

**THERAPEUTIC EFFICACY OF INTRATHECAL
ADMINISTRATION OF AAVRH10-HNEU3
VECTOR IN A MOUSE MODEL OF TAY-SACHS
DISEASE**

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ABSTRACT

THERAPEUTIC EFFICACY OF INTRATHECAL ADMINISTRATION OF AAVRH10-HNEU3 VECTOR IN A MOUSE MODEL OF TAY-SACHS DISEASE

Tay-Sachs disease is a neurodegenerative disorder that results from the mutated *HEXA* gene. This gene encodes the α -subunit of the β -hexosaminidase A (HexA) enzyme, which catalyzes the degradation of GM2 ganglioside. HexA deficiency causes the accumulation of GM2 and progressive neurodegeneration in Tay-Sachs patients. Interestingly, a mild disease manifestation in *Hexa*^{-/-} mice limits the occurrence of neuropathological aspects observed in patients. The early-onset Tay-Sachs disease mouse model, *Hexa*^{-/-}*Neu3*^{-/-}, shows a shortened lifespan of 20 weeks, together with neuropathological and behavioral impairments resembling Tay-Sachs patients. In mice, sialidase Neu3 has been shown to facilitate GM2 degradation through a "bypass" pathway, while the potential role of human Neu3 (hNeu3) in this context remains unexplored. Moreover, in this study, an anti-inflammatory drug, Istradefylline, was evaluated to reveal its potential to alleviate neuroinflammation in *Hexa*^{-/-}*Neu3*^{-/-} mice. To elucidate the therapeutic efficacy of hNeu3 alongside Istradefylline using molecular biological, immunohistochemical, and behavioral analyses, AAVrh10-hNeu3 was intrathecally administered to 8-week-old *Hexa*^{-/-}*Neu3*^{-/-} mice. Consequently, we found that hNeu3 and Istradefylline extended the lifespan to 28 weeks, resulting in detectable improvements in the body weight of *Hexa*^{-/-}*Neu3*^{-/-} mice. We observed reduced GM2 accumulation, lysosomal LAMP1, and TUNEL-positive cells and elevated levels of CNPase in 20-week-old mice given AAV alone or with Istradefylline. Additionally, rotarod and footprint analyses indicated enhancement in treated mice at 20 weeks. Our results demonstrated the first *in vivo* evidence for the therapeutic potential of human Neu3 gene delivery and Istradefylline to mitigate disease progression and ameliorate neuropathological and motor deficits shown in *Hexa*^{-/-}*Neu3*^{-/-} mice.

ÖZET

TAY-SACHS HASTALIĞI FARE MODELİNDE AAVRH10-HNEU3 VEKTÖRÜNÜN INTRATEKAL UYGULAMASININ TERAPÖTİK ETKİSİ

Tay-Sachs hastalığı, *HEXA* genindeki mutasyonlardan sonucu olan nörodejeneratif bir bozukluktur. Bu gen, GM2 gangliositinin yıkımını katalize eden β -hekzosaminidaz A (HexA) enziminin α -alt birimini kodlamaktadır. HexA eksikliği, Tay-Sachs hastalarında GM2'nin birikmesine ve ilerleyici nörodejenerasyona sebep olmaktadır. İlginç bir şekilde, *Hexa*^{-/-} farelerinde, hastalığın hafif düzeyde seyretmesi hastalarda gözlemlenen nöropatolojik bulguların ortaya çıkmasını sınırlamaktadır. Erken başlangıçlı Tay-Sachs hastalığı fare modeli, *Hexa*^{-/-}*Neu3*^{-/-}, Tay-Sachs hastalarına benzeyen nöropatolojik ve davranışsal bozukluklarla birlikte 20 haftalık kısa bir yaşam süresi göstermektedir. Farelerde, sialidaz Neu3 enziminin GM2'nin yıkımı bir "bypass" yolağı ile kolaylaştırdığı gösterilmişken, insan Neu3'ün (hNeu3) bu bağlamdaki potansiyel rolü henüz keşfedilmemiştir. Ayrıca, bu çalışmada, anti-inflamatuar bir ilaç olan İstradefilinin *Hexa*^{-/-}*Neu3*^{-/-} farelerinde nöroinflamasyonu azaltma potansiyeli incelenmiştir. İstradefilin ile birlikte hNeu3'ün terapötik etkinliğini moleküler biyolojik, immunohistokimyasal ve davranışsal analizler kullanarak açıklamak amacıyla AAVrh10-hNeu3, 8 haftalık *Hexa*^{-/-}*Neu3*^{-/-} farelerine intratekal olarak uygulanmıştır. Bunun neticesinde, *Hexa*^{-/-}*Neu3*^{-/-} farelerinde hNeu3 ve İstradefilinin yaşam süresini 28 haftaya kadar uzattığı ve farelerin vücut ağırlığında tespit edilebilir iyileşmelere yol açtığını bulduk. Özellikle sadece AAV veya İstradefilin ile birlikte AAV verilen 20 haftalık farelerde, GM2 birikiminde, lizozomal LAMP1 ve TUNEL pozitif hücrelerde azalma ve CNPase seviyelerinde artış gözlemlendik. Bunun yanı sıra, Rotarod ve ayak izi analizleri, tedavi edilen farelerde 20.haftada iyileşme olduğunu göstermektedir. Bulgularımız, insan Neu3 gen iletimi ve İstradefilinin, *Hexa*^{-/-}*Neu3*^{-/-} farelerinde görülen hastalık ilerlemesini hafifletme ve nöropatolojik ve motor eksikliklerini iyileştirme konusundaki terapötik potansiyeline ilişkin ilk *in vivo* kanıtı ortaya koymaktadır.



Dedicated to my precious family...

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CHAPTER 1

INTRODUCTION

1.1. Gangliosides

Gangliosides, a type of glycosphingolipid, include a ceramide tail linked to a glycan headgroup containing one or more sialic acid residues through a glycosidic linkage. They are abundantly located on the plasma membrane, where they play important roles in cell signaling and cell-to-cell interactions.^{1,2} Gangliosides are generated initially in the Endoplasmic Reticulum, then the Golgi apparatus, from where they are directed to the plasma membrane.³ They have heterogeneity in the sugar composition found on the glycan head, resulting in more than 200 ganglioside compositions. Moreover, ceramide residues can be diverse, such as long-chain sphingosine and fatty acyl chains with distinct lengths, giving rise to enhanced molecular diversity.⁴

Kolter⁵ showed a ganglioside biosynthesis pathway that started from the synthesis of Lactosyl Ceramide (LacCer). After LacCer production, simple gangliosides GM3, GD3, and GT3 were produced. The complex gangliosides were then generated through glycosyltransferases as 0-, a-, b-, and c-series gangliosides. This ganglioside biosynthesis pathway is shown in Figure 1.1.^{5,6}

Ganglioside composition and even their total mass vary among tissues and different cell types. Gangliosides are 10- to 30-fold higher in the human brain than in other tissues.⁷ Simple gangliosides, such as GM3, are mostly localized in peripheral tissues.⁸ However, more than 90% of the brain gangliosides are four complex gangliosides, GM1, GD1a, GD1b, and GT1b.⁹ According to previous studies, the abundances of gangliosides are ordered as myelin, followed by neuronal cells. Myelin is constituted of high amounts of GM4 and GM1, whereas adult oligodendrocytes contain GM1 and GD3.^{10,11}

Gangliosides are required for both the maturation and maintenance of the central nervous system (CNS). Thus, any mutations causing a loss in the function of genes encoding enzymes for ganglioside biosynthesis leads to severe neurodegenerative and neurodevelopmental disorders in humans, as well as in mouse models.^{12–14}

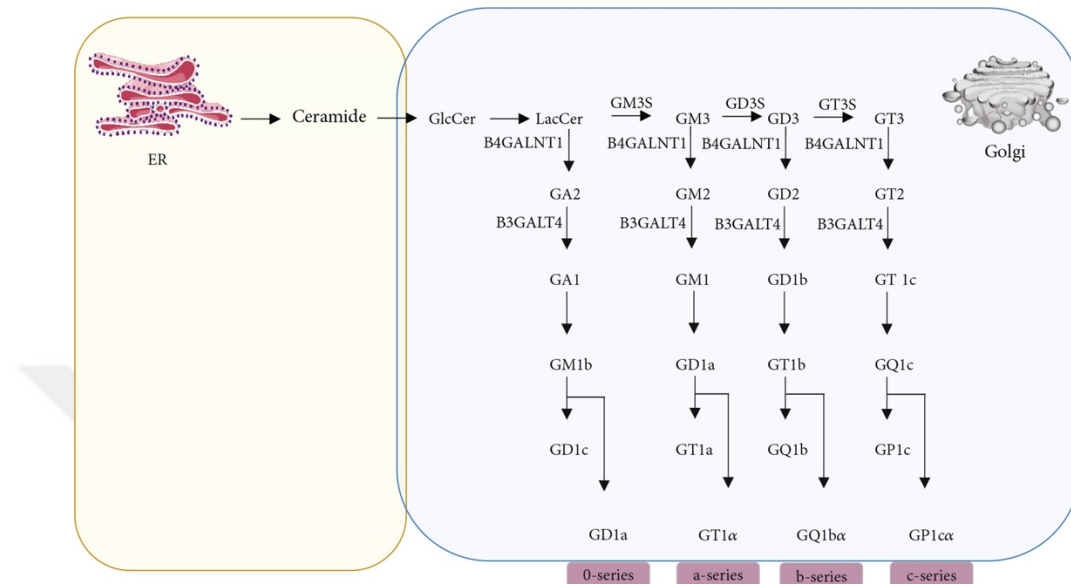


Figure 1.1. Illustration of the ganglioside biosynthetic pathways by the courtesy of İnci et al.⁶ Svennerholm's nomenclature is used for ganglioside identification.

1.2. Lysosomal Storage Disorders

Lysosomal storage diseases (LSDs) are heritable monogenic metabolic disorders due to lysosomal dysfunction. Approximately 70 LSDs that are identified with impaired lysosomal catabolism are inherited in an autosomal recessive manner, except for three LSDs that are inherited in an X-linked manner. The dysfunctionality in lysosomes results from the mutations in genes expressing several lysosomal proteins, such as proteases, glycosidases, transporters, or enzyme activators. These disruptions cause progressive accumulation of substrates within the lysosomes, leading to cellular dysfunction and death.¹⁵ In most LSDs, CNS involvement is prevalent along with neurodegeneration across multiple brain regions, except for a few LSDs without involvement of the CNS.¹⁶ The other common pathology in LSDs is the activation of the innate immune system in patients' brains.¹⁷ Astrogliosis, which is a form of neuroinflammation characterized by astrocyte activation, is also a hallmark of LSDs. This process results in neuronal damage

due to inflammation-directed glial scarring.^{17,18} The accumulation of different molecules is identified for different LSDs, including glycogen in Pompe disease, glycosaminoglycans in mucopolysaccharidoses (MPC), glycosylceramide in Gaucher's disease (GD), GM2 ganglioside in GM2 gangliosidoses, and cholesterol in Niemann-Pick disease (NPD) due to dysfunctionality in the lysosomal enzymes and catabolism.¹⁹

1.3. GM2 Gangliosidoses

Gangliosides, a type of glycosphingolipid, are mainly located in the neuronal cell membranes to perform their essential roles in the central nervous system (CNS).²⁰ Approximately 5% of all brain gangliosides are accounted for by GM2 gangliosides.²¹ Under normal conditions, GM2 ganglioside is degraded by β -hexosaminidases (Hex, EC 3.2.1.52) in lysosomes. Three forms of Hex enzymes, HexA ($\alpha\beta$), HexB ($\beta\beta$), and HexS ($\alpha\alpha$), hydrolyze N-acetylgalactosamine residues from GM2 ganglioside for its catalysis (Figure 1.2).²² The Hex enzymes are formed by the dimerization of α and β subunits. In addition, GM2 ganglioside degradation by the α subunit of HexA is facilitated by the GM2 activator protein (GM2AP).²⁰

Any mutation in the genes encoding α (HEXA), β (HEXB), or GM2-AP (GM2A) subunits results in the dysfunctional Hex enzymes and the accumulation of GM2 gangliosides in lysosomes. The accumulation of the GM2 gangliosides in lysosomes causes GM2 gangliosidoses, Tay-Sachs disease (TSD, OMIM #272800), Sandhoff disease (SD, OMIM #268800), and GM2-activator protein deficiency (AB variant; OMIM #272750), all of which are categorized under LSDs.²³ The severe build-up of GM2 gangliosides leads to cytotoxicity, especially in nerve cells, eventually causing neurodegeneration.²⁴ GM2 gangliosides function as a precursor for the production or degradation of complex brain gangliosides, especially GM1a, GD1a, GD1b, and GT1b, which are also abundantly located in neuronal cell membranes.²⁵ Those gangliosides are required for the axon-myelin interactions, transmembrane signaling pathways, and calcium homeostasis in the cell membranes of neurons.^{21,26} Thus, the central nervous system (CNS) is the primarily affected organ due to excessively accumulating GM2 ganglioside.²⁷

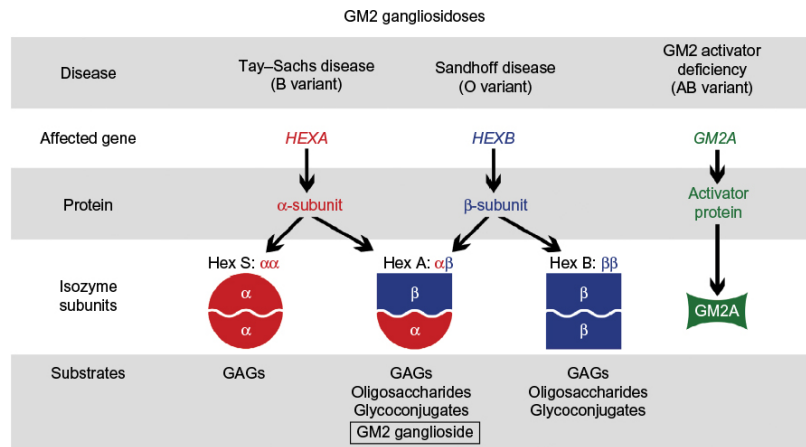


Figure 1.2. Three forms of Hex enzymes, HexA ($\alpha\beta$), HexB ($\beta\beta$), and HexS ($\alpha\alpha$), their substrates, and related GM2 gangliosidosis, by courtesy of Lawson and Martin.²² GAGs, N-acetylgalactosamine; HexA, hexosaminidase A; HexB, hexosaminidase B; HexS, hexosaminidase S

In patients with GM2 gangliosidosis, progressive neuronal loss and neurological abnormalities, such as hypotonia, seizures, motor deficits, and progressive weakness in muscles, are characterized.²⁸ Patients with SD, unlike patients with TSD, develop systemic manifestations like organomegaly.²⁹ The diagnosis of GM2 gangliosidosis requires an initial clinical assessment, after which enzyme activity of Hex enzymes and genetic mutation analysis are performed.^{30,31}

The use of animal models, including mice, sheep, and cats, replicating key biochemical and physiological aspects of GM2 gangliosidosis, is essential for understanding the pathophysiology of the diseases and developing effective therapeutic strategies.²² Seyrantepe et al.³² recently generated a TSD mouse model with the combined deficiency of Hexa and Neu3 genes (*Hexa*^{-/-}*Neu3*^{-/-}) that closely mimics the neuropathological aspects of early-onset Tay-Sachs disease. Hence, using the approved mouse model of TSD offers a valuable tool for developing efficient therapeutic strategies.³²

To date, several treatment approaches have been demonstrated for GM2 gangliosidosis, such as enzyme replacement therapy, pharmacological chaperones, substrate reduction therapy, hematopoietic stem cell transplantation, and gene therapy; however, there is currently no approved treatment strategy for the GM2 gangliosidosis. The most challenging problem in treating these diseases is the blood-brain barrier (BBB) that restricts the penetrance of intravenously administered drugs into the CNS.³³ Using

chimeric recombinant enzymes, direct brain administrations, and targeted delivery systems for a therapeutic protein aims to overcome the BBB.³⁴

1.4. Tay-Sachs Disease

Tay-Sachs disease (TSD) emerged because of a mutation in the *HEXA* gene encoding β -hexosaminidases A, which is an enzyme responsible for the catabolism of GM2 ganglioside. In the enzyme deficiency, the catabolism of GM2 ganglioside deteriorates, and the GM2 ganglioside accumulates in the brain and spinal cord.³⁵ The progressive build-up of GM2 ganglioside results in increased neuron size and destruction of nerve cells in the brain and spinal cord. Patients with TSD develop muscle weakness, seizures, and motor deficits due to cellular disruption in the central and peripheral nervous systems. The disease is categorized under lysosomal storage disorders since the accumulation of GM2 ganglioside due to an impaired enzyme occurs in lysosomes.³²

The clinical representation of TSD depends on the enzyme activity in the affected individuals. In the infantile form of TSD, a cherry-red spot on the retina, seizures, dysphagia, muscle weakness, motor dysfunction, and eventually, death at 4 years of age are characterized in the patients.³² In patients with the juvenile form of TSD, less than 1% of β -hexosaminidases A activity is detected, and the disease progression is slower than that of infantile TSD. Symptoms of juvenile TSD include a gradual loss of motor and cognitive abilities, seizures, loss of movement, and death, which generally occur due to infections.³⁶ The extremely rare form of TSD, the late-onset/adult form, appears between 20 and 30 years of age, but some individuals live up to 60-80 years of age. This form is associated with schizophrenia-like symptoms, and less than 10% of β -hexosaminidases A activity is measured. Clinical aspects observed in patients are tremors, muscle spasms, and neurological impairments.³⁷

1.4.1. Tay-Sachs Disease Mouse Model

Two different studies, it was aimed to generate mouse models of GM2 gangliosidoses by disrupting *Hexa* and *Hexb* genes, but the outcomes of the studies were unexpected.^{38,39} Whereas disruption of the *Hexb* gene resulted in the severe

neuropathological aspects observed in SD patients, *Hexa* knock-out mice showed almost normal phenotype with the absence of the neurological impairments of patients with the early-onset TSD.^{40,41} This discrepancy was attributed to the activities of mouse sialidases, by which GM2 ganglioside can be degraded to GA2 ganglioside by the removal of sialic acid moieties. GA2 ganglioside can then be further degraded by HEXB ($\beta\beta$).^{40,41} Although preliminary studies of potential therapeutic strategies for GM2 gangliosidosis have been performed in the SD mouse model, *Hexa*^{-/-} mice have limited utility as a model for TSD due to the absence of significant GM2 accumulation and behavioral abnormalities seen in TSD patients.³² Sialidases or neuraminidases (EC 3.2.1.18) are categorized under exo-glycosidases that remove sialic acid residues from glycolipids, glycoproteins, or oligosaccharides. They have critical roles in different cellular activities, such as cell proliferation, glycoconjugate metabolism, myelination control, and cell adhesion.⁴²

Human sialidases are encoded by multiple genes and have different optimal conditions, including substrate specificity, pH, and kinetic properties. Since they have different subcellular locations, they are classified as lysosomal (NEU1), cytosolic (NEU2), plasma membrane-associated (NEU3), or lysosomal/mitochondrial and intracellular membrane-associated (NEU4).⁴³ Hydrolysis of sialic acids from gangliosides is conducted by NEU1, NEU3, and NEU4.⁴⁴ In a previous study, Seyrantepe et al.³² demonstrated the enzymatic activity of NEU4 toward complex gangliosides and GM2 gangliosides.⁴⁵ In this study, *Hexa*^{-/-}*Neu4*^{-/-} mice were generated to show compensating effects for the metabolic defects in *Hexa*^{-/-} mice. However, a mild phenotype was observed in the double-deficient mice; hence, further studies were required to understand the preliminary enzymes in the GM2 catabolism in *Hexa*^{-/-} mice.⁴⁶ In 2018, Seyrantepe et al.³² demonstrated the role of Neuraminidase 3 (NEU3) in the catabolism of GM2 ganglioside in a mutant mouse line of double deficiency of *Hexa* and *Neu3*, *Hexa*^{-/-}*Neu3*^{-/-} mice. Using *Hexa*^{-/-}*Neu3*^{-/-} mice, the metabolic bypass in the ganglioside catabolism pathway was identified (Figure 1.3). Since the mice showed neuropathological, biochemical, clinical, and behavioral aspects seen in early-onset TSD patients, along with excessive buildup of GM2 gangliosides (Figure 1.4), *Hexa*^{-/-}*Neu3*^{-/-} mice serve as a powerful tool to understand pathophysiology under the pathophysiology of TSD.

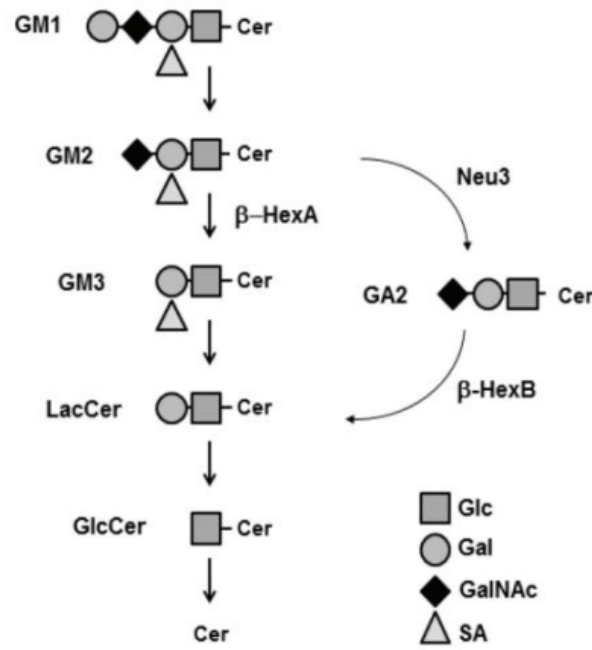


Figure 1.3. Schematic representation of the degradation pathway of GM2 ganglioside. NEU3 is identified as the primary enzyme mediating the metabolic bypass in the *Hexa*^{-/-} mice by courtesy of Seyrantepe et al.³².

Neuroinflammation, oxidative stress, autophagy, and neurodegeneration were all associated with the massive lysosomal storage of GM2 gangliosides in neurons of *Hexa*^{-/-}/*Neu3*^{-/-} mice.³² The increased mRNA levels of pro-inflammatory cytokines and chemokines, including *Ccl2*, *Ccl3*, *Ccl4*, and *Cxcl10*, and microgliosis and severe astrogliosis were revealed in *Hexa*^{-/-}/*Neu3*^{-/-} mice.⁴⁷ Additionally, according to electron microscopic analyses, excessively elevated levels of lysosomes and ring-like structures in neuronal lysosomes were demonstrated in *Hexa*^{-/-}/*Neu3*^{-/-} mice (Figure 1.5). Growth retardation and sudden death at 20 weeks of age were visualized in *Hexa*^{-/-}/*Neu3*^{-/-} mouse model.³²

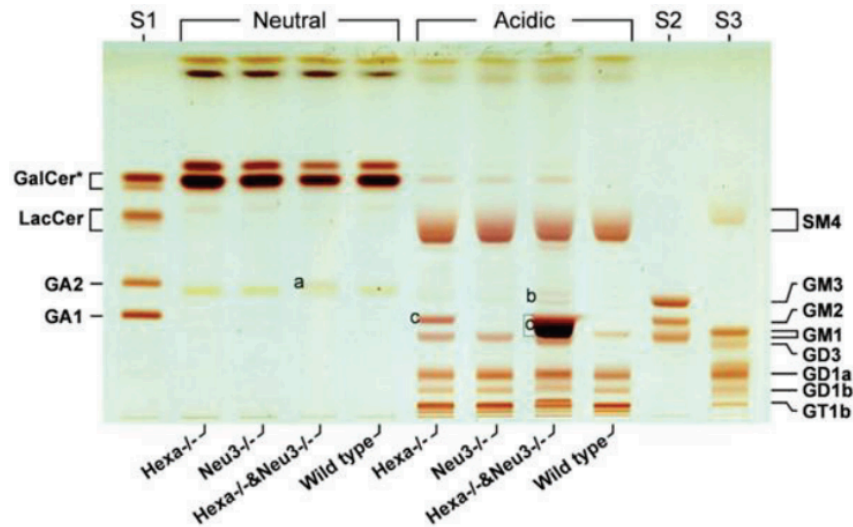


Figure 1.4. The abnormal accumulation of GM2 ganglioside in the brain of *Hexa*^{-/-}*Neu3*^{-/-} mice (d) compared to *Hexa*^{-/-} mice (c). The presence of GA2 ganglioside (a) and GM3 ganglioside (b) in the brains of *Hexa*^{-/-}*Neu3*^{-/-} mice, by courtesy of Seyrantepe et al.³².

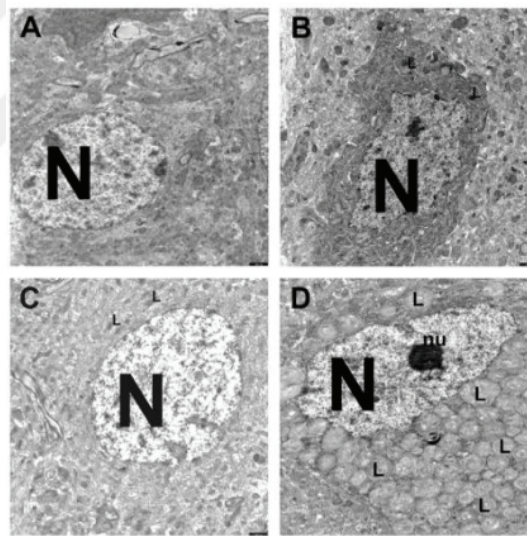


Figure 1.5. The images from the electron microscope of elevated levels of lysosomes and neuronal inclusions in the cortex of *WT* (A), *Hexa*^{-/-} (B), *Neu3*^{-/-} (C), and *Hexa*^{-/-}*Neu3*^{-/-} (D) mice, by courtesy of Seyrantepe et al.³². N: nucleus; L: lysosomes; nu: nucleolus

1.5. Strategies for the Treatment of LSDs

1.5.1. Enzyme Replacement Therapy

Enzyme replacement therapy (ERT) has demonstrated its efficacy in treating several lysosomal storage disorders, including Gaucher disease⁴⁸, Mucopolysaccharidosis (MPS) types³³, Fabry disease⁴⁹, and Pompe disease⁵⁰. The first ERT approved by the Food and Drug Administration (FDA) in 1991 was for Gaucher disease, and it was generated by purifying glucocerebrosidase (GC) enzymes from the human placenta.⁵¹ It was then superseded by imiglucerase, which has been widely used as a recombinant human glucocerebrosidase in the ERT of Gaucher's disease. The ERT strategies utilized recombinant human enzymes to enhance therapeutic efficacy and reduce immunogenicity.⁵²

Recombinant human enzymes are administered intravenously, after which they enter lysosomes via endocytosis and exert their functions to mitigate disease phenotypes in patients. However, their effectiveness varies among individuals, and not all organs and tissues show the same levels of responsiveness to ERTs.⁵³ Since recombinant human enzymes cannot cross the blood-brain barrier (BBB), they are considered ineffective in addressing neuropathologic manifestations of lysosomal storage disorders. In addition, their short half-life necessitates frequent intravenous administration of substantial protein doses.^{54,55} Furthermore, the development of an immune response and the production of neutralizing antibodies against ERT result in significant challenges and, thus, therapy discontinuation.⁵⁶ Despite these challenges, ERT is still a critical therapeutic approach for LSDs due to the limited availability of alternative strategies. To penetrate the BBB, nano-particle-based formulations of glucocerebrosidase (GC) have been investigated for Gaucher disease, but this approach remained in the preclinical phase.⁵⁷ CNS administration of ERT has been studied in certain LSDs, such as intrathecal injection for MPS I³³ and intraventricular infusion for neuronal ceroid lipofuscinosis type 2 (CLN2).⁵⁸ The long-term clinical and therapeutic benefits of ERT via CNS administration remain under investigation.⁵⁹

Enzyme replacement therapy for TSD poses significant challenges since an effective ERT must contain both a functional alpha and beta subunit of HexA. Several research groups have developed functional HexA enzymes showing improvement *in*

vitro^{60,61}. Akeboshi et al.⁶⁰ produced recombinant HexA enzymes using a methylotrophic yeast *O. minuta*, and showed the effectiveness of ERT in TS and SD fibroblasts by diminishing GM2 ganglioside.⁶⁰ Since the HexA enzyme has a large size, preventing BBB cross, traditional intravenous administration becomes ineffective in mitigating neuropathological symptoms observed in TSD.⁵² Alternative routes for the BBB entrance have been explored in the HexA administration. For instance, intracerebroventricular (ICV) administrations of a modified HexB containing both HexA and HexB activities in mouse models of Sandhoff disease showed a remarkable reduction in GM2 ganglioside levels and a two-fold increase in Hex activity.⁶² The modified form of HexB might serve as a treatment strategy for both TSD and Sandhoff disease, whereas ICV administration has not yet been tested in TSD patients, as it is not a practical route for drug delivery.⁵²

1.5.2. Enzyme Enhancing Therapy: Pharmacological Chaperones

Pharmacological chaperones are small-molecule drugs binding to proteins to correct their conformation and enable the proper transport of the proteins into their cellular sites for biological functions.⁵² In several LSDs, a missense mutation in the enzymes causes a failure of the enzymes to reach lysosomes due to misfolding, and thus, the enzymes remain in the endoplasmic reticulum (ER). These may enable suitable conditions to treat LSDs with pharmacological chaperones.⁵³ Small-molecule chaperone therapy shows potential since it allows oral administration, easier penetration into the BBB, reduction in immunogenicity, and lowered manufacturing cost, in contrast to the challenges observed in ERT. A common approach for characterizing specific chaperones includes screening for enzyme inhibitors, which bind to enzymes tightly to stabilize enzymes' tertiary structures and facilitate their correct folding so that enzymes can be transported into lysosomes from the ER.⁵³ Using this strategy, migalastat was initially characterized as a small molecule inhibitor with chaperone-like properties of alpha-galactosidase (alpha-GalA).⁶³ Migalastat was the first pharmacological chaperone approved by the FDA for the treatment of patients with Fabry disease.⁶⁴ Likewise, ambroxol has been identified as a pharmacological chaperone for some types of glucocerebrosidase (GC) mutants, and thus, it has been tested clinically in patients with Gaucher disease, showing improvement in disease phenotypes in some individuals.^{65,66}

In the case of Tay-Sachs disease, certain mutations cause misfolded HexA enzymes to be rapidly degraded before being transported into the lysosomes. The expected role of pharmacological chaperones in the patient cells is to facilitate the proper folding of misfolded enzymes. Pyrimethamine (PYR), a pharmacological chaperone, has been identified to inhibit HexA degradation long enough for the enzyme to reach the lysosome and facilitate GM2 hydrolysis *in vitro*.⁶⁵ The PYR specifically binds to the active site of domain II in HexA to stabilize cells with enzymes carrying the late-onset alphaG269S mutation.⁶⁵ The clinical trials of PYR showed only a temporary increase in HexA activity, accompanied by undesirable side effects, without a significant symptomatic improvement in patients with TSD.⁶⁷ Recently, certain pharmacological chaperones have exhibited their functions as small-molecule enzyme binders without exhibiting inhibitory activity. The identification of PYR was performed using *in vitro* screening of approved drugs, and hence, by expanding this screening to a larger scale, more promising chaperones to target HexA might be discovered.^{52,65}

1.5.3. Substrate Reduction Therapy

Substrate reduction therapy (SRT), another therapeutic approach, involves the inhibition of substrate synthesis by targeting the mutant enzymes responsible for the substrate accumulation in LSDs. As an example of SRT, the inhibitor of glucosylceramide synthase, miglustat (N-butyldeoxynojirimycin, NB-DNJ), ameliorates accumulating substrates due to glucocerebrosidase mutations in Gaucher disease.⁶⁸ SRT in TSD necessitates the reduction in the accumulating GM2 ganglioside. In the mice model of the late-onset TSD (*Hexa*^{-/-}), miglustat treatment partially reduced GM2 ganglioside levels in the brain by 50% via the competitive inhibition of glucosylceramide synthase.⁶⁹ Even though the preclinical findings presented promising results, clinical trials showed limited success for miglustat.³⁵ To test clinical efficacy and penetrance through the BBB, two infantile TSD patients were given miglustat, which failed to prevent the disease progression and neuropathological symptoms.⁷⁰ In a parallel strategy, a two-year trial in five juvenile GM2 gangliosidosis patients was administered with miglustat, and there were no improvements in neurological function.⁷¹ Similarly, a three-year miglustat treatment of adult Sandhoff disease (SD) patients demonstrated no mitigation of neurodegeneration.⁷²

Despite demonstrating an absence of clinical efficacy for treating the neurological impairments of chronic GM2 gangliosidosis, early intervention with miglustat or similar SRTs remains an open question. At the earliest stages of disease progression, using miglustat or similar SRTs might help to prevent neuropathological symptoms.⁵² Genz-529468, another example of SRT, which is more potent than miglustat, also functions as a glucosylceramide synthase inhibitor. Preclinical studies demonstrated that *Hexb*^{-/-} mice, Sandhoff disease mice, given Genz-529468 had a delayed loss of motor function and a prolonged lifespan, even without a decrease in GM2 ganglioside levels. When mice were given both miglustat and Genz-529468, anti-inflammatory responses were triggered in addition to delayed neuronal apoptosis. These results suggested that anti-inflammatory SRTs can cross the BBB, and thus, they might serve as promising therapeutic strategies.⁷³

1.5.4. Hematopoietic Stem Cell Transplantation

Using stem cells obtained from bone marrow, peripheral blood, or umbilical cord blood, hematopoietic stem cell transplantation (HSCT) is performed.³⁵ HSCT might be used in GM2 gangliosidosis since Hex enzymes can transport between cells using a mannose-6-phosphate receptor (M6PR)-mediated pathway.⁷⁴ The therapeutic efficacy of HSCT has been indicated in different LSDs, such as Gaucher disease types 1 and 2 and a mouse model of MPS I.^{75,76} Preclinical studies of bone marrow transplantation (BMT) using *Hexb*^{-/-} mice demonstrated an increased survival rate⁷⁷ and mitigation of neurodegeneration accompanied by reduced microgliosis.⁷⁸ After the BMT of a 3-year-old pre-symptomatic TSD patient, clinical results showed an elevation in HexA activity in leukocytes and plasma without preventing the onset of neuropathology.⁷⁹ In another clinical case involving a 15-year-old late-onset TSD patient receiving a BMT from a healthy sibling donor, the levels of HexA activity were enhanced in leukocytes and somatic tissues compared to healthy controls. Although it was not a complete elimination, the prevention of further neurodegeneration and a manageable life quality were shown in the patient.⁸⁰

Despite these promising results, there are still significant challenges in HSCT, including identifying suitable donors and potential immunogenic complications if suitable donors are unavailable. Recently, the transplantation of either healthy donor-derived stem cells or genetically corrected stem cells from patients has been an ongoing

effort, yet there have been immune response complications for allogeneic donor-derived stem cell transplantations.⁷⁴ Unlikely, the use of patient-specific autologous gene therapy-corrected stem cells in clinical trials has indicated a promising strategy for LSDs. However, this strategy has not yet been tested in TSD patients.⁸¹

1.5.5. Gene Therapy

Gene therapy, a promising therapeutic strategy for treating LSDs, involves the use of viral vectors, including a functional gene, to correct genetic defects. Given that TSD is a monogenic disorder, the use of gene therapy holds critical potential as a treatment approach. Different strategies exist for gene therapy, including *ex vivo* and *in vivo* administration. The *ex vivo* approach includes isolating the patient's autologous cells, which are genetically modified using viral vectors and then administered into the patient's body. In contrast, the *in vivo* strategy directly delivers the gene of interest to the patient, bypassing the need for cell isolation and modification outside the body.⁵² Performing the *in vivo* gene therapy necessitates the administration of viral vectors, especially adeno-associated viruses (AAVs). However, for the *ex vivo* approaches, retroviral vectors, such as lentiviruses or gamma-retrovirus vectors, are primarily utilized. To date, 250–300 clinical trials have been applied using AAV vectors, demonstrating a safety record for human studies.⁸² Numerous clinical trials of *in vivo* gene therapy have been utilized for LSDs, such as Sanfilippo Syndrome B and Late Infantile Neuronal Ceroid Lipofuscinosis. Given that AAVs do not cross the BBB naturally, the administrations have relied on intracerebral or intrathecal strategies, with promising safety and tolerability results.^{83,84}

1.6. Adeno-Associated Viral Vector Strategies

1.6.1. Adeno-Associated Viral Vectors

Adeno-associated viruses (AAVs) are categorized within the *Parvoviridae* family, of which the replication cycle depends on a helper virus, such as adenovirus (AdV). AAVs are not associated with any characterized human diseases, and they are identified in both humans and non-human primates.⁸² They were initially characterized

through serological methods. However, the molecular cloning of AAV genes has led to the identification of several different AAV strains across various species. Furthermore, they have lower immunogenicity compared to other viruses. AAV has a single-stranded genome, including three genes: *rep* (Replication), *cap* (Capsid), and *aap* (Assembly). Two T-shaped inverted terminal repeats (ITRs) flank this viral genome and serve as the origins of replication and packaging signals.⁸⁵ The viral replication is controlled by the *rep* gene encodes Rep78, Rep68, Rep52, and Rep40 proteins, whereas the *cap* gene encodes three capsid subunits via alternative splicing or translation using distinct start codons. The icosahedral protein capsid of AAVs is composed of three types of subunits: VP1, VP2, and VP3.⁸² The *aap* gene, on the other hand, is an assembly-activating protein (AAP) functioning as an alternative reading frame. The gene serves as a scaffold for capsid assembly.⁸⁶

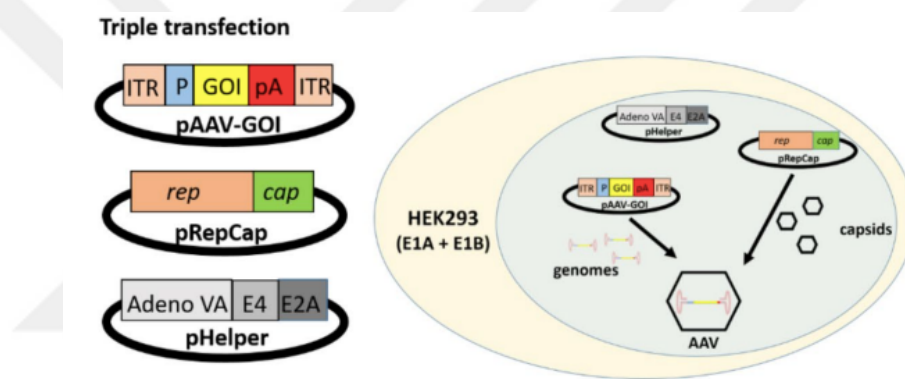


Figure 1.6. The triple transfection method with a transfection reagent in HEK293 cells, by courtesy of Robert et al.⁸⁷. The essential regions for adenovirus are located in the pHelper plasmid, while the *rep* and *cap* genes reside on the pRep/Cap plasmid. Different *cap* sequences are available for different AAV serotypes that determine the tropism.

AAV2, AAV5, and AAV9 are widely examined and primarily used in many clinical trials for various therapeutic applications. Since there is a lack of information on the biology of wild-type AAV serotypes, it is not the form used for gene therapy applications. Instead of wild-type AAV serotypes, recombinant AAV (rAAV) serotypes have been used in genetic manipulations. The lack of viral DNA and the ability to efficiently penetrate cell membranes make rAAVs an ideal system for delivering DNA cargo into the nucleus.⁸⁵ Without Rep proteins, the ITR-flanked transgenes within rAAV can form concatemers persisting as episomes in the nucleus of transduced cells, so they are diluted over time with successive rounds of replication. Since rAAV vectors have a

packaging capacity, for efficient packaging and increasing viral production yield, sequences under 5 kb can be used for designing an rAAV vector and triple transfection strategy with a suitable transfection reagent (Figure 1.6).⁸⁵⁻⁸⁷ Furthermore, single-stranded AAV-delivered transgene is a rate-limiting step in nuclear delivery due to the requirement of a double-stranded form. Hence, to solve this issue, self-complementary AAV (scAAV) is designed to accelerate gene expression; however, its packaging capacity is decreased to approximately 3.3 kb.⁸⁵

For higher levels of gene expression, strong, constitutively active promoters such as CMV (cytomegalovirus), EF1a (elongation factor 1a), SV40 (simian virus 40), chicken β -actin, and CAG (a hybrid promoter of CMV and chicken β -actin) should be used to enable robust gene expression across various cell types. For instance, the CMV promoter is prone to silencing in the central nervous system (CNS).⁸⁷

Driven by the goal of using rAAV vectors to insert genes into highly selective cell and tissue types, novel variants from human and primate tissues were designed. Each variant has unique capsid sequences for obtaining specific tropism.⁸⁸ AAV variants enter cells through carbohydrate interactions on the surface of target cells; hence, the difference in capsid sequences influences the transduction efficiency and cell-type specificity of the variant.⁸⁹ For example, AAV9 selectively recognizes galactose residues via amino acid variations in its capsid sequence.⁹⁰ The selective binding to the galactose residues enables AAV9 to cross the BBB and efficiently transduce CNS cells.⁹¹

1.6.2. A New AAV Serotype: AAVrh10

Previous studies identified the systemic delivery of self-complementary (sc) adeno-associated virus serotype 9 (scAAV9). The scAAV9 enables widespread gene transfer to the CNS, showing its non-invasive effect on neurological disorders. More recently, AAV serotype rh10 (AAVrh10), a serotype from rhesus monkeys, has been demonstrated to mediate an efficient CNS transduction in mice.⁹² Since exposure to the virus is low, exposure to preexisting antibodies has been reduced, as well. It has potential for gene therapy, and efficient transduction of neurons, and to a lesser extent, oligodendrocytes after intracranial or intrathecal injections. In late-infantile neuronal ceroid lipofuscinosis⁹³, amyotrophic lateral sclerosis⁹⁴, and metachromatic leukodystrophy⁹⁵, the ability of AAVrh10 to reverse the neuropathological symptoms in

the central and peripheral nervous system. Previously, the approval of rAAVrh.10 for two clinical trials was given for targeting late-infantile neuronal ceroid lipofuscinosis (NCT01161576) and Sanfilippo Type A syndrome.⁹⁶ Since intravenous vector delivery is the least invasive method, it has also been studied for AAVrh10 serotypes. The widespread transduction of a diverse range of cell types and BBB penetrance make both serotypes usable in neurological disorders.⁹⁷ Furthermore, Zhang et al.⁹⁸ showed widespread distribution and gene expression throughout the brain after intravenous delivery of AAVrh10. Belur et al.⁹⁷ demonstrated that the single intravenous administration of AAVrh10-IDUA, expressing human IDUA enzyme, to MPS I mice (*IDUA*^{-/-}) resulted in high levels of enzyme expression and reduction in GAG levels in the brain. In another study, lumbar intrathecal injections of AAVrh10 to mice indicated a widespread CNS transduction.

1.6.3. Adeno-Associated Viral Vector Strategies for Tay-Sachs Disease

Since TSD is also a neurodegenerative disorder, the use of AAV-based gene therapy suggests a viable path for treating this disorder. However, the challenge of recruiting both subunits of the heterodimeric HexA enzyme to produce an active enzyme, each encoded by *HEXA* and *HEXB* separately, must be overcome in TSD gene therapy.⁵² There have been several strategies explored in TSD and SD animal models, including co-administration of separate AAVs expressing *HEXA* and *HEXB*, administration of the modified *HEXM* subunit forming stable homodimers, and bicistronic AAV vector for simultaneous administration of both *HEXA* and *HEXB*.^{99–101} AAV-based therapies in mouse and feline models of TSD and SD have demonstrated reduced levels of GM2 ganglioside, while the same effect has not been revealed in non-human primates.⁷⁴

Furthermore, intracranial administration of recombinant AAV (rAAV) serotypes 2/1 or 2/2 expressing *HEXA* and *HEXB* genes in the SD mice model (*Hexb*^{-/-}) resulted in widespread HexA distribution throughout the nervous system along with the prolonged survival rate without cytotoxicity.¹⁰² There is a packaging capacity (2.1–4.5 kb) in AAV-based vectors; therefore, the administration of full-length cDNA of both subunits of HexA is limited. In addition, the co-transfection efficacy of both α and β subunits of HexA enzyme is remarkably lowered compared to single transfection.¹⁰³ To address this problem, Tropak et al.¹⁰³ utilized a self-complementary AAV9.47 vector encoding a

hybrid μ subunit (scAAV9.47-*HEXM*). It was then administered intracranially to 4-month-old TSD mice (*Hexa*^{-/-}), where a decreasing GM2 ganglioside accumulation in brains was observed.¹⁰³ In another study, scAAV9.47-*HEXM*, which was injected intravenously into newborns of TSD mice (*Hexa*^{-/-}), demonstrated a sustained reduction in GM2 ganglioside accumulation in the CNS.¹⁰⁴ Compared to other AAV serotypes, AAV9, AAVrh10, or AAVrh8, a lower liver distribution was shown in scAAV9.47-*HEXM*.¹⁰⁴ Additionally, in the newborns of SD mice (*Hexb*^{-/-}), intravenous injection of scAAV9.47-*HEXM* resulted in a 2.5-fold increase in survival rate, together with an efficient CNS transduction compared to control groups.¹⁰⁵

The therapeutic efficacy of AAVrh8 was also tested using a 2-4-month Jacob sheep model of TSD, which was administered intracranially either with AAVrh8-*HEXA* or a combination of AAVrh8-*HEXA* and AAVrh8-*HEXB*. After treatment, there was a delayed onset and/or attenuation of symptoms in the sheep model.¹⁰⁰ Combined administration of AAVrh8-*HEXA* and AAVrh8-*HEXB* revealed a widespread brain distribution of HexA, but there was a limited distribution for AAVrh8-*HEXA* alone.

1.7. An Anti-Inflammatory Agent: Istradefylline

Istradefylline (ISD) is a novel drug developed for the treatment of Parkinson's disease (PD). It acts as a selective antagonist of the A2A (adenosine) receptor (A2AR) and a therapeutic option for patients with Parkinson's disease.¹⁰⁶ It was approved by the FDA as an adjunctive treatment to levodopa/carbidopa (L/C) treatment in adult Parkinson's patients.¹⁰⁷

Astrogliosis, microgliosis, and cytokine/chemokine expression are common neuropathological symptoms observed in CNS disorders. Especially, astrogliosis is mostly demonstrated as a characteristic symptom of CNS disorders, acting primarily or contributorily in distinct neurodegenerative disorders, including Sandhoff disease (SD), Huntington's disease (HD), Alzheimer's disease (AD), multiple sclerosis (MS), epilepsy, and Parkinson's disease (PD).^{108–111} Recently, Orr et al.¹¹² identified an elevation in the expression of astrocytic A2A receptor in patients with AD, AD mouse models, and kainic acid-induced epilepsy models. The memory deficits in the AD mice model have been shown to improve after treatment with Istradefylline, so the changes in A2AR expression might represent its role in the modulation of active astrocytes.¹¹³ Similarly, in the studies

with *Hexb*^{-/-} mice, the SD model, the expression of A2AR on activated astrocytes in the cerebral cortices was detected; hence, the therapeutic potential of astrocytic A2AR in SD was demonstrated. Active A2AR in cultured astrocytes triggers CCL2 expression, which is a primary inflammatory modulator in CNS disorders. Inhibiting the receptors with Istradefylline resulted in the downregulation of chemokine and cytokine production, together with a decrease in microglial activation, in the cerebral cortices of *Hexb*^{-/-} mice. These results highlight the therapeutic efficacy of Istradefylline to target astrocytic A2AR in SD for the prevention of astrocyte and microglial activation, as well as CCL2 expression¹¹⁴, so Istradefylline might serve as an anti-inflammatory agent in CNS disorders.

1.8. Neuroinflammation

Neuroinflammation occurring in the brain or spinal cord is the inflammatory response of cells driven by the release of chemokines, cytokines, secondary messengers, and reactive oxygen species. Glial cells, such as microglia, astrocytes, endothelial cells, and peripheral immune cells of the central nervous system (CNS), generate those molecules. Those neuroinflammatory responses result in immune, biochemical, and physiological effects.¹¹⁵ The pro-inflammatory cytokines, IL-1 β , IL-6, and TNF- α , and chemokines, CCL2, CCL3, and CXCL1, are the key regulators of neuroinflammatory responses. The mediators are mainly generated from activated resident CNS cells, including astrocytes and microglia.¹¹⁶

Under normal conditions, neuroinflammation is mainly induced as a protective mechanism in response to CNS diseases, injuries, or infections. On the other hand, chronic or excessive inflammation could damage neural cells.^{117,118} Once the resident microglia and astrocytes detect pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs), peripheral leukocytes are recruited into the CNS to respond to chronic inflammation for preventing unnecessary damage (Figure 1.7).¹¹⁸ The prolonged inflammation, if not prevented, exacerbates damage and contributes to CNS pathology.¹¹⁹

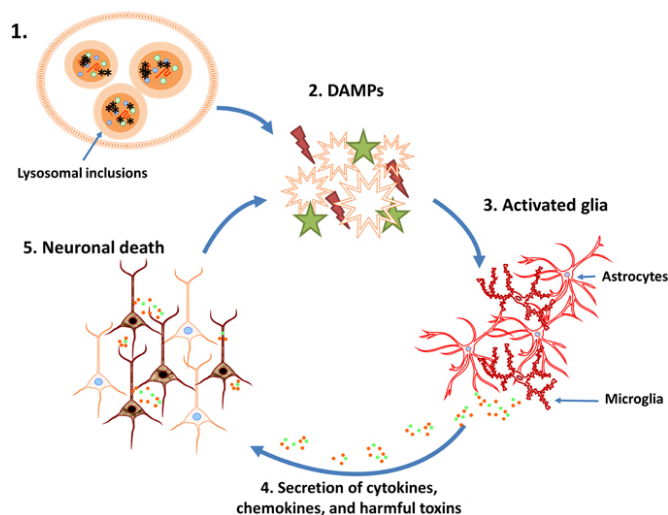


Figure 1.7. The schematic representation of neuroinflammation associated with LSDs where lysosomal inclusions and dysfunctional lysosomes cause the release of DAMPs, activation of astrogliosis, and microgliosis, and eventually neuronal death, by courtesy of Bosch and Kielian¹¹⁹.

1.8.1. Microgliosis

Microgliosis is a response of microglia of the CNS in response to pathogenic insults. It is characterized by an evident increase in the number of microglia at the site of injury. Microglia, resident cells of the CNS, are found in the resting state and dispersed throughout the intact brain. Microglia have a dynamic nature, and this feature is thought to maintain CNS homeostasis and neural plasticity.¹²⁰ They engulf misfolded proteins, dying cells, or cellular debris for CNS homeostasis. In addition, they crosstalk with neurons to monitor neuronal function and protection.¹²¹

Under pathological conditions, including neurodegenerative diseases, ischemic stroke, and traumatic brain injuries, the microglia gradually convert from the resting form into the active state.¹²⁰

Microglia are divided into two contrasting groups: classical M1, neurotoxic and pro-inflammatory, or alternative M2, neuroprotective and anti-inflammatory.¹²¹ In neuroinflammation, microglia are activated and polarized into either the M1 pro-inflammatory phenotype or the M2 anti-inflammatory phenotype in response to changes in the microenvironment.¹²² M1 microglia release inflammatory cytokines, such as IL-6, IL-1 β , IL-12, and CCL2, and results in neuronal death due to inflammation, while M2 microglia have a role in tissue repair by producing anti-inflammatory cytokines, such as

IL-10, TGF- β , and BDNF.¹²³⁻¹²⁵ In contrast to the anti-inflammatory and neuroprotective functions of M2 microglia, activation of M1 microglia leads to neurotoxicity and inflammation.¹²⁶ These two types of microglia are involved in the pathogenesis of neurodegenerative disorders.¹²⁷ Hence, the microglial activation should be finely modulated to keep brain homeostasis and prevent neurodegenerative disorders.¹²⁸

1.8.2. Astrogliosis

Astrocytes are the major subtype of glial cells of the CNS, having important roles in brain homeostasis, neuroprotection, neuronal support, and maintenance of BBB and neuronal plasticity.¹²⁹⁻¹³¹ Astrocytes, which have crucial roles in the maintenance of the healthy CNS, respond to various forms of CNS injury and disease through changes in cellular structure, function, and gene expression. Astrogliosis, which is a neuroimmunological deterioration, is characterized by a range of functional, molecular, and cellular changes in the astrocytes due to CNS diseases or injuries. The malformations in astrocyte structure or function are associated with the severity of the injury or disease. These alterations associated with astrogliosis are modulated through intra- and intercellular signaling mechanisms.^{111,132}

In reactive astrogliosis conditions, cellular signals from neurons, microglia, and synapses at the site of injury stimulate the remodeling of astrocytes into reactive forms. As a response to those signals, reactive astrocytes trigger the release of pro-inflammatory cytokines IL-6, and TGF- α by which expression of GFAP and vimentin is driven to enable neuroprotective functions. The primary role of astrogliosis is to preserve healthy regions, repair the BBB, and form glial scars, whereas dysfunctional astrogliosis could result in neuropathological damage.^{133,134} Liddel and Barres¹³⁵ showed the presence of A1 and A2 reactive astrocytes, from which A1 is involved in the secretion of pro-inflammatory molecules and neurotoxins, causing neurodegeneration. However, A2-type reactive astrocytes stimulate the expression of anti-inflammatory molecules and neurotrophic factors for neuronal survival.^{135,136}

1.8.3. Neuroinflammation in Lysosomal Storage Diseases

In several LSDs, the presence of neuroinflammation along with inflammatory changes and activated molecular pathways has been reported. LSDs are known to occur primarily by genetic mutations in the enzymes, causing the accumulation of lysosomal material, while they share common downstream characteristics. Despite the differences in the biochemical composition of accumulating material, there is emerging evidence suggesting the induction of neuroinflammation contributes to neurodegeneration.¹⁵

In Niemann-Pick type C disease (NPC), the lysosomal accumulation of cholesterol and sphingolipids occurs due to mutations in NPC1/NPC2 genes. *NPC*^{-/-} mice model of NPC showed an increase in microglial activation and expression of TNF- α and IL-1 β along with a neuronal loss in certain brain regions.¹³⁷ In Gaucher disease (GD) types 2 and 3, which is caused by the dysfunctional glucosylceramidase (GlcCerase) enzyme and accumulation of glucosylceramide (GlcCer) in the brain, the elevation of pro-inflammatory cytokines IL-1 α , IL-1 β , TNF- α , and IL-6 has been revealed.^{17,138} In addition to those LSDs, there is emerging evidence indicating the presence of neuroinflammation in the pathology of the Tay-Sachs disease. TSD results from the HEXA mutation, causing the deficiency of lysosomal β -hexosaminidase α and accumulation of GM2 ganglioside.³² In the TSD mice model, *Hexa*^{-/-}*Neu3*^{-/-}, the overexpression of pro-inflammatory cytokines, *Ccl2*, *Ccl3*, *Ccl4*, and *Cxcl10*, in the cortex, and *Ccl4*, *Cxcl13* in the cerebellum was indicated. The expression levels of anti-inflammatory cytokines, *IL10*, *IL2*, and *IL11*, were shown to decrease in the cortices and cerebellum of *Hexa*^{-/-}*Neu3*^{-/-} mice. Likewise, activated microglia were detected using Moma2 antibody to implicate increased Moma2-positive cells in the cortex, thalamus, and cerebellum of *Hexa*^{-/-}*Neu3*^{-/-} mice.⁴⁷

1.9. Aim of the Study

The aim of this study was the elucidation of the therapeutic efficacy of intrathecal AAVrh10-human Neu3 gene delivery in the early-onset Tay-Sachs disease mice model, *Hexa*^{-/-}*Neu3*^{-/-}. Additionally, a combination with or without an anti-inflammatory drug, Istradefylline, was aimed to investigate whether Istradefylline treatment alleviates the neuroinflammation pathology in the early-onset Tay-Sachs disease mouse model.

CHAPTER 2

MATERIALS AND METHODS

2.1. The Production of AAVrh10-hNeu3 Particles

2.1.1. Vectors

Confirmation of the AAV helper, AAV rep/cap, and mammalian expression vectors used for virus production was performed in this thesis study. The mammalian expression vector expressing the human Neu3 (hNeu3) gene under the control of a CMV promoter was designed and purchased from the VectorBuilder company as pAAV-[Exp]-CMV-mHexa-3xGGGGs-mCherry-oPRE (mammalian expression vector expressing hNeu3) (Figure 2.1). The mammalian expression vector expressed the hNeu3 as a fusion protein with mCherry, a reporter gene with the red signal. The AAV helper vector, pAdΔF6, and the AAV rep/cap vector for replication and capping virus genes, pAAV2/rh10, were designed and purchased from the Addgene company (Figure 2.2).

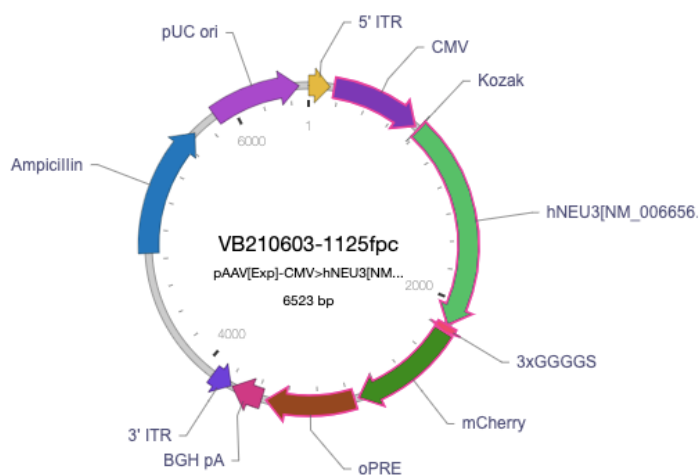
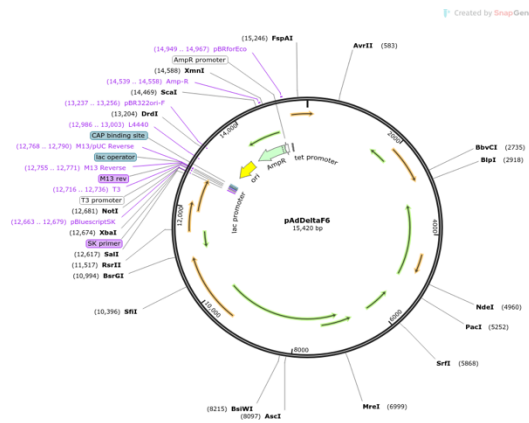


Figure 2.1. The representation of mammalian expression vector expressing human Neu3 under the control of CMV promoter, pAAV-[Exp]-CMV-mHexa-3xGGGGs-mCherry-oPRE (mammalian expression vector expressing hNeu3).

A.



B.

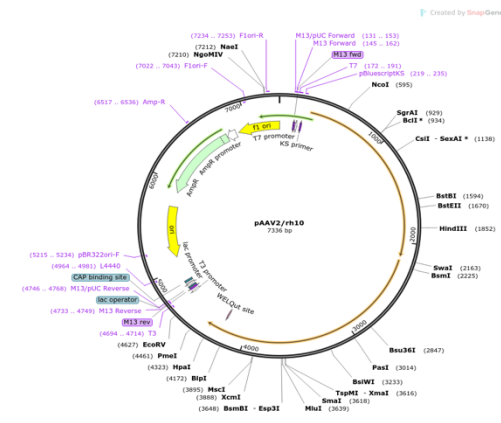


Figure 2.2. The representation of the AAV helper vector, pAdΔF6 (A), and the AAV rep/cap vector for replication and capping virus genes, pAAV2/rh10 (B).

2.1.2. Confirmation of The Vectors

The pAdΔF6, pAAV2/rh10, and mammalian expression vector expressing hNeu3 were verified after they were received in *E. coli* stocks, to show whether they expressed the desired genes. All three plasmids had an Ampicillin resistance gene; therefore, they were streaked over the LB agar plates with ampicillin using an inoculation loop. After the incubation at 37°C overnight, a single colony from each was selected and inoculated into LB Broth with ampicillin (Sigma) (100mg/ml). LB broths with selected colonies were incubated at 37°C for 16 hours, and the following day, the change in turbidity was observed. The vectors in LB broth were then isolated by performing the Alkaline Lysis (ALS) purification. 3 ml of the liquid was centrifuged at 12,000 g at +4°C for 30 seconds, and the resulting pellets were dissolved by vortexing in 200 µl of pre-cooled ALS Solution 1 (Table 2.1). Immediately after, 400 µl of the freshly prepared ALS Solution 2 was added to the samples. Then, 300 µl of pre-cooled ALS Solution 3 was added to the tubes, mixed by vortexing, and incubated on ice for 5 minutes. After the incubation, the mixtures were centrifuged at 12,000 g and +4°C for 5 minutes. The supernatants were transferred into new tubes, after which two volumes of 100% ethanol were added for precipitation. The mixtures were centrifuged again to obtain the vectors in the pellets, which were washed with 70% ethanol by centrifuging at 12,000 g and +4°C for 5 minutes. The resulting pellets were air-dried for 10 minutes and fully dissolved in 20 µl of dH₂O. The concentrations of vector DNAs were measured using a Nanodrop spectrophotometer

(IMPLEN), and 6000 ng of each vector DNA was used to utilize the restriction enzyme digestions.

Table 2.1. The solutions prepared for the Alkaline Lysis Purification protocol

ALS Solution 1	ALS Solution 2	ALS Solution 3
50 mM Glucose	0.4 N NaOH (500 μ l)	5M Potassium Acetate (60 mL)
25 mM Tris-HCl pH=8.0	%10 SDS (100 μ l)	Glacial Acetic acid (11.5 mL)
10 mM EDTA pH=8.0	dH ₂ O (400 μ l)	dH ₂ O (28.5 mL)
dH ₂ O (50 mL)		

To perform restriction enzyme digestion, each of the pAd Δ F6, pAAV2/rh10, and mammalian expression vector expressing hNeu3 was used as 6000 ng of plasmid DNA. For the restriction digestion of either pAd Δ F6 (Figure 2.3A) or pAAV2/rh10 (Figure 2.3B) vector, 1 μ l of BamHI enzyme (NEB, 10 units), 2 μ l of Biolab NE buffer 4 (10X), and vector DNA calculated to be 6000 ng were mixed with dH₂O to complete the total volume of 20 μ l. On the other hand, to perform the restriction digestion of the mammalian expression vector expressing hNeu3, 1 μ l of either EcoRI enzyme (NEB, 10 units) (Figure 2.4A) or PstI enzyme (NEB, 10 units) (Figure 2.4B), 2 μ l of Biolab NE buffer 4 (10X), vector DNA calculated to be 6000 ng were added together with dH₂O to complete the total volume of 20 μ l.

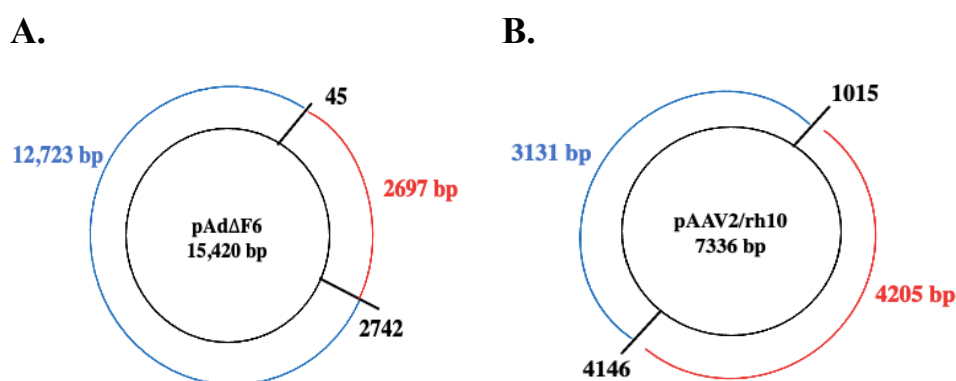


Figure 2.3. The representation of the restriction enzyme cut size for BamHI in the pAd Δ F6 (A) and pAAV2/rh10 (B) vectors.

All restriction enzyme digestion reactions were incubated at 37°C for 1.5 hours. The samples were run on a 1% agarose gel for 30 minutes at 95 Volts, and band sizes were determined. After the verification, the vectors were amplified in the Terrific broth (Fisher Scientific) for isolation using the manufacturer's instructions for the QIAGEN Plasmid Mega Kit.

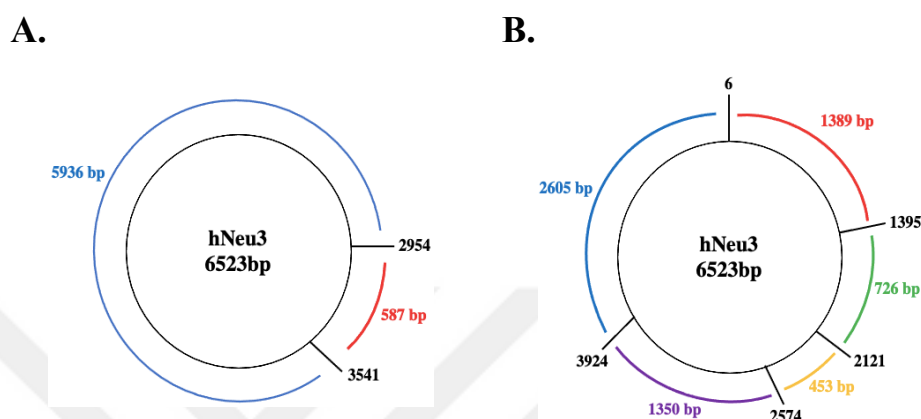


Figure 2.4. The representation of the restriction enzyme cut size for EcoRI (A) and PstI (B) in the mammalian expression vector expressing hNeu3 (B).

Before the Mega Kit isolation of the vectors, 3 mL from the pAdΔF6, pAAV2/rh10, or mammalian expression vector expressing hNeu3 was confirmed using the ALS protocol, and was inoculated into 3 L of the Terrific broth, including ampicillin, and incubated at 37°C overnight. The following day, the cultured bacteria were centrifuged to collect bacterial pellets at 6000 g at +4°C for 15 minutes. Using 50 ml of P1 solution with RNase A added to a final concentration of 100 µg/mL and LyseBlue in a 1:1000 dilution, the bacterial pellets were dissolved by vortexing. Then, the mixture was transferred to FEP centrifuge tubes, and 50 ml of P2 solution was added. After incubating the samples at room temperature for 5 minutes, 50 ml of pre-cooled P3 solution was added to the mixtures, which were incubated on ice for 30 minutes. The samples were centrifuged to obtain supernatants at 30,000 g and +4°C for 45 minutes. By that time, the columns for isolation were equilibrated using 35 ml of QBT, the equilibrium solution. The supernatants were collected and transferred through the columns, after which 200 mL of wash solution was added to the columns. To elute the vectors, 35 ml of QF solution was passed through the columns, and the vectors were collected in the flow-through in new tubes. The vector DNA separation was utilized using 25 ml of isopropanol

and centrifuging at 5000 g and +4°C for 60 minutes. The pellet was washed with 7 ml of 70% ethanol by centrifuging at 5000 g and +4°C for 15 minutes. After discarding the supernatant, the resulting pellets were air-dried at room temperature and dissolved in 1 mL of dH₂O. The concentrations of vector DNAs were measured using a Nanodrop spectrophotometer (IMPLEN), and then, using 1000 ng of each vector DNA and specific restriction enzymes, the restriction enzyme digestions were utilized. The restriction enzyme digestion reactions using BamHI for pAdΔF6 and pAAV2/rh10, and EcoRI or PstI enzymes for the mammalian expression vector expressing hNeu3, were performed in the QIAGEN Plasmid Mega Kit isolation. The samples were run on a 1% agarose gel for 30 minutes at 95 Volts, and band sizes were determined. After the confirmation of each plasmid, they were subjected to single and triple transfection using the HEK293T cell line to control the red fluorescent signal of the reporter gene, mCherry.

2.1.3. Single and Triple Transfection to Confirm the Vectors

The HEK293T cells from the laboratory stock were grown in three wells of a six-well culture plate (Greiner, bio-one) in DMEM (Gibco) containing 10% FBS (Gibco) and penicillin/streptomycin (Sigma) up to 70-80% confluency. For the single transfection, the medium over the HEK293T cells was replaced with 500 µl of empty DMEM and incubated at 37°C for 30 minutes. By then, solutions A and B for the transfection were prepared in two separate tubes. Into tube A, 40 µl of NaCl and 3 µl of polyethyleneimine (PEI, Sigma) were mixed, while 40 µl of NaCl and 1000 ng of mammalian expression vector expressing hNeu3 isolated with the QIAGEN Plasmid Mega Kit were added into tube B. Both tubes were incubated at room temperature for 10 minutes. After 10 minutes of incubation, the mixtures in tubes A and B were mixed, and the mixture was incubated for 20 minutes at room temperature. By the end of incubation, 20 µl of the mixture was added to each well, and the cells were incubated at 37°C for 3.5 hours, followed by 2 ml of serum-containing DMEM. The cells were incubated at 37°C for 16 hours. The following day, the medium was replaced with fresh medium with FBS and incubated at 37°C for 72 hours. Afterward, the mCherry red signal was monitored using a fluorescence microscope (BX53, Olympus).

To perform the triple transfection using isolated the pAdΔF6 vector, pAAV2/rh10 vector, or mammalian expression vector expressing hNeu3 in HEK293T cells, the cells

were grown in 100 mm cell culture plates (Greiner, bio-one) with DMEM containing 10% FBS and penicillin/streptomycin up to 70-80% confluency. On the day of transfection, the serum-containing DMEM was removed and replaced with 5 ml of serum-free DMEM. The cells were incubated at 37°C for 30 minutes, during which Tube A and Tube B were prepared. Into tube A, 350 µl of NaCl and 49 µl of polyethyleneimine (PEI, Sigma) were mixed, while 350 µl of NaCl and 7000 ng of pAdΔF6, 3500 ng of pAAV2/rh10, and 3500 ng of mammalian expression vector expressing hNeu3, all isolated with the QIAGEN Plasmid Mega Kit, were added into tube B. Both tubes were incubated at room temperature for 10 minutes. After 10 minutes of incubation, the mixtures in tubes A and B were mixed, and the final mixture was incubated at room temperature for 20 minutes. By the end of incubation, the mixture was added to the HEK293T cells, and they were incubated at 37°C for 3.5 hours, followed by the addition of 7 ml of serum-containing DMEM. The cells were incubated at 37°C for 16 hours. The next day, the medium was replaced with fresh serum-containing DMEM, and the cells were incubated at 37°C for 72 hours. Afterward, the mCherry red signal was monitored using a fluorescence microscope (BX53, Olympus).

2.1.4. Triple Transfection for AAVrh10-hNeu3 Production

The production of the AAVrh10-hNeu3 virus required the triple transfection of pAdΔF6, pAAV2/rh10, and a mammalian expression vector expressing hNeu3 into the HEK293T cells (Figure 2.5A). For this purpose, the HEK293T cells were grown in 50 150 mm plates (Greiner, bio-one) with DMEM containing 10% FBS and penicillin/streptomycin up to 70-80% confluency. On the day of transfection, the media were removed, and DMEM without serum was added to the plates. The cells were incubated at 37°C for 30 minutes, during which the tubes for triple transfection were prepared. Tube A with 1428 µl NaCl and 200 µl PEI, and Tube B with 1428 µl NaCl, 20,000 ng of pAdΔF6, 10,000 ng of pAAV2/rh10, and 10,000 ng of mammalian expression vector expressing hNeu3 were mixed and incubated at room temperature for 10 minutes. After they were mixed and waited for 20 minutes, the mixture was added, and the cells were incubated at 37°C for 3.5 hours. 10 ml of DMEM with serum was added to the cells for incubation at 37°C for 16 hours. The following day, the medium was replaced with fresh serum-containing DMEM, and the cells were incubated at 37°C

for 72 hours. The mCherry red signal was monitored using a fluorescence microscope (BX53, Olympus). At the end of the triple transfection, the HEK293 T cells with the AAVrh10-hNeu3 particles were collected by cell-scraping and centrifuging at 500x g for 5 minutes at +4°C (Figure 2.5B) and stored at -80°C for the isolation of the AAVrh10-hNeu3 particles.

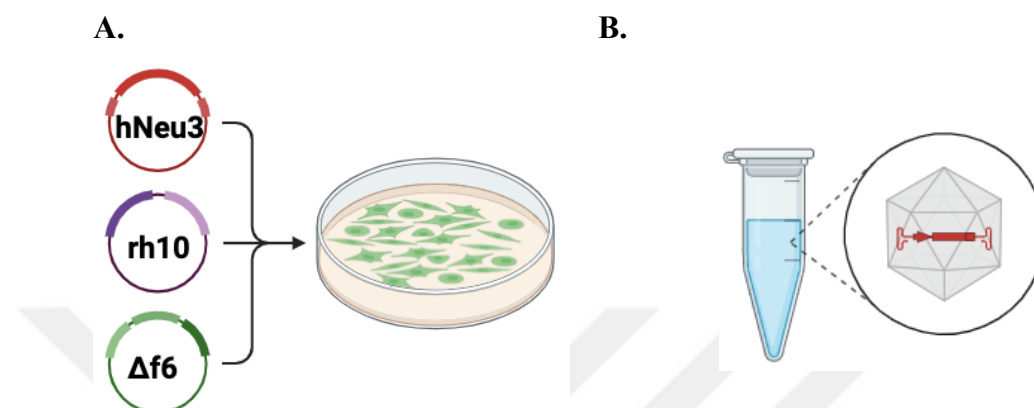


Figure 2.5. The schematic representation of the triple transfection of pAdΔF6, pAAV2/rh10, and mammalian expression vector expressing hNeu3 into HEK293T cells (A) and collection of HEK293T cells containing the AAVrh10-hNeu3 particles (B).

2.1.5. The Purification of the AAVrh10-hNeu3 Particles Using Iodixanol Gradient Centrifugation Method

The HEK293T cell pellets with the AAVrh10-hNeu3 particles collected and stored at -80°C were dissolved in the lysis solution (5M NaCl, 1M Tris-HCl, and ultra-pure dH₂O) by pipetting. The samples were then freeze-thawed by three repetitions of incubation at 37°C for 5 minutes and subsequently at -80°C for 5 minutes to facilitate the lysis. After that, the samples were centrifuged at 1,167 x g for 15 minutes at 4°C, after which the supernatants were collected into new tubes. Benzonase nuclease (Sigma), with a final concentration of 16.67 u/ml for each plate, was added to the supernatants, which were incubated at 37°C for 30 minutes. At the end of the incubation, the samples were centrifuged at 13,490 g for 20 minutes at +4°C, and the resulting supernatants were collected and filtered using 0.45 μm filters. The filtered solutions were ready for purification following the OptiPrep density gradient centrifugation protocol, and the solutions are shown in Table 2.2.

Table 2.2. The solutions required for the purification of the AAVrh10-hNeu3 particles using the Iodixanol gradient centrifugation method.

Solutions	Ingredients
10x PBS solution	40 g NaCl, 1 g KCl, 7.2 g Na ₂ HPO ₄ , and 1.2g KH ₂ PO ₄ , filled up to 500 ml of ultra-pure dH ₂ O
1M MgCl ₂ solution	20.33 g MgCl ₂ .6H ₂ O, filled up to 100 ml of ultra-pure dH ₂ O
1M KCl solution	7.45 g KCl, filled up to 100 ml of ultra-pure dH ₂ O
5x PBS magnesium and potassium solution (PBS-MK)	250 mL 10x PBS, 2.5 mL 1 M MgCl ₂ , and 6.25 mL 1 M KCl, filled up to 500 ml of ultra-pure dH ₂ O
%15 Iodixanol Gradient	12.5 mL OptiPrep density gradient solution, 10 mL 5 M NaCl, and 10 mL 5x PBS-MK filled up to 17.5 mL of ultra-pure dH ₂ O
%25 Iodixanol Gradient	20.8 mL OptiPrep density gradient solution, 10 mL 5x PBS-MK, and phenol red (Sigma), filled up to 19.2 mL of ultra-pure dH ₂ O
%40 Iodixanol Gradient	33.3 mL of OptiPrep density gradient solution, 10 mL 5x PBS-MK, filled up to 6.7 mL of ultra-pure dH ₂ O
%60 Iodixanol Gradient	50 mL of OptiPrep density gradient solution and phenol red (Sigma)

The OptiPrep density gradients using the solutions prepared according to Table 2.2 and ultracentrifugation were done at the Izmir Institute of Technology, Integrated Research Centers, Biotechnology and Bioengineering Research and Application Center, Urla/Izmir (IYTE-BIOMER). To prevent any contamination during the isolation of the AAVrh10-hNeu3 particles, a Class II-type cell culture cabinet where the gradients were prepared in the QuickSeal centrifuge tubes (16x76 mm, Beckman Instruments) was used. The total volume of the QuickSeal centrifuge tubes was 13.1 ml, and they were suitable for the ultracentrifuge (Beckman Coulter). During the OptiPrep density gradient preparation, the iodixanol solutions with 2.882 ml of 15% iodixanol solution, 2.148 ml of 25% iodixanol solution, 2.148 ml of 40% iodixanol solution, and 1.808 ml of 60% iodixanol solution were added to the QuickSeal centrifuge tubes sequentially. On top of the final solution, 4.114 ml of the virus lysate was added, and the resulting gradients are

shown in Figure 2.6A. The tubes were then subjected to ultracentrifugation at 250,000x g and 12°C for 2 hours. After centrifugation was completed, different layers were observed, as shown in Figure 2.6B. The AAVrh10-hNeu3 particles were collected from the 40% layer by puncturing the tube with a syringe. After that, the AAVrh10-hNeu3 particles were concentrated using a Concentrator (100K, Millipore).

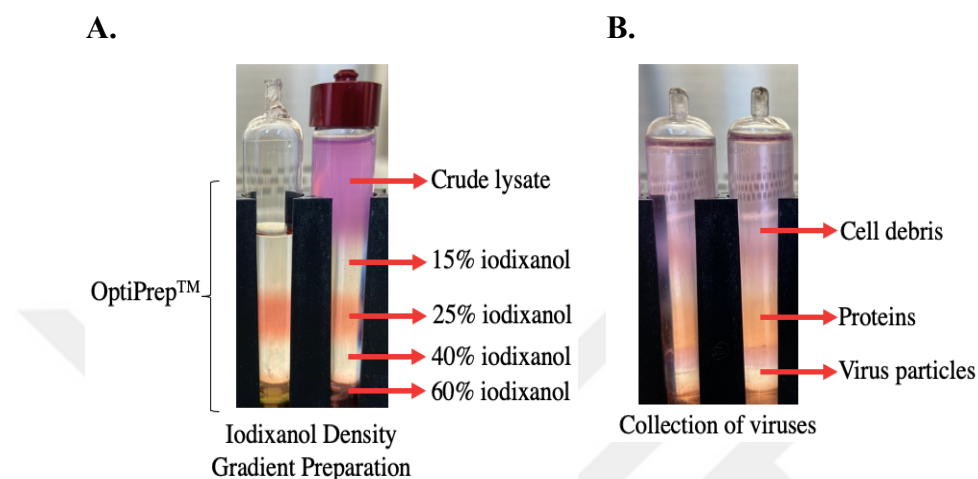


Figure 2.6. The representation of the OptiPrep iodixanol density gradients (A) and collection of the AAVrh10-hNeu3 particles from the 40% layer of iodixanol gradient (B).

2.1.6. The Concentration of the AAVrh10-hNeu3 Particles

The AAVrh10-hNeu3 particles isolated after iodixanol density gradient centrifugation were concentrated and purified using a 100 kDa Concentrator. The concentrator was a filter to remove iodixanol and other molecules below 100 kDa to purify the AAVrh10-hNeu3 particles. The procedure was initiated by centrifuging the filter at 4000x g for 5 minutes with 1x PBS-MK solution (Table 2.2). Subsequently, the AAVrh10-hNeu3 particles were mixed with 5 mL of 1x PBS-MK solution and added to the filter. The filter with the AAVrh10-hNeu3 particles was centrifuged at 4000x g till the final volume became 1 ml. 13 ml of 1x PBS-MK solution was added to the obtained solution and centrifuged, and these steps were repeated three times. Finally, to prevent aggregation of the AAVrh10-hNeu3 particles, a non-ionic detergent, 0.01% Pluronic F68 (v/v), in 13 ml of PBS, was added to the AAVrh10-hNeu3 particles in the concentrator. Until the final volume reached 300 μ L, the centrifugations were continued. The resulting

solutions, the AAVrh10-hNeu3 particles, were collected into a new tube and stored at -20°C.

2.1.7. Quantification of the AAVrh10-hNeu3 Particles

The quantification of AAVrh10-hNeu3 particles was performed by RT-PCR using specific primers for CMV promoters. Before the RT-PCR analysis, the cellular and viral proteins were removed from the solution. 2 µl of the collected AAVrh10-hNeu3 particles were diluted in 24.5 µl of dH₂O. This mixture was added 0.5 µl of DNase I (Thermo Scientific) and 3 µl of 10x DNase Buffer (Thermo Scientific) solution. The mixture was incubated at 37°C for 30 minutes, after which the reaction was stopped using 6 µl of EDTA (25 mM, Thermo Scientific) by incubating it at 65°C for 10 minutes. Later, 4 µl of 10x Proteinase K buffer (Sigma) and 1 µl of Proteinase K (PK) were added to the previous solution to degrade the proteins. The resulting 36 µl solution was incubated at 50°C for 60 minutes and then at 95°C for 20 minutes.

Table 2.3. The calculations required for the quantification of the AAVrh10-hNeu3 particles using the standard curve graph.

Formula 1	Plasmid molecular weight [g/mol] = plasmid size [bp] x 650 [Da/bp] 6724 bp x 650 Da/bp = 4370600 Da
Formula 2	Mole of plasmid in microliters = plasmid concentration [g/µl] / Molecular weight of plasmid [g/mol] $516 \times 10^{-9} \text{ g/}\mu\text{l} / 4370600 \text{ g/mol} = 11,806 \times 10^{-17}$
Formula 3	Molecule number of plasmid in microliters = Mole of plasmid in microliters [mol/µl] x Avogadro number [molecule/mol] $11806 \times 10^{-17} \text{ mol/}\mu\text{l} \times 6.022 \times 10^{23} = 7.1 \times 10^{10} \text{ molecule/}\mu\text{l}$
Formula 4	Plasmid Stock (100 µl) = (required concentration [molecule/µl] x 100 µl) / plasmid molecules [molecule/µl] $(1 \times 10^9 \text{ molecule/}\mu\text{l} \times 100 \mu\text{l}) / 7.1 \times 10^{10} \text{ molecule/}\mu\text{l} = 1.408 \mu\text{l (up to 100 } \mu\text{l)}$

(cont. on next page)

Table 2.3 (cont.).

	$1.4 \mu\text{l} + 98.6 \mu\text{l dH}_2\text{O} = 1 \times 10^9 \text{ vg}/\mu\text{l} = 4.48 \text{ ng}/\mu\text{l}$ $10 \mu\text{l from } 1 \times 10^9 \text{ vg}/\mu\text{l} + 90 \mu\text{l dH}_2\text{O} = 1 \times 10^8 \text{ vg}/\mu\text{l} = 7.405 \text{ ng}/\mu\text{l}$ $10 \mu\text{l from } 1 \times 10^8 \text{ vg}/\mu\text{l} + 90 \mu\text{l dH}_2\text{O} = 1 \times 10^7 \text{ vg}/\mu\text{l} = 10.93 \text{ ng}/\mu\text{l}$ $10 \mu\text{l from } 1 \times 10^7 \text{ vg}/\mu\text{l} + 90 \mu\text{l dH}_2\text{O} = 1 \times 10^6 \text{ vg}/\mu\text{l} = 14.405 \text{ ng}/\mu\text{l}$
Formula 5	Standard Curve Equation, $y = -1.512\ln(x) + 36,505$
Formula 6	Titer = $e^{(C_t-b)/a} \times 2$ (for ssAAV)

RT-PCR for CMV was performed by first preparing standards using a plasmid of known concentration for drawing a standard curve, as shown in Table 2.3. The AAVrh10-hNeu3 particles made ready by DNase I and PK treatments were quantified using the Roche-LightCycler® 480 system with LightCycler 480 SYBR Green I Master Mix (Roche) and CMV promoter-specific primers (10 mM) with the sequences shown in Table 2.4.

Table 2.4. Primer sequences specific for the CMV promoter region for CMV RT-PCR

Gene	Primer	Primer Sequences
CMV	CMV-F	5'-TCATATGCCAAGTACGCCCC-3'
	CMV-R	5'-CCCGTGAGTCAAACCGCTAT-3'

Using the optimized conditions as follows: 95°C for 10 minutes, followed by 45 cycles of 95°C for 20 seconds, 58°C for 15 seconds, and 72°C for 22 seconds in the Roche-LightCycler® 480 RT-PCR device, the expression levels of CMV were measured. The expression values obtained were used to calculate the virus quantity as viral genome per μl using the standard curve graph and equation shown in Figure 2.7, which was used to perform transduction experiments with equal amounts of the AAVrh10-hNeu3 particles. For intrathecal injections of $2.04 \times 10^{11} \text{ vg}/\mu\text{l}$ of AAVrh10-hNeu3 particles to *Hexa-/-Neu3-/-* mice, 30 μl of the sample was used in this thesis study according to calculations represented in Figure 2.7. Before the *in vivo* studies, the *in vitro* transduction assays were done to validate the expression of mCherry reporter gene along with the AAVrh10-hNeu3 particles.

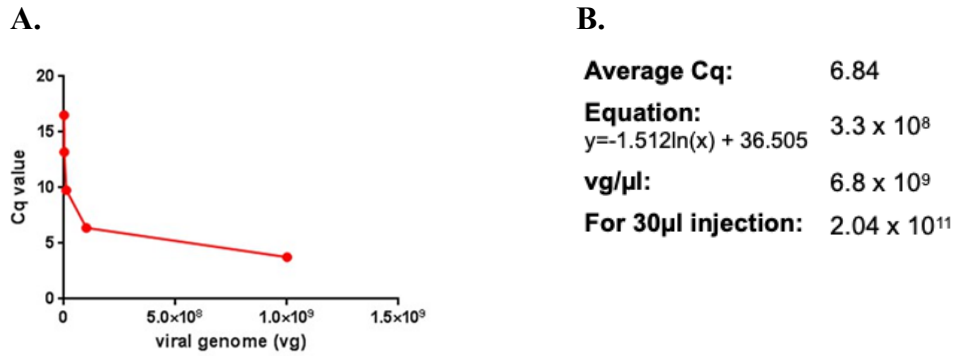


Figure 2.7. The standard curve (A) was obtained using RT-PCR primers targeting the CMV promoter in the hNeu3 plasmid. Virus titer as viral genome per microliter (vg/ μ l) and vg/ μ l for intrathecal injection of *Hexa*^{-/-}*Neu3*^{-/-} mice were calculated on average for Cq values and standard curve equation (B).

2.2. Transduction Analyses for the AAVrh10-hNeu3 Particles

2.2.1. Transduction of HEK293T Cells with the AAVrh10-hNeu3 Particles

The transduction test was first applied to HEK293T cells using a Multiplicity of Infection (MOI) of 10,000 AAVrh10-hNeu3 particles per 100,000 HEK293T cells, which were counted and plated to a 24-well cell-culture plate. According to the equation, AAV Genome copy particles used = Multiplicity of infection (MOI) x # of cells infected, 10^9 AAV genome copy (GC) particles were used to infect 100,000 HEK293T cells. Since one μ l of the original stock contained a 6.8×10^9 vector genome of AAVrh10-hNeu3 particles, to infect the HEK293T cells, 0.5 μ l from the original stock was added to get 10^9 AAV genome copies. Before the transduction assay, the serum-containing DMEM was removed from the HEK293T cells, and the cells were washed in 1x PBS for the complete removal of the remaining DMEM. Later, 0.5 μ l from the original stock of AAVrh10-hNeu3 particles dissolved in 500 μ l DMEM with 2% FBS and 1% Penicillin/Streptomycin was added to the HEK293T cells. The cells were incubated with virus-containing media for approximately 20 hours. The next day, DMEM with 10% FBS and 1% Penicillin/Streptomycin was added to the infected cells to incubate for 7 days. After 7 days of transduction, the mCherry expression was obtained using a fluorescence microscope (BX53, Olympus).

2.2.2. Transduction of the Neuroglia Cells of *Hexa*^{-/-}*Neu3*^{-/-} mice with the AAVrh10-hNeu3 Particles

Additionally, the neuroglia cells of 20-week-old *Hexa*^{-/-}*Neu3*^{-/-} mice, which were isolated with the MACS Adult Brain Dissociation Kit (Miltenyi Biotec) according to the manufacturer's protocol, were transduced using 500,000 MOI of AAVrh10-hNeu3 particles per 100,000 neuroglia cells in a 24-well cell culture plate. The use of higher MOI was to increase the transduction efficacy of the neuroglia cells; therefore, 1.5 µl from the original stock of AAVrh10-hNeu3 particles dissolved in 500 µl DMEM with 2% FBS and 1% Penicillin/Streptomycin, was used to get 5×10^{10} GC for infection, which was calculated from the same equation used for the transduction of HEK293T cells. The same procedure applied for the transduction of the HEK293T cells was performed for the transduction of the neuroglia cells of 20-week-old *Hexa*^{-/-}*Neu3*^{-/-} mice. At the end of 7 days of transduction, the mCherry expression was obtained using a fluorescence microscope (BX53, Olympus).

2.2.3. Confirmation of the Transduction Using Anti-GM2 Staining

The neuroglia cells of 20-week-old *Hexa*^{-/-}*Neu3*^{-/-} mice transduced with the AAVrh10-hNeu3 particles were used to confirm the transduction efficacy by performing anti-GM2 staining. For this purpose, the medium containing virus particles was removed, and the cells were washed with 1x PBS for 5 minutes at room temperature. Then, the cells were incubated in 4% paraformaldehyde (PFA, Sigma) dissolved in 1x PBS (pH 7.4) at room temperature for 30 minutes. The cells were washed in 1x PBS for 5 minutes to remove the remnants of the PFA solution. 500 µl of 1x PBS containing 0.5% Triton X-100 (Sigma) was added to the cells for membrane permeabilization and incubated at room temperature for 30 minutes. After this incubation, the solution was removed, and the cells were incubated in a blocking buffer (0.3% Triton X-100, 0.3M Glycine, 4% BSA in mg, and 10% normal goat serum in 1xPBS) for 1 hour at room temperature. Afterward, the sections were incubated with 50 µl of the primary antibody (KM966) diluted in blocking buffer (1:250) at +4°C overnight. The next day, the cells were incubated in the secondary antibody, Goat anti-Human Cross DyLight-488 (Thermo Scientific, 1:250), in the blocking solution for 1 hour at room temperature in the dark after removing the primary

antibody. The cells were washed thrice in 1x PBS for 5 minutes each and then mounted with Fluoroshield mounting medium containing DAPI (Abcam). Then, they were covered with coverslips and stored at +4°C until fluorescence images were taken using a fluorescence microscope (BX53, Olympus). The fluorescent images were taken with specific filters (green for anti-GM2 and blue for DAPI), and analyzed by the NIH Image J program (1.48v).

2.3. Animals

Hexa^{-/-} mice were generated by the backcrossing of *Neu4*^{-/-}*Hexa*^{-/-}*Neu1*^{-/-} triple knockout mice with wild-type C57/Black6 mice.¹³⁹ To maintain a colony with the same genetic background, brothers and sisters with the *Hexa*^{-/-} genotype were mainly mated. The mice with Neu3 deficiency, *Neu3*^{-/-}, were obtained by deleting a region of exon 3 within the *Neu3* and donated by Prof. Taeko Miyagi (Division of Cancer Glycosylation Research, Institute of Molecular Biomembrane and Glycobiology, Tohoku Pharmaceutical University, Sendai, Japan).¹⁴⁰ Early-onset Tay-Sachs disease mice, *Hexa*^{-/-}*Neu3*^{-/-}, were generated as previously described.³² Since *Hexa*^{-/-}*Neu3*^{-/-} mice were sterile, female *Hexa*^{+/-}*Neu3*^{-/-} and male *Hexa*^{+/-}*Neu3*^{-/-} mice were bred to obtain a colony (Table 2.5). All mice were housed under stable temperature and humidity on a 12-hour light-dark cycle as a group of five in a cage and mated in the Turkish Council on Animal Care (TCAC) accredited animal facility of Izmir Institute of Technology according to the TCAC guidelines. The mice were given food and water *ad libitum*. The experiments performed in the thesis were done following the Turkish Institute of Animal Health guide and approved by the Institutional Animal Care and Use Committee of the Izmir Institute of Technology.

2.4. DNA Isolation for Genotyping

Genotyping by Polymerase Chain Reaction (PCR) with specific primers for *Hexa* and *Neu3* requires the isolation of genomic DNA from mice tails. For genomic DNA isolation from the tails of mice, an already optimized protocol in our laboratory was used. After weaning mice at one month, their tails were taken and incubated in 250 µl of tissue lysis buffer (10% 1M Tris pH: 7.6, 2.5% 0.2M EDTA, 20% SDS, 4% 5M NaCl) with 6

μl of proteinase K solution (20 mg/ml, EcoTech Biotechnology) overnight in a shaking incubator at 55°C and 70 rpm. The next day, the tails were centrifuged for 10 minutes at 13,000 rpm. The supernatants were transferred to a new tube including 1 volume of 100% isopropanol to precipitate DNA by gently inverting and centrifuging the tubes at 15,000 rpm for 1 minute. Then, precipitated DNA pellets were washed in 1 volume of 70% ethanol by centrifuging at 15,000 rpm for 1 minute. After discarding 70% ethanol and air-drying the remaining solution, the genomic DNA pellets were dissolved in 100 μl of dH₂O and incubated at 55°C for 1 hour. The genomic DNAs were stored at -20 °C for later use in genotyping by PCR.

2.4.1. Genotyping of Mice

The genotypes of the mouse groups were determined using PCR with primers specific for the wild-type and mutant alleles of *Hexa* and *Neu3* independently. For this aim, the isolated genomic DNAs and previously designed primers of *Hexa* and *Neu3* were used in the PCR mixtures. The optimized conditions of *Hexa* PCR were 1 cycle of 3 minutes at 94°C; 35 cycles of 45 seconds at 94 °C, 45 seconds at 60 °C, 1 minute at 72 °C; and 1 cycle of 10 minutes at 72°C. The ingredients of *Hexa* PCR were 100 ng of genomic DNA mixed with 0.4 mM of HexaF, HexaR, and PGKR primers (Table 2.5), 0.4 mM of each dNTP, 10X reaction buffer with 2 mM MgCl₂, and 2.5 units of Taq polymerase (GeneDireX). The conditions optimized for *Neu3* were 1 cycle of 3 minutes at 94 °C; 35 cycles of 50 seconds at 94 °C, 45 seconds at 58 °C, 2 minutes at 72 °C; and 1 cycle of 10 minutes at 72°C. The *Neu3* PCR mixture was prepared by adding 100 ng of genomic DNA, 0.4 M of Neu3F, Neu3R, and NeoR primers (Table 2.5), 0.4 mM of each dNTP, 10X reaction buffer with 2 mM MgCl₂, and 2.5 units of Taq polymerase (GeneDireX). The PCR products of either *Hexa* or *Neu3* PCR were visualized by running the samples on a 1% agarose gel for 30 minutes at 95 Volts.

Table 2.5. The sequences of the primers for genotyping

Gene	Primer	Primer Sequence	Product Size
<i>Hexa</i>	HexaF	5'-GGCCAGATACAATCATACAG-3'	WT allele: 420 bp KO allele: 210 bp
	HexaR	5'-CTGTCCACATACTCTCCCCACAT-3'	
	PGKR	5'-CACCAAAGAAGGGAGCCGGT-3'	
<i>Neu3</i>	Neu3F	5'-AAGCAGAGAACATTCTTGAGAGAGCACAGC-3'	WT allele: 1022 bp KO allele: 601 bp
	Neu3R	5'-TCGTGCTTTACGGTATCGCCGCTCCCGATT-3'	
	NeoR	5'-GTGAGTTCAAGAGCCATGTTGCTGATGGTG-3'	

2.5. Injections

2.5.1. Administration of AAVrh10-hNeu3 Particles

The intrathecal injections of 1x PBS (vehicle) or AAVrh10-hNeu3 (abbreviated as AAV) were utilized using 8-week-old *Hexa*^{-/-}*Neu3*^{-/-} mice. Before the intrathecal injections, the mice were anesthetized by the intravenous injection of a mixture of Xylazine and Ketamine. Then, the spinal region of the mice was cleansed of fur with a razor, and the area was sterilized with 70% ethanol and Batticon. A small incision was opened in the spinal region to expose the L5-6 lumbar region, by which 30 µl of 1x PBS (Figure 2.8A) or 30 µl from the original stock containing 2.04x10¹¹ vg/µl of AAV (Figure 2.8B) was administered using an insulin syringe. After completing the injection, the incision was closed with the URGO spray. The *Hexa*^{-/-}*Neu3*^{-/-} mice injected with either AAV or the vehicle were housed individually in new, clean cages to monitor their recovery and weekly weight changes. A prolonged lifespan of mice up to 28 weeks was observed.

2.5.2. Intraperitoneal Injection of Istradefylline

Two weeks after the intrathecal administration of AAV, when the *Hexa*^{-/-}*Neu3*^{-/-} mice reached 10 weeks of age, half of them were administered Istradefylline (SML0422, Sigma), an inflammatory agent. Istradefylline, which was prepared at a concentration of 5mg/ml, was injected intraperitoneally into mice for 21 days (Figure 2.8C). A prolonged lifespan of mice up to 28 weeks was observed.

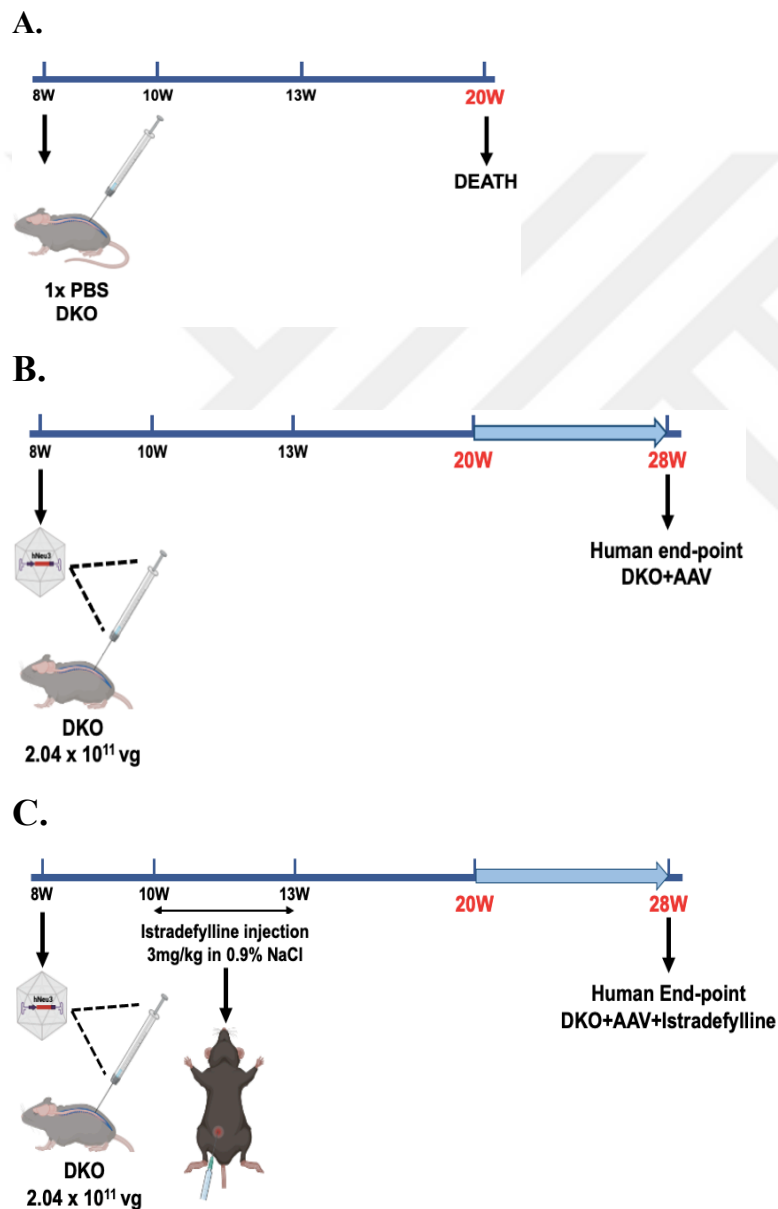


Figure 2.8. The representation of the intrathecal administration of either 1x PBS (vehicle) (A) or AAVrh10-hNeu3 (AAV) (B) and the combined treatment of AAV and Istradefylline (C) to *Hexa*^{-/-}*Neu3*^{-/-} mice was shown.

2.6. Experimental Groups

In this thesis study, 20-week-old *Hexa*^{-/-} (abbreviated as SKO) mice were used to compare the results with the 20-week-old *Hexa*^{-/-}*Neu3*^{-/-} (abbreviated as DKO) mice. Since the DKO mice administered with AAV with or without Istradefylline lived up to 28 weeks of age, 28-week-old SKO mice were used as the other age-matched control. Therefore, the experimental groups for the study were 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and 20- and 28-week-old DKO mice administered with AAV and Istradefylline.

2.7. Body Weight Measurements

Weekly body weight measurements of DKO mice (n=15) injected with were started at 8 weeks of age and continued weekly until their death at 20 weeks. For DKO mice administered with AAV (n=6) and those administered with AAV and Istradefylline (n=6), the body weight measurements were initiated at 8 weeks when the AAV injections were performed and continued weekly until 28 weeks. The body weight of SKO control mice (n=20) was also measured weekly until they reached 28 weeks of age. The body weight of both female and male mice was calculated for each mouse group.

2.8. Tissue Handling

The brain and visceral organs of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and 20- and 28-week-old DKO mice administered with AAV and Istradefylline were obtained by performing tissue dissection or PFA fixation.

2.8.1. Tissue Dissection

The cortex, cerebellum, liver, kidney, and spleen tissues of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and 20- and 28-week-old DKO mice administered with AAV and Istradefylline

were used in this thesis study for molecular biological and biochemical analyses. All mouse groups were sacrificed using CO₂ at the specified ages. After removing the total brain, the cortex and cerebellum tissues were dissected into their right and left hemispheres. The dissected cortex, cerebellum tissues, and visceral organs were frozen at -80°C.

2.8.2. Fixation

To perform immunohistochemical analyses, 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and 20- and 28-week-old DKO mice administered with AAV and Istradefylline were PFA-fixed at their specified ages. At first, the mice were anesthetized with the intraperitoneal injection of a mixture of Xylazine and Ketamine. An incision was made on the rib cage of anesthetized mice to open the thoracic cavity and expose their heart. After cutting the top-right atrium of the heart, a needle was inserted into the bottom-left ventricle, through which 0.9% NaCl solution (pH 7.4) was sent to the circulation system to drain the blood from the organs. Then, 4% PFA dissolved in 1x PBS (pH 7.4) was replaced with NaCl for the fixation of the mice. The fixed brain was incised and incubated in 4% PFA dissolved in 1x PBS (pH 7.4) overnight at +4°C. The next day, the sucrose gradient was conducted through incubation of the fixed brains in 10% and 20% sucrose in 1x PBS (pH 7.4) consecutively for 2 hours in each solution at +4 °C. The fixed brains were then incubated in 30% sucrose in 1x PBS (pH 7.4) overnight at +4 °C, after which they were embedded in OCT (optimal cutting temperature) and stored at -80 °C. The OCT-embedded fixed brains for each experimental group were cut coronally in 10 µm thicknesses using a Leica Cryostat (CM1850-UV, Leica) at -20°C, and sections were collected on adhesive-coated HistoBond slides. The brain sections were stored at -80 °C for the analyses that will be performed in this thesis study.

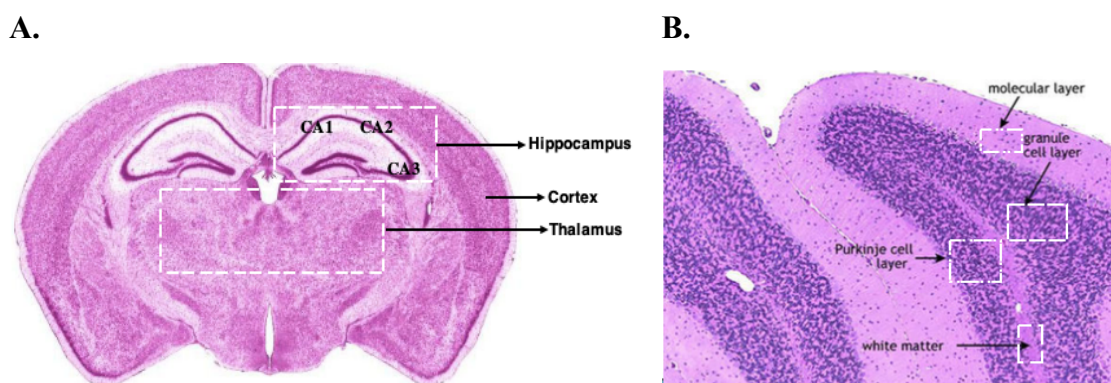


Figure 2.9. The coronal sections of the mouse brain showing different regions of the cortex, hippocampus, thalamus (A), and cerebellum (B) by courtesy of Woods et al.¹⁴¹

2.9. Ganglioside Isolation and Thin Layer Chromatography (TLC) analysis

Isolation of the acidic and neutral gangliosides and Thin Layer Chromatography (TLC) analysis were conducted using the cortex of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and 20- and 28-week-old DKO mice administered with AAV and Istradefylline. The isolation protocol was applied as described previously.¹⁴²

2.9.1. Neutral and Acidic Ganglioside Isolation

Using 50 mg of cortex from each experimental group, ganglioside isolation was performed. First, tissue samples were placed in borosilicate tubes with 2 mL dH₂O for homogenization using an Ultra-Turrax homogenizer (IKA T10, Sigma) at 6000 rpm for 30 seconds. After homogenization, the samples were sonicated using a sonicator (Bandelin-Sonopuls) for four cycles of 1.5 minutes each. The sonicated samples were then dried using nitrogen gas (N₂ flow) and heated at 55°C for rapid evaporation. The resulting pellets were dissolved in 3 mL of 100% acetone by vortexing and centrifuged at 2000 rpm for 5 minutes. This step was repeated to completely remove phospholipids and membrane lipids. The pellets were then dissolved in 1.5 mL of chloroform: methanol: water (10:10:1) mixture, vortexed, and centrifuged at 2000 rpm for 5 minutes. The

resulting upper phases were drawn off into new neutral glass tubes. These steps were repeated twice for the initial extraction of total gangliosides. The pellets were dissolved in 2 mL of chloroform: methanol: water (30:60:8) mixture, vortexed, and centrifuged at 2000 rpm for 5 minutes. The resulting supernatants were put into the tube containing the previously collected supernatants. These steps were also repeated twice for the final collection of total gangliosides.

The collected supernatants were used for the separation of acidic and neutral gangliosides through DEAE Sephadex A-25 ion exchange columns. For these columns, 1 gram of DEAE Sephadex A-25 resin (GE Health Care) was incubated in 10 mL of chloroform: methanol: 0.8M sodium acetate (30:60:8) mixture for 5 minutes. The chloroform: methanol: 0.8M sodium acetate mixture was removed from the resin, and the resin was incubated in the same mixture two more times. After that, the resin was incubated overnight in 10 mL of chloroform: methanol: 0.8M sodium acetate (30:60:8) mixture at room temperature. When the initial activation of the resin was done, the next day, it was washed in 10 mL of chloroform: methanol: water (30:60:8) mixture twice and left in the same solution for the acidic-neutral ganglioside separation. DEAE Sephadex A-25 ion exchange columns were prepared by closing the tip of Pasteur pipettes with some glass woolen and then loading approximately 2 ml of freshly prepared DEAE Sephadex A-25 resin on top. When the columns were washed with 1 ml solution of chloroform: methanol: water (30:60:8), new neutral tubes were placed under the columns, and the total ganglioside samples were loaded over the resin. 4 ml of 100% methanol was placed onto the columns and collected in the neutral tubes. The solutions collected in the neutral tubes were stored at +4 °C for later use as neutral gangliosides. To obtain acidic gangliosides, 5 ml of 500 mM potassium acetate in methanol was put onto the resins, and flow-through was collected into the new neutral tubes below.

The desalting procedure was applied for the acidic gangliosides using the Supelclean LC-18 column (Supelco, Sigma) through the Chromabond Vacuum Manifold (Macherey-Nagel) set to 5Hg. Before desalting, the LC-18 columns were equilibrated by washing them with 2 mL of methanol and 2 mL of 500 mM potassium acetate in methanol sequentially. Onto the LC-18 columns, the acidic gangliosides were loaded and allowed them bind to the column resins. The columns were then washed using 10 ml of dH₂O, after which new neutral tubes were located under the columns for elution of desalted acidic gangliosides with 4 ml of methanol and then 4 ml of chloroform: methanol (1:1). The solutions used for the elution of acidic gangliosides were vaporized using an N₂ flow

and water bath at +55°C for rapid evaporation. The resulting pellets were stored at +4 °C for the Thin Layer Chromatography (TLC) analysis.

2.9.2. Thin Layer Chromatography (TLC)

TLC analyses for this thesis study were conducted to characterize acidic gangliosides using the differences in their weights and structures in the cortex of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and 20- and 28-week-old DKO mice administered with AAV and Istradefylline. First, a TLC tank was readied by incubating a TLC running buffer, a mixture of chloroform, methanol, and 0.2% CaCl₂ in a 30:65:8 ratio for 2.15 hours at room temperature. Moreover, a silica TLC plate (20cm x 20cm, Merck) was dehumidified at 100°C for 30 hours before loading the samples. Once the TLC tank and the silica plate were arranged, the acidic gangliosides were prepared by dissolving the pellets in 100 µl of chloroform: methanol: water (10:10:1). 45 µl of the samples were loaded onto the silica plate automatically using a Hamilton syringe with the Linomat 5 (Camag) machine. After finishing the loading of each experimental group and a ganglioside marker, the silica plate was placed into the TLC tank, which was incubated with the TLC running buffer to run the samples at room temperature.

2.9.3. Orcinol Staining of the TLC Plates

To visualize the migration of acidic gangliosides and identify the differences among the experimental groups, the orcinol (Sigma) stain was freshly prepared by mixing 0.06 grams of orcinol, 3.75 ml of sulfuric acid, and 11.25 ml of dH₂O in a TLC sprayer (Sigma). Using the sprayer, the orcinol stain was sprayed over the TLC plate, and then the plate was put onto a TLC plate heater (Camag) heated at 120°C to make the acidic ganglioside bands visible. The silica plate was scanned using the HP scanner system. The bands of each sample were compared with a mixture of brain ganglioside standards (Avanti Polar Lipids), and the differences in band intensities were measured using the NIH ImageJ (1.48v) program.

2.10. The mRNA Expression Analysis by total RNA isolation and Real-Time PCR (RT-PCR)

Total RNA isolation and Real-Time PCR (RT-PCR) analyses were utilized using the cortex, cerebellum, kidney, liver, and spleen tissues collected from 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, 20- and 28-week-old DKO mice administered with AAV and Istradefylline to evaluate the differences among the relative gene expression levels of inflammation-related markers, lysosomal marker, and human *NEU3* (hNeu3).

2.10.1. Total RNA isolation

Total RNA isolation was started by homogenizing the 30 ng of tissue samples in 250 μ l of Trizol Reagent (ABM) in 2 ml Eppendorf tubes containing RNase-free beads. To homogenize the tissues, the tissue homogenizator (Retsch MM100) was run for 30 seconds at a frequency of 1⁻²⁵. The homogenized samples were then incubated in Genezol Reagent for 5 minutes at room temperature and transferred into new 1.5 ml Eppendorf tubes. 1/5 volume of chloroform was added over the supernatants for phase separation, and the mixtures were centrifuged for 15 minutes at +4°C and 12000xg for phase separation. The liquid phase containing the total RNAs was transferred into the new tubes, and 1/2 volume of 100% isopropanol was added for RNA precipitation after incubating the samples at room temperature for 10 minutes and centrifuging them for 10 minutes at 12000xg and +4°C. The resulting pellets of total RNAs were then washed with 1 volume of 70% ethanol by centrifuging them at 7500xg for 5 minutes at +4°C. After discarding and then air-drying the 70% ethanol from the RNA pellets, they were resuspended in 20 μ l of nuclease-free dH₂O and incubated in a water bath at 50°C for 10-15 minutes. The RNA concentrations were measured using a Nanodrop spectrophotometer (IMPLEN) at 260nm, and the RNA samples were stored at -80°C until the cDNA synthesis and RT-PCR analysis.

2.10.2. The cDNA Synthesis

The cDNA synthesis from the RNA samples was performed using the iScript™ cDNA Synthesis Kit (BioRad), by which 50 ng/μl of total cDNA was converted from each sample. The reverse transcription reactions were prepared as a total mixture of 20 μl using 1x RT buffer, 1x RT Random primers, and 50 units of iScript reaction with suitable amounts of RNAs and RNase-free dH₂O. The RT reaction was one cycle of 5 minutes at 25°C, one cycle of 20 minutes at 46°C, and one cycle of 1 minute at 95°C. After cDNA conversion was completed, the PCR for the housekeeping gene, GAPDH, was performed with 50 ng of each cDNA, preparing 25 μl of reaction with 0.8 mM of GAPDH gene primers, 10 mM of each dNTP, 1x reaction buffer with MgCl₂, and 1.75 units of GeneDireX Taq Polymerase. The PCR reaction was run for one cycle of 2 minutes at 95 °C, 30 cycles of 20 seconds at 95 °C, 15 seconds at 65 °C, 22 seconds at 72 °C, and one cycle of 3 minutes at 72 °C. The bands were visualized after running the samples on 1% agarose gel for 30 minutes at 95 Volts.

2.10.3. Real-Time PCR

The cDNAs of each experimental group were used for analyzing the mRNA expression levels of the genes given in Table 2.6 using RT-PCR. For the RT-PCR reaction, the Roche LightCycler 96 system was used. A reaction of a total of 20 μl including 1x Roche LightCycler 480 SYBR Green I Master Mix, mixed with 0.4 μM of each primer for the genes shown in Table 2.6 and 50 ng of cDNA was directed to 1 cycle of 10 minutes at 95°C and 45 cycles of 20 seconds at 95°C; 15 seconds at 60°C; and 22 seconds at 72°C. Each sample was loaded into 96-well plates in three replicates for averaging the expression values.

Table 2.6. The RT-PCR primer sequences used for mRNA expression analyses

Gene	Primer Sequence
CCL2	F:5'-ATGCAGTTAATGCCCCACTC -3' R:5'-TTCCTTATTGGGGTCAGCAC -3'
CCL3	F:5'-TCTGTACCATGACACTCTGC -3' R:5'- AATTGGCGTGGAATCTTCCG-3'
CCL5	F:5'-AGTGCTCCAATCTTGCAGTC-3' R:5'-AGCTCATCTCCAAATAGTTG-3'
CXCL10	F:5'-CACCATGAACCCAAGTGCTGCCGT-3' R:5'-AGGAGCCCTTTTAGACCTTTTTTG-3'
IL1 β	F:5'-TGAGTCACAGAGGATGGGCTC-3' R:5'-CCTTCCAGGATGAGGACATGA-3'
IL-6	F:5'-AAGTGCATCATCGTTGTTTCATACA-3' R:5'-GAGGATACCACTCCCAACAGACC-3'
IBA1	F: 5'-TCTGCCGTCCAAACTTGAAGCC-3' R: 5'-CTCTTCAGCTCTAGGTGGGTCT-3'
GFAP	F:5'-AGTAACATGCAAGAGACAGAG - 3' R:5'- TAGTCGTTAGCTTCGTGCTTG-3'
Lamp1	F:5'- CCAGGCTTTCAAGGTGGACAGT-3' R:5'- GGTAGGCAATGAGGACGATGAG-3'
hNeu3	F:5'-TTGGGCAGTTGGTACAGTGG-3' R:5'-TACGCAGGGATGACCAGTCT-3'
GAPDH	F: 5'-CCCCTTCATTGACCTCAACTAC-3' R: 5'-ATGCATTGCTGACAATCTTGAG-3'

2.11. The Protein Expression Analysis by protein isolation and Western Blot

Total protein isolation and Western Blot were performed using the cortex, cerebellum, kidney, liver, and spleen tissues collected from 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV,

and 20- and 28-week-old DKO mice administered with AAV and Istradefylline to evaluate the differences among the protein expression levels of inflammation-related markers.

2.11.1. Protein Isolation

Total proteins were isolated from each experimental group by homogenizing 30 µg of tissue samples in 100 µl of tissue lysis buffer (1% Triton X-100, 50 mM HEPES, 150 mM NaCl, 10% Glycerol, 50 mM Tris-Base, 1% PMSF, and 1% protease inhibitor) using a mini homogenizer. Then, homogenized samples were incubated on ice for 1 hour and vortexed every 10 minutes during this incubation. The samples were centrifuged after incubation for 15 minutes at 14000 rpm and +4 °C to collect the supernatant with total proteins into new tubes. These proteins were stored at -80 °C to prolong their use.

2.11.2. Bradford Assay

The concentration of isolated proteins was quantified with the Bradford assay. First, the proteins diluted with dH₂O, different concentrations of BSA (100µg/ml, 80µg/ml, 40µg/ml, and 20µg/ml), blank samples (dH₂O and tissue lysis buffer) were loaded into the 96-well plates in 3 replicates for each. 200 µl of the 5x Bradford reagent (SERVA Electrophoresis) was added over the protein samples, and the mixtures were incubated for 10 minutes at room temperature. Later, the absorbances were measured using the iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories) at 595 nm. Using the BSA absorbances in different concentrations, a BSA standard curve was drawn, and protein concentrations were calculated using the standard curve. Each protein sample was prepared as a stock of 20 µg protein in dH₂O mixed with loading buffer (40% Glycerol, 24 0mM Tris-HCl pH 6.8, 8% SDS, 0.04% Bromophenol Blue, and 5% β-mercaptoethanol) for Western Blot analyses. Before loading the protein samples to SDS-PAGE, they were boiled at 95°C for 10 minutes. They were stored at +4 °C for further use.

2.11.3. Western Blot

The stacking (upper) resolving (lower) polyacrylamide gels for Western Blot were prepared using the amounts of ingredients written in Table 2.7 below. At first, the resolving gel was poured between 2 glass plates and waited for its polymerization at room temperature. After that, the stacking gel was poured over the resolving gel, and the combs were placed in the gel for the formation of wells. When the stacking gel became solidified at room temperature, the combs were removed to load protein samples. The gel was placed into the running buffer (0.25 M Tris-Base, 1.92 M Glycine, and 1% SDS), and 12 μ l of protein stocks were loaded into the wells. The proteins were run at 80V for ~2 hours at room temperature. For transferring the resulting protein bands onto the 0.2 μ m pore-sized nitrocellulose paper (Bio-Rad Laboratories), a transfer sandwich system of the protein gel and nitrocellulose membrane was prepared. The transfer reaction was utilized in a transfer buffer containing 48 mM of Tris-Base, 39 mM of Glycine, and 20% Methanol (pH 9.2) at 0.25A for 1 hour. The resulting protein blots were then blocked using 5% milk in 1x PBS-T (0.05% Tween 20) for 1 hour at room temperature. After removing the blocking buffer with 1x PBS-T wash for 5 minutes thrice, the blots were readied for primary antibody incubation at +4 °C overnight. The primary antibodies, anti-NF κ B (Cell Signalling), anti-I κ B- α (Cell Signalling), anti-IL-1 β (Cell Signalling), and anti- β -actin (Cell Signalling) were diluted in a dilution of 1/1000 in a red solution (5% BSA, 0.02% NaAzide, Phenol Red, in PBS-T pH 7.5). After the primary antibody incubation, the blots were washed with 1x PBS-T wash for 5 minutes thrice to remove excess antibodies. They were then incubated in a secondary antibody, HRP-conjugated secondary antibody (Jackson ImmunoResearch Lab), for 1 hour at room temperature. The excess secondary antibody was removed from the blots by washing them in 1x PBS-T wash for 5 minutes three times. The specific protein bands were visualized by a chemiluminescence imaging system (Fusion SL, Vilber) using LuminataTM Forte Western HRP Substrate (Millipore). The band intensities were measured by the NIH Image J program (1.48v).

Table 2.7. The quantities of the reagents for the preparation of resolving gel (10%) and stacking gel (5%)

Resolving Gel (10%)	Stacking Gel (5%)
3 ml Lower buffer (1.5M Tris-HCl, pH: 8.8)	1.5 ml Upper buffer (1M Tris-HCl, pH: 6.8)
4 ml Acrylamide (30%)	1 ml Acrylamide (30%)
5 ml Water	3.5 ml Water
60 µl SDS (10%)	60 µl SDS (10%)
60 µl APS (10%)	60 µl APS (10%)
6 µl TEMED	6 µl TEMED

2.12. Immunohistochemical and *In Situ* Apoptosis Analyses

Immunohistochemical (IHC) and *in situ* Apoptosis (TUNEL) analyses were utilized using fixed brain specimens of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, 20- and 28-week-old DKO mice administered with AAV and Istradefylline to evaluate the fluorescence intensity changes in GM2 ganglioside, LAMP1, NeuN, CNPase, GFAP, and apoptotic cells.

2.12.1. Anti-GM2 Staining

For anti-GM2 (GM2 ganglioside marker) staining, the fixed brain sections of each experimental group were first incubated on ice for 15 minutes and then incubator at 55°C for 15 minutes. The slides were located in a Coplin jar with ice-cold acetone at -20°C for 10 minutes. The removal of acetone was performed using 1x PBS for 5 minutes at room temperature. The sections were encircled using a liquid blocker PAP pen (Sigma) after drying the sections. They were incubated in the blocking buffer (0.3% Triton X-100, 0.3M Glycine, 4% BSA in mg, and 10% normal goat serum in 1xPBS) for 1 hour at room temperature in a humidified chamber. The sections were incubated with 20 µl of the primary antibody (KM966) diluted in blocking buffer (1:250) at +4°C overnight in a

humidified chamber. The following day, after the removal of the primary antibody from the sections, they were incubated in the secondary antibody, Goat anti-Human Cross DyLight-488 (Thermo Scientific, 1:250), in the blocking solution for 1 hour at room temperature in the dark. When the slides were washed in 1x PBS three times for 5 minutes each, they were mounted with Fluoroshield mounting medium DAPI (Abcam). Then, they were covered with coverslips and stored at +4°C until fluorescence images were taken using a fluorescence microscope (BX53, Olympus). The fluorescent images of the cortex, hippocampus, thalamus, cerebellum, and pons at specific filters (green for anti-GM2 and blue for DAPI). The fluorescence images were measured using the NIH Image J program (1.48v).

2.12.2. Anti-Lamp1 Staining

The fixed brain sections of each experimental group were readied for anti-Lamp1, lysosomal marker (Abcam), staining by first incubating them on ice for 15 minutes and then placing the slides into the incubator at 55°C for 15 minutes. The slides were subjected to ice-cold methanol at -20°C for 10 minutes. The remaining methanol was removed using 1x PBS for 5 minutes at room temperature. After drying the sections, they were encircled using a liquid blocker PAP pen (Sigma), after which the sections were blocked in the blocking buffer (0.3% Triton X-100, 0.3M Glycine, 4% BSA in mg, and 10% normal goat serum in 1xPBS) for 1 hour at room temperature in the humidified chamber. The next day, the sections were covered with 20 µl of the primary antibody diluted in blocking buffer (1:250) and incubated at +4°C overnight in a humidified chamber. After the removal of the primary antibody from the sections, they were incubated in the secondary antibody, Alexa Fluor®-488 (Abcam, 1:500), in the blocking solution for 1 hour at room temperature at dark. The slides were washed in 1x PBS three times for 5 minutes each, and then they were mounted with Fluoroshield mounting medium DAPI (Abcam). The slides were covered with coverslips and stored at +4°C until fluorescence images were taken using a fluorescence microscope (BX53, Olympus). The fluorescent images of the cortex, hippocampus, thalamus, cerebellum, and pons at specific filters (green for anti-Lamp1 and blue for DAPI). The fluorescence images were measured using the NIH Image J program (1.48v).

2.12.3. Anti-NeuN Staining

The fixed brain slides of each experimental group were prepared by placing them on ice for incubation of 15 minutes and then into the incubator at 55°C for 15 minutes consecutively. For IHC staining with the anti-NeuN (neuronal marker) antibody, initially, the slides were washed in 1x PBS for 10 minutes at room temperature. Afterward, the cell membrane permeabilization was performed in ice-cold acetone at -20°C for 15 minutes. To remove the remaining acetone, the slides were washed in 1x PBS twice for 5 minutes each at room temperature. After drying the sections, they were encircled with a liquid blocker PAP pen (Sigma) to prevent the leakage of the blocking buffer. Then, 10 µl of blocking buffer (0.3% Triton X-100, 0.3M Glycine, 4% BSA in mg, and 10% normal goat serum in 1xPBS) was added to the sections in a humidified chamber, and the slides were incubated for 1 hour at room temperature. The primary antibody, anti-NeuN (Cell Signaling), was prepared by diluting it in a 1:50 ratio in the blocking buffer. 20 µl of the antibody was placed over each section in a humidified chamber, and the slides were incubated at +4°C overnight. The next day, the antibody was eliminated after washing in 1x PBS three times for 5 minutes each. The sections were then incubated in the secondary antibody, Alexa Fluor®-488 (Abcam), diluted in the blocking solution in a 1:500 ratio for 1 hour at room temperature at dark. To remove excess antibodies, the slides were washed in 1x PBS three times for 5 minutes each. Using Fluoroshield mounting medium DAPI (Abcam), the slides were mounted and covered with coverslips. The slides were stored at +4°C until the images were taken with a fluorescence microscope (BX53, Olympus). The fluorescent images of the cortex, thalamus, hippocampus, cerebellum, and pons at specific filters (green for anti-NeuN and blue for DAPI). The fluorescence images were measured using the NIH Image J program (1.48v).

2.12.4. Anti-CNPase Staining

The fixed brain slides for each experimental group were prepared by placing them on ice for incubation of 15 minutes and then into the incubator at 55°C for 15 minutes sequentially. For anti-CNPase, oligodendrocyte marker staining, at first, the slides were put into Coplin jars containing 4% PFA in 1x PBS for 15 minutes. The slides were then washed to remove PFA three times for 5 minutes each in 1x PBS at room temperature.

After fixation, they were placed into ice-cold 100% methanol for 10 minutes to permeabilize the cell membranes. The excess methanol was eliminated by washing the slides in 1x PBS for 5 minutes thrice. The surroundings of the sections were encircled with a liquid blocker PAP pen (Sigma), by which leakage of the blocking buffer was prevented. The blocking was performed by putting 10 μ L of the blocking buffer (0.3% Triton X-100, 0.3M Glycine, 4% BSA in mg and 10% normal goat serum in 1xPBS) onto the sections and incubating the slides in a humidified chamber for 1 hour at room temperature. After that, the primary antibody, anti-CNPase (Cell Signaling), was prepared by dissolving it in a dilution of 1:100 in the blocking buffer and put onto the sections in 20 μ L in a humidified chamber at +4°C overnight. The following day, the slides were washed in 1x PBS three times for 5 minutes each to remove excess antibodies. The sections were readied to be incubated in the secondary antibody, Alexa Fluor®-488 (Abcam), diluted in the blocking solution in a 1:500 ratio for 1 hour at room temperature in a humidified chamber at dark. The excess antibodies were eliminated over the sections by washing them in 1x PBS three times for 5 minutes each. Using Fluoroshield mounting medium DAPI (Abcam), the slides were mounted and covered with coverslips. The slides were stored at +4°C until the images were taken via a fluorescence microscope (BX53, Olympus). The fluorescent images of the cortex, thalamus, cerebellum, and pons at specific filters (green for anti-CNPase and blue for DAPI). The fluorescence images were measured using the NIH Image J program (1.48v).

2.12.5. Anti-GFAP Staining

The fixed brain slides of each experimental group were prepared by placing them on ice for incubation of 15 minutes and then into the incubator at 55°C 15 minutes consecutively. For staining with the anti-GFAP (active astrocyte marker) antibody, firstly, the slides were washed in 1x PBS for 10 minutes at room temperature. Later, when the sections were dried, they were encircled with a liquid blocker PAP pen (Sigma) to prevent the leakage of the blocking buffer. 10 μ L of blocking solution (0.3% Triton X-100, 0.3M Glycine, 4% BSA in mg, and 10% normal goat serum in 1xPBS) was added to each section in a humidified chamber to incubate them for 1 hour at room temperature. The primary antibody, anti-GFAP (Cell Signaling), was diluted (a ratio of 1:200) in the blocking buffer and placed in 20 μ L/section. Then, the slides were incubated at +4°C

overnight in a humidified chamber. Subsequently, the primary antibody was washed thrice with 1x PBS for 5 minutes each. The sections were incubated in the secondary antibody, Alexa Fluor®-488 (Abcam), prepared in the blocking solution (a 1:500 ratio) for 1 hour at room temperature at dark. After removing the excess antibodies by washing in 1x PBS three times for 5 minutes each, the slides were mounted with Fluoroshield mounting medium DAPI (Abcam). The slides were covered with coverslips and stored at +4°C until fluorescence images were taken using a fluorescence microscope (BX53, Olympus). The fluorescent images of the cortex, hippocampus, cerebellum, and pons at specific filters (green for anti-GFAP and blue for DAPI). The fluorescence images were measured using the NIH Image J program (1.48v).

2.12.6. *In situ* Apoptosis (TUNEL) Assay

The brain slides for each experimental group were taken from -80°C and incubated on ice for 15 minutes and at 55°C for 15 minutes in a humidity chamber sequentially. The manufacturer's protocol for the ApopTag® Fluorescein *In situ* Apoptosis Detection Kit S7110 (TUNEL) kit was followed in the *in situ* Apoptosis (TUNEL) Assay. The slides were then incubated in 1% PFA (in 1x PBS) for 10 minutes for further fixation of brain sections. To remove excess PFA from the sections, they were washed in 1x PBS two times for 5 minutes. Post-fixation was applied to brain sections in pre-chilled ethanol: acetic acid (2:1) for 5 minutes at -20°C. Subsequently, the slides were washed in 1x PBS twice for 5 minutes each to remove excess solutions. The equilibration buffer (75 µL/5 cm²) was immediately added to each section, and the slides were incubated for at least 10 seconds at room temperature. To remove excess liquid, the slides were gently tapped, and the Working Strength TdT enzyme (55 µL/5 cm²) was applied to each section. The slides were incubated with the enzyme in a humidity chamber at 37°C for 1 hour, after which the reaction was stopped using the working-strength stop/Wash Buffer for 10 minutes at room temperature. Removal of excess liquid was performed by washing the slides three times in 1x PBS for 1 minute each at room temperature. Later, a pre-warmed Anti-Digoxigenin Conjugate (65 µL/5 cm²) was added to each section, and slides were incubated at room temperature for 30 minutes in a humidified chamber, protected from light. Excess Anti-Digoxigenin Conjugate was eliminated by washing the slides twice in 1x PBS for 2 minutes each at room temperature. In the final step, the slides were mounted

with 15 μ L of mounting medium with 0.5-1.0 μ g/mL Propidium Iodide (PI), and they were covered with a coverslip. The slides were kept at +4°C until the images with a fluorescence microscope (BX53, Olympus). The fluorescent images of the cortex, thalamus, hippocampus, cerebellum, and pons at specific filters (green for apoptotic cells and red for PI). The fluorescence images were measured using the NIH Image J program (1.48v).

2.13. Behavioral Assays

Footprint and Rotarod analyses were performed using 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and 20- and 28-week-old DKO mice administered with AAV and Istradefylline to address the potential improvements in their gait abilities and locomotor activities.

2.13.1. Footprint Analysis

20- and 28-week-old SKO mice (n=3 for each), 20-week-old DKO mice (n=3), 20- and 28-week-old DKO mice administered with AAV (n=3 for each), 20- and 28-week-old DKO mice administered with AAV and Istradefylline (n=3 for each) were subjected to the footprint test at their specific ages. A non-toxic ink was used to stain the fore and hind paws of mice. Subsequently, the mice were allowed to walk along a runway (50 cm long, 10 cm wide, and 10 cm high walls) inside an enclosed box on white paper. The walking patterns were recorded using an HP scanner.

2.13.2. Rotarod Analysis

20- and 28-week-old SKO mice (n=6 and 7, respectively), 20-week-old DKO mice (n=6), 20- and 28-week-old DKO mice administered with AAV (n=6 for each), 20- and 28-week-old DKO mice administered with AAV and Istradefylline (n=6 for each) were subjected to the rotarod test at the specified ages. First, the mice were trained on a rotating rod at a constant speed of 5 rpm. Then, the mice were tested on the rod at an increasing speed (4 to 49 rpm over 5 min). During the testing, the latency to fall was

monitored using Sedacom version 2.0 (Harvard Apparatus). The mice underwent three trials each, with 15-minute rest periods in between, and the mean latency of fall was calculated.

2.14. Statistical Analyses

Data analyses were performed using GraphPad Prism 8.0 (GraphPad Software Inc.), and the results were shown as mean $\pm$ SEM. Pairwise comparisons were utilized using the one-way ANOVA and Tukey's multiple comparison test as a posttest by setting the statistical significance as $p < 0.05$. A minimum of three independent experiments were conducted to obtain consistent statistical comparisons for all analyses.

CHAPTER 3

RESULTS

3.1. The Production of AAVrh10-hNeu3 Particles

3.1.1. Confirmation of The Vectors

The confirmation of the pAd Δ F6, pAAV2/rh10, and mammalian expression vector expressing hNeu3 were performed after being delivered in *E.coli* stocks to understand whether they expressed the desired genes.

After the MegaPrep isolation, the control of the pAAV2/rh10 and pAd Δ F6 vectors was performed using restriction enzyme digestion with BamHI. Subsequently, agarose gel electrophoresis was done to show the expected bands after BamHI digestion (Figure 3.1). The expected band sizes for the pAAV2/rh10 vector after BamHI digestion were 4,205 bp and 3,131 bp, giving the vector size of a total of 7,336 bp (Figure 3.1A). Additionally, the restriction enzyme digestion of the pAd Δ F6 vector with a total size of 15,420 bp resulted in 2 bands with 12,723 bp and 2,697 bp on the agarose gel (Figure 3.1B).

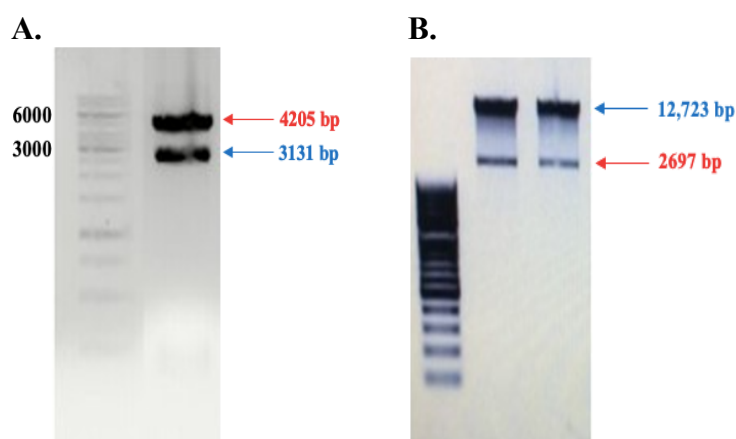


Figure 3.1. The agarose gel electrophoresis images after the MegaPrep isolation of pAAV2/rh10 (A) and pAd Δ F6 (B) and restriction enzyme digestion of the vectors with BamHI.

The restriction enzyme digestion of the mammalian expression vector expressing hNeu3 with EcoRI and PstI (Figure 3.2) and agarose gel electrophoresis were conducted to verify the vector after the MegaPrep isolation. Using EcoRI digestion, 2 expected bands, 5,936 bp, and 587 bp, were observed. Further confirmation with PstI resulted in band sizes of 2,605 bp, 1389 bp, 1350 bp, 726 bp, and 453 bp. A total vector size of 6,523 bp was given by VectorBuilder, and both enzymes proved the presence of the vector in the *E.coli* stocks being delivered (Figure 3.2).

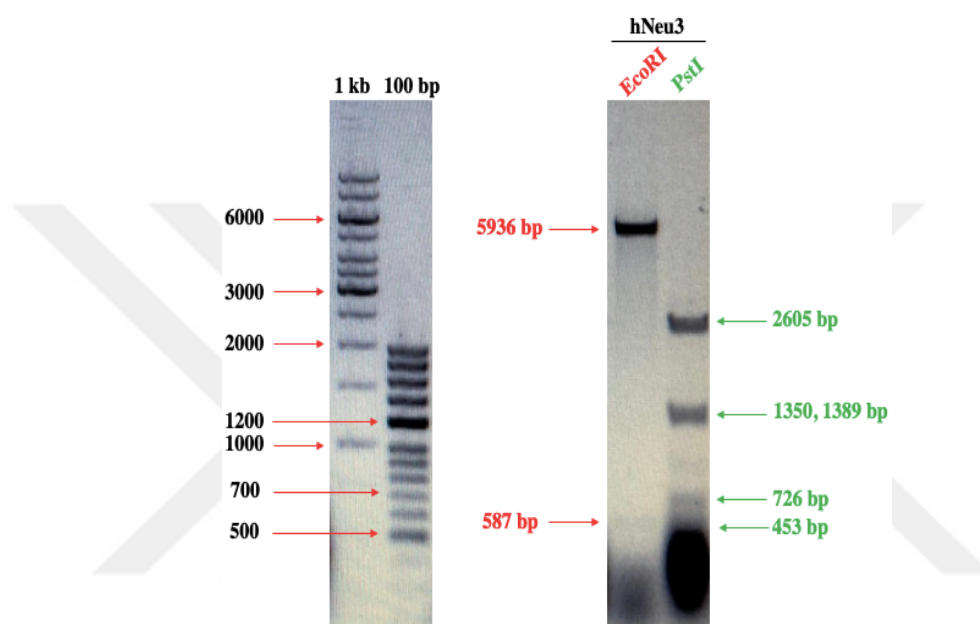


Figure 3.2. The agarose gel electrophoresis image after MegaPrep isolation and restriction enzyme digestion with EcoRI and PstI of the mammalian expression vector expressing hNeu3.

3.1.2. Single and Triple Transfection to Confirm the Vectors

Using the mammalian expression vector expressing hNeu3, the single transfection was conducted in the HEK293T cell line. Additionally, triple transfection of the HEK293T cells with the pAdΔF6, pAAV2/rh10, and mammalian expression vector expressing hNeu3 was performed. In both single and triple transfection, human Neu3 expression was controlled under a fluorescence microscope using the mCherry red fluorescent marker, which was fused to the protein of interest. The red signal was observed in the cells 72 hours after the single (Figure 3.3A) and triple (Figure 3.3B) transfections.

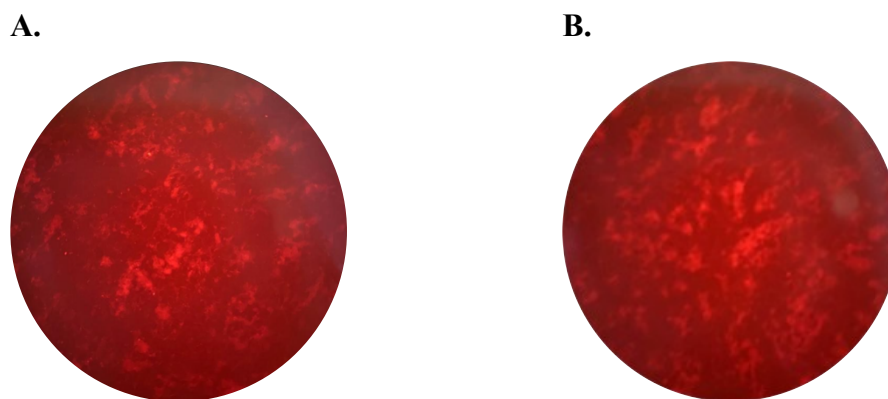


Figure 3.3. The red signal was visualized under a fluorescent microscope 72 hours after the single transfection with the mammalian expression vector expressing hNeu3 (A) and triple transfection with the pAdΔF6, pAAV2/rh10, and mammalian expression vector expressing hNeu3 (B) of HEK293T cells (20x magnification).

3.2. Transduction Analyses for the AAVrh10-hNeu3 Particles

3.2.1. Transduction of HEK293T Cells with the AAVrh10-hNeu3 Particles

The transduction test using the HEK293T cells with a Multiplicity of Infection (MOI) of 10,000 AAVrh10-hNeu3 particles per 100,000 HEK293T cells was done to check the presence of the isolated viral particles and their transduction efficiency. After 7 days of transduction, mCherry expression in red signal under a fluorescent microscope was visualized (Figure 3.4).

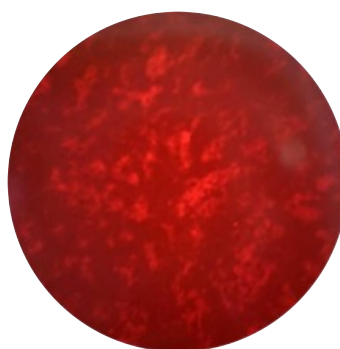


Figure 3.4. The red signal was visualized under a fluorescent microscope 7 days after the transduction of 100,000 HEK293T cells with an MOI of 10,000 AAVrh10-hNeu3 particles (20x magnification).

In addition to the HEK293T cells, the neuroglia cells of 20-week-old *Hexa*^{-/-} *Neu3*^{-/-} mice, which were previously isolated with the MACS Adult Brain Dissociation Kit (Miltenyi Biotec) according to the manufacturer's protocol, were transduced using 500,000 MOI of AAVrh10-hNeu3 particles per 100,000 neuroglia cells. Increasing the MOI, the increase in the transduction efficacy of the neuroglia cells was aimed. Seven days after the transduction, the mCherry expression in red signal was observed under a fluorescence microscope (Figure 3.5), indicating the presence of AAVrh10-hNeu3 particles in the isolate.

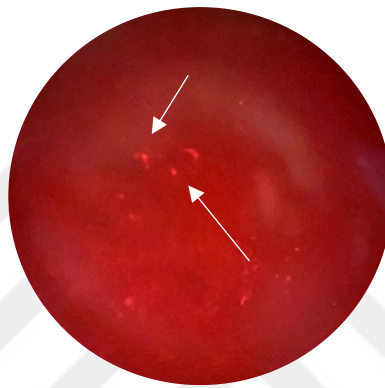


Figure 3.5. The red signal was visualized under a fluorescent microscope 7 days after the transduction of 100,000 neuroglia cells of 20-week-old *Hexa*^{-/-} *Neu3*^{-/-} mice cells with an MOI of 500,000 AAVrh10-hNeu3 particles (20x magnification).

3.2.2. Confirmation of the Transduction Using Anti-GM2 Staining

The neuroglia cells of 20-week-old *Hexa*^{-/-} *Neu3*^{-/-} mice transduced with an MOI of 500,000 AAVrh10-hNeu3 particles were stained immunocytochemically by performing anti-GM2 staining (Figure 3.6A). A statistically significant decrease was observed in the neuroglia cells of 20-week-old *Hexa*^{-/-} *Neu3*^{-/-} mice transduced with AAVrh10-hNeu3 particles compared to the non-transduced control, indicating the GM2 ganglioside degradation of the Human Neu3 in the neuroglia cells (Figure 3.6B).

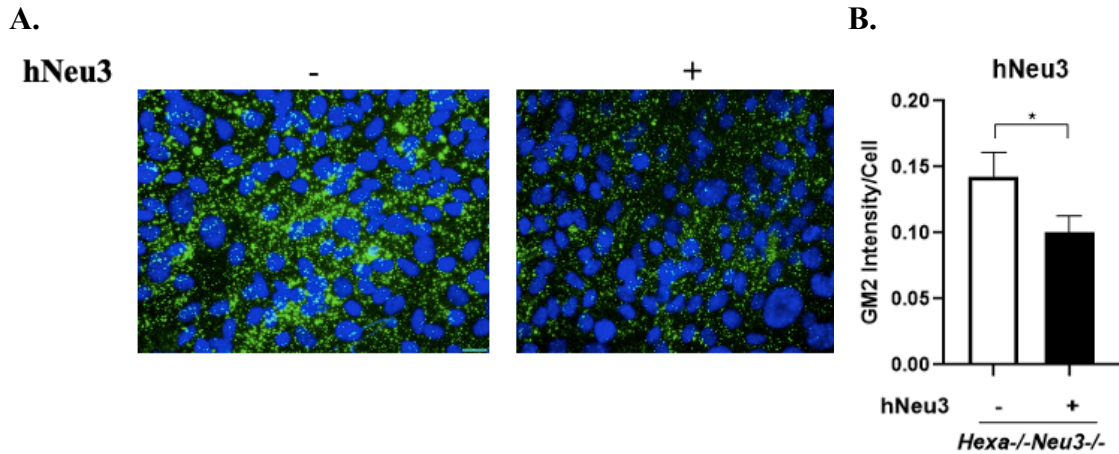


Figure 3.6. Immunocytochemical analysis was performed in non-transduced neuroglia cells of 20-week-old *Hexa*^{-/-}*Neu3*^{-/-} mice (-) and transduced neuroglia cells of 20-week-old *Hexa*^{-/-}*Neu3*^{-/-} mice with the AAVrh10-hNeu3 particles (+). The cells were stained with anti-GM2 antibody (green; GM2 ganglioside marker) and DAPI (blue; nuclei) (A). Total GM2 ganglioside intensity was shown in non-transduced neuroglia cells of 20-week-old *Hexa*^{-/-}*Neu3*^{-/-} mice and transduced neuroglia cells of 20-week-old *Hexa*^{-/-}*Neu3*^{-/-} mice with the AAVrh10-hNeu3 particles (B). The data were represented as the mean $\pm$ SEM (n = 3; *p < 0.05). Scale bar: 100 μ m.

3.3. Genotyping of Mice

The genotypes of the *Hexa*^{-/-} and *Hexa*^{-/-}*Neu3*^{-/-} mice were controlled by performing Hexa and Neu3 PCR with their specific primers (Table 2.1). Using HexaF and HexaR primers, the WT allele of the Hexa gene with a band size of 420 bp was visualized, while the KO allele of 210 bp was observed using HexaF and PGKR primers (Figure 3.7A). The Neu3 PCR with Neu3F and Neu3R primers resulted in the WT allele with a band size of 1022 bp, whereas the KO allele with a band size of 601 bp was shown using Neu3F and NeoR primers (Figure 3.7B)

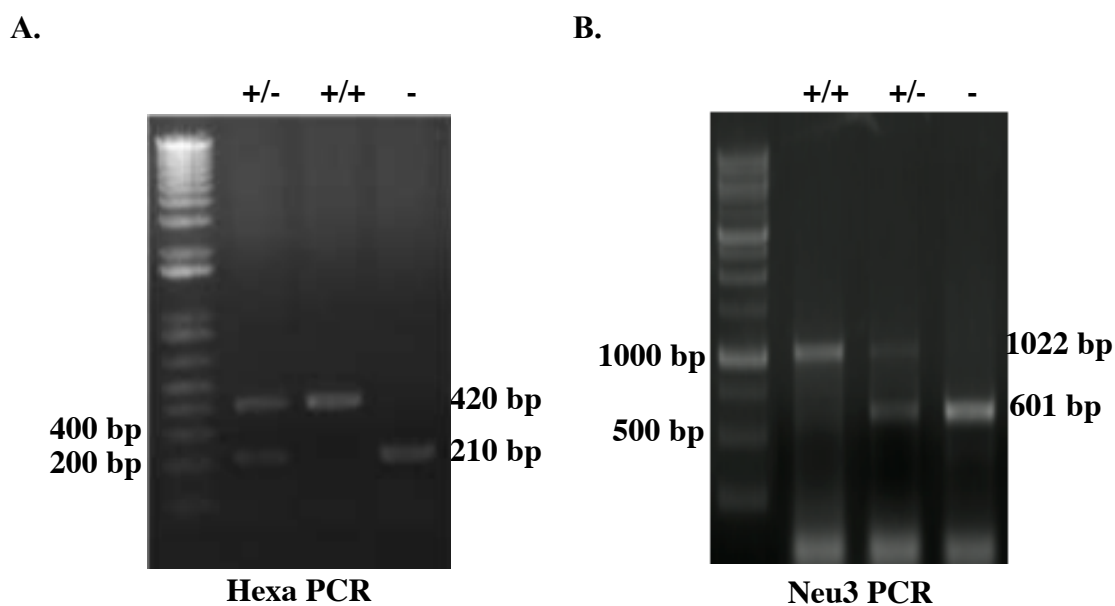


Figure 3.7. Agarose gel electrophoresis images of Hexa (A) and Neu3 (B) PCR products.

3.4. Body Weight Measurements

The survival plot revealed a sudden death at 20 weeks for DKO mice, while DKO mice with AAV and those with AAV and Istradefylline died at around 28 weeks (Figure 3.8A). Weekly body weight measurements of SKO mice, DKO mice, and DKO mice with AAV and those with AAV and Istradefylline were initiated at 8 weeks and continued up to the week they died. A decrease in the body weight of DKO mice was shown after 14 weeks, and the loss in weight continued until their death at 20 weeks (Figure 3.8B), in contrast to other groups. The DKO mice with AAV and those with AAV and Istradefylline started to lose weight after 20 weeks of age and continued until their death at 28 weeks, compared to the SKO mice (Figure 3.8B). SKO mice maintained a normal body weight for up to 28 weeks (Figure 3.8B).

The box plot indicating the changes in body weight at 20 weeks of SKO mice, DKO mice, DKO mice with AAV, and those with AAV and Istradefylline represented a significant loss in the body weight of DKO mice in contrast to other groups (Figure 3.8C). Although there was a loss in body weight of DKO mice with AAV and those with AAV and Istradefylline after 20 weeks, there was no significant difference in their body weight compared to that of SKO mice (Figure 3.8C).

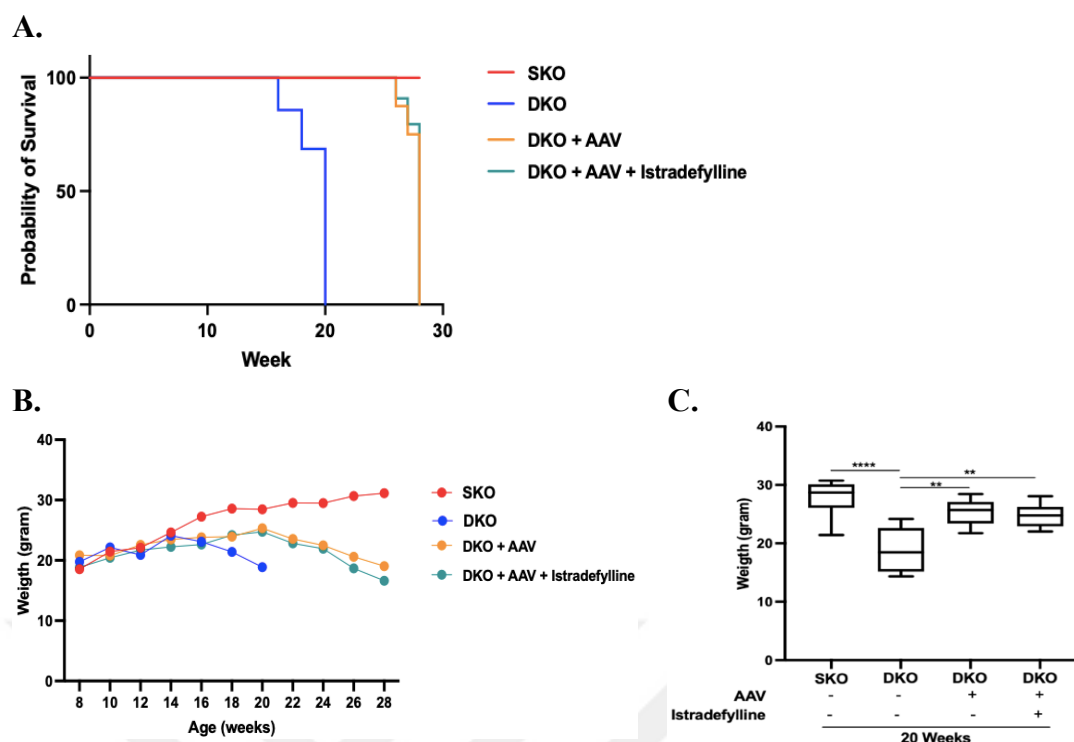


Figure 3.8. Kaplan-Meier survival curve of SKO mice, DKO mice, and DKO mice administered with AAV and those administered with AAV and Istradefylline (A). The changes in the body weight starting from 8 weeks up to 20 and 28 weeks of SKO mice (n=20), DKO mice administered with AAV (n=6), those administered with AAV and Istradefylline (n=6), and 20 weeks of DKO mice (n=15) (B). The box plot of the body weights of SKO mice, DKO mice, and DKO mice administered with AAV and those administered with AAV and Istradefylline at 20 weeks (C). The p-values were calculated with GraphPad via one-way ANOVA analysis. The data are presented as mean $\pm$ SEM (**p<0.025 and ****p<0.001).

3.5. Thin Layer Chromatography Analysis

Thin layer chromatography analysis (TLC) using the cortex tissues of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and those with AAV and Istradefylline to understand the changes in GM2 ganglioside levels. For this aim, the intensity of GM2 ganglioside was normalized using the intensity of GM1 ganglioside in this tissue. The results showed a remarkable increase in the GM2 ganglioside levels in the cortex of 20-week-old DKO mice compared to age-matched SKO mice (Figure 3.9B). Although it was not significant, there was a decrease in GM2 ganglioside levels in the cortex of 20-week-old DKO mice administered with AAV and those with AAV and Istradefylline compared to 20-week-

old DKO mice (Figure 3.9B). Moreover, there was no significant change in the GM2 ganglioside levels in the cortex of 28-week-old DKO mice administered with AAV alone and combined with Istradefylline compared to both their 20-week-old counterparts and DKO mice (Figure 3.9B).

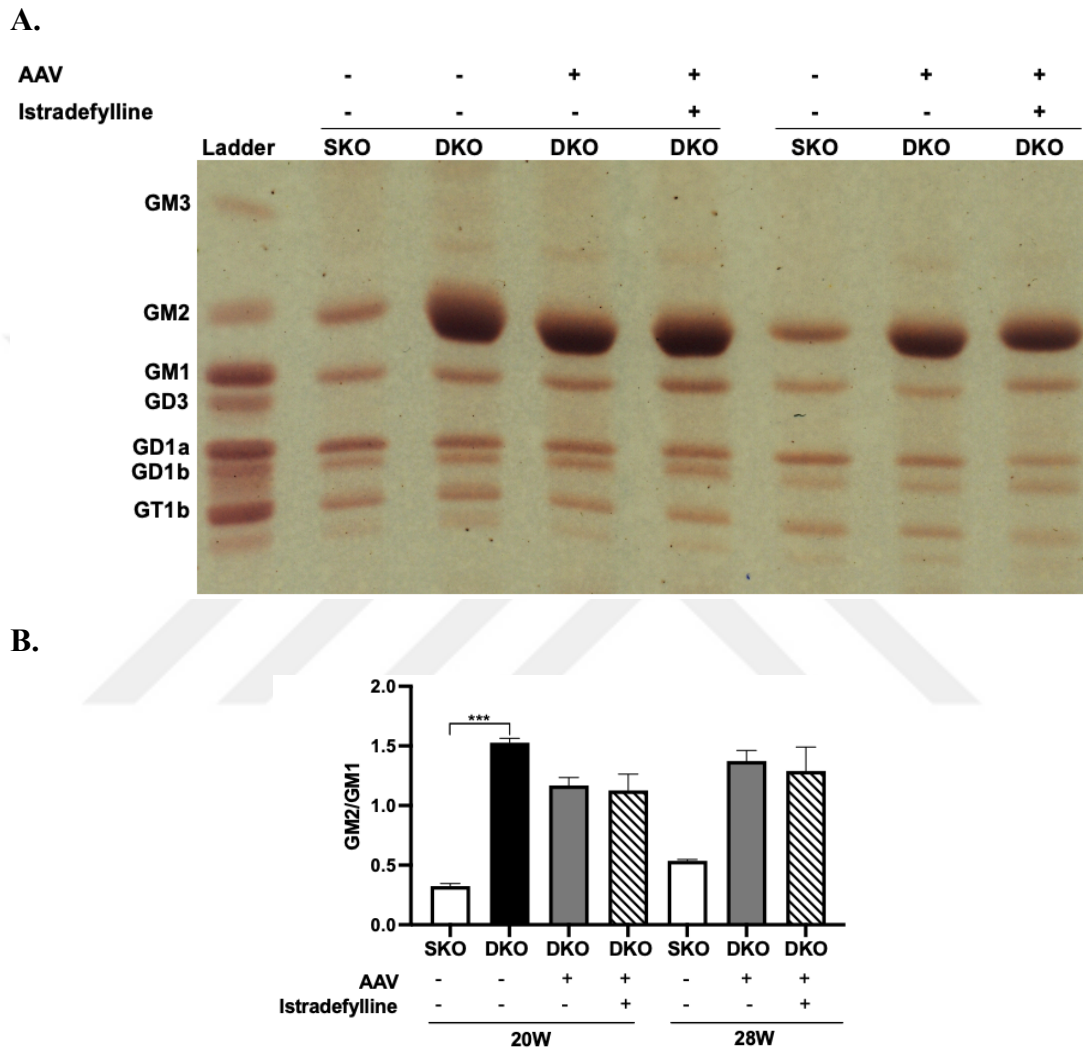


Figure 3.9. Thin layer chromatography (TLC) image (A) of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline for acidic GSLs isolated from the cortex. The ratio of GM2 ganglioside to GM1 ganglioside (B) was indicated. The p-values were calculated with GraphPad via one-way ANOVA analysis. The data are presented as mean $\pm$ SEM (n=3; ***p<0.01).

3.6. The mRNA Expression Analysis

3.6.1. Real-Time PCR Analyses for Inflammation-related Markers

Total RNA isolation and Real-Time PCR (RT-PCR) analyses were conducted using the cortex, cerebellum, kidney, liver, and spleen tissues of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV and those with AAV and Istradefylline to analyze the changes in the mRNA expression levels of human *NEU3* (hNeu3), inflammation-related markers, and lysosomal marker *Lamp1*.

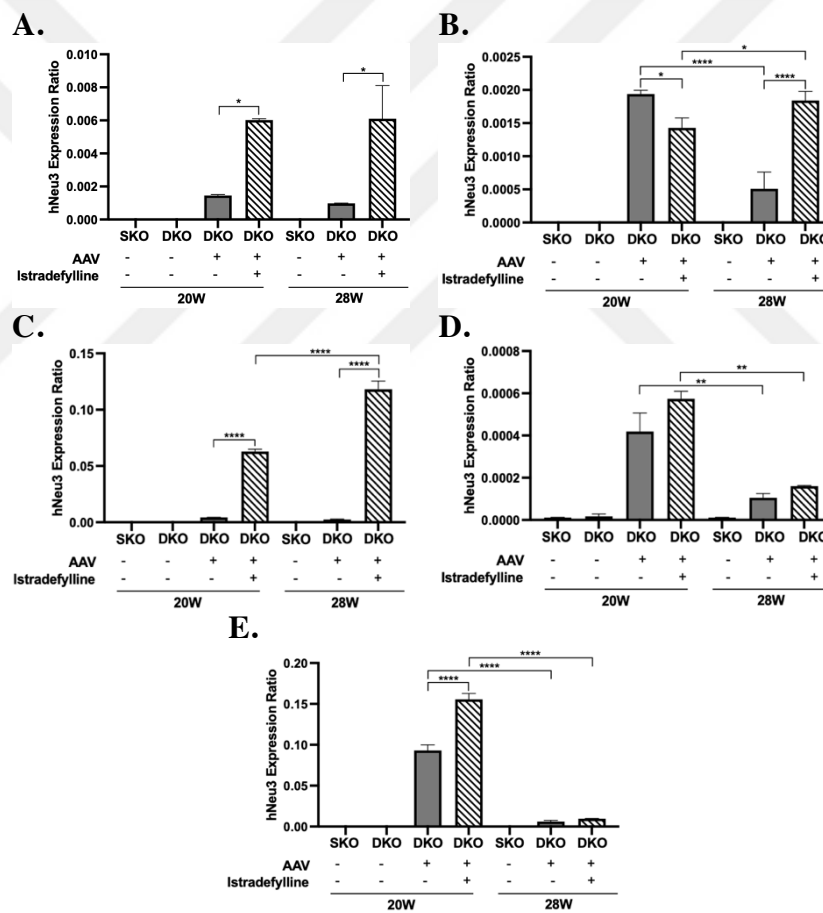


Figure 3.10. The expression ratios of human Neuraminidase 3, hNeu3, in the cortex (A), cerebellum (B), kidney (C), liver (D), and spleen (E) of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline were calculated by the ΔCT method. The p-values were calculated with GraphPad via one-way ANOVA analysis. The data are presented as mean $\pm$ SEM (n=3; *p<0.05, **p<0.025, and ****p<0.001).

In Figure 3.8, increases in the mRNA expression of hNeu3 were shown in the cortex, cerebellum, kidney, liver, and spleen tissues of 20- and 28-week-old DKO mice administered with AAV and those with AAV and Istradefylline, in contrast to the SKO and DKO mouse groups. Significantly increased levels were observed in 20- and 28-week-old DKO mice administered with AAV and Istradefylline compared to their age-matched counterparts, especially in the cortex (Figure 3.10A) and kidney (Figure 3.10C). Moreover, a significant increase in the kidney was measured in 28-week-old DKO mice administered with AAV and Istradefylline compared to its counterpart at 20 weeks (Figure 3.10C). However, in the cerebellum, there was a significant decrease in hNeu3 mRNA levels in 20-week-old DKO mice administered with AAV and Istradefylline compared to 20-week-old DKO mice administered with AAV and 28-week-old DKO mice administered with AAV and Istradefylline (Figure 3.10B). A significant down-regulation was shown in 28-week-old DKO mice administered with AAV compared to those administered with AAV and Istradefylline and 20-week-old DKO mice administered with AAV (Figure 3.10B). Interestingly, at 28 weeks of age, the decrease in the mRNA expression levels of hNeu3 was indicated in the liver (Figure 3.10D) and spleen (Figure 3.10E) of DKO mice administered with AAV and those with combined administration compared to their 20-week-old counterparts. Besides, there was a significant up-regulation of hNeu3 expression in the spleen of 20-week-old DKO with combined administration compared to only AAV-administered DKO mice at the same age (Figure 3.10E).

In Figure 3.11, the mRNA expression levels of neuroinflammatory markers *Ccl2*, *Ccl3*, *Ccl5*, *Cxcl10*, *IL-1 β* , *Iba1*, and *GFAP* in the cortex of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and those with AAV and Istradefylline were measured in the cortex. An increase of *Ccl3* (Figure 3.11B), *Cxcl10* (Figure 3.11D), *Iba1* (Figure 3.11F), and *GFAP* (Figure 3.11G) levels was measured in the cortex of 20-week-old DKO compared to age-matched SKO mice. Additionally, the elevation of *Ccl3* (Figure 3.11B), *Ccl5* (Figure 3.11C), *Iba1* (Figure 3.11F), and *GFAP* (Figure 3.11G) mRNA levels were observed in the cortex of 20-week-old DKO mice administered with AAV and Istradefylline compared to DKO mice. In contrast, decreasing *IL-1 β* levels were shown among the same groups (Figure 3.11E). For all markers, significant upregulation was identified in the cortex of 28-week-old DKO mice administered with AAV compared to those of DKO mice and 20-week-old DKO mice administered with AAV (Figure 3.11).

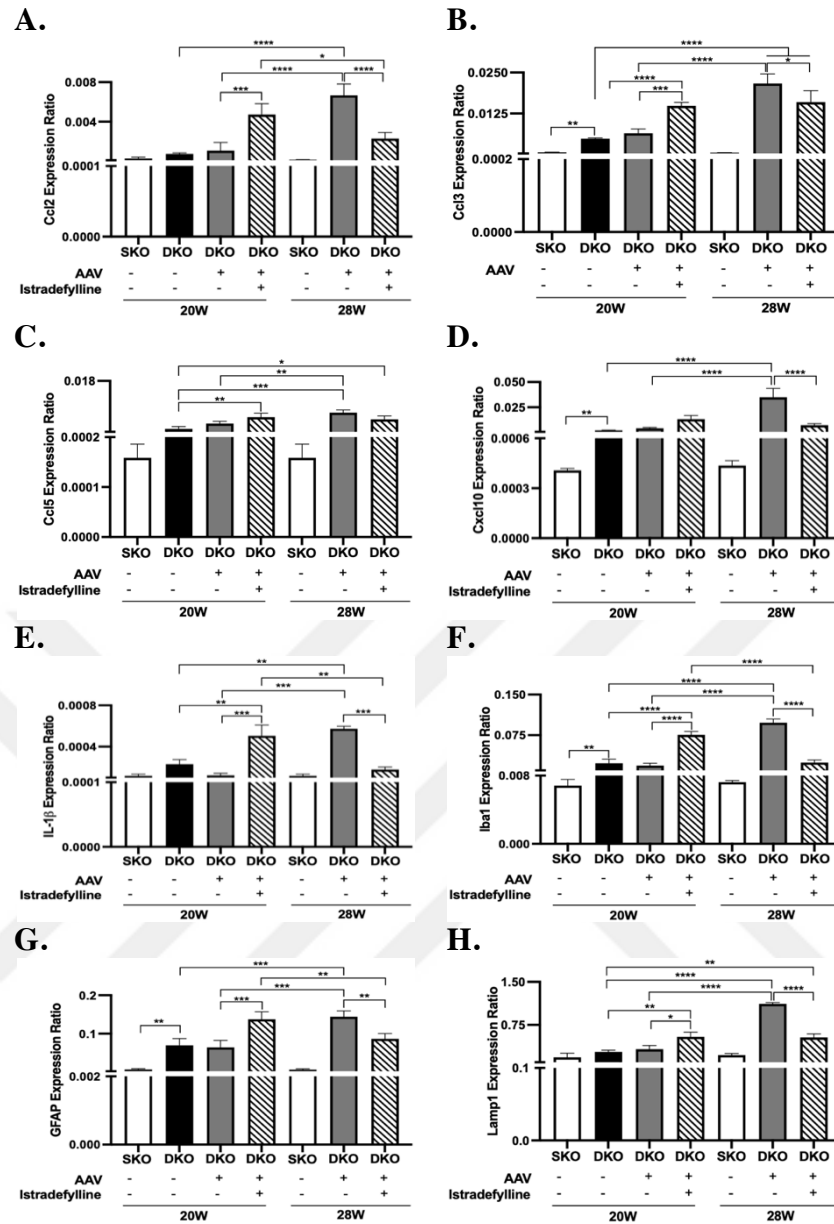


Figure 3.11. The expression ratios of neuroinflammation-related *Ccl2* (A), *Ccl3* (B), *Ccl5* (C), *Cxcl10* (D), *IL1- β* (E), *Iba1* (F), *GFAP* (G), and lysosome-related *Lamp1* (H) were calculated by the $\Delta\Delta\text{CT}$ method in the cortex of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline. The p-values were calculated with GraphPad via one-way ANOVA analysis. The data are presented as mean $\pm$ SEM (n=3; **p<0.025, ***p<0.01 and ****p<0.001).

All neuroinflammatory markers, except for *Ccl5* and *Cxcl10*, which showed no change, increased significantly in the cortex of 20-week-old DKO mice with the combined administration, in contrast to age-matched DKO mice administered with AAV

only (Figure 3.11). In the cortex of 28-week-old DKO mice administered with AAV, an up-regulation only in *Ccl3* (Figure 3.11B) and *Ccl5* (Figure 3.11C) markers was measured compared to DKO mice, while in the same group, a decrease was observable in the mRNA expression levels of *Ccl2* (Figure 3.11A), *IL-1 β* , (Figure 3.11F), and *GFAP* (Figure 3.11G) compared to those of 20-week-old DKO mice with combined administration

In addition to the neuroinflammation regulators, the lysosomal *Lamp1* expression was also analyzed in all experimental groups (Figure 3.11). When compared to 20-week-old DKO mice with combined administration, 28-week-old DKO mice administered with AAV, and 20- and 28-week-old DKO mice administered with AAV and Istradefylline, the *Lamp1* expression was significantly lower in 20-week-old DKO mice (Figure 3.11H). Compared to 20-week-old DKO mice administered with AAV, the *Lamp1* levels were increased in 20-week-old DKO mice with combined administration and 28-week-old DKO mice administered with AAV (Figure 3.11H). In the cortex of 28-week-old DKO mice with combined administration, the *Lamp1* expression was down-regulated compared to the age-matched DKO mice given only AAV (Figure 3.11H).

In Figure 3.12, *Ccl2*, *Ccl3*, *Ccl5*, *Cxcl10*, *IL-1 β* , *Iba1*, *GFAP*, and *Lamp1* mRNA levels in the cerebellum of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and those with AAV and Istradefylline were analyzed. The mRNA expression levels of *Ccl2* (Figure 3.12A), *Ccl3* (Figure 3.12B), *Cxcl10* (Figure 3.12D), *Iba1* (Figure 3.12F), and *GFAP* (Figure 3.12G) significantly increased in DKO mice compared to 20-week-old SKO mice. Surprisingly, compared to 28-week-old DKO mice with AAV, all markers except *IL-1 β* and *Iba1* were upregulated (Figure 3.12), whereas only *IL-1 β* (Figure 3.12E) levels were higher in 28-week-old DKO mice with combined administration. There was an increase in the mRNA levels of *Ccl2* (Figure 3.12A), *Ccl3* (Figure 3.12B), *Cxcl10* (Figure 3.12D), and *Iba1* (Figure 3.12F) in the cerebellum of 28-week-old DKO mice with AAV compared to those of 20-week-old counterparts. With the Istradefylline administration, the levels of *Ccl3* (Figure 3.12B), *Ccl5* (Figure 3.12C), *Cxcl10* (Figure 3.12D), *Iba1* (Figure 3.12F), and *Lamp1* (Figure 3.12H) were remarkably downregulated compared to the age-matched DKO mice with AAV.

In Figure 3.13, *Ccl2*, *Ccl3*, *Ccl5*, *Cxcl10*, *IL-1 β* , and *Lamp1* mRNA levels in the kidney of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and those with AAV and Istradefylline were

analyzed. The increase in mRNA levels of *Ccl2* (Figure 3.13A), *Ccl3* (Figure 3.13B), *Ccl5* (Figure 3.13C), and *Lamp1* (Figure 3.13F) was demonstrated in DKO mice in contrast to SKO mice at 20 weeks. On the other hand, the same markers were significantly reduced in 20-week-old DKO mice with AAV compared to those of DKO mice (Figure 3.13).

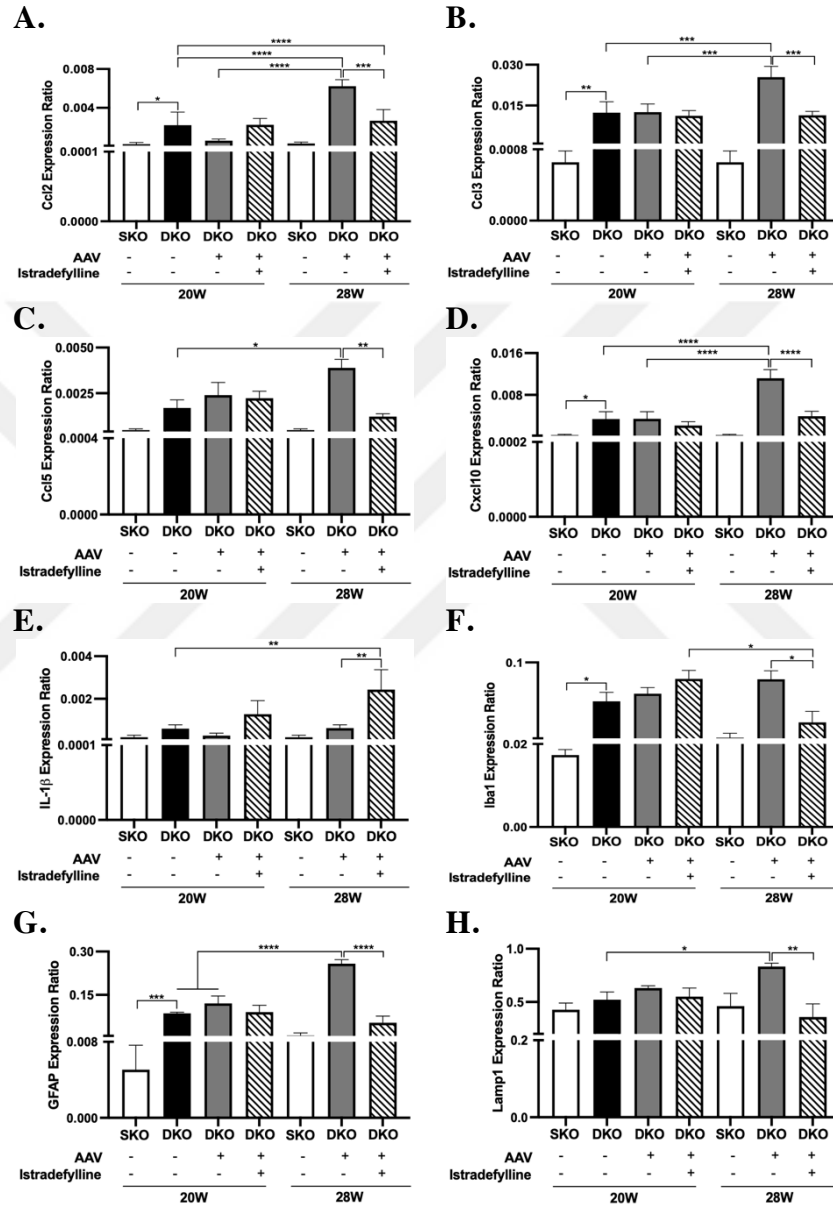


Figure 3.12. The expression ratios of neuroinflammation-related *Ccl2* (A), *Ccl3* (B), *Ccl5* (C), *Cxcl10* (D), *IL1-β* (E), *Iba1* (F), *GFAP* (G), and lysosome-related *Lamp1* (H) were calculated by the ΔCT method in the cerebellum of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline. The p-values were calculated with GraphPad via one-way ANOVA analysis. The data are presented as mean $\pm$ SEM (n=3; **p<0.025, ***p<0.01 and ****p<0.001).

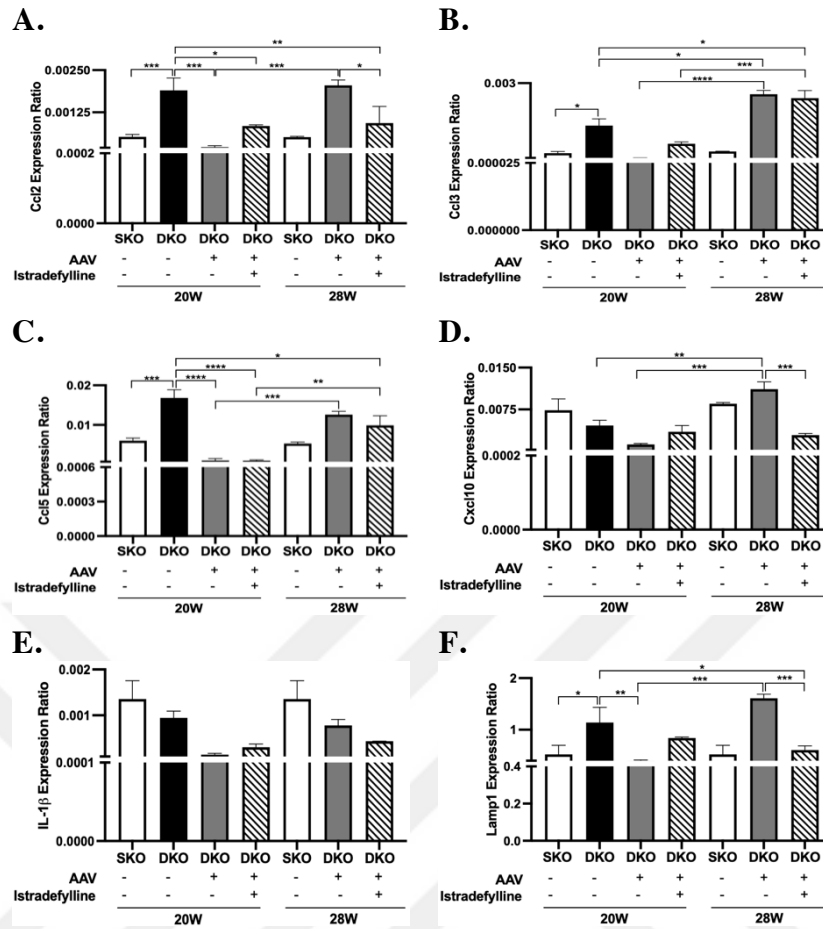


Figure 3.13. The expression ratios of inflammation-related *Ccl2* (A), *Ccl3* (B), *Ccl5* (C), *Cxcl10* (D), *IL1-β* (E), and lysosome-related *Lamp1* (F) were calculated by the ΔCT method in the kidney of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline. The p-values were calculated with GraphPad via one-way ANOVA analysis. The data are presented as mean $\pm$ SEM (n=3; *p<0.05, **p<0.025, ***p<0.01 and ****p<0.001).

Moreover, a significant decrease was indicated in the levels of *Ccl2* (Figure 3.13A) and *Ccl5* (Figure 3.13C) in the kidney of 20-week-old DKO mice with combined administration compared to those of DKO mice. At 28 weeks, the DKO mice with only AAV showed an increase in the levels of *Ccl3* (Figure 3.13B) compared to DKO mice. While *Ccl3* (Figure 3.13B) and *Ccl5* (Figure 3.13C) levels were upregulated in 28-week-old DKO mice with combined administration, the *Ccl2* (Figure 3.13A) levels decreased in contrast to DKO mice. *Lamp1* (Figure 3.13F) and all inflammatory markers except *IL-1β* (Figure 3.13E) in the kidney of 28-week-old DKO mice administered with AAV compared to the 20-week-old counterparts. Increased mRNA levels of *Ccl3* (Figure

3.13B) and *Ccl5* (Figure 3.13C) were observed in 28-week-old DKO mice administered with AAV and Istradefylline compared to their counterparts at 20 weeks. The Istradefylline treatment resulted in a decrease in the mRNA levels of *Ccl2* (Figure 3.13A), *Cxcl10* (Figure 3.13D), and *Lamp1* (Figure 3.13F) in 28-week-old DKO mice administered with AAV and Istradefylline compared to age-matched DKO mice with only AAV.

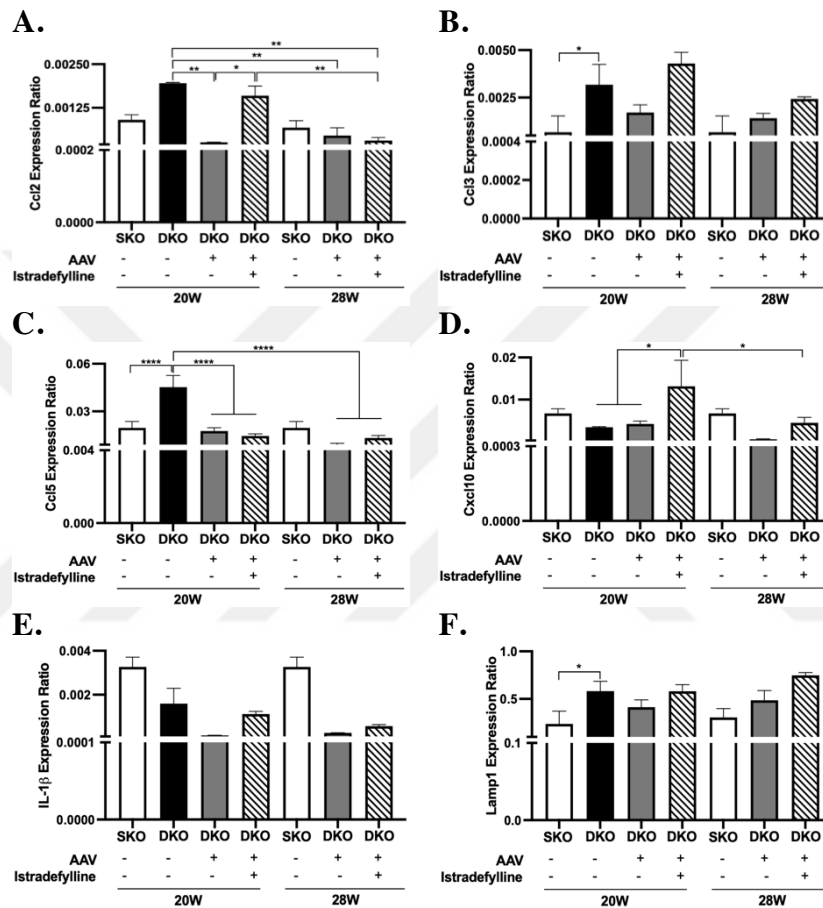


Figure 3.14. The expression ratios of inflammation-related *Ccl2* (A), *Ccl3* (B), *Ccl5* (C), *Cxcl10* (D), *IL-1 β* (E), and lysosome-related *Lamp1* (F) were calculated by the $\Delta\Delta\text{CT}$ method in the liver of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline. The p-values were calculated with GraphPad via one-way ANOVA analysis. The data are presented as mean $\pm$ SEM (n=3; *p<0.05, **p<0.025, and ***p<0.001).

In Figure 3.14, the mRNA expression levels of *Ccl2*, *Ccl3*, *Ccl5*, *Cxcl10*, *IL-1 β* , and *Lamp1* markers were analyzed in the liver of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and those

with AAV and Istradefylline. Significantly elevated levels of *Ccl3* (Figure 3.14B), *Ccl5* (Figure 3.14C), and *Lamp1* (Figure 3.14F) were measured in the liver of DKO mice compared to those of age-matched SKO mice. With the AAV administration, 20-week-old DKO mice showed a decrease in the mRNA levels of *Ccl2* (Figure 3.14A) and *Ccl5* (Figure 3.14C) compared to 20-week-old DKO mice. The expression levels of *Ccl5* (Figure 3.14C) and *Cxcl10* (Figure 3.43D) were downregulated in 20-week-old DKO mice with combined administration, in contrast to the levels of the markers in the liver of the 20-week-old DKO mice. Both the *Ccl2* (Figure 3.14A) and *Ccl5* (Figure 3.14C) mRNA expression levels were remarkably decreased in 28-week-old DKO mice administered with AAV and those with AAV and Istradefylline compared to DKO mice. Although *Ccl2* (Figure 3.14A) and *Cxcl10* (Figure 3.14D) expression levels increased in 20-week-old DKO mice with combined administration compared to 20-week-old DKO mice administered with AAV, their levels were downregulated in the 28-week-old DKO mice with combined administration.

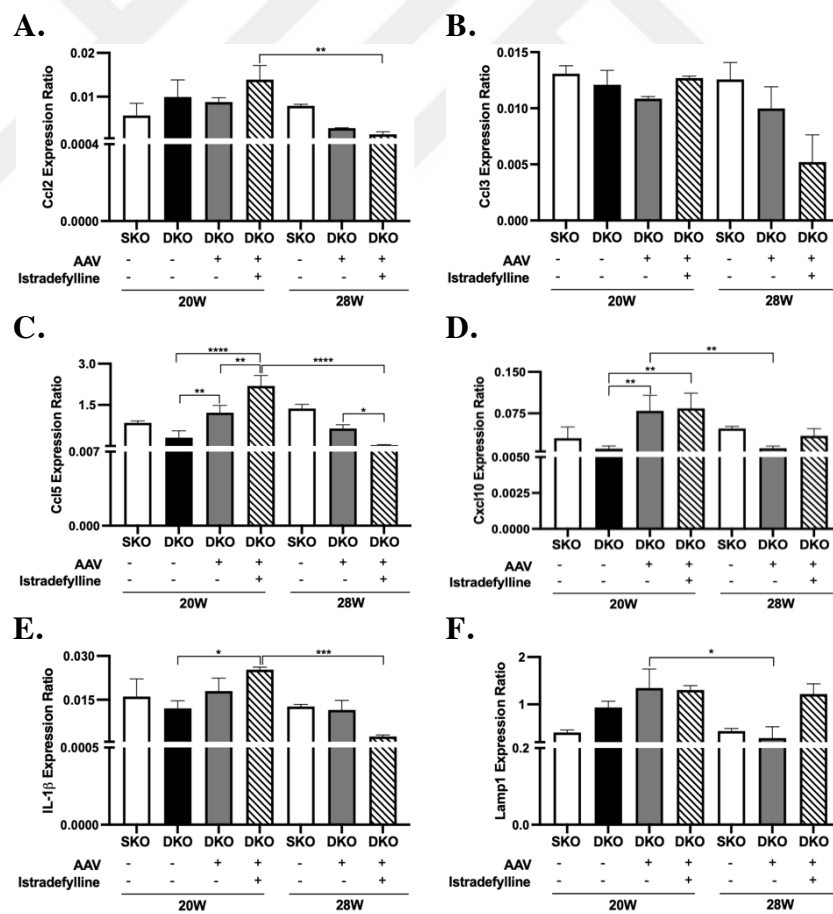


Figure 3.15. The expression ratios of inflammation-related *Ccl2* (A), *Ccl3* (B), *Ccl5* (C), *Cxcl10* (D), *IL1-β* (E), and lysosome-related *Lamp1* (F) were calculated by the $\Delta\Delta CT$ method in the spleen of 20- and 28-week-old SKO mice, 20-week-

old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline. The p-values were calculated with GraphPad via one-way ANOVA analysis. The data are presented as mean $\pm$ SEM (n=3; *p<0.05, **p<0.025, ***p<0.01, and ****p<0.001).

In Figure 3.15, the changes in the expression levels of *Ccl2*, *Ccl3*, *Ccl5*, *Cxcl10*, *IL-1 β* , and *Lamp1* mRNAs were identified in the spleen of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and those with AAV and Istradefylline. Interestingly, the levels of *Ccl5* (Figure 3.15) and *Cxcl10* (Figure 3.15D) were significantly elevated in 20-week-old DKO mice administered with AAV and those with AAV and Istradefylline compared to DKO mice. Decreasing expression levels of *Cxcl10* (Figure 3.15D) and *Lamp1* (Figure 3.15F) were shown in 28-week-old DKO mice administered with AAV compared to their counterparts at 20 weeks. Additionally, the *Ccl5* (Figure 3.15C) and *IL-1 β* (Figure 3.15E) levels were lower in 28-week-old DKO mice with combined administration when compared to their 20-week-old equivalents.

3.7. The Protein Expression Analysis

3.7.1. Western Blot Analyses for Inflammation-related Markers

Total protein isolation and Western Blot were performed using the cortex, cerebellum, kidney, liver, and spleen tissues of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, 20- and 28-week-old DKO mice administered with AAV and Istradefylline to evaluate the differences among the protein expression levels of inflammation-related markers, I κ B- α , NF κ B, and IL-1 β .

In Figure 3.16, the protein expression levels of I κ B- α was assessed in the cortex of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and those administered with AAV and Istradefylline. The only significant change with an increase was observed among 20- and 28-week-old DKO mice administered with AAV and Istradefylline (Figure 3.16B).

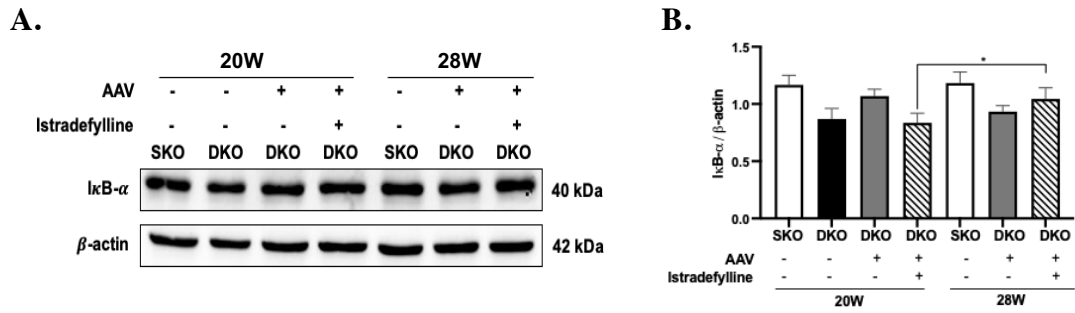


Figure 3.16. Immunoblot image (A) and densitometric analysis of anti-IκB-α (B) in the cortex of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline. β-actin served as an internal control. Band intensities were measured using ImageJ, and p-values were calculated with GraphPad via one-way ANOVA analysis. The data are presented as mean ± SEM (n=3; *p<0.05).

In Figure 3.17, the protein expression levels of NFκB were visualized in the cortex of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and those administered with AAV and Istradefylline. A significant decrease was observed in 20-week-old DKO mice administered with AAV compared to DKO and, interestingly, 20-week-old DKO mice with combined administration (Figure 3.17B). Additionally, a significant upregulation in the protein expression levels of NFκB was indicated in the cortex of 28-week-old DKO mice administered with AAV compared to their counterparts at 20 weeks (Figure 3.17B).

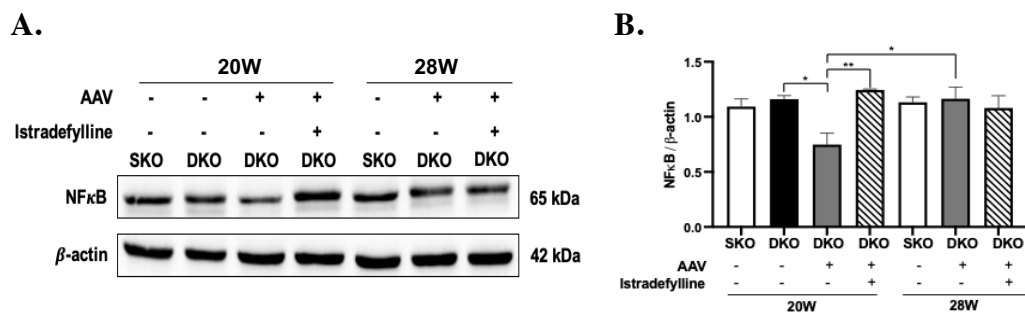


Figure 3.17. Immunoblot image (A) and densitometric analysis of anti-NFκB (B) in the cortex of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline. β-actin served as an internal control. Band intensities were measured using ImageJ, and p-values were calculated with GraphPad via one-way ANOVA analysis. The data are presented as mean ± SEM (n=3; *p<0.05 and **p<0.025).

In Figure 3.18, the protein expression levels of IL-1 β were evaluated in the cortex of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and those administered with AAV and Istradefylline. According to the densitometric analyses, there was no significant change among the groups (Figure 3.18B).

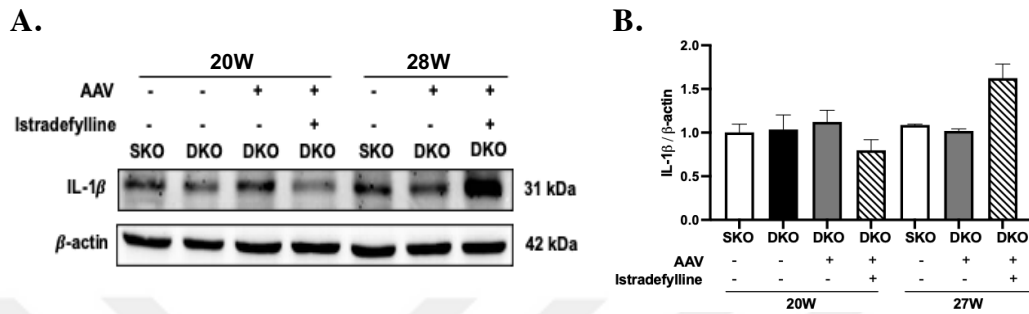


Figure 3.18. Immunoblot image (A) and densitometric analysis of anti-IL-1 β (B) in the cortex of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline. β -actin served as an internal control. Band intensities were measured using ImageJ, and p-values were calculated with GraphPad via one-way ANOVA analysis. The data are presented as mean $\pm$ SEM (n=3).

In Figure 3.19, the I κ B- α protein expression levels were measured in the cerebellum of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and those administered with AAV and Istradefylline. The results showed that there were no changes in the I κ B- α protein expression levels among the experimental groups (Figure 3.19B).

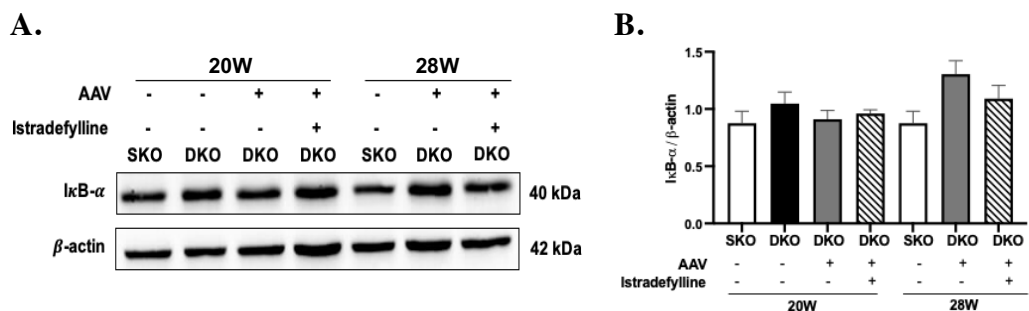


Figure 3.19. Immunoblot image (A) and densitometric analysis of anti-I κ B- α (B) in the cerebellum of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline. β -actin served as an internal control. Band intensities were measured using ImageJ, and p-values

were calculated with GraphPad via one-way ANOVA analysis. The data are presented as mean $\pm$ SEM (n=3).

In Figure 3.20, the NF κ B protein expression levels were assessed in the cerebellum of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and those administered with AAV and Istradefylline. The levels of anti-NF κ B did not present any significant changes among the experimental groups (Figure 3.20B).

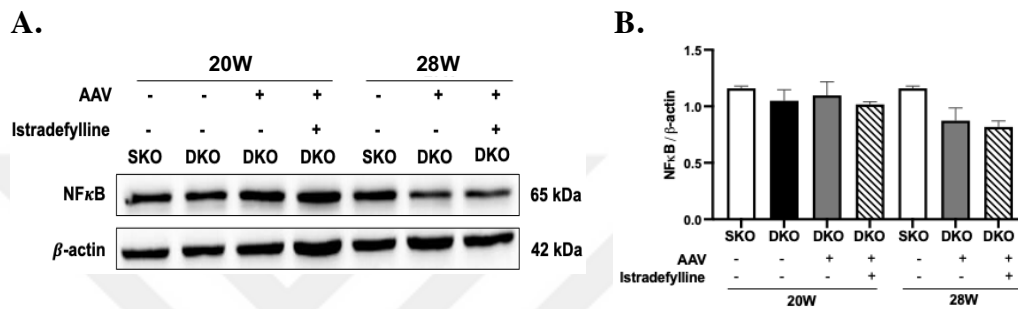


Figure 3.20. Immunoblot image (A) and densitometric analysis of anti-NF κ B (B) in the cerebellum of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline. β -actin served as an internal control. Band intensities were measured using ImageJ, and p-values were calculated with GraphPad via one-way ANOVA analysis. The data are presented as mean $\pm$ SEM (n=3).

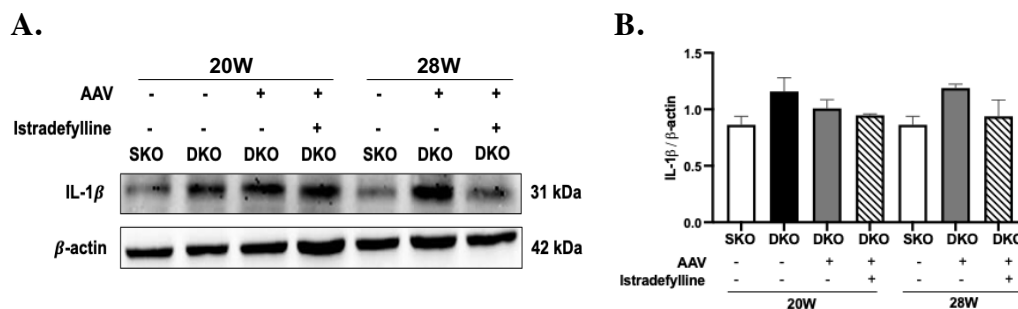


Figure 3.21. Immunoblot image (A) and densitometric analysis of anti-IL1- β (B) in the cerebellum of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline. β -actin served as an internal control. Band intensities were measured using ImageJ, and p-values were calculated with GraphPad via one-way ANOVA analysis. The data are presented as mean $\pm$ SEM (n=3).

In Figure 3.21, the expression levels of IL1- β protein were measured in the cerebellum of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and those administered with AAV and Istradefylline. There was no significant change observed among the experimental groups for anti-IL1- β (Figure 3.21B).

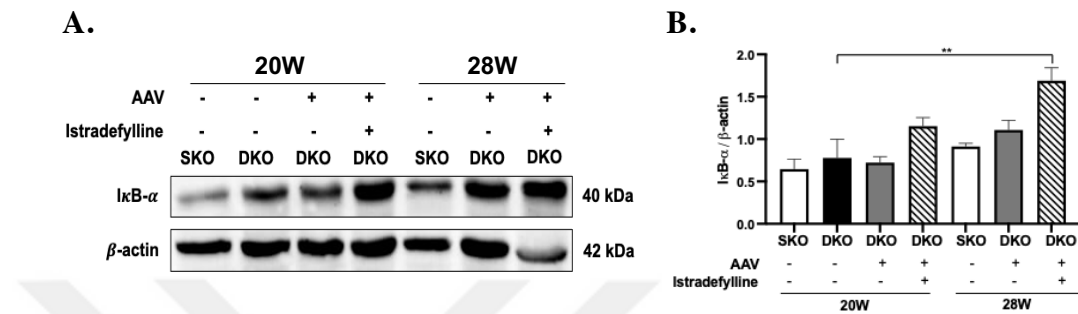


Figure 3.22. Immunoblot image (A) and densitometric analysis of anti-IκB-α (B) in the kidneys of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline. β-actin served as an internal control. Band intensities were measured using ImageJ, and p-values were calculated with GraphPad via one-way ANOVA analysis. The data are presented as mean ± SEM (n=3; **p<0.025).

In Figure 3.22, the IκB-α expression levels were characterized in the kidney of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and those administered with AAV and Istradefylline. Surprisingly, the IκB-α protein expression levels were higher in 28-week-old DKO mice administered with AAV and Istradefylline compared to those of DKO mice (Figure 3.22B).

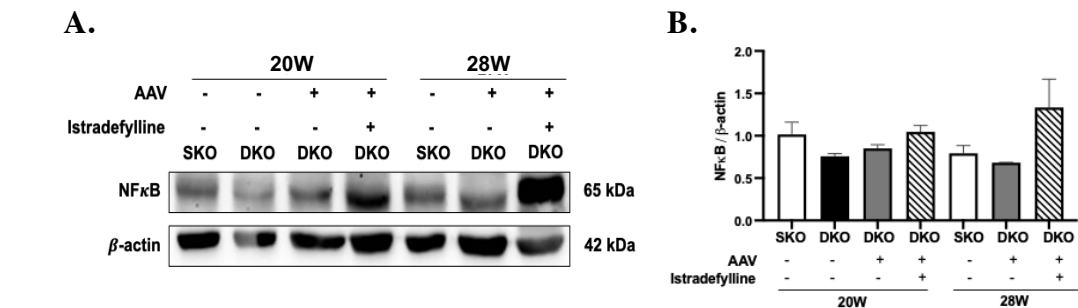


Figure 3.23. Immunoblot image (A) and densitometric analysis of anti-NFκB (B) in the kidney of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline. β-actin served as an internal control. Band intensities were measured using ImageJ, and p-values

were calculated with GraphPad via one-way ANOVA analysis. The data are presented as mean $\pm$ SEM (n=3).

In Figure 3.23, the anti-NF κ B was used to understand changes in the expression levels of protein in the kidney of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and those administered with AAV and Istradefylline. However, there was no significant change in NF κ B protein expression levels among the mouse groups (Figure 3.23B).

In Figure 3.24, the IL1- β expression levels were compared in the kidneys of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and those administered with AAV and Istradefylline. It was observed that the IL1- β levels were higher in 20-week-old DKO mice compared to age-matched SKO mice (Figure 3.24B). Moreover, remarkably increasing expression levels of IL1- β were visualized in the kidney of 28-week-old DKO mice administered with AAV along with Istradefylline in contrast to the levels shown in DKO mice and DKO mice with combined administration at 20 weeks (Figure 3.24B).

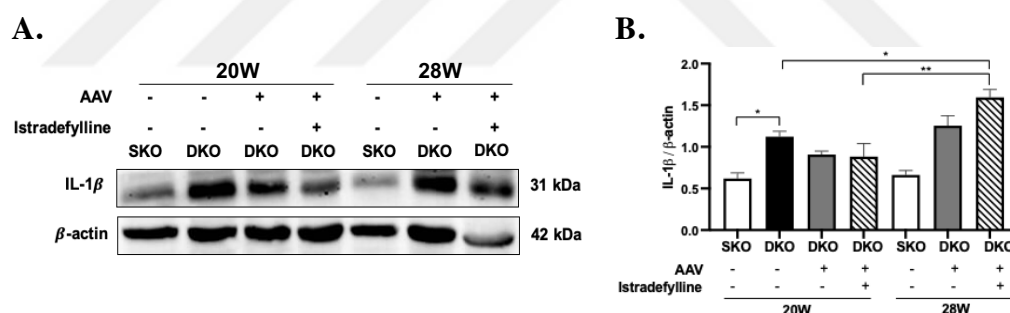


Figure 3.24. Immunoblot image (A) and densitometric analysis of anti-IL1- β (B) in the kidneys of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline. β -actin served as an internal control. Band intensities were measured using ImageJ, and p-values were calculated with GraphPad via one-way ANOVA analysis. The data are presented as mean $\pm$ SEM (n=3; *p<0.05 and **p<0.025).

In Figure 3.25, the analysis of I κ B- α protein expression levels was performed in the liver of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and those administered with AAV and Istradefylline. Compared to DKO mice, an elevation was observed in I κ B- α levels was

represented in 20- and 28-week-old DKO mice with combined administration and 28-week-old DKO mice administered only with AAV (Figure 3.25B).

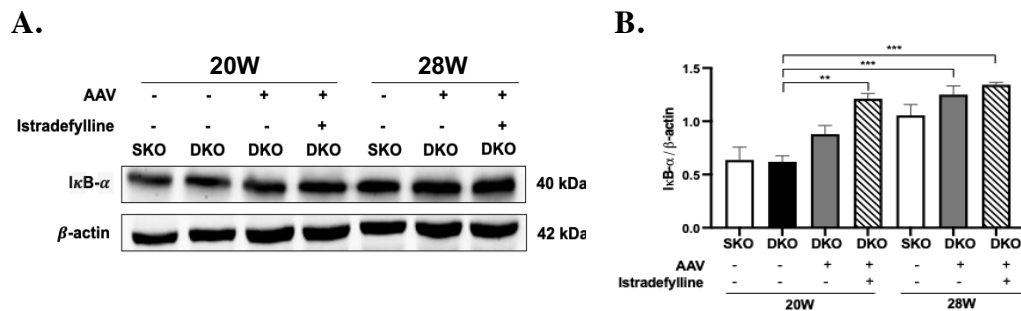


Figure 3.25. Immunoblot image (A) and densitometric analysis of anti-IκB-α (B) in the liver of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline. β-actin served as an internal control. Band intensities were measured using ImageJ, and p-values were calculated with GraphPad via one-way ANOVA analysis. The data are presented as mean ± SEM (n=3; *p<0.05 and ***p<0.01).

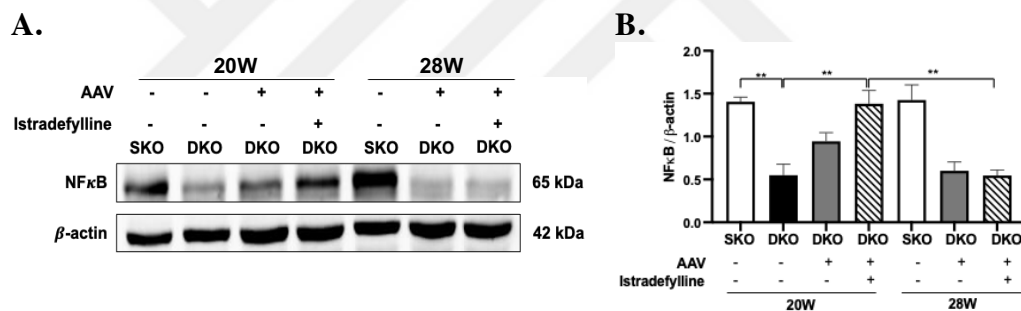


Figure 3.26. Immunoblot image (A) and densitometric analysis of anti-NFκB (B) in the liver of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline. β-actin served as an internal control. Band intensities were measured using ImageJ, and p-values were calculated with GraphPad via one-way ANOVA analysis. The data are presented as mean ± SEM (n=3; **p<0.025).

In Figure 3.26, the NFκB protein expression levels were measured in the liver of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and those administered with AAV and Istradefylline. Interestingly, the levels were significantly decreased in DKO mice compared to SKO mice of the same age (Figure 3.26B). A remarkable upregulation was observed in 20-week-old DKO mice with combined administration compared to DKO mice, while the

levels were decreased compared to 28-week-old DKO mice administered with AAV and Istradefylline (Figure 3.26B).

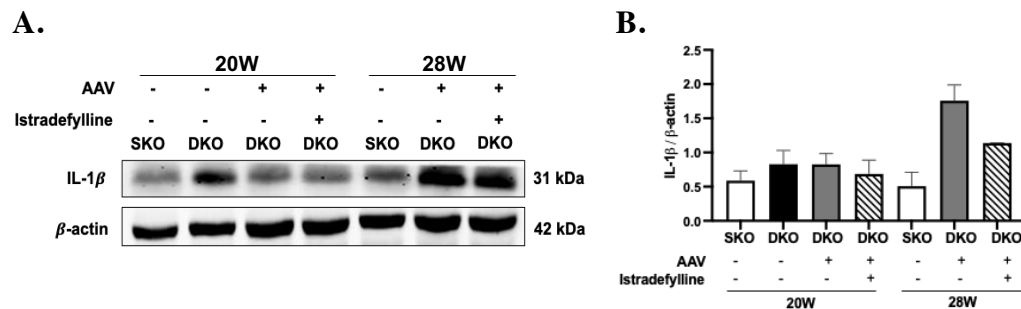


Figure 3.27. Immunoblot image (A) and densitometric analysis of anti-IL1- β (B) in the liver of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline. β -actin served as an internal control. Band intensities were measured using ImageJ, and p-values were calculated with GraphPad via one-way ANOVA analysis. The data are presented as mean $\pm$ SEM (n=3).

In Figure 3.27, the IL1- β expression levels were evaluated in the liver of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and those administered with AAV and Istradefylline. Although it was not significant, there was a decrease in 28-week-old DKO mice administered with AAV and Istradefylline compared to age-matched counterparts with only AAV (Figure 3.27B).

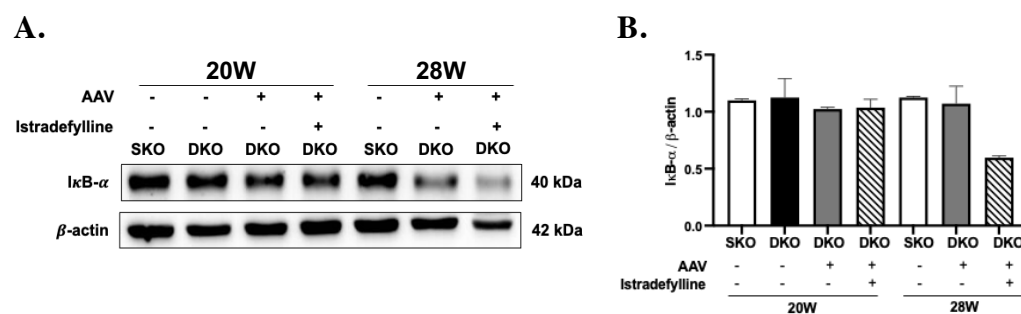


Figure 3.28. Immunoblot image (A) and densitometric analysis of anti-I κ B- α (B) in the liver of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline. β -actin served as an internal control. Band intensities were measured using ImageJ, and p-values were calculated with GraphPad via one-way ANOVA analysis. The data are presented as mean $\pm$ SEM (n=3; *p<0.05).

In Figure 3.28, in the spleen of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline, the $\text{I}\kappa\text{B-}\alpha$ protein expression levels were evaluated. Even though a slight change, a decrease after Istradefylline treatment was observed in 28-week-old DKO mice administered with AAV and Istradefylline compared to age-matching counterparts with only AAV (Figure 3.28B).

In Figure 3.29, the protein expression levels of $\text{NF}\kappa\text{B}$ in the spleen of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline, were measured. There was a significant decline in DKO mice compared to age-matching SKO mice (Figure 3.29B). The significant elevation in the levels of $\text{NF}\kappa\text{B}$ protein was shown in 20-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline compared to DKO mice (Figure 3.29B). Unexpectedly, a downregulation in $\text{NF}\kappa\text{B}$ protein levels was observed in the spleen of 28-week-old DKO mice administered with AAV, and those with combined administration compared to their counterparts at 20 weeks (Figure 3.29B).

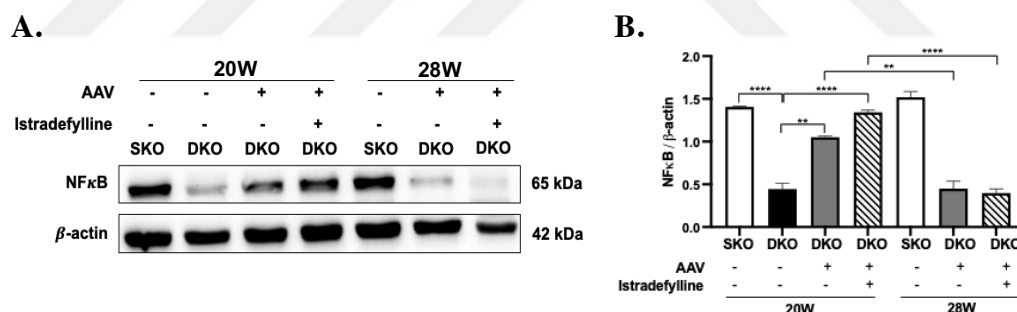


Figure 3.29. Immunoblot image (A) and densitometric analysis of anti- $\text{NF}\kappa\text{B}$ (B) in the spleen of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline. β -actin served as an internal control. Band intensities were measured using ImageJ, and p-values were calculated with GraphPad via one-way ANOVA analysis. The data are presented as mean $\pm$ SEM (n=3; **p<0.025 and ****p<0.001).

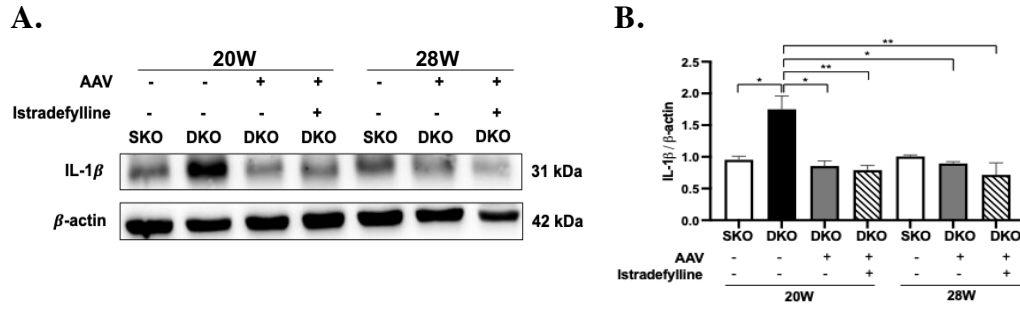


Figure 3.30. Immunoblot image (A) and densitometric analysis of anti-IL1- β (B) in the spleen of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline. β -actin served as an internal control. Band intensities were measured using ImageJ, and p-values were calculated with GraphPad via one-way ANOVA analysis. The data are presented as mean $\pm$ SEM (n=3; *p<0.05 and **p<0.025).

In Figure 3.30, the protein expression levels of IL1- β in the spleen of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline, were evaluated. A significant increase between 20-week-old SKO mice and age-matching DKO mice was observed in the IL1- β expression levels (Figure 3.30B). In the spleen of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline, the protein expression levels of IL1- β declined compared to those of DKO mice (Figure 3.30B).

3.8. Immunohistochemical and *In Situ* Apoptosis Analyses

Immunohistochemical (IHC) and *in situ* Apoptosis (TUNEL) analyses were utilized using fixed brain specimens of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline to measure the fluorescence intensities using anti- GM2, anti-LAMP1, anti-NeuN, anti-CNPase, anti-GFAP, and TUNEL assay.

3.8.1. Anti-GM2 Staining

In Figure 3.31, the anti-GM2, GM2 ganglioside marker, levels showed a significant increase in the DKO mice compared to SKO mice in all brain regions (Figure 3.31). After AAV administration, the GM2 ganglioside levels were downregulated at 20 weeks compared to DKO mice in all sections except for the pons (Figure 3.31). In the cortex (Figure 3.31B), hippocampus (Figure 3.31C), and thalamus (Figure 3.31D), GM2 ganglioside levels significantly decreased in 20-week-old DKO mice with combined administration compared to DKO mice. Only in the cortex (Figure 3.31B) did the GM2 ganglioside levels decrease in 28-week-old DKO mice with AAV compared to DKO mice. In comparison, the levels were lower in 28-week-old DKO mice with AAV and Istradefylline compared to DKO mice in both cortex (Figure 3.31B) and hippocampus (Figure 3.31C). Surprisingly, the GM2 ganglioside levels were upregulated in the hippocampus (Figure 3.31C) and cerebellum (Figure 3.31E) of 20-week-old DKO mice with combined administration in contrast to those of DKO mice with AAV only at the same age. In the thalamus (Figure 3.31D), cerebellum (Figure 3.31E), and pons (Figure 3.31F) of 28-week-old DKO mice administered with AAV, the GM2 ganglioside levels were higher compared to their counterparts at 20 weeks. Likewise, in the thalamus (Figure 3.31D) of 28-week-old DKO mice with combined administration, the increase in GM2 ganglioside levels was observed compared to 20-week-old DKO mice with combined administration. However, in the hippocampus (Figure 3.31C) of 28-week-old DKO mice with combined administration, there was a decrease in the GM2 ganglioside levels compared to that of 28-week-old DKO mice with only AAV.

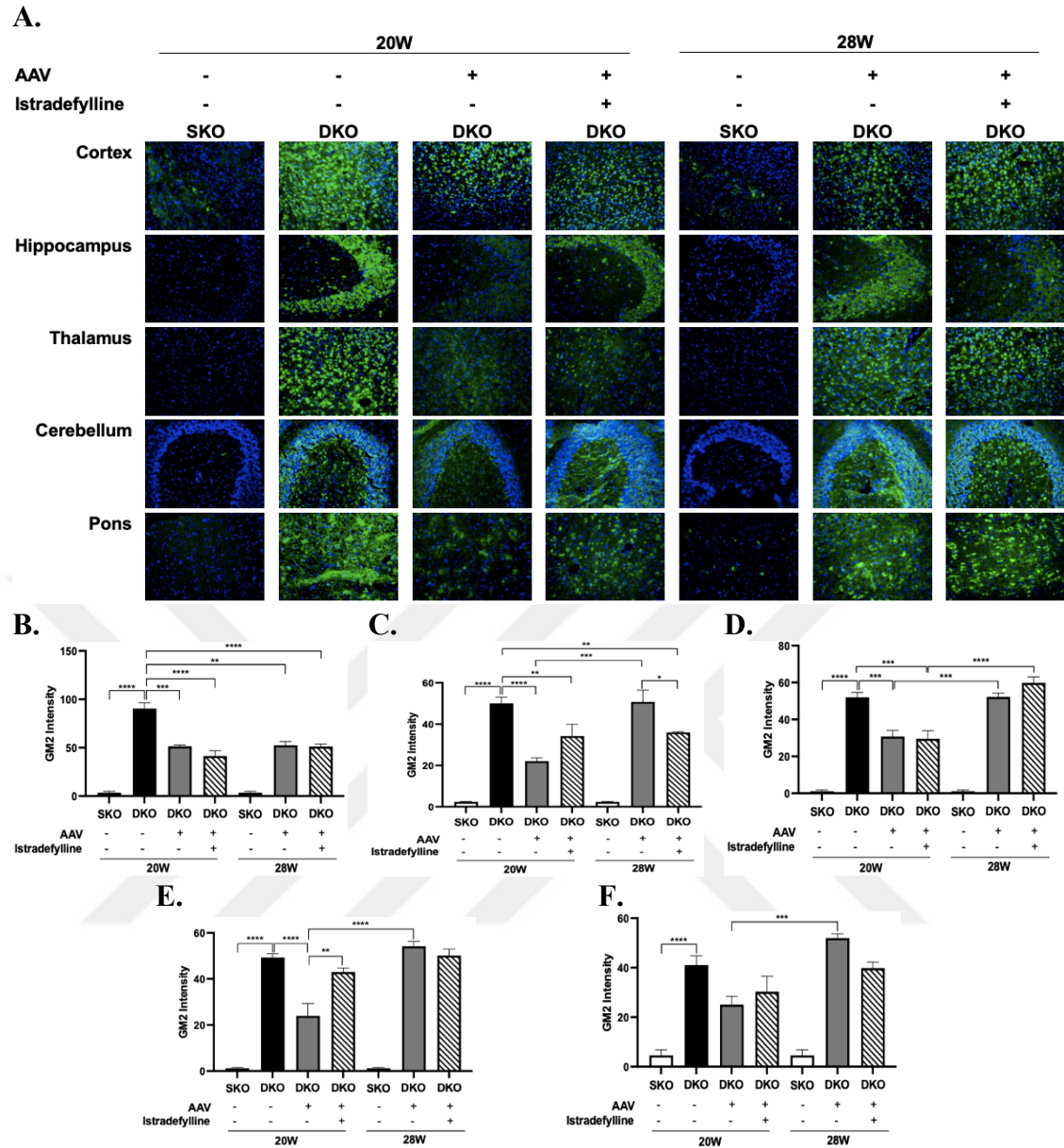


Figure 3.31. The sections (A) from the cortex, hippocampus, thalamus, cerebellum, and pons of 20- and 28-week-old SKO, 20-week-old DKO, 20- and 28-week-old DKO administered with AAV, and 20- and 28-week-old DKO administered with AAV and Istradefylline were stained with anti-GM2 (green) and DAPI (blue). The histograms represent the quantification of GM2 ganglioside in the cortex (B), hippocampus (C), thalamus (D), cerebellum (E), and pons (F). Scale bar = 50 μ m. One-way ANOVA analysis was used to determine p-values by GraphPad. The data were represented as mean $\pm$ SEM. (n=3; **p<0.025, ***p<0.01, and ****p<0.001).

3.8.2. Anti-Lamp1 Staining

In Figure 3.32, the Lamp1, a lysosomal marker, fluorescent intensity analyses were performed in different brain sections of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline. According to the results, increasing Lamp1 levels in all brain regions of DKO mice were observed compared to those of SKO mice (Figure 3.32). Moreover, in the thalamus (Figure 3.32D) and pons (Figure 3.32F) of 20-week-old DKO mice administered with AAV, the Lamp1 levels significantly decreased compared to DKO mice, whereas only in the pons (Figure 3.32F) of 20-week-old DKO mice with combined administration was the decrease observed. In all brain regions except for the cerebellum of 28-week-old DKO mice with AAV, the Lamp1 levels were downregulated in contrast to those of DKO mice (Figure 3.32). The Lamp1 intensity was lower in the pons of 28-week-old DKO mice administered with AAV, as well as those with combined administration, compared to DKO mice (Figure 3.32F). Higher Lamp1 levels were measured in the thalamus (Figure 3.32D) and cerebellum (Figure 3.32E) of 20-week-old DKO mice administered with AAV and Istradefylline in contrast to age-matching DKO mice administered with AAV. In the cerebellum (Figure 3.32E) of 28-week-old DKO mice administered with AAV, the Lamp1 fluorescent intensity was upregulated compared to 20-week-old DKO mice administered with AAV. The Lamp1 levels increased in the cortex (Figure 3.32B), hippocampus (Figure 3.32C), and thalamus (Figure 3.32D) of 28-week-old DKO mice with combined administration in contrast to their age-matched counterparts with AAV.

3.8.3. Anti-NeuN Staining

In Figure 3.33, using anti-NeuN antibody the changes in the number of neurons were assessed in distinct brain sections of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline. In all brain regions except for the pons, the neuron numbers were higher in 20-week-old SKO mice compared to DKO mice (Figure 3.33). Significantly elevated NeuN levels were observed in the cortex (Figure

3.33B), thalamus (Figure 3.33D), and pons (Figure 3.33F) of 20-week-old DKO mice with AAV compared to DKO mice.

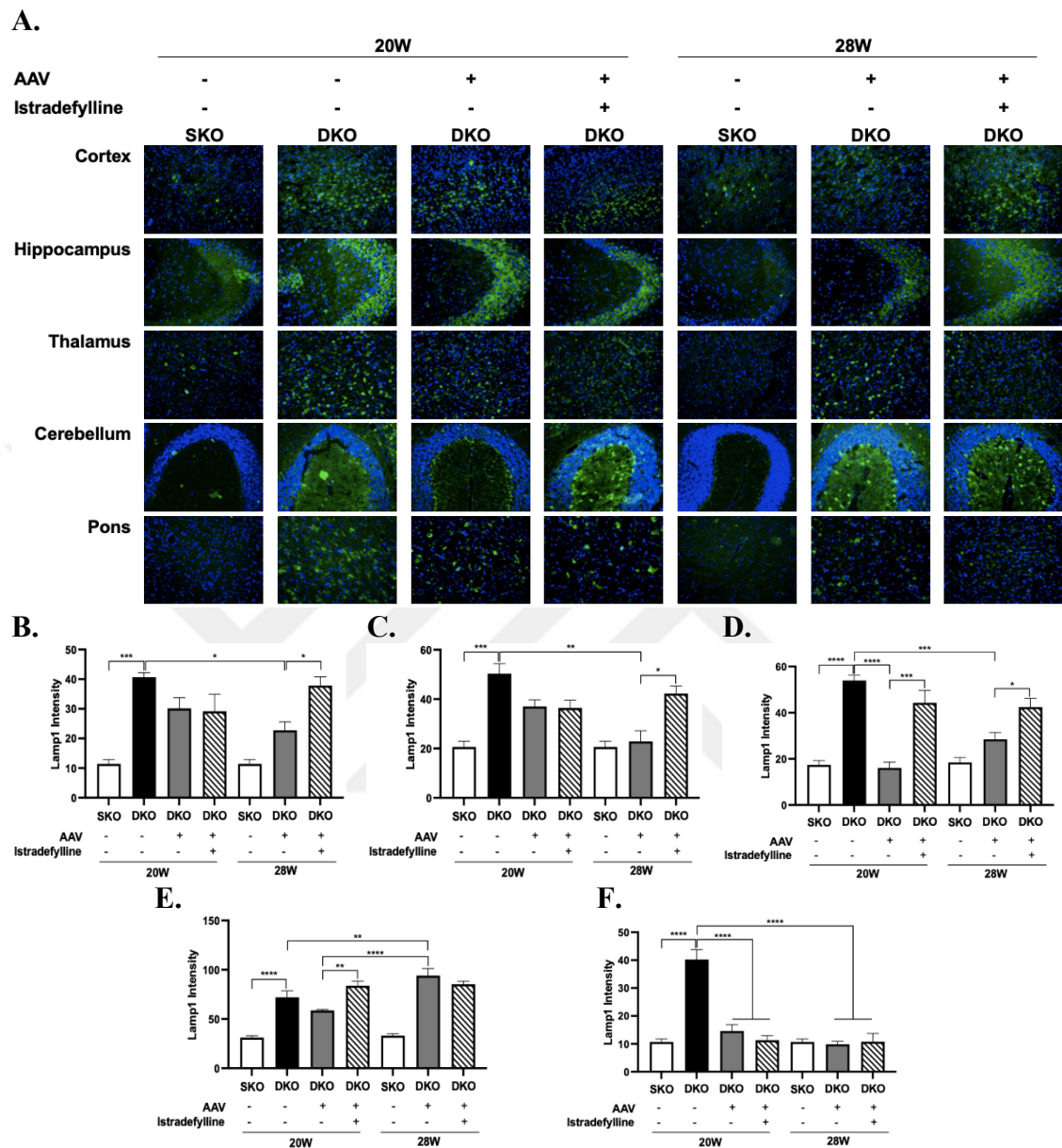


Figure 3.32. The sections (A) from the cortex, hippocampus, thalamus, cerebellum, and pons of 20- and 28-week-old SKO, 20-week-old DKO, 20- and 28-week-old DKO administered with AAV, and 20- and 28-week-old DKO administered with AAV and Istradefylline were stained with anti-Lamp1 (green) and DAPI (blue). The histograms represent the quantification of Lamp1 in the cortex (B), hippocampus (C), thalamus (D), cerebellum (E), and pons (F). Scale bar = 50 μ m. One-way ANOVA analysis was used to determine p-values by GraphPad. The data were represented as mean $\pm$ SEM. (n=3; *p<0.05, **p<0.025, ***p<0.01, and ****p<0.001).

In contrast to lower neuron levels in DKO mice, a remarkable increase in the cortex (Figure 3.33B), hippocampus (Figure 3.33C), and thalamus (Figure 3.33D) of 20-week-old DKO mice with combined administration was indicated. In the cerebellum of 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline, neuron numbers were lowered compared to DKO mice (Figure 3.33E). Between 20-week-old DKO mice administered with AAV and those administered with AAV and Istradefylline, the neuron levels were increased in the hippocampus of the latter (Figure 3.33C). The neuron number in the cortex (Figure 3.33B), cerebellum (Figure 3.33E), and pons (Figure 3.33F) of 28-week-old DKO mice with AAV decreased compared to their counterparts at 20 weeks. Likewise, a reduction in the neuron number in the cortex (Figure 3.33B), thalamus (Figure 3.33D), cerebellum (Figure 3.33E), and pons (Figure 3.33F) of 28-week-old DKO mice with combined administration compared to those of their younger counterparts.

3.8.4. Anti-CNPase Staining

In Figure 3.34, the fluorescent intensity of anti-CNPase antibody, the oligodendrocyte marker, was evaluated in distinct brain sections of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline. The levels of CNPase were lower in all brain regions of DKO mice compared to 20-week-old SKO mice (Figure 3.34). Furthermore, the oligodendrocyte levels were elevated in 20-week-old DKO mice with combined administration compared to DKO mice in the cortex (Figure 3.34B) and thalamus (Figure 3.34C). In the thalamus (Figure 3.34C), cerebellum (Figure 3.34D), and pons (Figure 3.34E) of 28-week-old DKO mice administered with AAV, higher levels of CNPase were shown compared to DKO mice. Elevation in the oligodendrocyte levels in the thalamus (Figure 3.34C) and cerebellum (Figure 3.34D) was indicated in 28-week-old DKO mice administered with AAV and Istradefylline compared to DKO mice. The lower oligodendrocyte levels were detected in the thalamus (Figure 3.34C) of 20-week-old DKO with only AAV compared to age-matching DKO with combined administration, while the same difference was observable in both thalamus (Figure 3.34C) and cerebellum (Figure 3.34D) compared to those of 28-week-old DKO administered with AAV.

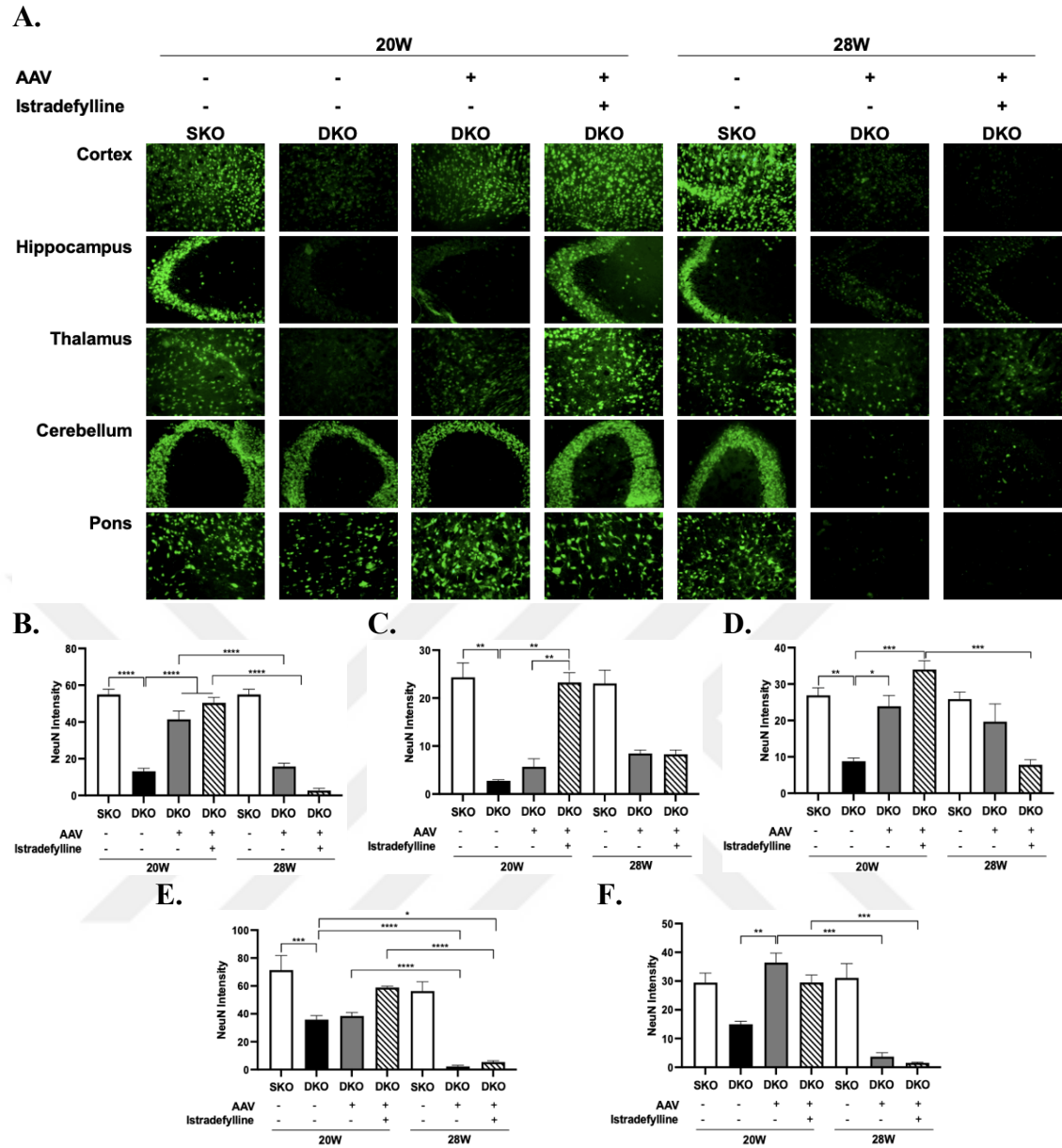


Figure 3.33. The sections (A) from the cortex, hippocampus, thalamus, cerebellum, and pons of 20- and 28-week-old SKO, 20-week-old DKO, 20- and 28-week-old DKO administered with AAV, and 20- and 28-week-old DKO administered with AAV and Istradefylline were stained with anti-NeuN (green) and DAPI (blue). The histograms represent the quantification of NeuN in the cortex (B), hippocampus (C), thalamus (D), cerebellum (E), and pons (F). Scale bar = 50 μ m. One-way ANOVA analysis was used to determine p-values by GraphPad. The data were represented as mean $\pm$ SEM. (n=3; *p<0.05, **p<0.025, and ****p<0.001).

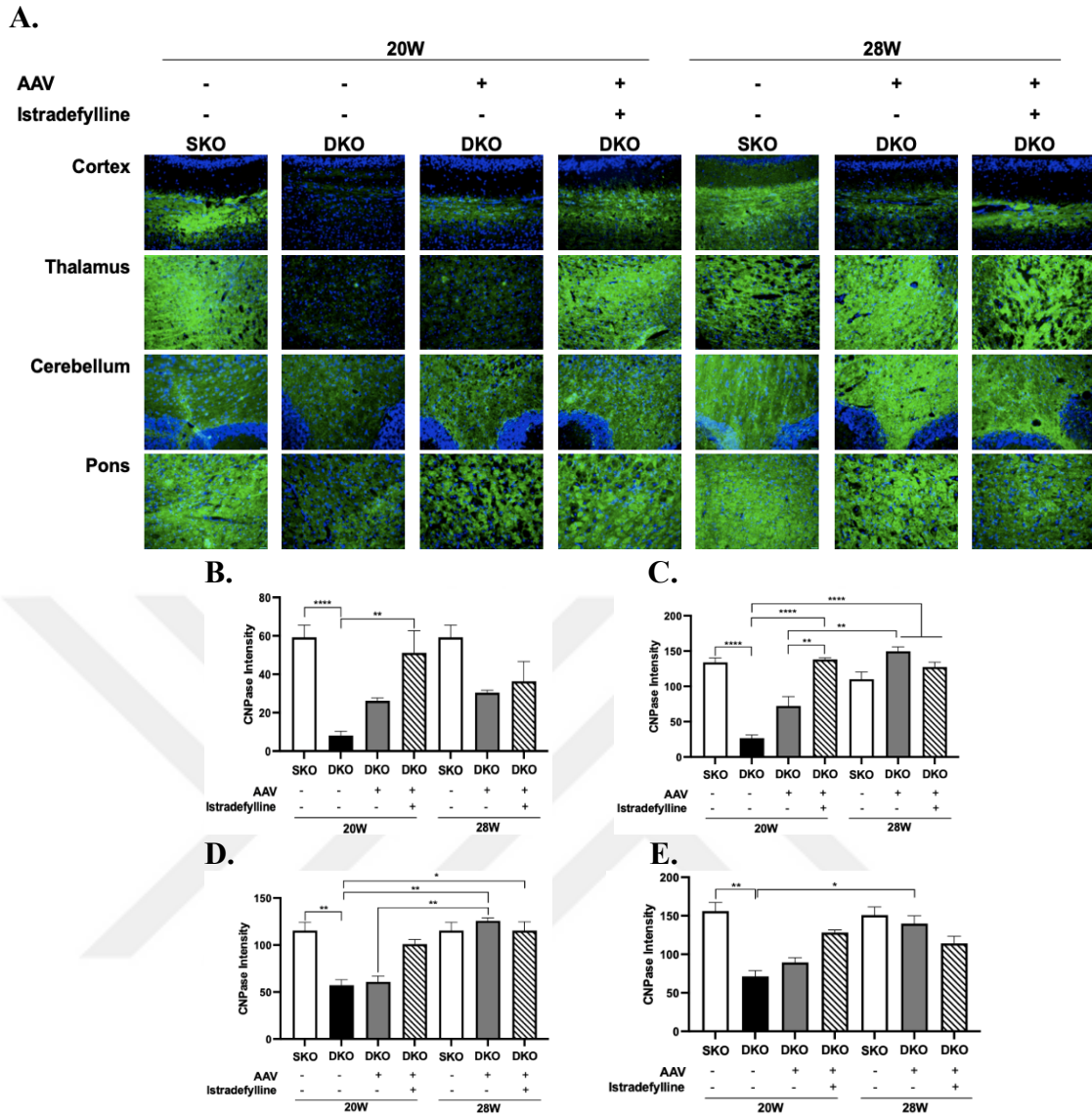


Figure 3.34. The sections (A) from the cortex, thalamus, cerebellum, and pons of 20- and 28-week-old SKO, 20-week-old DKO, 20- and 28-week-old DKO administered with AAV, and 20- and 28-week-old DKO administered with AAV and Istradefylline were stained with anti-CNPase (green) and DAPI (blue). The histograms represent the quantification of oligodendrocytes in the cortex (B), thalamus (C), cerebellum (D), and pons (E). Scale bar = 50 μ m. One-way ANOVA analysis was used to determine p-values by GraphPad. The data were represented as mean $\pm$ SEM. (n=3; *p<0.05, **p<0.025, and ****p<0.001).

3.8.5. Anti-GFAP Staining

In Figure 3.35, the astrocyte marker, GFAP, was analyzed in distinct brain sections of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and both 20- and

28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline. The active astrocyte levels were elevated in DKO mice compared to SKO mice in all brain regions (Figure 3.35). Furthermore, the active astrocyte levels were higher in all brain regions of DKO mice in contrast to 20-week-old DKO mice administered with AAV (Figure 3.35). Interestingly, the active astrocyte levels were elevated in the cerebellum and pons of 20-week-old DKO mice with combined administration compared to age-matching counterparts with AAV (Figure 3.35). In all brain regions except for the hippocampus of 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline, the active astrocyte levels were higher compared to DKO mice (Figure 3.35). In addition, active astrocyte levels were upregulated in 28-week-old DKO mice administered with AAV and those administered with AAV and Istradefylline compared to their younger counterparts (Figure 3.35).

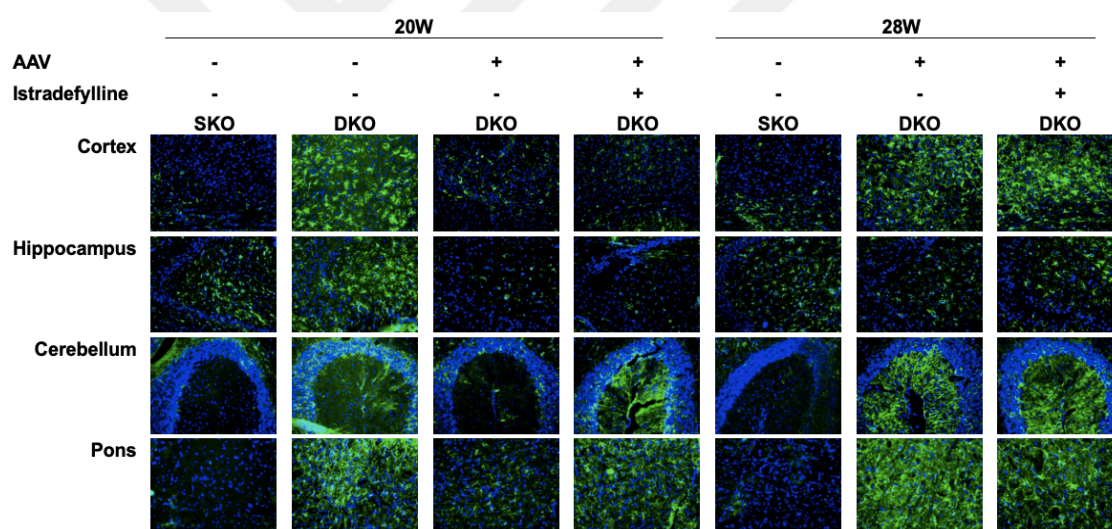


Figure 3.35. The sections from the cortex, thalamus, cerebellum, and pons of 20- and 28-week-old SKO, 20-week-old DKO, 20- and 28-week-old DKO administered with AAV, and 20- and 28-week-old DKO administered with AAV and Istradefylline were stained with anti-GFAP (green) and DAPI (blue). Scale bar = 50 μ m. (n=3).

3.8.6. *In Situ* Apoptosis (TUNEL) Analyses

In Figure 3.36, the number of TUNEL-positive apoptotic cells was measured in distinct brain sections of 20- and 28-week-old SKO mice, 20-week-old DKO mice, and

both 20- and 28-week-old DKO mice administered with AAV, as well as those administered with AAV and Istradefylline.

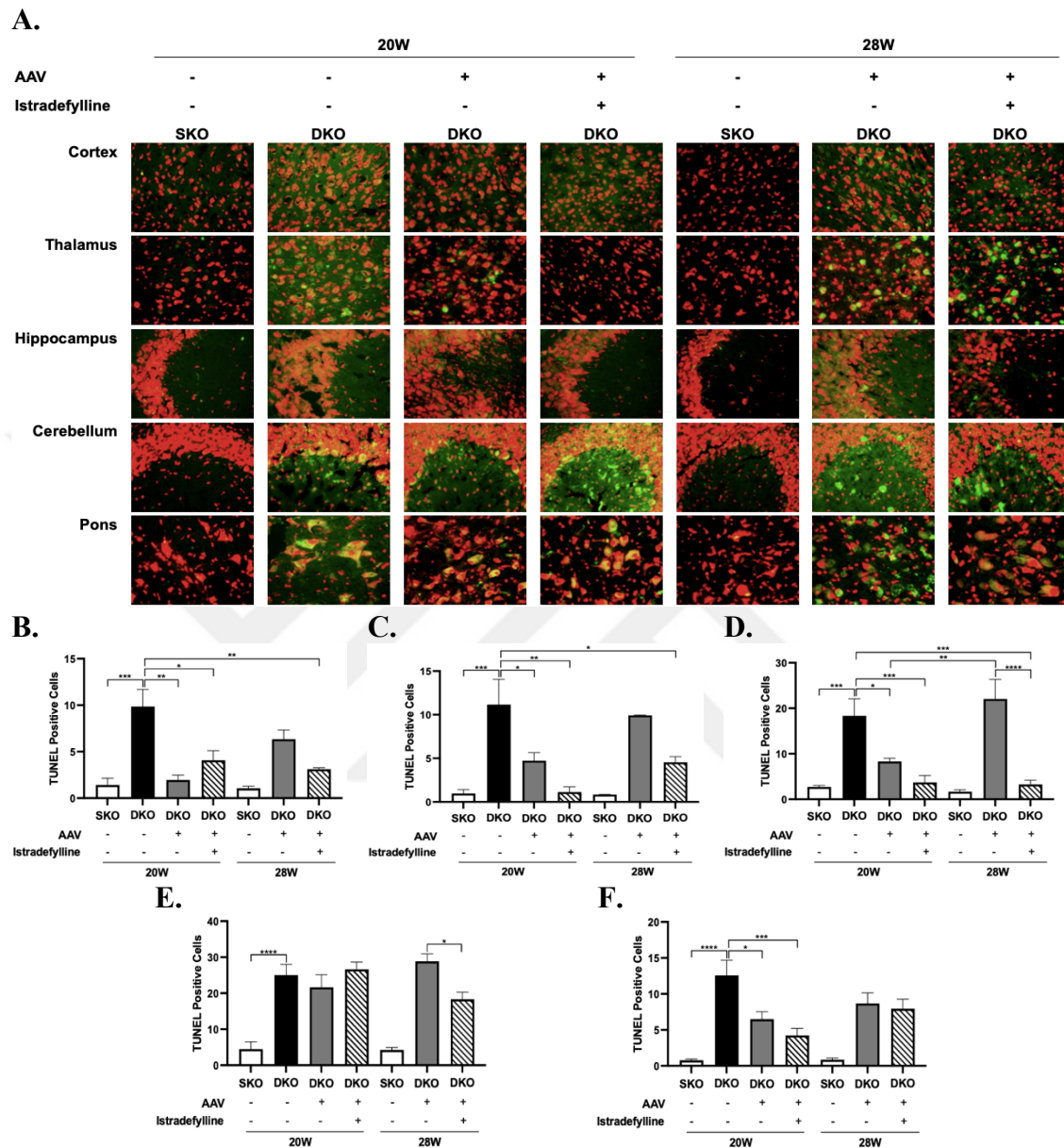


Figure 3.36. The sections (A) from the cortex, thalamus, cerebellum, and pons of 20- and 28-week-old SKO, 20-week-old DKO, 20- and 28-week-old DKO administered with AAV, and 20- and 28-week-old DKO administered with AAV and Istradefylline were stained with TUNEL (green) and PI (red). The histograms represent the quantification of TUNEL-positive cells per area in the cortex (B), thalamus (C), cerebellum (D), and pons (E). Scale bar = 50 μ m. One-way ANOVA analysis was used to determine p-values by GraphPad. The data were represented as mean $\pm$ SEM. (n=3; *p<0.05, **p<0.025, and ***p<0.001).

The number of TUNEL-positive apoptotic cells was lowest in all brain regions of 20-week-old SKO mice (Figure 3.36). At the same time, a prominent elevation was observed across all brain regions of DKO mice in the number of TUNEL-positive apoptotic cells compared to SKO mice (Figure 3.36). Additionally, the apoptotic cells were substantially decreased in all brain regions except for the cerebellum of 20-week-old DKO mice administered with AAV, and those administered with AAV and Istradefylline in contrast to age-matching DKO mice (Figure 3.36). With Istradefylline treatment, at 28 weeks, DKO mice with AAV reduced the number of apoptotic cells compared to 20-week-old DKO mice across all brain regions except for the cerebellum and pons (Figure 3.36). Surprisingly, there was a significant increase in the numbers of TUNEL-positive apoptotic cells in the thalamus of 28-week-old DKO mice administered with AAV compared to those of 20-week-old DKO mice (Figure 3.36D).

3.9. Behavioral Assays

Footprint and Rotarod analyses were performed using 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and 20- and 28-week-old DKO mice administered with AAV and Istradefylline to address the potential improvements in their gait abilities and locomotor activities.

3.9.1. Footprint Analysis

The footprint analyses (Figure 3.37A) revealed a significant reduction in stride length (Figure 3.37B), sway length (Figure 3.37C), and stance length (Figure 3.37D) of 20-week-old DKO mice compared to those of 20-week-old SKO mice. Although there was no significant change in sway length among the experimental groups, the decrease in stride and stance length of 28-week-old DKO mice with combined administration compared to their younger counterparts was indicated (Figure 3.37A, D). Furthermore, a remarkable decline in the stride length of (Figure 3.37B) 28-week-old DKO mice with AAV was shown when compared to 20-week-old DKO mice administered with AAV.

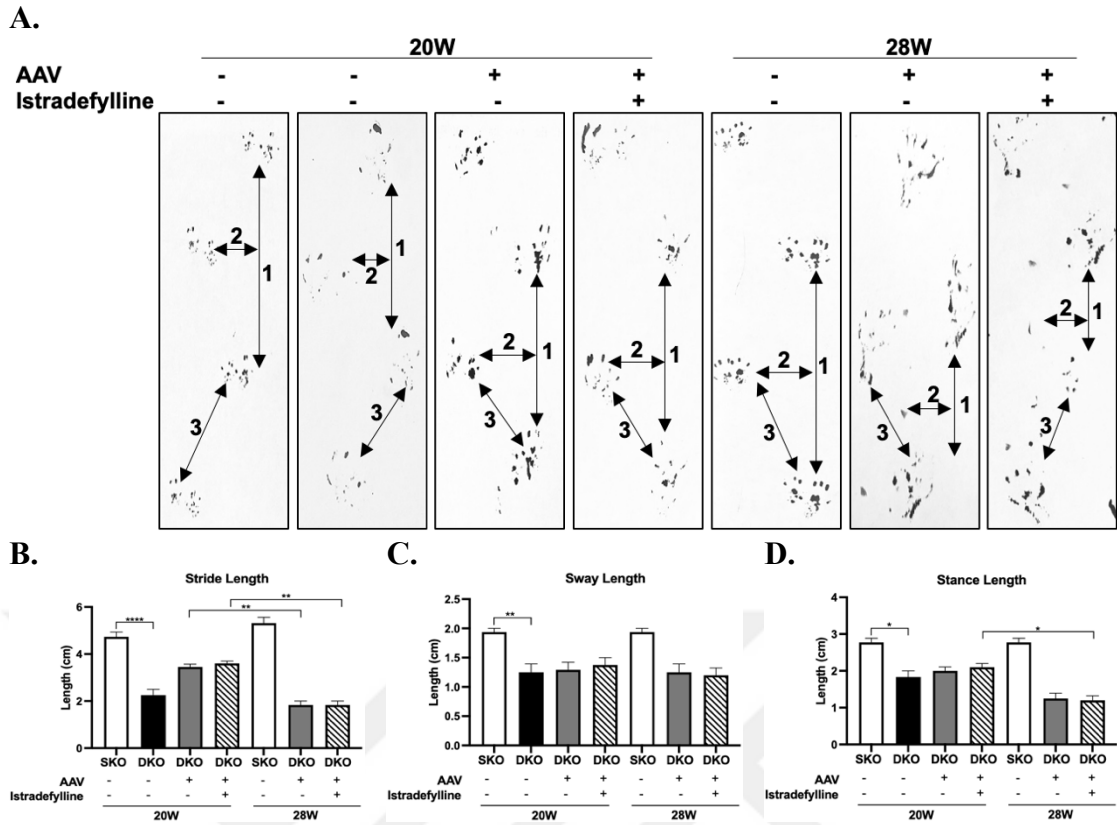


Figure 3.37. Footprint test to analyze the walking patterns of 20- and 28-week-old SKO (n=12 and n=8), 20-week-old DKO (n=5), 20- and 28-week-old DKO mice administered with AAV (n=11 and n=4), and those administered with AAV and Istradefylline (n=4 for each) (in terms of stride length (1), sway length (2), and stance length (3)). The histograms show the mean values of stride length (B), sway length (C), and stance length (D). One-way ANOVA analysis was used to determine p-values by GraphPad. The data were represented as mean $\pm$ SEM. (n=; *p<0.05, **p<0.025, and ****p<0.001).

3.9.2. Rotarod Analysis

To show the changes in the locomotor activities of 20- and 28-week-old SKO mice, 20-week-old DKO mice, 20- and 28-week-old DKO mice administered with AAV, and those administered with AAV and Istradefylline, the rotarod test was performed. A remarkable increase in the time spent on the rod was observed in 20-week-old DKO mice administered with AAV, as well as those with AAV and Istradefylline compared to the age-matching DKO mice, which showed a significant reduction in the locomotor activity compared to 20-week-old SKO mice (Figure 3.38). In the locomotor activity of 28-week-old counterparts of 20-week-old DKO mice administered with AAV, as well as those with AAV and Istradefylline, a slight reduction was observed (Figure 3.38).

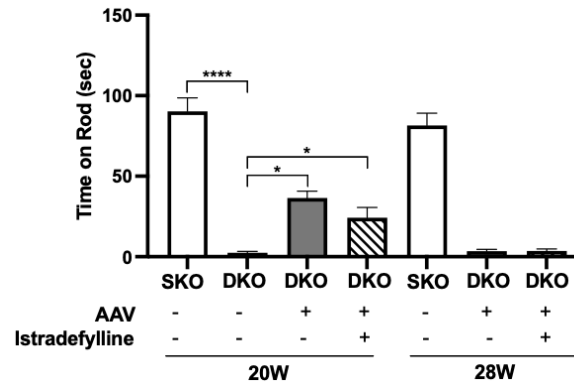


Figure 3.38. Rotarod test to analyze the locomotor activities of 20- and 28-week-old SKO (n=12 and n=8), 20-week-old DKO (n=5), 20- and 28-week-old DKO administered with AAV (n=11 and n=4), and those administered with AAV and Istradefylline (n=4 for each). The histogram shows the mean values of time spent on the rotating rod. The speed was accelerated from 4 rpm to 40 rpm over five minutes. One-way ANOVA analysis was used to determine p-values by GraphPad. The data were represented as mean $\pm$ SEM. (n=; *p<0.05 and ****p<0.001).

CHAPTER 4

DISCUSSION

Lysosomal storage diseases (LSDs) are heritable monogenic metabolic disorders due to lysosomal dysfunction and accumulating lysosomal inclusions. Among 70 types of LSDs with different lysosomal accumulation and CNS involvement, GM2 gangliosidoses are characterized by an abnormal buildup of GM2 ganglioside in the neuronal lysosomes, causing neurodegeneration.^{15,22} Tay-Sachs disease, a type of GM2 gangliosidosis, occurs due to a mutation in the *HEXA* gene encoding β -hexosaminidases A and thus, excessive accumulation of GM2 gangliosides.³⁵ Due to progressively accumulating GM2 ganglioside in patient neurons, neuron size increases, and neurons are destroyed in the brain and spinal cord of TSD patients.³² As a result, patients with TSD develop a cherry-red spot on the retina, seizures, dysphagia, muscle weakness, motor dysfunction, and eventually, death at 4 years of age. Up to now, there has been no effective cure developed for preventing the symptoms and sudden death in patients with TSD.³² Seyrantepe et al.³² showed that *Hexa* and *Neu3* doubly deficient mice exhibit similar neuropathological, biochemical, clinical, and behavioral aspects seen in early-onset TSD patients, associated with an excessive buildup of GM2 ganglioside in CNS and PNS.³² Previous studies with *Hexa*^{-/-}*Neu3*^{-/-} mice revealed the presence of disrupted cellular pathways, including neuroinflammation, oxidative stress, autophagy, and neurodegeneration due to massive GM2 ganglioside buildup.³² Moreover, Demir et al.⁴⁷ emphasized the release of pro-inflammatory cytokines and chemokines due to reactive astrocytes and microglia in different brain regions of *Hexa*^{-/-}*Neu3*^{-/-} mice.⁴⁷ Progressive neurodegeneration and altered biological pathways in *Hexa*^{-/-}*Neu3*^{-/-} mice resulted in sudden death at 20 weeks of age.³² Among several treatment strategies, Adeno-associated viral (AAV)-based gene therapy is the most promising approach to cure Tay-Sachs disease since AAVs can cross the BBB easily. Moreover, combining an anti-inflammatory agent, such as Istradefylline, with AAV-based gene therapy could increase the effectiveness of this therapeutic strategy targeting neuroinflammatory responses.

Although the role of mouse Neu3 in a bypass mechanism for the degradation of GM2 ganglioside has been understood using *Hexa*^{-/-}*Neu3*^{-/-} mice (DKO), the potential contribution of human Neu3 (hNeu3) in GM2 degradation remains unclear. In this study,

intrathecal administration of an AAV serotype, AAVrh10, to express human *NEU3* (hNeu3) combined with Istradefylline treatment was performed in DKO mice. By administration of AAVrh10-hNeu3 virus particles at 8 weeks of age, the efficacy of AAVrh10-human Neu3-mediated gene therapy in the early-onset TSD mice model was elucidated. In addition, whether Istradefylline treatment alleviates the neuroinflammation pathology in DKO mice was aimed to understand.

The results of the study demonstrated a prolonged lifespan of up to 28 weeks in DKO administered with AAV combined with or without Istradefylline (Figure 3.8A). In addition, according to the results of weekly body weight measurements, DKO mice administered with AAV and those with AAV and Istradefylline showed delayed weight loss, starting at 20 weeks and persisting until a sudden death at 28 weeks in contrast to DKO mice exhibiting a progressive weight loss (Figure 3.8B, C). These observations suggest that AAV with or without Istradefylline contributed to an extended lifespan and delayed weight loss, possibly by mitigating some underlying disease mechanisms. However, since the mice died suddenly at 28 weeks, the intervention might not fully prevent pathological progression leading to mortality.

According to TLC analyses, a slight decrease in GM2 ganglioside levels were observed in the cortex of 20-week-old DKO mice administered with AAV and those with combined administration compared to DKO mice, whereas at 28 weeks the GM2 levels reached the levels observed in DKO mice. The efficacy of human Neu3 sialidase for partial degradation of GM2 ganglioside at 20 weeks was observed. However, this effect might diminish in time as the disease progress. Moreover, immune response against the AAV administration could lead to suppression of AAV efficacy (Figure 3.9).

The mRNA expression analyses were performed using different brain regions and visceral organs of the experimental groups. Since the AAVrh10 serotype shows tropisms for the liver, kidney, and brain, those organs were evaluated in terms of human Neu3 and inflammation-related marker expression. According to the results, human Neu3 expression was observed in the cortex, cerebellum, kidney, and liver, all of which AAVrh10 shows tropism. Even though the AAVrh10 serotype does not exhibit tropism for the spleen, the human Neu3 expression was also upregulated in this tissue. This might be related to the immune function of this organ.¹⁴³

In the cortex of DKO mice, the most severe neuroinflammatory response was identified with significantly increased expressions of *Ccl3*, *Cxcl10*, *Iba1*, and *Gfap* (Figure 3.10). Likewise, significantly increased expression levels of *Ccl2*, *Ccl3*, and

Cxcl10 in the cortex were demonstrated in 20-week-old DKO mice previously.⁴⁷ Furthermore, astrogliosis with GFAP staining and microglial activation with IBA1 staining were characterized in 20-week-old DKO mice.^{32,47} AAV treatment alone increased the expression of neuroinflammatory markers further at 28 weeks, especially *Ccl3*, *Ccl5*, and *Iba1* in the cortex. Istradefylline administration reduced *IL-1 β* and *Gfap* levels, suggesting an anti-inflammatory effect, possibly due to adenosine A2A receptor antagonism, which reduced the microglial activation.¹¹⁴

Unlike the cortex, the neuroinflammatory response was exhibited at moderate levels in the cerebellum of 20-week-old DKO mice. Upregulation of *Ccl2*, *Ccl3*, *Cxcl10*, *Iba1*, and *Gfap* mRNAs was observed in 20-week-old DKO mice (Figure 3.11). Demir et al.⁴⁷ revealed the increased expression of *Ccl3* and *Cxcl10* and astrogliosis and microgliosis in 20-week-old DKO mice.⁴⁷ At 28 weeks, the expression levels of *Ccl2*, *Ccl3*, *Cxcl10*, and *Iba1* were higher in the cerebellum of DKO mice administered with AAV, but Istradefylline decreased *Ccl3*, *Ccl5*, *Cxcl10*, and *Iba1* expression, demonstrating its anti-inflammatory role (Figure 3.11).¹¹⁴

Increasing expression of *Iba1* and *Gfap* mRNAs reveals the neurodegenerative effects of astrogliosis and microgliosis in DKO mice. Additionally, the downregulation of neuroinflammatory markers in the cerebellum but not fully in the cortex after Istradefylline treatment in 28-week-old DKO mice administered with AAV might suggest that its effects might vary between different brain regions due to distinct levels of receptor expression or immune cell activation. Taken together, these results suggest the presence of chronic neuroinflammation and microglial activation in DKO mice, which are hallmarks of LSDs, including Niemann-Pick type C disease¹⁴⁴, Gaucher disease¹⁴⁵, and neuronal ceroid-lipofuscinoses¹⁴⁶. Interestingly, lysosomal *Lamp1* expression increased in DKO mice administered with AAV at 28 weeks in both the cortex and cerebellum tissues (Figures 3.10 and 3.11). These results signify the presence of an adaptive response to prolonged neurodegenerative stress to improve lysosomal function and potential therapeutic efficacy.

In the kidney, a distinct pattern of inflammation was observed in 20-week-old DKO mice, where pro-inflammatory cytokines *Ccl2*, *Ccl3*, *Ccl5*, and lysosomal marker *Lamp1* were all upregulated (Figure 3.12). These changes implicated the presence of chronic inflammation along with lysosomal dysfunction in the visceral organs of DKO mice. The elevated *Lamp1* expression might represent abnormal lysosomal storage in the kidney, as well. Although in DKO mice with AAV at 20 weeks of age, there was reduced

inflammation, at 28 weeks, pro-inflammatory cytokines, *Ccl3* and *Ccl5*, were increased, suggesting a delayed inflammatory resurgence. The protective effects of Istradefylline on kidneys were observed in 28-week-old DKO mice with AAV, where further reduction in *Ccl2*, *Cxcl10*, and *Lamp1* was revealed.

Similarly, in the liver of 20-week-old DKO mice, significantly increased expression of pro-inflammatory cytokines, *Ccl3*, *Ccl5*, and lysosomal marker *Lamp1* demonstrated a chronic immune activation (Figure 3.13). AAV treatment downregulated the mRNA levels of *Ccl2* and *Ccl5* at 20 weeks, whereas the chronic inflammation reappeared at 28 weeks. The Istradefylline treatment might suppress the inflammatory effects of *Ccl5* and *Cxcl10* at 20 weeks, but was less effective at 28 weeks. These results indicated a transient anti-inflammatory activity of Istradefylline at 28 weeks.

Unexpectedly, in the spleen, a major immune organ, of 20-week-old DKO given AAV with or without Istradefylline, elevated mRNA levels of *Ccl5* and *Cxcl10* were shown. However, at 28 weeks, a decrease in *Cxcl10* and *Lamp1* was observed in the spleen of AAV-treated DKO mice (Figure 3.14). These results might demonstrate a terminal decline, where reductions in those markers result from widespread cell death, tissue degradation, or systemic failure. Istradefylline, on the other hand, reinforced its anti-inflammatory effects in the spleen of 28-week-old DKO mice with AAV by reducing the expression of *Ccl5* (Figure 3.14).

For further understanding of inflammatory pathways in different organs of the experimental groups of this study, western blot analyses were performed. In the cortex of 28-week-old DKO mice administered with both AAV and Istradefylline, a significant increase in I κ B- α levels but no change in NF κ B levels was shown compared to 20-week-old counterparts, suggesting potential anti-inflammatory feedback or compensatory responses at later stages (Figure 3.15 and 3.16). At 20 weeks, DKO mice administered with only AAV revealed a decrease in NF κ B levels compared to DKO mice, whereas combining AAV with Istradefylline paradoxically reversed this situation (Figure 3.16). Istradefylline might initially promote NF κ B activation as a compensatory mechanism to clear accumulating stress conditions in the cortex. Moreover, NF κ B might regulate cell survival in the cortex of these mice in addition to its pro-inflammatory activities.

In the kidneys of 28-week-old DKO mice treated with AAV and Istradefylline, I κ B- α levels were elevated (Figure 3.22), pointing to anti-inflammatory attempts toward prolonged inflammation at later stages. Significantly increased IL-1 β levels in 28-week-

old DKO mice with combined treatment might reflect an unresolved inflammation or the inflammatory effects of AAV administration in a tissue-specific manner (Figure 3.24).

Elevation of I κ B- α levels in the liver of 20- and 28-week-old DKO mice with AAV and combined treatment might suggest the activation of anti-inflammatory pathways, while DKO mice might not respond to chronic inflammation in a tissue-dependent manner (Figure 3.25). Since NF κ B levels were increased in the liver of 20-week-old DKO mice with combined treatment compared to DKO mice, the cellular survival mechanisms could be activated. The decrease in NF κ B levels at later ages could indicate a progression toward systemic failure along with sudden death (Figure 3.26).

In the spleen of DKO mice, there was a significant reduction in NF κ B levels compared to SKO mice and 20-week-old DKO mice with AAV only or with combined treatment (Figure 3.28). These results might represent the activation of cell survival mechanisms. However, at 28 weeks, NF κ B protein expression was downregulated up to levels similar to DKO mice, reflecting exhaustion or organ shutdown (Figure 3.29). Increased expression of IL-1 β identified in DKO mice was downregulated after AAV and Istradefylline treatments (Figure 3.30), aligning with the potential of the treatments to reduce splenic inflammation. Taken together, these results might demonstrate transient suppression of inflammation with AAV alone, whereas combined treatment might offer more sustained modulation with varying effects in different tissues. Unexpected increases in AAV and Istradefylline treatment conditions highlight the complexity and potential treatment-induced stress in different organs.

Immunohistochemical (IHC) and *in situ* Apoptosis (TUNEL) analyses were performed in this study to reveal the changes in the levels of GM2 ganglioside and LAMP1, numbers of neurons, oligodendrocytes, astrocytes, and apoptotic cells in DKO mice with AAV only or combined treatment. The results of anti-GM2 staining indicated an elevated buildup of GM2 ganglioside due to lysosomal dysfunction in all brain regions (Figure 3.31). Seyrantepe et al.³² previously identified significantly increased levels of GM2 ganglioside in the hippocampi and cerebella of 20-week-old DKO mice.³² In 20-week-old DKO mice, treatment with AAV appeared to affect GM2 accumulation partially, especially in the cortex and hippocampus, where neuronal activity and turnover are high (Figure 3.31B, C). AAV therapy is less effective in the pons, which might be related to different cellular compositions (Figure 3.31F). Especially in the cortex, hippocampus, and thalamus of 20-week-old DKO mice with combined therapy, significantly decreased GM2 ganglioside levels suggested the potential of the combined

treatment. Interestingly, the cerebellum and pons showed unexpected upregulation in GM2 ganglioside levels, representing a regional variability (Figure 3.31). Furthermore, at 28 weeks, AAV only or with Istradefylline continued to decrease GM2 ganglioside accumulation, particularly in the cortex, whereas GM2 levels in the hippocampus were reduced with the combined treatment compared to AAV alone, reflecting the responsiveness of those tissues when inflammation is modulated. On the contrary, in the thalamus, cerebellum, and pons, higher GM2 ganglioside levels were observed in 28-week-old DKO mice with AAV compared to their younger counterparts, indicating ongoing neurodegeneration or slower therapeutic effect. The transient upregulation of GM2 in certain brain regions following AAV and Istradefylline administration might demonstrate a complex interplay between neuroinflammation and lipid homeostasis. In a previous study, the correlation of the storage of GM1 and GM2 gangliosides in the CNS with microgliosis and astrogliosis was demonstrated. The degree of inflammation is associated with elevated levels of ganglioside buildup.¹⁴⁷

Since GM2 ganglioside accumulation is associated with lysosomal dysfunction and neuroinflammatory responses in CNS, Demir et al.⁴⁷ showed increased levels of LAMP1 and its colocalization with microglia in all brain regions of 20-week-old DKO mice.^{47,119} In this study, increased levels of LAMP1 were also identified in all brain regions of 20-week-old DKO mice, which is correlated with increased GM2 ganglioside accumulation and thus, lysosomal burden (Figure 3.32). In especially thalamus and cerebellum, decreasing LAMP1 levels were observed in 20-week-old DKO with AAV, suggesting a better response due to regional differences in AAV transduction efficiency or baseline lysosomal stress. Although AAV alone contributed to lysosomal clearance in these tissues, combined therapy paradoxically decreased lysosomal degradation temporarily, causing compensatory upregulation of LAMP1. In the cortex, hippocampus, and thalamus of 28-week-old DKO mice with combined treatment, there was an elevation in LAMP1 levels compared to AAV alone. Considering those results, the potential efficacy of AAV to reduce lysosomal burden in DKO mice, whereas combining AAV with Istradefylline resulted in complex modulation of neuroinflammatory and lysosomal pathways in a region- and age-specific manner, could be understood.

Neurodegeneration and reduced neuron number were the hallmarks of TSD. Therefore, the loss of NeuN levels in the brain of 20-week-old DKO mice was explained by an elevation in neuronal death and the number of apoptotic cells.⁴⁷ In this current study, regional change in the levels of NeuN immunostaining was also performed.

Significantly decreasing neuron number was identified in 20-week-old DKO mice compared to control mice (Figure 3.33). Those results reflected neurodegeneration correlated with lysosomal dysfunction and neuroinflammatory responses in DKO mice. Notably, AAV administration at 20 weeks advanced neuron number in the cortex, thalamus, and pons regions. These results were consistent with LAMP1 levels since AAV enhanced lysosomal clearance in these regions. The rescue of neuron number in the cortex and thalamus is particularly significant since they are involved in motor and cognitive functions. In the case of combined administration at 20 weeks, a further enhancement in neuron number was demonstrated in the cortex, hippocampus, and thalamus. Since Istradefylline antagonizes A2A receptors to perform anti-inflammatory actions, decreased inflammatory signaling might protect neurons from cytokine-induced cell death.¹¹⁴ The neuron numbers were lower in all brain regions of DKO mice at 28 weeks, correlating with progressive neurodegeneration and sudden death (Figure 3.33).

Oligodendrocytes, a type of glial cell, have crucial roles in the formation of myelin sheaths, by which axons are insulated and supported.¹⁴⁸ Demir et al.⁴⁷ demonstrated a significant loss in the numbers of 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase)-positive cells in all brain regions of 20-week-old DKO mice, reflecting a decrease in myelination.⁴⁷ Previously, the gene expression profile in the cerebral cortex of TSD and SD patients indicated a significant reduction in the levels of the myelin basic protein gene, expressed by oligodendrocytes.¹⁴⁹ Here, the oligodendrocyte levels were characterized using CNPase immunostaining (Figure 3.34). Substantially decreased CNPase intensity was observed in all brain regions of 20-week-old DKO mice. Lysosomal impairment and pro-inflammatory signals probably contributed to this global oligodendrocyte loss in DKO mice. With the combined treatment, at 20 weeks, increased oligodendrocyte survival, and thus, myelin production, was re-established in almost all brain regions, indicating the potential of the anti-inflammatory microenvironment. Although at 28 weeks, oligodendrocyte recovery seemed to increase, neurodegeneration and neuronal loss were faster than the glial recovery. Oligodendrocytes might attempt to force remyelination at later stages, as a compensatory mechanism to increased axonal injury.

To highlight dynamic changes in astrocyte reactivity, Glial fibrillary acidic protein (GFAP) immunostaining was performed across different brain regions and experimental groups. A pronounced increase in the levels of active astrocytes in 20-week-old DKO mice was revealed, consistent with the previous results.⁴⁷ The astrogliosis likely reflected the neuroinflammatory responses due to lysosomal impairment and GM2

ganglioside accumulation in the mouse model. The AAV administration alone appeared to attenuate astrocyte activation at 20 weeks across all brain regions compared to DKO mice (Figure 3.35). The neuroprotective effects of AAV therapy by enhancing GM2 ganglioside clearance or indirectly reducing neuronal stress signals might be understood. Unexpectedly, in the cerebellum and pons of 20-week-old DKO mice with combined treatment, GFAP levels were elevated compared to age-matched DKO with AAV alone, suggesting regional modulatory effects of the drug. Moreover, the age-dependent progression of GFAP elevation in nearly all brain regions of 28-week-old DKO mice treated with AAV alone or in combination with Istradefylline represented a response to prolonging neuronal degeneration, lysosomal stress, or neuroinflammatory stimuli.

In addition to IHC analyses, *in situ* apoptosis analysis was performed in this study to show changes in TUNEL-positive apoptotic cells in distinct brain regions (Figure 3.36). Through TUNEL analysis, novel insights into the potential mitigation of the neurodegenerative process after AAV and Istradefylline treatments. The highest number of TUNEL-positive cells was observed in DKO mice across all brain regions compared to SKO mice, indicating pronounced neurodegeneration.³² Interestingly, AAV alone or in combination with Istradefylline resulted in a substantial reduction of apoptotic cells across all brain regions, except for the cerebellum. Through gene delivery, AAV could modulate disease pathology and perform neuroprotective roles, whereas, in combination with Istradefylline, this effect was potentiated. However, the lack of improvement in the cerebellum might reflect region-specific changes to these interventions. A more complex pattern occurs at 28 weeks in DKO mice with AAV and Istradefylline treatment as compared to AAV-treated counterparts, showing higher numbers of TUNEL-positive cells. This could be explained by the substantial loss in the number of neurons, but not by the neuroprotective effects of the interventions. The significant elevation in the number of apoptotic cells in the thalamus of 28-week-old DKO mice treated with AAV compared to their 20-week-old counterparts could be related to the vulnerability of this region to the disease course, which could not be mitigated by AAV alone. Moreover, these increases could be further correlated with the pronounced chronic inflammation in this region.

Previously, it was depicted that abnormal GM2 ganglioside accumulation in the CNS of 20-week-old DKO mice leads to progressive neurodegeneration, astrogliosis, and neurobehavioral deterioration, including slow movement, gait abnormalities, and tremors.^{32,47} Therefore, to show the effectiveness of AAV and Istradefylline treatments

on the neurobehavioral capabilities of DKO mice, footprint and rotarod analyses were performed across different experimental groups of this study. A significant reduction in stride length, sway length, and stance length of 20-week-old DKO mice compared to age-matched control mice reflected gait abnormalities due to underlying neurodegeneration or glial activation (Figure 3.37). In later stages, mice with AAV alone or combined therapy exhibited a decrease in stride and stance lengths compared to their younger counterparts, which correlated with the neuroinflammatory signals and motor neuron loss. The neuronal loss and astrogliosis in the cerebellum and cortex contributed to observed motor deficits at later ages despite therapeutic interventions. The rotarod analyses revealed a remarkable improvement in 20-week-old DKO mice administered with AAV or AAV and Istradefylline compared to DKO mice, indicating the neuroprotective effects of AAV to improve neuronal function. By mitigating the deleterious effects of neuroinflammation, AAV with or without Istradefylline might recover the motor coordination of 20-week-old DKO mice. Although a slight improvement in motor functions of 28-week-old DKO mice with AAV and with combined treatment was observed, it does not fully restore the motor activities seen in their younger counterparts. Since the progression of neurodegeneration and chronic neuroinflammation is more pronounced at 28 weeks, the decline in rotarod performance could be explained (Figure 3.38).

CHAPTER 5

CONCLUSION

In conclusion, this study gave valuable insights into the potential of AAVrh10-hNeu3-based gene therapy, in combination with Istradefylline, since the neuropathological and neurobehavioral abnormalities in *Hexa*^{-/-}*Neu3*^{-/-} (DKO) mice were mitigated. The extension of the lifespan of DKO mice from 20 to 28 weeks was observed in AAV- and Istradefylline-administered conditions, along with a delay in weight loss in the treated mouse groups. These results suggest that AAV-mediated expression of human Neu3 could partially alleviate GM2 ganglioside accumulation and potentially decelerate the disease progression. However, sudden death at 28 weeks might indicate a partial prevention of neurodegeneration and other disease mechanisms seen in DKO mice.

Furthermore, the importance of neuroinflammation in TSD pathology was highlighted in this study by analyzing a variety of inflammation-related markers across different brain regions of mice groups with AAV alone or with combined treatment. Although it was not uniform among brain regions, the reduction of pro-inflammatory cytokines, such as IL-1 β , and inhibition of astrogliosis and microgliosis were demonstrated in experimental groups with Istradefylline treatments. The regional modulation of neuroinflammatory responses could be correlated with differential responses to the treatments or region-specific immune cell activation.

The changes in the expression of lysosomal marker LAMP1 at later ages reflect a compensatory response to prolonged neurodegenerative stress. In the case of Istradefylline treatment, at 20 weeks, the elevation in LAMP1 expression might be a response to temporarily enhance lysosomal function. Despite the partial clearance of GM2 ganglioside, the thalamus and cerebellum exhibited persistent accumulation, which indicated a variability in AAV transduction efficacy or cellular composition of those regions. The enhancement in NeuN-positive neuron number in the cortex, thalamus, hippocampus, and pons of 20-week-old DKO mice administered with AAV or with AAV and Istradefylline further supports the efficacy of therapeutic interventions. However, the neuronal loss at later stages, together with the progression of neurodegeneration shown by *in situ* apoptosis analyses and eventually sudden death at 28 weeks, represented a

temporary benefit of those treatments. While at 28 weeks, the amount of CNPase-positive glia and remyelination was increased in response to the anti-inflammatory effects of Istradefylline, it was not sufficient to counteract the ongoing neurodegeneration.

In summary, the combination of AAVrh10-hNeu3 and Istradefylline demonstrates the potential to delay disease progression, mitigate neuroinflammation, and slow neurodegeneration in the mouse model of Tay-Sachs disease. Despite the promising results, further investigation is needed to optimize the therapeutic conditions, address the limitations in therapeutic efficacy, and explore possible long-term solutions for TSD and similar lysosomal storage disorders.



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Inci, O. K., Basırlı, H., Can, M., Yanbul, S., & Seyrantepe, V. (2024). Gangliosides as therapeutic targets for neurodegenerative diseases. *Journal Of Lipids*, 2024(1), 4530255.2024

Basirli, H., Can, M., Sengul, T., & Seyrantepe, V. (2024). Lithium treatment rescues dysfunctional autophagy in the cell models of Tay-Sachs disease. *Molecular Genetics and Metabolism*, 141(3), 108140.2024

Can, M., Sengül, T., Demir, S. A., İnci, O. K., Basırlı, H., & Seyrantepe, V. (2022). Analysis of brain lipids in the early-onset Tay–Sachs disease mouse model with the combined deficiency of β -hexosaminidase A and Neuraminidase 3. *Frontiers in Molecular Biosciences*, 9, 892248.

SCHOLARSHIPS

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AWARDS

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