T.C.

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INVESTIGATION OF ELECTRICAL AND BEHAVIORAL PROPERTIES OF DENTATE GYRUS PRODYNORPHIN NEURONS IN AB-INDUCED ALZHEIMER'S MODEL TRANSGENIC MICE

MASTER THESIS

HABİBE GÖREN

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Istanbul, 2024

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APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated ..... and numbered .....

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Director of Institute of Health Sciences

DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

16.02.2024

Habibe GÖREN

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LIST OF SYMBOLS AND ABBREVIATIONS

AAV: Adeno Associated Virus

Aβ: Amyloid Beta

aCSF: Artificial Cerebrospinal Fluid

AChE: Acetylcholinesterase

AD: Alzheimer's Disease

AMPA: α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid

APP: Amyloid Precursor Protein

APOEε4: apolipoprotein E gene

ATP: Adenosine Triphosphate

BACE: Beta-Site Cleaving Enzyme

CaCl2: Calcium Chloride

CBF: Cholinergic Basal Forebrain

ChAT: Choline Acetyltransferase

ChR: Channel-Rhodopsin

CNO: Clozapine-N-Oxide

CNS: Central Nervous System

Cre: Causes of Recombination

CSF: Cerebrospinal Fluid

DG^{Pdyn}: Prodynorphin neurons in the dentate gyrus

DOR: Delta Opioid Receptor

ddH_2O: Double Distilled Water

DNA: Deoxyribonucleic Acid

DREAAD: Designer Receptors Exclusively Activated by Designer Drugs

DTI: Diffusion Tensor Imaging

EDTA: Ethylenediaminetetraacetic Acid

EPM: Elevated Plus Maze

EOAD: Early-Onset Alzheimer's Disease

FDA: Food and Drug Administration

FLEX: Flip-Excision

GABA: Gamma (γ)-Aminobutyric Acid

GFP: Green Fluorescent Protein

GPCRs: G-Protein-Couple Receptors

hM3D(Gq): human M3 Muscarinic Receptor

hM4D(Gi): human M4 Muscarinic Receptor

Ip: Intraperitoneal

KCl: Potassium Chloride

KOR: Kappa Opioid Receptor

Lox: Locus of Crossing

LOAD: Late-Onset Alzheimer's Disease

MAP: Microtubule-Associated Protein

MgSO4: Magnesium Sulfate

MOR: Mu Opioid Receptor

MWM Test: Morris Water Maze Test

NaCl: Sodium Chloride

NaHCO3: Sodium Bicarbonate

NaHPO4: Sodium Phosphate

NAOH: Sodium Hydroxide

NFTs: Neurofibrillary Tangles

NMDA: N-Methyl-D-Aspartate

NMDG: N-methyl-D-Glucamine

NOR Test: Novel Object Recognition Test

OFT: Open Field Test

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PD: Parkinson disease

Pdyn: Prodynorphin

PET: Positron Emission Tomography

PENK: Pre-Proenkephalin

POMC: Pro-Opiomelanocortin

PSEN1: presenilin 1 gene

PSEN2: presenilin 2 gene

REM: Rapid-Eye-Movement

RNA: Ribonucleic Acid

ROS: Reactive Oxygen Species

TBE: Tris Borate EDTA

TBI: Traumatic Brain Injury

TUBITAK: The Scientific and Technological Research Council of Türkiye

WHO: World Health Organization

ABSTRACT

Gören, H. (2024). Investigation of Electrical and Behavioral Properties of Dentate Gyrus Prodynorphin Neurons in Aβ-Induced Alzheimer's Model Transgenic Mice. Yeditepe University, Institute of Health Science, Department of Physiology, Master Thesis. Istanbul.

Alzheimer's Disease (AD) is a neurodegenerative disorder characterized by progressive cognitive decline and the accumulation of abnormal protein aggregates in the brain. The endogenous opioid system is a complex neurochemical system in the body that plays a crucial role in some physiological and psychological processes. Prodynorphin neurons, a part of the endogenous opioid system, are nerve cells that synthesize and release prodynorphin, the precursor polypeptide for various dynorphin opioid peptides. Today, the effect of the opioid system on AD and its role in the mechanism of dementia are not still fully understood. Therefore, this study aimed to investigate a possible relationship between AD and Prodynorphin neurons in the dentate gyrus (DG^{Pdyn}), which is a critical region for learning and memory. In this study, ten-weeks-old seventy Pdyn-Cre male mice were used. For electrophysiological studies; Cre-dependent GFP virus, for chemogenetics activation/inhibition studies; hM3D and hM4D viruses, and for optogenetic stimulations; ChR2 virus were injected bilaterally into the dentate gyrus of mice. To create AD-like model, mice were given amyloid beta oligomer (Aβ), while PBS was given intracerebroventricularly to the control groups. Mice were taken into four different behavioral tests. The locomotor levels, the anxiety-like behaviors, the short- and long-term memory states, and the spatial memory/learning levels of the mice were evaluated by performing the open field, the elevated plus maze, the novel object recognition, and Morris water maze (MWM) tests, respectively. In behavioral tests, Chronic inhibition of DG^{Pdyn} neurons in the PBS group caused a decrease in locomotor activity, while chronic activation of these neurons in the AB group caused a decrease in velocity ($p<0,0001$). No effect of these neurons on anxiety-like behavior was observed. In addition, in the AB group, chronic activation of DG^{Pdyn} neurons caused a reduction in novel object exploration duration for both short-term memory ($p<0,01$) and long-term memory ($p<0,05$), also, the chronic inhibition of these neurons improved spatial memory and learning, which were impaired in the AB-GFP group ($p<0,01$). Finally, the firing frequency of DG^{Pdyn} neurons increased significantly in Aβ -induced mice ($p<0.01$). These results suggested that Aβ accumulation may disrupt locomotor-related synaptic transmissions of DG^{Pdyn} neurons by altering prodynorphin signaling. Furthermore, with an increased DG^{Pdyn} electrical activity in AD-like modeling, these neurons may be involved in the progression of AD by inducing cognitive impairment.

Key words: Alzheimer's Disease, Hippocampus, Prodynorphin, Chemogenetics, Optogenetics

The study was supported by The Scientific and Technological Research Council of Türkiye (TUBITAK).

ÖZET

Gören, H. (2024). Aβ ile İndüklenmiş Alzheimer Modeli Transgenik Farelerde Dentat Girus Prodinorfin Nöronlarının Elektriksel ve Davranışsal Özelliklerinin Araştırılması Yeditepe Üniversitesi Sağlık Bilimleri Enstitüsü, Fizyoloji ABD., Yüksek Lisans Tezi. İstanbul.

Alzheimer Hastalığı (AH), ilerleyici bilişsel gerileme ve beyinde anormal protein agregatlarının birikmesi ile karakterize edilen nörodejeneratif bir hastalıktır. Endojen opioid sistemi vücutta bazı fizyolojik ve psikolojik süreçlerde önemli rol oynayan karmaşık bir nörokimyasal sistemdir. Endojen opioid sisteminin bir parçası olan prodinorfin nöronları, çeşitli dinorfin opioid peptitlerinin öncü polipeptiti olan prodinorfini sentezleyen ve salgılayan sinir hücreleridir. Günümüzde, opioid sisteminin AH üzerindeki etkisi ve demans mekanizmasındaki rolü halen tam olarak anlaşılamamıştır. Bu nedenle bu çalışmada AH ile öğrenme ve hafıza için kritik bir bölge olan dentat girustaki (DG^{Pdyn}) Prodinorfin nöronları arasındaki olası ilişkinin araştırılması amaçlanmıştır. Bu çalışmada on haftalık yetmiş adet Pdyn-cre erkek fare kullanıldı. Elektrofizyolojik çalışmalar için; cre'ye bağımlı GFP virüsü, kemogenetik aktivasyon/inhibisyon çalışmaları için; hM3D ve hM4D virüsleri ve optogenetik uyarımlar için; ChR2 virüsü, farelerin dentat girusuna iki taraflı olarak enjekte edildi. AH'ye benzer bir model oluşturmak için farelere amiloid beta oligomer verilirken, kontrol gruplarına intraserebroventriküler olarak PBS verildi. Fareler dört farklı davranış testine tabi tutuldu. Farelerin lokomotor seviyeleri, kaygı benzeri davranışları, kısa ve uzun süreli hafıza durumları ve uzamsal hafıza/öğrenme seviyeleri sırasıyla açık alan testi, yükseltilmiş artı labirent testi, yeni nesne tanıma testi ve Morris su labirenti testi yapılarak değerlendirildi. Davranış testlerinde PBS grubunda DG^{Pdyn} nöronlarının kronik inhibisyonu lokomotor aktivitede azalmaya neden olurken, AB grubunda bu nöronların kronik aktivasyonu hızda azalmaya neden oldu ($p<0,0001$). Bu nöronların kaygı benzeri davranışlar üzerinde herhangi bir etkisi gözlemlenmedi. Ayrıca AB grubunda DG^{Pdyn} nöronlarının kronik aktivasyonu hem kısa süreli bellek ($p<0,01$) hem de uzun süreli bellek ($p<0,05$) için yeni nesneyi keşfetme süresinde bir azalmaya neden oldu. bu nöronların kronik inhibisyonu, AB-GFP grubunda bozulan uzaysal hafıza ve öğrenmeyi iyileştirebildi ($p<0,01$). Son olarak amiloid beta ile indüklenen farelerde DG^{Pdyn} nöronlarının ateşlenme frekansı önemli ölçüde arttı ($p<0.01$). Bu sonuçlar, amiloid beta birikiminin, prodinorfin sinyalini değiştirerek DG^{Pdyn} nöronlarının lokomotorla ilişkili sinaptik iletimlerini bozabileceğini öne sürmektedir. Ayrıca, AH-benzeri modellemede DG^{Pdyn} nöronlarının elektriksel aktivitesinin artmasıyla birlikte, bu nöronlar bilişsel bozukluğu indükleyerek AH'nin ilerlemesinde rol oynayabilir.

Anahtar Kelimeler: Alzheimer Hastalığı, Hipokampus, Prodinorfin, Kemogenetik, Optogenetik

Bu çalışma Türkiye Bilimsel ve Teknolojik Araştırma Kurumu (TÜBİTAK) tarafından desteklenmiştir.

1. INTRODUCTION AND PURPOSE

Dementia is a chronic and progressive condition associated with deterioration in cognitive function and everyday activities. It is characterized by progressive impairment of memory, thinking, speech, problem-solving abilities and personality behaviors (1,2). AD represents a neurodegenerative condition characterized by gradual deterioration in cognitive function and the buildup of abnormal protein aggregates in the brain (1). This irreversible and progressive neurodegenerative disease is the most common cause of dementia worldwide and accounting for 60-70% of all dementia diagnoses in the world (1). The endogenous opioid system is a complex neurochemical system in the body that plays a crucial role in regulation pain, reward, stress and various other physiological and psychological processes (39). This system functions through the interactions of endogenous opioid peptides and opioid receptors. Endogenous opioid system consists of three major endogenous opioid peptides: endorphins, enkephalins and dynorphins, as well as three main types of opioid receptors: mu (μ), delta (δ), and kappa (κ) (39). Prodynorphin neurons, a part of the endogenous opioid system, are nerve cells that synthesize and release prodynorphin, the precursor polypeptide for various dynorphin opioid peptides.

The modulation of cognitive processes by prodynorphin and its derived peptides involves complex neurobiological mechanisms. Some study findings suggest that upregulation of dynorphins in the aged brain contributes to age-related cognitive impairment and emotional dysfunction (42,43). On the contrary, in 1993 a study suggested that dynorphin A-(1-13) which is an agonist kappa opioid receptor (KOR) plays a role in preventing memory impairment in mice (44). Hauser and colleagues showed that dynorphin peptides can act as an excitotoxic factor through glutamate receptors under pathophysiological conditions (40). Moreover, in another study, higher PDYN expression was found in the hippocampus of rats with learning disabilities than in healthy control animals (46). In addition, aged PDYN knockout mice have been reported to perform better than healthy control mice in the spatial water maze test (47). In addition to animal experiments, a clinical study detected that the increased dynorphin A levels in the postmortem brains of AD patients is correlated with the increased Aβ levels (43). As it can be seen, the effect of the opioid system on AD and its role in the mechanism of dementia are not still fully understood. Therefore, this study aimed to investigate a possible relationship between AD and DG^{Pdyn}, which is a critical region for learning processes and memory status in Pdyn-Cre mice.

2. LITERATURE REVIEW

2.1 Alzheimer's Disease

Dementia is a chronic and progressive condition associated with deterioration in cognitive function and everyday activities. It is characterized by progressive impairment of memory, thinking, speech, problem-solving abilities and personality behaviors (1,2). Common forms of dementia include AD, vascular dementia, Lewy body dementia and frontotemporal dementia (2). By a German physician, Alois Alzheimer, AD was first described in 1907. This irreversible and progressive neurodegenerative disorder stands as the predominant etiology of dementia globally, encompassing 60-70% of all diagnosed cases of dementia worldwide (1). AD has been acknowledged as a significant global public health concern by the World Health Organization (WHO). It has been reported as the fifth leading cause of death among adults aged 65 years and above (3).

A study projected that around 10 million new cases of dementia are anticipated to emerge annually in the forthcoming years, and the number of these individuals is expected to double every 20 years (Figure 1) (2). Moreover, according to the Alzheimer's epidemiology report prepared in 2022, WHO reported that the global numbers of people who will face a dementia diagnosis will be approximately 154 million by 2050 (4). AD is an economic burden for many countries, and its economic impact goes beyond individual and family costs. The direct and indirect costs of healthcare for AD are estimated to be approximately $500 billion annually (1,3). Women make up two-thirds of people diagnosed with AD dementia. Considering that the risk of Alzheimer's dementia increases with advancing age and that the life expectancy of women is higher in compared to men, it appears that the risk and incidence of AD dementia is greater for women (5).

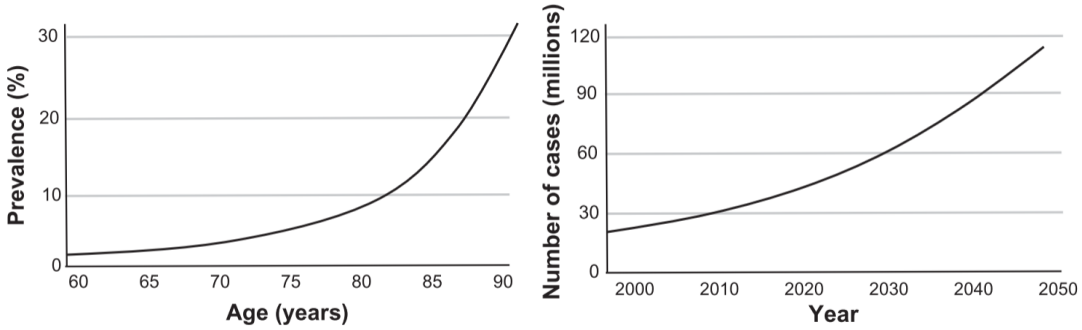


Figure 1. Global prevalence of AD as a function of age and total number of worldwide cases per year (2).

AD can be categorized into Early-Onset AD (EOAD) and Late-Onset AD forms based on the age at which symptoms first appear (6). EOAD accounts for a smaller percentage of Alzheimer's cases, approximately 5-10% and this case may progress more rapidly than LOAD (7). In the patients with EOAD, symptoms appear before the age of 65, often individuals in their 40s or 50s and there may be a higher likelihood of a family history of Alzheimer's (6,7). A stronger genetic predisposition is evident in Early-Onset cases, characterized by autosomal dominant inheritance of coding mutations in specific genes such as presenilin genes (PSEN1 and PSEN2) and amyloid precursor protein (APP), which are associated with familial forms of EOAD (8). In the patients with LOAD, genetics also play a role but these people are more influenced by complex interactions between genetic, environmental and life style factors. LOAD patients which are represents the majority of Alzheimer's cases, about 90-95%, often occurs sporadically and show typically slower progression with a more gradual decline in cognitive function (8). Both forms share the common features of cognitive decline and memory impairment. Understanding the differences is crucial for diagnosis, treatment and support strategies to specific characteristics of EOAD or LOAD.

The clinical symptoms and stages of AD involve a range of cognitive, behavioral, and functional impairments that progressively worsen over time. AD can basically classified into 3 stages; Early stage (mild AD) is consisting of mild symptoms which are enough to be noticed by close associates or family members, middle stage (moderate AD) is the longest stage where person requires greater assistance and supervision, here cognitive and functional impairments are more evident, and finally in Late stage (Severe AD), Individuals experience a progressive loss of ability to respond to their environment, communication abilities, and eventually motor control, requiring full-time care (8). Early stages of AD, most of the people may show memory impairments, underact changes in their personalities and lapses of judgment. In the course of the disease, their memory and speaking abilities worsen, and these patients begin to have difficulty in performing their routine activities in daily life, such as forgetting to take medications regularly, they may also have problems with navigation they are unfamiliar since they may have visuospatial problems (8).

During the late stage of this neurodegenerative disease, the ability to control motor functions worsen in AD patients' day by day, they may have difficulties to recognize family members and close friends. As AD progresses, patients can be affected as emotional and behavioral like developing aggression, depression and delusions (8).

There are more than 20 risk factors associated with AD, these factors can be classified under 6 groups including demographic (age, gender, race etc.) , genetics (due to mutations in

APP, PSEN1/2, APOE genes) , lifestyle (consuming alcohol, smoking, lack of exercise and cognitive activity etc.), medical (being cancer, having a cardiovascular disease or a congestive heart failure, traumatic brain injury, type2 diabetes and obesity etc.), psychiatric (being in depression and being exposed to early stress), environmental (air pollution, calcium and vitamin deficiency, being exposed to heavy metals; copper, zinc etc.) and infections because of viruses, bacteria, or fungi (9,10).

There is still no certain cure for AD, but present treatments and medications applied AD patients can only slow down the progression of dementia. Currently, only two classes of pharmacological treatments are available for patients with AD. One of them is cholinesterase inhibitors (donepezil, rivastigmine and galantamine) which can be used for mild, moderate or severe AD dementia, the other one is glutamate receptor antagonist and dopamine agonist (memantine) used for moderate-to-severe patients with AD (10,11). In addition, AD necessitates various medications targeting risk factors, such as supplements for vitamin D deficiency, non-steroidal anti-inflammatory drugs, and omega-3 fatty acid supplements like fish oil (10).

The hallmark characteristics of AD include the abnormal accumulation of Aβ protein fragments outside nerve cells, forming plaques that disrupt intercellular communication, formation of twisted tangles of tau proteins within nerve cells that leads to dysfunction of cell transportation systems (Figure 2 &3) (13,14). In addition to these, progressive loss of neurons especially in brain regions related with memory and cognitive functions, shrinkage of brain tissues particularly hippocampus and cortex which are crucial for higher cognitive and memory functions, decline in language skills, some physiological and behavioral symptoms such as anxiety, agitation etc. having difficulties for remembering recent events and for managing daily tasks are hallmark characteristics of AD (2,5).

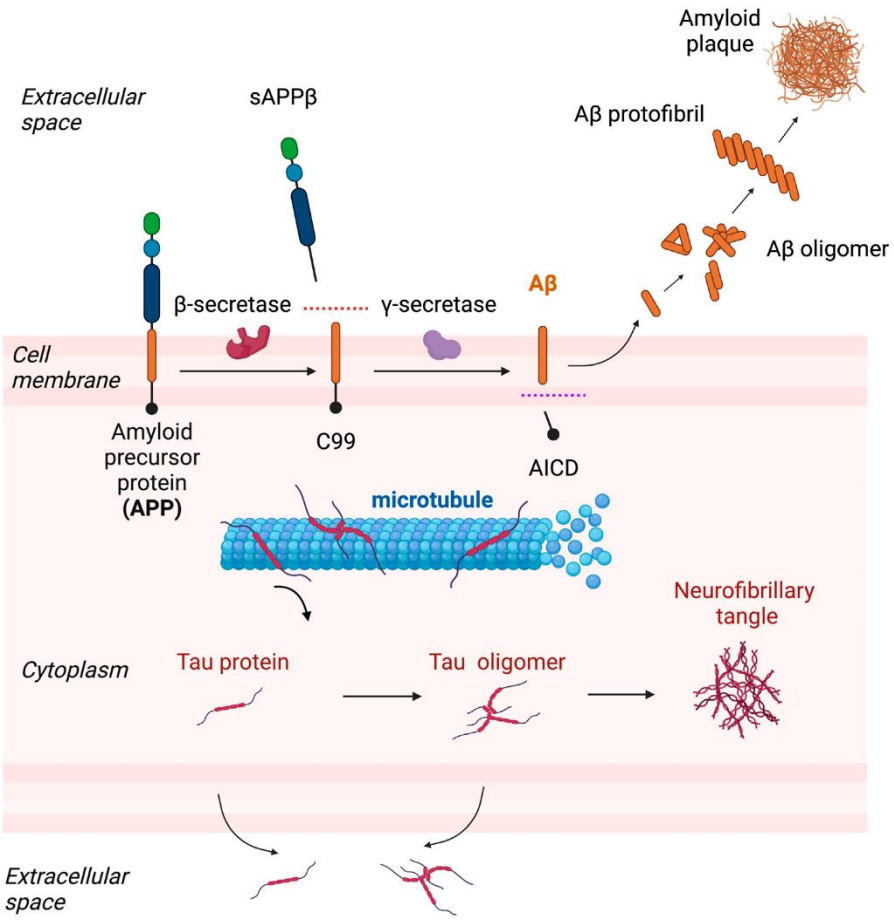


Figure 2. Aggregation process of both Aβ protein outside of the cell and tau protein inside of the cell (13).

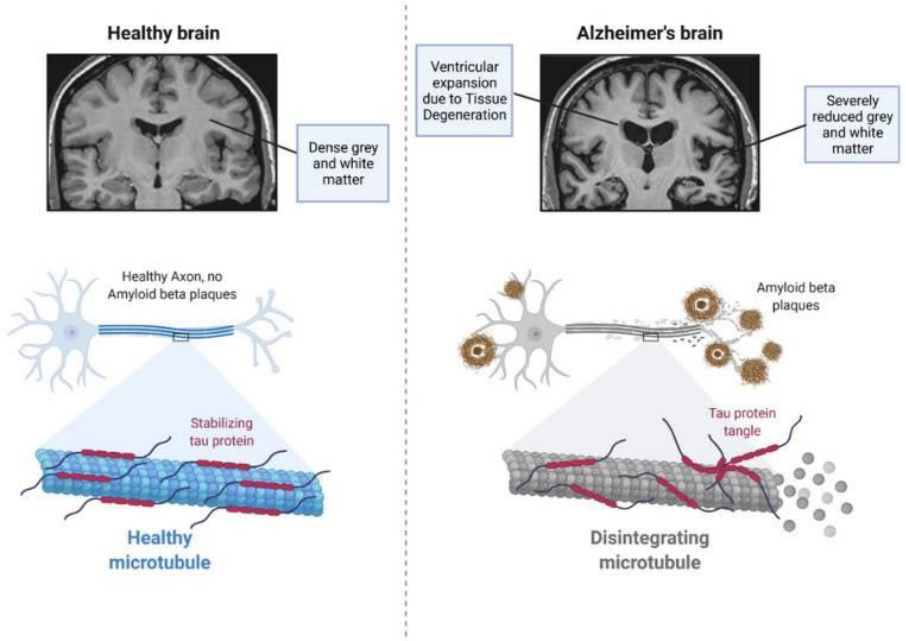


Figure 3. Pathophysiological differences between the brain from healthy and AD patients (14).

2.1.1 Pathological Features of Alzheimer's Disease

AD can be characterized by cognitive and non-cognitive dysfunctions. While memory loss and language difficulties are cognitive dysfunctions, psychiatric symptoms, depression and delusion are non-cognitive dysfunctions (19). In prodromal phase, at which cognitive deficits are visible long before AD symptoms, neuropathological changes are observed in specific part of the brain related to long term episodic memory such as hippocampal formations, gyrus and entorhinal cortex (16,17)

Various hypotheses have been posited to elucidate the etiology and advancement of AD. The hypotheses encompassing the etiology and progression of AD include the amyloid cascade hypothesis, tau hypothesis, cholinergic hypothesis, inflammation hypothesis, genetic factors, mitochondrial dysfunction, vascular factors, and environmental factors.

2.1.1.1 Amyloid Cascade Hypothesis

The amyloid cascade hypothesis posits that the neurodegeneration observed in AD is attributed to the abnormal accumulation of Aβ plaques in various regions of the brain, crucial for learning and memory (19). The hypothesis postulate that the Aβ accumulation and its aggregation are the primary factor for the courses of AD (figure 4) (18,19). According to this hypothesis, Aβ accumulation is believed to be toxic to neurons and disrupt communication between brain cells by playing roles in neurotic injury, neuronal dysfunction and cell death as a pathological trigger (23).

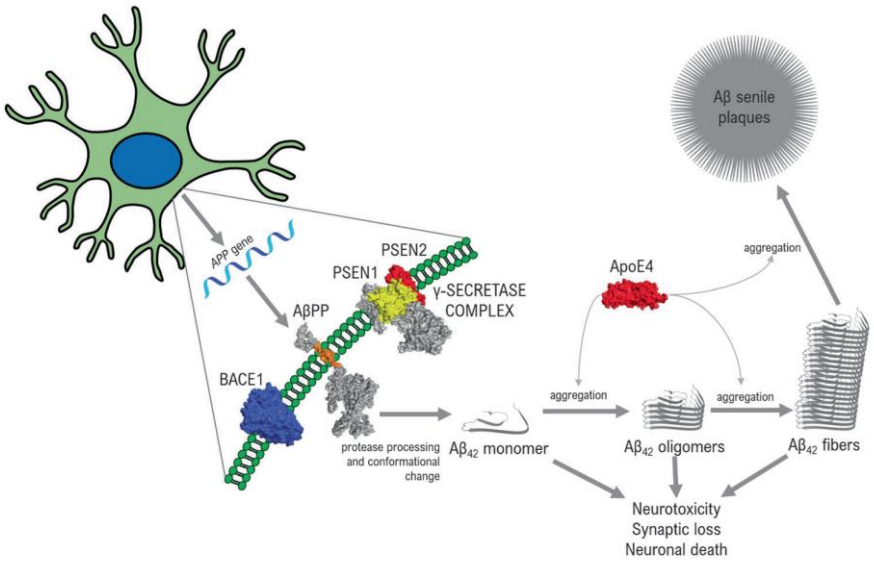


Figure 4. Amyloid cascade hypothesis (18).

The Aβ peptides, which constitute the Aβ plaques, are peptides consisting of 39-43 amino acid residues. These peptides are enzymatically derived from the transmembrane APP through the activity of β-secretase and γ-secretase enzymes (19,20). There two APP isoforms resulted from enzymatic actions on widely distributed APP, these are Aβ1–42 and Aβ1–40 isoforms. Aggregation rate of Aβ1–42 isoform is faster than Aβ1–42 (Figures 5 & 6) (20,21). In addition, based on the cleavage site of APP, length of the isoforms varies. While production of Aβ is normal process, uncontrolled production of all Aβ and increased 42 acid form can result in EOAD (20). Basically, the increased total production of Aβ and the dysregulation between its production and clearance lead to the deposition of Aβ and the formation of diffuse plaques, subsequently triggering inflammatory responses such as microglial and astrocytic activation (22,24). Afterwards, this environmental changes surroundings axons, dendrites and cell bodies leads to progressive synaptic loss and neuronal injury, this condition induces alterations in neuronal ionic homeostasis which then causes aberrant oligomerization and tau hyperphosphorylation (23,24). Consequently, neuronal dysfunction and cell death, coupled with deficits in neurotransmitters, contribute to the onset of dementia characterized by the presence of plaques and tangle pathology (24).

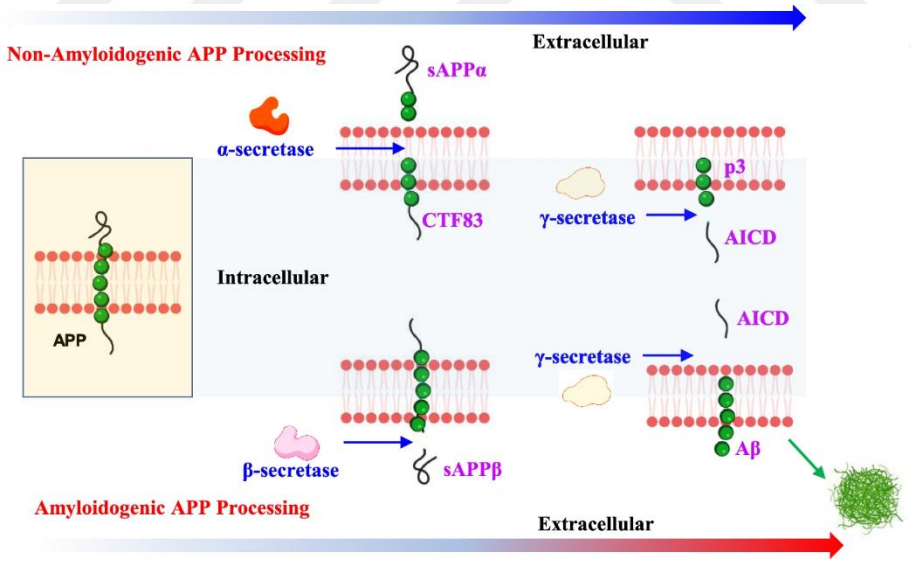


Figure 5. Amyloid Plaque Formation Process (21).

As already known, Genetic factors are involved in the development and progression of AD. Specifically, autosomal mutations in the APP, presenilin-1 (PSEN-1), and presenilin-2 (PSEN-2) genes cause abnormal Aβ peptide production in early-onset familial AD (19). In addition to these genes, ε4allele of the apolipoprotein E gene (APOEε4) which increases was

identified as a dose-dependent risk factor for late-onset FAD Aβ peptide aggregation and its clearance (19) APOE is primarily produced and then released from both astrocytes and microglial cells, and later it is lapidated and binds to soluble Aβ in the extracellular space, finally effects amyloid plaques formation and its transportation within Central Nervous System (CNS) (15,19).

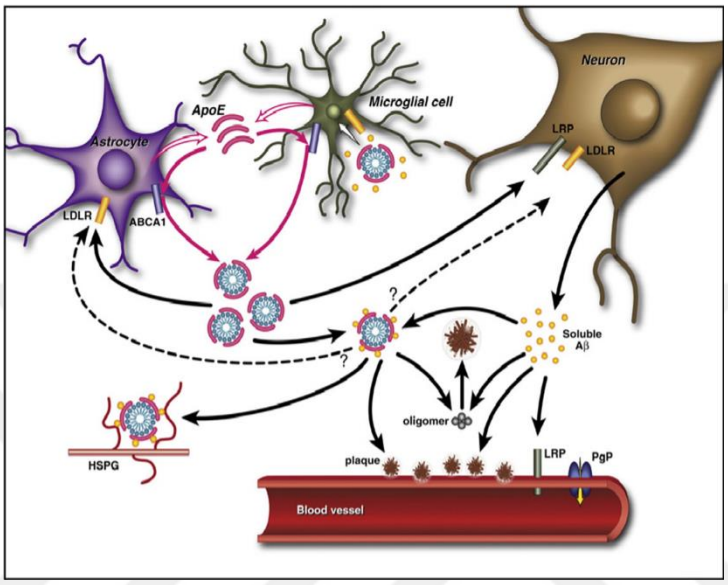


Figure 6. Represents the effects of ApoE released by astroglia and microglial cell on Aβ metabolism and deposition (15).

2.1.1.2 Tau Cascade Hypothesis

Tau is a protein associated with microtubules, known as a microtubule-associated protein (MAP), protein that forms a part of a microtubule structure which helps maintain the cell's shape, plays role in axonal transportation and transport of nutrients (21). Tau cascade hypothesis postulates that abnormal and excessive phosphorylation of Tau proteins lead to the conversion of normal adult tau proteins into neurofibrillary tangles (NFTs) in the brain (Figure 7) (21,26). Normally, Tau proteins promotes stabilization of microtubules inside the cells, however in AD these proteins undergo abnormal changes and form twisted tangles interfere with the normal functioning of neuronal cells and contribute to cell death. Tau proteins form a family of six isoforms and these isoforms consist of 352-441 amino acids, if a mutation takes place in the way of altering the function and expression of these 6 isoforms, tau hyperphosphorylation appears (24). This pathological situation leads to formation of NFTs, and finally interruption of axonal transport and cell-to-cell communication (21).

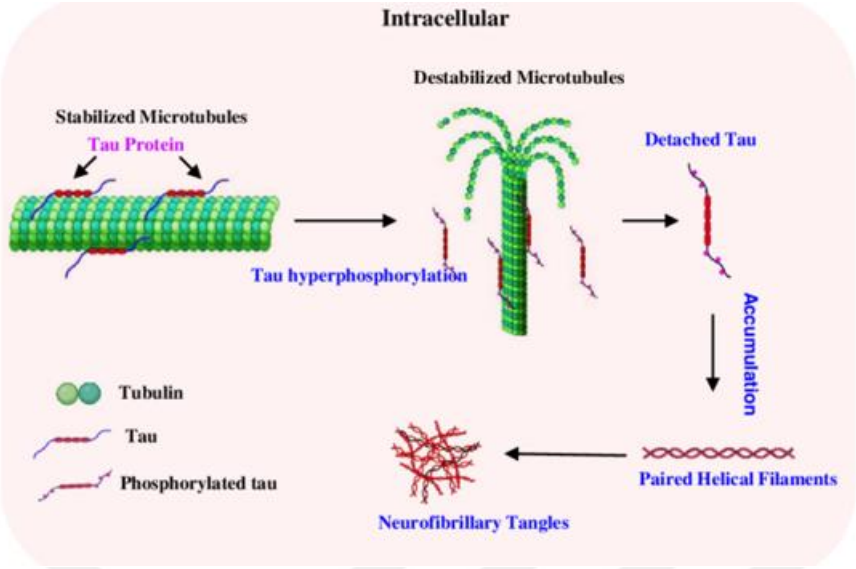


Figure 7. Formation of Neurofibrillary Tangles (21).

2.1.1.3 Cholinergic Dysfunction in AD

The cholinergic system refers to the structure and function of neurons that use acetylcholine as their neurotransmitter, which is crucial for many brain functions including arousal, attention, memory and motivation (25). Although various neurotransmitter-containing cell bodies decline in the end-stage of AD pathology, the most consistent losses throughout the development and progression of neurodegenerative Alzheimer's pathology are observed in long projection neurons, particularly cholinergic neurons located in the basal forebrain, which include the nucleus basalis of Meynert, the horizontal and vertical diagonal bands of Broca (HDBB and VDBB, respectively), and the medial septal nucleus (MS) (26). Most of the cholinergic projections into the hippocampus and cerebral cortex come from nucleus basalis and septal diagonal band forebrain regions, these cholinergic innervations play crucial role in attention, learning and memory functions (25,26). During the progression of AD, cholinergic basal forebrain (CBF) cortical projection neurons undergo chemical phenotypic alterations (25). In a study, it was stated that in AD, a decrease in both choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) activity in the cortex was observed, as well as cell loss in the CBF region (27). Most researchers postulate that progressive disruption of cholinergic system within the brain may underlie short-term memory loss and cognitive symptoms observed in AD (25,26,28).

2.1.1.4 Mitochondrial Dysfunction

Mitochondria, vital organelles present in nearly all eukaryotic cells, serve as the primary source of adenosine triphosphate (ATP), which functions as the cell's primary energy currency (21). Beyond energy production, mitochondria have several other important roles in the cell. The principal role of mitochondria is the generation of ATP via oxidative phosphorylation. Furthermore, mitochondria are integral in calcium signaling processes within the cell, help regulate the metabolism of carbohydrates, fats and proteins, and plays role in apoptosis or programed cell death (21,29). Mitochondrial dysfunction, characterized by reduced energy production and increased reactive oxygen species (ROS) generation, plays a pivotal role in inducing oxidative stress, inflammation, and excitotoxicity. These factors are common risk factors associated with various neurological disorders such as AD, traumatic brain injury (TBI), Parkinson's Disease (PD), and depression (29). There are two dynamic and continuous processes that maintain the functional integrity of mitochondria; mitochondrial fission and fusion which are crucial for responding to metabolic changes, regulating mitochondrial function and ensuring the health and survival of cells (29). Mitochondrial fusion is the process by which two mitochondria join together, this process is important for mitochondrial health as it allows the mixing of mitochondrial contents including mitochondrial DNA (Deoxyribonucleic Acid), proteins and lipids (21). On the other hand, mitochondrial fission is the division of a single mitochondrion into two or more independent structures, this process is essential for mitochondrial quality control in terms of removing damaged mitochondria by autophagy (21). The balance between these two dynamic processes is crucial for cellular homeostasis, failing these processes can lead to various pathological conditions, including aging-related diseases and neurodegenerative disorders. In the AD brain, there is imbalance between dynamic and continuous mitochondrial fusion and fission in terms of increased expression of fission protein and decreased of fusion protein expression (figure 8) (21).

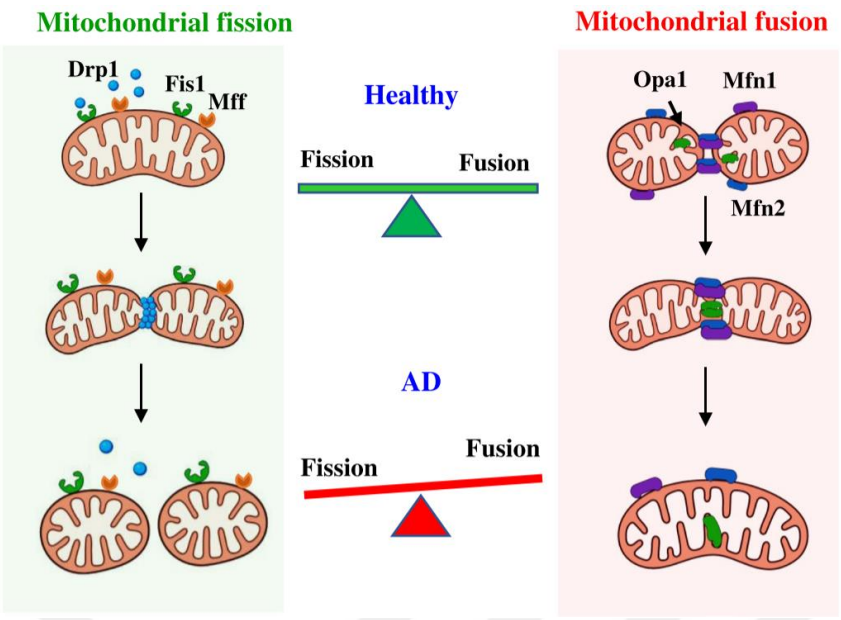


Figure 8. Mitochondrial fission and fusion process, their balance in healthy and AD situations (21).

2.1.1.5 Oxidative Stress

Oxidative stress is characterized by an imbalance between the production of ROS, which are reactive oxygen-containing molecules, and the body's capacity to detoxify these harmful molecules or repair the resulting damage (21). Antioxidants are the molecules produced in body to counteract oxidative stress, these molecules can safely interact with free radicals and terminate the chain before vital molecules are damaged (21). ROS can activate signaling pathways that lead to inflammatory responses, while inflammation can increase ROS production, creating a vicious cycle (21). When the body's ability fails to neutralize and eliminate ROS, oxidative stress occurs and then contribute to the development and progression of many diseases, including neurodegenerative disorders like AD (21,30).

2.1.1.6 Neuroinflammation

Besides other risk factors, neuroinflammation can be the one of the key neuropathological factors leading to neurodegeneration and cognitive decline in AD (31). In AD, neuroinflammation is primarily characterized by the activation of glial cells, such as microglia and astrocytes, within the brain. Normally, microglial cells are act as immune cells, they get activated and recruited to the injured or infected site of the body during an inflammatory response and clears the debris and pathogens (31). In the early stages of AD, the accumulation of Aβ initiates microglial cells activation to engulf and clear these Aβ plaques through phagocytosis (32). During this process, the microglial activation works as a protective response

against AD progression, their initial response maybe be protective but persistent activation of lead to release an array of pro-inflammatory mediators, following chronic inflammation and neuronal damage, finally, the clearing Aβ plaques declines as Alzheimer's progresses (31). The activated microglia can be classified into two functional subtypes; M1 (Pro-inflammatory subtype) and M2 (anti-inflammatory phenotype (21). M1 phenotype produces proinflammatory cytokines, including IL-1β, TNF-α, IL-6, and IL-12 which plays role in AD progression, however, M2 anti-inflammatory phenotype provides neuroprotective effects in AD by releasing anti-inflammatory cytokines such as IL-4, IL-10, IL-13, and TGF-β (21). Similar to microglia, astrocytes which are another sub-type of glial cells in CNS are correlated with cognitive deficits in AD (21). They are found near the sites of Aβ plaques, that are subdivided into A1(neurotoxic phenotype) and A2 (neuroprotective) phenotypes (21,33). To sum up, both microglia and astrocytes actively participate in and influence the pathological processes of AD, contributing to the accumulation of Aβ and tau, synaptic dysfunction, and neuronal loss.

2.1.2 Diagnosis and Current Treatment Approaches

2.1.2.1 Diagnostic Methods

Diagnosis and treatment of AD involve a combination of medical evaluation, cognitive testing and symptom management strategies. The diagnosis methods for AD can be divided into Volumetric Data Analysis, Diffusion Tensor Imaging (DTI), Positron Emission Tomography (PET) Scan, cerebrospinal fluid (CSF) and Blood Tests. While limited, some of these diagnostic tools and applications are available for people. The volumetric data method is utilized to identify volume changes in specific critical regions of the brain, enabling the prediction of the likelihood of mild cognitive impairment progressing to AD through radiological assessments. Hippocampal and cortical abnormalities in terms of volumetric changes are regarded as a significant AD biomarker (34). DTI, an advanced neuroimaging technique, is used to visualize the structure of vertical cellular micro-circuits based on the diffusion properties of water molecules to produce magnetic resonance images, this makes DTI to be used as a marker for neurodegeneration (34). PET scans can show patterns of brain activity and amyloid accumulation, which is helpful against AD progression. This technique is able to assess for both Aβ and hyperphosphorylated tau proteins and serve as a reliable potential biomarker (34). Research is ongoing into biomarkers like tau or amyloid proteins in blood or CSF which is accessible through puncture and surrounds the brain, and This approach may aid in the diagnosis of AD by utilizing the CSF biomarkers, such as the Aβ42: Aβ40 ratio and the phosphorylated tau at threonine analysis (34,35).

2.1.2.2 Current Therapeutic Strategies and Options

There is currently no cure for AD. Treatments strategies for AD primarily focus on managing symptoms, decreasing the progression rate and improving quality of life.

2.1.2.2.1 Cholinesterase Inhibitors

Current AD medications were developed to reduce the effect resulted from cholinergic deficit, an early pathologic finding observed in AD. Learning and memory neuronal circuitry is affected by the selective loss of cholinergic neurons which leads to severe reduction in the production and release of Acetylcholine neurotransmitter (34).

One of the primary treatment strategies for AD involves the use of cholinesterase inhibitors which prevents the breaking down of acetylcholine in the brain synapses (34). There are 3 Food and Drug Administration-approved (FDA-approved) cholinesterase inhibitor drugs currently used to restore the decline in memory and cognitive function seen in AD; donepezil, rivastigmine and galantamine (34,36). Current pharmacological interventions can enhance cognitive symptoms and delay the decline in activities of daily living for some individuals with AD, particularly in the early to moderate stages of the condition. However, these medications do not arrest the progression of AD and may not be universally effective for all patients (36). As the disease progresses, the continued loss of cholinergic neurons and other pathological changes in the brain may reduce the effectiveness of these medications.

2.1.2.2.2 NMDA Receptor Antagonist

Glutamate, the predominant excitatory neurotransmitter in the brain, exerts a multifaceted and crucial role in AD pathology. As similar to acetylcholine neurotransmitter, it is also crucial for normal brain functions, including learning, memory and attention. It operates through various receptors, including NMDA (N-methyl-D-aspartate) receptors, which plays significant roles in glutamate synaptic transmission and are key for synaptic plasticity and memory formation (34). In AD, the regulation of glutamate can become dysfunctional by overstimulation of glutamate neurons which leads to overload of calcium flux into neurons, and eventual excitotoxicity (34, 37). This leads to the gradual loss of synaptic function damage and death of nerve cells caused by prolonged exposure to excessive levels of excitatory neurotransmitter (37). Memantine, an FDA-approved AD drug, is a low-affinity NMDA receptor antagonist, it reduces glutamate-induced excitotoxicity and blocks the function of extra synaptic NMDA receptors, thus slows intracellular calcium accumulation and may treat

moderate to severe neurodegenerative AD (8,34,37). Treatments of AD often involve a combination of these approaches.

2.1.2.2.3 Antibody Therapies & Active Vaccinations

Passive immunotherapeutic in AD involves the use of monoclonal antibodies to target and neutralize specific proteins or pathological elements that are believed to contribute to the disease's progression (34). In the case of AD, these therapies primarily focus on two hallmarks proteins; Aβ and tau (14,34). Aβ is a protein that accumulates in the brains of Alzheimer's patients, forming Aβ plaques which are thought to disrupt cell function and trigger a cascade of neurodegenerative processes (20). Passive Immunotherapies targeting Aβ seek to reduce its accumulation and plaque formation (34). Monoclonal antibodies like aducanumab (FDA-approved), lecenemab, donanmab, ganteranumab and crenezumab have been developed for this purpose due to their abilities to reduce amyloid plaques and progression of cognitive impairment in the brain (14,34). Tau is another protein that becomes abnormally modified in AD, leading to the formation of NTFs inside the neurons, which are associated with neuron death and cognitive decline (8). The therapies including gosuranumab and semorinemab target tau proteins and aim to slow its aggregation and facilitate its clearance from the brain. Besides antibody therapies, in active vaccination therapy vaccine containing specific antigen for Aβ is administered to make the patients produce their own antibodies specific to the vaccine (Figure 9) (14). The vaccines produced against AD including AV-1959D, Y-5a15, AN1792, and protollin, however there is no currently FDA-approved AD vaccine (14). While these treatments have been shown promise in early-stage trials, particularly in terms of biomarker changes like reductions in amyloid plaque, translating these changes into clinical benefits such as improved cognition or slowed disease progression has been challenging.

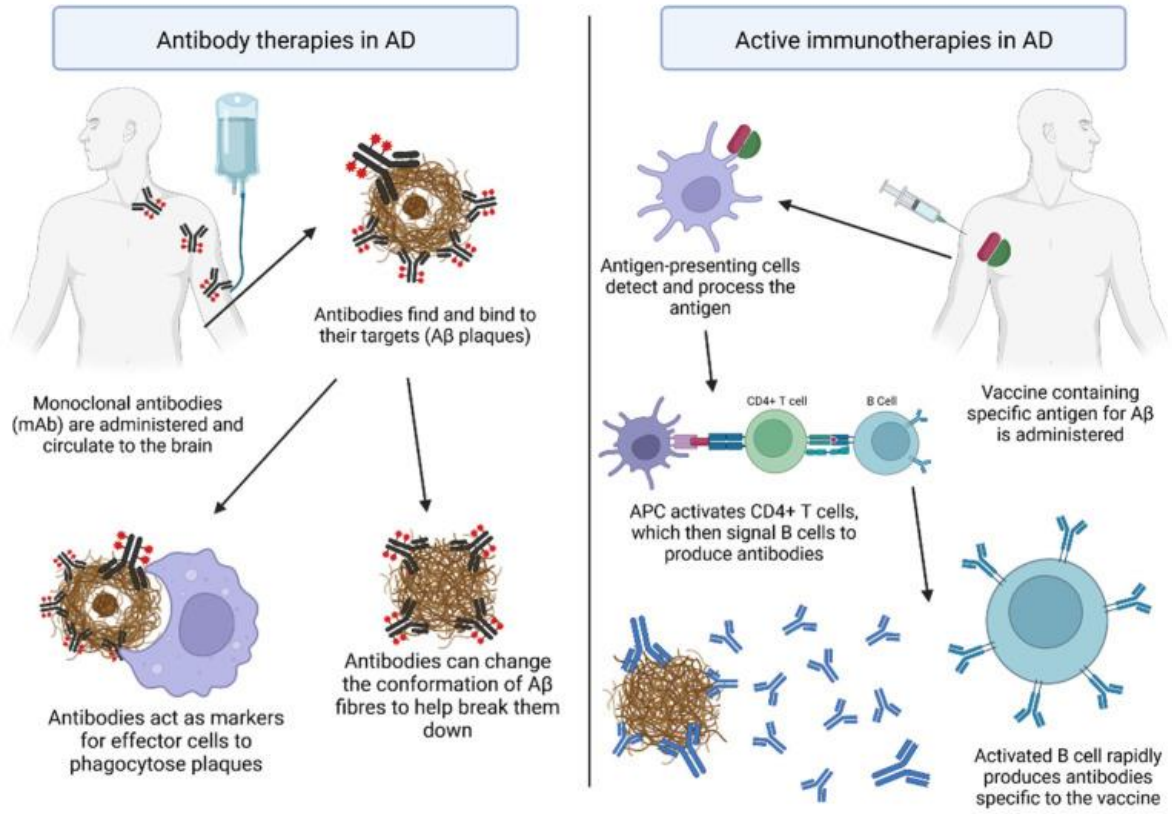


Figure 9. Immunotherapies against AD pathophysiology (14).

2.1.2.2.4 BACE Inhibitors

BACE inhibitors, or Beta-Site Cleaving Enzyme inhibitors, are a class of drugs designed to treat AD by targeting and inhibiting the BACE1 enzyme (14). This enzyme is involved in the production of beta-amyloid from proteolysis of APP by enzymatic action of BACE1 enzyme (Figure 10) (14,38). The therapeutics strategy behind BACE inhibitors is based on the hypothesis that reducing the production of beta-amyloid will slow the progression of AD (34). Although BACE inhibitors have demonstrated significant success in preventing Aβ formation, they have not been shown to provide cognitive, clinical, or functional benefits in AD patients in large randomized controlled trials (34).

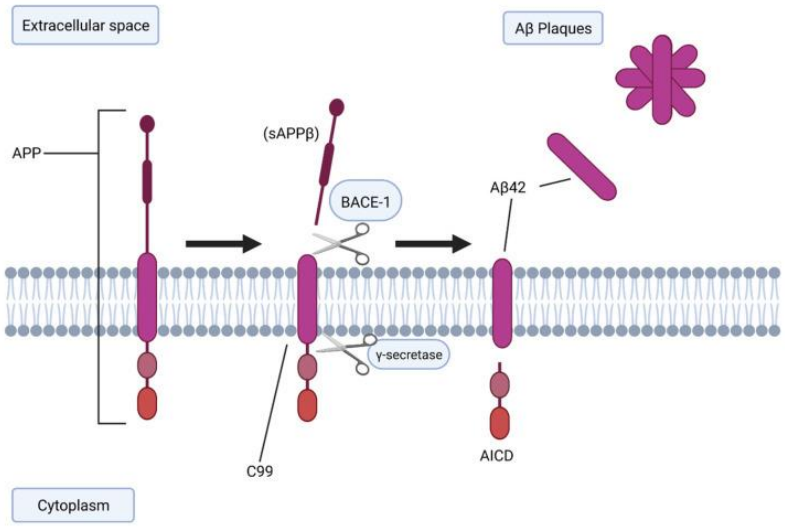


Figure 10. Generation of free Aβ from APP by enzymatic action of BACE1 and γ-secretase (14).

2.2 Endogenous Opioid System

The endogenous opioid system is a complex neurochemical system in the body that plays a crucial role in regulation pain, reward, stress and various other physiological and psychological processes (39). This system functions through the interactions of endogenous opioid peptides and opioid receptors. The endogenous opioid system comprises three primary endogenous opioid peptides: endorphins, enkephalins, dynorphins, and endo-morphine, along with three main types of opioid receptors: mu (μ), delta (δ), and kappa (κ) (39). The endogenous opioid system consists of three primary endogenous opioid peptides: beta-endorphin, leucine (Leu)- and methionine (Met)-enkephalins, dynorphins A and B, and neoendorphins, and finally nociception. These peptides are derived from proteolytic cleavage of different protein precursors: proopiomelanocortin (POMC), pre-proenkephalin (PENK), pre-prodynorphin (PDYN), and pronociceptin (PNOC) (39). Each of these peptides has its own specific regions of action and roles, but broadly, they are involved in modulating pain, emotion, stress response, immune function, reward system and other physiological processes, in addition each type of these opioid receptor has a different distribution in the body and mediates different physiological effects including pain relief, mood regulation and control of stress and anxiety (figure 11 & Table 1). These opiodergic neurons have heterogenous distribution within the brain; PDYN is abundantly expressed in hippocampus, hypothalamus, and nucleus accumbens, PENK in thalamus, and POMC in hypothalamic arcuate nucleus (39).

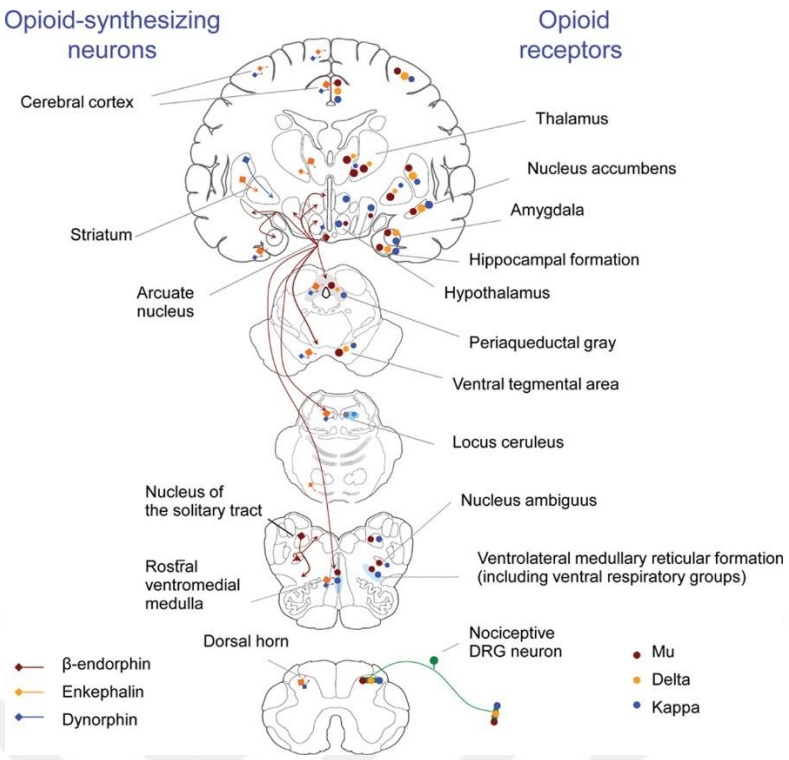


Figure 11. General distribution of 3 main endogenous opioid peptides and their receptors in the CNS (39).

Table 1. Represents the main distribution of opioid receptors and their effects (39).

Table **Opioid receptors**

	μ	δ	κ
Alternative nomenclature	MOR, MOP, OP3	DOR, DOP, OP1	KOR, KOP, OP2
Main distribution	Cerebral cortex	Cerebral cortex	Hippocampus
	Brainstem	Thalamus	Striatum
	Dorsal horn	Striatum	Hypothalamus
	DRG neurons	Dorsal horn	Brainstem
		DRG neurons	Dorsal horn
Main endogenous agonist	β-Endorphin (Met-enkephalin)	Enkephalins	Dynorphin A
Effect in analgesia	Supraspinal and spinal analgesia	Supraspinal and spinal analgesia	Spinal analgesia
	Acute and chronic pain	Moderate in chronic pain	Hyperalgesia
Effect on reward mechanisms and drug addiction	Facilitates	Facilitates	Inhibits
Behavioral effects	Sedation	Sedation	Psychotomimetic
	Euphoria	Antidepressant	Sedation
			Dysphoria
Other effects	Respiratory depression[a]	Neuroprotection	Seizure protection?
	Miosis[a]		
	Vomiting[a]		
	Urinary retention		
	Constipation		

[a] Mediated by μ2 receptors.

2.2.1 Prodynorphin Neurons

Prodynorphin neurons are nerve cells that synthesize and release prodynorphin, the precursor polypeptide for various dynorphin peptides. These neurons are a part of the endogenous opioid system and play significant roles in numerous physiological and psychological processes. They are particularly prevalent in areas involved in pain processing, stress response, and emotional regulation, such as hypothalamus, amygdala, hippocampus and spinal cord. Prodynorphin undergoes conversion into multiple bioactive peptide derivatives, including alpha-Neoendorphin (α-NE), Big Dynorphin (Dyn A/B 1-32), Leumorphin (Dyn B 1-29), dynorphin A (Dyn A 1-17), dynorphin B (Dyn B 1-16), Leucine-enkephalin-arginine (Leu-enkephalin-Arg), and potentially Leucine-enkephalin (Leu-enkephalin). These peptides are the result of proteolytic processing of prodynorphin and contribute to the diverse functional roles within the endogenous opioid system (Figure 12) (40). These prodynorphin-derived peptides show inhibitory neurotransmitter roles especially in pain reduction mechanisms (40).

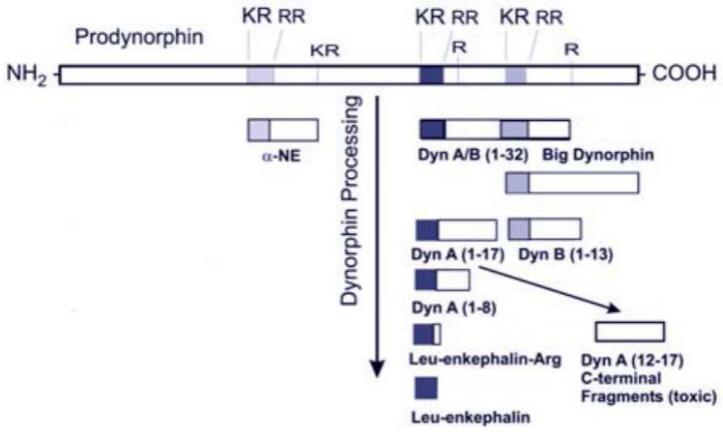


Figure 12. Major bioactive peptides converted from Prodynorphin (40).

Dynorphin A which is an important posttranslational product of Pdyn gene acts as neurotransmitters or neuromodulators by showing high affinity to primarily KOR and this binding process typically results in analgesic effects, then modulates stress response and can affect mood and behavior. Dynorphin A shows intrinsic activity at both opioid and non-opioid receptors, including KOR, MOR and DOR, and glutamate receptors (NMDAR and AMPA), receptors, respectively (40). N-terminal peptide derivatives of Dynorphin A exhibits activity on opioid receptors, while fragments derived from the C-terminus of the peptide have been shown to activate glutamate receptors, and exert excitotoxic effects on neurons, astroglia and oligodendroglia (Figure 13) (40).

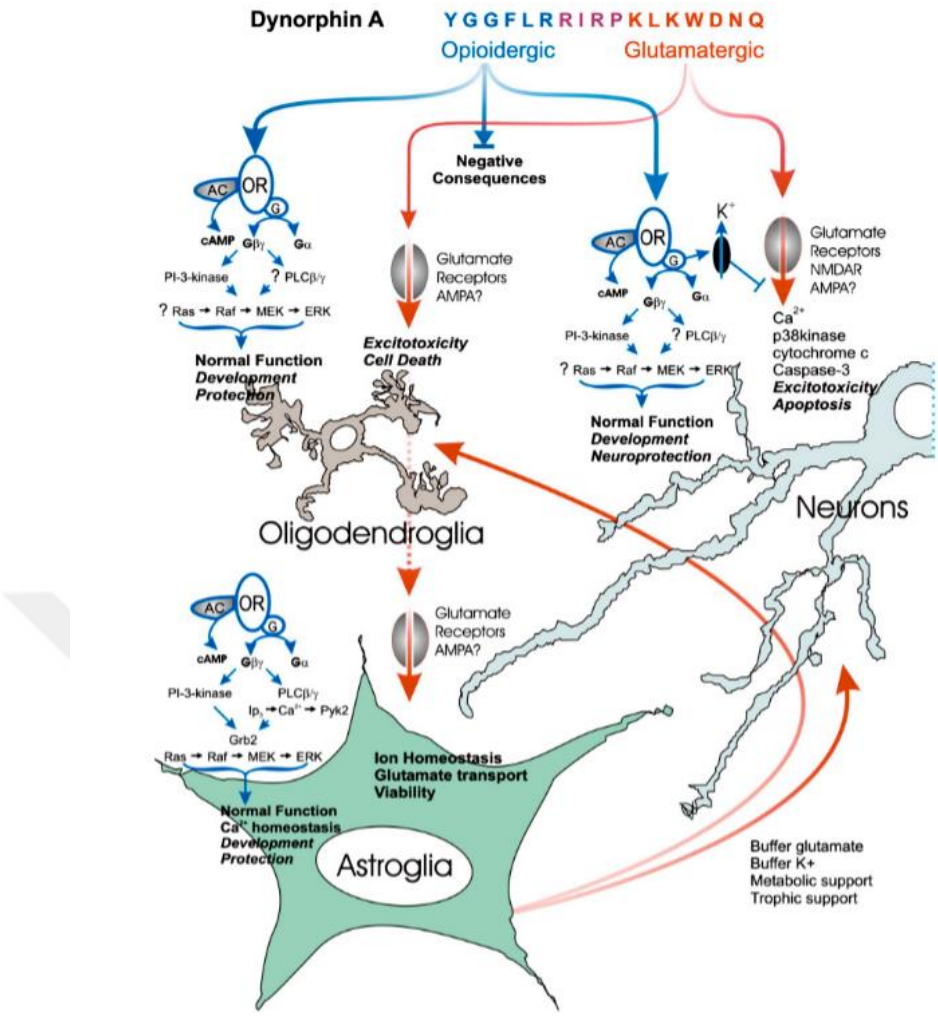


Figure 13. Summary the effects of dynorphin A (40).

The relationship between prodynorphin neurons and AD is a topic of ongoing research, as scientists continue to explore the complex interactions between various neurotransmitter systems and neurodegenerative processes. Dynorphins encoded by PDYN gene have been related with cognitive processes, stress response and emotional control (41).

2.2.2 Cognitive Modulation

Prodynorphin is the precursor peptide to dynorphins. These peptides primarily bind to and activate KORs, playing a significant role in modulating various physiological processes, including pain, stress, emotional response, and cognitive functions. The modulation of cognitive processes by prodynorphin and its derived peptides involves complex neurobiological mechanisms.

Some study findings suggest that upregulation of dynorphins in aged brain contributes to the age-related cognitive impairment and emotional dysfunction (42,43). On the contrary, a study from 1993 suggested that an endogenous KOR agonist, dynorphin A-(1-13), plays role in preventing mice from memory dysfunction (44). Hauser and his friends showed that dynorphin peptides can act as an excitotoxic factor under pathophysiological conditions through glutamate receptors (40). In a clinical study, dynorphin A levels was detected in high concentration in the AD patients' brains, suggesting a potential role of dynorphins in neuronal disorders (43). In addition to clinic studies, animal studies revealed that the activity of dynorphin signaling influences cognitive abilities. Kotz and his friends showed that the PDYN expression was increased in the hippocampus of aged rats compared with young rats suggesting that dynorphin signaling may have a higher impact on aging-induced decline cognitive abilities in aged animals (45). Moreover, another study found higher PDYN expression in their hippocampus of learning-deficient rats than in healthy control animals (46). In addition, aged PDYN knockout mice have been reported to perform better in the spatial water maze test compared to healthy control mice (47). And finally, Reducing PDYN gene expression has been shown to protect aged mice against impaired cognition and increased anxiety-related behaviors (48).

KOR are widely distributed throughout the CNS and are crucial in modulating various physiological processes such as stress, mood, reward, pain, inflammation, and remyelination. Demyelination, the loss of damage of protective myelin sheath that surrounds nerve fibers, is a pathological factor occurs in AD and KOR agonism is thought to have protective role in AD (49,50). Hypermethylation withing the promoter region of KOR-encoded gene, OPRK1, was determined to be an increased risk factor for AD (51). Some studies conducted with the KOR agonist U50488H have reported that this agonist significantly reduces cognitive impairment and promotes improved learning and memory (52,53). The current study done by Song and his friends investigated the effect of this against on spatial memory and synaptic plasticity in AD mice model (APP/PS1), they were able to successfully demonstrate that U50,488 reduced Aβ plaque accumulation in the prefrontal cortex and hippocampus and damage to hippocampal neurons in AD mice, caused improvement in synaptic plasticity, and these mice had a significant improvement in cognitive abilities in the MWM test (52). In addition to the studies showed the beneficial effects of KOR agonists on cognitive dysfunction, Takahashi and his friends reported that the administration of U50,488 or dynorphin A via intracerebroventricular (i.c.v) decreased cognitive dysfunction in mice (53).

In contrast to the studies reported that KOR agonists ameliorate cognition, some studies showed that KOR agonists can also disrupt cognition (54,55). Abraham and colleagues

discovered that dynorphin-induced activation of KORs in the medial prefrontal cortex led to cognitive impairment in mice experiencing acute morphine withdrawal (54). Furthermore, it has also been shown that exposure to U50,488 inhibits novel object recognition in mice through the effect of KOR activation (55). Collectively, these findings suggest that KOR agonists and its endogen ligand dynorphin can have both beneficial and detrimental effects on cognition. Hence, additional research is warranted to comprehensively elucidate the intricate functions and potential implications of these mechanisms in AD.

2.2.3 Opioid system dysregulation in Alzheimer's Disease

The opioid system comprises three major opioid peptides, namely beta-endorphin, enkephalins, and dynorphins, which bind to three distinct types of opioid receptors: mu, delta, and KORs, respectively. They play a significant role in the brain's functioning, affecting processes such as pain modulation, stress response, mood regulation, and cognitive functions. Altered functioning of the opioid system has been implicated in a range of neurological and psychiatric disorders, including AD. Consequently, AD may be linked to changes in the opioid system within the brain.

A clinical study found differential alterations in mu, delta, and KOR populations in specific areas of postmortem brains from AD patients compared to age-matched, dementia-free individuals (56). Specifically, reductions in mu-opioid receptor binding were observed in the subiculum and hippocampus, areas critical for memory and navigation. The study reported a significant increase in delta-opioid receptor binding in the dorsal and ventral putamen, as well as in the cerebral cortex, in postmortem brains from AD patients compared to age-matched, dementia-free individuals (56). These alterations indicate the potential involvement of the opioid system in the neuropathological mechanisms of AD, potentially influencing the disease's cognitive and emotional symptoms. Moreover, the increased expression of endogenous kappa-opioid peptide, dynorphin, has been linked to cognitive deficits in aging and AD (57). Researchers showed that activation of KOR by increased dynorphin lead to stress-related memory impairments (57).

These findings underscore the complexity of the opioid system's involvement in AD, pointing to its potential as a target for therapeutic intervention. The altered opioid receptor expression and the role of endogenous opioid particularly dynorphin in the disease's pathology highlight the need for further research into how modulating this system could contribute to treatment strategies for AD.

3. MATERIALS AND METHODS

All experiments were performed at Yeditepe University Faculty of Medicine Department of Physiology Brain Research Laboratories. The experimental protocol was approved by Yeditepe University Animal Experiments Ethics Committee with the decision numbered 2023-09 in 2023. GraphPad Prism v.9.0 program was used to perform all statistical analyzes. All scientific figure illustration were done by using BioRender program.

3.1 Experimental Animal Used in Study

In this study, 70 male (10 weeks) Pdyn-Cre transgenic mice were used (Jackson Lab: #027958). Production of the Pdyn-Cre mouse line obtained from Jackson Laboratory (USA) were provided by Yeditepe University Faculty of Medicine Experimental Research Center (YÜDETAM) as homozygous x homozygous mating strategy. Mice were housed as 3-4 animals/cage, they were kept at 21±2 °C with %40 humidity and 12 hours light/dark cycle were followed. Mice were allowed to access to water and food (standard diet) *ad libitum*.

3.2 Genotyping

The newborns (3-4 weeks) from homozygous x homozygous mating strategy were separated into different cages according to their gender. After the lactation period, ear tissues with 2cm diameter were taken and then collected into DNase free Eppendorf tubes which then stored at -21°C until performing DNA isolation and the standard PCR (Polymerase Chain Reaction) protocols.

3.3 DNA Isolation Protocol

DNA isolation from taken-ear samples was performed by adding 100 µl alkaline buffer (25mM NaOH-Sigma and 0.2 mM EDTA) into the PCR sample-containing tubes. Then the tubes were placed in PCR machine (Labnet International, TC9610) and 98 °C for 1hour protocol was followed to boil the tissue samples. After the boiling process is completed, 100µl of neutralization buffer (40mM Tris HCl, Sigma-T5941) was added into boiled-alkaline buffer containing tissue tubes and then these tubes were placed in a centrifuge device (SIGMA 1-14 microcentrifuge) at 4000 rpm for 4 minutes. After centrifuge process, the supernatant from sample mixture was transferred into new separate tubes and used for PCR protocol.

3.4 Polymerase Chain Reaction Protocol

For polymerase chain reaction, 2X master mix containing Taq polymerase enzyme (New England Biolabs, MO270S), Cre/Forward primers (table 2) and double distilled water (ddH_2O) were used. The mixture for standard PCR protocol were prepared with specific concentration and volume shown in table 3. The prepared PCR solution was poured into empty tubes, as 10.5µl in each tube. Finally, the isolated DNA samples were added to separate tubes in an amount of 2µl. To check the presence of possible contamination in the prepared PCR solution, ddH_2O was used instead of DNA for the negative control (-C), and a DNA sample previously proven to be Pdyn-cre by PCR was used as a positive control (+C). The entire PCR protocol was performed on ice at 4 °C. Finally, the prepared tubes were placed in PCR machine and the thermal cycle for PCR protocol was followed as shown in Table 4.

Table 2. Cre Primer Sequences.

Primers	Primer Sequences (5' ➡ 3')
Cre Forward	GCC AGC TAA ACA TGC TTC ATC
Cre Reverse	ATT GCC CCT GTT TCA CTA TCC

Table 3. Standard PCR Protocol

Chemicals	Concentration	Volume
Taq Polymerase	2X	6 µl
Forward Primer	20 µM	0.125 µl
Reverse Primer	21 µM	0,125 µl
Double Distilled Water		5,75 µl
Template DNA		0,5 µl

Table 4. Standard PCR Program.

Number of Cycle	Temperature (°C)	Time (s)
1	95	30
38	95	30
38	60	30
38	68	60
1	68	300

3.5 Agarose Gel Imaging

To prepare % 2 agarose gel mixture, 2g agarose (Bioshop AGA001.1) was measured and poured into 100ml 1X TBE buffer, then the mixture was left in the microwave until it

obtained a homogeneous appearance. After adding Ethidium bromide to the gel mixture, it was mixed gently and poured into the electrophoresis cassette under a hood, the combs were placed, and the solution was left to cool for 20 minutes. During this time, 1xTBE buffer was poured in an amount sufficient to cover the gel to be placed in the electrophoresis tank. 1ul of 5X loading dye (Sigma-G7654) was added to each of the PCR tubes containing the samples taken from the PCR machine. After 20 minutes, the cooled gel was placed in the electrophoresis tank and 3ul Hyper Ladder (Bioline, H4-215108) was loaded into the first well. Afterwards, PCR samples with dye added to the wells were loaded into each well, 10µl respectively. DNA execution protocol was followed as 120V, 300 mA, 18 minutes. Finally, the gel was visualized by BioRAD ChemiDoc imaging device (figure 14).

Figure 14. PCR results for Pdyn-Cre mice.

Ladder (L), Negative control (-C), Positive control (+C).

3.6 Experimental Groups & Set-up

In this study, 70 male (10 weeks old) Pdyn-Cre transgenic mice were used and divided into 10 groups: Control, chemogenetic and optogenetic groups (tables 5 and 6).

Table 5. Experimental Groups.

CONTROL		**OPTOGENETIC**		**CHEMOGENETIC**			
Groups		**Acute Activation Groups**		**Chronic Activation Groups**		**Chronic Inhibition Groups**	
Group 1	GFP-Alzheimer (n=7)	**Group4**	ChR2-Alzheimer (n=7)	**Group7**	hM3D-PBS (n=7)	**Group9**	Hm4D-PBS (n=7)
Group2	GFP-PBS (n=7)	**Group5**	ChR2-PBS (n=7)	**Group8**	hM3D-Alzheimer	**Group10**	Hm4D-Alzheimer (n=7)
Group3	GFP-Control (n=7)	**Group6**	GFP-PBS (n=7)				

Table 6. The experimental groups planned in the study and the applications to be applied.

Groups		**Treatments**	**Number of Mice (n)**
Electrophysiology and Functional Imaging Groups	**Group 1**	The virus containing the green fluorescent protein (GFP) transgene was injected into the hippocampus dentate gyrus region, and 7 days later, icv Aβ1-42 oligomer was administered to the ventricles. The group to be used in imaging after behavioral and electrophysiology experiments.	7
	Group 2	The virus containing GFP transgene was injected into the hippocampus dentate gyrus region, and 7 days later, icv phosphate-buffered saline (PBS) was administered to the ventricles. The group to be used in imaging after behavioral and electrophysiology experiments.	7
	Group 3	The Virus containing GFP transgene was injected into the hippocampus dentate gyrus region. The group to be used in imaging after behavioral and electrophysiology experiments	7
	Group 4	The virus containing the ChR2 (Channel-Rhodopsin2) transgene was injected into the hippocampus dentate gyrus region, and 7 days later, icv Aβ1-42 oligomer was administered to the ventricles. The group in which behavioral experiments will be conducted.	7
		The virus containing the ChR2 transgene was injected into the hippocampus dentate	

Optogenetic Acute Activation Groups	**Group 5**	gyrus region, and 7 days later icv PBS was administered to the ventricles. Behavioral experiments will be conducted in this group.	7
	Group 6	The virus containing GFP transgene was injected into the hippocampus dentate gyrus region, and 7 days later, icv PBS was administered to the ventricles and an optogenetic ferula will be inserted. Behavioral tests were conducted.	7
Chemogenetic Chronic Activation Groups	**Group 7**	The virus containing the human M3 Muscarinic Receptor (hM3D(Gq)) transgene was injected into the hippocampus dentate gyrus, and after 7 days, icv PBS was administered to the ventricles. Behavioral tests were done by applying 1mg/kg Clozapine-N-Oxide (CNO) intraperitoneally (i.p.) before the experiments.	7
	Group 8	The virus containing the human M3 Muscarinic Receptor (hM3D(Gq)) transgene was injected into the hippocampus dentate gyrus, and after 7 days, icv Aβ1-42 oligomer was administered to the ventricles. Behavioral tests were performed by applying 1mg/kg i.p. CNO before the experiments.	7
	Group 9	The virus containing the human M4 Muscarinic Receptor (hM4D(Gi)) transgene was injected into the hippocampus dentate gyrus, and after 7 days, icv PBS was administered to the ventricles. Behavioral tests were	7

Chemogenetic Chronic Inhibition Groups		performed by applying 1mg/kg i.p. CNO before the experiments.	
	Group 10	The virus containing the human M4 Muscarinic Receptor (hM4D(Gi)) transgene was injected into the hippocampus dentate gyrus, and after 7 days, icv Aβ1-42 oligomer was administered to the ventricles. Behavioral tests were performed by applying 1mg/kg i.p. CNO before the experiments.	7

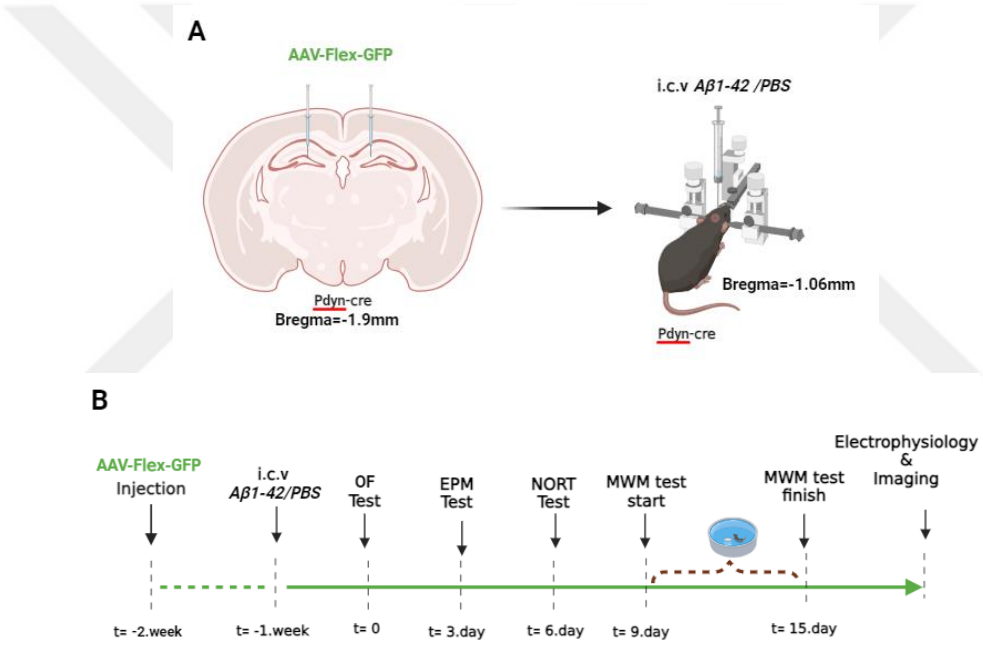
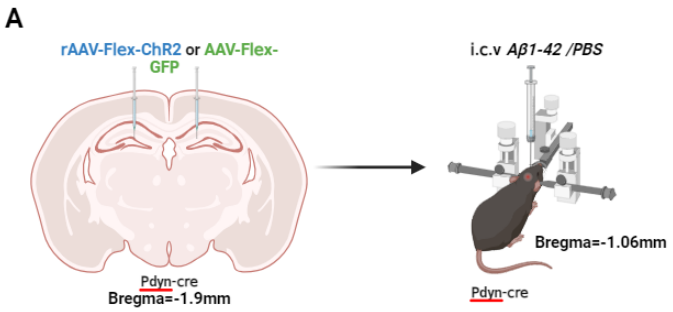


Figure 15. A) Stereotaxic injection of AAV-GFP (Adeno Associated Virus-Green Fluorescent Protein) virus and i.c.v Aβ1-42 or PBS for group 1, 2, and 3 mice. B) Experimental behavior, electrophysiology and imaging program to be followed for these groups.



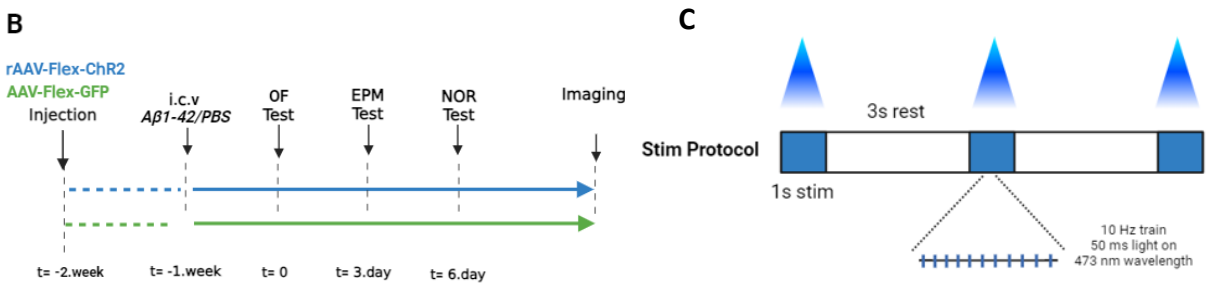


Figure 16. A) Stereotaxic injection of AAV-ChR2 (Adeno Associated Virus-ChannelRhodopsin2) virus and i.c.v Aβ1-42 or PBS for group 4, 5, and 6 mice. B) Experimental behavioral tests and imaging schedule to be applied to these groups. C) Represents the experimental stimulation protocol followed for optogenetic experiments.

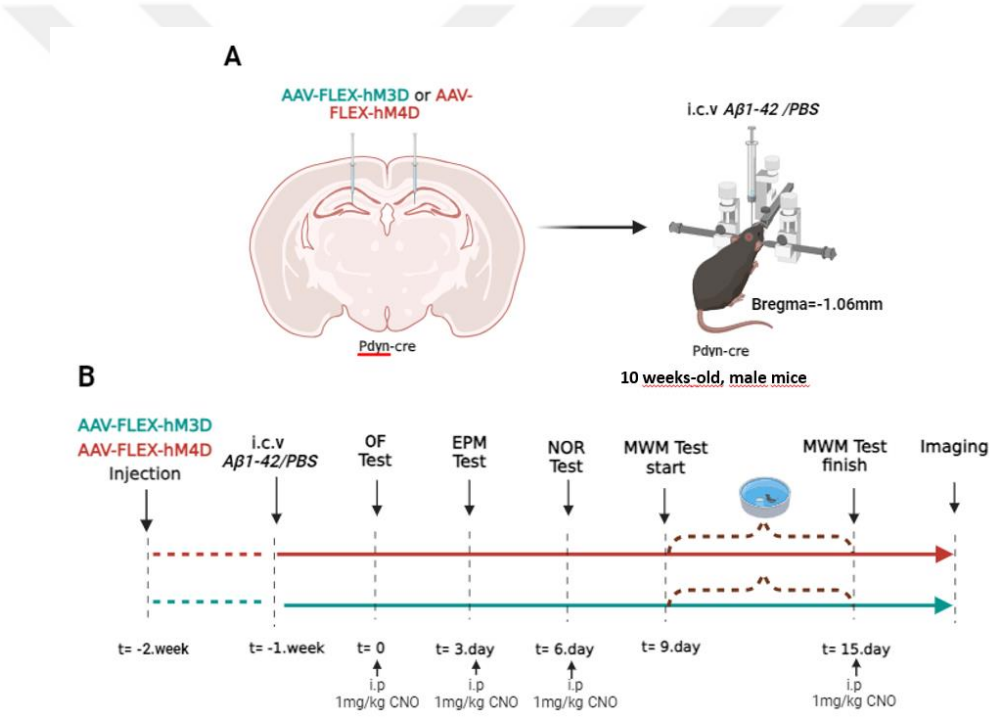


Figure 17. A) Stereotaxic injections of AAV-hM3D (Adeno Associated Virus- human M3 Muscarinic Receptor transgene) or AAV-hM4D (Adeno Associated Virus- human M4 Muscarinic Receptor transgene) viruses and i.c.v Aβ1-42 or PBS for group 7, 8, 9, and 10 mice. B) Experimental behavioral tests and imaging program to be applied to these groups.

3.7 Aβ1-42 induced Alzheimer's Disease Model

Senile plaques represent a distinctive hallmark of AD pathology, forming due to the aggregation of Aβ 40 and 42 peptides, which are directly responsible for the production of free

radicals that are toxic to the brain (58). Amyloid β-Peptide (1-42), also known as Aβ42, is a 42-amino acid peptide that holds a pivotal role in the pathogenesis of AD (59). In a study, soluble Aβ42 oligomer in 0.1 M PBS solution was able to induce oxidative stress, neurodegeneration and anxiety-related behaviors in icv-injected rats (59). Therefore, the fact that intracranial Aβ42 oligomer exposure results in dysregulated cognitive effects provides an example of achieving Alzheimer's-like modeling. In this study, we aimed to examine the effects of hippocampal DG prodynorphin neurons on spatial learning and memory impairment caused by Aβ1-42. First of all, Amyloid β Protein Fragment 1-42 (Lot no: SLBJ2877V) was used to prepare Aβ42 oligomer. To develop the mouse model of AD, amyloid-beta (Aβ1–42, 1.2μL/10 min/mouse) oligomers were injected icv into the brain of mice under the isoflurane/oxygen anesthesia system using a stereotaxic frame (60).

Briefly, a stock solution of Aβ1-42 protein fragment (Sigma-Aldrich) was prepared at a concentration of 1 mg/ml in sterile 0.1 M PBS (pH 7.4). Afterwards, this Aβ1-42 protein fragment taken into PBS was incubated at 37°C for 4 days for Aβ1-42 oligomerization (figure 18). This aggregated form of Aβ 1-42 was then administered i.c.v to 10-week-old adult Pdyn-cre male mice at a dose of 160 pmol/mouse (1.2μL/10 min/mouse) into the 3 dorsal lateral ventricles using a Hamilton micro syringe. Stereotaxic coordinates for injection were made according to the Paxinos and Franklin Mouse Brain Atlas (AP: -1.06mm, ML: 0mm, DV: -2mm) (61). AD control group mice were injected with PBS at an amount of 1.2ul/10 min/mouse icv.

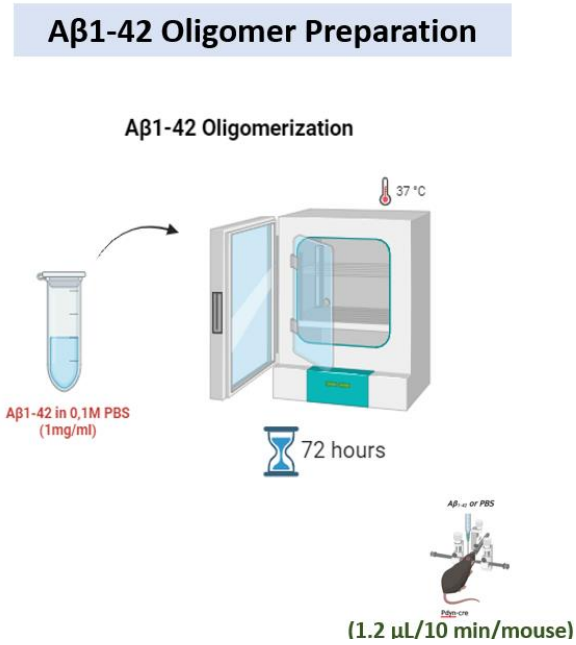


Figure 18. The Aβ oligomerization protocol followed for AD-like modelling.

3.8 Stereotaxic Intracranial GFP Virus Injection

As it was stated before (62), the coordinates for bilateral AAV-Flex-GFP virus injection into Pdyn-Cre mice were determined according to the Paxinos and Franklin Mouse Brain Atlas (AP: -1.9mm, ML: +/- 1.1mm, DV: -2.0mm) (61). The genetic map of this virus includes the GFP gene and LoxP sites to which the Cre-recombinase enzyme binds. Thus, in the Cre-dependent transgenic mouse line, neurons containing Cre-recombinase enzyme were marked and GFP expression of Pdyn (+) neurons was achieved. Intracranial injection was made to the hippocampal Dentate Gyrus (DG) region using a stereotaxic device under anesthetic isoflurane gas and oxygen gas (Figure 19).

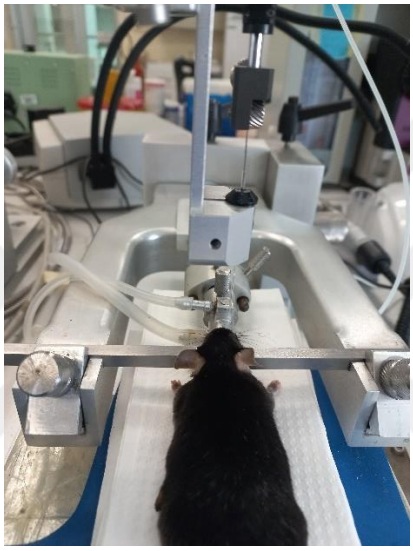


Figure 19. Intracranial virus injection by using stereotaxic frame.

3.9 Stereotaxic Intracranial Chemogenetic Virus Injection

The DREAAD (Designer Receptors Specially Activated by Designer Drugs) technology used in this study is based on the principle of sensitizing muscarinic receptors to the developed CNO instead of self-antigen (63). Among these specially produced channel proteins, AAV-FLEX-hM3D, a cation channel, is used for activation, and AAV-FLEX-hM4D, an anion channel, is used for inhibition (63). By inserting channel proteins into the virus DNA, infection of the targeted neuron group will be achieved. By using a stereotaxic frame, AAV-FLEX-hM3D activation and AAV-FLEX-hM4D inhibition viruses were bilaterally injected into the DG region of Prodynorphin-Cre transgenic mice according to the Paxinos and Franklin Mouse Brain Atlas (AP: -1.9mm, ML: +/- 1.1mm, DV: -2.0mm) (61). At the end of the two-week infection period, channel proteins that can be activated by CNO will be present in DG prodynorphin neurons. CNO, a muscarinic receptor type 3 receptor agonist, will be administered i.p to the chemogenetic groups 30 minutes before the experiments. CNO

application provides the opportunity to stimulate the target area acutely (approximately 2 hours) (64). Anxiety and locomotor activity states that may occur in mice were measured with OF (Open Field) and EPM (Elevated Plus Maze) tests, and learning and memory levels were evaluated with NOR (Novel Object Recognition) and MWM (Morris Water Maze) tests.

3.10 Optogenetic Virus Injection

ChRs located on the cell membrane are light-sensitive non-specific ion channels. They allow ions to pass through the cell membrane structure when exposed to light of a certain wavelength. By intracranially injecting Cre-specific rAAV-Flex-ChR2 virus into Cre-dependent neuron lines and then exposing this neuron population to blue light at a wavelength of 473nm, ChR2 ion channels are opened, thus an influx of cation ions into the cell occurs, resulting in cell depolarization (65,66). In this study, for acute manipulation of hippocampal DG^{Pdyn} neurons to perform optogenetic manipulation, Pdyn-Cre mice were given bilateral ChR2 virus injection according to the Paxinos and Franklin Brain Atlas (AP: -1.9mm, ML: +/-1.1mm, DV: -2.0mm) (61). Then, the optogenetic ferule was placed in the injection area, using the Mouse Brain Atlas as a reference, with the depth coordinate 0.2-0.5 mm above the target area. In order to prevent the ferrule cable placed inside the brain from being damaged and moving while the mouse continues its normal vital functions, the ferule was attached to the skull with the help of dental acrylic (figure 20). Finally, OF, EPM and NOR tests were performed while this neuron line was stimulated with a 473 nm blue laser.

Figure 20. Placement of an optic ferule on the head of mice for Optogenetic stimulation.

3.11 Patch Clamp Electrophysiology Recordings

As we stated in our other studies (67) for electrophysiological recordings, Pdyn-cre mice were deeply anesthetized with isoflurane/oxygen. Afterwards, cardiac perfusion was performed with N-methyl-D-glucamine (NMDG) based solution (92 mM NMDG, 1.25 mM NaHPO4, 2.5 mM KCl, 30 mM NaHCO3, 25 mM glucose, 20 mM HEPES, 2 mM thiourea, 3 mM sodium pyruvate, 5 mM sodium ascorbate, 10 mM MgSO4.7H2O and 0.5 mM CaCl2.2H2O) kept at -80°C for 40 minutes, and after the blood was removed from the brain, brain removal was performed with surgical equipment. The removed brain was incubated in aCSF prepared under laboratory conditions (124 mM NaCl, 1.25 mM NaHPO4, 2.5 mM KCl, 5 mM HEPES, 12.5 mM glucose, 24 mM NaHCO3, 2 mM CaCl2.2H2O and 2 mM MgSO4.7H2O). First of all, 250μm thick coronal brain slices were obtained in NMDG solution gassed with 95% Oxygen & 5% Carbon Dioxide using a vibratome device. Brain slices containing the DG region were placed in 34°C NMDG solution gassed with 95% Oxygen & 5% Carbon Dioxide for 15 minutes. Finally, these brain slices were incubated for 1 hour in the holding HEPES solution (92 mM NaCl, 1.25 mM NaH2PO4, 2.5 mM KCl, 2 mM thiourea, 30 mM NaHCO3, 25 mM glucose, 20 Mm HEPES, 3 mM sodium pyruvate, 5 mM sodium ascorbate, 2 mM CaCl2.2H2O and 2 mM MgSO4.7H2O) at room temperature and were ready for the Patch Clamp recording process. For recording, one of the incubated DG brain sections was placed in a bath system perfused with aCSF solution. Then, cell-attached recordings of DG^{Pdyn} neurons were taken. Accordingly, the MultiClamp 700B Amplifier by Molecular Devices, San Jose, CA, in conjunction with the Axon pCLAMP 11.3 software, was utilized to analyze the firing frequencies of DG^{Pdyn} neurons in the study.

3.12 Elevated Plus Maze Test

The EPM test was used to measure anxiety-like behavior in Pdyn-cre mice. The mice used in the experiment were left facing the same arm on a plus-shaped platform (110cm*110cm*40cm) with two arms open and two arms closed, and were allowed to spend 5 minutes of free time. Only chemogenetic groups were subjected to EPM testing 30 minutes after CNO administration. The parameters of the EPM test include the total number of entries to open and closed arms, the percentage of time spent in open arms relative to the total time spent in arms, and the percentage of open arm entries to total entries (68). By using the CCD camera and Ethovision-XT video tracking system, the number of seconds the mouse spent in which arm and the frequency of entering the arms were recorded and analyzed. After the mice were removed from the platform, the entire platform was cleaned with 70% ethanol to prevent any odor stimulus (65).

3.13 Open Field (OF) Test

The OF test was employed to assess locomotor activity and anxiety levels in mice. Mice exhibiting anxiety-like behavior prefer to stay close to walls and spend more time in corners and edges, while mice with lower anxiety prefer to spend more time in the center of the open space (69). The mice were left in the middle of a white 80x80x50cm^3 wide platform with a high surrounding area. Only chemogenetic groups were subjected to OF testing 30 minutes after CNO administration. The performance of the mice was evaluated with a CCD camera and Ethovision-XT video tracking computer system. After each mouse was taken from the platform, the entire platform was cleaned with 70% ethanol (62).

3.14 Novel Object Recognition (NOR) Test

In this study, the Novel Object Recognition (NOR) test was utilized to evaluate short- and long-term memory status in mice. To determine short-term memory levels, the experimental design was made as a 5-minute habitation, 10-minute training and 10-minute test procedure after 1 hour. During the 5-minute habituation period, the mice were positioned in the center of a white, empty platform (60x30x30cm^3) and permitted to explore the surroundings. During the 10-minute training phase, two identical objects were placed in the field and the mice were allowed to explore, and in the test, phase performed 1 hour later, one of the identical objects was substituted with a new object (70,71). To test long-term memory levels, 24 hours after the short-term memory performed the previous day, the animals were taken to the area where two different objects were located, and the time they spent in the objects was analyzed with a CCD camera and Ethovision XT video tracking computer system (71). The platform and objects were cleaned with 70% ethanol to prevent the formation of odor clues (67). The experimental set-up followed for short-term and long-term memory levels evaluation was showed in figure 21.

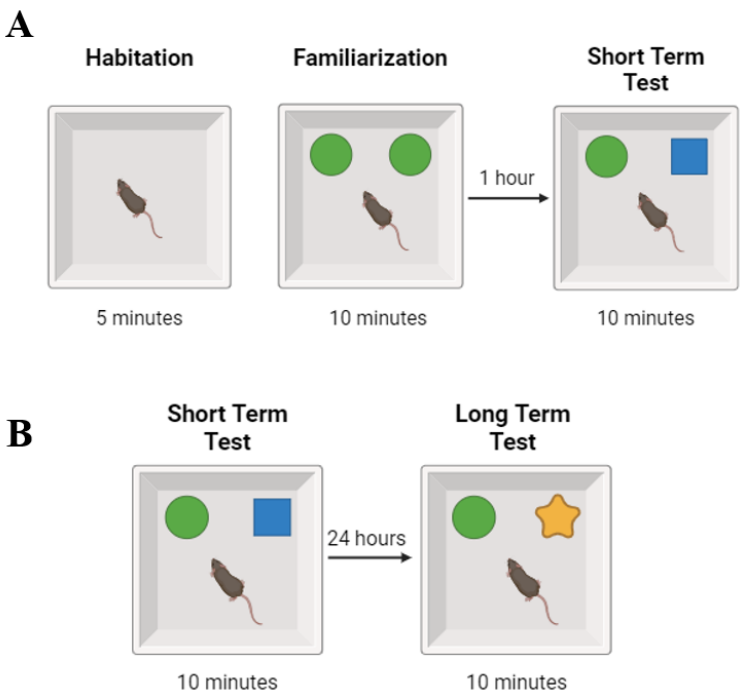


Figure 21. The illustration of the experimental setup to evaluate short-term and long-term memory levels in Pdyn-Cre mice.

3.15 Morris Water Maze Test

MWM Test is employed to assess spatial memory and hippocampal learning capabilities. In this test, a circular tank with a height of approximately 60 cm (35-90 cm) and a diameter of 117 - 210 cm was filled with 22°C water up to a height of 45 cm, and several black figures were placed around the pool as signs, in a way that the mouse could see (Figure 22) (72). The pool was imaginatively divided into eight imaginary axes (such as N, S, E, W, NE, NW, SE and SW), and an escape platform was placed in a fixed place, 1 cm below the water. This test consists of 3 different stages; The experiment, including the pre-training trial, acquisition trial, and probe trial, was completed in 6 days (72). In the pretraining trial of the MWM experiment, on day 1, mice were allowed to habituate to the pool and explore the visibly placed escape platform. The acquisition trial was performed 4 trials per day for days 2-5 of the experiment, with each mouse swimming for 60 seconds, and the mouse was released from different points of the pool and allowed to learn the platform hidden in a fixed location. Finally, the probe trial was performed on the last day of MWM, with the escape platform removed from the pool to assess memory acquisition. In this experiment, on the Probe experiment day, the swimming performance of the swimming mice towards the location where the platform was

previously hidden was evaluated within 60 seconds with the help of a CCD camera and Ethovision XT video tracking computer system.

Figure 22. Morris Water Maze Arena with several black figures around.

3.16 Immunohistochemistry and Confocal Imaging

Since the viruses injected into mice contain the fluorescent protein transgene along with the transgenes of the relevant ion channels and receptors, DG^{Pdyn} neurons infected with the virus will also synthesize the fluorescent protein. This feature will allow us to confirm that the targeted area is infected and will provide information about the Pdyn neuron population in the infected area. In order to image the infected neurons under a confocal microscope, the brains of anesthetized mice were removed by cardiac perfusion and incubated in 4% paraformaldehyde (PFA) solution for 4 hours at +4 °C. After cardiac perfusion, 70 µm thick coronal (lateral-vertical) sections were obtained from the brains in cold 1 X PBS by using a vibratome device. Immunofluorescence staining was performed by using c-fos primary antibody and Alexa Fluor 594 and antirabbit Alexa Fluor 488 secondary antibodies to confirm that the transfected neurons were Pdyn neurons, that chemogenetic manipulations had occurred, and to examine neuronal activation (with c-fos protein marker). For this purpose, coronal sections containing the DG region taken at a thickness of 70 µm were washed 3 times for 10 minutes with 1X PBS washing solution. Then, 0.1% TritonX-100 based permeabilization buffer was added to the sections and the sections were incubated on the shaker at 4 ºC for 1 hour. The blocking solution was prepared as 0.3% BSA in 1X PBS, 0.05% TritonX-100 and poured onto the sections that were incubated for 1 hour beforehand, and the sections were incubated on a shaker at room temperature for 1 hour. After the incubation process, the sections were added to c-fos primary antibody and incubated overnight at 4 ºC. Next day, primary antibody solution (1% BSA in 1X PBS, 0.05% TritonX-100) was poured onto the sections and sections were washed for 10 minutes.

Subsequently, a secondary antibody was applied to the sections, followed by an incubation period of 1 hour in darkness at room temperature. As the final step of the staining process, the sections were washed 3 times for 10 minutes with 1X PBS on the shaker. After the staining process was completed, the sections were placed on polylysine-coated slides and covered with Flouromount sealing solution. Then, prepared sections were examined under a confocal microscope and neurons synthesizing c-fos fluorescent protein were visualized. In this way, the modulation of Pdyn neurons in the targeted region was confirmed.

3.17 Congo Red Staining

Congo red staining protocol was applied to confirm AD-like modelling through detection of Aβ deposition. First, 70 μm brain sections taken with a vibratome device were adhered to a coverslip. A circle was drawn around the brain sections with the help of a pub-pen to prevent the sections from falling off the coverslip, and then these sections were washed with 1ml PBS. after that, 1ml of Congo red dye was dropped onto the sections, enough to cover the sections, and they were incubated for 20 minutes at room temperature. After staining, the sections were washed 3 times with PBS, following this process, 1ml of alkaline alcohol was poured onto the sections and sections incubated at room temperature for 2 minutes. Afterwards, PBS was washed and enough hematoxylin was poured onto the sections to cover them, and the sections were incubated at room temperature for 2 minutes. After PBS washing, 95% alcohol was poured onto the sections and the sections were incubated for 2 minutes at room temperature. After PBS washing, 100% alcohol was poured onto the sections and the sections were incubated for 2 minutes at room temperature. Afterwards, PBS washing was performed, and xylene was poured onto the sections and the sections were incubated at room temperature for 3 minutes. Finally, the xylene was removed from the sections and the sections were covered with mounting medium and left to dry for 20 minutes at room temperature. Thus, Congo-red stained brain sections were imaged under polarized light.

3.18 Statistical Analysis

Statistical analysis was conducted using the GraphPad Prism software program. Following the normality test, data that exhibited normal distribution were subjected to analysis using Two-way ANOVA with Tukey's multiple comparisons test and Bonferroni's multiple comparisons test. Data are expressed as median (minimum-maximum) and arithmetic mean ± standard deviation, and $p<0.05$ was considered to be statistically significant.

4. RESULTS

4.1 The effect of manipulation of DG^{Pdyn} neurons on locomotor activity in mice

OF test was performed to evaluate the locomotor activities of mice. According to the OFT results, no significant difference was found between GFP-PBS and GFP-AB mice (**figure 23B)**. However, chemogenetic inhibition of DG^{Pdyn} neurons in the PBS group caused significant locomotor impairment compared to the GFP-PBS group ($p<0.0001$), whereas chemogenetic activation of these neurons in the AB group reduced the locomotor activity of these mice compared to the GFP-AB group ($p<0.0001$). GFP-AB mice entered the central zone less than GFP-PBS mice ($p<0.05$) (**figure 23C)**. Chemogenetic activation and inhibition of DG^{Pdyn} neurons in the PBS group resulted in decreased entries to the center in both groups compared to the GFP-PBS group ($p<0.05$ and $p<0.0001$, respectively). However, in the AB groups, chemogenetic activation of these neurons caused the mice to enter the center less ($p<0.05$) (**figure 23C)**.

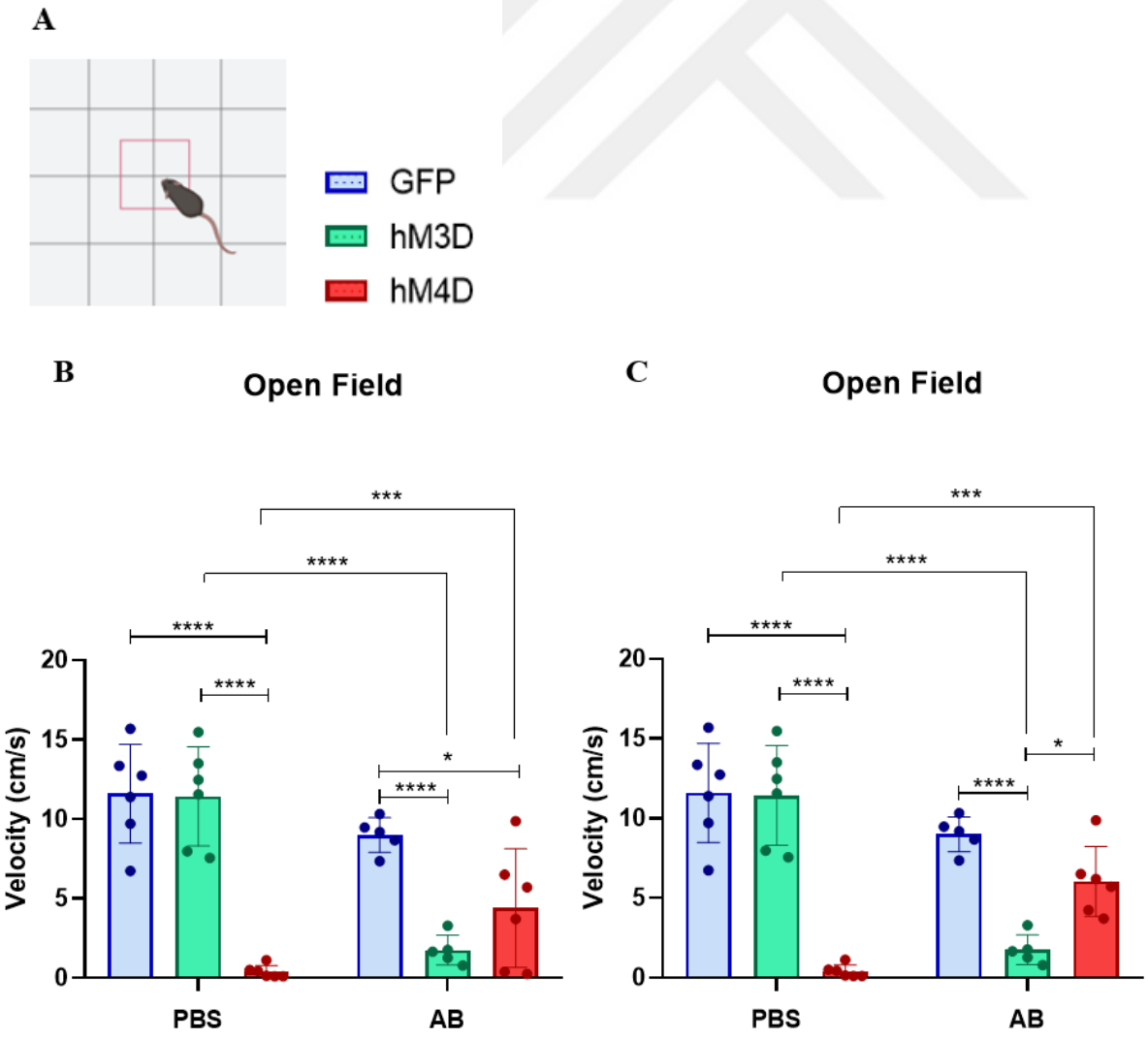


Figure 23. The effect of chemogeneticaly manipulated DG^{Pdyn} neurons on locomotor activity of mice. A, B, C; the illustration of OFT, the speed of the mice in the arena and the center entries number between PBS and AB groups, respectively (Two-way ANOVA TUKEY's multiple comparisons test and Bonferroni's multiple comparisons test, ****p<0,0001, ***p<0,001, **p<0,01, *p<0,05).

For optogenetic stimulations, no significant difference was found between the mice in terms of locomotor activity, the number of times entering the center, and the number of times being in the center, respectively (**figure 24**).

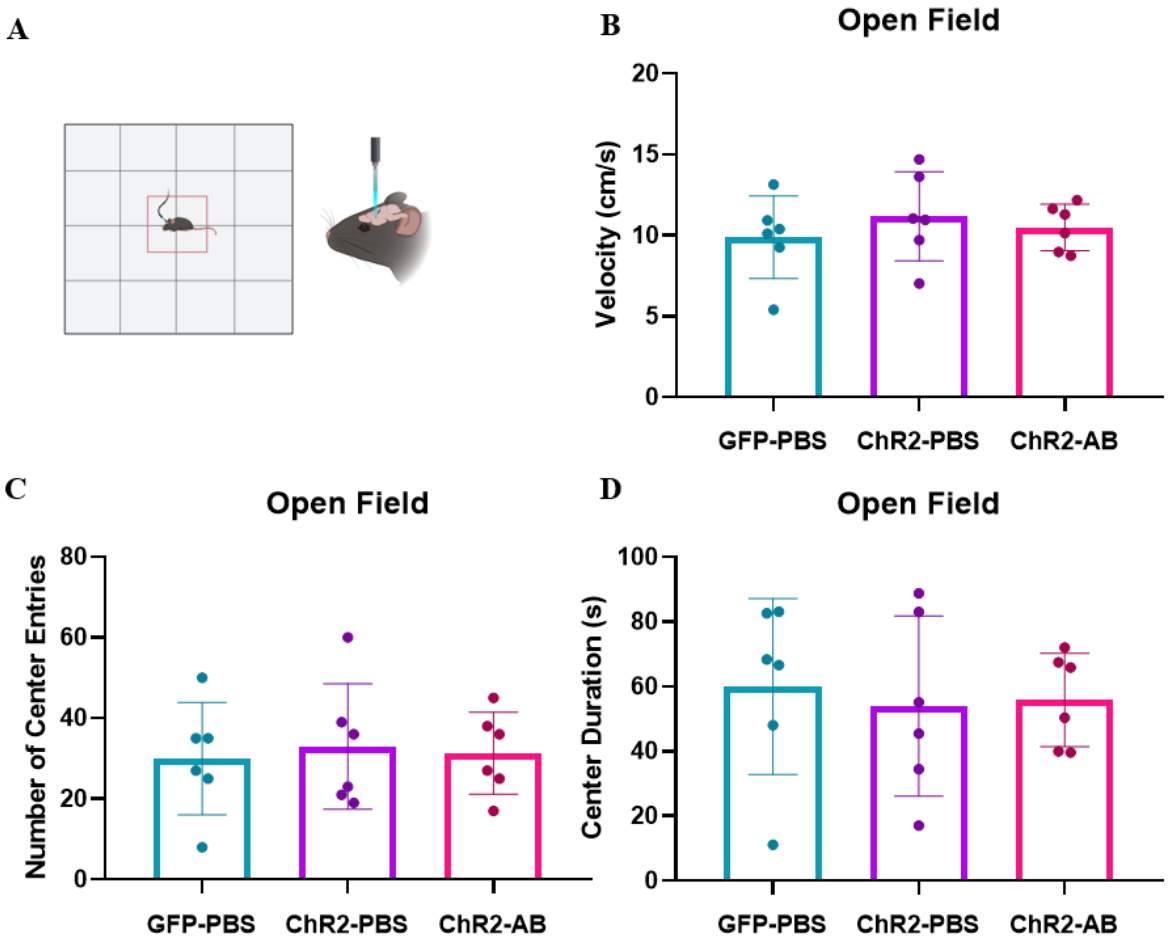


Figure 24. The effect of optogeneticaly manipulated DG^{Pdyn} neurons on locomotor activity of mice. A, B, C, D; the illustration of OFT, the speed of the mice in the arena, center entries number and spent time in the center between PBS and AB groups (One-way ANOVA Tukey's multiple comparisons test).

4.2 The effect of manipulation of DG^{Pdyn} neurons on anxiety-like behaviors in mice

EPM Test was performed to evaluate the anxiety-like behaviors of mice. According to EPM Test results, no significant difference was found between GFP-PBS and GFP-AB mice but chemogenetic activation of these neurons with AB exposure showed that hM3D-AB mice entered less open arm compared to hM3D-PBS mice ($p<0.05$) (**figure 25B)**. In the PBS groups, with chemogenetic inhibition of DG^{Pdyn} neurons, hM4D-PBS mice entered open arm less frequently than GFP-PBS mice ($p<0.05$). However, in the AB groups, hM3D-AB mice were observed to enter the open arm less frequently compared to GFP-AB mice ($p<0.001$). The locomotor levels of mice were also examined during the EPM test. The locomotor levels of the hM4D-PBS group were revealed to be significantly lower than the GFP-PBS group during the test ($p<0.05$), this difference was not observed when AB groups were compared within themselves or when PBS groups were compared with AB groups (**figure 25C)**. In addition, when the open arm durations of the mice were compared, no significant difference was found within the groups or between the PBS/AB groups (**figure 25D)**.

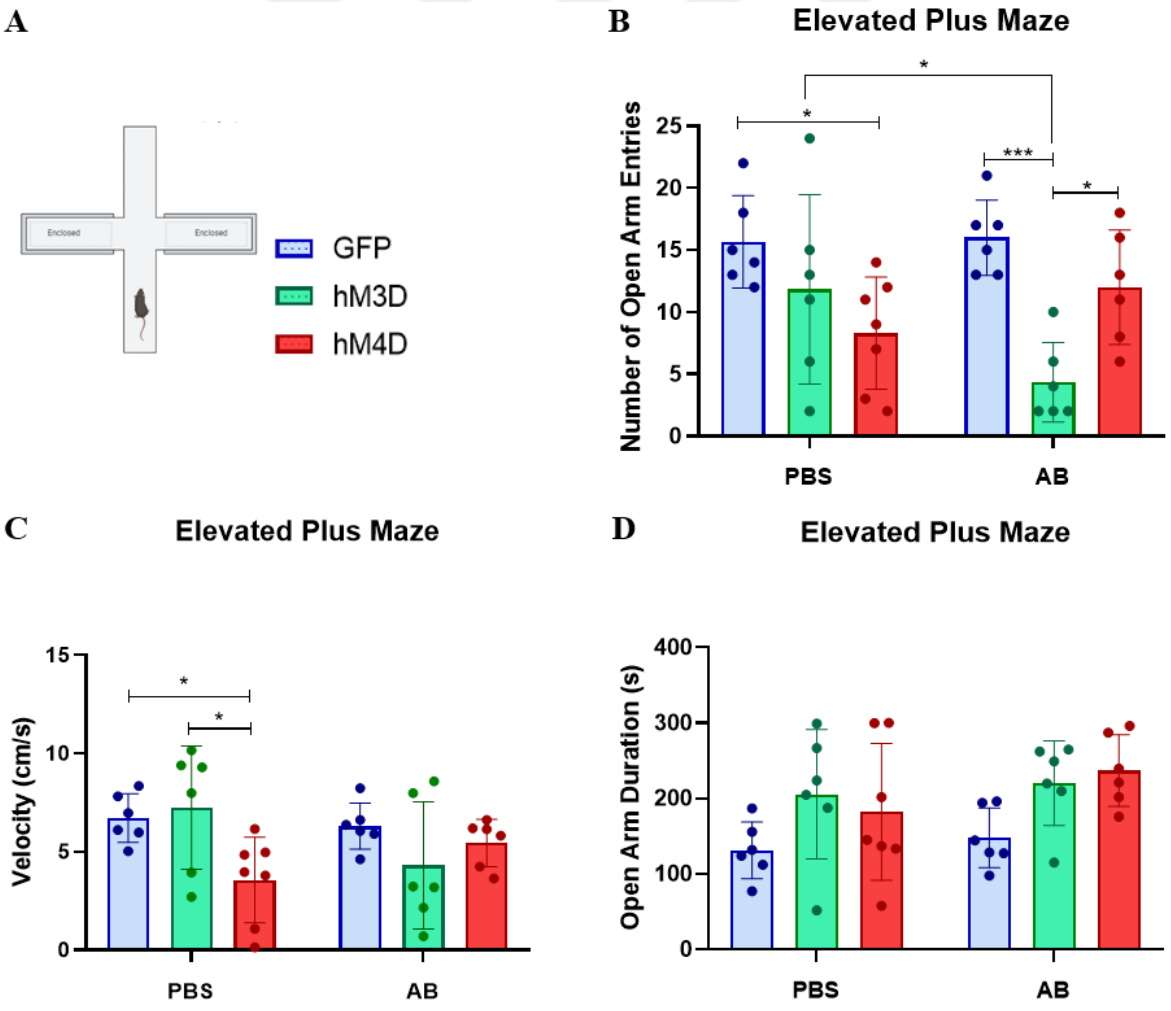


Figure 25. The effect of chemogeneticaly manipulated DG^{Pdyn} neurons on anxiety-like behaviors of mice. A, B, C, D; the illustration of EPM Test, the number of open arm entries, velocity of mice, and open arm duration, respectively (Two-Way ANOVA Tukey's multiple comparisons test and Bonferroni's multiple comparisons test Tukey, ***p<0,001, *p<0,05).

For optogenetic stimulations**,** no significant difference was found between the mice in terms of the number of open arm entries and open arm duration, respectively (**figures 26B-C)**.

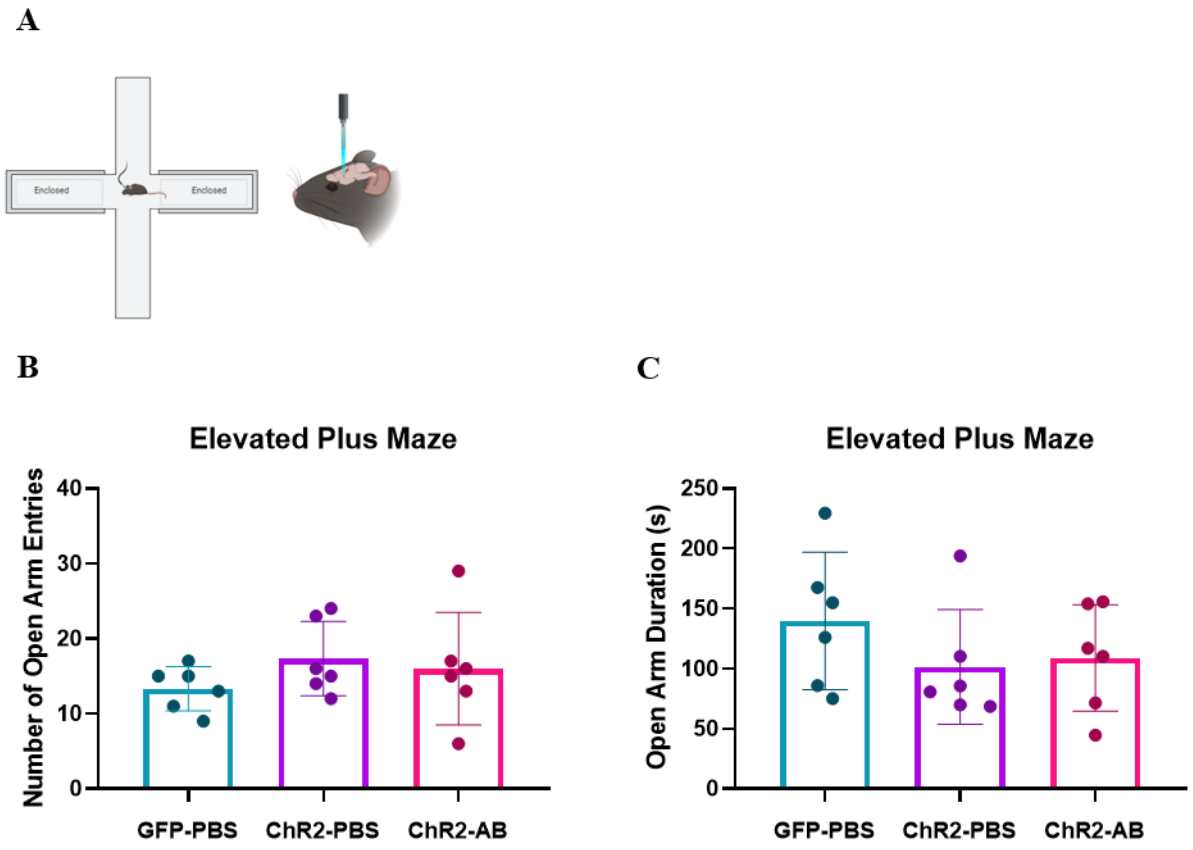


Figure 26. The effect of optogenetically manipulated DG^{Pdyn} neurons on anxiety-like behaviors of mice A, B, C; the illustration of EPM Test, the number of open arm entries and open arm frequency, respectively (One-Way ANOVA Tukey's multiple comparisons test).

4.3 The effect of manipulation of DG^{Pdyn} neurons on short-term and long-term memory levels in mice

NOR test was performed to evaluate the short-term and long-term memory levels of Pdyn-cre mice.

4.3.1 The effect of manipulation of DG^{Pdyn} neurons on short-term level in mice

NOR test was employed to assess short-term memory capacities in mice. No short-term memory damage was observed in the GFP-AB group compared with the GFP-PBS group (**figure 27B)**. However, with Aβ oligomer exposure, hM3D-AB mice were less oriented to the novel object compared to hM3D-PBS mice ($p<0.01$), whereas hM4D-AB mice were more oriented to the novel object compared to hM4D-PBS mice ($p<0,01$). Moreover, in the PBS group, chemogenetic inhibition of DG^{Pdyn} neurons significantly reduced the number of orientations to the new object ($p<0.01$), while chemogenetic activation of these neurons in the AB group showed a trend towards decreased orientation. The locomotor levels of mice were also examined during the Short-Term NOR test., During the test, there was no significant difference between GFP-PBS and GFP-AB mice, but the locomotor levels of the hM4D-PBS group appeared to be significantly lower than those of the GFP-PBS group ($p<0.01$) (**figure 27C)**. In addition, the speed of hM3D-AB mice was lower than that of hM3D-PBS mice ($p<0.01$), while the locomotor levels of the hM4D-AB group were higher than the hM4D-PBS group ($p<0.05$). Finally, when the AB groups were compared within themselves, no difference in locomotor levels was detected. When comparing the mice's exploration duration of the new object, there was no significant difference between the GFP-PBS and GFP-AB groups, while with chemogenetic activation of the DG^{Pdyn} neurons, hM3D-AB mice spent significantly less time with the new object compared to GFP-AB mice ($p<0,01$) (**figure 27D)**.

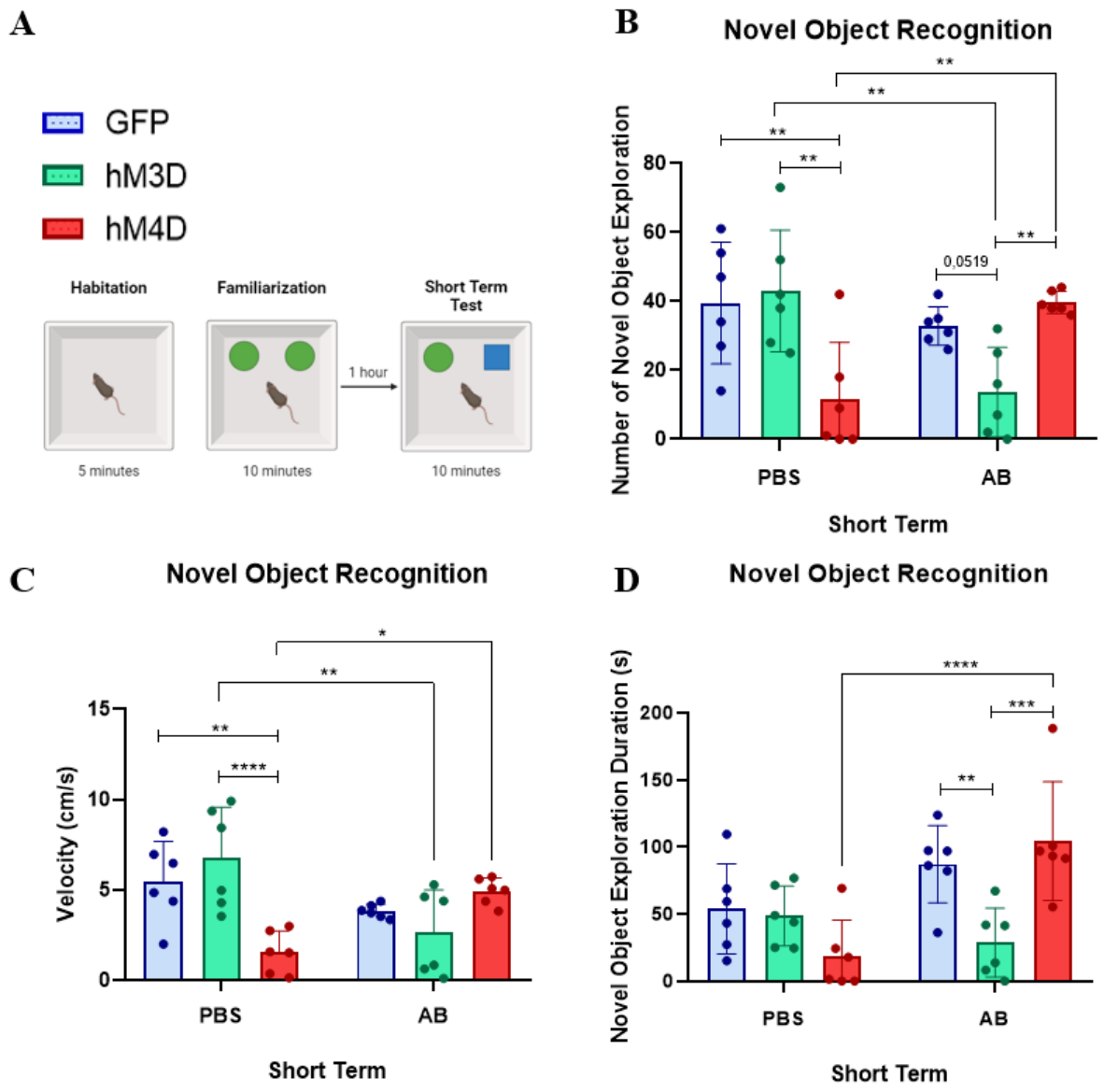


Figure 27. The effect of chemogeneticaly manipulated DG^{Pdyn} neurons on short-term memory of mice. A, B, C, D; the illustration of Short-Term Memory NOR test protocol, the number of novel object exploration, the velocity of mice during the test, and novel object exploration duration, respectively (Two-Way ANOVA Tukey's multiple comparisons test and Bonferroni's multiple comparisons test, ****p<0,0001, **p<0,01, *p<0,05).

For optogenetic stimulations, no significant difference was found between the mice in terms of the number of novel object exploration and novel object exploration durations (**figure28)**.

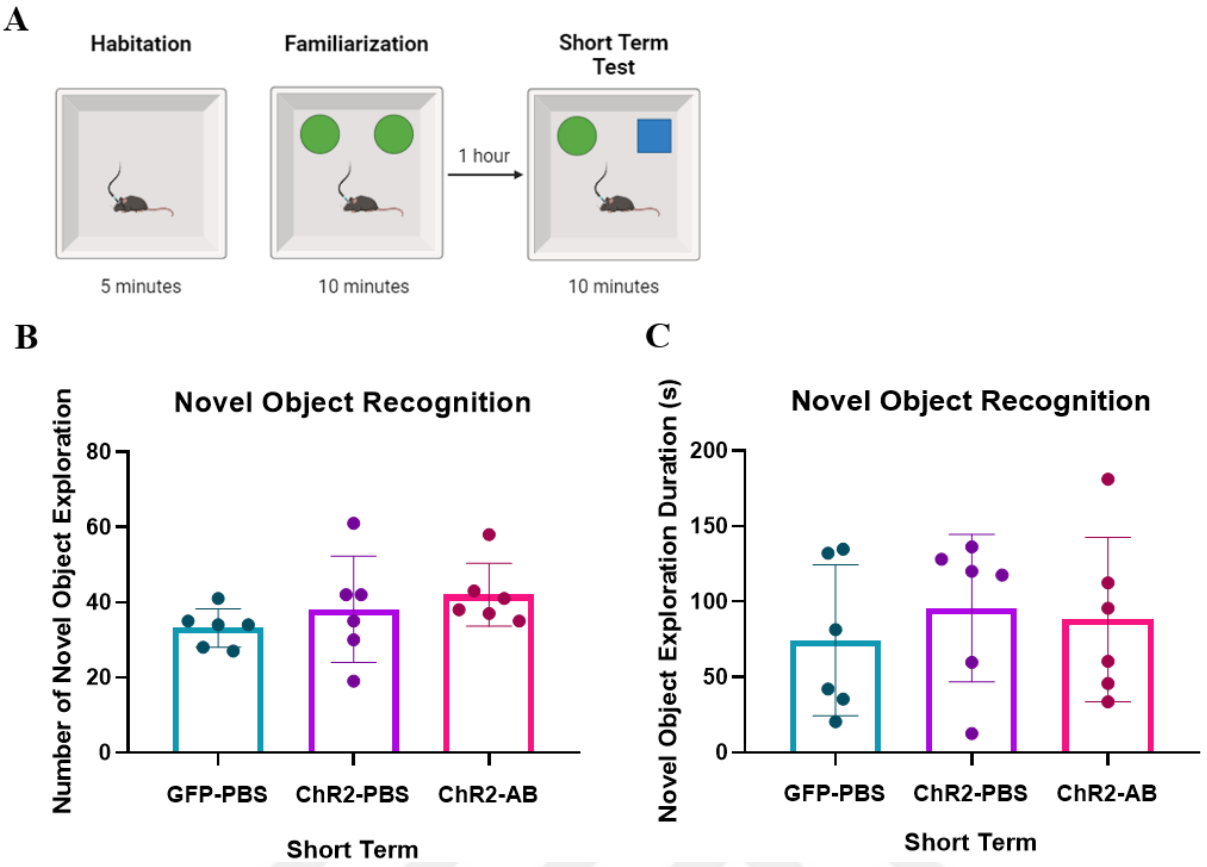


Figure 28. The effect of optogenetically manipulated DG^{Pdyn} neurons on short-term memory of mice. A, B, C; the illustration of Short-Term Memory NOR test protocol, the number of novel object exploration and novel object exploration durations, respectively (One-Way ANOVA Tukey's multiple comparisons test).

4.3.2 The effect of manipulation of DG^{Pdyn} neurons on long-term level in mice

When looking at long-term memory levels in mice, as well as short-term memory, long-term memory damage was observed in the GFP-AB group compared with the GFP-PBS group (**figure 29B**). However, with Aβ oligomer exposure, hM3D-AB mice were less oriented to the novel object compared to hM3D-PBS mice ($p<0.01$). Moreover, in the PBS group, chemogenetic inhibition of DG^{Pdyn} neurons significantly reduced the number of orientations to the new object ($p<0.05$). The locomotor levels of mice were also examined during the Long-Term NOR test. During the test, there was no significant difference between GFP-PBS and GFP-AB mice, but the locomotor levels of the hM4D-PBS group appeared to be significantly lower than the GFP-PBS group ($p<0.001$) (**figure 29C)**. In addition, the speed of hM3D-AB mice was lower than that of hM3D-PBS mice ($p<0.05$). Finally, when the AB groups were compared within themselves, no difference in locomotor levels was detected. When comparing

the mice's exploration duration of the new object in the long-term memory test, there was no significant difference between the GFP-PBS and GFP-AB groups, while with chemogenetic activation of the DG^{Pdyn} neurons, hM3D-AB mice spent significantly less time with the new object compared to GFP-AB mice ($p<0,05$) (**figure 29D)**.

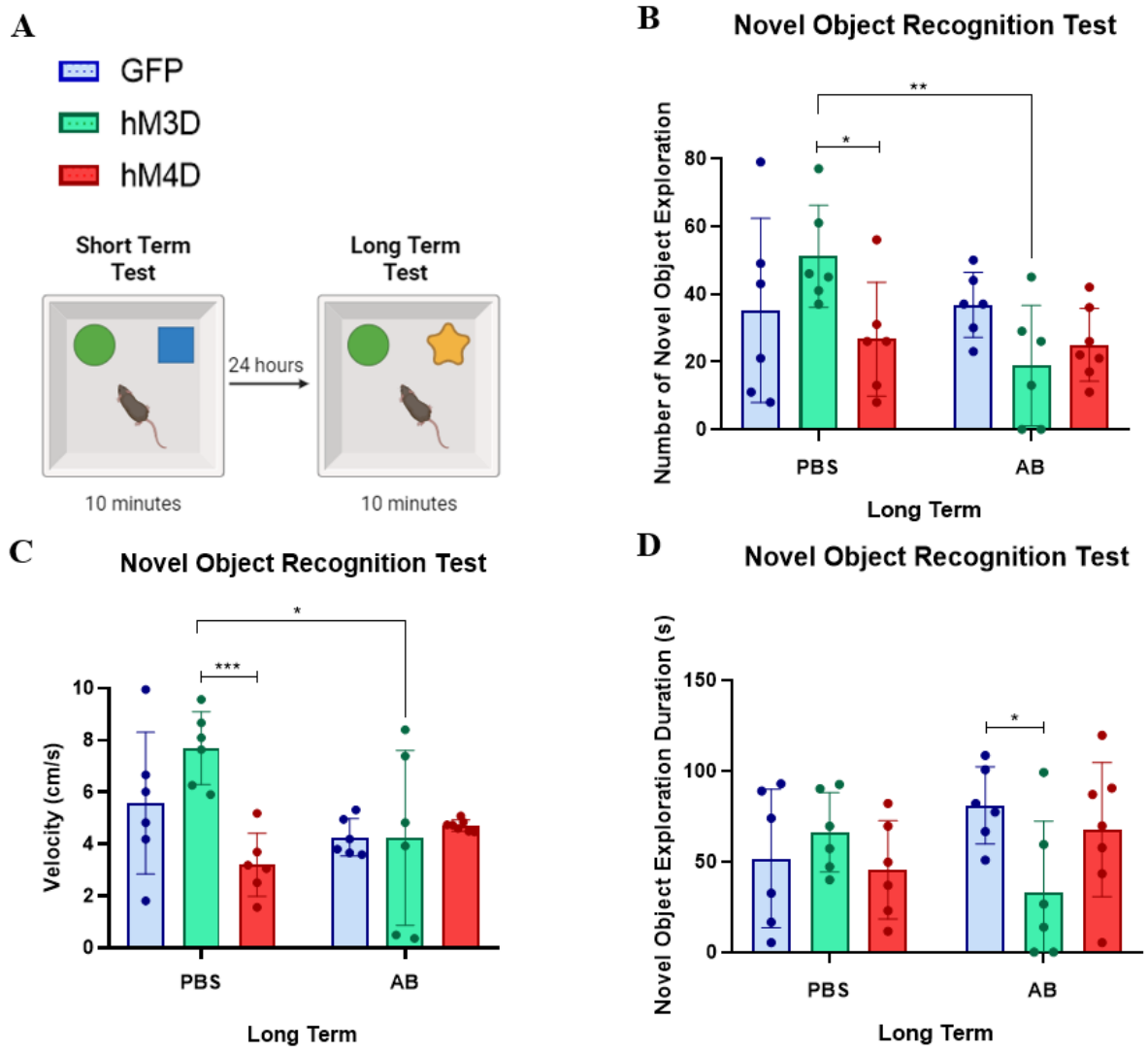


Figure 29. The effect of chemogeneticaly manipulated DG^{Pdyn} neurons on long-term memory of mice. A, B, C, D; the illustration of Long-Term Memory NOR test protocol, the number of novel object exploration, the velocity of mice during the test, and novel object exploration duration, respectively (Two-Way ANOVA Tukey's multiple comparisons test and Bonferroni's multiple comparisons test, ***$p<0,001$, **$p<0,01$, *$p<0,05$).

For optogenetic stimulations, no significant difference was found between the mice in terms of the number of novel object exploration and novel object exploration durations (**figure30)**.

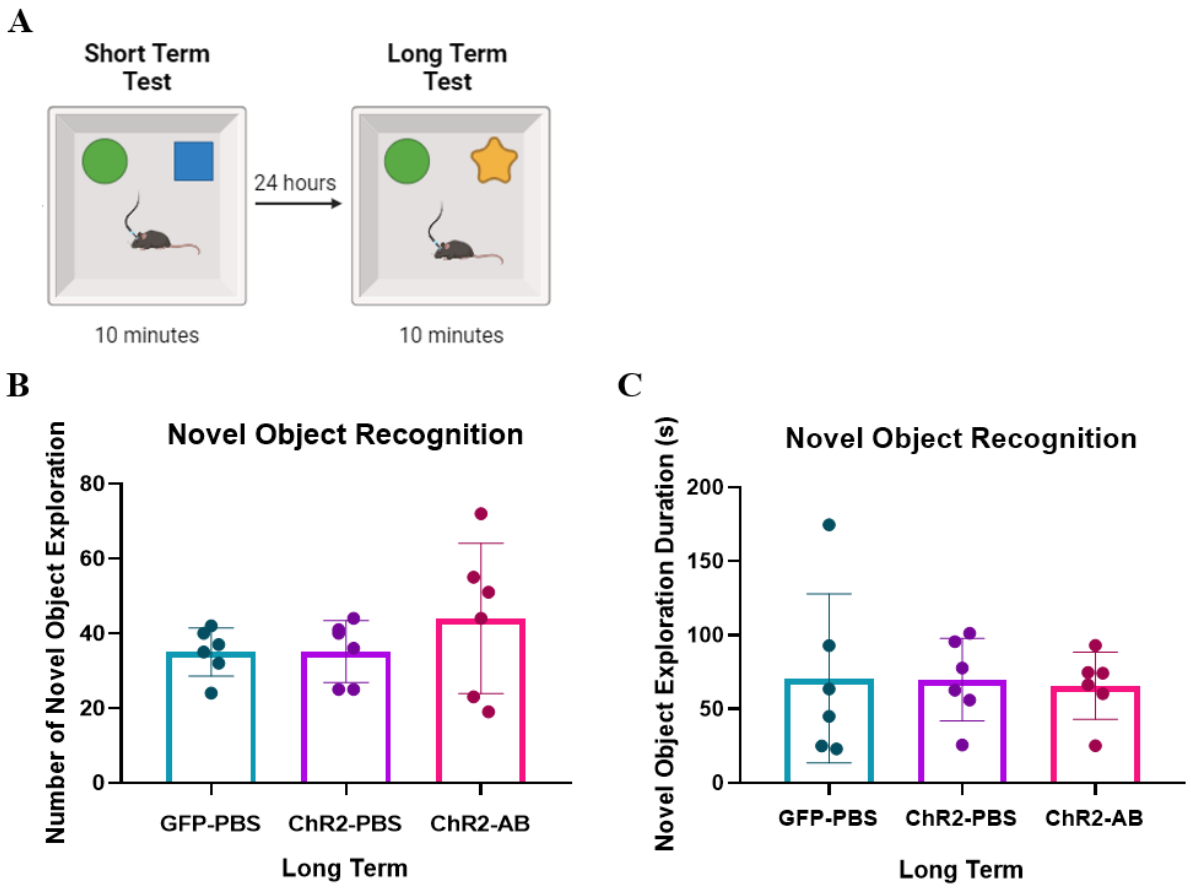


Figure 30. The effect of optogenetically manipulated DG^{Pdyn} neurons on long-term memory of mice. A, B, C; the illustration of Long-Term Memory NOR test protocol, the number of novel object exploration and novel object exploration durations, respectively (One-Way ANOVA Tukey's multiple comparisons test).

4.4 The effect of manipulation of DG^{Pdyn} neurons on spatial memory and learning levels in mice

MWM test was conducted to assess the spatial memory and learning abilities of mice. GFP-AB group showed a significant decrease in the number of entries to the platform compared to the GFP-PBS group ($p<0,05$), while this decrease showed an increasing trend with chemogenetic inhibition of DG^{Pdyn} neurons (**figure 31B)**. Additionally, GFP-AB mice entered the platform zone significantly less than GFP-PBS mice ($p<0,05$), whereas the number of entries into the platform zone increased significantly with inhibition of these neurons in the AB group ($p<0,01$) (**figure 31C)**.

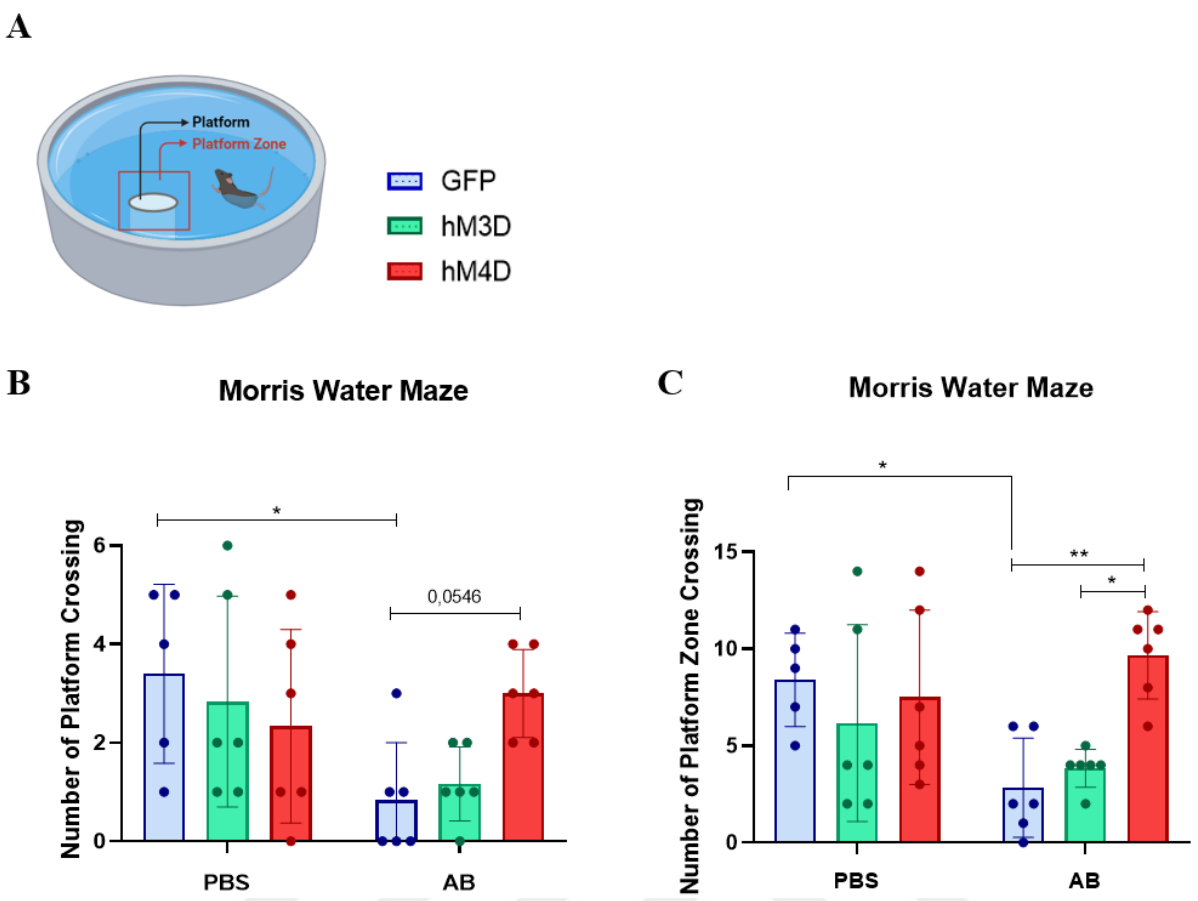


Figure 31. The effect of chemogeneticaly manipulated DG^{Pdyn} neurons on spatial memory and learning levels of mice. A, B, C; the illustration of MWM test, the number of platforms crossing of mice, and the duration in the platform zone, and the platform zone frequency between PBS and AB groups, respectively (Two-Way ANOVA Tukey's multiple comparisons test and Bonferroni's multiple comparisons test, **p<0,01, *p<0,05).

4.5 The effect of Aβ1-42 oligomers on the electrical activity of DG^{Pdyn} neurons

The patch clamp technique was used to determine whether Aβ oligomers alter the electrical properties of DG^{Pdyn} neurons. The cell-attached recordings were taken from GFP-labelled Pdyn neurons in DG by using the recording pipette (**figure 32B-C)**. The effect of Aβ oligomers on DG^{Pdyn} neurons was investigated and the spontaneous firing frequency of DG^{Pdyn} neurons was significantly higher in GFP-AB mice compared to GFP-PBS mice (p<0,01) **(figure 32D**).

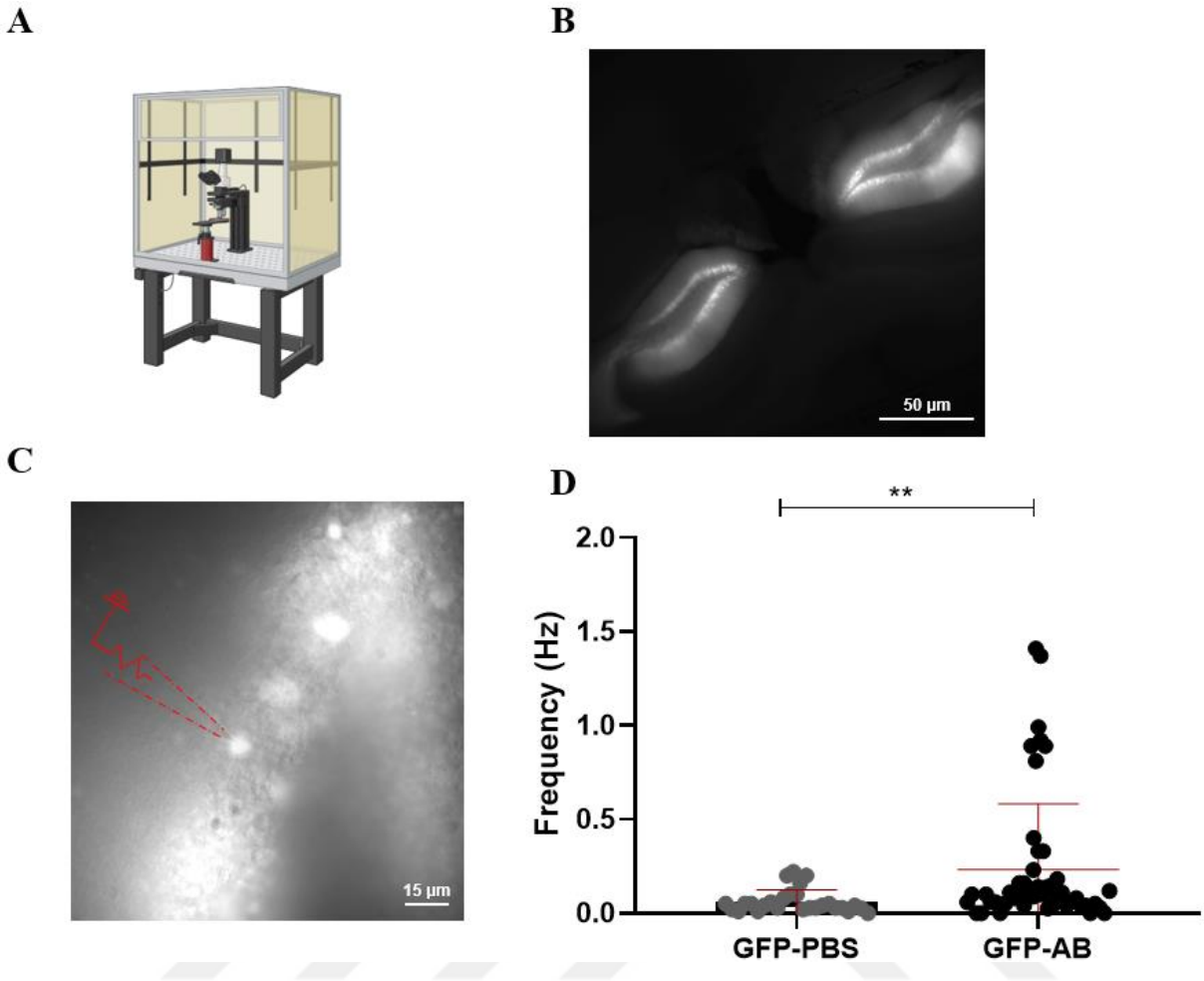


Figure 32. The effect of Aβ1-42 oligomers on the electrical activity of DG^{Pdyn} neurons. A, B, C, D; the illustration of patch clamp system, Pdyn neurons infected by GFP virus in Hippocampal dentate gyrus, a specific DG^{Pdyn} neuron attached by recording pipette (in red) and the electrophysiological cell-attached recordings taken from DG^{Pdyn} neurons of GFP-PBS and GFP-AB mice (GFP-PBS: n=27 neurons/3 mice, GFP-AB: n=50 neurons/4 mice, Mann Whitney T-test, **p<0,01).

4.6 The demonstration of Aβ deposits in Aβ-induced mice.

Congo red staining was performed to confirm Aβ modeling. While amyloid accumulation was observed in the hippocampus of mice given Aβ (reddish color)**,** no Aβ accumulation was observed in the hippocampus of mice injected with PBS **(figure 33A-B).**

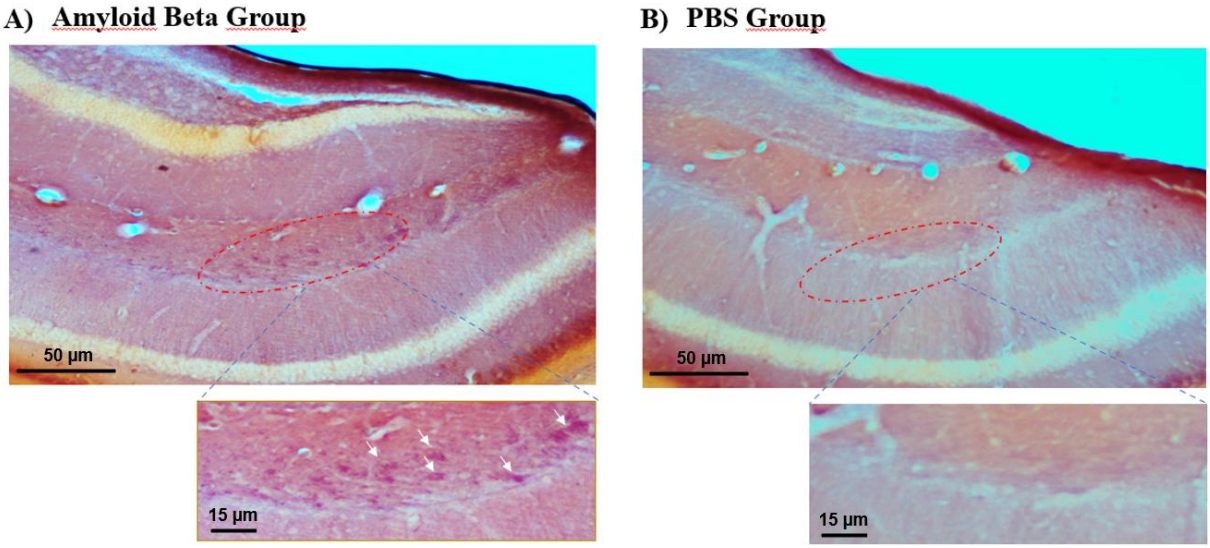


Figure 33. The demonstration of Aβ deposits in AD-like modelling. A) amyloid deposition in hippocampus of an Aβ injected mouse (reddish) which is shown by white arrows. B) No amyloid deposition in the hippocampus of PBS-injected mice.

4.7 The validation of chemogenetic manipulation of DG^{Pdyn} neurons by confocal microscopy

To demonstrate the confirmation of chemogenetic activation of hM3D virus and chemogenetic inhibition of hM4D viruses by CNO ligand, c-Fos activation protein staining was performed and visualized under the confocal microscopy. While overlap was observed in c-Fos and DG^{pdyn} neurons in the chemogenetic activation group, no overlap was observed in the chemogenetic inhibition group (**figures 34&35A)**. In addition, to examine the proximity of the implanted area to the dentate gyrus, the brain section of the mouse with optogenetic ferule was imaged under the confocal microscope (**figure 35B)**.

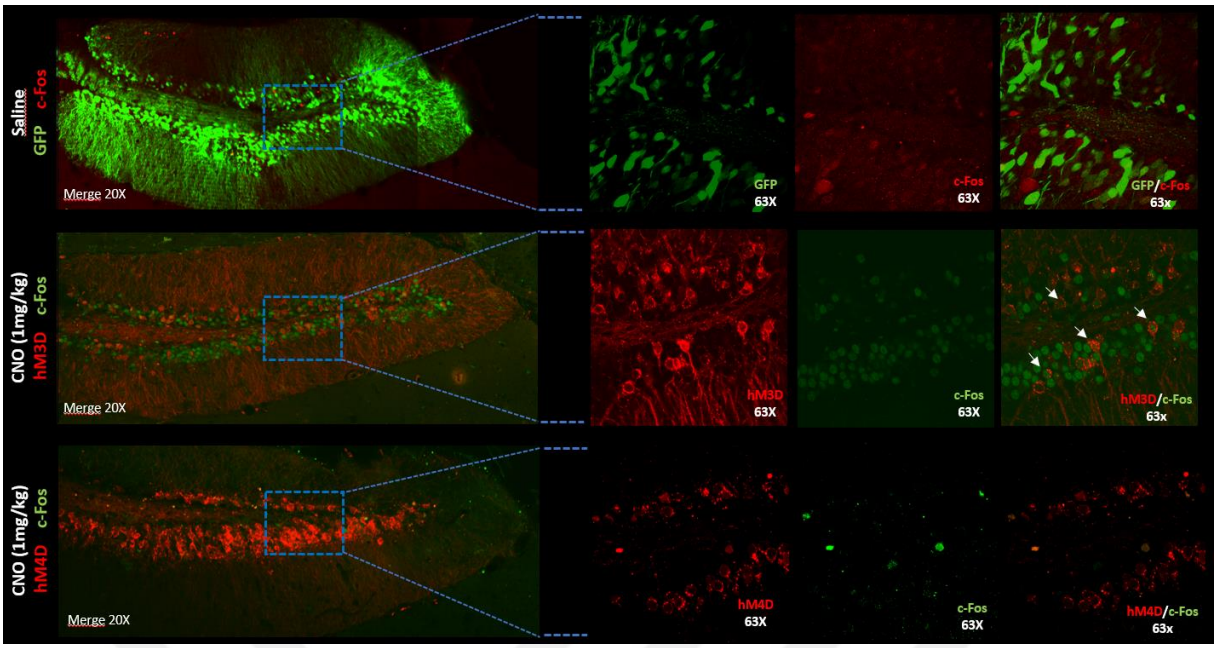


Figure 34. The validation of chemogenetic manipulation of DG^{Pdyn} neurons by confocal microscopy. mCherry-labelled Pdyn neurons (in red) and c-Fos protein in DG were observed at 20X and 63X. while overlap was observed in c-fos and DG^{pdyn} neurons in hM3D group (shown by white arrows), no overlap was observed in hM4D group.

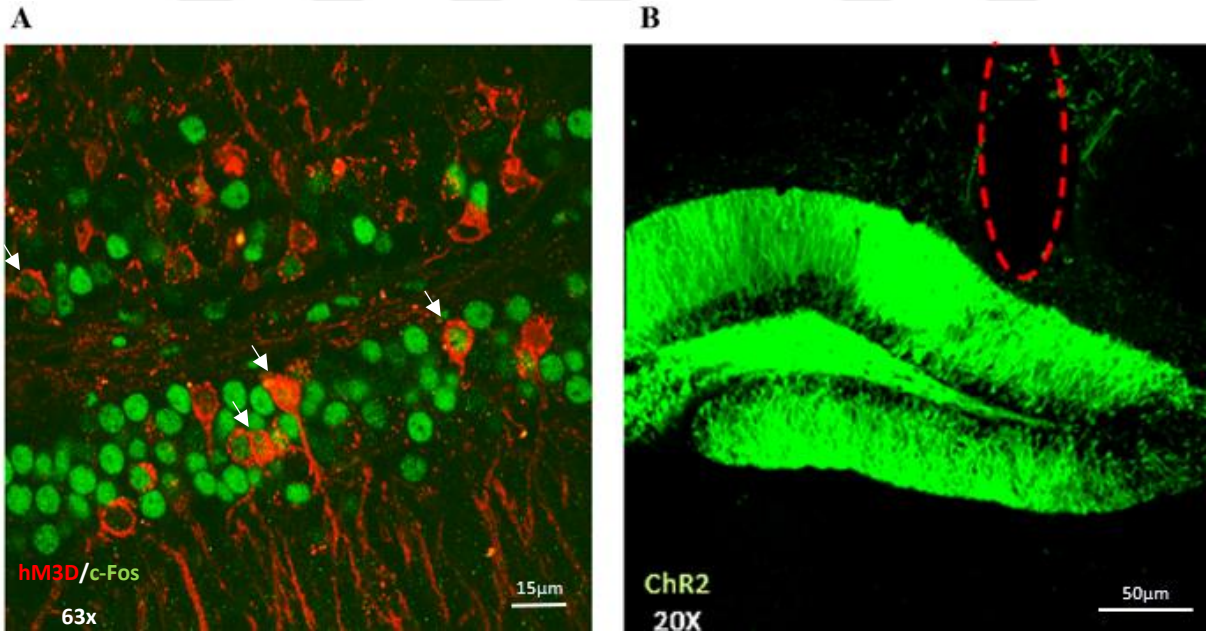


Figure 35. The validation of chemogenetic manipulation of DG^{Pdyn} neurons and ferule scar by confocal microscopy. A) mCherry-labelled Pdyn neurons (in red) and c-Fos protein in DG were visualized at 63X, the overlap of c-fos and DG^{pdyn} neurons in hM3D group was observed (shown by white arrows). B) Ferule scar (red dots) placed surgically after administration of AAV-ChR2 virus (green).

5. DISCUSSION AND CONCLUSION

AD is a neurodegenerative condition typified by gradual cognitive deterioration and the buildup of atypical protein aggregates within the brain. This irreversible and advancing neurodegenerative disorder stands as the predominant etiology of dementia globally, encompassing 60-70% of all dementia cases worldwide (1). The endogenous opioid system is a complex neurochemical system in the body that plays a crucial role in regulation pain, reward, stress and various other physiological and psychological processes (39). This system functions through the interactions of endogenous opioid peptides and opioid receptors. Prodynorphin neurons, a part of the endogenous opioid system, are nerve cells that synthesize and release prodynorphin, the precursor polypeptide for various dynorphin opioid peptides.

Today, the effect of the opioid system on AD and its role in the mechanism of dementia are not fully understood. Therefore, this study aimed to investigate a possible relationship between AD and DG^{Pdyn} neurons, which is a critical region for learning and memory. For this purpose, the potential role of DG^{Pdyn} neurons in Alzheimer's progression was investigated by providing acute activation with optogenetics and chronic activation and inhibition with chemogenetic techniques. In this direction, four different behavioral tests were applied to Pdyn-cre mice and finally, the effect of Aβ exposure on DG^{Pdyn} neurons was examined in terms of these neurons' electrical activity.

According to the OFT results, no locomotor changes were observed in mice receiving Aβ alone, but the locomotor levels of the mice decreased significantly with chemogenetic activation of DG^{Pdyn} neurons. Conversely, chemogenetic inhibition of these neurons in mice receiving PBS resulted in a noticeable locomotor decrease. These results revealed that Aβ exposure alone did not cause any locomotor changes in mice, but that DG^{Pdyn} neurons regulated locomotor levels in mice. In addition, chemogenetic activation and inhibition of these neurons altered locomotor levels differently in the PBS and AB groups, suggesting that Aβ accumulation may disrupt locomotor-related synaptic transmissions of DG^{Pdyn} neurons by altering prodynorphin signaling. In the literature, alterations in the opioid system have been associated with various neurological and psychiatric disorders, including AD (56,57). For example, a clinical study found that mu, delta, and KOR populations in certain areas of the postmortem brain of patients with AD had different expression levels and distribution compared to age-matched non-demented individuals (56). In addition, increased expression of the endogenous

kappa-opioid peptide dynorphin was associated with cognitive deficits in aging and AD (57). These changes suggest that the altered opioid system may be involved in the neuropathological processes of AD and potentially affect the cognitive and emotional symptoms of the disease. The findings indicated that the locomotor levels of mice may have an effect on the number of center entries of mice, however, the center preference numbers of GFP-AB and hM3D-PBS mice were locomotor independent. For optogenetic stimulations, no significant differences were found between the mice in terms of the locomotor activity, number of center entries, and number of centers. Stimulation of acute Pdyn neurons may not be sufficient to trigger a physiological response in terms of locomotor activity in mice.

According to the EPM results, no significant differences were found between GFP-PBS and GFP-AB mice, but by chemogenetic activation and inhibition of DG^{Pdyn} neurons, interestingly both hM4D-PBS and hM3D-AB mice entered the open arm less. Since it is understood that DG^{Pdyn} neurons have a large effect on locomotor activity, the speed of the mice during the test was also examined to see whether the open arm preference of the mice in the EPM test was a locomotor-dependent behavior. The locomotor levels of the hM4D-PBS group appeared to be significantly lower during testing, so the less open arm preference of hM4D mice may be a locomotor-dependent behavior, but the hm3d-AB mice may have shown a locomotor-independent open arm preference. When the open arm durations of the mice are compared, it is difficult to say that these neurons have an effect on anxiety-like behaviors, since no significant difference was found within the groups or between the PBS/AB groups. Thus, this study showed that DG^{Pdyn} neurons may not have any effect on anxiety-like behaviors. Since no significant difference was found between the mice in terms of the number of open arm entries and open arm durations by the optogenetic stimulations, the acute dynorphin release may not have an effect on regulating anxiety-related behaviors. According to the literature, a decrease in open arm activity of animals in terms of duration or entries reflects anxiety-like behaviors (73). Thus, this study showed that DG^{Pdyn} neurons may not have any effect on anxiety-like behaviors in mice.

No short-term memory damage was observed in the GFP-AB group compared to the GFP-PBS group. However, in the AB group, with chemogenetic activation of DG^{Pdyn} neurons, mice showed fewer orientations to the novel object and a decrease in spending time in the novel object area. This finding suggests that AB mice may be experiencing short-term memory loss when DG^{Pdyn} neurons are activated so they may have difficulty recalling the information they

remember about objects. Since it was recognized that the effect of DG^{Pdyn} neurons on locomotor activity is great, the speed of the mice during the test was examined to see whether the smaller number of new object orientations of hM4D-PBS mice in the NOR test is a locomotor-dependent behavior. The speed of hM4D-PBS mice was lower during the test, leading us to think that less orientation to the novel object can be a locomotor-related behavior in hM4D-PBS mice. When looking at the long-term memory levels of mice, there was no damage in GFP-AB mice but with the Aβ oligomer exposure, hM3D-AB mice were less oriented to the novel object and spent less time to discover it. When the locomotor levels of the mice were analyzed during testing, hM4D-PBS mice appeared to show a reduced speed, which may restrict the orientation of these mice to the novel object, while hM3D-AB mice may have long-term memory damage regardless of their velocity. Based on the literature, since mice have an innate novelty preference, a mouse that remembers a previously shown object is expected to gravitate towards the novel object and spend more time with it in order to explore the new object (74). In the present study, it was revealed that the chemogenetic activation of DG^{Pdyn} neurons may have prevented the recall of old information in AB mice. There are some studies in the literature on the relationship of dynorphin with memory damage. In a study conducted by Cheng et al., it was reported that increased dynorphin/KOR system activation in the prelimbic cortex may be a possible mechanism for methamphetamine-induced cognitive impairment (75). Another study reported that after exposing the dynorphin gene-disrupted mice to repeated forced swimming stress, these mice did not show learning and memory deficits in the NOR test (76). In addition, for optogenetic stimulations, the acute release of dynorphin may have been insufficient to produce a physiological effect on both short-term and long-term memories in mice.

According to MWM test, GFP-AB mice entered the platform area less frequently compared to the GFP-PBS group. This result shows that the spatial memory and learning levels of mice with Aβ are impaired, therefore it indicates that they may not have learned the location of the platform exactly or may have difficulty remembering it, as supported by the literature (77,78). Interestingly, this memory damage tended to be recovered by chemogenetic inhibition of DG^{Pdyn} neurons. Besides, with the chemogenetic inhibition of DG^{Pdyn} neurons, these mice showed a better performance during the test, and ameliorated the impaired spatial memory state. This finding is supported by the literature. Old PDYN knockout mice have been reported to perform better in the spatial water maze test compared to healthy control mice (47). And, reducing PDYN gene expression has been shown to protect aged mice against impaired cognition and increased anxiety-related behaviors (48).

In order to see whether Aβ accumulation in the brain has an effect on the electrical activity of DG^{Pdyn}, cell-attached recording was taken from these neurons. The spontaneous firing frequency of DG^{Pdyn} neurons was significantly increased in GFP-AB mice compared to GFP-PBS mice, which suggests that the Aβ oligomers may alter the electrical properties of DG^{Pdyn} neurons in AD-like modeling. We supported the behavioral experiments by showing the increased electrical activity of DG^{Pdyn} neurons in AD-like modeling. Apparently, further chemogenetic activation of DG^{Pdyn} impaired both the short-term and long-term memory levels of mice, while inhibiting these neurons was able to improve the mice's impaired spatial memory and learning levels. In addition, the AD-like modeling utilized in this study was validated by the demonstration of Aβ plaque accumulation in the hippocampus with the help of Congo red staining.

In conclusion, the behavioral and electrophysiological properties of DG^{Pdyn} neurons in the AD-like Pdyn-cre mouse model were investigated for the first time in the present study. Our findings demonstrated that DG^{Pdyn} neurons play a potential role in AD-like progression by inducing cognitive impairment. Additionally, investigating the synaptic connections of DG^{Pdyn} neurons may be important in elucidating the potential roles of these neurons in the pathology and progression of AD and may suggest different approaches for the treatment of the disease.

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7. APPENDICES

7.1 Ethical Approval

YEDİTEPE ÜNİVERSİTESİ HAYVAN DENEYLERİ YEREL ETİK KURULU	HAYVAN DENEYLERİ YEREL ETİK KURULU KARAR FORMU	Doküman No : FR83 Yayın Tarihi : 06.12.2022 Revizyon No : 00 Revizyon Tarihi : 00.00.0000 Sayfa : 1 / 1

ETİK KURUL KARARI				
Protokol No	**Toplantı Tarihi**	**Toplantı Sayısı**	**Karar No**	**Proje Yürütücüsü**
2023-09	27.03.2023	2023/03	2023/03-01	Prof.Dr.Bayram Yılmaz
'Aβ ile İndüklenmiş Alzheimer Modeli Transgenik Farelerde DG PDYN Nöronlarının Elektriksel ve Davranışşsal Özelliklerinin Araştırılması'isimli proje oy birliğiyle etik açıdan uygun görülmüştür.				
Hayvan Türü / Irkı	**Toplam Hayvan Sayısı**		**Hayvan Cinsiyeti**	
Fare/Pdyn-Cre	81		Erkek	

Görevi	Adı Soyadı	Onay/Ret Durumu	Katılım Durumu
Başkan	Prof. Dr. Bayram YILMAZ	☐ Onay ☐ Ret	
Başkan Vekili	Prof. Dr. Erdem YEŞİLADA	☐ Onay ☐ Ret	
Üye	Dr. Engin SÜMER	☑ Onay ☐ Ret	
Üye	Prof. Dr. M. Ece GENÇ	☑ Onay ☐ Ret	
Üye	Prof. Dr. Rukset ATTAR	☑ Onay ☐ Ret	
Üye	Prof. Dr. Gamze TORUN KÖSE	☑ Onay ☐ Ret	
Üye	Prof. Dr. Özlem MALKONDU	☑ Onay ☐ Ret	
Üye	Doç. Dr. Aylin YABA UÇAR	☑ Onay ☐ Ret	
Üye	Doç. Dr. Burcu GEMİCİ BAŞOL	☑ Onay ☐ Ret	
Üye	Atakan Mücahit YAVUZ	☑ Onay ☐ Ret	
Üye	Ahmet ŞENKARDEŞLER	☑ Onay ☐ Ret	

Hazırlayan	Sistem Onayı	Yürürlük Onayı
YÜDETAM SORUMLU YÖNETİCİ	YÜDETAM MÜDÜRÜ	YÜDETAM MÜDÜRÜ

7.2 Curriculum Vitae

Personal Informations

Name	Habibe	**Surname**	GÖREN

Education

Degree	Department	The name of the Institution Graduated From	Graduation year
Doctorate			
Master			
University	Molecular Biology and Genetics	**Izmir Institute of Technology**	2021
High school	-	**Körfez Oruç Reis Anatolian High School**	2016