglia.....	78

<u>Figure</u>	<u>Page</u>
Figure 3.37. Dot plot of KEGG enrichment analysis of the significantly up and down-regulated miRNAs in ML-SA1 treated <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} neuroglia compared to ML-SA1.....	79
Figure 3.38. Dot plot of Reactome enrichment analysis of the significantly up and down-regulated miRNAs in ML-SA1 treated <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} neuroglia compared to ML-SA1.....	80
Figure 3.39. Dot plot of Reactome enrichment analysis of the significantly upregulated mmu-miR-672-5p and mmu-miR-5624-3p in both untreated <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} neuroglia	82
Figure 3.40. Dot plot of Reactome enrichment analysis of the significantly upregulated miRNAs mmu-miR-375-3p and mmu-miR-672-5p in both ML-SA1 treated <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-}	83

LIST OF TABLES

<u>Table</u>	<u>Page</u>
Table 2.1. <i>Hexa</i> and <i>Neu3</i> gene-specific genotyping primer.....	23
Table 2.2. Extracellular β -hexosaminidase B enzyme.....	29
Table 2.3. Intracellular β -hexosaminidase B enzyme.....	29
Table 2.4. Intracellular β -hexosaminidase A enzyme.....	30
Table 2.5. Expression primer sequences and product sizes of autophagy-related genes.....	33
Table 2.6. Overview of the pairwise comparison groups used for DE analysis.....	39
Table 3.1. Common upregulated and downregulated miRNAs between <i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} fibroblasts and neuroglia in.....	81
Table 3.2. Reactome pathway enrichment results of common downregulated miRNA mmu-miR-7091-5p in.....	84

CHAPTER 1

INTRODUCTION

1.1 Lysosomal Storage Disorders (LSDs)

Lysosomes are organelles in animal cells that contain several different (approximately 50) acid hydrolase enzymes used in the degradation of macromolecules.^{1,2} These organelles have critical roles in many cellular events like calcium signaling, cell death, autophagy, etc.³ Lysosomal storage disorders (LSDs) are described as metabolic errors caused by dysfunctional lysosomal activity. These are the monogenic diseases resulting from mutations in genes encoding lysosomal enzymes or in genes that code for enzyme activator proteins, membrane transporters, and membrane proteins. Most LSDs are inherited in an autosomal recessive manner, but some of them have X-linked inheritance.⁴ Their prevalence is very rare, with 1:5000 live births. In all LSDs, malfunctioning hydrolytic enzymes or proteins cause macromolecules or substrates (mucopolysaccharides, sphingolipids, glycoproteins...) to progressively accumulate in the lysosomal compartments instead of being degraded. This unwanted storage negatively affects cellular homeostasis and leads to cell death and dysfunctionality of organs. More than 70 different types of LSD have been identified. The age of onset of these diseases and the symptoms patients exhibit depend on several variables: the level of enzyme/protein activity, the biochemical structure of the accumulated substrate, and the type of cell in which it is stored.^{1,5} The central nervous system (CNS) pathology (neurodegeneration and neuroinflammation) is seen in seventy percent of LSDs. In LSDs that manifest at birth or in childhood, the symptoms are typically most severe compared to those observed in adults. Common neurological symptoms of most LSD patients can be listed as difficulties in cognition, brain atrophy, decline in motor activities, behavioral problems, loss of vision, deafness, difficulty in controlling movements, and seizures. In addition to CNS, several other organs can be involved in different LSDs. For example, in Gaucher disease, cytopenia, skeletal abnormalities (pain followed by bone involvement), and enlargement of the spleen and liver are typical clinical symptoms, while in Fabry disease, the cardiovascular system is affected.^{5,6}

LSDs can be diagnosed with several methods. The most common approach is to measure enzyme activity in dried blood spots (DBS) by using tandem mass spectrometry (MS/MS) or fluorometric assays. Accumulation of specific substrates in blood or urine samples of patients can also be detected. In recent years, next-generation sequencing (NGS) has also been considered as a promising and reliable tool for screening.⁷

1.2 Gangliosides

Gangliosides are an amphiphilic subclass of glycosphingolipids that contain at least one sialic acid residue. There are more than 200 different gangliosides in vertebrates, depending on the structure of the ceramide part (length and saturation level of fatty acid chains) they contain. Gangliosides are predominantly found in the nervous system. They are located in microdomains known as lipid rafts, caveolae and glycosphingolipid enriched domains on the outer surface of the plasma membrane together with sphingomyelin and cholesterol. Their hydrophobic part, ceramide moieties, are placed into the lipid bilayer while the hydrophilic part, oligosaccharide chains extend outward from the membrane into the extracellular area. They play roles in the regulation of ion channels, intercellular communication, and recognition, adhesion, and signal transduction processes. It has also been shown that these lipids are present in the nuclear membrane. Their role here is to maintain calcium homeostasis within the cell and nucleus.^{8–10}

Glycosphingolipid and ganglioside synthesis is initiated in the inner leaflet of the endoplasmic reticulum (ER), where ceramide moieties are formed. These ceramides are subsequently transported to the Golgi apparatus, where the sequential action of multiple glycosyltransferases catalyzes the addition of sugar residues, leading to the formation of diverse ganglioside species.¹¹ The origin of all gangliosides is mainly lactosylceramide (LacCer) but there is an exception. GM4 ganglioside is generated from the series of modifications that occurred in galactosylceramide (GalCer) instead of LacCer.⁸

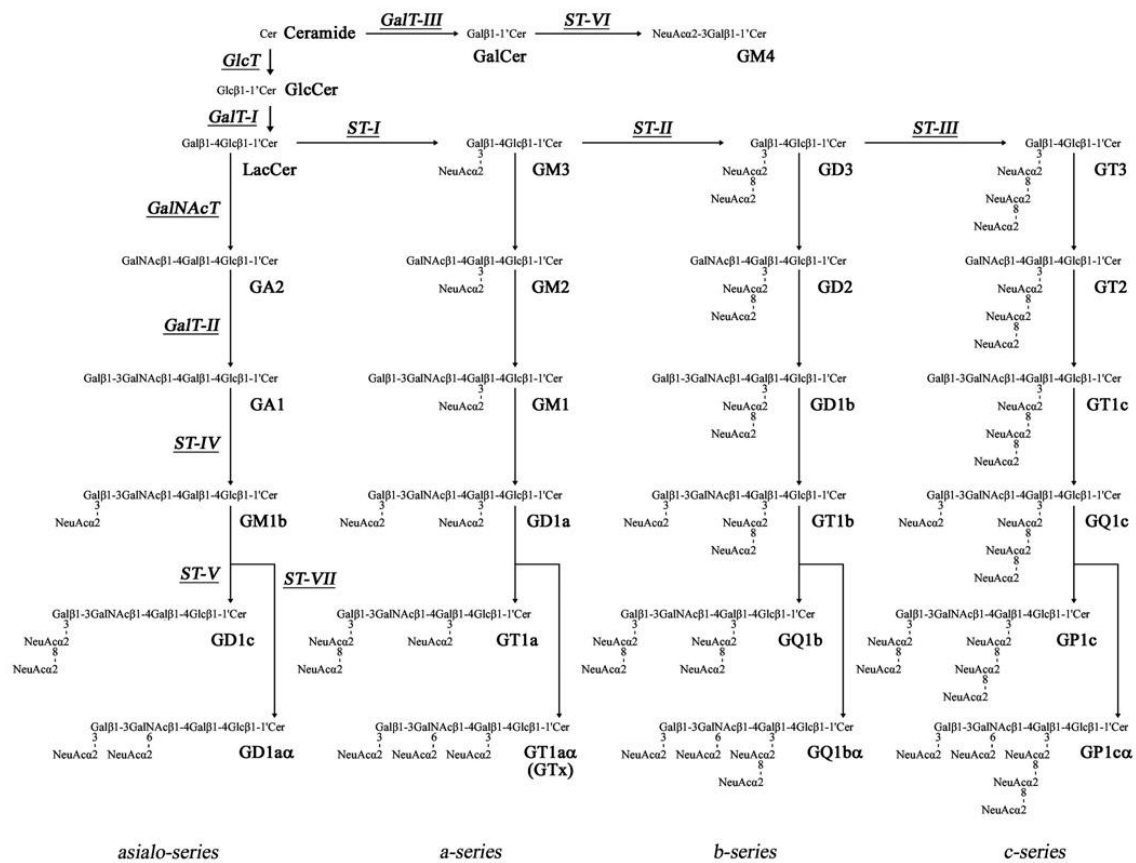


Figure 1.1. Biosynthesis pathways of ganglio-series gangliosides and their structures.⁸

There are 10 to 30 times more gangliosides in the brain compared to other tissues in the human body. Therefore, gangliosides primarily affect the nervous system. More than ninety percent of the brain gangliosides consist of GM1, GD1a/b, and GT1b (complex gangliosides). These gangliosides are mostly enriched in myelin and neurons. In astrocytes, generally, GM3, GD3, and GM4 are exhibited as simple forms of gangliosides. Their regulatory functions in the nervous system rely on the formation of functional synapses, maintenance of synaptic plasticity, neurotransmitter dynamics, neuronal development, and maturation that are crucial for memory and learning.^{10,12}

1.2.1 GM2 Gangliosidoses

GM2 gangliosidoses are the autosomal recessively inherited subclass of LSDs that comprise three different diseases, each caused by a mutation in three distinct genes. HEXA gene codes for the alpha subunit of the β -hexosaminidase A enzyme and the *HEXB*

gene encodes its beta subunit. The function of this heterodimeric enzyme is diminished in terms of GM2 degradation when there is a mutation in one of these genes.¹³ Mutations in the *HEXA* and *HEXB* genes lead to Tay-Sachs disease (OMIM 272800) and Sandhoff disease (OMIM 268800) in order. The last type of GM2 gangliosidoses is called GM2 Activator Deficiency.¹⁴ As the name indicates, this disease is caused by a mutation in the GM2 activator protein coding *GM2A* gene. Even if the β -hexosaminidase A enzyme is working properly, without activator protein action GM2 cannot be degraded because this protein works like a cofactor of the enzyme. Activator protein binds to the GM2 ganglioside in the membrane and directs the β -hexosaminidase A enzyme to degrade GM2 into GM3 ganglioside. As a result of these deficiencies, GM2 ganglioside is stored in the lysosomes of neuronal cells.¹⁵

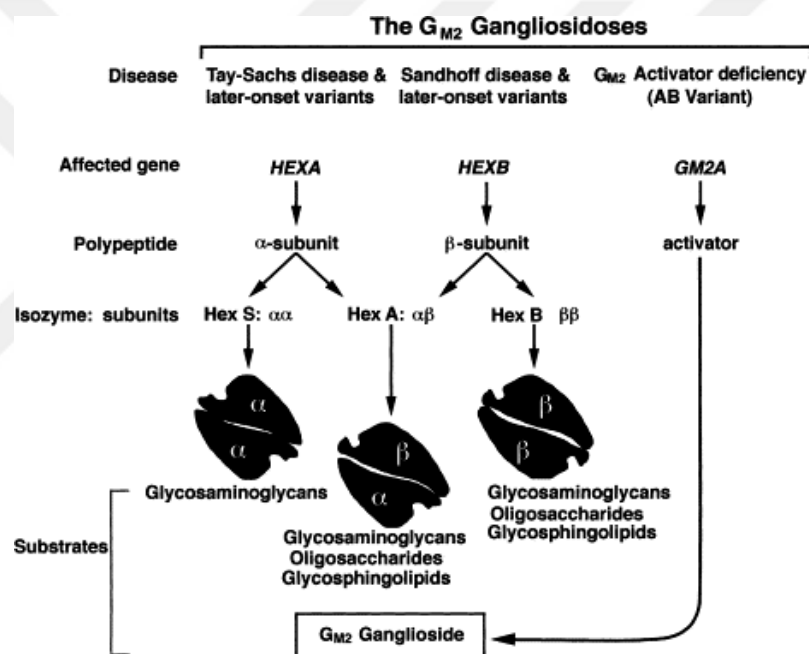


Figure 1.2. Schematic explanation of GM2 gangliosidoses.¹³

Abnormal storage of GM2 ganglioside primarily affects the cells of the central nervous system (microglia, oligodendrocytes, neurons). Therefore, progressive neurodegeneration is the characteristic feature of these diseases. There are three forms of GM2 gangliosidoses: acute infantile, sub-acute juvenile, and late-onset. The highest severity of the disease is shown in infantile onsets, with the lowest residual enzyme activity.¹⁵

1.2.2 Tay-Sachs Disease

Tay-Sachs disease (TSD) is a neurodegenerative, autosomal recessive disorder. It arises from the deficiency of β -N-acetylhexosaminidase A (HEXA) enzyme due to a mutated *HEXA* gene. The *HEXA* gene is responsible for the expression of the alpha subunit of the HEXA enzyme. Nearly 130 mutations that cause the TSD have been identified. Types of mutations are single base substitutions, small indels (insertions and deletions), duplications, rearrangements, and splicing.¹⁶ HEXA enzyme plays a key role in the degradation of GM2 into GM3 ganglioside. It hydrolyzes the N-acetylgalactosamine part of the GM2 to accomplish the conversion. Since the amount of residual enzyme activity varies depending on the type of mutations in the *HEXA* gene, the severity of the disease can differ clinically. The enzyme activity should be between 10 and 15 % to prevent disease. Consequently, mutations (mentioned above) cause an absent or dysfunctional enzyme, and GM2 ganglioside is highly accumulated in neuronal cells.¹⁷ TSD is one of the rare genetic diseases. The probability of occurrence is 1 in 100,000 live births. Being a carrier is a more common situation, with an incidence rate of 1 in 250 people. When looked at worldwide, the rate of having TSD is higher in the Ashkenazi Jewish population, and especially in people of Eastern European origin.^{17,18}

There are three types of clinical phenotypes depending on the age of onset. These can be listed as infantile, juvenile, and late-onset. Infantile is the type of TSD that occurs in babies at 6 months of age and has the most severe symptoms. The residual activity of the HEXA enzyme is lower than 0.5%. As an outcome, they display neurodegenerative abnormalities like uncontrolled eye and muscle movements, reduced blood pressure, deficient myelination, difficulties in sitting and holding the head without help, and impaired swallowing. Another characteristic feature is the cherry-red spot formation in their retina. Generally, these patients die at the age of 5.^{17,19} The juvenile type of disease is seen around the age of 3-5. They present swallowing and speaking difficulties, decreased muscle tone, seizures, and reduced cognition as time passes, and survive until the age of 15.^{19,20} Lastly, in the late-onset type, in addition to symptoms of the juvenile form, psychological problems like manic depression and cerebellar ataxia can be shown.²¹ Symptoms begin to appear at the age of 25-30, but they can survive up to the age of 60-65.¹⁷

Substrate reduction therapy²², enzyme replacement/enhancing therapy^{23,24} hematopoietic stem cell transplantation²⁵, mRNA and antisense oligonucleotide-mediated therapy²⁶, and gene therapies²⁷ are the most studied treatment methods for GM2 gangliosidoses. However, there is no curative treatment available for TSD patients to date because the delivery of vectors or molecules to the CNS is obstructed by the blood-brain barrier. Also, clinical trials are very infrequent and expensive for families.^{17,28}

1.2.3 Mouse Model of Tay-Sachs Disease

Many studies were conducted to establish a TSD mouse model. Firstly, the *Hexa* gene was targeted, and a *Hexa*^{-/-} mouse model was generated in two different studies. As a result, these mice did not exhibit severe neurological and behavioral symptoms. GM2 accumulation was not observed at all over the CNS, unlike in the human patients. Cerebellum and spinal ganglia were clear in terms of GM2 storage.²⁹ In *Hexa*^{-/-} mouse, GA2 ganglioside, which is the asialylated type of GM2, was also observed in addition to GM2.³⁰ Therefore, these findings suggested the presence of possible differences in the metabolic pathways of GM2 in different species, like the action of sialidases in mice.^{31,32} Sialidases, also called neuraminidases, are glycohydrolase enzymes that cleave terminal sialic acids of glycoconjugates. According to areas that are specifically expressed, they are categorized as lysosomal neuraminidase (NEU1), cytosolic neuraminidase (NEU2), plasma membrane neuraminidase (NEU3), and intracellular neuraminidase (NEU4).³³

To generate a better mouse model of TSD, the unknown roles of sialidases were studied in the GM2 degradation pathway. First, the *Neu4* gene was chosen to disrupt, and a *Hexa*^{-/-}*Neu4*^{-/-} double knock-out mouse was generated. According to results, epileptic seizures and accumulation of GM2 in cortical layers, pointing to a more severe form of the disease than *Hexa*^{-/-}, were observed. However, this genotype did not represent the real state of disease severity, and neuraminidase 4 was evaluated as a modifier sialidase.³⁴ Finally, the mouse model of the early-onset form of the disease was successfully obtained with the disruption of the *Neu3* gene expression in addition to *Hexa*. *Hexa*^{-/-}*Neu3*^{-/-} mice showed a most severe form of the disease, together with tremors, ataxia, growing abnormalities, weight loss, and excessive GM2 accumulation in the brain (mostly in cerebellum, cortex, and hippocampus). Moreover, they exhibited reduced motor

activities, progressive neurodegeneration, and neuroinflammation. Therefore, it was understood that neuraminidase 3 is the major player in the bypass mechanism of sialidases in GM2 metabolism of mice.³⁵

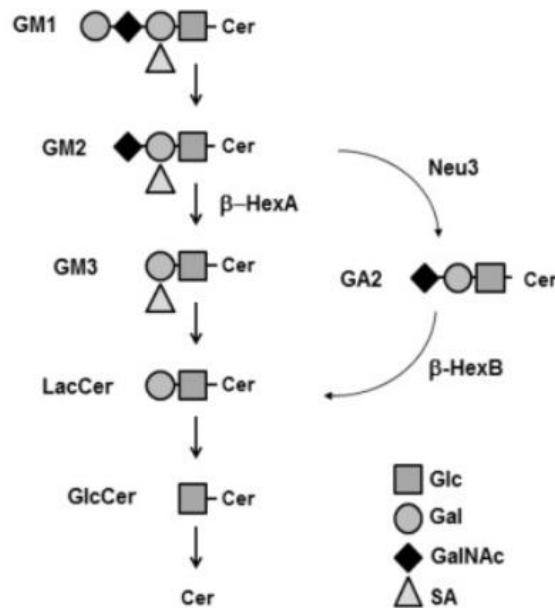


Figure 1.3. A pathway illustration representing the role of Neuraminidase 3 (Neu3) as the main contributor to the metabolic bypass mechanism in the context of the *Hexa*^{-/-} mouse model.³⁵

As shown in Figure 1.3, neuraminidase 3 hydrolyzes the GM2 to GA2 ganglioside with the removal of sialic acid (SA), and GA2 is degraded to lactosylceramide (LacCer) by the action of Hexb enzyme in *Hexa*^{-/-} mice.³⁵

1.3 Lysosomes

Lysosomes are acidic organelles surrounded by a single membrane. They can have different sizes, morphologies, contents, and locations within the cell.³⁶ Lysosomal membrane contains different membrane proteins such as lysosome-associated membrane proteins (LAMP1 and LAMP2A mostly), calcium ion channels (TRPMLs and TPCs), and lipid transporters.³⁷ In these organelles, more than 50 acid hydrolases are found and activated by the acidic pH (4.5-5.5) of the lumen. Vacuolar ATPases (v-ATPases) found

in the lysosome membrane hydrolyze ATP and pump generated protons to the lumen to maintain this low pH range inside. Lysosomal hydrolases are sulfatases, proteases, phosphatases, nucleases, lipases, and glycosidases.^{36,38,39} Lysosomal hydrolases are produced in the ER and transferred to the Golgi apparatus. They are tagged with the mannose-6-phosphate (M6P) moiety and recognized by the M6P receptors in the trans-Golgi network. After that, they are covered by clathrin-coated vesicles to be transferred to the late-endosomes and lysosomes.⁴⁰ Depending on the positions in the cell, degradation capacities and luminal pH of these organelles can vary. Those lysosomes placed near the nucleus have higher hydrolase activity with lower pH, while the lysosomes near the plasma membrane have reduced catalytic activity with higher pH. This shows their heterogeneous identity.⁴¹

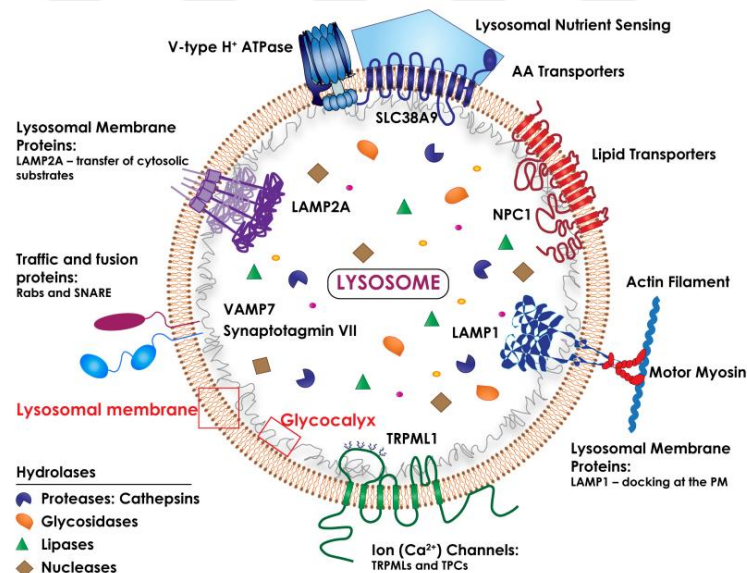


Figure 1.4. The illustration of the lysosome structure.³⁷

The well-known function of the lysosomes is the digestion of small molecules or surface receptors that are taken up via endocytic pathways; phagocytosed bacteria or cell debris, as well as damaged organelles, pathogens, or cytosolic proteins targeted by autophagy. At the end of their digestive action on the macromolecules, monomers are provided to the cell to reuse.^{36,38} In addition to digestion and recycling, they have a role in secretion to the extracellular space, thus enabling plasma membrane repair.^{39,42,43} New insight about the lysosomes is that they are the metabolic hub of the cell, controlling signal transduction and maintaining cell homeostasis.⁴⁴

1.4 Lysosomal Exocytosis

Exocytosis refers to the fusion of intracellular vesicles involved in the secretion process with the cell membrane. As a result of this fusion, the contents of the vesicles are expelled from the cell, and the molecules (lipids and proteins) in the vesicular membrane are integrated into the cell membrane. There are 2 types of exocytosis: constitutive and regulated. As the name suggests, constitutive exocytosis happens without any stimulation in all cell types. However, regulated exocytosis requires a signal for activation, like a high level of Ca^{2+} concentration in the cytosol, and typically occurs in neurons, endocrine, and exocrine cells.⁴⁵

Lysosomal exocytosis is a subtype of regulated exocytosis, but it is known that this process is seen in all cell types.^{42,46} In this pathway, lysosomes move from the perinuclear area toward the plasma membrane and fuse with it. Finally, lysosomal content is secreted into the extracellular space. It is mainly coordinated by the free Ca^{2+} in the cytoplasm. Lysosomal exocytosis plays a crucial role in cellular clearance and repair of the cell membrane. It is involved in antigen presentation, bone resorption, and neurotransmitter secretion processes.⁴⁷

The cellular mechanism of lysosomal exocytosis includes several proteins. First of all, in the presence of stimuli, lysosomes are brought close to the plasma membrane from the perinuclear area by the activity of kinesin motor proteins (Kinesin-1,2,3 but generally Kinesin-1) on the microtubules (anterograde transport). This interaction between Kinesin1 and lysosome is regulated by the small-GTPase Arl8 (Arf-like protein 8). Active (GTP state) Arl8 interacts with the membrane of the lysosome, then recruits SKIP (SifA (Salmonella-Induced Filaments A) and Kinesin-Interacting Protein) adaptor protein, which binds to kinesin-1 to the lysosome.⁴⁷ Also, actin filament-lysosome interactions can be important for the localization of lysosomes around the cell membrane before docking. Arp2/3 complex is activated by WASH (Wiskott–Aldrich syndrome protein and SCAR homolog) protein, and new actin filaments are polymerized. A study showed that in WASH mutant cells, exocytosis was diminished.⁴⁸ The next step is the docking of the lysosomes to the plasma membrane by the interaction of target and vesicular soluble NSF attachment protein receptors (t-SNARE & v-SNARE). Lysosomes have VAMP7 (vesicle-associated membrane protein 7), a type of v-SNARE, on their surface. First, VAMP7 links to syntaxin-4 and SNAP23 (synaptosome-associated protein

of 23 kDa) that are t-SNARE proteins found in the cytosolic portion of the plasma membrane to form the trans-SNARE complex. This complex brings the two membranes closer, but does not touch.⁴⁹ After the formation of the complex, an increment in the intracellular Ca^{2+} concentration results in the fusion of the lysosomal and plasma membranes. There are different types of proteins that participate in Ca^{2+} signaling and facilitate fusion. Syt-VII (Synaptotagmin-VII) is one of them found on the lysosomal membrane.^{50,51} In addition, some studies stated that small-GTPases like Rab3a, Rab10, Rab27, and Rab11 are involved and required for lysosomal exocytosis.^{52–54}

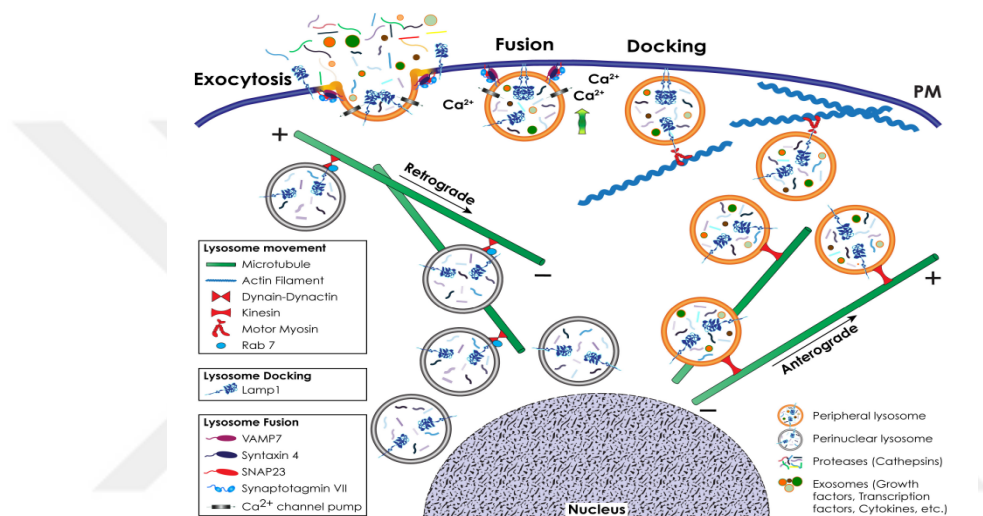


Figure 1.5. The representative illustration of lysosomal exocytosis.³⁷

Lysosomal exocytosis is tightly controlled and regulated by several factors and molecules. TFEB is a transcription factor of genes related to lysosomal biogenesis and autophagy. If there are enough nutrients in the cell, mTORC1 (mammalian target of rapamycin complex 1) is in the active state as bound to the lysosome. Active mTORC1 inactivates TFEB by phosphorylation. Thus, TFEB cannot penetrate the nucleus and stays in the cytosol, inhibiting lysosomal exocytosis. However, when nutrients are deprived, mTORC1 is deactivated and dissociates from the lysosomes. TFEB is dephosphorylated and translocated to the nucleus, resulting in enhanced lysosomal exocytosis.^{47,55,56} However, according to a study, lysosome positioning is related to the nutritional state. Lysosomes are placed around the nucleus in starvation conditions and are localized in the periphery in the presence of nutrients.⁵⁷ This two-way regulation of TFEB is explained as both increasing the degradation capacity of lysosomes and causing secretory lysosomes to undergo fusion. Ca^{2+} release is another positive regulator for that process. TFEB

activity in the nucleus leads to activation Ca^{2+} channel of lysosomes and endosomes, TRPML1 (transient receptor potential cation channel, mucolipin subfamily, member 1), thus triggering the Ca^{2+} release to the cytosol. Consequently, the fusion of lysosomes with the plasma membrane is supported.^{47,58}

1.4.1 Lysosomal Exocytosis in LSDs

LSDs mainly occur due to a deficiency of hydrolases or mutated activator proteins that lead to abnormally accumulated lipids (in the lipidoses classes of LSDs) and other molecules in lysosomal compartments. At the end, lysosomes are enlarged and become dysfunctional. However, secondary accumulation of lipids can also arise due to defects in endosome-lysosomal membrane trafficking inside the cell. This situation is explained by the term ‘trafficking jam’, which leads to impaired delivery of hydrolases to the lysosome within vesicles. It was also stated that primary insoluble lipid accumulation might result in ‘trafficking jam’.^{4,59} Lysosomal exocytosis can be examined by several methods, like surface Lamp1 immunostaining, intracellular calcium measurement, extracellular lysosomal enzyme activity assays, and live cell imaging by total internal reflection fluorescent microscopy.⁵⁰

Mucopolidoses (MLs) are a group of LSDs that present similar clinical symptoms but are caused by different mutated genes, leading to the accumulation of sphingoglycolipids, cholesterol, and phospholipids in the lysosomes.^{50,60} Several organs (visceral organs like liver, kidney, spleen; bone, muscle, and brain) are enlarged and damaged in these patients. There are 4 categories of MLs: MLI (Sialidosis, defective lysosomal enzyme Neurominidase1), MLII & MLIII (defective lysosomal enzyme N-acetylglucosamine-1-phosphotransferase / GlcNAc phosphotransferase), and MLIV (mutated *MCOLN1* gene).⁶¹ The *MCOLN1* gene encodes mucolipin-1 protein. Mucolipin-1, also called TRPML1, is a membrane channel, as mentioned in previous sections. Although it is known to be permeable to many cations, its most critical function is serving as a Ca^{2+} channel localized to the late endosomal and lysosomal membranes. Therefore, loss-of-function mutations in this gene alter calcium signaling, which then gives rise to disrupted late endosome/autophagosome fusion with the lysosome. In addition, reconstructions and exocytosis of lysosomes, and lysosome-related regulatory signals

(mTORC1 and TFEB) are negatively affected by this situation.^{50,60,62,63} In the study, it was shown that endocytosis-lysosome fusion declined, and large vacuoles were formed as a result of *cup-5* (human *MCOLN1* gene ortholog) mutation in *C.elegans*.⁶⁴ In the fibroblasts of MLIV patients, lysosomal exocytosis was induced by ionomycin. However, LAMP1 cannot be detected on the plasma membrane. Then it was understood that lysosomal exocytosis was still diminished without TRPML1 activity.⁶⁵

Niemann-Pick disease type C is an LSD that arises due to mutations in *NPC1* or *NPC2* genes, which bind to cholesterol. Endocytosed cholesterol cannot be transported properly and instead builds up specifically in lysosomes and late endosomes.⁶⁶ Wolman disease is another LSD related to cholesterol deposition in lysosomes, as a consequence of mutations in the *LIPA* gene coding for lysosomal acid lipase.⁶⁷ Inducing lysosomal exocytosis with δ -Tocopherol and treatment decreased the lysosomal accumulation of cholesterol and the size of lysosomes in the fibroblast cells of NPC1 and Wolman patients.⁶⁸ 2-hydroxypropyl- β -cyclodextrin was also used to enhance lysosomal exocytosis in NPC1 mouse models.^{69,70} Finally, with the simultaneous application of tocopherol and cyclodextrin, induced lysosomal exocytosis followed by reduced GM2 storage in lysosomes was shown in TSD patients and *Hexa*^{-/-}*Neu3*^{-/-} mice cells.⁷¹

1.5 Autophagy

The process of sending accumulated or misfolded proteins, damaged organelles (ER, mitochondria, peroxisomes etc.), and invaded pathogens into the lysosomal system to break down for reuse is called autophagy. Autophagy balances cellular homeostasis by providing monomers to the cell in cases of nutrient deficiency and development. Also, this self-eating mechanism of eukaryotic cells serves an antiviral function (defense mechanism) by targeting and degrading virion components or entire virions within lysosomal compartments. In addition, innate and adaptive immune responses can be triggered by autophagy at the time of infection.^{72,73} It is known that autophagy is divided into three classes as follows: macroautophagy, chaperone-mediated autophagy, and microautophagy. First, in the macroautophagy, cytoplasmic macromolecules are directed and collected in the double-membraned autophagosome vesicles. Autophagosomes fuse with lysosomes and form autolysosomes for the degradation of these materials. Second,

chaperone-mediated autophagy is quite different because proteins are targeted and transferred to the lysosomes selectively. Cytosolic chaperon proteins, such as Hsc-70, recognize and bind to targeted proteins, then LAMP2A receptors of lysosomal membranes unfold and take them into lysosomes for degradation. Lastly, during microautophagy, lysosomes directly take in portions of the cytoplasm through the formation of tubular structures (non-selective degradation pathway). This process involves the invagination and protrusion of the lysosomal membrane, allowing the internalization of random proteins, molecules, and organelles.^{74,75}

Autophagy generally refers to macroautophagy if there is no additional statement. It is stimulated by several conditions, such as hypoxia, low levels of ATP, insulin, and amino acids in the cells and tissues.⁷⁵ There are lots of proteins and protein complexes involved in the machinery of autophagy. Studies focused on the autophagy mechanism started with the determination of *Atg* genes referred to as core autophagy-related genes in the *Saccharomyces cerevisiae*.⁷⁴ The main steps of autophagy are categorized as initiation, elongation, maturation, fusion, and degradation. Different *Atg* proteins play roles in all these steps.⁷⁶ AMPK (AMP-activated protein kinase) triggers autophagy by sensing nutrient levels, while mTORC1 inhibits.⁷⁷ However, in starvation conditions, mTORC1 is inactivated by TFEB as described in previous sections. Upon the mTORC1 inactivation, ULK1/2 complex (scaffold including UNC-51-like kinase 1 and 2, FIP200 (or named as RB1CC1), Atg13, Atg101) is activated and marks the area of the ER membrane where the phagophore formation will start (phagophore assembly site, PAS).^{74,75,78} After that, class III PI3K kinase complex (including Beclin1 (Atg6) and VPS34) and Atg9-containing vesicles are recruited by the ULK1/2 complex to the PAS, to make PI3P (phosphatidylinositol-3-phosphate) and provide a membrane source, respectively, for elongation of the phagophore. Atg9 proteins work with the Atg2 proteins that transport phospholipids from the outer side of the ER membrane to the outer side of the phagophore. Atg2 proteins are localized to the PAS via PI3P binding receptors, such as WIPI1 and WIPI2.⁷⁴ In addition, two different ubiquitin-like binding cascades, LC3 (microtubule-associated protein 1A/1B light chain) and Atg12-Atg5, are involved in the elongation and maturation steps. In the LC3-binding cascade, LC3 (ubiquitin-like protein) binds to phosphatidylethanolamine (PE) with the help of E1-like enzyme Atg7 and E2-like enzyme Atg3. In the Atg12-Atg5 binding cascade, Atg12 (ubiquitin-like protein) interacts with Atg5 covalently with the help of Atg7 and Atg10 (E2-like enzyme). This complex binds to Atg16l1 and acts as an E3 enzyme on the LC3-PE complex. At the end,

lipidation of LC3 (conversion of cytosolic LC3-I to LC3-II that is conjugated to the autophagosome membrane) occurs.^{73,75} After the closure of the phagophore and maturation into the autophagosome, the SNARE complex (SNAP29/VAMP7-8/STX17 or SNAP29/YKT6/STX7, depending on the cell type) is generated, and fusion with the lysosome is mediated. Upon fusion, the autolysosome structure is generated, and materials are degraded via lysosomal enzymes.⁷⁹

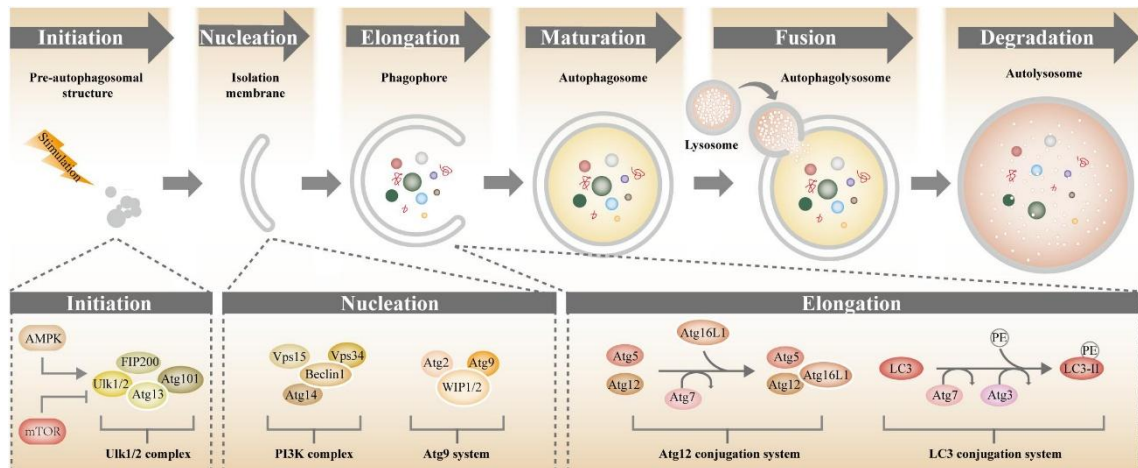


Figure 1.6. Schematic representation of macroautophagy.⁷⁶

Autophagy can be selective and also non-selective in terms of recognition of materials that will be degraded.⁷² p62/SQSTM1 is a ubiquitous protein and one of the several receptors of selective autophagy. It has a ubiquitin-associated (UBA) domain and an LC3-interacting (LIR) domain in its structure. Therefore, ubiquitinated proteins are recognized by p62. At the same time, p62 interacts with the LC3-II in the autophagosome membrane from its LIR domain. Ultimately, targeted materials are directed to the autophagosomes in a selective manner and are digested in autolysosomes.⁸⁰

1.5.1 Autophagy in LSDs

Autophagy is a self-eating mechanism that results in lysosomal degradation of autophagic cargos. Therefore, there is a strong relationship between autophagy and lysosomal function. Many studies have shown that LSDs are not related only to dysfunctional lysosomes but also to alterations in the autophagy mechanism, which was not surprising when the interaction of autophagosomes and lysosomes is considered.⁸¹

Autophagy can be investigated by several autophagic markers. For example, abnormally high levels of p62 and LC3-II and p62-containing inclusion bodies indicate defects in autophagic clearance. However, LC3-II can be upregulated due to newly synthesized autophagosomes upon induced autophagy not necessarily from impaired autophagy. Thus, examination of LC3-II levels should be made via autophagic flux analysis.^{73,82} Accordingly, in the thalamus, brain stem, and basal ganglia of the neuronopathic mouse model of Gaucher disease, p62 accumulation was detected.⁸³ In addition, neurons that were differentiated from iPSCs of Gaucher patients showed a high amount of autophagosomes and an increased level of p62 and LC3-II.⁸⁴ iPSCs of Danon disease (DD) patients were used to generate cardiomyocytes to monitor autophagy, and it was stated that the defective LAMP-2B isoform impaired autophagosome-lysosome fusion⁸⁵ and muscle fiber biopsies of DD patients showed accumulated p62 and LC3-II⁸⁶. Distorted autophagy was also observed in the rat model of Mucopolysaccharidosis VI and fibroblast cells of patients.⁸⁷ Lastly, in the brain regions of the TSD mouse model (*Hexa-/-Neu3-/-*) autophagy defects were demonstrated via increased levels of p62 and LC3-II.⁸⁸

1.6 Effect of ML-SA1 on Lysosomal Exocytosis and Autophagy

As mentioned before, TRPML1 is a non-selective calcium channel found in the endosome and lysosome membranes. This channel controls several processes like late-endosome maturation into lysosomes, movement and trafficking within the endolysosomal system, nutrient sensing, exocytosis, lysosome reformation, autophagy, etc.⁸⁹

The TRPML1 channel is activated via PI(3,5)P₂, and it is inhibited via PI(4,5)P₂ endogenously.⁹⁰ Activation of lysosomal TRPML1 channel increases the release of Ca²⁺ into the cytosol. Ca²⁺-dependent phosphatase calcineurin (CaN) receives an increased cytosolic calcium as a signal and is activated. Active CaN triggers the nuclear transport of TFEB via dephosphorylation. Ultimately, expressions of genes referred to as CLEAR (Coordinated Lysosomal Expression and Regulation) are enhanced, and lysosomal biogenesis and autophagy are activated.⁹¹ The additional mechanism of increased TRPML1 activity results in activation of CaMKK β (calcium/calmodulin-dependent protein kinase kinase β) and AMPK, followed by the ULK1 and VPS34 induction to form a phagophore.⁹²

ML-SA1 (Mucolipin synthetic agonist 1) is a small synthetic molecule that binds and activates the TRPML1.⁸⁹ In a recent study, it was shown that, S configuration of ML-SA1 bound to lysosomal TRPML1 channel 10 times strongly than other configurations and led to its activation. Consequently, autophagy flux was induced (expression of autophagy-markers was increased) via nuclear localization of TFEB.⁹³ Also, the ability of ML-SA1 to trigger autophagy was shown by the decrease in LC3-positive autophagic accumulations in rat primary cortical neurons in which TRPML1 function was blocked.⁸⁹

Lysosomal exocytosis and biogenesis are also regulated by cytosolic Ca^{2+} , CaN, and TFEB. Therefore, exogenous activation of the TRPML1 channel by ML-SA1 can regulate lysosomal exocytosis.⁹⁴ Accordingly, increased lysosomal exocytosis and release of α -synuclein were shown in dopaminergic neurons of Parkinson's disease patients with ML-SA1.⁹⁵ Also, uranium-induced nephrotoxicity in mouse kidney and renal proximal tubular epithelial cells could be reversed by the increased lysosomal exocytosis of uranium upon ML-SA1 treatment.⁹⁶

1.7 microRNAs (miRNAs)

The genomes of nearly all species (archaea to human) encode not only protein-coding RNAs but also non-coding RNAs (ncRNAs) serving as a gene expression regulatory hub, and microRNAs (miRNAs) are members of this class.⁹⁷ miRNAs are categorized as single-stranded small ncRNAs because they have 19-23 nucleotides (nts) in length.⁹⁸ The first miRNA discovered is 'lin-4' (22 nts), which was found to be able to pair with the LIN-14 protein-coding mRNA in *C. elegans*. After this finding, several miRNAs were identified across many species. It is known that there are more than 2000 miRNAs in humans. They can be present in plasma, urine, stool, and saliva as well as cells.⁹⁹ However, they are encapsulated in high-density lipoproteins (HDLs), exosomes, or bound to Ago 2 proteins to be protected from RNase function in these fluids. Due to their presence in body fluids, several miRNAs have been considered and studied as promising biomarkers of many diseases in recent years.¹⁰⁰

Roles of miRNAs in gene expression regulation generally occur at the posttranscriptional level; they interact with the 3'UTR region of particular mRNAs. However, they can also bind to the 5'UTR, even to the promoter and coding area.¹⁰¹

miRNAs-based regulation of mRNA transcription and translation processes can be categorized as canonical and non-canonical. In the canonical process, miRNA binds the 3'UTR of targeted mRNA from its seed sequence (a short sequence starting from the 5' of miRNA) and results in deadenylation and finally degradation of target mRNA. On the other hand, in a non-canonical process, there is no total match between miRNA and their target mRNAs. It is known that in human cells, 60% of miRNA-target interactions (MTI) occur through noncanonical binding. Therefore, one miRNA generally targets several different mRNAs at the same time, additionally, several miRNAs can target the same mRNA.^{100,102} The general idea about miRNA action on the target mRNAs is the inhibition of expression or translation post-transcriptionally. Nevertheless, it has been reported that they can also enhance gene expression/translation directly or indirectly.¹⁰³

In mammals, miRNAs are generated in a set of processes called canonical biogenesis. Initially, RNA polymerase II transcribes the primary miRNA, having a hairpin structure (pri-miRNA) from a particular DNA locus (miRNA genes). After the generation of pri-miRNA, a complex called 'microprocessor' consisting of DROSHA (a type of RNase III) and DGCR8 (DiGeorge Syndrome Critical Region 8) proteins chops the hairpin structure of pri-miRNA, and pre-miRNA (60-90 nt) is generated. This pre-miRNA still has a stem-loop structure and is translocated to the cytoplasm from the nucleus with the help of exportin 5 protein, XPO5. This transportation is dependent on RAN-GTP. In the cytoplasm, the loop structure of pre-miRNA is degraded by DICER (a type of RNase III) in complex with TRBP (a type of RNA-binding protein). At the end, Argonaute 2 (Ago2) protein binds to double-stranded miRNA (19-22 nt) and separates its two strands. Generally, one of these two strands is selected as functional miRNA according to its high purine content and modest thermodynamic stability of the 5' end, and the other strand can be degraded or also selected. miRNAs can be identified as 5p or 3p according to the ends of the pre-miRNA from which they are generated. If the miRNA strand is generated from the 3' end of the pre-miRNA, then it is called 3p. Conversely, if it is generated from the 5' end of pre-miRNA is called 5p. Selected strand (5p or 3p) interacts with RISC (RNA-induced silencing complex) and regulates posttranscriptional gene expression via degradation/destabilization of mRNA and inhibition of translation.¹⁰⁴

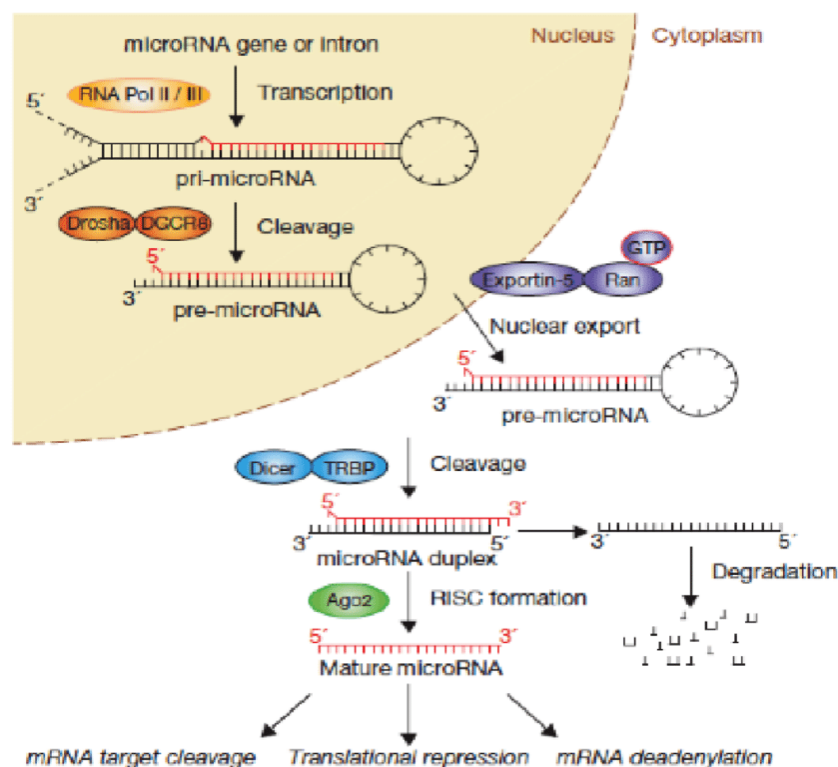


Figure 1.7 Canonical pathway of miRNA biogenesis.¹⁰⁴

miRNAs can also be generated with non-canonical pathways such as DICER-independent and DROSHA-independent biogenesis.^{105–107} Examples of miRNAs that are generated via these non-canonical pathways are mirtrons and 7-methylguanine capped (m^7G)-pre-miRNA. Their biogenesis occurs independently of DROSHA in the nucleus. The only difference between them is their transport protein. Mirtrons are exported to the cytoplasm by Exportin5 while m^7G -pre-miRNA is exported by Exportin1.¹⁰¹ miR-451 is a unique miRNA that is generated via a DICER-independent pathway because it is cleaved by the Ago2 protein in the cytoplasm.¹⁰⁸

These small ncRNAs have wide-ranging functions in the regulation of many biological events like cell fate determination, differentiation, apoptosis, cell signaling, metabolism, and maintaining tissue homeostasis. Accordingly, miRNAs are involved in nearly all cellular pathways; thus, their dysregulation is found in many diseases such as cancer, rheumatoid arthritis, neurodegenerative diseases like Alzheimer's disease, LSDs, etc.^{102,109}

1.7.1 miRNAs in LSDs

Since miRNAs play a role in a wide variety of cell mechanisms, as mentioned before, the possible roles of these small molecules in LSDs have also been investigated for the last 15 years. The first study on miRNA profile in LSDs was conducted in 2010 using fibroblast cells from Niemann Pick Type C (NPC) patients. In this study, miRNA profiling comparing patient fibroblasts and healthy fibroblasts revealed that miR-196a, miR-196b, and miR-296 were significantly upregulated while miR-143 and miR-98 were significantly downregulated in the disease state. Also, it was stated that these dysregulated miRNAs target genes in cholesterol metabolism and protein ubiquitination mechanism, parallel to the NPC pathology.¹¹⁰ miRNA profiling was also examined in the mouse model of NPC1, and it was shown that in the spleen, miR-155 was downregulated while it was upregulated in the liver compared to *WT* littermates. miR-155 was known to play a regulatory role in immunity and inflammatory mechanisms; therefore, it was thought that it may contribute to the disease progression.¹¹¹ In another study, multiple sulfatase deficiency (an LSD resulting from mutation of the sulfatase-modifying factor 1 (SUMF1), which is a post-transcriptional sulfatase activator) was found to be related to miR-95 overexpression and its downregulatory effect on the SUMF1 expression. Additionally, miR-95 distorted autophagic flux in vitro.¹⁰⁹ Gaucher disease is a type of LSD arising due to a deficiency of the glucocerebrosidase enzyme. Transfection of miR-195-5p and miR-16-5p on the Gaucher disease patient fibroblast cells and it was shown that these miRNAs increased glucocerebrosidase enzyme activity, while miR-127-5p, miR-19a-5p, and miR-1262 transfection decreased the enzyme activity.¹¹² In vivo miRNA profiling in the ventral mesencephalon area of the Gaucher disease mouse model revealed that miR-142 and miR-29b were upregulated, and miR-let7b was downregulated. Also, their involvement in the regulation of inflammatory pathway genes was found.¹¹³ In addition, upregulated miR-10a-5p in the midbrain of neuronopathic Gaucher disease mouse model repressed the expression of *Arhgef11* and *Epha8* genes involved in neuroprotection mechanisms like axon guidance.¹¹⁴ Another LSD, Fabry disease, was also studied in terms of miRNA profiling from blood samples of patients. miR-696 was significantly upregulated and miR-1 was downregulated compared to control samples. Upregulation of miR-696 inhibited the expression of mitochondrial biogenesis genes, and downregulation of miR-1 decreased the translation of mitochondria-related genes.

Therefore, Fabry disease was found to be associated with mitochondrial miRNA dysregulation.¹¹⁵ Small-scale miRNA analysis was performed in the subventricular zone and the striatum area of GM2 ganglioside mouse models (SD and TSD). Results showed that among 12 miRNAs examined, miR-9, miR-29a, miR-33, miR-19a, miR-34a, miR-124, miR-126a, miR-128, and miR-137 were dysregulated in both brain areas found in the SD and TSD mouse models.¹¹⁶

1.8 The Aim of the Study

The primary aim of this thesis study was to identify miRNAs and their biological roles in the Tay-Sachs disease mouse model (*Hexa*^{-/-}*Neu3*^{-/-}) fibroblast and neuroglia cells, in normal conditions and ML-SA1 treatment conditions, for the first time. For this purpose, comprehensive miRNA profiling with NGS (Illumina) followed by bioinformatic analyses on pairwise comparisons was performed. In this way, miRNAs that might be associated with the TSD pathology and progression were investigated. As a sub-aim of the study, the effect of the ML-SA1 on the previously shown impaired autophagic flux in *Hexa*^{-/-}*Neu3*^{-/-} mice was investigated simultaneously with the lysosomal exocytosis mechanism by using the same cell lines.

CHAPTER 2

MATERIALS & METHODS

2.1 Mouse Breeding

All animals used in this study were housed by the Turkish Animal Care Council (TCAC), an accredited animal facility of the Izmir Institute of Technology. Animals were kept in a controlled environment, under constant temperature and humid conditions. Also, they were maintained under a 12-h light/dark cycle. All animals were provided with food and water in an ad libitum way. Animal care and handling were carried out following the Turkish Animal Health Institute's laboratory animal handling and use guide. In the study, the *Hexa*^{-/-} mouse was crossed with the *Neu3*^{-/-} mouse. As a result of the first crossover, heterozygous (*Hexa*^{+/-}*Neu3*^{+/-}) male and female mice were obtained in the F1 generation. Then, heterozygous female and male mice in the F1 generation were crossed with each other to create a GM2 gangliosidosis mouse model with the *Hexa*^{-/-}*Neu3*^{-/-} genotype. All genotyping and cell line generation experiments were performed with the approval of the ethics committee. The principles of animal welfare were strictly followed.

2.2 Mouse Genotyping

2.2.1 Isolation of DNA

Tail tissues taken from 1-month-old mice were incubated at 55°C and 50 rpm overnight by adding 6 µl of proteinase K (EcoTech) and 250 µl of tissue lysis solution (10% 1M Tris-pH 7.6, 2.5% 0.2M EDTA, 20% SDS, 4% 5M NaCl). After incubation, the disrupted tails were centrifuged at 13000 rpm for 10 minutes at room temperature. The supernatant was taken into a new Eppendorf tube, 250 µl of 100% isopropanol was added, and the tubes were inverted to form a DNA pellet. DNA pellets were taken into

new Eppendorf tubes, 250 µl of 70% ethanol was added, and centrifuged at 15000 rpm for 1 minute at room temperature. After DNA pellets were completely freed from ethanol, they were allowed to dry at 55°C for 20 minutes. 100 µl of dH₂O was added to the DNA pellets and incubated at 55°C for 1 hour to dissolve. DNA samples were kept at -20°C for polymerase chain reactions.

2.2.2 Polymerase Chain Reaction (PCR) for Genotyping

For the genotyping of mice, PCR of *Hexa* and *Neu3* genes were conducted from isolated DNAs. Specific primer sequences for *Hexa* and *Neu3* PCR were listed in Table 2.1 below. In *Hexa* PCR, 48.5 µl solution consisting of 5 µl buffer solution (GeneDirex), 1 µl dNTP, 2 µl F primer, 1 µl R primer and 1 µl PGK primer, 0.5 µl Taq polymerase (GeneDirex), 38 µl dH₂O was prepared for each sample and 1.5 µl of DNA sample was added. The prepared tubes were placed in the PCR machine (BioRad) and the reaction was carried out under the conditions of 94°C for 3 minutes (initial denaturation); 35 cycles of 94°C for 45 seconds (denaturation), 60°C for 45 seconds (annealing), 72°C for 45 seconds (extension) and 72°C for 5 minutes (final extension). In *Neu3* PCR, 48.5 µl solution consisting of 5 µl buffer solution (GeneDirex), 1 µl dNTP, 1 µl F primer, 1 µl 552R primer and 1 µl 553R primer, 0.5 µl Taq polymerase (GeneDirex), 39 µl dH₂O was prepared for each sample and 1.5 µl of DNA sample was added. The prepared tubes were placed in the PCR machine. The reaction was carried out under the conditions of 94°C for 3 minutes (initial denaturation), 35 cycles of 94°C for 50 seconds (denaturation), 58°C for 45 seconds (annealing), 72°C for 2 seconds (extension), and 72°C for 10 minutes (final extension). Then, all samples were run on a 1% agarose gel at 90 volts for 30 minutes, and images were taken under UV light with the Fusion SL device.

Table 2.1. *Hexa* and *Neu3* gene-specific genotyping primer sequences.

Gene Name	Primer	Primer Sequences	Product Size
<i>Hexa</i>	Forward	5'-GGCCAGATACAATCATACAG-3'	WT Allele: 420 bp Mutant Allele: 210 bp
	Reverse	5'-TGTCCACATACTCTCCCCACAT-3'	
	PGK	5'-CACCAAAGAAGGGAGCCGGT-3'	
<i>Neu3</i>	Forward	5'- AAGCAGAGAACATTCTTGAGAGAG CACAGC -3'	WT Allele: 1022 bp Mutant Allele: 601 bp
	552 Reverse	5'- TCGTGCTTTACGGTATCGCCGCTCC CGATT-3'	
	553 Reverse	5'- GTGAGTTCAAGAGCCATGTTGCTGA TGGTG-3'	

2.3 Generation of Immortal Cell Lines

2.3.1 Generation of Primary Neuroglia Cell Lines

After genotyping the mice, primary neuroglia cell lines from the cortex and hippocampus regions of 5-month-old *WT* and *Hexa*^{-/-}*Neu3*^{-/-} mice were obtained according to the protocols of an Adult Brain Dissociation Kit (MACS Miltenyi Biotec) and Adult Neuron Isolation Kit (MACS Miltenyi Biotec). First, the mouse brains were removed in laminar hood (SCANLAF), washed in cold Dulbecco's phosphate buffered

saline, (D-PBS, Biowest), removed from the excess blood, and the cortex and hippocampus regions were carefully obtained by dissection. These tissues were then cut into small pieces and placed in a gentleMACS™ C tubes (Miltenyi Biotec) containing 1960 µl of enzyme mixture 1 (50 µl enzyme P in 1910 buffer Z). The C tubes were placed in the m_brain_01 program registered in the gentleMACS™ Dissociator device (Miltenyi Biotec). After that, tubes were taken from the dissociator, placed in the MACSmic™ Tube Rotator (Miltenyi Biotec), and rotated slowly and continuously for 15 minutes at 37°C. After 15 minutes, 30 µl of enzyme mixture 2 (30 µl enzyme Y and 15 µl enzyme A) was added and mixed but not vortexed. Then, the C tubes were placed in the m_brain_02 program registered in the dissociator. Tubes were taken from the dissociator again, placed in the rotator, and turned slowly and continuously for 15 minutes at 37°C. After the rotation was completed, C tubes were placed in the m_brain_03 program registered in the dissociator. Then, the remaining 15 µl of enzyme mixture 2 was added into C tubes and mixed without vortexing. After the MACS SmartStrainers (70 µm, Miltenyi Biotec) were placed on the 50 ml falcon tubes and wetted with 3 ml of D-PBS containing MACS® BSA solution (Miltenyi Biotec). Stock solution of BSA was diluted as 1:20 with D-PBS. The suspensions obtained were passed through the MACS SmartStrainer. Then, 10 ml of cold D-PBS containing BSA was added to each C tube. All homogenized tissue particles were collected and quickly added to MACS SmartStrainers. Centrifugation was performed at 300 g for 10 minutes at room temperature. The supernatants were removed carefully. After dissolving the pellets by adding 1550 µl of cold D-PBS, they were placed into 15 ml falcon tubes. In addition to D-PBS, 450 µl of debris removal solution was added to each falcon. Pipetting was done gently. Then, 2 ml of cold D-PBS was added to the resulting mixture to create a gradient very slowly. Centrifugation was performed at 3000 g for 10 minutes at +4°C in full acceleration and brake. Then, the upper 2 phases were removed, and cold D-PBS was added onto the remaining phase until it reached 15 ml. Tubes were inverted 3-4 times, and the solutions were centrifuged for 15 min at 1000 g, +4°C. The supernatants were discarded totally. After this stage, the method of red blood cell removal was continued. For that purpose, 500 µl of 1X Red Blood cell removal solution was mixed with the obtained cell pellets and tubes were incubated in the +4°C for 10 min. Then, 5 ml of cold PB (phosphate buffer) solution was added and centrifuged for 10 min at 300 g, +4°C. The upper phase was completely removed. The obtained cells were allowed to grow in T25 flasks (Greiner) in 5 ml of complete Duplecco's Modified Eagle Medium (DMEM, Gibco) containing

10% Fetal Bovine Serum (FBS, Gibco), 1% Penicillin-Streptomycin (Gibco). For growth, all cells were incubated at cell culture incubator (Thermo Scientific) serving 37°C, 5% CO₂, and 75% humidity.

2.3.2 Generation of Primary Fibroblast Cell Lines

Primary fibroblast cell lines were simultaneously generated from the same 5-month-old *WT* and *Hexa*^{-/-}*Neu3*^{-/-} mice whose brain tissues were used to generate primary neuroglia cells. Each mouse was killed using the cervical dislocation procedure. They were cleaned with 70% ethanol on their outer surface and placed in a laminar hood. Their rib cages were shaved with a scalpel and their hairs were removed as much as possible. A tiny piece of their skin was cut. The skin tissues were placed in complete medium and cut into small pieces with a scalpel. The dissected tissues were placed in a T25 flask with complete medium and left to grow in a cell culture incubator. The cells were washed with 1X PBS (Biowest) and grown in a complete medium until they reached the appropriate confluency and showed morphology. The cells at the appropriate density were washed with 1X PBS, 500 µl of 0.25% (w/v) trypsin-0.53mM EDTA solution (Gibco) was added, and kept in the incubator at 37°C for approximately 5 minutes. The cells separated from the surface were seeded again in the flask for equal distribution and continued to be cultured in 5 ml of complete medium.

2.3.3 Immortalization of Primary Cell Lines

To immortalize previously generated primary neuroglia and fibroblast cells, 1 ml of the viral supernatant (Human Papilloma Virus Type 16, LXS_N16E6E7) routinely used in our laboratory was mixed with 3 ml complete DMEM and 8 µg/ml polybrene (Sigma-Aldrich) and given to the cells. After overnight incubation in the cell culture incubator, the viral medium was removed from the cells. The cells were washed with 1X PBS and grown for 2 days in complete DMEM. Then, to select the immortal cells, they were grown

for 10 days in complete DMEM containing 4 µg/ml Geneticin (G418, Gibco). The cell media was refreshed every 4 days during the selection process.

2.4 MTT Assay

Cell viability and proliferation were measured using the MTT Cell Proliferation Assay Kit (Cayman Chemical) under the effect of ML-SA1. Neuroglia and fibroblast cells generated from *WT* and *Hexa^{-/-}Neu3^{-/-}* mouse were seeded at equal rates (nearly 2×10^4 cells per well) in 96-well cell culture plates as 3 replicate wells. They were grown in the cell culture incubator. The next day, the different concentrations of ML-SA1 (10 µM and 20 µM) were applied to each cell line, and 3 wells of each cell line were kept as untreated (control group). Treatment was done for 24 hours in the incubator. At the end of the treatment, the DMEM was removed from the cells, and fresh complete DMEM (without ML-SA1) was added to each well. 10 µl of MTT solution was added to wells as recommended by the kit protocol and mixed with the medium by pipetting. Then, the 96-well plate was incubated for 4 hours in the cell culture incubator. After formazan crystal formation was observed in each well, 100 µl of crystal solvent solution was added to the wells and incubated for an additional 18 hours in the incubator. Colorimetric measurements were made using 570 nm with a multipurpose microplate reader (Thermo Scientific) located in our school's Integrated Research Center (TAM). According to the measurements obtained, cell viability was calculated as a percentage for each cell line and analyzed using the GraphPad Prism program. The optimal dosage of ML-SA1 was determined for further experiments based on these results.

2.5 ML-SA1 Treatment

After the correct dosage was determined, 5-month-old *WT* and *Hexa^{-/-}Neu3^{-/-}* mouse neuroglia and fibroblast cell lines were grown in the cell culture incubator. Once the cells reached 80% confluency in T25 flasks, complete DMEM was removed. They were washed with 1X PBS to remove dead cells, and trypsin was applied to detach the

cells from the surface to which they adhered. Cells were incubated in trypsin for 3 minutes at 37°C, and they were collected with complete DMEM and transferred to new T75 flasks (Greiner) and left to grow in an incubator. After the cells were raised to 90% confluency, they were passaged with the same procedure, reseeded to 100-mm cell culture dishes (Greiner), and waited to reach 70% confluency. ML-SA1 powder (Cayman Chemical) was dissolved in the appropriate amount of dimethyl sulfoxide (DMSO, Sigma-Aldrich) to obtain a 70 mM final concentration. Before treatment, DMEM was removed, and cells were washed with 1X PBS. 7 ml of complete DMEM with 1 µl of the 70 mM ML-SA1 stock solution to reach a final concentration of 10 µM ML-SA. This mix was given to the cells. Cells were incubated at 37°C, 5% CO₂, and 75% humidity for 24 hours. When the treatment needed to be done in 6-well, 24-well, 96-well plates, or even in the 150-mm cell culture dishes, all steps were performed without any change in the final ML-SA1 concentration (10 µM).

2.6 Enzyme Activity Assays

The specific activities of the acid phosphatase, β-Hexosaminidase B, and β-Hexosaminidase A enzymes were quantified from the cell culture media (complete DMEM), and also the cell lysate of 5-month-old *WT* and *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblast cell lines after the 10 µM, 24 hours ML-SA1 treatment by comparing to untreated conditions. In this way, the effect of ML-SA1 on lysosomal exocytosis was investigated.

2.6.1 Sample Preparation

WT and *Hexa*^{-/-}*Neu3*^{-/-} fibroblast and neuroglia cells were grown in 6-well cell culture plates and treated with 10 µM ML-SA1 for 24 hours. For the measurement of acid phosphatase, β-Hexosaminidase B, and β-Hexosaminidase A enzyme activities from the cell culture medium (extracellular) and cell lysate (intracellular), the media and cells were collected simultaneously from each well. Cells were harvested from wells with ice-cold

1X PBS and centrifuged for 5 min at 860 rpm. After one more 1X PBS wash, supernatants were removed from the cell pellets. Mediums were centrifuged at 12000 g for 10 minutes at +4°C to get rid of the dead cells, and they were transferred to the new 1.5 ml Eppendorf tubes. Cell pellets were resuspended in 20 µl dH₂O and sonication was performed at 60 V for 3 x 5 seconds with a Sonicator (Bandelin).

2.6.2 β-hexosaminidase B (Hexb) Assay

As a substrate of β-hexosaminidase B, fluorescent tagged 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide (Sigma-Aldrich) was used. Tubes for measuring extracellular and intracellular β-Hexosaminidase B enzyme activity were prepared as indicated in Table 2.2 and Table 2.3, respectively. Then, the mouths of the tubes were closed with parafilm, and the samples were incubated in a water bath at 37°C for 1 hour. To stop the reaction, the 0.4 M Glycine buffer (pH-10.8) was added to each tube until the total volume was 4 ml. Fluorometric measurements were performed by the RF-5301PC (Shimadzu Europe) spectrofluorometer device at an emission wavelength of 365 nm and excitation wavelength of 445 nm. A standard curve generated based on the 4-Methylumbelliferyl was used during measurements. In addition, the total protein levels of each sample were determined by the Bradford assay as described previously. Then, the measurement results were calculated as specific enzyme activity and analyzed with the GraphPad Prism software.

All calculations were made according to the following formulas:

$$\text{Enzyme Activity (nmol/hour/ml)} = \frac{\text{Fluorometric measurement} \times (\text{Volume}_{\text{Final reaction}} / \text{Volume}_{\text{Sample}}) \times (1/\text{incubation time (h)})}{1}$$

$$\text{Specific Enzyme Activity (nmol/mg protein/hour)} = \frac{\text{Enzyme Activity}}{\text{Total Protein Concentration}}$$

Table 2.2. Extracellular β -hexosaminidase B enzyme activity assay

Blank Tube	Sample Tube
- 0.5 μl Substrate - 74.5 μl Sodium Citrate-Phosphate Buffer (pH-4.5) - 75 μl dH₂O	- 0.5 μl Substrate - 74.5 μl Sodium Citrate-Phosphate Buffer (pH-4.5) - 75 μl Cell Culture Medium

Table 2.3. Intracellular β -hexosaminidase B enzyme activity assay

Blank Tube	Sample Tube
- 0.5 μl Substrate - 144.5 μl Sodium Citrate-Phosphate Buffer (pH-4.5) - 5 μl dH₂O	- 0.5 μl Substrate - 144.5 μl Sodium Citrate-Phosphate Buffer (pH-4.5) - 5 μl Cell Lysate

2.6.3 β -hexosaminidase A (Hexa) Assay

In this assay substrate of the enzyme was 4-Methylumbelliferyl-6-sulfo-2-acetamido-2-deoxy- β -D-glucopyranoside potassium salt (SantaCruz). For the measurement of intracellular β -Hexosaminidase A enzyme activity, tubes were prepared as shown in Table 2.4. The mouths of the tubes were closed with parafilm, and the

samples were incubated in a water bath at 37°C for 1 hour. To stop the reaction, the 0.4 M Glycine buffer (pH-10.8) was added to each tube until the total volume reached 4 ml. Fluorometric measurements were done by the RF-5301PC (Shimadzu Europe) spectrofluorometer device at an emission wavelength of 365 nm and excitation wavelength of 445 nm. A standard curve obtained based on the 4-Methylumbelliferyl was used. The total protein levels of each sample were found with Bradford Assay. Specific enzyme activities were calculated. Data was analyzed with the GraphPad Prism software.

All calculations were made according to the following formulas:

Enzyme Activity (nmol/hour/ml) = Fluorometric measurement x (Volume_{Final reaction} / Volume_{Sample}) x (1/incubation time (h))

Specific Enzyme Activity (nmol/mg protein/hour) =
$$\frac{\text{Enzyme Activity}}{\text{Total Protein Concentration}}$$

Table 2.4. Intracellular β -hexosaminidase A enzyme activity assay

Blank Tube	Sample Tube
- 50 μ l Substrate - 40 μ l 0.1 M Sodium Acetate Buffer (pH-4.5) - 10 μ l dH ₂ O	- 50 μ l Substrate - 40 μ l 0.1 M Sodium Acetate Buffer (pH-4.5) - 10 μ l Cell Lysate

2.7 Real-Time Polymerase Chain Reaction (RT-PCR)

The effect of ML-SA1 treatment on the expression levels of autophagy-related genes (*Atg7*, *Beclin1*, *p62*, and *Lamp1*) was investigated by RT-PCR in the treated and untreated neuroglia and fibroblast cells of 5-month-old *WT* and *Hexa*^{-/-}*Neu3*^{-/-} mice.

2.7.1 Total RNA Isolation

Firstly, control groups and ML-SA 1 treated 5-month-old *WT* and *Hexa*^{-/-}*Neu3*^{-/-} mouse fibroblast and neuroglia cells were collected from 100-mm cell culture dishes to 15 ml Falcon tubes, and each cell pellet was dissolved in 1 ml RiboEx (GeneAll) solution. Mixed cells were transferred to 1.5 ml Eppendorf tubes and incubated for 5 minutes at room temperature. Then, 200 µl of chloroform was added to the tubes, inverted gently to ensure mixing, and incubated for another 2 minutes at room temperature. After the incubation was done, the tubes were centrifuged for 5 minutes at 12000 g, +4°C, and the upper liquid phase was transferred to a new 1.5 ml Eppendorf tube. After adding 500 µl of isopropanol, they were mixed by gently turning upside down and incubated for 10 minutes at room temperature. Then, they were centrifuged for 10 minutes at 12000 g, +4°C, and pellet formation was observed. The supernatant portions were removed from the pellets, and 1 ml of 75% ethanol was added to the RNA pellet and centrifuged for 5 minutes at 7500 g, +4°C. The ethanol was removed, and the tubes were left to dry with their caps open. After all remaining ethanol was air dried, 30 µl of RNase-free dH₂O was added to the RNA pellets, and they were dissolved in a 50°C water bath. The RNA concentration of the dissolved pellets was measured with the Implen Nanophotometer N50 nanodrop device and recorded.

2.7.2 cDNA Conversion

All RNA samples were converted to cDNA using the Iscript cDNA conversion kit (Bio-Rad) in accordance with the conditions specified by the vendor. With this aim, 4 µl 5x Iscript Mix, 1 µl Reverse Transcriptase enzyme, and 15 µl RNA + RNase-free dH₂O (total 20 µl) were mixed for each RNA sample separately. The amount of RNA was arranged as 1000 ng during the reaction preparation. The condition for this reaction starts with 5 minutes at 25°C (primer annealing) and continues with 20 minutes at 46°C (reverse transcription), and completes with 1 minute at 95°C (enzyme halting). After the conversion of cDNA, PCR for *Gapdh*, a housekeeping gene, was conducted to ensure that RNA samples were properly converted to cDNA in every sample. PCR mixture was prepared as follows; 2.5 µl buffer solution, 1.5 µl 50 mM MgCl₂, 1 µl dNTP, 1 µl Forward primer, 1 µl Reverse primer, 16 µl dH₂O and 0.5 µl Taq polymerase. As a template 1.5 µl cDNA was added into the mix. The prepared tubes were placed in the PCR machine. The reaction was carried out under the conditions of 95°C for 3 minutes (initial denaturation), 30 cycles of 95°C for 20 seconds (denaturation), 63°C for 15 seconds (annealing), 72°C for 20 seconds (extension) and 72°C for 5 minutes (final extension). Then, all samples were run on 1% agarose gel at 90 volts for 30 minutes and images were taken under UV light with Fusion SL device.

2.7.3 RT-PCR

To measure the expression levels of the autophagy-related genes listed below (Table.2.5), qPCR reaction mixes were prepared separately by using iTaq™ Universal SYBR® Green Supermix (Bio-Rad). In addition, *Gapdh* was used as an internal control to normalize the expression levels of target genes. According to the protocol, 5 µl SYBR Green mix, 3.6 µl dH₂O, 0.2 µl forward primer and 0.2 µl reverse primer were mixed and 1 µl cDNA (50 ng/µl) was added for control groups and ML-SA1 treated 5-month-old *WT* and *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblast cells. These reactions were placed in 96-well PCR plate. For each gene and genotype/treatment conditions, 3 wells were used as replicates to get more accurate results. After sealing the 96-well plate with a plate sticker,

it was placed in the LightCycler 96 (Roche) machine. RT-PCR conditions for these genes involve 1 cycle of 10 minutes at 95°C, 45 cycles of 20 seconds at 95°C, 15 seconds at 57°C, and 22 seconds at 72°C. After the reaction was completed, the data were analyzed and visualized by using GraphPad Prism software. During statistical analysis, two-way ANOVA was used to identify significant changes.

Table 2.5. Expression primer sequences and product sizes of autophagy-related genes.

Gene Name	Primer Sequence		Product Size (bp)
<i>Beclin1</i>	F	5'- GAGGAGCAGTGGACAAAAGC -3'	112
	R	5'- CAAACATCCCCTAAGGAGCA -3'	
<i>p62</i>	F	5'- TGTGGAACATGGAGGGAAGAG-3'	67
	R	5'- TGTGCCTGTGCTGGAAC TTTC-3'	
<i>Atg7</i>	F	5'- TGCCTATGATGATCTGTGTC -3'	146
	R	5'- CACCAACTGTTATCTTTGTCC -3'	
<i>Lamp1</i>	F	5'- CCAGGCTTTCAAGGTGGACAGT -3'	134
	R	5'- GGTAGGCAATGAGGACGATGAG -3'	
<i>Gapdh</i>	F	5'- CCCCTTCATTGACCTCAACTAC-3'	347
	R	5'- ATGCATTGCTGACAATCTTGAG-3'	

2.8 Western Blot Analysis

Western blot analysis was performed to assess autophagy by measuring the protein levels of Lamp1, p62, Beclin1, and LC3 in untreated and ML-SA1 treated 5-month-old *WT* and *Hexa-/-Neu3-/-* mouse fibroblast and neuroglia cells.

2.8.1 Protein Isolation

100 μ l of RIPA lysis solution including 1% TritonX100, 150 mM NaCl, 10% Glycerol, 50 mM TrisBase and HEPES, 1% PMSF (Sigma-Aldrich) and 1% PIC (protease inhibitor cocktail, Sigma-Aldrich) was added to each cell pellets and kept on ice for 1 hour by vortexing every 10 minutes to ensure cell lysis. Then centrifugation was done for 10 minutes at 14000 rpm and +4°C. The obtained supernatants were taken into new Eppendorf tubes and used as total protein.

2.8.2 Bradford Assay

Protein stocks were prepared from each isolated total protein, as they would have the same concentrations. For that purpose, a Bradford assay was conducted. Firstly, total protein solutions and RIPA buffer (without PMSF, PIC) were diluted in dH₂O with a ratio of 1/10 (4 μ l of solution into 36 μ l dH₂O). Also, bovine serum albumin (BSA), which has several concentrations (0.1, 0.2, 0.4, 0.8, and 1.0 mg/ml), was used to obtain a standard curve during concentration measurement. After that, 10 μ l of each diluted sample, lysis buffer, dH₂O, and BSA solutions were loaded to the 96 well plates as 3 replicas. 200 μ l Bradford solution was added into each well simultaneously by using a multichannel micropipette. 7 minutes of incubation was applied in the dark, then spectrophotometric measurement was done at 595 nm by iMark™ Microplate Absorbance Reader (BioRad). Absorbance results were recorded for each well. To create a standard curve, firstly, the absorbance value of water was subtracted from the average absorbance values of BSA solutions, and the graph was drawn. The concentrations of the isolated proteins were found by subtracting the average value of the lysis buffer and using the equation from this curve. In this way, 4X protein stocks (15 μ g/ μ l) were prepared for each sample by using isolated protein, dH₂O, and loading dye (40% glycerol, 8% SDS, 240 mM Tris-HCl pH: 6.8, 0.04% bromophenol blue, 5% β -mercaptoethanol). 1X stock includes 9 μ l protein+dH₂O and 3 μ l loading dye. At the end, 4X protein stocks were boiled at 95°C for 10 minutes and stored at +4°C until the SDS-PAGE step.

2.8.3 SDS-PAGE

In this step, a vertical SDS-PAGE gel system was prepared using a 12% resolving gel and a 10% stacking gel. The resolving gel solution was prepared by mixing 3.3 ml dH₂O, 2.5 ml 40% Acrylamide, 2 ml pH-8.8 Tris Buffer, 80 µl 10% SDS, 80 µl 10% Ammonium persulfate (APS), and 80 µl N,N,N',N'-Tetramethylethylenediamine (TEMED) while the stacking gel was made by combining 1.55 ml dH₂O, 0.5 ml 40% Acrylamide, 0.625 ml pH-6.8 Tris Buffer, 25 µl 10% SDS, 25 µl 10% APS, and 2.5 µl TEMED. Once the SDS-PAGE gel system was set, 3 µl of the protein ladder (BioRad) and 12 µl of each prepared protein stock were loaded into the wells, and the chamber was filled with running buffer (0.25 M Tris, 1.92 M Glycine, 1% SDS). Electrophoresis was carried out at 80V for 20 minutes, followed by 120V for 90 minutes. After the run was completed, proteins were transferred onto a nitrocellulose membrane at 250 mA for 75 minutes in an ice-cold transfer buffer (48 mM Tris, 39 mM, 20% MeOH) using the sandwich method. In this method, the membrane and SDS gel were carefully stacked between sponges and filter papers to ensure efficient transfer. After that, blocking was done by incubating the membrane within the 5% non-fat dry milk in 1X PBS-T on a shaker (Cole-Parmer™ Stuart™) for 60 minutes at room temperature. Next, the membrane was washed three times for 5 minutes each with 1X PBS-T solution. It was then cut into several pieces according to the molecular weights (kDa) of the targeted proteins. Primary antibodies (anti-Lamp1 (Cst-E5N9z), anti-LC3B (Cst-2775s), anti-p62 (Cst-39749s) anti-Beclin1 (Cst-3495S) and anti-β-actin (Cst-4970s) were diluted in the red solution (5% BSA, 0.02% NaAzide, Phenol Red in PBS-T pH-7) with the ratio of 1/1000 before being added to the corresponding membrane pieces. The membranes were then incubated overnight at +4°C on a shaker. The next day, all membranes were washed with 1X PBS-T again three times for 5 minutes and allowed to be incubated for 60 minutes in the HRP-conjugated secondary antibody (Jackson ImmunoResearch Lab) that was diluted in the 5% non-fat dry milk within 1X PBS-T as 1/10000. Finally, the washing was applied as described previously and blot images were captured by Fusion SL chemiluminescence visualization machine. To make the protein bands visible, an enhancer solution (2 ml of 100 mM Tris-HCl pH-8.5, 4.4 µl coumaric acid, 10 µl luminol, 0.5 µl H₂O₂) was added to the membranes and allowed to incubate for 30 seconds in the dark room. This procedure was repeated 3 times for each protein to perform statistical

analysis. Analysis of the images was done by using NIH ImageJ program. Statistically significant changes were determined through two-way ANOVA, and results were graphed using GraphPad software.

2.9 Immunocytochemical (ICC) Staining

ICC staining was made to analyze the effect of ML-SA1 in autophagy and lysosomal exocytosis pathways. To make these observations, total p62, total LC3, total Lamp1, LC3-Lamp1, and surface Lamp1 staining were performed in ML-SA1 treated and untreated 5-month-old *WT* and *Hexa-/-Neu3-/-* mouse neuroglia and fibroblast cells.

Firstly, cells were seeded on a 24-well plate containing round glass coverslips in each well. They were grown in the incubator until they reached 50 % confluency and treated with 10 μ M ML-SA1 for 24 hours. At the end of the treatment, all medium was discarded completely, and cells were gently washed with 1X PBS for 5 minutes. This step was repeated 3 times. Then they were fixed with 500 μ l 4% paraformaldehyde (PFA) for 30 minutes at room temperature. After 3 washes with 1X PBS for 5 minutes each, they were incubated with 1X PBS containing 0.5% Triton X-100 for 30 minutes at room temperature to permeabilize the cell membranes. However, for the surface Lamp1 staining, this step was not performed and was skipped directly to the blocking step. The blocking solution was prepared with 10% goat serum, 4% BSA, 0.3 M glycine, and 0.003% Triton X-100 in 1X PBS. To each well, 200 μ l blocking solution was added dropwise and waited for 1 hour at room temperature. In addition. Then, the cells were incubated overnight at +4°C with the primary antibodies anti-Lamp1 (Thermo-Scientific, PA5-95849), anti-p62 (Cst-39749s), and anti-LC3 (Rdsystems-Mab8558/ Cst-2775s) dissolved in blocking solution with appropriate dilution ratios. The next day, after being washed 3 times for 5 minutes with 1X PBS, the secondary antibodies Alexa Fluor®-488 (anti-rabbit, ab150077) and Alexa Fluor®-568 (anti-rat, ab175476) were diluted in a blocking solution. 200 μ l from Alexa Fluor®-568 was used for LC3B and p62-stained cells, while Alexa Fluor®-488 was added to the LC3A-Lamp1 and surface Lamp1-stained cells. Secondary antibody incubation was made at room temperature, for 1 hour. During this incubation, the 24-well plate was protected from the light. After incubation, the cells were washed with 1X PBS 3 times for 5 minutes, and the cells on the coverslips

were mounted and fixed on the slides with 8 µl DAPI-fluoroshield mounting medium (Abcam). Images of the slides were taken using a Fluorescence Microscope (Olympus BX53). Qualitative and quantitative analyses were performed using NIH ImageJ and GraphPad Prism software.

2.10 microRNA (miRNA) Profiling

To identify differentially expressed (DE) miRNAs and their target genes and cellular pathways, comprehensive miRNA profiling was performed on both ML-SA1 treated and untreated fibroblasts and neuroglia cells of 5-month-old *WT* and *Hexa*^{-/-} *Neu3*^{-/-} mice.

2.10.1 Sample Preparation for miRNA Sequencing

For whole microRNA profiling, ML-SA1 treated and untreated *WT* and *Hexa*^{-/-} *Neu3*^{-/-} fibroblast and neuroglia cells were sent to the FASTERIS company (Plan-les-Ouates, Switzerland) in DNA/RNA shield solution (Zymo Research; R1100-50). Sample preparation was carried out according to the manufacturer's guidelines. Cells were treated in 100 mm cell culture flasks and collected as described previously. Cell pellets were washed with 1 ml 1X PBS and resuspended in 800 µl DNA/RNA shield solution. All samples were kept at -80°C until the shipping day, then they were sent at room temperature conditions due to the preservation feature of the shield solution.

2.10.2 miRNA Isolation and Sequencing

The isolation of miRNAs from the sent cell pellets and their sequencing using Illumina Next Generation Sequencing (NGS) were performed by Fasteris Life Sciences SA (Geneva, Switzerland) through service procurement.

2.10.3 Raw Data Analysis

In order to perform comparative analyses of ML-SA1 treated and untreated neuroglia and fibroblast cells of *WT* and *Hexa-/-Neu3-/-* mice, differential gene expression analyses (Differential Expression Analysis) were applied. Before proceeding with these analyses, the raw sequencing data of the relevant samples were obtained in FASTQ format, and quality controls were examined in detail using FastQC (v 0.12) software. As a result of the quality assessment, it was observed that adapter sequences were highly represented. In order not to negatively affect data analyses, preprocessing was performed using fastp (v 0.23.4) software. In this way, adapter contaminations were eliminated from the sequences and data quality was enhanced. The cleaned and high-quality sequences were mapped using the R programming language (Rsubread (v3.17) library) using the mm10 (*Mus musculus*) version as the reference genome. To make comparisons at the miRNA level, expression determination was performed at the gene level using the annotation files of the miRBase (v22). Using the obtained data, expression levels between sample groups were calculated, and biologically significant gene differences were determined. Finally, in order to determine differentially expressed genes, a threshold value of $|\log_2FC| > 2$ and a False Discovery Rate (FDR) < 0.05 were applied to reveal significant differences between genes. As a result of these filters, statistically significantly increased (upregulated) and decreased (downregulated) genes were successfully detected.

In gene enrichment analyses, target genes of the relevant miRNAs were determined using two different online tools, MIENTURNET and miRNet. In the first stage, potential target mRNAs of miRNAs were identified using these tools, and then miRNA-mRNA interaction networks were created based on the obtained data. In order to increase the validity of these interactions, the Minimum Target Interaction (MTI) value was generally determined as 3; however, this threshold was applied as 2 and even 1 when the number of pathways obtained was below 20.

miRNA-target gene matches were taken from two different reliable databases, namely TargetScan and miRTarBase. While the TargetScan database was used with default parameters, “Strong” and “Weak evidence” categories were taken into consideration together while selecting miRTarBase data. The identified miRNA-target gene interactions were subjected to functional enrichment analysis using KEGG and

Reactome pathway databases. In the obtained result tables, $p < 0.05$ (if needed $p < 0.1$) filter was applied as the significance threshold, and only statistically significant pathways were taken into account. Enrichment results were visualized using in R programming language (ggplot2 package) and differences at the pathway level were presented graphically.

To find out the significant DE miRNAs, 8 comparisons were made between samples as shown in Table 2.6.

Table 2.6. Overview of the pairwise comparison groups used for DE analysis.

	Group 1		Group 2
1.	<i>WT</i> fibroblast (untreated)	$\leftrightarrow$	<i>WT</i> fibroblast + ML-SA1
2.	<i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} fibroblast (untreated)	$\leftrightarrow$	<i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} fibroblast + ML-SA1
3.	<i>WT</i> fibroblast (untreated)	$\leftrightarrow$	<i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} fibroblast (untreated)
4.	<i>WT</i> fibroblast + ML-SA1	$\leftrightarrow$	<i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} fibroblast + ML-SA1
5.	<i>WT</i> neuroglia (untreated)	$\leftrightarrow$	<i>WT</i> neuroglia + ML-SA1
6.	<i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} neuroglia (untreated)	$\leftrightarrow$	<i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} neuroglia + ML-SA1
7.	<i>WT</i> neuroglia (untreated)	$\leftrightarrow$	<i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} neuroglia (untreated)
8.	<i>WT</i> neuroglia + ML-SA1	$\leftrightarrow$	<i>Hexa</i> ^{-/-} <i>Neu3</i> ^{-/-} neuroglia + ML-SA1

Finally, shared DE miRNAs were examined between fibroblasts and neuroglia cells in untreated and ML-SA1 treated conditions by using $\log_2FC \geq 2$ for upregulation and $\log_2FC \leq -2$ for downregulation. After that, their gene and pathway enrichment analysis were performed.

CHAPTER 3

RESULTS

3.1 Mouse Genotyping

PCR and agarose gel electrophoresis methods were applied to isolated DNA samples for each mouse to detect their genotypes for *Hexa* and *Neu3* genes. Wild-type and mutant alleles for each gene were analyzed by comparing the band size of their PCR products with a 1 kb DNA ladder.

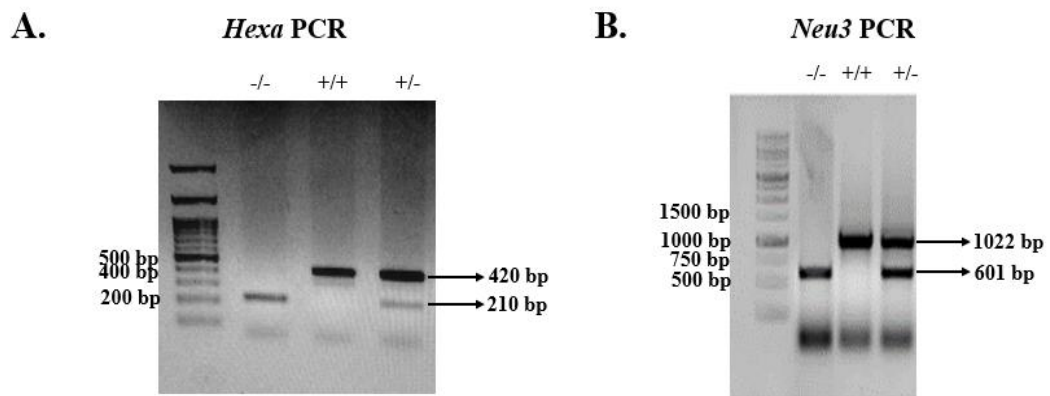


Figure 3.1. The images of agarose gel electrophoresis after PCR of *Hexa* (A), and *Neu3* (B) genes. The wild-type allele of the *Hexa* gene is 420 bp and the mutant allele is 210 bp in length. For the *Neu3* gene, wild-type allele is produced as 1022 bp, and the mutant allele is produced as 601 bp in length.

The primers used in the *Hexa* PCR were PGK, forward, and reverse primers specifically designed for the *Hexa* gene, while in the *Neu3* PCR, specific forward, 552 reverse, and 553 reverse primers were used. Genotypes of each mouse in terms of *Hexa* and *Neu3* genes were indicated as *-/-*, *+/+*, *+/-* (Figure 3.1 A and Figure 3.1 B respectively) in the gel image.

3.2 MTT Assay

Proliferation and viability levels of *WT* and *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblast cells were measured in 10 and 20 μ M ML-SA1 treatments by MTT assay to determine the most proper ML-SA1 dosage for the cells. Treatment time was arranged as 24 hours for both concentrations.

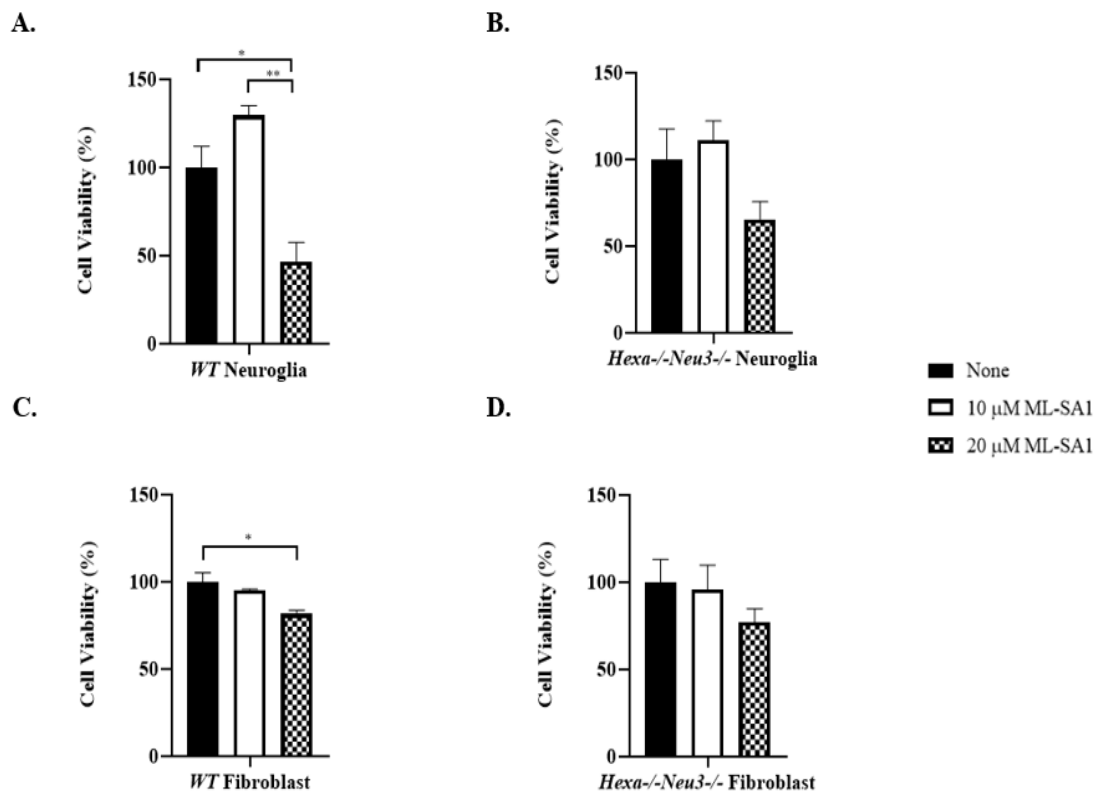


Figure 3.2. Comparative representation of the viability percentages of 5-month-old *WT* neuroglia (A), *Hexa*^{-/-}*Neu3*^{-/-} neuroglia (B), *WT* fibroblast (C), *Hexa*^{-/-}*Neu3*^{-/-} fibroblast (D) after 24 h of 10 μ M and 20 μ M ML-SA1 treatments compared to the untreated (none) condition. The data were represented as the mean $\pm$ SEM, and one-way ANOVA analysis was used to determine p-values by using GraphPad. (n=3, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001).

According to Figure 3.2, it was observed that 20 μ M ML-SA1 treatment caused a significant decrease in cell viability of *WT* neuroglia compared to 10 μ M (Figure 3.2 A). Although not statistically significant, 20 μ M ML-SA1 reduced cell viability more than 10 μ M in all cell lines (Figure 3.2 A-D). In addition, 10 μ M ML-SA1 increased the cell

viability in neuroglia of *WT* and *Hexa*^{-/-}*Neu3*^{-/-} compared to untreated conditions; however, these increments were not statistically significant (Figure 3.2 A and Figure 3.2 B).

3.3 Enzyme Activity Assays

Specific activities of Hexb and Hexa enzymes were measured from intracellular and extracellular areas after the treatment of neuroglia and fibroblast cells of *WT* and *Hexa*^{-/-}*Neu3*^{-/-} mice with ML-SA1. In this way, the effect of ML-SA1 in the lysosomal exocytosis pathway was investigated.

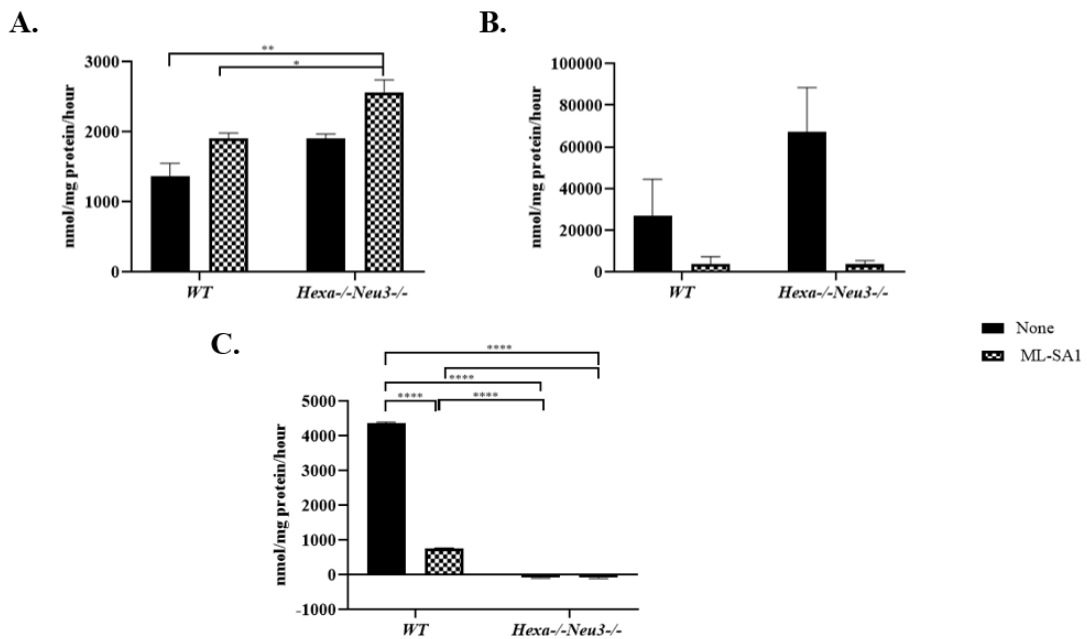


Figure 3.3. Specific activity of extracellular Hexb (A), intracellular Hexb (B), and intracellular Hexa (C) in 5-month-old *WT* and *Hexa*^{-/-}*Neu3*^{-/-} neuroglia under the untreated (none) and ML-SA1 treated conditions. The data were represented as the mean $\pm$ SEM, and two-way ANOVA analysis was used to determine p-values by using GraphPad. (n=3, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001).

The specific Hexb enzyme activity was elevated in the media (extracellular) of both *WT* and *Hexa*^{-/-}*Neu3*^{-/-} neuroglia after ML-SA1 treatment compared to the media of untreated cells. However, this increase was not statistically significant. In addition, a

non-significantly increased specific Hexb activity was observed in the media of untreated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia compared to *WT* (Figure 3.3 A). On the other hand, the intracellular specific activity of Hexb enzyme showed a decline, which was not significant, in *WT* and *Hexa*^{-/-}*Neu3*^{-/-} neuroglia under the effect of ML-SA1. Again, the intracellular level of specific activity was higher in the *Hexa*^{-/-}*Neu3*^{-/-} neuroglia when compared to the *WT* neuroglia, but the observed difference was not statistically significant (Figure 3.3 B). There was a statistically significant decrease for intracellular Hexa enzyme activity in ML-SA1 treated *WT* neuroglia. However, Hexa enzyme was not detectable in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia, and there was no change after the treatment (Figure 3.3 C).

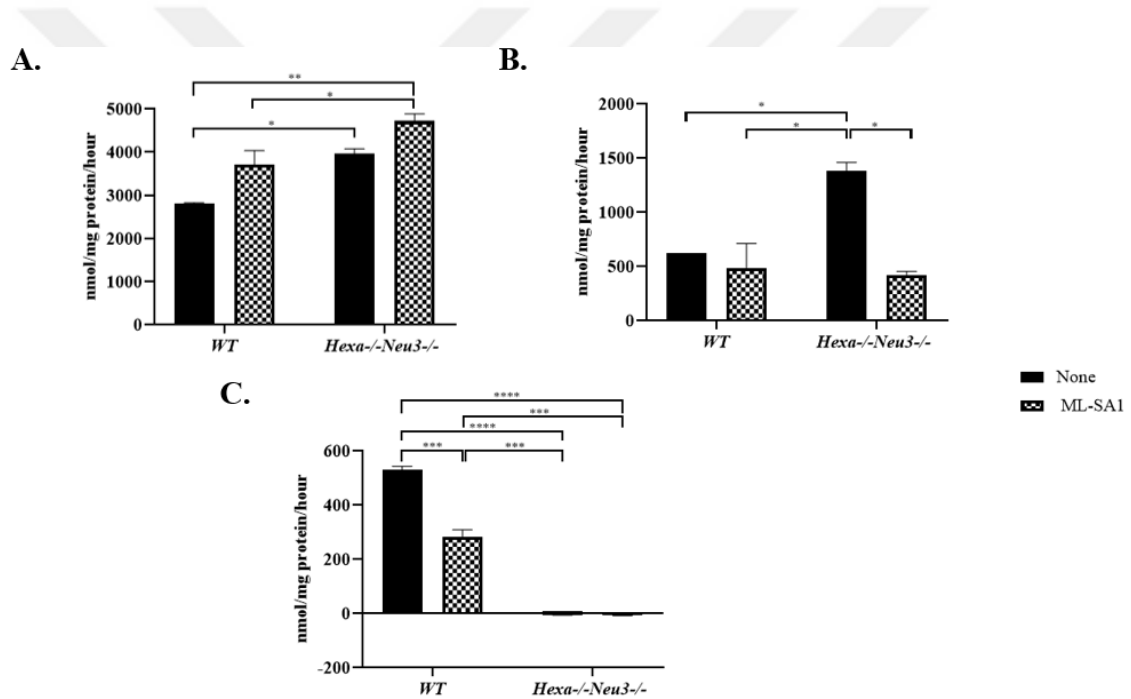


Figure 3.4. Specific activity of extracellular Hexb (A), intracellular Hexb (B), and intracellular Hexa (C) in 5-month-old *WT* and *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts under the untreated (none) and ML-SA1 treated conditions. The data were represented as the mean $\pm$ SEM, and two-way ANOVA analysis was used to determine p-values by using GraphPad. (n=3, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001).

The extracellular specific activity of the Hexb enzyme increased in the *WT* and *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts in ML-SA1 treatment conditions compared to untreated cells,

but neither was statistically significant (Figure 3.4 A). When it comes to the intracellular activity of Hexb, it was observed that with the presence of ML-SA1, there was a decrease in specific enzyme activity compared to the untreated condition for both genotypes. However, this decline was only significant for *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts. Specific activity of intracellular Hexa enzyme was significantly decreased in *WT* fibroblasts after ML-SA1 treatment, nevertheless, it could not be detected in *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts, and no change was observed (Figure 3.4 C).

3.4 RT-PCR

Expression levels of autophagy-related genes *Beclin1*, *Atg7*, *p62*, and *Lamp1* were examined by using the RT-PCR technique, in the context of ML-SA1 treated neuroglia and fibroblast cells of 5-month-old *WT* and *Hexa*^{-/-}*Neu3*^{-/-} mice.

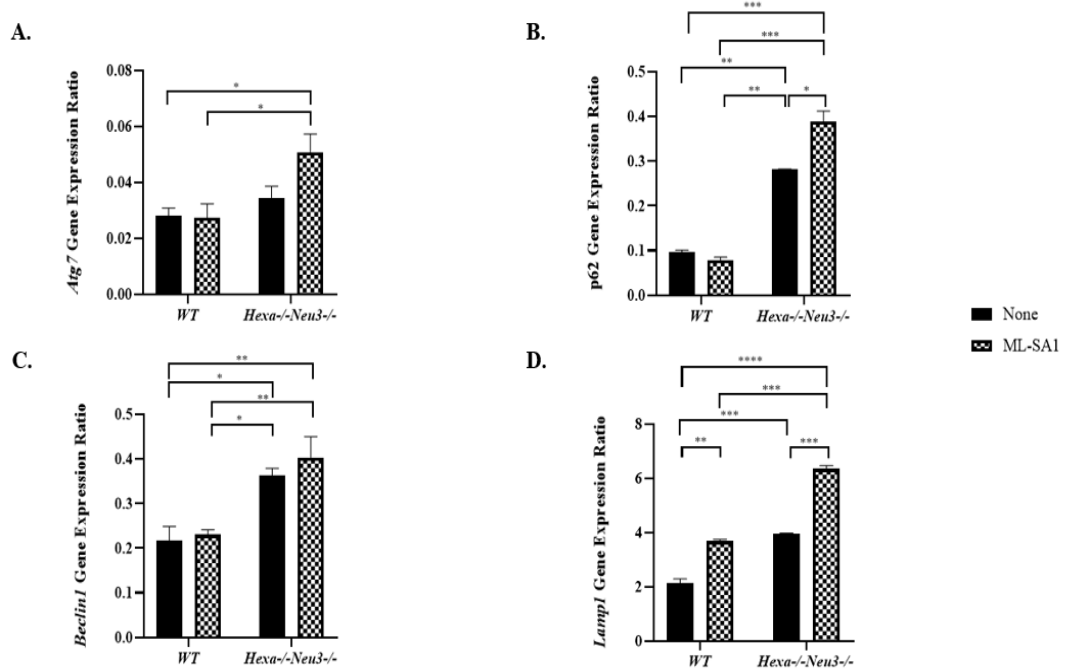


Figure 3.5. Relative expression levels of autophagy-related genes *Atg7* (A), *p62* (B), *Beclin1* (C), and *Lamp1* (D) in 5-month-old *WT* and *Hexa*^{-/-}*Neu3*^{-/-} neuroglia cells under the untreated (none) and ML-SA1-treated conditions. The expression ratio of each gene was normalized to *Gapdh*. The data were represented as the mean $\pm$ SEM, and two-way ANOVA analysis was used to determine p-values by using GraphPad. (n = 3, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001).

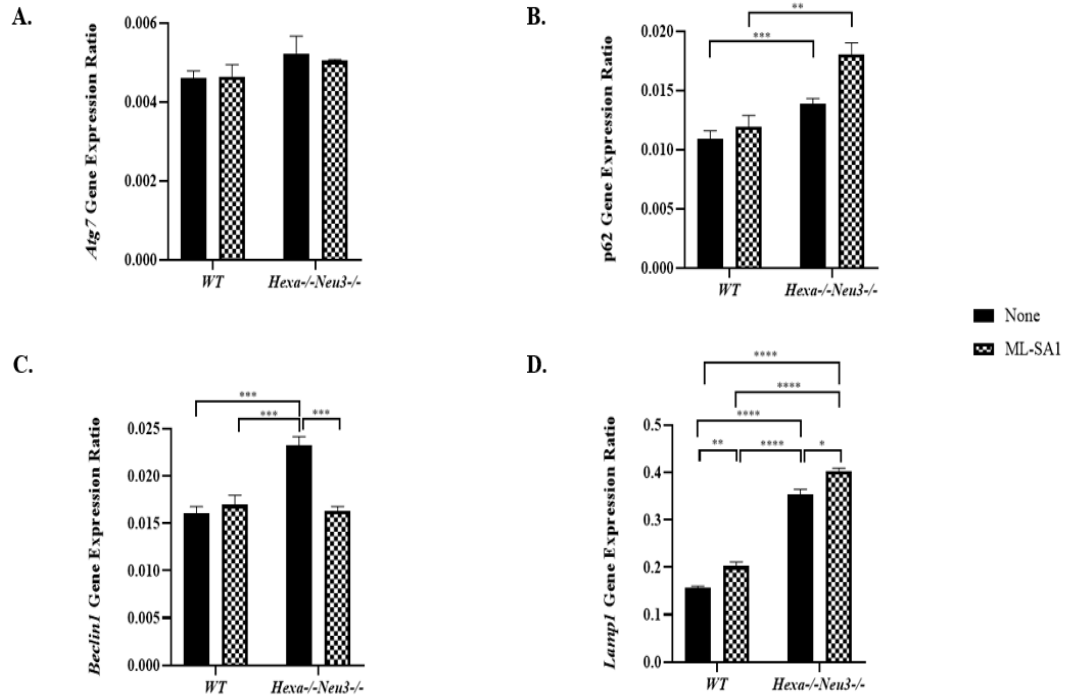


Figure 3.6. Relative expression levels of autophagy-related genes *Atg7* (A), *p62* (B), *Beclin1* (C), and *Lamp1* (D) in 5-month-old WT and *Hexa*^{-/-}*Neu3*^{-/-} fibroblast cells under the untreated (none) and ML-SA1-treated conditions. The expression ratio of each gene was normalized to *Gapdh*. The data were represented as the mean $\pm$ SEM, and two-way ANOVA analysis was used to determine p-values by using GraphPad. (n = 3, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001).

In the level of *Atg7* gene expression, there was no change between ML-SA1 treated and untreated WT neuroglia, WT and *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts (Figure 3.5 A and Figure 3.6 A). *Hexa*^{-/-}*Neu3*^{-/-} neuroglia showed non-significantly increased *Atg7* expression after ML-SA1 treatment compared to the untreated condition of *Hexa*^{-/-}*Neu3*^{-/-} neuroglia (Figure 3.5 A). Moreover, a non-significantly higher *Atg7* gene expression level was detected in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblasts than in WT neuroglia and fibroblasts, respectively, under untreated conditions (Figure 3.5 A and Figure 3.6 A).

The *p62* gene expression was found to be non-significantly decreased in WT neuroglia after ML-SA1 treatment, while it was non-significantly increased in the WT fibroblasts with ML-SA1 treatment compared to the untreated condition. ML-SA1 treatment increased the *p62* expression in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblasts compared to untreated conditions. However, this increment was statistically significant only in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia. In addition, a significant increase of *p62* expression

level was observed in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblasts than in *WT* neuroglia and fibroblasts, respectively, in untreated conditions (Figure 3.5 B and Figure 3.6 B).

When *Beclin1* gene expression levels were examined, it was observed that there was a non-significant minor increase in the ML-SA1 treated *WT* neuroglia and fibroblasts compared to untreated conditions. ML-SA1 treatment resulted in a non-significant increase in the level of *Beclin1* expression in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia, while it decreased *Beclin1* expression significantly in *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts compared to untreated conditions. Finally, significantly increased *Beclin1* expression was detected in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblast cells compared to *WT* neuroglia and fibroblast cells, respectively, in untreated conditions. (Figure 3.5 C and Figure 3.6 C)

The expression level of the *Lamp1* gene significantly increased with the presence of ML-SA1 in neuroglia and fibroblasts of both genotypes compared to untreated conditions of these cells. In untreated conditions, *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblasts showed significantly greater *Lamp1* gene expression than *WT* neuroglia and fibroblasts in order (Figure 3.5 D and Figure 3.6 D)

3.5 Western Blot

The effect of ML-SA1 treatment on the Beclin1, p62, and LC3 protein levels was assessed in neuroglia and fibroblast cells of 5-month-old *WT* and *Hexa*^{-/-}*Neu3*^{-/-} mice by Western Blot analysis.

The Beclin1 protein level showed a non-significant decrease in *WT* neuroglia, whereas it showed a non-significant increase in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia after ML-SA1 treatment compared to untreated conditions. Also, in untreated conditions, a non-significant minor increase was observed in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia compared to *WT* neuroglia (Figure 3.7 A and B).

The p62 protein level was significantly increased in both genotypes of neuroglia under ML-SA1 treatment conditions. In addition, the increased level of p62 was detected in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia compared to *WT* neuroglia in untreated conditions, but this increment was not statistically significant (Figure 3.7 C and D).

In ML-SA1 treatment conditions, an increased LC3-II level was detected compared to the untreated neuroglia of both genotypes; however, this increase was statistically significant in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia only. Furthermore, untreated *Hexa*^{-/-}

Neu3^{-/-} neuroglia showed a non-significant increase of LC3-II when compared to untreated *WT* neuroglia (Figure 3.7 E and F)

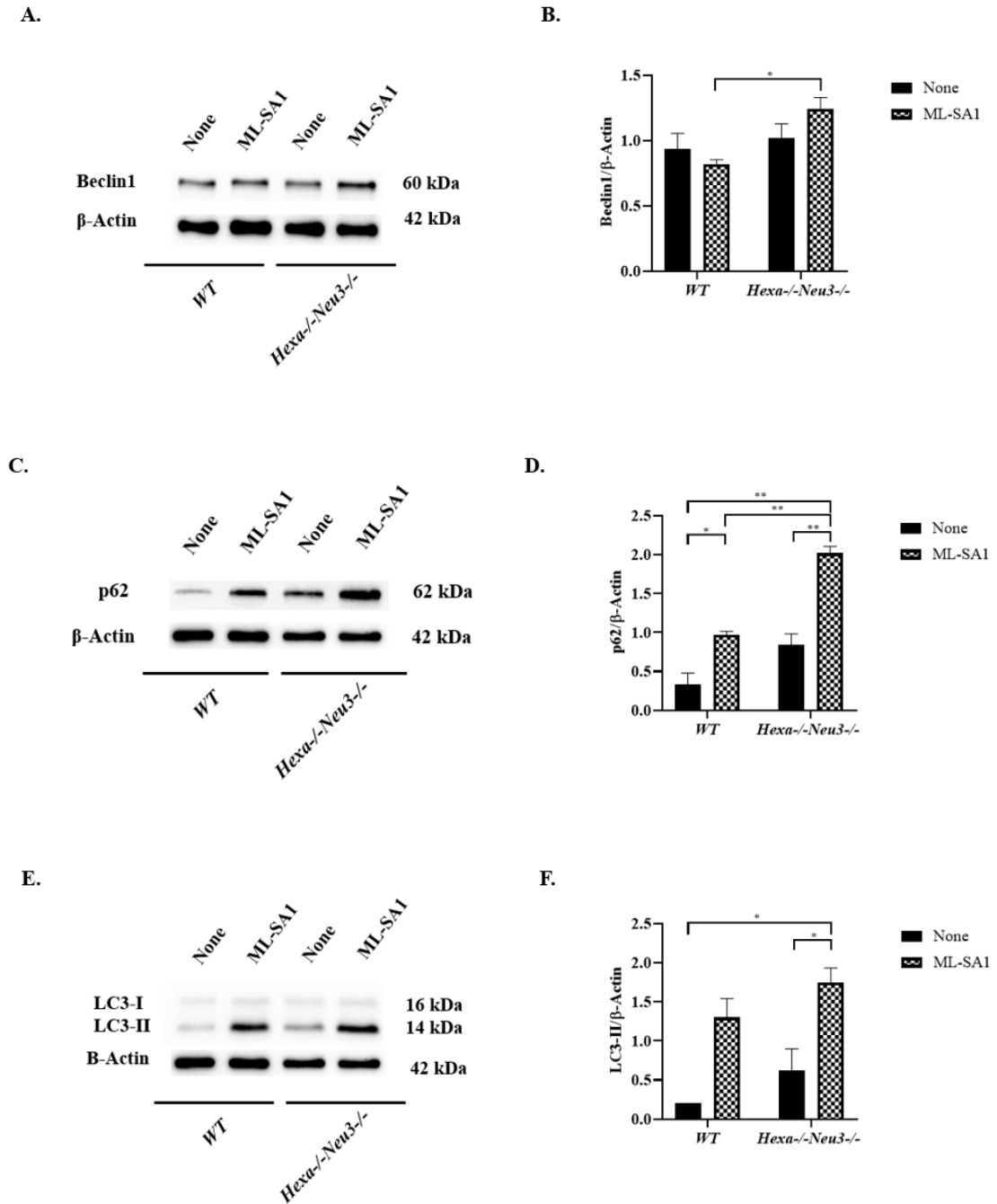


Figure 3.7. Representative Western Blot results for autophagy markers Beclin1 (A), p62 (C), and LC3 (E) in 5-month-old *WT* and *Hexa*^{-/-}*Neu3*^{-/-} neuroglia cells under the untreated (none) and ML-SA1-treated conditions. Quantification of Beclin1 (B), p62 (D), and LC3 (F) band intensities normalized to β -actin. The data were represented as the mean $\pm$ SEM, and two-way ANOVA analysis was used to determine p-values by using GraphPad. (n = 3, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001).

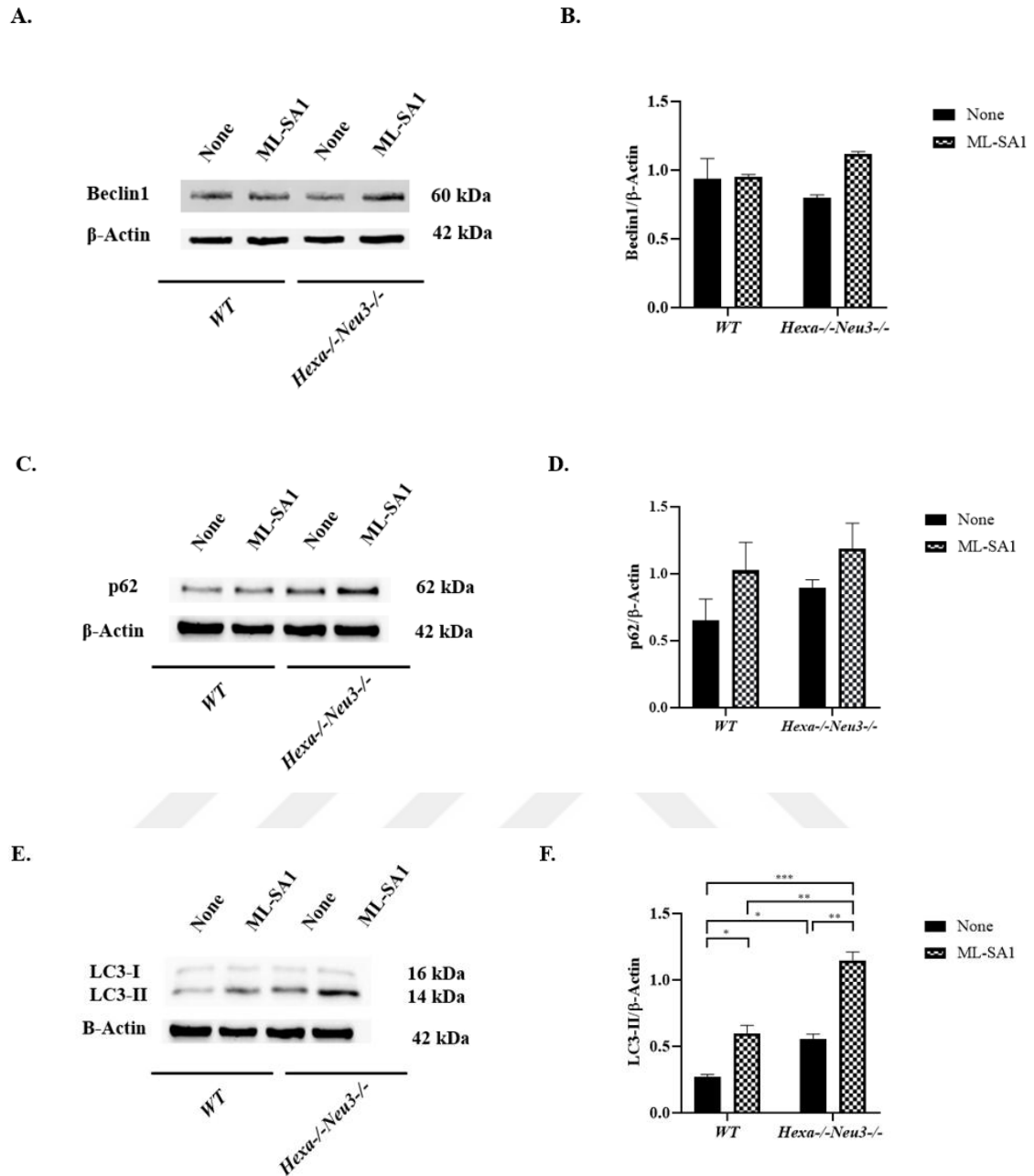


Figure 3.8. Representative Western Blot results for autophagy markers Beclin1 (A), p62 (C), and LC3 (E) in 5-month-old *WT* and *Hexa*^{-/-}*Neu3*^{-/-} fibroblast cells under the untreated (none) and ML-SA1-treated conditions. Quantification of Beclin1 (B), p62 (D), and LC3 (F) band intensities normalized to β-actin. The data were represented as the mean ± SEM, and two-way ANOVA analysis was used to determine p-values by using GraphPad. (n = 3, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001).

The Beclin1 protein level did not show any change in *WT* fibroblasts under ML-SA1 effect, whereas it showed a non-significant increase in *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts with ML-SA1 compared to untreated conditions. A non-significant decrease was detected

between *WT* and *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts, in untreated conditions (Figure 3.8 A and B).

The p62 protein level was non-significantly increased in *WT* and *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts under ML-SA1 treatment conditions compared to untreated cells. Moreover, the p62 protein level was found at a higher level in *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts than in *WT* fibroblasts, but this difference was not statistically significant (Figure 3.8 C and D).

In ML-SA1 treated fibroblasts of both genotypes, a significantly increased LC3-II level was detected compared to the untreated conditions. Additionally, a statistically significant increase of LC3-II was observed in untreated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts compared to untreated *WT* fibroblasts (Figure 3.8 E and F).

3.6 Immunocytochemical (ICC) Staining

Observation of ML-SA1 effect in autophagy and lysosomal exocytosis pathways was done by conducting ICC staining for p62, LC3, total Lamp1, LC3-Lamp1, and surface Lamp1 (lysosomal exocytosis marker) in neuroglia and fibroblast cells of 5-month-old *WT* and *Hexa*^{-/-}*Neu3*^{-/-} mice.

Intensity of p62 in *WT* neuroglia was non-significantly increased in the ML-SA1 treatment condition compared to untreated cells. On the other hand, in the *Hexa*^{-/-}*Neu3*^{-/-} neuroglia, p62 intensity was significantly increased in the ML-SA1 treatment conditions compared to untreated cells. Additionally, p62 protein intensity was increased in the untreated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia than untreated *WT* neuroglia; however, this increment was not statistically significant (Figure 3.9 A and B). In fibroblasts, the intensity of p62 was significantly increased in both genotypes with ML-SA1 treatment compared to untreated cells. As in neuroglia cells, p62 protein intensity was non-significantly increased in the untreated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts compared to the untreated *WT* fibroblasts (Figure 3.9 C and D).

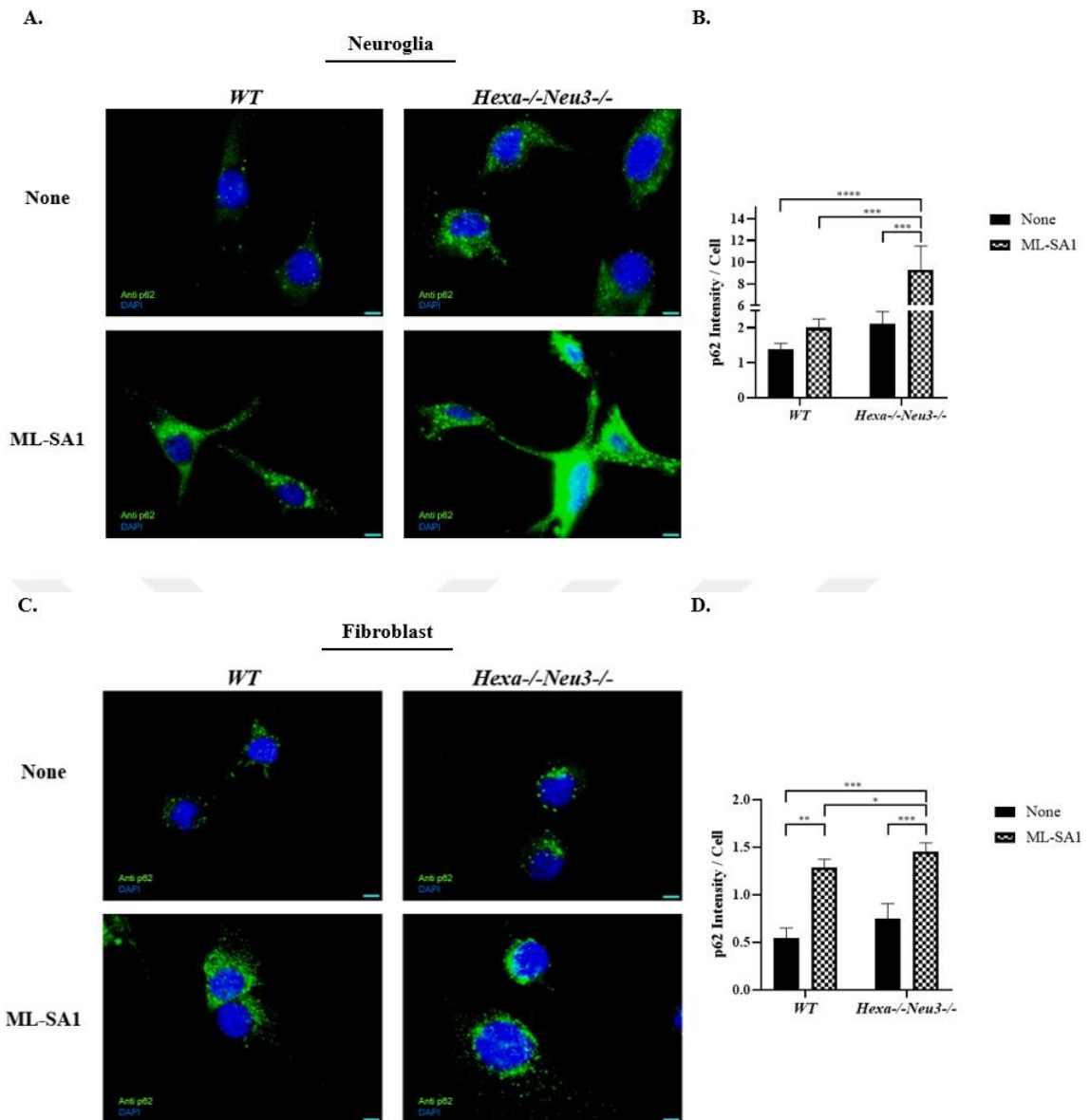


Figure 3.9. Immunostaining of p62 protein in 5-month-old *WT* and *Hexa^{-/-}Neu3^{-/-}* neuroglia (A) and fibroblast (C) cells under the untreated (none) and ML-SA1-treated conditions, p62 protein intensity/cell graphs of neuroglia (B) and fibroblasts (D). All images were taken at 100X magnification by using the same light intensity. Fluorescent signals of proteins were measured by using ImageJ. The data were represented as the mean $\pm$ SEM, and two-way ANOVA analysis was used to determine p-values by using GraphPad. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

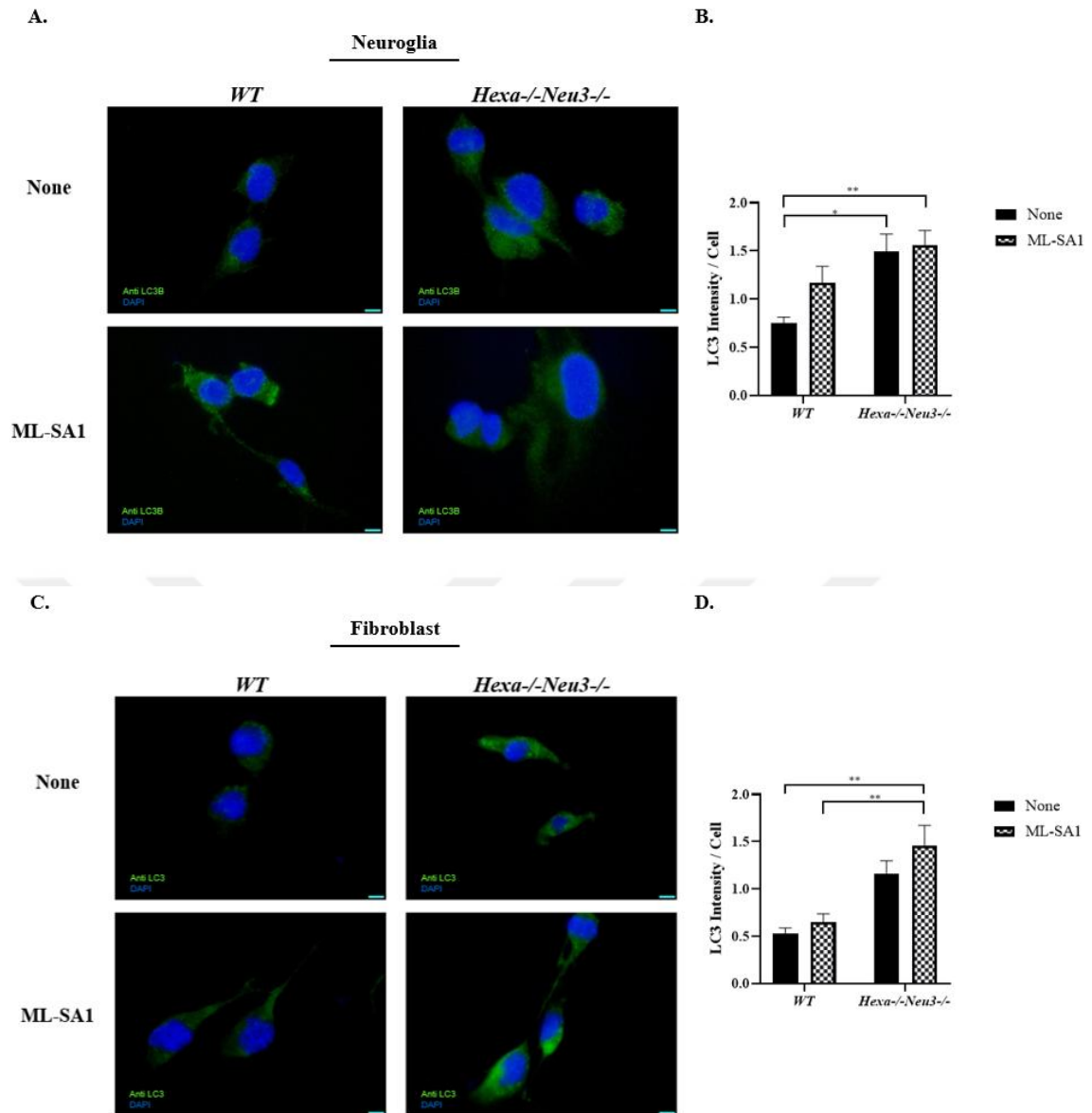


Figure 3.10. Immunostaining of LC3 protein in 5-month-old *WT* and *Hexa*^{-/-}*Neu3*^{-/-} neuroglia (A) and fibroblast (C) cells under the untreated (none) and ML-SA1-treated conditions, LC3 protein intensity/cell graphs of neuroglia (B) and fibroblasts (D). All images were taken at 100X magnification by using the same light intensity. Fluorescent signals of proteins were measured by using ImageJ. The data were represented as the mean $\pm$ SEM, and two-way ANOVA analysis was used to determine p-values by using GraphPad. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

LC3 intensity was non-significantly increased in *WT* and *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblast cells after ML-SA1 treatment compared to untreated conditions (Figure 3.10). In *Hexa*^{-/-}*Neu3*^{-/-} neuroglia, this increase was very minor (Figure 3.9 A

and B). In addition, untreated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblast cells showed increased LC3 intensity compared to untreated *WT* cells; however, this increment was found significant only in neuroglia cells (Figure 3.10)

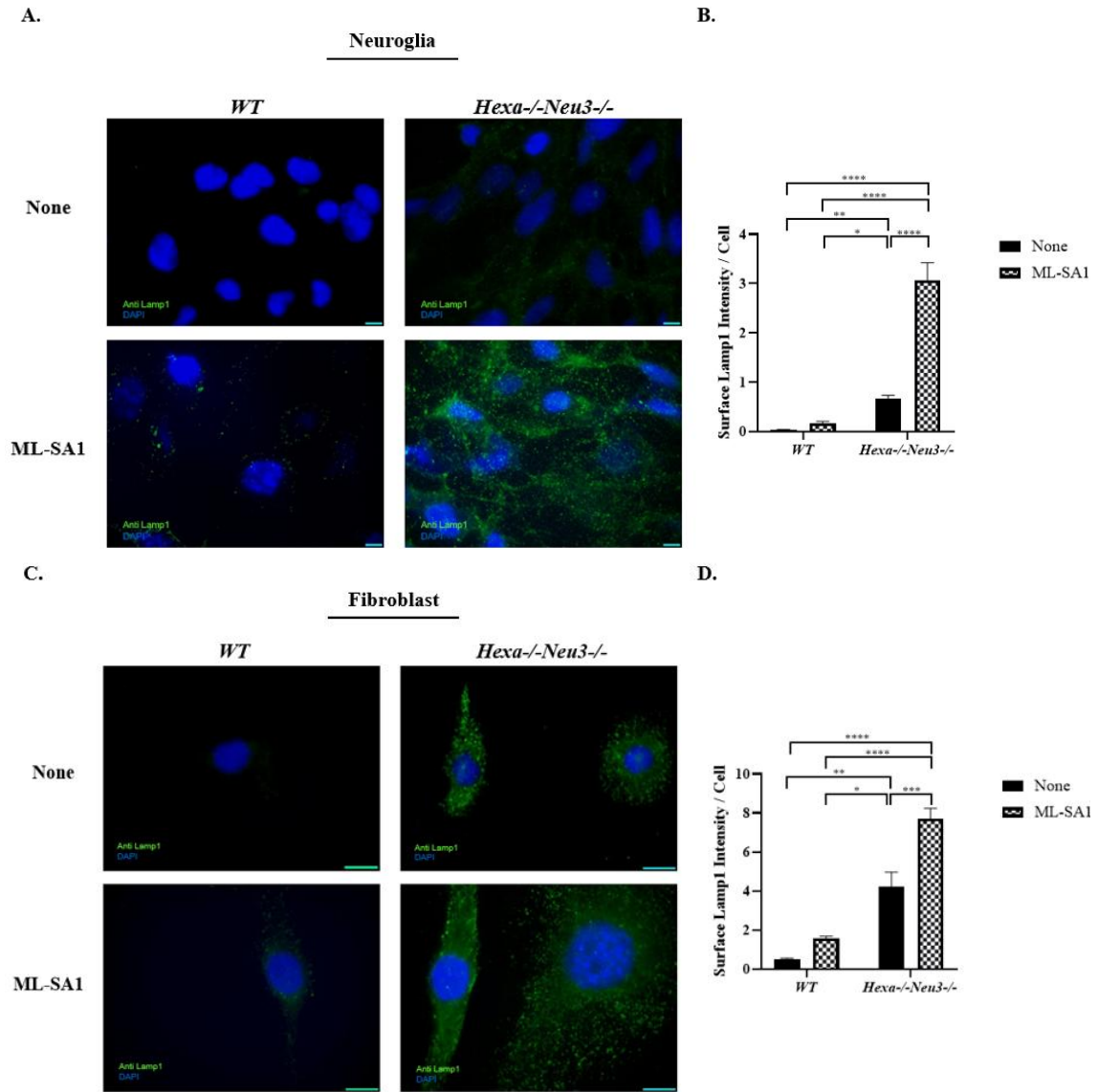


Figure 3.11. Immunostaining of surface Lamp1 protein in 5-month-old *WT* and *Hexa*^{-/-}*Neu3*^{-/-} neuroglia (A) and fibroblast (C) cells under the untreated (none) and ML-SA1-treated conditions, surface Lamp1 protein intensity/cell graphs of neuroglia (B) and fibroblast (D). Neuroglia images were taken at 40X magnification and fibroblast cells were taken at 100X magnification. In each staining, the same light intensity were used. Fluorescent signals of proteins were measured by using ImageJ. The data were represented as the mean $\pm$ SEM, and two-way ANOVA analysis was used to determine p-values by using GraphPad. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Lamp1 protein localized to the surface of *WT* neuroglia and fibroblasts was found to be non-significantly higher in the ML-SA1 treatment condition. In the *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblasts, surface Lamp1 intensity was significantly increased with ML-SA1 treatment compared to untreated *Hexa*^{-/-}*Neu3*^{-/-} cells. Moreover, surface Lamp1 intensity of *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblasts was significantly higher than *WT* neuroglia and fibroblasts, respectively, in untreated conditions (Figure 3.11).

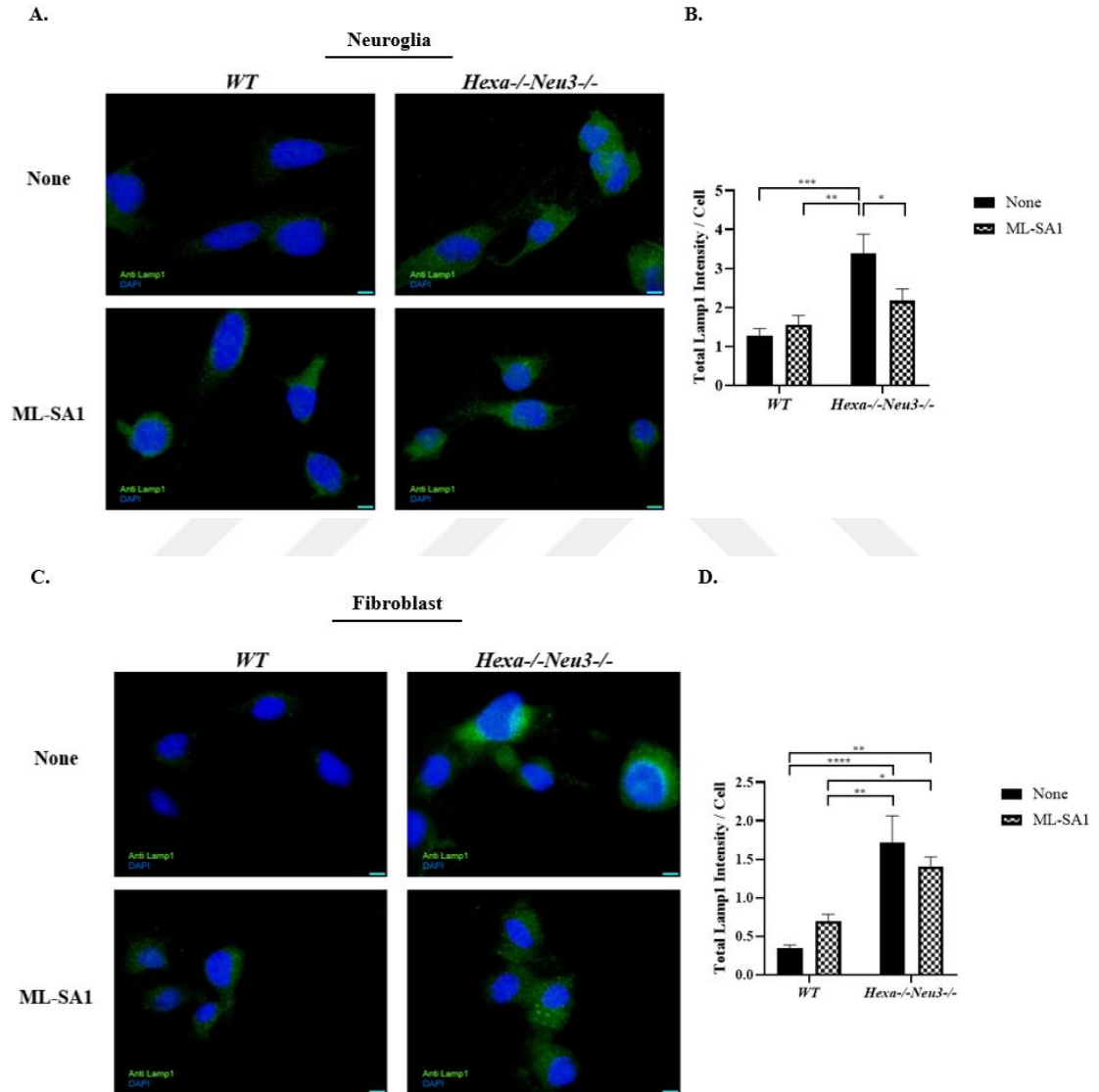


Figure 3.12. Immunostaining of total Lamp1 protein in 5-month-old *WT* and *Hexa*^{-/-}*Neu3*^{-/-} neuroglia (A) and fibroblast (C) cells under the untreated (none) and ML-SA1 treated conditions, total Lamp1 protein intensity/cell graphs of neuroglia (B) and fibroblast (D). All images were taken at 100X magnification by using the same light intensity with different fluorescent filters. Fluorescent signals of proteins were measured by using ImageJ. The data were represented as the mean $\pm$ SEM, and two-way ANOVA analysis was used to determine p-values by using GraphPad. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

In *WT* neuroglia, total Lamp1 protein intensity showed a non-significant minor increase in the presence of ML-SA1. However, in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia, total Lamp1 intensity decreased significantly under the ML-SA1 treatment conditions compared to untreated conditions. Untreated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia showed significantly increased total Lamp1 intensity level compared to untreated *WT* neuroglia (Figure 3.12 A and B). As seen in *WT* neuroglia, *WT* fibroblasts showed a non-significant increase in total Lamp1 intensity with ML-SA1 treatment compared to untreated fibroblasts. However, in *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts, the level of total Lamp1 intensity decreased non-significantly after ML-SA1 treatment compared to untreated fibroblasts. Finally, total lamp1 intensity was observed to be significantly higher in untreated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts than in untreated *WT* fibroblasts (Figure 3.12 C and D).

In *WT* neuroglia, there was no change in the colocalization intensity of LC3 and Lamp1 proteins between untreated and ML-SA1 treated conditions. However, in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia, colocalization intensity of these proteins was significantly higher in the ML-SA1 treated cells compared to untreated ones. Also, *Hexa*^{-/-}*Neu3*^{-/-} neuroglia showed non-significantly increased LC3-Lamp1 colocalization intensity compared to *WT* neuroglia under untreated conditions (Figure 3.13 A and B).

WT fibroblast showed no change in the colocalization intensity of LC3 and Lamp1 proteins between untreated and ML-SA1 treated conditions. In *Hexa*^{-/-}*Neu3*^{-/-} fibroblast, colocalization intensity of LC3 and Lamp1 proteins was significantly decreased in the ML-SA1 treated conditions compared to untreated ones. Additionally, untreated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts showed significantly increased LC3-Lamp1 colocalization intensity compared to untreated *WT* fibroblasts (Figure 3.14 A and B).

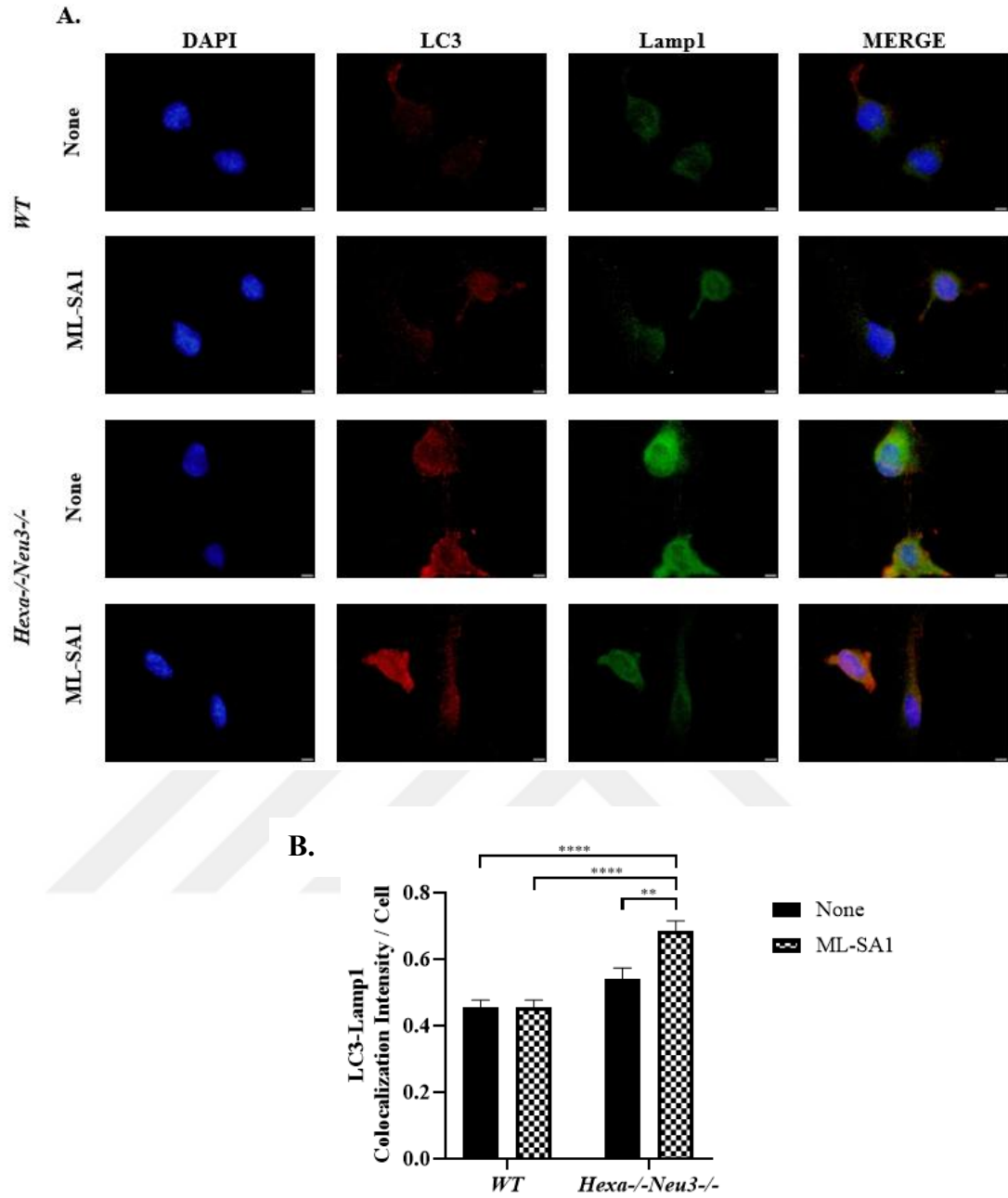


Figure 3.13. Immunostaining of LC3 and Lamp1 protein in 5-month-old *WT* and *Hexa-/-Neu3-/-* neuroglia cells under the untreated (none) and ML-SA1-treated conditions (A), LC3-Lamp1 colocalization intensity/cell graph (B). All images were taken at 100X magnification by using the same light intensity with different fluorescent filters. Colocalization signals of proteins were measured by using ImageJ. The data were represented as the mean $\pm$ SEM, and two-way ANOVA analysis was used to determine p-values by using GraphPad. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

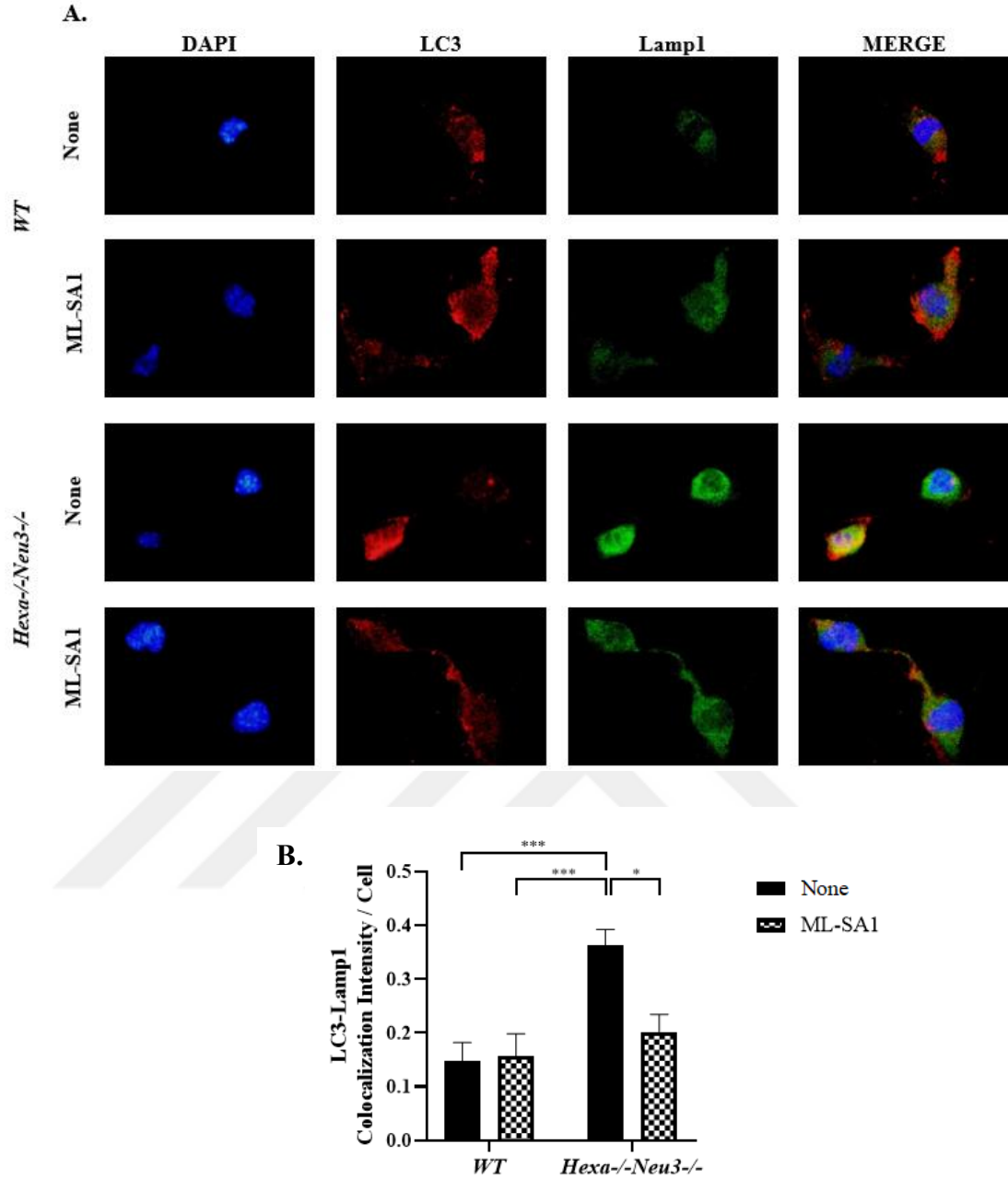


Figure 3.14. Immunostaining of LC3 and Lamp1 protein in 5-month-old *WT* and *Hexa^{-/-}Neu3^{-/-}* fibroblast cells under the untreated (none) and ML-SA1-treated conditions (A), LC3-Lamp1 colocalization intensity/cell graph (B). All images were taken at 100X magnification by using the same light intensity with different fluorescent filters. Colocalization signals of proteins were measured by using ImageJ. The data were represented as the mean $\pm$ SEM, and two-way ANOVA analysis was used to determine p-values by using GraphPad. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

3.7 miRNA Profiling

By comparing the ML-SA1 treated and untreated conditions in each cell group and comparing the *Hexa*^{-/-}*Neu3*^{-/-} and *WT* cells in both untreated and ML-SA1 treated conditions, significantly DE miRNAs were obtained as upregulated and downregulated then the pathways in which they are enriched were identified. Also, miRNAs showing the same pattern in both fibroblasts and neuroglia in the disease condition without treatment and in the disease condition with ML-SA1 effect were identified, and a separate pathway enrichment analysis was performed for these miRNAs.

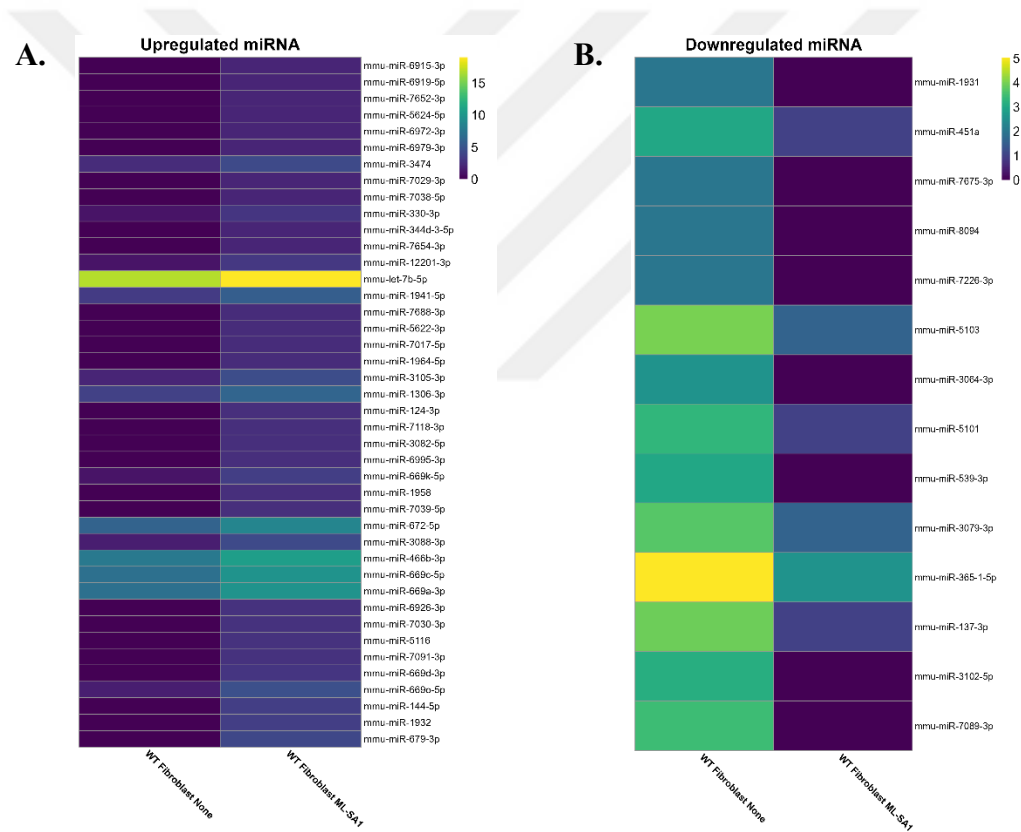


Figure 3.15. Heatmap of significantly upregulated (A) and downregulated (B) miRNAs in ML-SA1 treated *WT* fibroblasts compared to untreated (none) *WT* fibroblasts. Each column represents the treatment condition. Each row shows the identity of miRNAs (p-value < 0.05, log₂FC ≥ 2 for upregulation and log₂FC ≤ -2 for downregulation).

Significantly DE miRNAs in ML-SA1 treated *WT* fibroblasts compared to untreated *WT* fibroblasts were given as a heatmap (Figure 3.15). Accordingly, 42 upregulated miRNAs (mmu-miR-6915-3p, mmu-miR-6919-5p, mmu-miR-7652-3p, mmu-miR-5624-5p...) (Figure 3.15 A) and 14 downregulated miRNAs (mmu-miR-1931, mmu-miR-451a, mmu-miR-7675-3p, mmu-miR-8094...) (Figure 3.15 B) were identified.

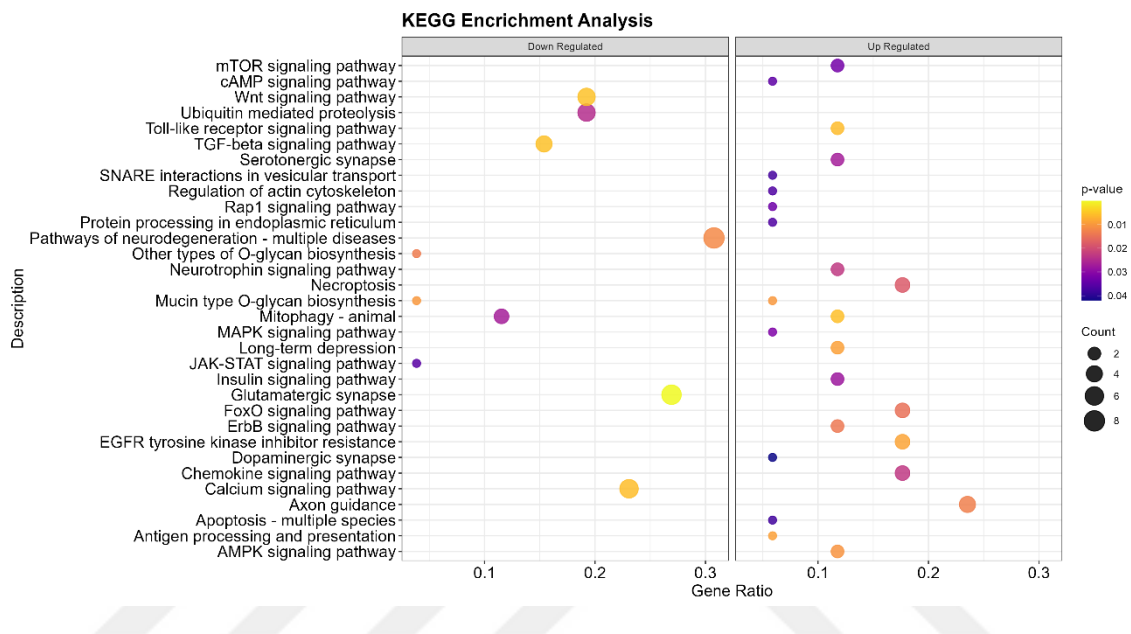


Figure 3.16. Dot plot of KEGG enrichment analysis of the significantly up and down-regulated miRNAs in ML-SA1 treated *WT* fibroblasts compared to untreated *WT* fibroblasts. Dot sizes represent the number of genes involved in the pathway targeted by miRNAs. The dot color represents p-values of pathways. The gene ratio was calculated as the number of pathway-related genes divided by the total number of processed genes. Raw p-value < 0.05 was statistically significant.

KEGG pathway enrichment analysis, visualized with a dot plot graphic, showed that 32 cellular pathways were targeted by miRNAs that were DE in ML-SA1 treated *WT* fibroblasts compared to the untreated *WT* fibroblasts. 2 pathways (mitophagy and mucin-type O-glycan biosynthesis) were found to be targeted by both significantly upregulated and downregulated miRNAs. When gene count, gene ratio, and p-value were considered, the most significant target pathway of downregulated miRNAs was detected as ‘pathways of neurodegeneration’. On the other hand, for the upregulated miRNAs, the most significant target pathway was ‘axon guidance’ (Figure 3.16).



Figure 3.17. Dot plot of Reactome enrichment analysis of the significantly up and down-regulated miRNAs in ML-SA1 treated *WT* fibroblasts compared to untreated *WT* fibroblasts. Dot sizes represent the number of genes involved in the pathway targeted by miRNAs. The dot color represents p-values of pathways. The gene ratio was calculated as the number of pathway-related genes divided by the total number of processed genes. Raw p-value < 0.05 was statistically significant.

According to the dot plot of Reactome pathway enrichment analysis, 43 pathways were targeted by DE miRNAs in ML-SA1 treated *WT* fibroblasts compared to the untreated *WT* fibroblasts. Among them, 4 cellular pathways (regulation of TP53 activity through phosphorylation, O-linked glycosylation of mucins, neuronal system, TNFR1 induced proapoptotic signaling) were found to be targeted by both significantly upregulated and downregulated miRNAs. Based on gene count, gene ratio, and p-value, the most significant pathway of downregulated miRNAs was found as ‘neuronal system’ while ‘signaling by receptor tyrosine kinases’ was the most significant pathway of upregulated miRNAs (Figure 3.17).

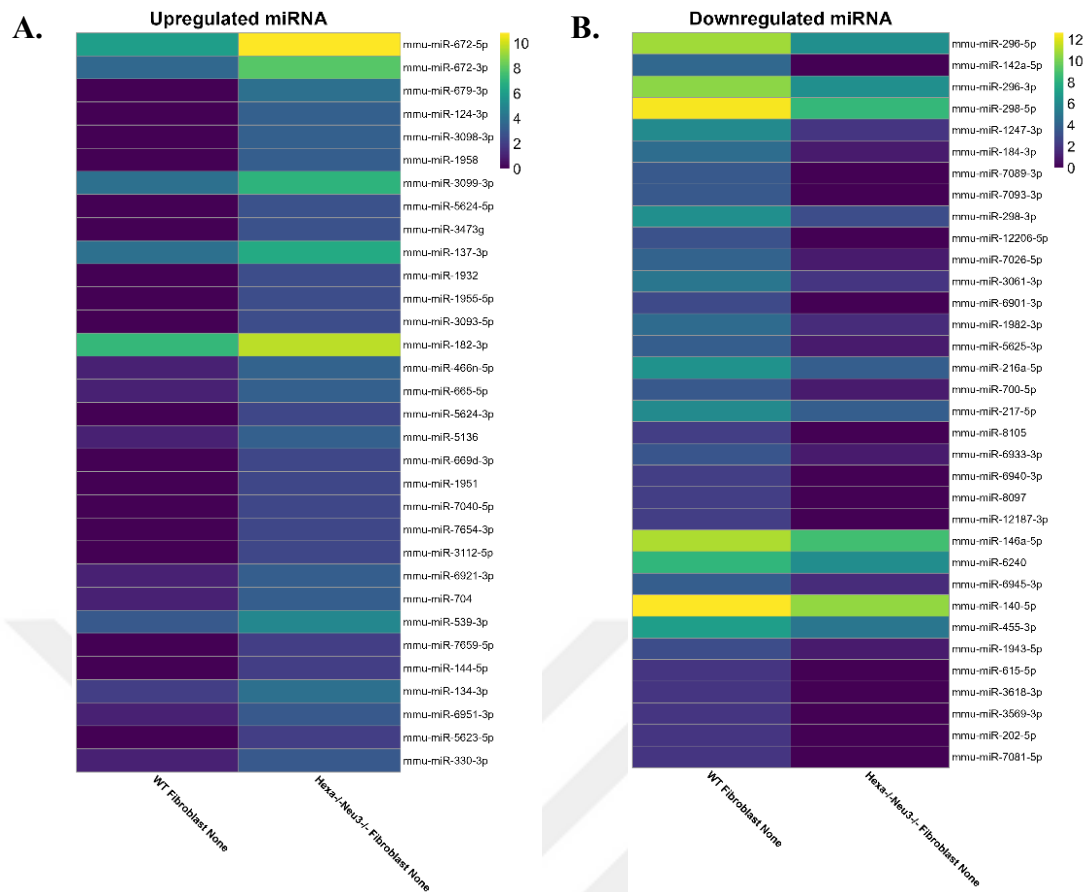


Figure 3.18. Heatmap of significantly upregulated (A) and downregulated (B) miRNAs in untreated (none) *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts compared to untreated (none) *WT* fibroblasts. Each column represents the genotype of cells. Each row shows the identity of miRNAs (p-value < 0.05, log₂FC ≥ 2 for upregulation and log₂FC ≤ -2 for downregulation).

Significantly DE miRNAs in untreated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts compared to untreated *WT* fibroblasts were given as a heatmap (Figure 3.18). Based on this heatmap, 32 upregulated miRNAs (mmu-miR-672-5p, mmu-miR-672-3p, mmu-miR-679-3p, mmu-miR-124-3p...) (Figure 3.18 A) and 34 downregulated miRNAs (mmu-miR-296-5p, mmu-miR-142a-5p, mmu-miR-296-3p, mmu-miR-298-5p...) (Figure 3.18 B) were detected.

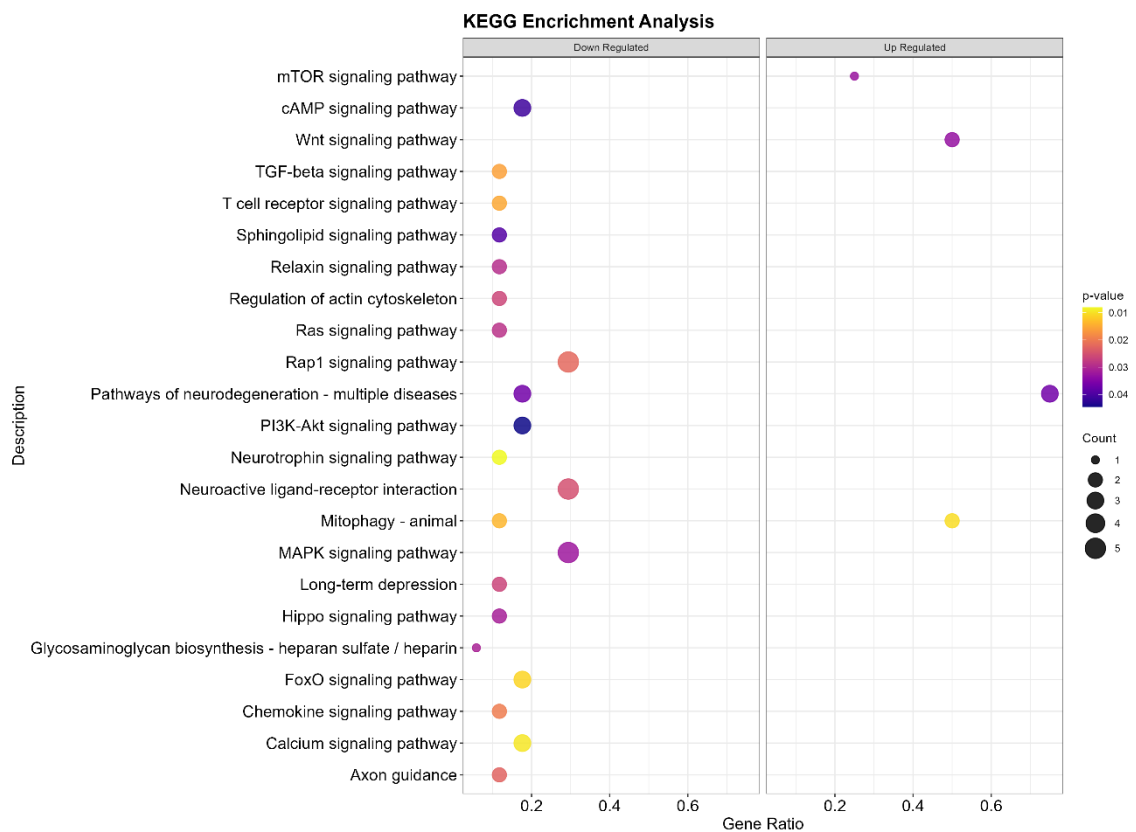


Figure 3.19. Dot plot of KEGG enrichment analysis of the significantly up and down-regulated miRNAs in untreated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts compared to untreated *WT* fibroblasts. Dot sizes represent the number of genes involved in the pathway targeted by miRNAs. The dot color represents p-values of pathways. The gene ratio was calculated as the number of pathway-related genes divided by the total number of processed genes. Raw p-value < 0.1 was considered statistically significant.

In the KEGG pathway enrichment analysis applied to the DE miRNAs of untreated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts compared to untreated *WT* fibroblasts, 23 cellular pathways were enriched. 2 of them (mitophagy and pathways of neurodegeneration) were found to be targeted by both significantly upregulated and downregulated miRNAs. When gene count, gene ratio, and p-value features were considered, it was found that the most significant pathway of downregulated miRNAs was ‘Rap1 signaling pathway’ and the most significant pathway of upregulated miRNAs was ‘pathways of neurodegeneration’ (Figure 3.19).

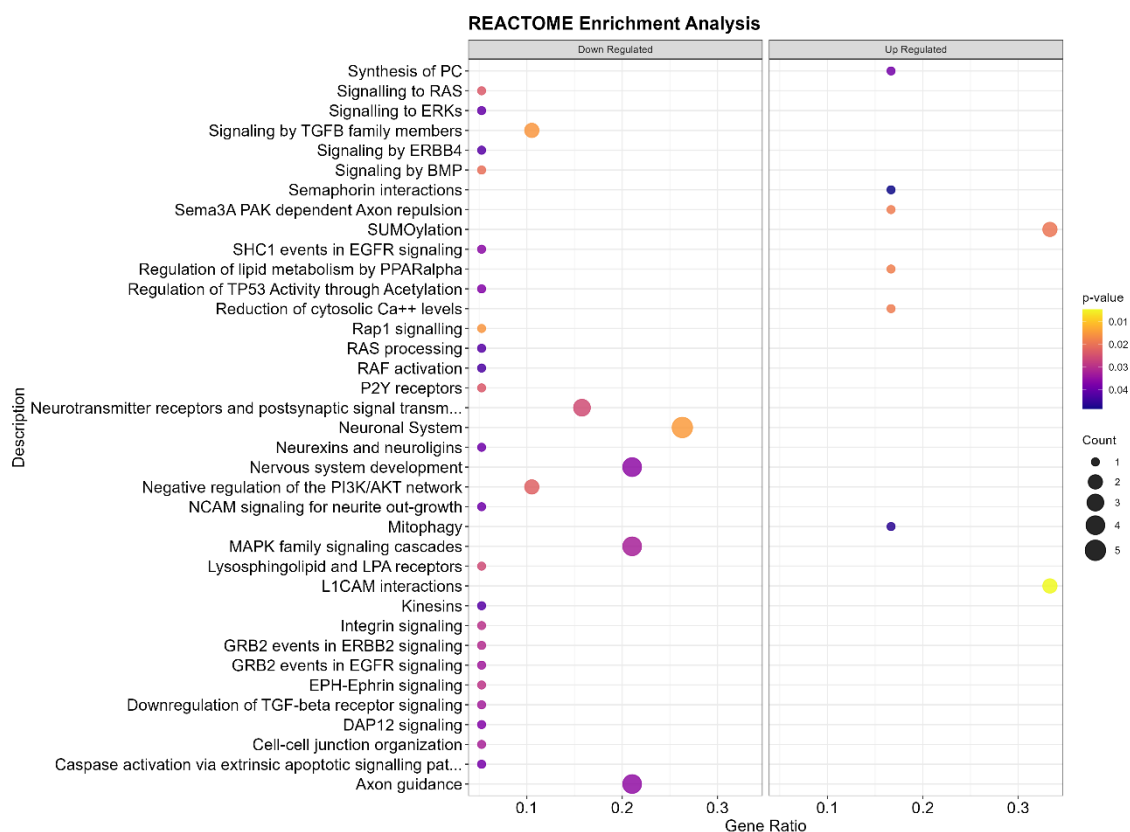


Figure 3.20. Dot plot of Reactome enrichment analysis of the significantly up and down-regulated miRNAs in untreated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts compared to untreated *WT* fibroblasts. Dot sizes represent the number of genes involved in the pathway targeted by miRNAs. The dot color represents p-values of pathways. The gene ratio was calculated as the number of pathway-related genes divided by the total number of processed genes. Raw p-value < 0.1 was statistically significant.

Reactome pathway enrichment analysis, visualized with a dot plot graphic, showed that 37 cellular pathways were targeted by miRNAs that were DE in untreated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts compared to untreated *WT* fibroblasts. There was no shared pathway between significantly upregulated and downregulated miRNAs. According to gene count, gene ratio, and p-value, the most significant pathway of downregulated miRNAs was detected as ‘neuronal system’. On the other hand, for the upregulated miRNAs, the most significant pathway was found as ‘L1CAM interactions’ (Figure 3.20).

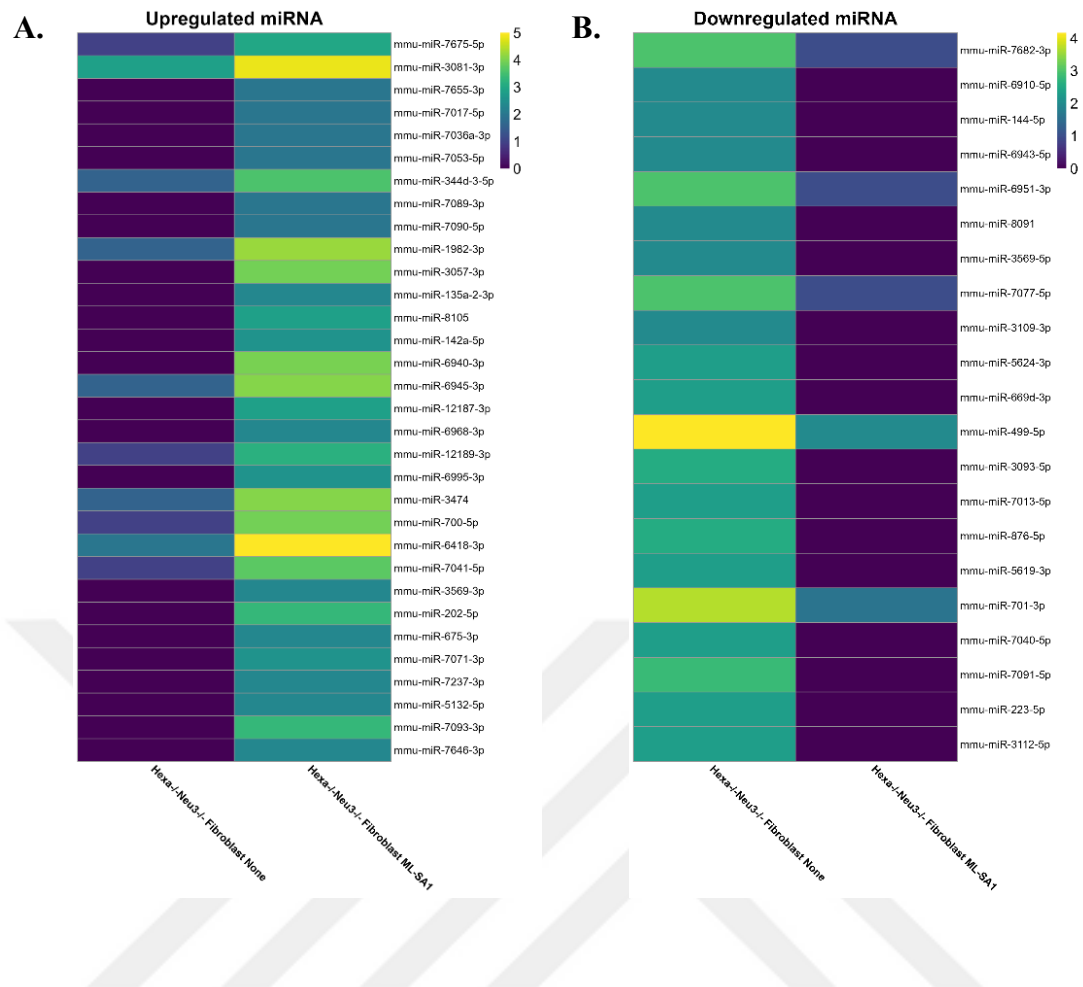


Figure 3.21. Heatmap of significantly upregulated (A) and downregulated (B) miRNAs in ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts compared to untreated (none) *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts. Each column represents the treatment condition. Each row shows the identity of miRNAs (p-value < 0.05, log₂FC ≥ 2 for upregulation and log₂FC ≤ -2 for downregulation).

Significantly DE miRNAs in ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts compared to untreated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts were shown in a heatmap (Figure 3.21). As a result, it was observed that 32 miRNAs (mmu-miR-7675-5p, mmu-miR-3081-3p, mmu-miR-7655-3p, mmu-miR-7017-5p...) were upregulated (Figure 3.21 A) and 21 miRNAs (mmu-miR-7682-3p, mmu-miR-6910-5p, mmu-miR-144-5p, mmu-miR-6943-5p...) were downregulated (Figure 3.21 B).

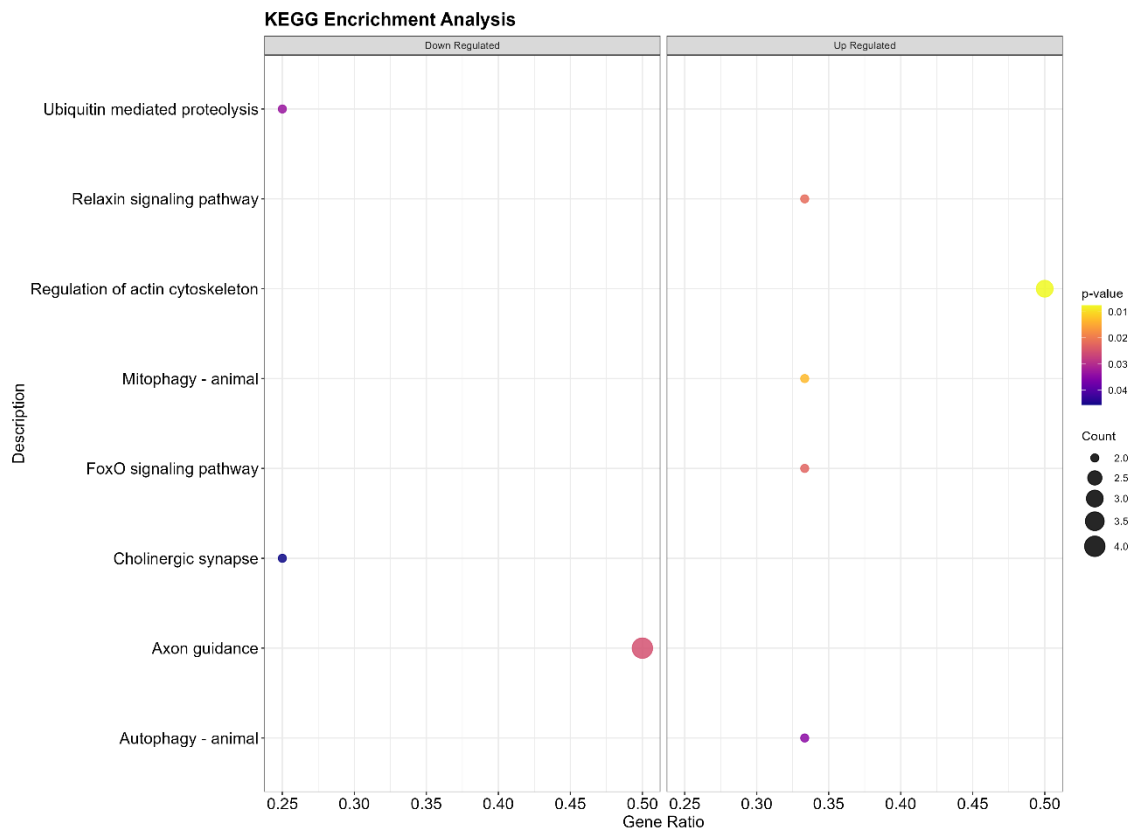


Figure 3.22. Dot plot of KEGG enrichment analysis of the significantly up and down-regulated miRNAs in ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts compared to untreated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts. Dot sizes represent the number of genes involved in the pathway targeted by miRNAs. The dot color represents p-values of pathways. The gene ratio was calculated as the number of pathway-related genes divided by the total number of processed genes. Raw p-value < 0.05 was statistically significant.

KEGG pathway enrichment analysis, visualized with a dot plot graphic, demonstrated that 8 cellular pathways were targeted by miRNAs that were DE in ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts compared to untreated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts. Additionally, there was no shared pathway between significantly upregulated and downregulated miRNAs. The most significant pathway of downregulated miRNAs was detected as ‘axon guidance’. For the upregulated miRNAs, the most significant pathway was identified as ‘regulation of actin cytoskeleton’ when the gene ratio, gene count, and p-value criteria were weighed (Figure 3.22).

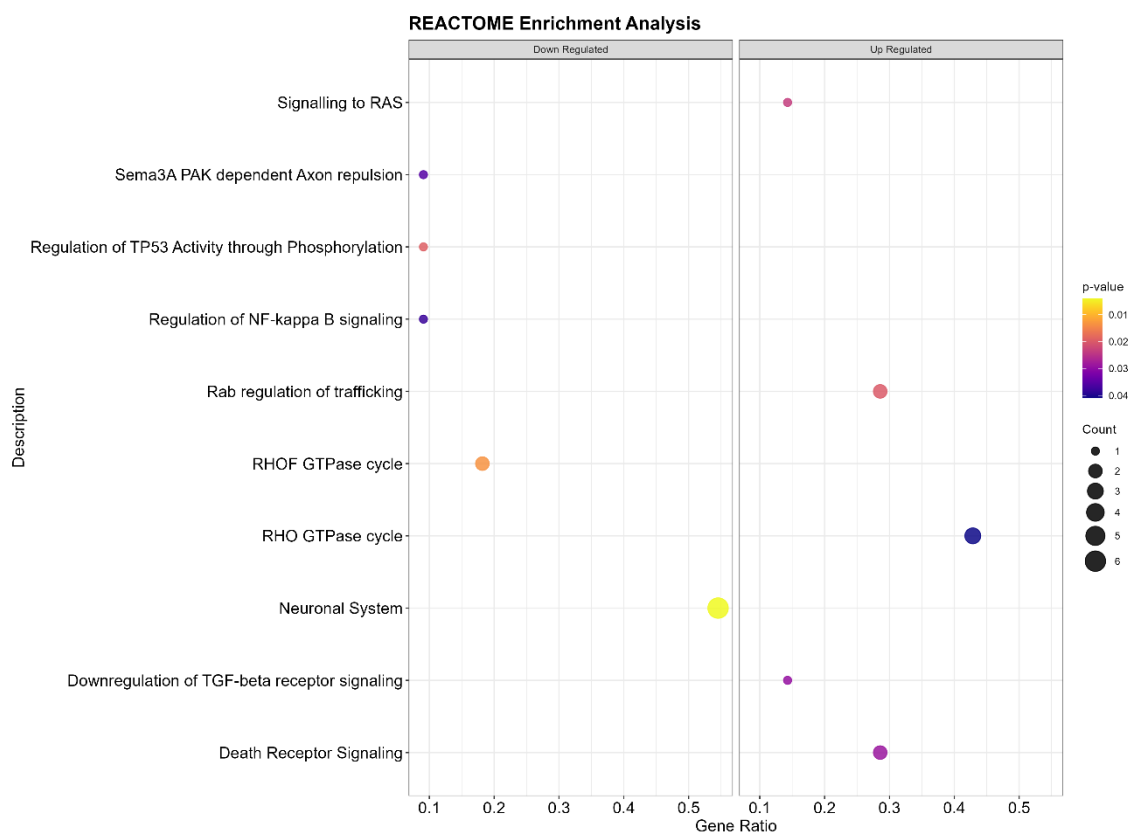


Figure 3.23. Dot plot of Reactome enrichment analysis of the significantly up and down-regulated miRNAs in ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts compared to untreated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts. Dot sizes represent the number of genes involved in the pathway targeted by miRNAs. The dot color represents p-values of pathways. The gene ratio was calculated as the number of pathway-related genes divided by the total number of processed genes. Raw p-value < 0.05 was statistically significant.

Reactome pathway enrichment analysis, visualized with a dot plot graphic, exhibited that 10 cellular pathways were targeted by miRNAs that were DE in ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts compared to untreated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts. Among these pathways, there was no pathway targeted by both significantly upregulated and downregulated miRNAs. The most significant pathway of downregulated miRNAs was found as ‘neuronal system’, while ‘RHO GTPase cycle’ was identified as the most significant pathway of upregulated miRNAs according to gene ratio, gene count, and p-value criteria (Figure 3.23).

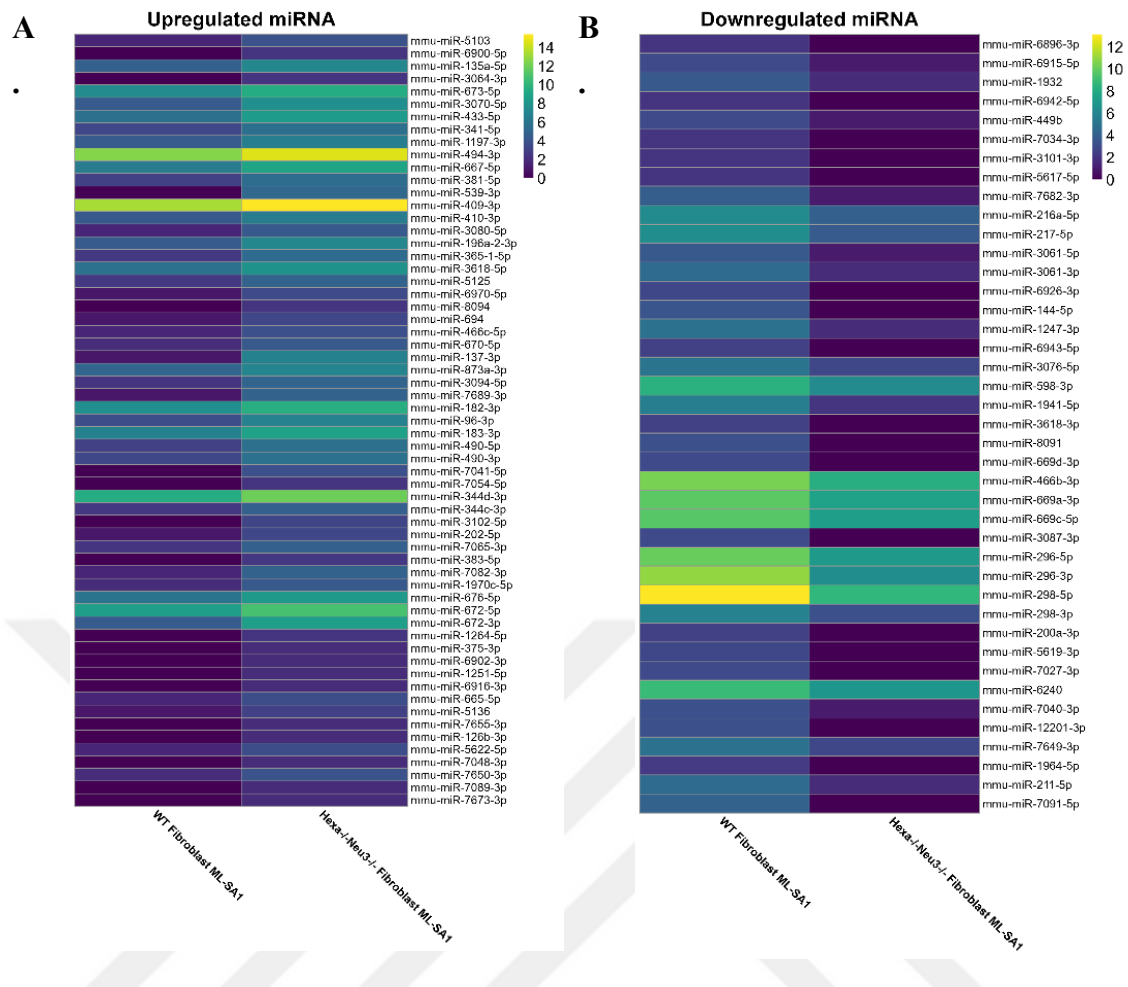


Figure 3.24. Heatmap of significantly upregulated (A) and downregulated (B) miRNAs in ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts compared to ML-SA1 treated *WT* fibroblasts. Each column represents the genotype of cells. Each row shows the identity of miRNAs (p-value < 0.05, log₂FC ≥ 2 for upregulation and log₂FC ≤ -2 for downregulation).

Significantly DE miRNAs in ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts compared to ML-SA1 treated *WT* fibroblasts were shown in a heatmap (Figure 3.24). As a result, it was observed that 61 miRNAs (mmu-miR-5103, mmu-miR-6900-5p, mmu-miR-135a-5p, mmu-miR-3064-3p...) were upregulated (Figure 3.24 A) and 41 miRNAs (mmu-miR-6896-3p, mmu-miR-6915-5p, mmu-miR-1932, mmu-miR-6942-5p...) were downregulated (Figure 3.24 B).

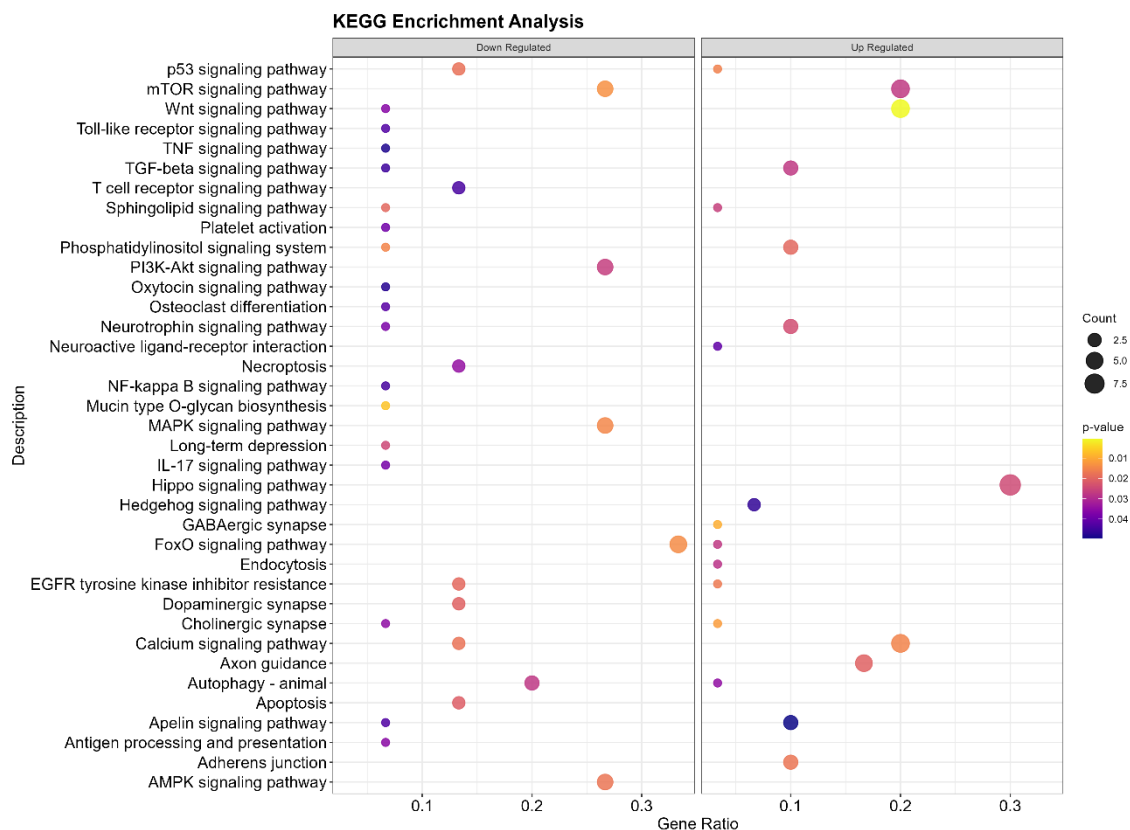


Figure 3.25. Dot plot of KEGG enrichment analysis of the significantly up and down-regulated miRNAs in ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts compared to ML-SA1 treated *WT* fibroblasts. Dot sizes represent the number of genes involved in the pathway targeted by miRNAs. The dot color represents p-values of pathways. The gene ratio was calculated as the number of pathway-related genes divided by the total number of processed genes. Raw p-value < 0.05 was statistically significant.

In the dot plot of KEGG pathway enrichment analysis applied to the DE miRNAs of ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts compared to ML-SA1 treated *WT* fibroblasts, 37 cellular pathways were seen in total. 13 of these pathways (p53 signaling pathway, mTOR signaling pathway, Wnt signaling pathway, TGF-beta signaling pathway, sphingolipid signaling pathways, etc.) were found as a target of both significantly upregulated and downregulated miRNAs. When gene count, gene ratio, and p-value were considered, the most significant pathway of downregulated miRNAs was detected as ‘FoxO signaling pathway’; for the upregulated miRNAs, the most significant pathway was found as ‘Hippo signaling pathway’ (Figure 3.25).

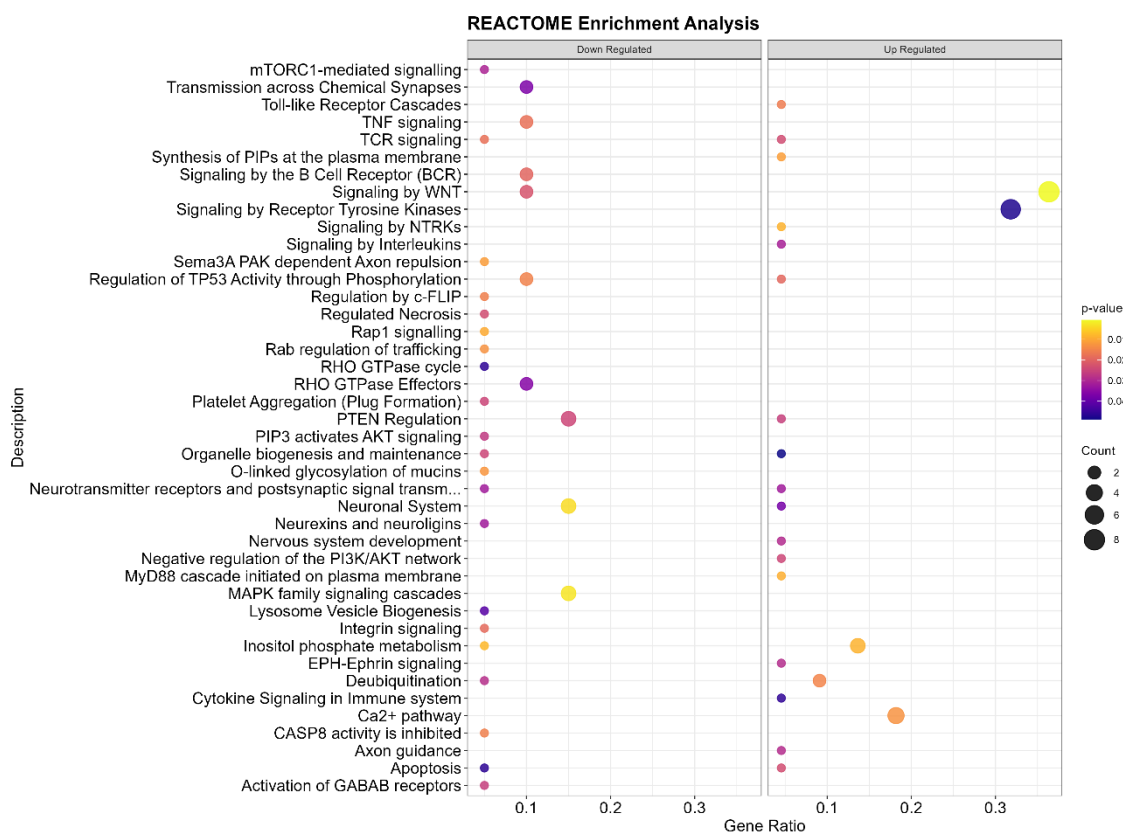


Figure 3.26. Dot plot of Reactome enrichment analysis of the significantly up and down-regulated miRNAs in ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts compared to ML-SA1 treated *WT* fibroblasts. Dot sizes represent the number of genes involved in the pathway targeted by miRNAs. The dot color represents p-values of pathways. The gene ratio was calculated as the number of pathway-related genes divided by the total number of processed genes. Raw p-value < 0.05 was statistically significant.

In the dot plot of Reactome pathway enrichment analysis applied to the DE miRNAs of ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts compared to ML-SA1 treated *WT* fibroblasts, 42 cellular pathways were seen in total. Among them, 10 pathways (TCR signaling, signaling by Wnt, regulation of TP53 activity through phosphorylation, PTEN regulation, organelle biogenesis and maintenance, etc.) were targeted by both significantly upregulated and downregulated miRNAs. When gene count, gene ratio, and p-value were considered, the most significant pathways of downregulated miRNAs were identified as ‘MAPK family signaling cascade’; for the upregulated miRNAs, the most significant pathway was found as ‘signaling by Wnt’ (Figure 3.26).

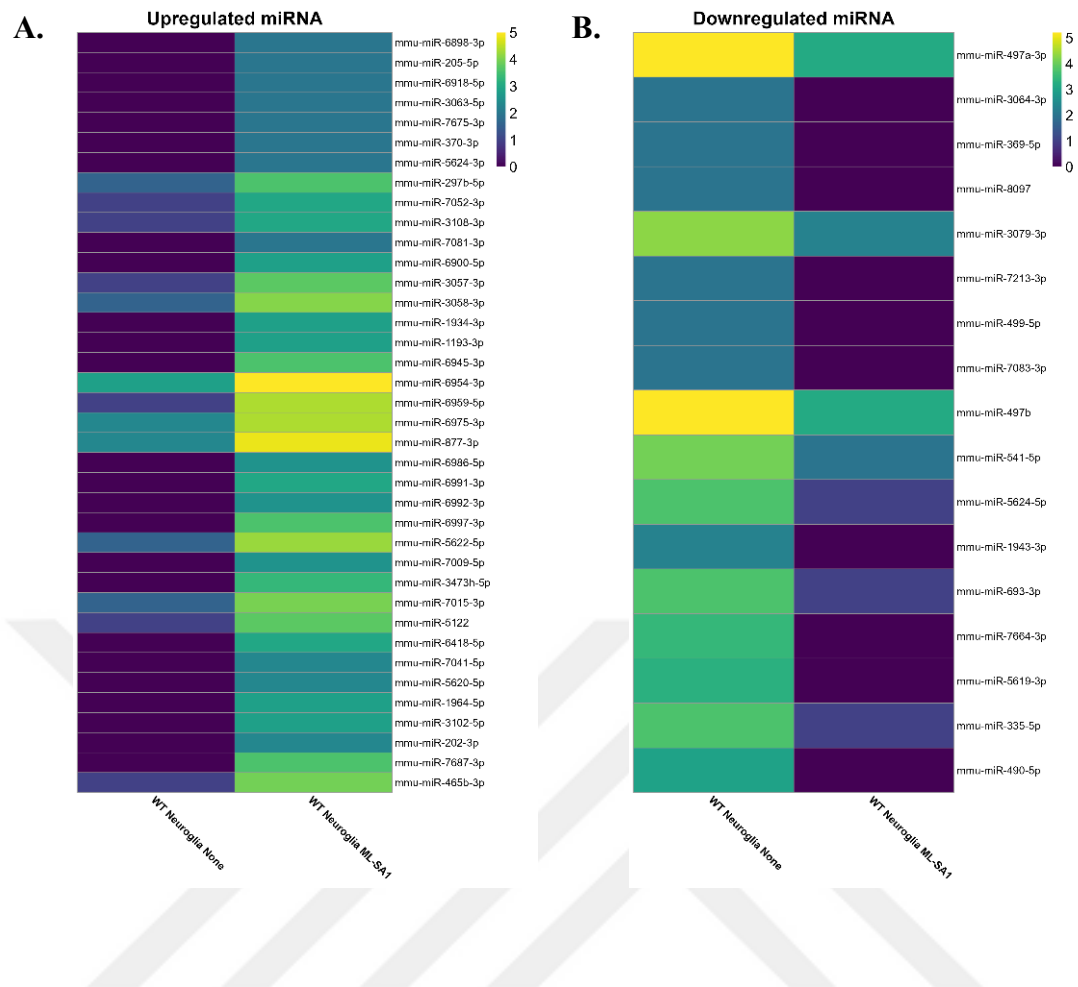


Figure 3.27. Heatmap of significantly upregulated (A) and downregulated (B) miRNAs in ML-SA1 treated *WT* neuroglia compared to untreated (none) *WT* neuroglia. Each column represents the treatment condition. Each row shows the identity of miRNAs ($p\text{-value} < 0.05$, $\log_2\text{FC} \geq 2$ for upregulation and $\log_2\text{FC} \leq -2$ for downregulation).

Significantly DE miRNAs in ML-SA1 treated *WT* neuroglia compared to untreated *WT* neuroglia were given as a heatmap (Figure 3.27). Accordingly, 38 upregulated miRNAs (mmu-miR-6898-3p, mmu-miR-205-5p, mmu-miR-6918-5p, mmu-miR-3063-5p...) (Figure 3.27 A) and 17 downregulated miRNAs (mmu-miR-497a-3p, mmu-miR-3064-3p, mmu-miR-369-5p, mmu-miR-8097...) (Figure 3.27 B) were identified.

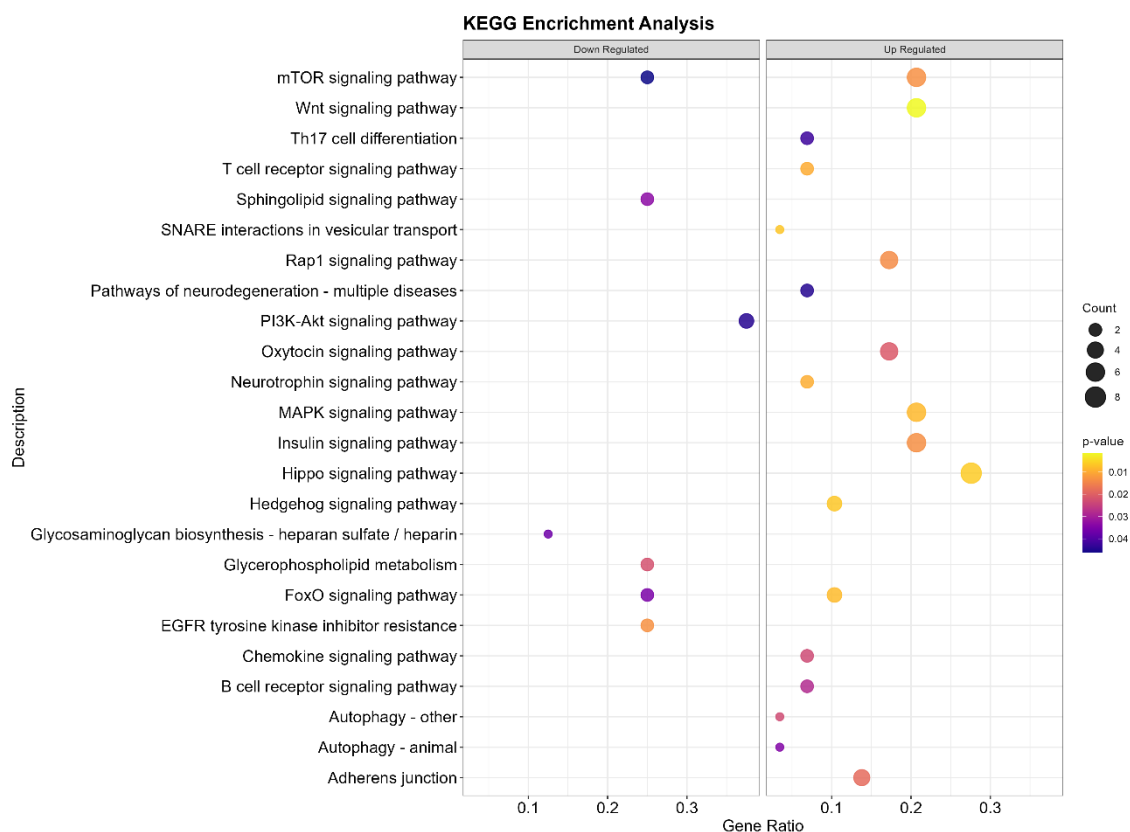


Figure 3.28. Dot plot of KEGG enrichment analysis of the significantly up and down-regulated miRNAs in ML-SA1 treated *WT* neuroglia compared to untreated *WT* neuroglia. Dot sizes represent the number of genes involved in the pathway targeted by miRNAs. The dot color represents p-values of pathways. The gene ratio was calculated as the number of pathway-related genes divided by the total number of processed genes. Raw p-value < 0.05 was statistically significant.

According to the KEGG pathway enrichment analysis, visualized with a dot plot graphic, 24 cellular pathways were targeted by miRNAs that were DE in ML-SA1 treated *WT* neuroglia compared to untreated *WT* neuroglia. In addition, 2 pathways (mTOR signaling pathway and FoxO signaling pathway) were found to be targeted by both significantly upregulated and downregulated miRNAs. The most significantly enriched pathway of downregulated miRNAs was ‘PI3K-Akt signaling pathway’, while ‘Hippo signaling pathway’ was identified as the most significant pathway of upregulated miRNAs according to gene ratio, gene count, and p-value criteria (Figure 3.28).

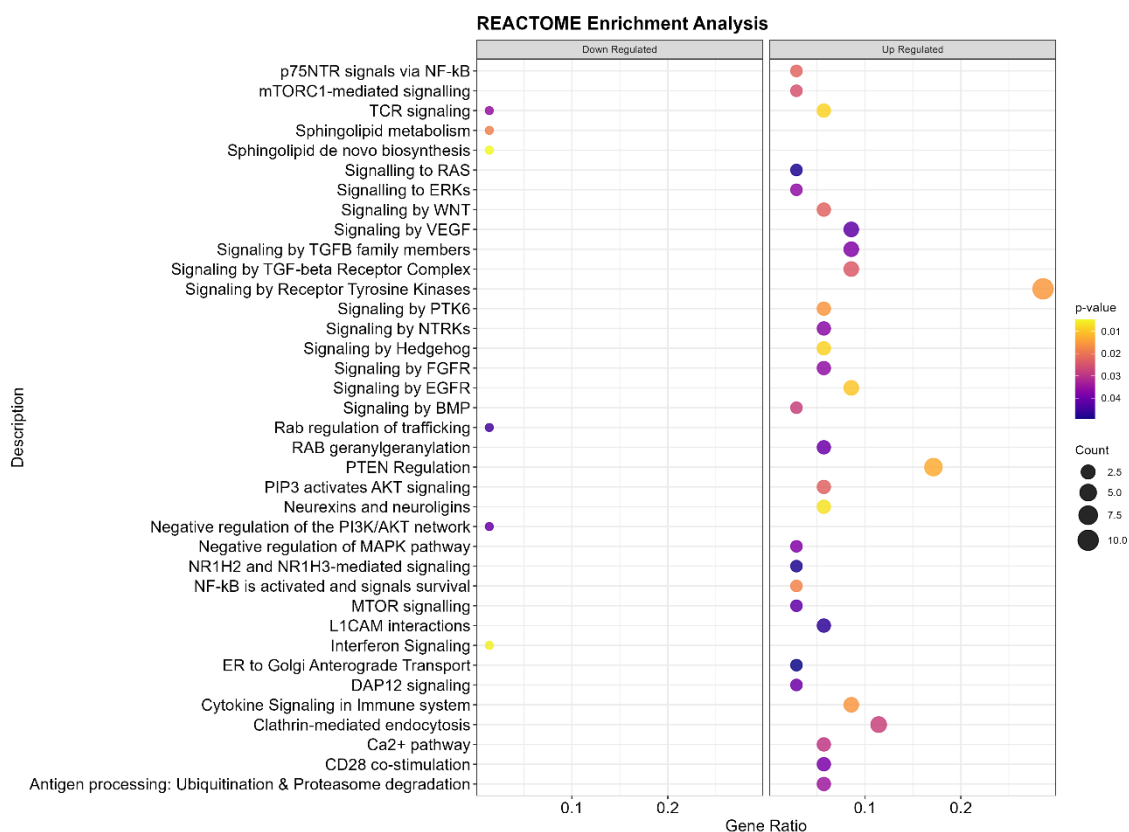


Figure 3.29. Dot plot of Reactome enrichment analysis of the significantly up and down-regulated miRNAs in ML-SA1 treated *WT* neuroglia compared to untreated *WT* neuroglia. Dot sizes represent the number of genes involved in the pathway targeted by miRNAs. The dot color represents p-values of pathways. The gene ratio was calculated as the number of pathway-related genes divided by the total number of processed genes. Raw p-value < 0.05 was statistically significant.

Based on the dot plot of Reactome pathway enrichment analysis, it can be said that 37 cellular pathways were targeted by miRNAs that were DE in ML-SA1 treated *WT* neuroglia compared to untreated *WT* neuroglia. The only overlapped pathway was TCR signaling between significantly upregulated and downregulated miRNAs. The most significantly enriched pathway of downregulated miRNAs was found as ‘sphingolipid de novo biosynthesis’ while ‘signaling by receptor tyrosine kinases’ was identified as the most significant pathway of upregulated miRNAs according to gene ratio, gene count, and p-value criteria (Figure 3.29).

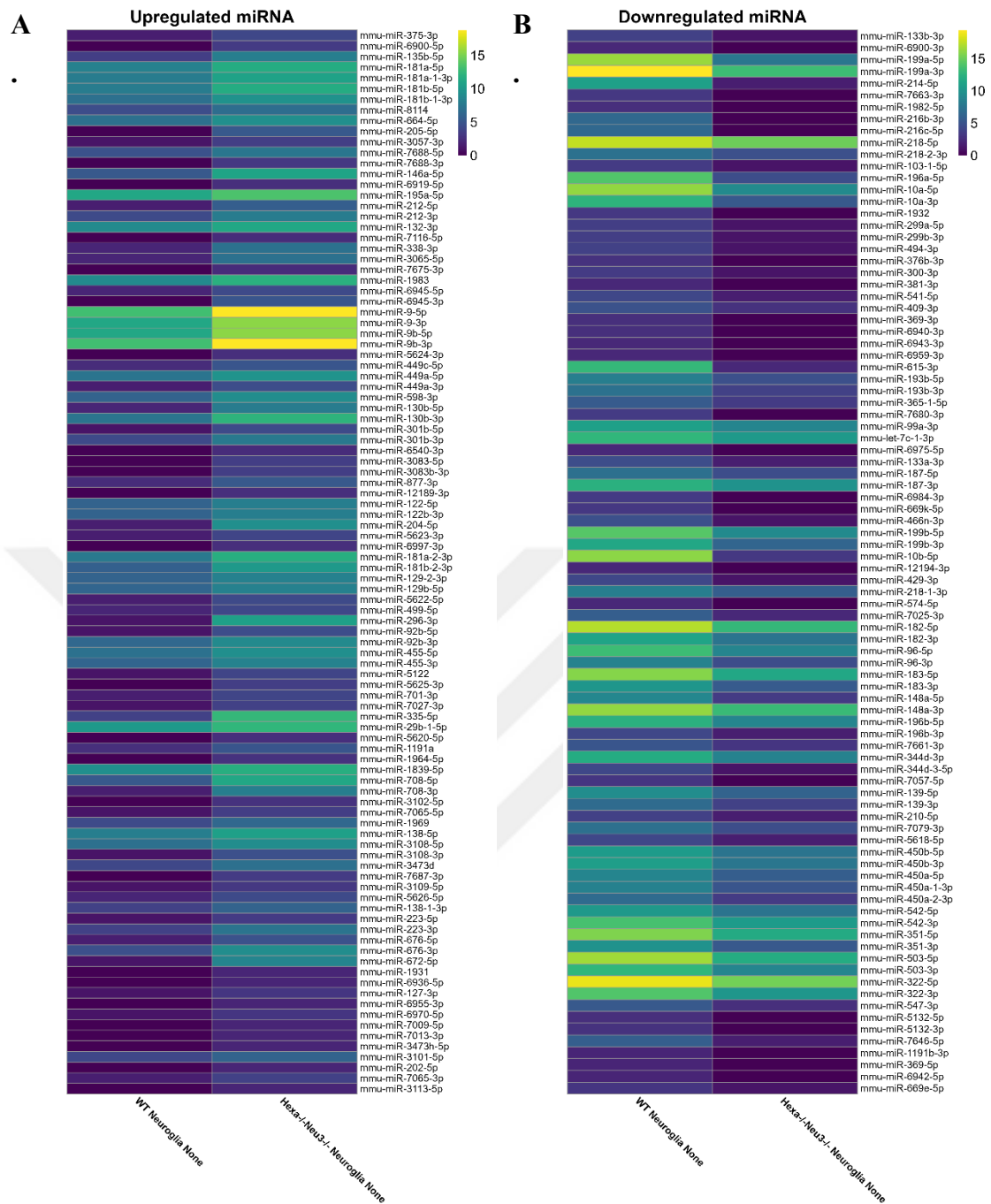


Figure 3.30. Heatmap of significantly upregulated (A) and downregulated (B) miRNAs in untreated (none) *Hexa*^{-/-}*Neu3*^{-/-} neuroglia compared to untreated (none) *WT* neuroglia. Each column represents the genotype of cells. Each row shows the identity of miRNAs (p-value < 0.05, log₂FC ≥ 2 for upregulation and log₂FC ≤ -2 for downregulation).

Significantly DE miRNAs in untreated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia compared to untreated *WT* neuroglia were given as a heatmap (Figure 3.30). Accordingly, 100

upregulated miRNAs (mmu-miR-375-3p, mmu-miR-6900-5p, mmu-miR-135b-5p, mmu-miR-181a-5p...) (Figure 3.30 A) and 90 downregulated miRNAs (mmu-miR-133b-3p, mmu-miR-6900-3p, mmu-miR-199a-5p, mmu-miR-199a-3p...) (Figure 3.30 B) were identified.

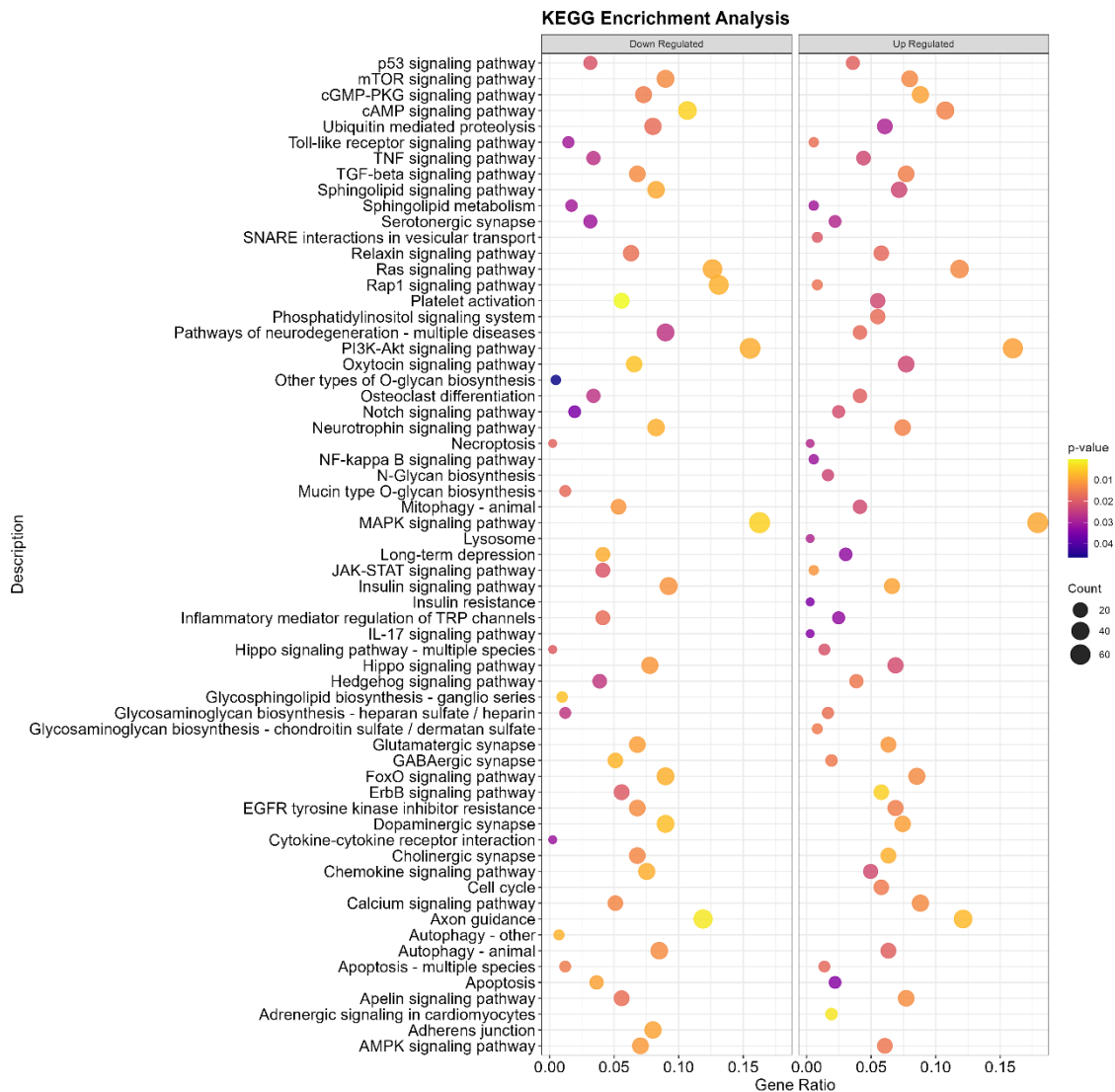


Figure 3.31. Dot plot of KEGG enrichment analysis of the significantly up and down-regulated miRNAs in untreated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia compared to untreated *WT* neuroglia. Dot sizes represent the number of genes involved in the pathway targeted by miRNAs. The dot color represents p-values of pathways. The gene ratio was calculated as the number of pathway-related genes divided by the total number of processed genes. Raw p-value < 0.05 was statistically significant.

KEGG pathway enrichment analysis, visualized with a dot plot graphic, demonstrated that 63 cellular pathways were targeted by miRNAs that were DE in untreated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia compared to untreated *WT* neuroglia. In total, 47 pathways (mTOR signaling pathway, TNF signaling pathway, sphingolipid metabolism, MAPK signaling pathway, insulin signaling pathway, etc.) were found to be targeted by both significantly upregulated and downregulated miRNAs. The most significant pathway of both downregulated and upregulated miRNAs was detected as ‘MAPK signaling pathway’ when the gene ratio, gene count, and p-value criteria were weighed (Figure 3.31).

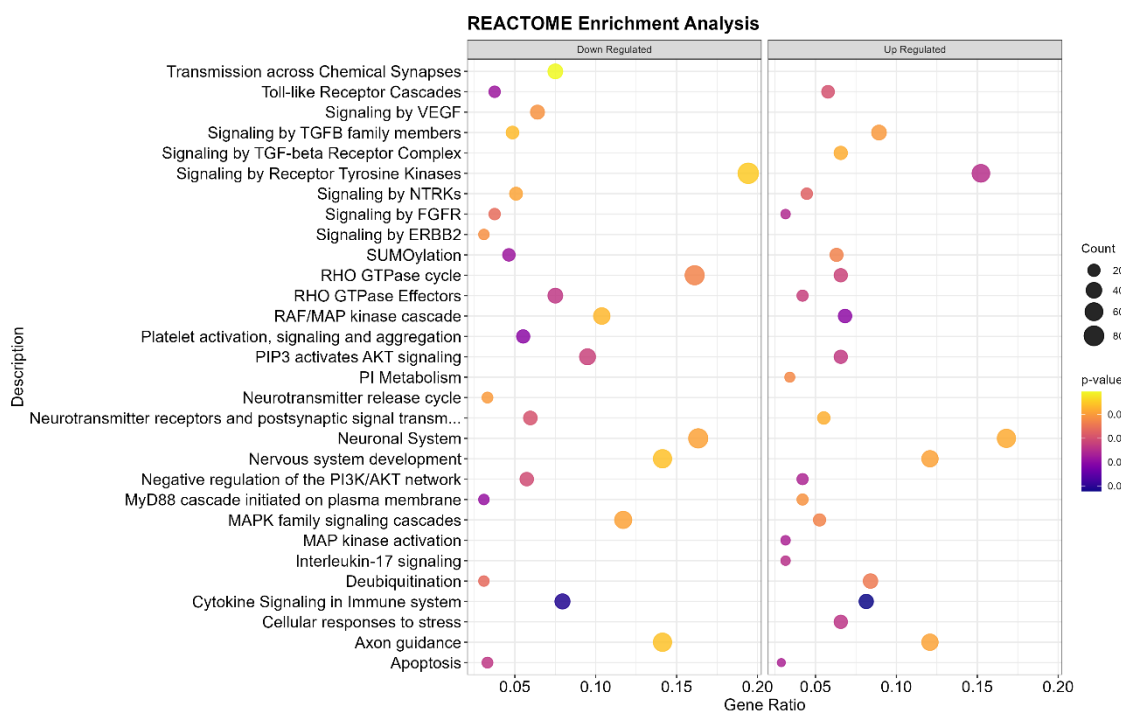


Figure 3.32. Dot plot of Reactome enrichment analysis of the significantly up and down-regulated miRNAs in untreated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia compared to untreated *WT* neuroglia. Dot sizes represent the number of genes involved in the pathway targeted by miRNAs. The dot color represents p-values of pathways. The gene ratio was calculated as the number of pathway-related genes divided by the total number of processed genes. The top 50 pathways based on gene ratio were selected. Raw p-value < 0.05 was statistically significant.

Reactome pathway enrichment analysis, visualized with a dot plot graphic, demonstrated that 30 cellular pathways (due to a great amount of enriched pathways, top 50 were filtered according to gene ratio) were targeted by miRNAs that were DE in ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts compared to untreated *Hexa*^{-/-}*Neu3* fibroblasts.

In total, 20 pathways (signaling by TGFB family members, Toll-like receptor cascades, RHO GTPase cycle, nervous system, RAF/MAP kinase cascade, etc.) were found to be targeted by both significantly upregulated and downregulated miRNAs. The most significant pathway of downregulated miRNAs was detected as ‘signaling by receptor tyrosine kinases’, and for the upregulated miRNAs, the most significant pathway was identified as the ‘neuronal system’ when the gene ratio, gene count, and p-value criteria were weighed (Figure 3.32).

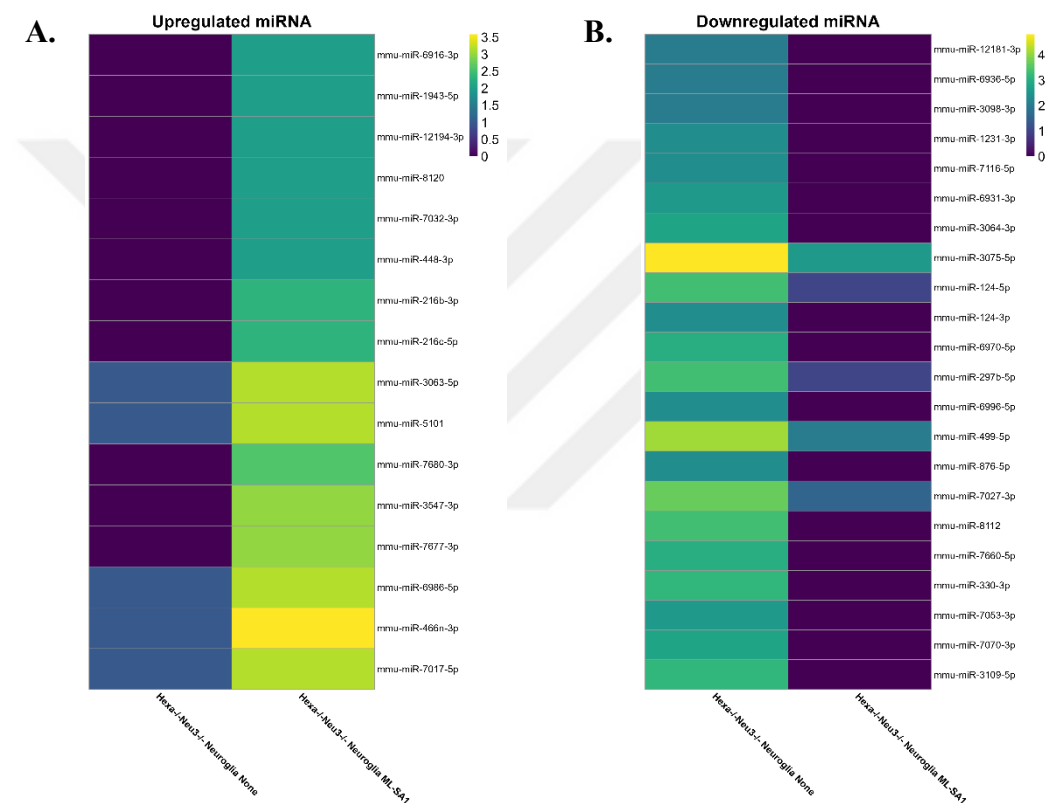


Figure 3.33. Heatmap of significantly upregulated (A) and downregulated (B) miRNAs in ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia compared to untreated (none) *Hexa*^{-/-}*Neu3*^{-/-} neuroglia. Each column represents the treatment condition. Each row shows the identity of miRNAs (p-value < 0.05, log₂FC ≥ 2 for upregulation and log₂FC ≤ -2 for downregulation).

Significantly DE miRNAs in ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia compared to untreated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia were shown in a heatmap (Figure 3.33). As a result, it was observed that 16 miRNAs (mmu-miR-6916-3p, mmu-miR-1943-5p, mmu-miR-12194-3p, mmu-miR-8120...) were upregulated (Figure 3.33 A) and 22

miRNAs (mmu-miR-12181-3p, mmu-miR-6936-5p, mmu-miR-3098-3p, mmu-miR-1231-3p...) were downregulated (Figure 3.33 B).

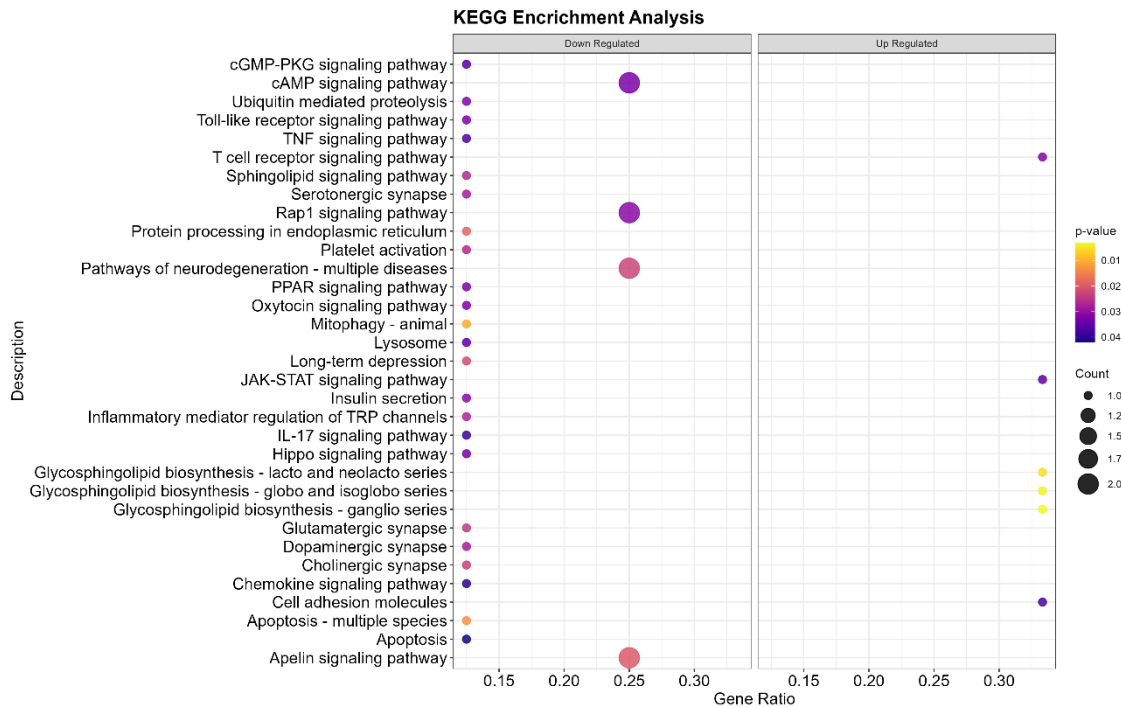


Figure 3.34. Dot plot of KEGG enrichment analysis of the significantly up and down-regulated miRNAs in ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia compared to untreated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia. Dot sizes represent the number of genes involved in the pathway targeted by miRNAs. The dot color represents p-values of pathways. The gene ratio was calculated as the number of pathway-related genes divided by the total number of processed genes. Raw p-value < 0.05 was statistically significant.

In the dot plot of KEGG pathway enrichment analysis applied to the DE miRNAs of ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia compared to untreated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia, 33 cellular pathways were seen in total. Among them, no pathway was targeted by both significantly upregulated and downregulated miRNAs. When gene count, gene ratio, and p-value were considered, the most significant pathway of downregulated miRNAs was identified as ‘apelin signaling pathway’. When it comes to the upregulated miRNAs, the most significant pathway was found as ‘glycosphingolipid biosynthesis – ganglio series’ (Figure 3.34).

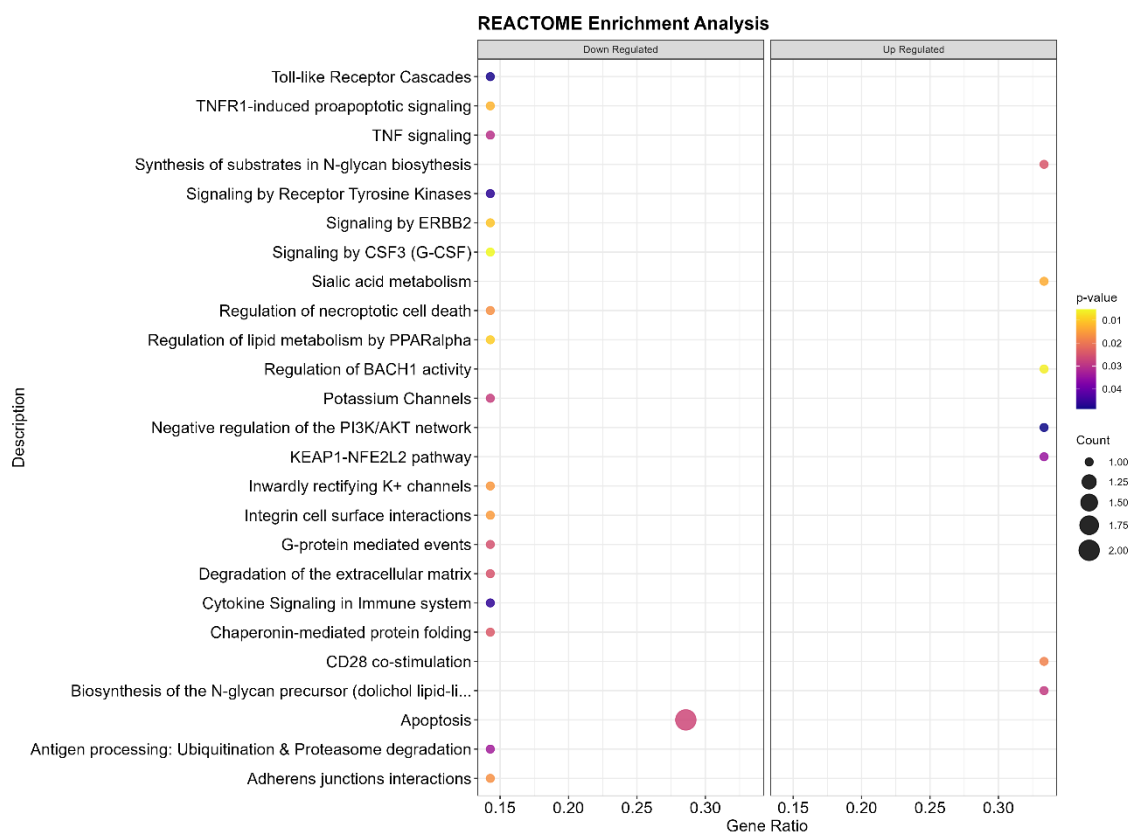


Figure 3.35. Dot plot of Reactome enrichment analysis of the significantly up and down-regulated miRNAs in ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia compared to untreated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia. Dot sizes represent the number of genes involved in the pathway targeted by miRNAs. The dot color represents p-values of pathways. The gene ratio was calculated as the number of pathway-related genes divided by the total number of processed genes. Raw p-value < 0.05 was statistically significant.

According to the dot plot of Reactome pathway enrichment analysis applied to the DE miRNAs of ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia compared to untreated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia, 25 cellular pathways were seen in total. Among them, there was no pathway as a target of both significantly upregulated and downregulated miRNAs. When gene count, gene ratio, and p-value were considered, the most significant pathway of downregulated miRNAs was identified as ‘apoptosis’; for the upregulated miRNAs, the most significant pathway was found as ‘regulation of Bach1 activity’ (Figure 3.35).

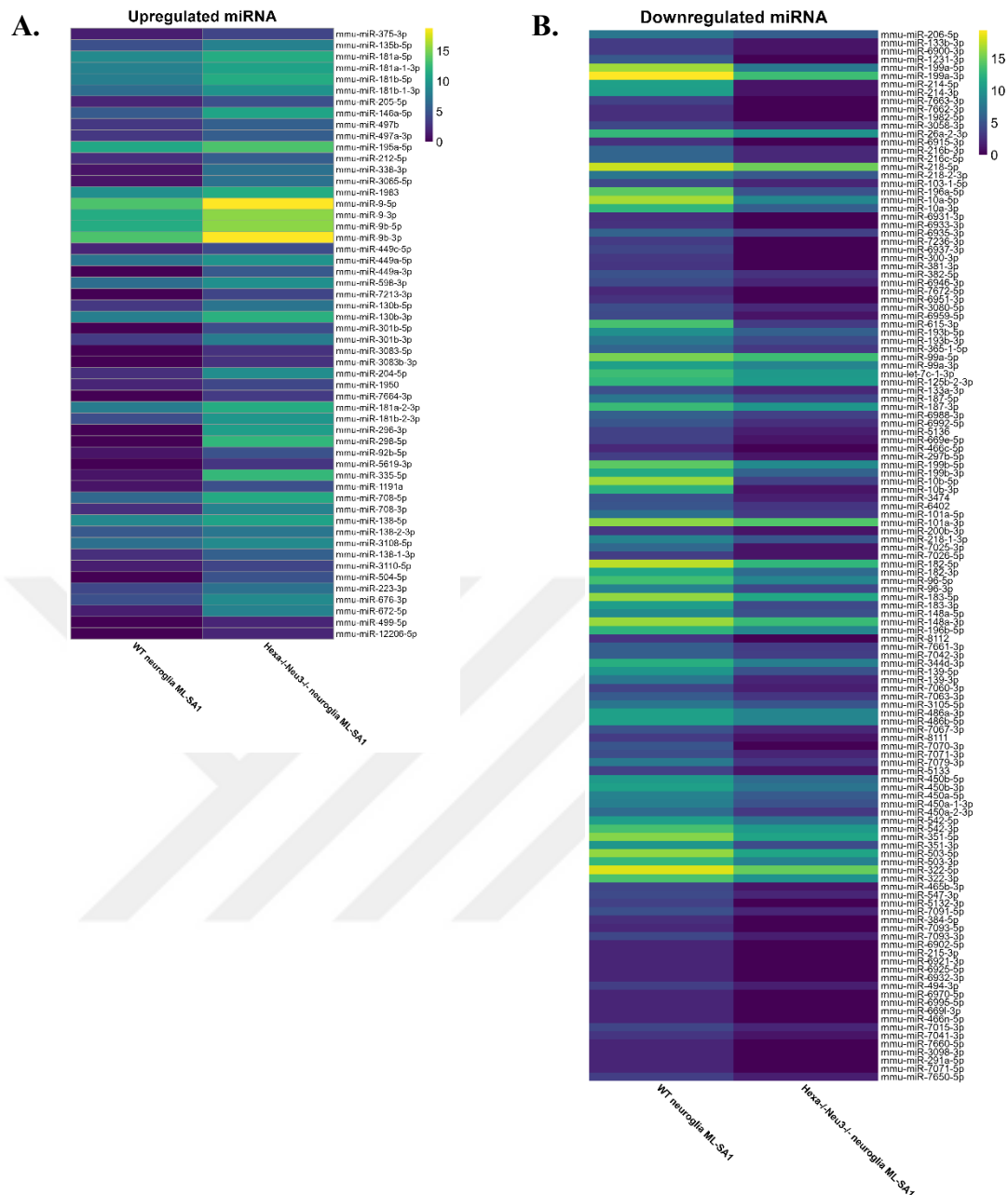


Figure 3.36. Heatmap of significantly upregulated (A) and downregulated (B) miRNAs in ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia compared to ML-SA1 treated *WT* neuroglia. Each column represents the genotype of cells. Each row shows the identity of miRNAs (p-value < 0.05, log₂FC ≥ 2 for upregulation and log₂FC ≤ -2 for downregulation).

Significantly DE miRNAs in ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia compared to ML-SA1 treated *WT* neuroglia were shown in a heatmap (Figure 3.36). As a result, it was observed that 54 miRNAs (mmu-miR-375-3p, mmu-miR-135b-5p, mmu-miR-181a-5p, mmu-miR-181a-1-3p...) were upregulated (Figure 3.36 A) and 127

miRNAs (mmu-miR-206-5p, mmu-miR-133b-3p, mmu-miR-6900-3p, mmu-miR-1231-3p...) were downregulated (Figure 3.36 B).

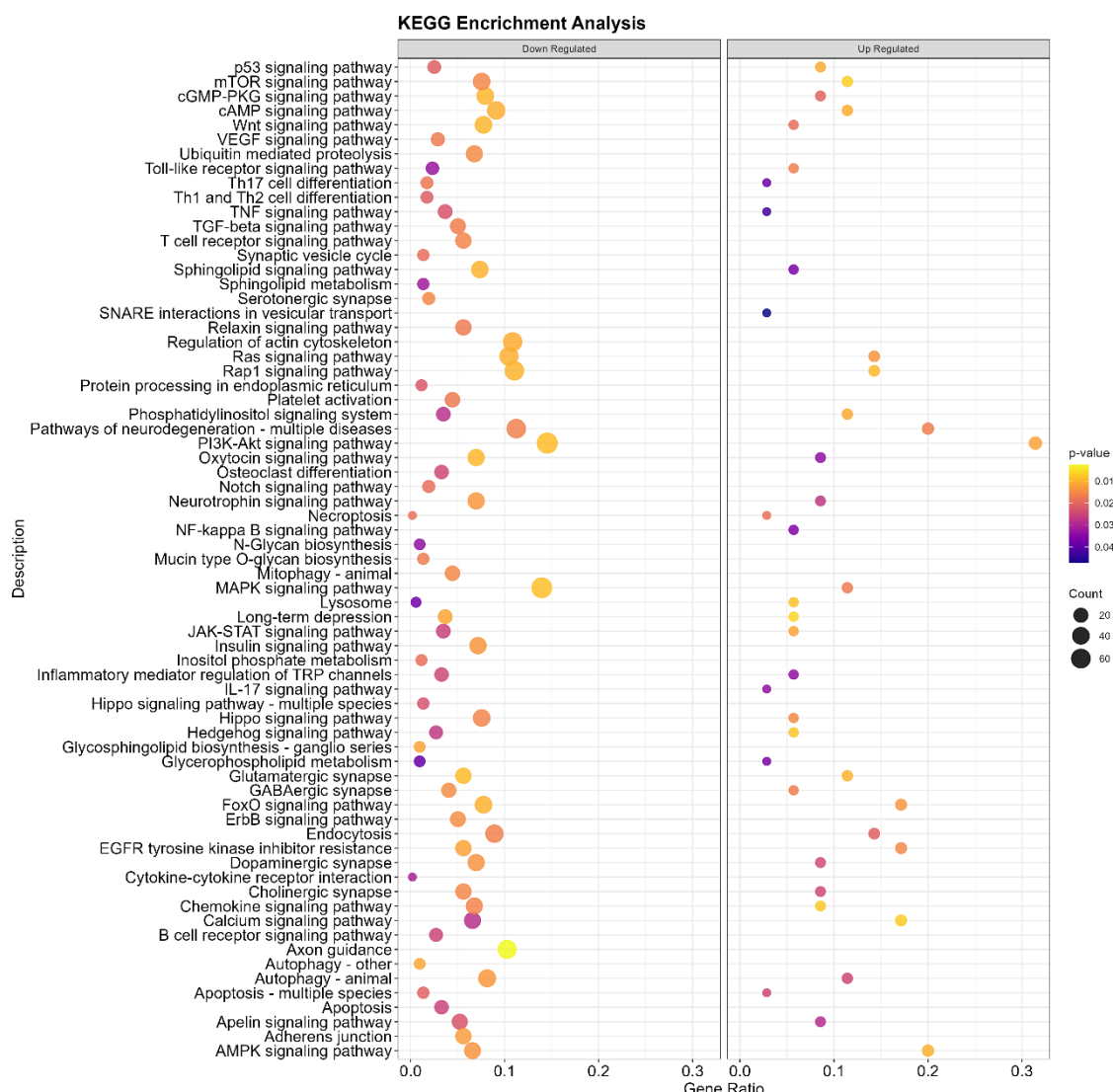


Figure 3.37. Dot plot of KEGG enrichment analysis of the significantly up and down-regulated miRNAs in ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia compared to ML-SA1 treated *WT* neuroglia. Dot sizes represent the number of genes involved in the pathway targeted by miRNAs. The dot color represents p-values of pathways. Gene ratio was calculated as pathway-related genes / all processed genes. Raw p-value < 0.05 was statistically significant.

KEGG pathway enrichment analysis, visualized with a dot plot graphic, exhibited that 69 cellular pathways were targeted by miRNAs that were DE in ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia compared to ML-SA1 treated *WT* neuroglia. Among them, 38 pathways (p53 signaling pathways, mTOR signaling pathway, cGMP-PKG signaling

pathway, cAMP signaling pathway, Wnt signaling pathway, etc.) were found to be targeted by both significantly upregulated and downregulated miRNAs. The most significant pathway of both downregulated and upregulated miRNAs was detected as ‘PI3K-Akt signaling pathway’, according to gene ratio, gene count, and p-value criteria (Figure 3.37).

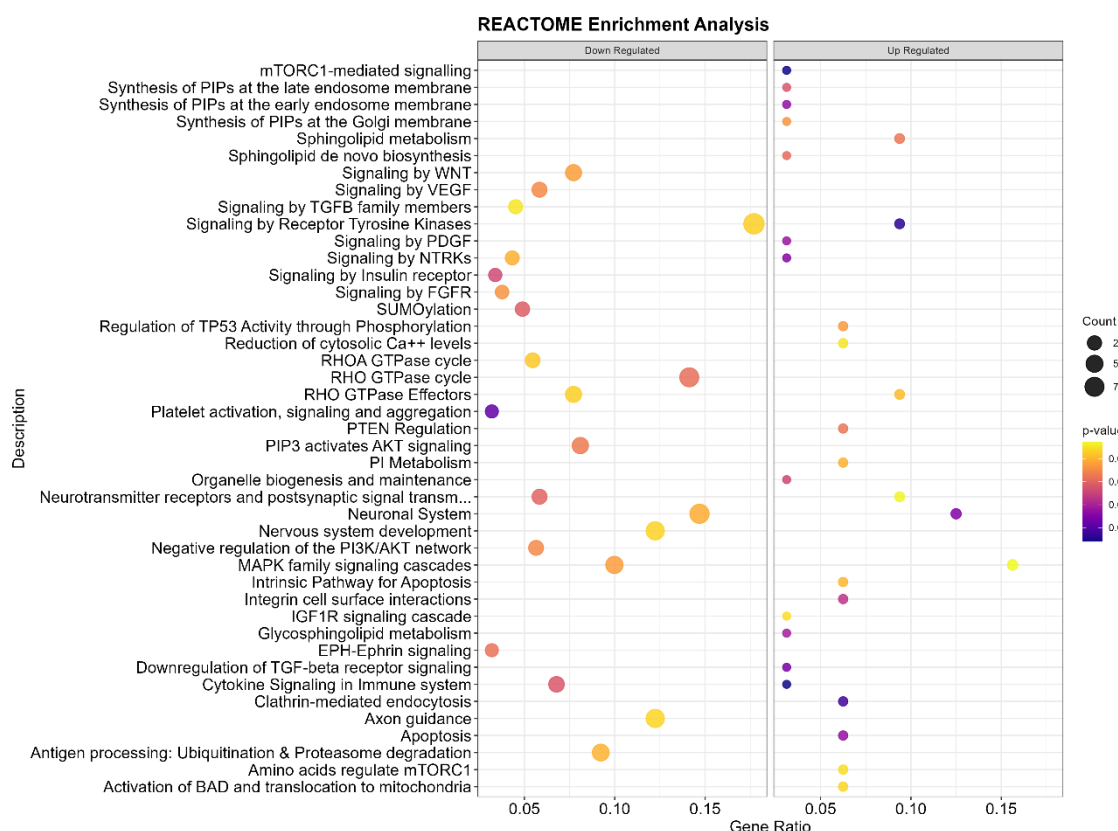


Figure 3.38. Dot plot of Reactome enrichment analysis of the significantly up and down-regulated miRNAs in ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia compared to ML-SA1 treated *WT* neuroglia. Dot sizes represent the number of genes involved in the pathway targeted by miRNAs. The dot color represents p-values of pathways. The gene ratio was calculated as the number of pathway-related genes divided by the total number of processed genes. The top 50 pathways based on gene ratio were selected. Raw p-value < 0.05 was statistically significant.

Reactome pathway enrichment analysis, visualized with a dot plot graphic, showed that 43 cellular pathways were targeted by miRNAs that were DE in ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia compared to ML-SA1 treated *WT* neuroglia. Among them, 7 pathways (signaling by receptor tyrosine kinases, signaling by NTRKs, RHO

GTPase effectors, neurotransmitter receptors and postsynaptic signal transmission, neuronal system, etc.) were found to be targeted by both significantly upregulated and downregulated miRNAs. The most significant pathway of downregulated miRNAs was ‘signaling by receptor tyrosine kinases’; for upregulated miRNAs, ‘MAPK family signaling cascades’ was the most significant pathway when the gene ratio, gene count, and p-value criteria were weighed (Figure 3.38).

Table 3.1. Common upregulated and downregulated miRNAs between *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts and neuroglia in only disease, as well as ML-SA1 treatment in disease conditions. (FC: fold change, $p < 0.05$ and $\log_2FC \leq -2$ for downregulation and $\log_2FC \geq 2$ for upregulation)

Condition Description	Commonly Upregulated miRNAs	Commonly Downregulated miRNAs
<i>Hexa</i>^{-/-}<i>Neu3</i>^{-/-} fibroblasts and neuroglia cells compared to their respective <i>WT</i> controls (disease effect only)	mmu-miR-672-5p (log ₂ FC: 4.9 in fibroblast) (log ₂ FC: 7.6 in neuroglia)	mmu-miR-6940-3p (log ₂ FC: -2.3 in fibroblast) (log ₂ FC: -2.6 in neuroglia)
	mmu-miR-5624-3p (log ₂ FC: 2.8 in fibroblast) (log ₂ FC: 2.6 in neuroglia)	
ML-SA1-treated <i>Hexa</i>^{-/-}<i>Neu3</i>^{-/-} fibroblasts and neuroglia cells compared to their ML-SA1-treated <i>WT</i> controls (treatment effect on disease)	mmu-miR-672-5p (log ₂ FC: 2.3 in fibroblast) (log ₂ FC: 7.6 in neuroglia)	mmu-miR-7091-5p (log ₂ FC: -4.1 in fibroblast) (log ₂ FC: -2.7 in neuroglia)
	mmu-miR-375-3p (log ₂ FC: 2 in fibroblast) (log ₂ FC: 2.4 in neuroglia)	

Significantly upregulated and downregulated miRNAs that were common between untreated and ML-SA1 treated neuroglia and fibroblast cells of *Hexa*^{-/-}*Neu3*^{-/-} mice compared to their respective *WT* controls were given in Table 3.1. According to Table 3.1, it can be understood that mmu-miR-672-5p ($\log_2FC = 4.9$ in fibroblasts and $\log_2FC = 7.6$ in neuroglia) and mmu-miR-5624-3p ($\log_2FC = 2.8$ in fibroblasts and $\log_2FC = 2.6$ in neuroglia) were significantly upregulated while mmu-miR-6940-3p ($\log_2FC = -2.3$ in fibroblasts and $\log_2FC = -2.6$ in neuroglia) was significantly

downregulated in untreated fibroblasts and neuroglia of *Hexa*^{-/-}*Neu3*^{-/-} mice. In addition, mmu-miR-672-5p ($\log_2\text{FC} = 2.3$ in fibroblasts and $\log_2\text{FC} = 7.6$ in neuroglia) and mmu-miR-375-3p ($\log_2\text{FC} = 2$ in fibroblasts and $\log_2\text{FC} = 2.4$ in neuroglia) were significantly upregulated while mmu-miR-7091-5p ($\log_2\text{FC} = -4.1$ in fibroblasts and $\log_2\text{FC} = -2.7$ in neuroglia) was significantly downregulated in ML-SA1 treated fibroblasts and neuroglia of *Hexa*^{-/-}*Neu3*^{-/-} mice.

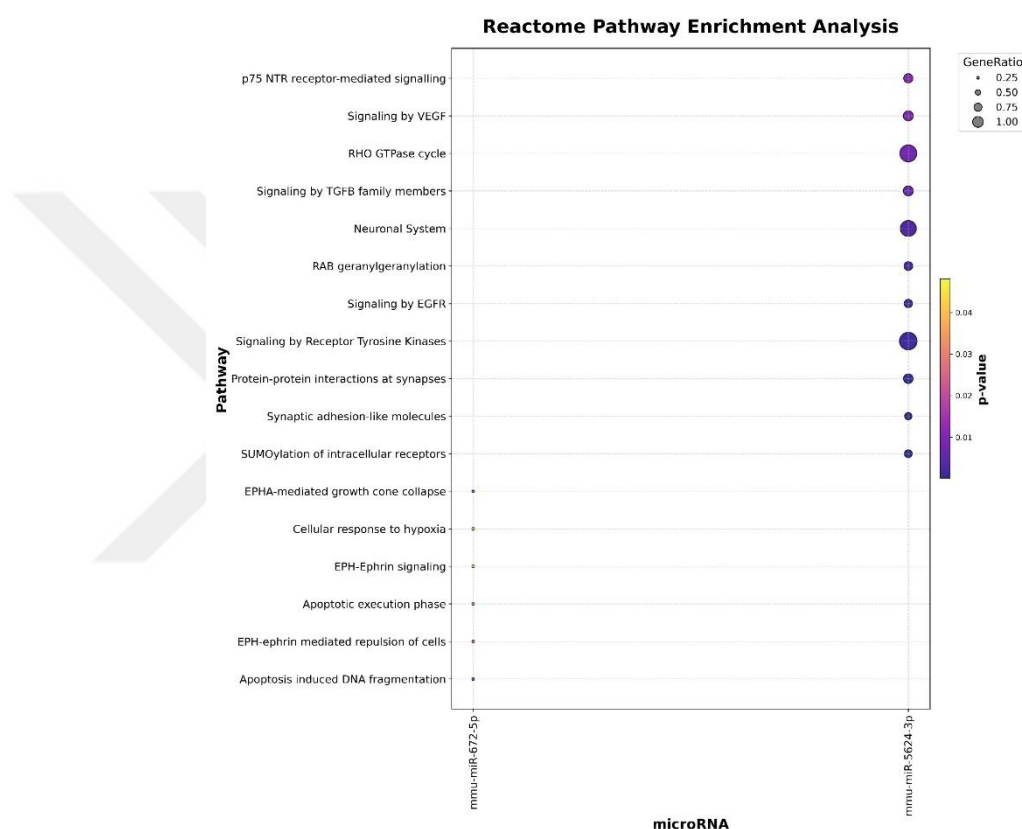


Figure 3.39. Dot plot of Reactome enrichment analysis of the significantly upregulated mmu-miR-672-5p and mmu-miR-5624-3p in both untreated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblasts compared to their untreated *WT* controls. Dot sizes represent the number of genes involved in the pathway targeted by miRNAs. The dot color represents p-values of pathways. The gene ratio was calculated as the number of pathway-related genes divided by the total number of processed genes. Raw p-value < 0.05 was statistically significant.

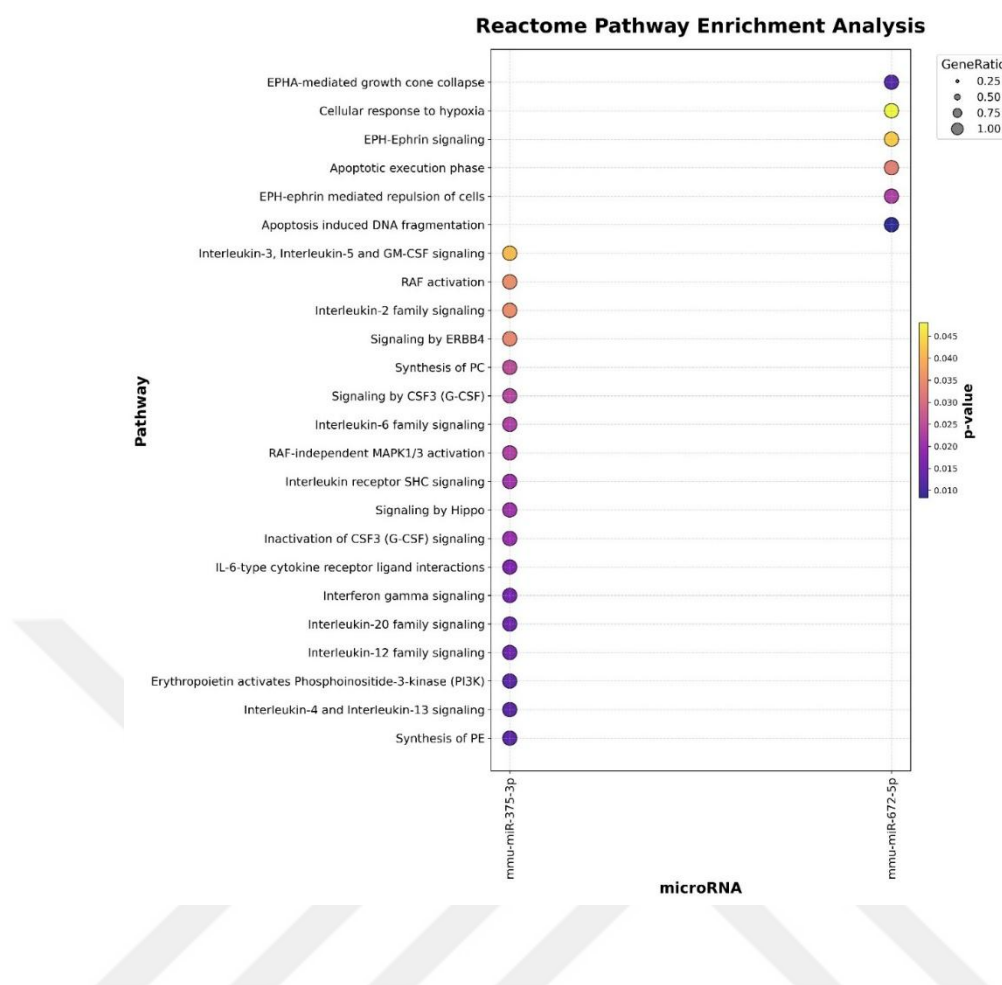


Figure 3.40. Dot plot of Reactome enrichment analysis of the significantly upregulated miRNAs mmu-miR-375-3p and mmu-miR-672-5p in both ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblasts compared to their ML-SA1 treated *WT* controls. Dot sizes represent the number of genes involved in the pathway targeted by miRNAs. The dot color represents p-values of pathways. The gene ratio was calculated as the number of pathway-related genes divided by the total number of processed genes. Raw p-value < 0.05 was statistically significant.

In the dot plot of Reactome pathway enrichment analysis applied to commonly upregulated miRNAs, mmu-miR-672-5p and mmu-miR-5624-3p, between untreated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblasts compared to their untreated *WT* controls 17 cellular pathways were seen in total. Among these pathways, 6 pathways (EPHA-mediated growth cone collapse, cellular response to hypoxia, EPH-Ephrin signaling, apoptotic execution phase, etc.) were enriched for mmu-miR-672-5p. 11 pathways (RHO-GTPase cycle, neuronal system, signaling by receptor kinases, etc.) were enriched for mmu-miR-5624-3p. According to gene count, gene ratio, and p-value criteria, it can be

said that the most significant pathway of mmu-miR-672-5p was ‘apoptosis induced DNA fragmentation’. For mmu-miR-5674-3p, the most significant pathway was ‘signaling by receptor tyrosine kinases’ (Figure 3.39).

In the dot plot of Reactome pathway enrichment analysis applied to the shared upregulated miRNAs, mmu-miR-375-3p and mmu-miR-672-5p, between ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblasts compared to their ML-SA1 treated *WT* controls, 24 cellular pathways were seen in total. Among these pathways, 6 pathways (EPHA-mediated growth cone collapse, cellular response to hypoxia, EPH-Ephrin signaling, apoptotic execution phase, etc.) were enriched for mmu-miR-672-5p. 18 pathways (interleukin-3, interleukin-5 and GM(CSF) signaling, IL-6-type cytokine receptor ligand interactions, interferon gamma signaling, etc.) were enriched for mmu-miR-375-3p. According to gene count, gene ratio, and p-value criteria, it can be said that the most significant pathways of mmu-miR-375-3p were ‘synthesis of PE, interleukin-4 and interleukin-13 signaling, erythropoietin activates Phosphoinositide-3-kinase (PI3K)’. For mmu-miR-672-5p, the most significant pathway was ‘apoptosis induced DNA fragmentation’ (Figure 3.40).

Table 3.2. Reactome pathway enrichment results of common downregulated miRNA mmu-miR-7091-5p in ML-SA1 treated fibroblasts and neuroglia of *Hexa*^{-/-}*Neu3*^{-/-} mice compared to their ML-SA1 treated *WT* controls.

miRNA ID	Enriched Pathway	Gene Count	P value	FDR	Target Genes
mmu-miR-7091-5p	Regulation of gene expression by Hypoxia-inducible Factor	1	0.000844	0.00422	<i>Epas1</i>
mmu-miR-7091-5p	Oxygen-dependent proline hydroxylation of Hypoxia-inducible Factor Alpha	1	0.00295	0.0044	<i>Epas1</i>
mmu-miR-7091-5p	Regulation of Hypoxia-inducible Factor (HIF) by oxygen	1	0.00352	0.0044	<i>Epas1</i>
mmu-miR-7091-5p	Cellular response to hypoxia	1	0.00352	0.0044	<i>Epas1</i>
mmu-miR-7091-5p	Cellular responses to stress	1	0.0346	0.0346	<i>Epas1</i>

Reactome pathway enrichment results showed that commonly downregulated miRNA, mmu-miR-7091-5p, between ML-SA1 treated fibroblasts and neuroglia of *Hexa*^{-/-}*Neu3*^{-/-} mice compared to their ML-SA1 treated *WT* controls targeted only 1 gene, *Epas1*. Consequently, related pathways were seen as regulation of gene expression by Hypoxia-inducible factor, oxygen-dependent proline hydroxylation of hypoxia inducible factor alpha and etc.

CHAPTER 4

DISCUSSION

In eukaryotic cells, lysosomes work as degradation machineries by including ~ 50 acid hydrolases.^{1,2} Also, they are involved in different processes like cell death, calcium signaling, autophagy, etc.³ Lysosomal storage disorders (LSDs) are rare, inherited, monogenic diseases arising from the distorted function of lysosomal enzymes or activators of hydrolase enzymes or transporters found in the lysosome membrane. These different dysfunctionalities mainly result in the gradual storage of macromolecules like sphingolipids in lysosomes and consequent impairment of several organs. More than 70 LSDs are known to exist.^{1,5} Gangliosides are amphiphilic glycosphingolipid members having at least one sialic acid moiety. Vertebrates have more than 200 types of gangliosides with diverse hydrophobic ceramide moieties, and the neuronal system has the greatest amount of them. In cells, gangliosides are enriched in plasma membrane microdomains (for ex., lipid rafts) along with sphingomyelin and cholesterol. They have regulatory effects on ion channels, cellular recognition and communication, signal transduction, etc.⁸⁻¹⁰ GM2 gangliosidosis is a subgroup of LSDs comprising Tay-Sachs disease (TSD), Sandhoff disease (SD), and GM2 activator deficiency. Each disease has different mutated genes, but the overall result is an abnormal accumulation of GM2 ganglioside in lysosomes.¹³⁻¹⁵ TSD is a rare, autosomal recessively inherited, neurodegenerative LSD that arises from deficient β -N-acetylhexosaminidase A (HEXA) enzyme due to a mutated *HEXA* gene responsible for encoding the alpha subunit of the heterodimeric HEXA enzyme.¹⁶ Normally, HEXA converts GM2 into GM3 ganglioside by hydrolyzing the N-acetylgalactosamine part of the GM2. However, in TSD, the absence or dysfunctionality of the enzyme prevents this conversion, and GM2 accumulation in the lysosomes of the central nervous system (CNS) is seen. Infantile TSD patients exhibit the most severe nervous system-related abnormalities, and they die at the age of 5.^{17,19} To obtain the mouse model of TSD, *Hexa*^{-/-} mice were generated, but they did not show severe pathological symptoms.²⁹ It was understood that, in mice, there is a bypass mechanism mediated by Neu3 sialidase. Therefore, with the generation of *Hexa*^{-/-}*Neu3*^{-/-} mice, TSD was mimicked with severe phenotypic features in parallel with the disease.³⁵

Lysosomal exocytosis is a regulated secretion mechanism seen in all cell types.^{42,46} During lysosomal exocytosis, lysosomes are directed from the perinuclear area to the plasma membrane and release their content to the extracellular space by membrane fusion. Lysosomal exocytosis provides cellular clearance and plasma membrane repair to the cell. The free Ca^{2+} level of the cytoplasm induces membrane fusion.⁴⁷ Therefore, late-endosome and lysosome-specific Ca^{2+} channel Mucolipin-1 or TRPML1 is a positive regulator of lysosomal exocytosis.¹¹⁷ ML-SA1 is a synthetic agonist of the TRPML1 channel.¹¹⁸ Previously, distorted lysosomal exocytosis followed by lysosomal accumulation of specific substrates was repaired after ML-SA1 treatment by restoring calcium homeostasis in dopaminergic neurons of Parkinson's disease patients⁹⁵, nephrotoxicity mimicking mouse kidney⁹⁶, and the *Fig4* deficient mouse model of Charcot-Marie-Tooth disease Type 4J¹¹⁹. In previous studies, it was stated that endosome-lysosomal membrane trafficking (trafficking jam) can cause secondary lipid accumulation in the lysosomes with the distorted transport of hydrolase enzymes to the lysosome via endocytic vesicles. In addition, a trafficking jam might occur due to the primary lipid storage.^{4,59} This impaired endo-lysosomal trafficking was observed in lysosomal storage conditions, such as Niemann Pick Type A, B, and C1¹²⁰⁻¹²² and Mucopolidoses Type IV^{123,124}. In earlier studies, ML-SA1 treatment in NPC1^{-/-} macrophages and Fabry disease fibroblast cells, late endosome-lysosome to Golgi trafficking was significantly improved, and lysosomal lipofuscin storage decreased, respectively. However, calcium flux did not increase as expected. Therefore, studies exhibited that the activity of the TRPML1 channel is compromised in several LSDs due to different lipid accumulations (Fabry disease, NPA, NPB, and NPC1).^{123,125} Although the effect of ML-SA1 on lysosomal exocytosis has been studied in neurodegenerative diseases and some LSDs, no study has been conducted in Tay-Sachs disease yet. Therefore, the first sub-aim of this study was to examine how ML-SA1 would cause changes in the lysosomal exocytosis pathway in TSD mouse cells. For this purpose, intracellular and extracellular specific activities of lysosomal Hexa and Hexb enzymes were measured in ML-SA1 treated and untreated 5-month-old *WT* and *Hexa*^{-/-}*Neu3*^{-/-} mouse fibroblast and neuroglia cells. In addition, the localization and level of Lamp1 protein were investigated by immunocytochemical staining of cell surface Lamp1 and total Lamp1. Before all treatments, the concentration of ML-SA1 was optimized by using the MTT cell viability assay. According to results, it was observed that 10 μM was less

toxic for all cells than 20 μ M ML-SA1 (Figure 3.2 A-D). Thus, all experiments were conducted with this dosage.

In enzyme activity assays, Hexb enzyme activity in cell media increased non-significantly in both *WT* and *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblast cells after ML-SA1 treatment (Figure 3.3 A and Figure 3.4 A). In parallel with these results, intracellular Hexb enzyme activity decreased in both genotypes of fibroblasts and neuroglia, but this decrement was significant only in *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts (Figure 3.3 B and Figure 3.4 B). Likely, intracellular Hexa enzyme activity decreased significantly in *WT* neuroglia and fibroblasts, while due to the disease condition, this enzyme could not be detected in the *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts and neuroglia (Figure 3.3 C and Figure 3.4 C). Overall, these results are a clear indication that ML-SA1 treatment can trigger the release of lysosomal enzymes via lysosomal exocytosis in cells and are consistent with studies in the literature.^{95,96,126}

When it comes to surface Lamp1 levels, immunocytochemical analysis showed that surface Lamp1 of *WT* neuroglia and fibroblasts non-significantly increased after ML-SA1 treatment. In *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblasts, surface Lamp1 was significantly increased compared to untreated cells. Surprisingly, surface Lamp1 intensity of *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblasts was significantly higher than *WT* neuroglia and fibroblasts, respectively, in untreated conditions (Figure 3.11). Surface localization of Lamp1 (lysosomal membrane protein) is a widely used lysosomal exocytosis marker.¹²⁷⁻¹²⁹ Therefore, the increase in Lamp1 localized to the neuroglia and fibroblast membranes under the influence of ML-SA1 suggests that exocytosis can be further induced, possibly via Ca²⁺ release from the TRPML1 channel, and fusion of lysosomes with the plasma membrane is supported in parallel with previous works.^{95,96} Non-significantly increased surface Lamp1 by ML-SA1 under healthy conditions (*WT* cells) can be explained by the fact that the cell already has balanced calcium homeostasis. On the other hand, the excess amount of Lamp1 on the surface of *Hexa*^{-/-}*Neu3*^{-/-} mouse fibroblasts and neuroglia under normal conditions suggests that the cell, which wants to get rid of the lysosomal storage, tends more towards exocytosis as a compensation mechanism. In total Lamp1 staining, *Hexa*^{-/-}*Neu3*^{-/-} neuroglia cells showed a significant decrease after ML-SA1 treatment (Figure 3.12 A and B) while a non-significant decrease was observed in *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts (Figure 3.12 C and D). In addition, a slight, nonsignificant increase was observed in *WT* neuroglia and fibroblasts (Figure 3.12 A-D). When considered together with the increased surface Lamp1 levels observed, these results

suggest that lysosomal exocytosis is increased in disease conditions in a better way compared to the healthy state. These findings are consistent with the decrease in total Lamp1 signaling observed upon induction of exocytosis through tocopherol and cyclodextrin in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblasts as shown in the previous study.⁷¹ This suggests that the translocation of lysosomal membranes to the cell surface may lead to depletion of the intracellular Lamp1 pool, and an active compensatory mechanism operates to reduce lysosomal accumulation.

Autophagy is the process of degradation of accumulated or misfolded proteins, damaged organelles, and pathogens via the lysosomal route for reuse. This mechanism is involved in cellular homeostasis maintenance, antiviral defense mechanisms, and innate and adaptive immune responses in different cellular states.^{72,73} Several conditions, like energy and nutrient states of cells and tissues, regulate autophagy (macroautophagy), and a bunch of proteins and protein complexes work coordinately in the autophagy process.⁷⁵ Previous studies have shown that many LSDs^{130–133}, including TSD⁸⁸, exhibit altered autophagic machinery and accumulated autophagic cargo. There is a great impact of the altered autophagy in neurodegeneration and lysosomal accumulation as a vicious cycle.¹³⁴ Similar to lysosomal exocytosis, TRPML1-mediated Ca²⁺ release can trigger autophagy via activation of calcineurin (CaN) followed by nuclear translocation of TFEB and increased expression of CLEAR, or by the activation of CaMKK β and AMPK, leading to phagophore formation.⁹¹ In this context, ML-SA1 has been used in different disease conditions, like ischemic brain injury¹³⁵, Parkinson's disease¹³⁶, Alzheimer's disease⁸⁹, epileptic seizures¹³⁷, Amyotrophic lateral sclerosis and Parkinson's dementia complex¹³⁸, retinal detachment¹³⁹, and results showed that ML-SA1-mediated TRPML1 activation restored defective autophagy mechanisms. In addition, in a previous study conducted on LSDs such as NPC1 and MLIV, the effect of ML-SA1 on autophagic markers was also investigated.¹⁴⁰ However, in the literature, there is no study on this objective specifically for Tay-Sachs disease. Therefore, the second sub-aim of this thesis study was to investigate how ML-SA1 would affect the previously stated impaired autophagy (p62 and LC3 accumulation) in TSD mouse cells.¹⁴¹ Following this aim, gene expression analysis of *Atg7*, *p62*, *Beclin1*, *Lamp1*; Western Blot analysis of Beclin1, p62, LC3; and ICC analysis of p62, LC3, Lamp1 were performed in ML-SA1 treated and untreated 5-month-old *WT* and *Hexa*^{-/-}*Neu3*^{-/-} mouse neuroglia and fibroblast cells.

Atg7 is the enzyme that is involved in the lipidation of cytosolic LC3-I to form autophagosome-bound LC3-II.^{73,75} According to RT-PCR results, *Atg7* expression did not

change clearly after ML-SA1 treatment in *WT* neuroglia and fibroblasts, and also in *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts (Figure 3.5 A and Figure 3.6 A). *Hexa*^{-/-}*Neu3*^{-/-} neuroglia showed non-significantly increased *Atg7* expression after ML-SA1 treatment (Figure 3.5 A). In untreated conditions, there were non-significant increase between *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblasts compared to *WT* neuroglia and fibroblasts respectively (Figure 3.5 A and Figure 3.6 A), although a previous study showed a significant increase in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia compared to *WT* neuroglia at the basal level.¹⁴¹ This can be explained by the fact that autophagy is a dynamic mechanism, and in vitro experiments can be affected by cell density, number of passages, etc. p62 is a receptor of selective autophagy that directs autophagic cargos to the autophagosome via interaction with the LC3-II.⁸⁰ Significantly increased *p62* expression was observed in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblasts than in *WT* neuroglia and fibroblasts, respectively, in untreated conditions (Figure 3.5 B and Figure 3.6 B) as shown and explained by a distorted termination of autophagy in previous studies conducted on several LSDs, including TSD.^{83,84,86,88,141} Additionally, ML-SA1 caused an increase in all cell types except *WT* neuroglia, but this was significant only in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia (Figure 3.5 B and Figure 3.6 B). In general, it was understood that ML-SA1 increased the already existing overexpression of *p62* in disease conditions. It has been previously reported that lithium treatment decreased *p62* expression in the same cell lines, indicating that autophagy flux was effectively progressing.¹⁴¹ This difference suggests that although ML-SA1 increased lysosomal gene expression via TFEB activation, it might not sufficiently support the completion of autophagy flux. Beclin1 is the critical member of the class III PI3K kinase complex responsible for autophagy initiation, especially in phagophore formation.⁷⁴ In *Beclin1* gene expression analysis, significantly increased expression was found in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblast cells compared to *WT* cells (Figure 3.5 C and Figure 3.6 C), although a previous study reported an increased *Beclin1* expression only in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia.¹⁴¹ This inconsistency might be attributed to differences in cellular stress level, passage number, or the severity of disease in the mice that were used to generate these cell lines. Moreover, compared to untreated conditions, a non-significant minor increase was detected in the ML-SA1 treated *WT* neuroglia and fibroblasts. ML-SA1 treatment caused a non-significant increase in *Beclin1* expression in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia, conversely, it decreased *Beclin1* expression significantly in *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts compared to untreated conditions (Figure 3.5 C and Figure 3.6 C) These results suggest the idea that in the disease condition cells try to induce autophagy as a

kind of compensatory mechanism. Also, the activation of TRPML1 may have triggered cell-type-specific feedback mechanisms in *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts, resulting in the decrease of *Beclin1* expression. Finally, as expected, untreated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblasts showed significantly increased *Lamp1* expression compared to untreated *WT* neuroglia and fibroblasts (Figure 3.5 D and Figure 3.6 D), as a consequence of TSD disease pathology (higher lysosome amount).³⁵ After ML-SA1 treatment, the expression level of *Lamp1* significantly increased in neuroglia and fibroblasts of both genotypes. (Figure 3.5 D and Figure 3.6 D) These results can be explained with the information that ML-SA1 mediated activation of the TRPML1 channel stimulates lysosomal biogenesis in cells via nuclear localization of TFEB.⁹¹

According to the Western Blot analysis of Beclin1 protein, in untreated conditions, a non-significant minor increase was shown in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia compared to *WT* neuroglia. Also, a non-significant decrease in *WT* neuroglia and a non-significant increase in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia after ML-SA1 treatment were observed (Figure 3.7 A and B). When these results are considered together with the RT-PCR results, the effect of ML-SA1 on *WT* neuroglia (increase in mRNA but decrease in protein level) may indicate post-translational regulations, which means the increase in mRNA level does not seem to be reflected in the protein level. The remaining neuroglia results appear to be consistent with each other. In fibroblasts, a non-significant decrease was observed in *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts compared to *WT* fibroblasts in untreated conditions. Beclin1 protein levels did not change in *WT* fibroblasts after ML-SA1 treatment. However, ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts showed a non-significant increase in Beclin1 levels, compared to untreated conditions (Figure 3.8 A and B). These results can be seen as inconsistent with the RT-PCR results, (for example, the significant decrease in mRNA level while an increase in protein level, as seen in ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts), however they might suggest the half-life of proteins or post-translational modifications and the effect of negative feedback mechanisms. The p62 protein level was non-significantly increased in untreated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia compared to untreated *WT* neuroglia, as consistent with the previous study.¹⁴¹ ML-SA1 treatment significantly increased the p62 protein level in both genotypes of neuroglia (Figure 3.7 C and D). Except for the ML-SA1 treated *WT* neuroglia, these Western Blot results are consistent with the RT-PCR results. When it comes to fibroblasts, the p62 protein level was non-significantly increased in *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts compared to *WT* fibroblasts in untreated conditions, in parallel with the already reported data.¹⁴¹ Also,

non-significantly increased p62 levels were detected in *WT* and *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts after ML-SA1 treatment (Figure 3.8 C and D). All Western Blot results of p62 performed in fibroblasts are consistent with the RT-PCR results. LC3 plays a role in autophagosome initiation and maturation (conversion of LC3-I to LC3-II by lipidation) during autophagy. Additionally, LC3-II bound to the mature autophagosome membrane is involved in lysosomal fusion. Therefore, simultaneous accumulation of LC3-II and p62 was considered as an impaired autophagic flux in different LSD studies, including TSD.^{83,84,86,88,141} Accordingly, the Western Blot results showed an increased LC3-II protein level in untreated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia (Figure 3.8 E and D) and fibroblasts (Figure 3.9 E and D) compared to untreated *WT* cells, which was significant only in neuroglia. These results are consistent with the previously published data and indicate impaired autophagic degradation in TSD mouse cells.¹⁴¹ ML-SA1 increased LC3-II protein level in both *WT* and *Hexa*^{-/-}*Neu3*^{-/-} neuroglia compared to untreated conditions, while this increase was significant only in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia (Figure 3.8 E and D). Similarly, ML-SA1 significantly increased the level of LC3-II protein in both genotypes of fibroblasts (Figure 3.9 E and D). ML-SA1-induced increase in LC3-II level indicates mature autophagosome accumulation, and ML-SA1 mediated LC3-II increment was also demonstrated on the NPC1 and MLIV cells previously.¹⁴⁰ However, additional p62 accumulation observed in this study suggests that this LC3-II accumulation is not due to the repaired autophagic flux but the block in the termination step.

In immunocytochemical (ICC) analyses, p62 intensity non-significantly increased in untreated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia (Figure 3.9 A and B) and fibroblasts (Figure 3.9 C and D) compared to untreated *WT* cells, consistent with the previous study.¹⁴¹ Also, with ML-SA1 treatment, p62 intensity increased in all genotypes and cells compared to untreated conditions. This increase was not significant only in the *WT* neuroglia (Figure 3.9). For LC3, *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblast cells showed an increased LC3 intensity compared to *WT* cells in untreated conditions, with the significant increase only in neuroglia cells (Figure 3.10). These results were also consistent with the data shown previously.¹⁴¹ According to these results, it can be said that ICC analyses results confirmed the Western Blot results for p62 and LC3 proteins. Measurement of LC3 and Lamp1 colocalization intensity is commonly used method to detect whether autophagolysosomes are accumulated or not during autophagy.¹⁴² In the colocalization analysis of LC3 and Lamp1, untreated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia and fibroblasts showed increased colocalization compared to untreated *WT* cells, with the significant increase

only in fibroblast cells (Figure 3.13 and Figure 3.14) suggesting the lysosomal dysfunction as already reported in previous study.¹⁴¹ There was no change in *WT* neuroglia and fibroblasts after ML-SA1 treatment compared to untreated conditions. However, in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia, colocalization intensity was significantly increased, while intensity was significantly decreased in *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts after ML-SA1 treatment. (Figure 3.13 and Figure 3.14). Overall, the results of colocalization analysis suggest the idea that the ML-SA1 increased autophagolysosomal formation, but post-fusion lysosomal degradation still failed to occur in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia. Lastly, in *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts, ML-SA1 might negatively affect autophagosome-lysosome fusion and cause a blockage in the termination of autophagy.

MicroRNAs (miRNAs) are single-stranded small (19-23 nts) non-coding RNAs serving a role in post-transcriptional gene expression regulation.⁹⁷ More than 2000 miRNAs are found in human cells and body fluids.⁹⁹ miRNA action on the target mRNAs is mostly the inhibition of translation.¹⁰³ They are involved in several processes like cell signaling and differentiation, apoptosis, metabolism, and tissue homeostasis. Due to that, their altered regulation was detected in cancer, rheumatoid arthritis, neurodegenerative diseases like Alzheimer's disease, LSDs, etc.^{102,109} Specifically, Niemann Pick Type C^{110,111}, Multiple Sulfatase Deficiency¹⁰⁹, Gaucher disease¹¹²⁻¹¹⁴, and Fabry disease¹¹⁵ were studied in terms of miRNA regulations. Also, small-scale miRNA analysis (containing only 12 miRNAs) was performed previously in the specific brain regions of the SD (*Hexb*^{-/-}) and TSD mouse model (*Hexa*^{-/-}). It was reported that miR-9, miR-29a, miR-33, miR-19a, miR-34a, miR-124, miR-126a, miR-128, and miR-137 were differentially expressed in both brain areas of these mouse models.¹¹⁶ Although a small-scale study was conducted on the *Hexa*^{-/-} mouse, a comprehensive miRNA profile of the *Hexa*^{-/-}*Neu3*^{-/-} mouse model that mimics the actual TSD disease severity is not available in the literature. Therefore, the primary aim of this thesis study was to evaluate the miRNAs and their biological roles in the TSD mouse model (*Hexa*^{-/-}*Neu3*^{-/-}) fibroblasts and neuroglia cells, in untreated and ML-SA1 treated conditions. To achieve this, firstly, ML-SA1 treated and untreated conditions were compared in each cell group. Additionally, by comparing *Hexa*^{-/-}*Neu3*^{-/-} and *WT* cells in both untreated and ML-SA1 treated conditions, significantly differentially expressed miRNAs were identified as both up and downregulated, and the biological pathways in which these miRNAs enriched were identified. At the end of these comparisons, common significantly up and downregulated miRNAs were identified for only ML-SA1 treatment condition, only

disease condition, and disease and ML-SA1 treatment conditions together. Finally, pathway enrichment analysis was performed on the target genes of these common miRNAs to reveal the relevant biological pathways.

In ML-SA1 treated *WT* fibroblasts, 42 miRNAs were significantly upregulated, and 14 miRNAs were significantly downregulated compared to untreated *WT* fibroblasts (Figure 3.15). According to pathway enrichment analyses, the most significant target pathways of downregulated miRNAs were ‘pathways of neurodegeneration’ for KEGG (Figure 3.16) and ‘neuronal system’ for Reactome (Figure 3.17). For the upregulated miRNAs, the most significant target pathways were ‘axon guidance’ for KEGG (Figure 3.16) and ‘signaling by receptor tyrosine kinases’ for Reactome (Figure 3.17). Since miRNAs generally reduce the translation of target mRNAs¹⁰³ it is thought that as a result of downregulation of miRNAs regulating these pathways, the relevant genes are less translationally repressed, leading to activation of these pathways. Conversely, by upregulation of miRNAs, their target pathways could potentially become less active. After ML-SA1 treatment, a downregulation of receptor tyrosine kinase signaling may cause reduced proliferative activity.^{143,144} Even though fibroblasts are not neuronal cells the enriched pathways of the neuronal system can be explained by the fact that some genes involved in these pathways generally are not specific to neurons but also include proteins expressed in other cell types and play a general role in cellular homeostasis (for ex., calcium channel subunits). Therefore, these results cannot be considered as unexpected. Calcium signaling was targeted by downregulated miRNAs, while SNARE interactions in the vesicular transport, which is a critical step for lysosomal fusion⁴⁹, was targeted by upregulated miRNAs (Figure 3.16). These opposing regulations (possible enhancement of calcium signaling and inhibited SNARE interactions) suggest that the *WT* fibroblasts might try to balance the excess triggering of lysosomal exocytosis via ML-SA1 treatment, to maintain cell physiology at the miRNA level. Lastly, increased MAPK activity causes an increase in the expression of proinflammatory cytokines.¹⁴⁵ Negative regulation of MAPK might be repressed by upregulated miRNAs (Figure 3.17), which can lead to increased inflammatory response in *WT* fibroblasts under the effect of ML-SA1.

In untreated *Hexa-/-Neu3-/-* fibroblasts, 32 miRNAs were significantly upregulated, and 34 miRNAs were significantly downregulated compared to untreated *WT* fibroblasts (Figure 3.18). According to their pathway enrichment analyses, the most significant targets of downregulated miRNAs were ‘Rap1 signaling pathway’ for KEGG

(Figure 3.19) and ‘neuronal system’ for Reactome (Figure 3.20). For the upregulated miRNAs, the most significant targets were ‘pathways of neurodegeneration’ for KEGG (Figure 3.19) and ‘L1CAM interactions’ for Reactome (Figure 3.20). Since these cells are fibroblasts, the acquisition of some pathways related to the CNS and neurodegeneration can be explained by cell-type-independent effects of miRNAs in addition to previous explanations. Therefore, cell type differences should be taken into consideration when commenting on the regulation of these pathways. Previously, the activation of Rap1 GTPase has been associated with the suppression of mTORC1 activation on the lysosome membrane, leading to activation of autophagy.¹⁴⁶ Therefore, it can be said that enhancement of the Rap1 activity in *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts might confirm the increased *Beclin1* expression in these cells (Figure 3.6 C). *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts may reduce the miRNA pressure on Rap1 to inhibit mTORC1 and induce autophagy as a compensatory mechanism. Another target of downregulated miRNAs was PIK3-Akt signaling. Upregulation of this pathway leads to repression of autophagy by activating mTORC1¹⁴⁷, which seems to contradict the previous statement. However, autophagy inhibition may become neutralized by upregulation of genes that play a role in negative regulation of PI3K-Akt (Figure 3.20) and downregulation of the mTOR activity (Figure 3.19). Finally, the genes involved in the reduction of cytosolic calcium levels were targeted by upregulated miRNAs (Figure 3.20), which suggests that *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts might develop an adaptive response to maintain cytosolic calcium homeostasis required for lysosomal exocytosis and autophagy induction¹⁴⁶ to offset the effects of lysosomal accumulation.

In ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts, 32 miRNAs were significantly upregulated, and 21 miRNAs were downregulated compared to untreated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts (Figure 3.21). Their pathway enrichment analyses showed that the most significant targets of downregulated miRNAs were ‘axon guidance’ for KEGG (Figure 3.22) and ‘neuronal system’ for Reactome (Figure 3.22). For the upregulated miRNAs, the most significant targets were ‘regulation of actin cytoskeleton’ for KEGG (Figure 3.22) and ‘RHO GTPase cycle’ for Reactome (Figure 3.23). Also, rab regulation of trafficking was the target of upregulated miRNAs (Figure 3.23). It is known that lysosomes perform their short-distance and near-plasma membrane movements via the actin skeleton⁴⁷, and RHO GTPase family member RhoA is involved in actin polymerization¹⁴⁸. Additionally, Rab GTPases (Rab3a, Rab10, Rab27, and Rab11) were reported as critical players of lysosomal exocytosis.^{52–54} Therefore, these results seem

surprising at first, but might suggest the fact that in the disease state, cells were already prone to exocytosis due to excess GM2 accumulation, (as confirmed by surface Lamp1 staining results, Figure 3.11 C-D) and the further induction of vesicular trafficking by ML-SA1, may have directed the *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts to a compensatory regulation at the post-transcriptional level.

In ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts, 61 miRNAs were significantly upregulated, and 41 miRNAs were significantly downregulated compared to ML-SA1 treated *WT* fibroblasts (Figure 3.24). According to their pathway enrichment analyses, the most significant targets of downregulated miRNAs were ‘FoxO signaling pathway’ for KEGG (Figure 3.25) and ‘MAPK family signaling cascades’ for Reactome (Figure 3.26). For the upregulated miRNAs, the most significant targets were ‘Hippo signaling pathway’ for KEGG (Figure 3.25) and ‘signaling by Wnt’ for Reactome (Figure 3.26). Cell cycle control, oxidative stress response, and apoptosis are regulated by FoxO signaling.¹⁴⁷ Targeting the FoxO and Wnt signaling pathways by both up and downregulated miRNAs (Figure 3.25 and 3.26) indicates that they are reprogrammed in a very dynamic and complex way. Therefore, it is hard to make a comment on their repression or activation states. As seen in the *WT* fibroblasts (Figure 3.17), ML-SA1 treatment might cause increased MAPK activity in *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts, leading to unwanted inflammatory response.¹⁴⁵ Hippo signaling is a crucial pathway for cell proliferation.¹⁴⁹ Consequently, the proliferation capacity of *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts may decrease due to the suppression of the Hippo signaling pathway after ML-SA1 treatment. However, considering the data that the Wnt pathway was suppressed by upregulated miRNAs in the absence of ML-SA1 effect (Figure 3.19), it can be said that ML-SA1 treatment reshapes this suppressive effect, and downregulated miRNAs also participate in pathway regulation. This may reflect the cell's adaptive response to treatment and its effort to reestablish homeostatic balance. Finally, enhanced lysosome biogenesis (Figure 3.26) can be explained by the nuclear translocation of TFEB^{47,55,56} after ML-SA1 treatment, as expected.

In ML-SA1 treated *WT* neuroglia, 38 miRNAs were significantly upregulated, and 17 miRNAs were significantly downregulated compared to untreated *WT* neuroglia (Figure 3.27). According to pathway enrichment analyses, the most significant target pathways of downregulated miRNAs were ‘PI3K-Akt signaling pathway’ for KEGG (Figure 3.28) and ‘sphingolipid de novo biosynthesis’ for Reactome (Figure 3.29). For the upregulated miRNAs, the most significant target pathways were ‘Hippo signaling

pathway' for KEGG (Figure 3.28) and 'signaling by receptor tyrosine kinases' for Reactome (Figure 3.29). Firstly, ML-SA1-induced cytosolic calcium elevation might relieve miRNA-mediated repression of sphingolipid biosynthesis enzymes like sphingomyelin synthases (SMS)¹⁵⁰, thereby enhancing sphingolipid biosynthesis in *WT* neuroglia. Therefore, ML-SA1 usage may halt membrane lipid homeostasis in healthy neuroglia cells and affect the autophagy-apoptosis crosstalk due to increased sphingomyelin/ceramide synthesis.¹⁵¹ This result suggests that calcium-sensitive post-transcriptional regulation can modulate lipid metabolism under lysosomal signaling conditions in a healthy state. On the other hand, autophagy might be repressed due to upregulation of PI3K-Akt signals¹⁴⁷, which can disrupt cellular physiology in *WT* neuroglia. However, miRNA-mediated repression of mTORC1 signaling (Figure 3.29) suggests the prevented inhibitory effect of PI3K-Akt signal on autophagy induction. For upregulated miRNAs, it can be said that ML-SA1 treatment might cause reduced cell proliferation in *WT* neuroglia by targeting the Hippo and receptor tyrosine kinase signaling pathways^{143,149}, in parallel with the ML-SA1 treated *WT* fibroblasts (Figure 3.17).

In untreated *Hexa-/-Neu3-/-* neuroglia, 100 miRNAs were significantly upregulated, and 90 miRNAs were significantly downregulated compared to untreated *WT* neuroglia (Figure 3.30). According to pathway enrichment analyses, the most significant target pathways of downregulated miRNAs were 'MAPK signaling pathway' for KEGG (Figure 3.31) and 'signaling by receptor tyrosine kinases' for Reactome (Figure 3.32). For the upregulated miRNAs, the most significant target pathways were 'MAPK signaling pathway' for KEGG (Figure 3.31) and 'neuronal system' for Reactome (Figure 3.32). Surprisingly, in this comparison, 75% of target pathways underwent bidirectional regulation as seen in the MAPK signaling pathway. Therefore, it is not possible to make a unidirectional conclusion regarding whether the activity of MAPK is stimulated or repressed in the context of miRNA regulation. Also, this situation is correct for the other 2 most significant signaling pathways listed above. From this perspective, focusing on the unidirectionally regulated pathways of KEGG and Reactome is safe to proceed. Accordingly, SNARE interactions in vesicular transport and lysosome pathway-related genes were targeted by only upregulated miRNAs (Figure 3.31) that might suggest a regulatory modulation of vesicular trafficking pathways between the Golgi, endosomes, lysosomes, and plasma membrane.^{79,152} Given their roles in membrane fusion and vesicle transport¹⁵², the downregulation of SNARE proteins might exacerbate lysosomal

dysfunction. In detail, transport of acid hydrolases (like cathepsin) from Golgi to lysosomes¹⁵² might be negatively affected directly or indirectly (retrograde transport of M6P receptors for recycling)¹⁵³, and this may be the cause of accumulated p62 in autophagolysosomes in *Hexa*^{-/-}*Neu*^{-/-} neuroglia as previously reported¹⁴¹ and confirmed by this study. Additionally, phosphatidylinositol (PI) signaling /metabolism was targeted by upregulated miRNAs only in KEGG (Figure 3.31) and Reactome (Figure 3.32). PIs are the critical regulators of cytoskeleton reorganizations, cell signaling, and membrane/vesicle trafficking.¹⁵⁴ Therefore, downregulation of PI metabolism and signaling is thought to be another mechanism supporting the distorted vesicular trafficking idea in the disease state.

In ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia, 16 miRNAs were significantly upregulated, and 22 miRNAs were significantly downregulated compared to untreated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia (Figure 3.33). According to pathway enrichment analyses, the most significant target pathways of downregulated miRNAs were ‘apelin signaling pathway’ for KEGG (Figure 3.34) and ‘apoptosis’ for Reactome (Figure 3.35). For the upregulated miRNAs, the most significant target pathways were ‘glycosphingolipid biosynthesis -ganglio series’ for KEGG (Figure 3.34) and ‘regulation of Bach1 activity’ for Reactome (Figure 3.35). Upregulation of the apelin signaling pathway and apoptosis, indicating bidirectional cellular effects of the ML-SA1 treatment. Activation of the apelin signaling can be considered as a compensatory mechanism. Indeed, the Apelin/Apj signaling has been previously identified with its effects supporting survival signals in neuronal cells.¹⁵⁵ On the other hand, activation of the apoptosis pathway suggests that ML-SA1 may trigger cellular stress responses by stimulating lysosomal Ca²⁺ release, thereby increasing lysosomal membrane permeability (LMP). Increased LMP may lead to the escape of lysosomal proteases (like cathepsin B and D) into the cytosol and the initiation of an apoptotic response.¹⁵⁶ Previously, it was reported that excessive sphingomyelin mediated LMP was the main reason for autophagolysosome accumulation observed in Niemann Pick disease type A (NPA).¹⁵⁷ In light of this information, ML-SA1 treatment may also lead to cell death response due to excessive Ca²⁺ signaling and lysosomal stress in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia. Moreover, these suggestions are highly confirmed by the observed exacerbating effect of ML-SA1 in autophagolysosomal accumulation in neuroglia cells (e.g., Figure 3.9 and Figure 3.13). The targeting of genes involved in the ganglioside biosynthesis pathway by upregulated miRNAs indicates that this biosynthetic pathway is being suppressed. This may indicate that the cell is trying to

slow down biosynthesis to reduce the accumulation of toxic GM2 ganglioside, which is the main pathology of TSD.¹⁷ These findings highlight the possible therapeutic effect of ML-SA1 in *Hexa*^{-/-}*Neu3*^{-/-} neuroglia. Bach1 is a transcription factor that suppresses the antioxidant response under normal conditions. The increase in miRNAs targeting BACH1 regulation after ML-SA1 treatment indicates that this suppressive effect might be eliminated or triggered according to the identity of the targeted regulator. For example, in the context of Fbxl17, which triggers the degradation of Bach1 via ubiquitination¹⁵⁸, it can be suggested that ML-SA1 may not always be protective; it may further increase the previously reported GM2 accumulation-mediated oxidative load in TSD¹⁵⁹.

In ML-SA1 treated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia, 54 miRNAs were significantly upregulated, and 127 miRNAs were significantly downregulated compared to ML-SA1 treated *WT* neuroglia (Figure 3.36). According to pathway enrichment analyses, the most significant target pathways of downregulated miRNAs were ‘PI3K-Akt signaling pathway’ for KEGG (Figure 3.37) and ‘signaling by receptor tyrosine kinases’ for Reactome (Figure 3.38). For the upregulated miRNAs, the most significant target pathways were ‘PI3K-Akt signaling pathway’ for KEGG (Figure 3.37) and ‘MAPK family signaling cascades’ for Reactome (Figure 3.38). However, these 3 pathways were under bidirectional miRNA regulation. On the other hand, while SNARE interactions in vesicular transport were targeted only by upregulated miRNAs, the same result was seen in the comparison made in untreated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia against untreated *WT* neuroglia (Figure 3.31). Therefore, it can be said that ML-SA1 did not cause a post-transcriptional regulatory effect on SNARE interactions in neuroglia, and this result was gained due to disease pathology. Additionally, glycosphingolipid biosynthesis -ganglio series was the target of downregulated miRNAs only, in comparison made in untreated *Hexa*^{-/-}*Neu3*^{-/-} neuroglia against untreated *WT* neuroglia (Figure 3.31) same with ML-SA1 treatment condition. Although ML-SA1 treatment could activate lysosomal exocytosis (Figure 3.11) the consistent targeting of the ganglio-series sphingolipid biosynthesis pathway by downregulated miRNAs across both untreated and ML-SA1-treated conditions of neuroglia cells in the context of disease suggests that TRPML1 activation does not significantly modulate this pathway at the miRNA level. This may indicate that ML-SA1 does not influence GM2 synthesis via miRNA-mediated regulation.

mmu-miR-672-5p and mmu-miR-5624-3p were significantly upregulated, and mmu-miR-6940-3p was significantly downregulated in untreated fibroblasts and

neuroglia of *Hexa*^{-/-}*Neu3*^{-/-} mice compared to their *WT* controls. mmu-miR-672-5p and mmu-miR-375-3p were significantly upregulated while mmu-miR-7091-5p (log₂FC = -4,1) was significantly downregulated in ML-SA1 treated fibroblasts and neuroglia of *Hexa*^{-/-}*Neu3*^{-/-} mice compared to ML-SA1 treated *WT* cells. According to these results, it can be said that ML-SA1 did not affect the regulation of mmu-miR-672-5p. However, it resulted in an increased expression of mmu-miR-375-3p in both cell types (Table 3.1).

Apoptosis induced DNA fragmentation, apoptotic execution phase, EPHA-mediated growth cone collapse, EPH-ephrin mediated repulsion of cells, EPH-Ephrin signaling, and cellular response to hypoxia were found as target pathways of mmu-miR-672-5p (Figure 3.39). Previously, it was reported that neurons underwent apoptosis due to toxic ganglioside accumulation in GM2 gangliosidosis.¹⁶⁰ Upregulation of mmu-miR-672-5p, which represses DNA fragmentation, may be a response mechanism against increased oxidative stress in neuroglia and fibroblast cells, and excessive apoptotic cell death in the *Hexa*^{-/-}*Neu3*^{-/-} mouse brain, as shown in a previous study.¹⁵⁹ Although this increase tries to suppress apoptosis, it is insufficient, or other pathological mechanisms are dominant than this miRNA regulation. On the other hand, hypoxia was not defined in *Hexa*^{-/-}*Neu3*^{-/-} mice; however, from these results, it can be understood that cells modulated hypoxia responses, which might need to be investigated. Ephrin-EPH (receptor tyrosine kinase) signaling regulates cell migration, adhesion, differentiation and matrix remodeling, synaptic plasticity (e.g., growth cone collapse during axon guidance¹⁶¹) processes.¹⁶² Neurodegeneration was previously reported in the *Hexa*^{-/-}*Neu3*^{-/-} mice.^{35,163} Therefore, downregulation of this signaling pathway may contribute to the course of the disease by causing disruption of neuronal connections, loss of synaptic function, and delay in tissue repair. On the other hand, suppression of receptor tyrosine kinase signaling, neuronal system and Rho GTPase cycle by mmu-miR-5624-3p can impair the cellular events like cell growth, proliferation and survival^{143,144}; synaptic function, neuronal communication, and plasticity¹⁶⁴; cytoskeletal dynamics, axon growth cone modulation, and cell migration^{148,165} in order, which possibly accelerate the disease progression in *Hexa*^{-/-}*Neu3*^{-/-} fibroblast and neuroglia cells. After ML-SA1 treatment, the synthesis of PE, interleukin-4 and interleukin-13 signaling, and erythropoietin activates phosphoinositide-3-kinase (PI3K) pathways were targeted by upregulated mmu-miR-375-3p. In a previous study, mmu-miR-375-3p has been found to be associated with reduced proliferation of rat pulmonary microvascular endothelial cells under hypoxic conditions.¹⁶⁶ Also increased level of this miRNA in mouse serum was reported under the

toxic effect of streptozotocin and radiation in different studies.^{167,168} Although PE production might be suppressed by mmu-miR-375-3p, there may still be basal expression of PE in the cell, which explains the increased PE-conjugated LC3 (LC3-II)^{73,75} levels after ML-SA1 treatment (Figure 3.7 E and Figure 3.8 E). Moreover, suppression of anti-inflammatory cytokine signals such as interleukin-4 and interleukin-13¹⁶⁹ suggests that ML-SA1 may further trigger the already existing neuroinflammation in the disease state.¹⁶⁴ In addition, decreased PI3K activation may explain why ML-SA1 induces autophagy better through the inability of PI3K to activate the Akt/mTORC1.¹⁴⁷ Finally, mmu-miR-7091-5p was found to target and possibly downregulate the *Epas1* (Endothelial PAS domain protein 1) gene (Table 3.2). This gene encodes the hypoxia-inducible factor 2 alpha protein, which triggers the expression of hypoxia-response genes that increase vascularization and limit intracellular oxygen consumption under hypoxic conditions.¹⁷⁰ In this context, it can be considered that ML-SA1 tries to reduce hypoxia that may exist under normal conditions. These results reinforce the need to investigate the hypoxia state of *Hexa*^{-/-}*Neu3*^{-/-} mouse cells.

CHAPTER 5

CONCLUSION

Tay-Sachs disease (TSD) is a type of lysosomal storage disorder (LSD) that arises due to a mutated *HEXA* gene and subsequent deficiency of β -N-acetylhexosaminidase A (HEXA) enzyme, causing an abnormal GM2 accumulation in lysosomal compartments of cells. This accumulation causes progressive neurodegeneration, declined motor skills, and behavioral abnormalities in TSD patients. *Hexa*^{-/-}*Neu3*^{-/-} mouse was generated as an animal model of TSD, and lysosomal enlargement and impaired autophagic flux were demonstrated in these mice in addition to other pathological symptoms. ML-SA1 is an agonist of lysosome and endosome-specific calcium channel (TRPML1) and known as an inducer of autophagy and lysosomal exocytosis processes according to previous studies conducted on neurodegenerative diseases, including some LSDs, but not TSD. MicroRNAs (miRNAs) are small non-coding RNAs playing critical roles in post-transcriptional gene expression regulation by degrading mRNAs or inhibiting translation. Their expression profile was studied in several diseases recently; however, there is no study about whole miRNA profiling for the TSD mouse model.

In this thesis study, it was aimed to investigate in which direction ML-SA1 would affect autophagy and lysosomal exocytosis pathways in *Hexa*^{-/-}*Neu3*^{-/-} mouse fibroblasts and neuroglia cells. Additionally, the primary aim was to determine miRNAs that may play a role in the pathology of the TSD and to examine the effect of ML-SA1 on miRNA regulation by performing comprehensive miRNA profiling of *WT* and *Hexa*^{-/-}*Neu3*^{-/-} mouse fibroblasts and neuroglia cells in untreated and ML-SA1 treated conditions. Results showed that ML-SA1 successfully increased lysosomal exocytosis rate in *WT* and *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts and neuroglia, as evidenced by increased extracellular enzyme activities and surface Lamp1 intensities. On the other hand, although autophagy induction was achieved by ML-SA1, the accumulation of autophagosome markers in both genotypes and cell types indicated that autophagic clearance was not achieved even in the presence of ML-SA1. Finally, miRNA profiling stated that several miRNAs are dysregulated in disease and treatment conditions. These differentially expressed miRNAs were found to play a critical role in modulating important cellular pathways including sphingolipid biosynthesis, oxidative stress responses, vesicular trafficking, and mTOR

activity. In untreated *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts and neuroglia, mmu-miR-672-5p and mmu-miR-5624-3p were significantly upregulated, whereas mmu-miR-6940-3p was significantly downregulated in comparison with *WT* controls. Upon ML-SA1 treatment, a distinct expression pattern was observed; mmu-miR-672-5p remained unaffected, whereas mmu-miR-375-3p was significantly upregulated in both *Hexa*^{-/-}*Neu3*^{-/-} fibroblasts and neuroglia. In contrast, mmu-miR-7091-5p was markedly downregulated in these cells compared to ML-SA1-treated *WT* counterparts. These observations suggested that lysosomal calcium modulation can differentiate miRNA regulation in the disease state. Overall, these findings reveal a complex miRNA-mediated post-transcriptional regulatory network, with both genotype- and treatment-specific effects, contributing to the altered molecular mechanisms observed in TSD mouse cells. To conclude, this study provides important information to the literature for the development of miRNA-based therapy methods for TSD patients. However, since this study does not include mechanistic experiments for miRNA expression regulation, further studies are required.

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