A Dissertation for the Degree of Doctor of Philosophy

Discovery of Anti-Alzheimer's Disease Agent Modulating Ca^{2+}-permeable TRPV1 Pain Receptors

Ca^{2+} 투과성 TRPV1 통증 수용체를 조절하는

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Discovery of Anti-Alzheimer's Disease Agent Modulating Ca^{2+}-permeable TRPV1 Pain Receptors

By

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Discovery of Anti-Alzheimer's Disease Agent modulating Ca^{2+}-permeable TRPV1 Pain Receptors

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Abstract

According to the World Health Organization, more than 55 million people have dementia worldwide, in addition to approximately 10 million new cases annually. Dementia mainly occurs in older people, and its incidence is expected to increase with the aging of the population worldwide. Cognitive function and memory decline are typical symptoms of dementia. Therefore, patients with dementia depend on the people around them. Thus, dementia requires not only treatment but also management, and its social cost is very high. Moreover, dementia affects not only patients but also the people around them, thus affecting society.

Alzheimer's disease (AD) is the most common form of dementia; 70%–80% of patients with dementia have AD. Although AD was reported by Alois Alzheimer more than 100 years ago, no proper treatment has been established. Because amyloid-β (Aβ) and phosphorylated tau (p-tau) levels are higher than normal in the brains of patients with AD, they have been used as diagnostic criteria for AD.

Currently, the cause of AD is unclear; however, two hypotheses are prominent: Aβ and p-tau. Aβ and p-tau accumulation causes toxicity,

which leads to neuronal apoptosis and inflammation in the brain, resulting in cognitive function and memory deficits. According to this hypothesis, several studies on drug development have been conducted with the mechanism of removing Aβ and p-tau by developing antibodies. Aducanumab (Aduhelm^{tm}) is an Aβ antibody drug that was approved by the Food and Drug Administration (FDA) for the first time in 2022. Aβ is removed by aducanumab, but it is not effective in improving memory and cognitive function.

Recently, studies have reported that calcium (Ca^{2+}) dyshomeostasis is the main cause of AD. Ca^{2+} affects various signals and enzyme activities in nerve cells. Specifically, it functions as a neurotransmitter and plays a vital role in memory formation. Ca^{2+} concentration in the brains of patients with AD is higher than normal. Memantine (approved as a treatment for AD) reduces intracellular Ca^{2+} concentration by inhibiting the N-methyl-D-aspartate Ca^{2+} receptor. Accordingly, reducing Ca^{2+} concentration shows therapeutic effects against AD.

The transient receptor potential vanilloid 1 (TRPV1) is a Ca^{2+}-permeable nonselective cation channel mainly expressed in the peripheral nervous system. Nevertheless, TRPV1 is expressed in the central nervous

system, such as the hippocampus and cerebrum. When affecting the nervous system, it causes depression, anxiety, etc. Studies have shown that TRPV1 deficiency causes neurogenesis and synaptic plasticity.

In this study, when TRPV1 was genetically deficient in an AD mouse model (3 × Tg-AD), the Ca^{2+} concentration in the brain decreased, and Aβ and p-tau production and neuronal apoptosis were suppressed. TRPV1 deficiency also improved memory and cognitive function of objects and space in behavioral experiments. These beneficial effects result from activating the brain-derived neurotrophic factor/cAMP-response element binding (BDNF/CREB) signaling pathway by Ca^{2+} homeostasis recovery.

Through these results, lowering the Ca^{2+} concentration through the TRPV1 antagonist would have therapeutic effects on AD. To develop a new drug agent for AD, I tried to confirm the efficacy of treating AD by using α-spinasterol, which is a known TRPV1 antagonist and a major component of spinach. Administering α-spinasterol to the hippocampus improved the memory and cognitive function of object and space and inhibited neuronal apoptosis by activating BDNF/CREB signaling pathway through lowered Ca^{2+} concentration.

This study is the first to find that genetic deficiency of Ca^{2+}-permeable TRPV1 receptor rescues memory deficits and Aβ and p-tau production and reveals that its mode of action is the activation of BDNF/CREB signaling pathway caused by decreased Ca^{2+} concentration. In addition, α-spinasterol, a TRPV1 antagonist and a major component of spinach, was confirmed to be an effective AD treatment. α-Spinasterol has a potential for use as a new AD drug agent. An integrated study developed a new AD drug agent from identifying the role of TRPV1 through genetic deficiency in AD mice model to revealing the efficacy of AD treatment using α-spinasterol, a TRPV1 antagonist.

Key Words: The transient receptor potential vanilloid 1 (TRPV1), Calcium, Alzheimer's Disease (AD), Apoptosis, Amyloid-β, p-tau, BDNF/CREB signaling pathway, α-spinasterol,

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Chapter 1

Therapeutic potential of TRPV1 antagonism for the treatment of Alzheimer's disease: A literature review

Abstract

Calcium (Ca^{2+}) regulates intercellular signaling and various functions in neuronal cells. In the brain, Ca^{2+} is involved in the activity of important signaling proteins such as brain-derived neurotrophic factor/cAMP-response element binding (BDNF/CREB) signaling pathway, which regulates the differentiation and activity of neuronal cells and memory formation. Therefore, Ca^{2+} homeostasis is very important for maintaining normal brain functions and the survival of neuronal cells. When Ca^{2+} homeostasis collapses, neuronal cells die because of the abnormal activities of various signals, which results in neurodegenerative diseases.

In patients with Alzheimer's disease (AD), the most common degenerative brain disease, Ca^{2+} concentration is upregulated in brain. Upregulated Ca^{2+} concentration promotes amyloid-β and p-tau production, which is a pathological marker of AD, and inactivates BDNF/CREB signaling pathway. These lead to neuronal cell death in brain. Therefore, Ca^{2+} homeostasis control is very important; thus, various Ca^{2+}-permeable receptors such as transient receptor potential vanilloid 1 (TRPV1), N-methyl-D-aspartate receptor, and voltage-gated calcium channel (VGCC)

are present in neuronal cells. This review will address the function of TRPV1 receptors, whose functions have not been identified in AD.

TRPV1 is a Ca^{2+}-permeable cation channel that is activated by noxious stimuli such as heat, acid, and capsaicin. It is mainly expressed in the sensory neurons of the peripheral and central nervous systems, such as the hippocampus, cortex, and cerebrum. It plays a vital role in the neurotransmission system. Recent clinical studies have shown that TRPV1 is involved in addiction, anxiety, depression, cognition, and learning. Specifically, the loss of TRPV1 function improves neurogenesis and synapse plasticity in the brain. This means that TRPV1 plays an important role in brain function. Accordingly, TRPV1 deficiency may alleviate AD. This review highlights the importance of TRPV1 antagonism in brain function and α-spinasterol (TRPV1 antagonist) as a therapeutic agent for AD prevention.

1.1 Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disease accompanied by memory and cognitive deficits [1]. Although Alois Alzheimer discovered AD more than 100 years ago, no suitable treatment has been established. The causes of AD are unclear; however, the most notable hypotheses are the amyloid-β (Aβ) and the highly phosphorylated tau (p-tau) hypotheses [2, 3].

These hypotheses point out that Aβ and hyperphosphorylated tau accumulation causes neuronal apoptosis, synaptic collapse, and neuroinflammation, which are representative symptoms of AD. This consequently decreases memory and cognitive function, which progresses into severe AD [4].

Therefore, the direction of the development of AD treatments has focused on developing antibodies of Aβ and p-tau and removing them. Until 2022, 20 clinical studies of Aβ antibody treatments and 13 clinical studies on p-tau antibody treatments were conducted [5]. Aducanumab, the Aβ antibody treatment, was approved by the FDA in 2021 as an AD treatment, which significantly reduced Aβ levels in clinical studies [6]. Although Aβ levels were reduced in clinical studies [7], no improvement

effect on memory and cognitive function was found [8]. Accordingly, a new AD hypothesis was proposed in 2015, which pointed out that AD treatment is difficult with just Aβ removal because it not only directly kills nerve cells but also promotes various signals related to AD development [9]. Therefore, patients who were clinical targets were also tailored to the preclinical stage before AD was diagnosed. Thus, the key is reducing the incidence of AD by blocking Aβ accumulation [10].

Aβ and hyperphosphorylated tau production is associated with high intracellular calcium (Ca^{2+}) concentrations. The excessive intracellular Ca^{2+} concentration activates enzyme-related Aβ production and promotes tau phosphorylation [11]. In patients with AD, Ca^{2+} concentration in nerve cells is higher than usual, which cannot form memory [12]. Ca^{2+} dyshomeostasis affects various signaling pathways, such as the brain-derived neurotrophic factor/cAMP-response element binding (BDNF/CREB) signaling pathway, and damages the nerve cells, eventually developing into severe AD [13, 14]. Therefore, if the Ca^{2+} concentration is lowered to suppress Aβ accumulation, its therapeutic effect on AD is demonstrated. Memantine, which antagonizes the Ca^{2+}-permeable N-methyl-D-aspartate (NMDA) receptor, has been approved by

the FDA for use in the treatment of patients with moderate-to-severe AD [15, 16]. In the clinical trial of memantine, Aβ accumulation was suppressed by lowering the intracellular Ca^{2+} concentration, and it showed practical AD treatment efficacy by restoring memory and cognitive ability [17].

As a target for the development of AD treatments, Ca^{2+} homeostasis control has great potential because it shows practical therapeutic effects on AD. As described above, Ca^{2+} performs a very important and fundamental function in nerve cell functioning. Therefore, the types of Ca^{2+}-permeable receptors involved in Ca^{2+} concentration control widely varied [18]. In this review, I will address Ca^{2+}-permeable transient receptor potential vanilloid 1 (TRPV1) receptors, known as pain receptors whose role in AD development has not been identified.

TRPV1 is a member of the TRP family channel, and the TRP channel is a receptor that recognizes external stimuli. Among them, TRPV1 is widely distributed in the peripheral nervous system and recognizes strong external stimuli such as heat and acid [19]. Heat and acid are deadly stimuli that cause protein denaturation. Accordingly, TRPV1 receptors were found to have released greater amounts of Ca^{2+}

than NMDA receptor, the target receptor of memantine, for the same stimulus [20, 21]. TRPV1 is also present in the brain [22]; therefore, TRPV1 is expected to play a large role in the regulation of Ca^{2+} homeostasis in the brain, and this role remains to be identified.

TRPV1 is the receptor for capsaicin, the pungent agent from chili peppers that elicits burning pain. TRPV1 is a Ca^{2+}-permeable nonselective cation channel that is highly expressed in primary sensory neurons [23]. Furthermore, it is expressed in many brain regions, including the hippocampus, cortex, cerebellum, olfactory bulb, mesencephalon, and hindbrain, where it may be involved in modulating neuronal function [24, 25]. TRPV1 is suggested to mediate anxiety-, depression-, and schizophrenia-related behaviors [26-28]. TRPV1 function loss induces increased expressions of neurogenesis and synaptic plasticity-related genes [29], thus probably modulating AD. I investigated the effect of TRPV1 function loss on AD pathogenesis in a 3 × Tg-AD mouse model. Four genotypes of mice were generated, namely, 3 × Tg-$AD^{-/-}$/$TRPV1^{+/+}$, 3 × Tg-$AD^{+/+}$/$TRPV1^{+/+}$, 3 × Tg-$AD^{+/+}$/$TRPV1^{+/-}$, and 3 × Tg-$AD^{+/+}$/$TRPV1^{-/-}$, which were analyzed for their neuroprotective effect, memory function, hippocampal Ca^{2+} levels, Aβ, and tau pathology.

Through the genetic TRPV1 deficiency, the possibility as a treatment target of AD was confirmed. In addition, I would like to confirm the possibility of a new AD treatment agent using α-spinasterol, which is a known TRPV1 antagonist [30, 31] and a major phytosterol component of spinach. Spinach contains 0.08% of α-spinasterol [32]. As α-spinasterol is a spinach-derived ingredient, its LD50 in a mouse model is 479 mg/kg, which is not toxic [33]. α-Spinasterol has functional effects, such as antidepressant, antianxiety, anti-insomnia, and anti-inflammatory activities [34, 35]. A big hurdle in the development of AD treatments is the blood–brain barrier (BBB) permeability. BBB permeability is a very important factor in the development of AD treatments because the brain is the target organ of AD treatments. In a pharmacokinetics study, α-spinasterol showed high BBB penetration [33]; thus, it can directly affect the brain. Therefore, I investigated the effect of α-spinasterol on the pathogenesis of AD in an AD mouse model ($A\beta_{1-42}$-treated mice) [36].

1.2 Dementia

Dementia is common in older people, and more than 55 million patients worldwide suffer from dementia. This is expected to significantly

increase as society ages, and by 2050, it is expected to increase to 139 million [37]. The World Health Organization defined dementia as “a brain disorder that slowly destroys memory and thinking skills and, eventually, the ability to carry out the simplest task.” As with the definition of dementia, patients with dementia can barely perform simple tasks; thus, help from around them is essential. Dementia requires management, help, and treatment of patients, and its social management cost is very high. In 2020, the cost of dementia management worldwide was $ 1.3 trillion, which is expected to increase about five times to $ 6.5 trillion in 2050 [37]. Therefore, dementia is a great social disaster worldwide, and to overcome it, its cause should be known, and its appropriate treatment should be established. The types of dementia are AD, vascular dementia, dementia with Lewy bodies, Parkinson’s disease dementia, frontotemporal dementia, and mixed dementias. Among them, Alzheimer’s disease is a representative disease of dementia, and approximately 80% of patients with dementia suffer from Alzheimer’s disease. Alzheimer’s disease was first made known by Alois Alzheimer’s in 1906 in Germany.

Alzheimer’s disease is a degenerative brain disease that develops very slowly and progresses gradually [38]. In the early stages of AD,

memory begins to decline, and language ability and spatial cognitive ability gradually decreased. If AD worsened, the ability to perform daily living activities such as eating and hygiene management wane. Thus, this study aimed to determine the causes of Alzheimer's disease and the current status of its treatment.

1.3 Diagnostic Markers for AD

Unlike normal people, the most prominent feature in the brain of patients with AD is the accumulation of Aβ and formation of tau fibers by tau hyperphosphorylation [4]. The first criterion for the diagnosis of patients with AD is Aβ accumulation. The amyloid precursor protein (APP), the parent of Aβ, is involved in the differentiation of neurons and intercellular interactions. Therefore, the expression and metabolic process of APP are very active, and Aβ is the by-product produced during the metabolic process [39]. If this Aβ is not removed and accumulated, it becomes toxic, causing neuronal apoptosis [40].

The second diagnostic marker is tau hyperphosphorylation. Tau proteins serve to form the structure of the axon of nerve cells. However, when tau is hyperphosphorylated, it aggregates and fails to form the

structure of nerve cells, leading to neuronal apoptosis [41]. The kinase that directly affects tau hyperphosphorylation is GSK3β, which is activated by Aβ accumulation [42]. To diagnose AD, these two AD diagnostic markers are confirmed through cerebrospinal fluid and positron emission tomography (PET) imaging.

The third diagnostic marker is neuronal apoptosis. The brain of patients with AD is more contracted than a normal brain. Therefore, it is used as an AD diagnosis criterion, i.e., the degree of neuronal apoptosis in the brain is measured through PET and magnetic resonance imaging (MRI) [43]. AD diagnosis is made based on the above three biomarkers; however, AD in the early stages is difficult to diagnose. During the AD onset phase, the above three markers can be diagnosed after AD has already progressed [44, 45]. Until now, studies have explored the cause of AD development and resulting treatments have been developed; however, no clear AD treatment has been developed. Accordingly, the prevention stage rather than the treatment stage is the focus in AD. These changes will be investigated by understanding the causes and developing treatments for AD.

1.4 Changes in the AD Hypothesis

AD is considered a great social disaster worldwide. Accordingly, many countries tried to develop AD treatments. A representative project is the A4 study, which was launched in 2012 with a budget of $775 million, with the United States declaring a war on dementia [46]. First hypothesis about the cause of AD is the amyloid cascade hypothesis, in which neurons are killed by Aβ accumulation [47]. Based on this hypothesis, the goal was to treat AD by developing Aβ antibodies to remove Aβ, which was a key study in the A4 study. Based on this study, a total of 20 new drugs were conducted in clinical trials by 2022. Through this, aducanumab treatment was approved by the FDA as an AD treatment for the first time in 2021. Aducanumab treatments significantly reduced Aβ accumulation in clinical trials [48]. However, the efficacy of this treatment is not yet clear. In recent clinical trials of AD treatment, aducanumab antibody treatments showed efficacy in improving memory and cognitive abilities [49].

A new hypothesis was proposed for the cause of AD in 2015. This is an Aβ-trigger hypothesis, i.e., AD occurs when various AD-related signals are activated by Aβ accumulation. Therefore, this hypothesis states

that when signals are already activated by Aβ, AD progression would not stop even if Aβ is removed [9]. Therefore, in the development of new AD treatments, the focus is to prevent Aβ accumulation, and clinical trials are underway at a clinical stage where the three markers of AD diagnosis do not appear.

I would like to discuss the pharmacological mechanisms and target molecules of newly developed drugs currently taking place.

1.5 Pharmacological Mechanisms and Target Molecules of Newly Developed Drugs

In AD, the pathophysiology is gradually revealed, and treatment development is accelerating. Several AD treatments are being developed, targeting the causes of AD. In the development of drugs targeting Aβ or tau proteins, neurotransmitters are becoming major targets. In addition, drug developments targeting intracellular signaling systems, oxidative stress, mitochondria, and cell Ca^{2+} metabolism are ongoing.

Table 1. AD treatment development targets and strategies.

Target	Therapeutic strategies
Neurotransmission	Cholinesterase inhibitors

Amyloid	Secretase enzyme modulation
	Amyloid transport
	Prevent amyloid aggregation
	Promote amyloid clearance
	Amyloid-based immunotherapy
Tau	Tau phosphorylation inhibition
	Microtubule stabilization
	Tau-based immunotherapy

1.5.1 AD treatment targeting neurotransmission

Acetylcholine synthesis and metabolism are reduced in the brain of patients with AD. Starting with the first acetylecholinesterase inhibitor tacrine developed in 1993, donepezil, rivastigmine, and galantamine were developed and have been approved by the FDA for use as mild and moderate AD agents [50].

Table 2. Status of AD Treatments based on neurotransmission.

Drug	**FDA approval**	**Mechanism**
Tacrine	1993	Cholinesterase inhibitors

Donepezil	1996	Cholinesterase inhibitors
Rivastigmine	1997	Cholinesterase inhibitors
Galantamine	2001	Cholinesterase inhibitors
Donepezil +Memantine	2014	Cholinesterase inhibitors + N-methyl D-aspartate receptor antagonism

Despite some cognitive functions and overall function improvements, the degree was not significant, and side effects such as gastrointestinal disorders and hallucinations were reported. The development of percutaneous formulations such as patch and microneedle types that can reduce side effects while maintaining the bioavailability and duration of drugs in the body is underway. However, this is not a fundamental treatment of AD, but a only symptom relief measure, and the degree of AD progression cannot be alleviated at a certain degree [51].

1.5.2 Amyloid-related dementia treatment status

The most basic pathophysiological phenomenon in AD is Aβ accumulation and the resulting neuronal apoptosis. Amyloid precursor protein (APP) is located in the cell membrane of a nerve cell, where Aβ is produced when it is cut off by a secretase. When this insoluble Aβ is not

removed and aggregated, it accumulates in the nerve tissue to form an amyloid plaque, leading to neuronal apoptosis [52]. Recently, in addition to Aβ antibodies, therapeutic drugs are being developed targeting each stage from generation, aggregation, and removal of factors related to Aβ production [53].

Table 3. Status of AD Treatments based on amyloid-β removal.

Drug	FDA approval	Mechanism
Verubecestat	Failure	BACE1, 2 inhibition
Semagacetat	Failure	γ-Secretase inhibition
Avagacetate	Failure	γ-Secretase inhibition
Alzhemed	Failure	Aβ aggregate inhibition
ELND005	Failure	Aβ aggregate inhibition
Bapineuzumab	Failure	Anti-Aβ monoclonal antibody
Solanezumab	Failure	Anti-Aβ monoclonal antibody
Gantenerumab	Phase 3	Anti-Aβ monoclonal antibody
Crenezumab	Phase 3	Anti-Aβ monoclonal antibody
Adcanumab	2021	Anti-Aβ monoclonal antibody

1.5.3 Current status of tau-related dementia treatment

Until recently, the development of Alzheimer's disease drugs has been mainly based on Aβ. However, due to a series of failures of Aβ target treatments, the development of tau-based treatments has recently attracted attention. Aβ induces tau phosphorylation, and the hyperphosphorylated tau damages the microskeletal structure in the cell and fails to maintain biological functions such as maintaining cell shape or metabolite movement, eventually leading to neuronal apoptosis [54]. The strategy in developing tau-based treatments is to develop new drugs that inhibit tau phosphorylation, stabilization of the microskeletal structure, tau removal by immunotherapy, and inhibition of tau aggregation [55]. However, the development of tau-based treatments is still in the early stage compared with the development of amyloid target treatments.

Table 4. Status of AD Treatments based on tau fibril removal

Drug	FDA approval	Mechanism
AADvac1	Phase 2	Anti-tau monoclonal antibody
Tilavonemab	Phase 2	Anti-tau monoclonal antibody
Semorinemab	Phase 2	Anti-tau monoclonal antibody
ACI-35	Phase 1	Phospho-tau specific vaccine
TRx0237	Phase 3	Tau aggregate inhibition

1.6 Strategy for the Treatment of Alzheimer's disease: Calcium Homeostasis

Cognitive and memory impairments are typical symptoms of AD [56]. Aβ and p-tau are representative pathological markers of AD, which cause neuronal apoptosis [57]. The cause of AD is unclear; however, studies have reported that Ca^{2+} dyshomeostasis is the primary cause. As the target stage of AD treatment development is prevention, a mechanism to prevent AD pathology is needed before these two markers progressed. Mild cognitive impairment occurs in the early stages of AD; however, Aβ accumulation and tau hyperphosphorylation are not found [58]. Recent studies have shown that Ca^{2+} homeostatic collapse was first observed before Aβ accumulation and tau hyperphosphorylation appeared in asymptomatic or early AD stages. Synaptic pathology appeared because of collapsed Ca^{2+} homeostasis. Therefore, Ca^{2+} homeostasis regulation is expected to be a highly likely target for AD prevention. Ca^{2+} activates or deactivates various signaling pathways in neurons. If Ca^{2+} homeostasis control is impaired, various signal transmissions collapse and learning and memory impairments occur [59]. According to an earlier study, patients with AD have higher Ca^{2+} concentrations than usual. Accordingly, long-

term potentiation cannot occur, and memory is not formed [60, 61]. Memantine, the NMDA Ca^{2+} receptor antagonist, is approved by the Food and Drug Administration (FDA) as a drug for AD treatment [62]. Memantine lowers the intracellular Ca^{2+} concentration by inhibiting the NMDA Ca^{2+} receptor [63]. Memantine-induced decrease in Ca^{2+} concentration improved neuroprotection, learning, and cognitive functions, showed inhibitory effects on Aβ and p-tau production, and activated the BDNF/CREB signaling pathway [64, 65]. Decreased intracellular Ca^{2+} concentration is beneficial for AD treatment and can be a therapeutic target mechanism.

1.7 Relationship Between Ca^{2+} Homeostasis and Neuronal Apoptosis

Neuronal apoptosis is a prominent feature of AD, which is currently a marker of AD diagnosis. Neuronal apoptosis is highly associated with Ca^{2+} homeostatic collapse [66]. An example is the BDNF/CREB signaling pathway, which is involved in neuronal differentiation, synaptic plasticity, and neuronal survival. BDNF is expressed throughout the central nervous system, with the highest level in the hippocampus. The hippocampus is divided into cornu ammonis 1

(CA1), cornu ammonis 3 (CA3), and dentate gyrus (DG) as the central region of learning and memory. The expression of BDNF is high in these areas [67]. However, in AD, the expression level of BDNF and the activity of BDNF/CREB signaling pathway are decreased [68]. The FDA introduces BDNF as an evaluation marker for the efficacy of AD treatments [69]. Several experiments have shown that BDNF overexpression or its injection into AD animal models demonstrated therapeutic efficacy for AD [70]. The BDNF/CREB signaling pathway is regulated by Ca^{2+} [71]. p-CREB is a transcription factor involved in BDNF expression and is activated by Ca^{2+} influx. However, excessive Ca^{2+} influx causes dephosphorylation of p-CREB and reduction of BDNF expression [72]. As a result, apoptotic markers such as caspase-3, PARP, BAX, and BCL-2, which are submechanisms of the BDNF/CREB signaling pathway, are activated, causing neuronal apoptosis [73].

Here, I would like to discuss the Ca^{2+} receptors involved in Ca^{2+} homeostasis that have been targeted so far and the development stage of new drugs targeting them.

1.8 Status of the Target Receptors of New Drugs Related to Ca^{2+} Homeostasis

Ca^{2+} homeostasis is very important in nerve cell functioning. Various receptors are related to this because the activities of various signals change even with a small change in Ca^{2+} concentration [74]. Ca^{2+} homeostasis and AD are closely related. Therefore, the development of new AD drugs targeting Ca^{2+} homeostasis recovery is actively taking place.

I will discuss Ca^{2+} receptors that have been targeted so far and their development stages.

Table 5. Status of AD treatments based on Ca^{2+} homeostasis recovery.

Drug	FDA approval	Mechanism
DAOIB	Phase 2	N-methyl-D-aspartate R: antagonist
Troriluzole	Phase 3	N-methyl-D-aspartate R: glutamate modulator
amlodipine	Phase 3	L-type voltage-gated calcium channel: blocker
Levetiracetam	Phase 2	N-type calcium channel: blocker

1.8.1 NMDA receptors

NMDA receptors are sodium-, potassium-, and Ca^{2+}-permeable receptors activated by glutamate. It is expressed throughout the central nervous system and is involved in nerve development and synaptic plasticity [75]. However, as aging progresses, the efficiency of the NMDA receptor decreases, increasing Ca^{2+} concentration in nerve cells. The continuous influx of Ca^{2+} by NMDA gradually damages the nerves and further leads to neuronal apoptosis [76]. The inhibition of NMDA receptors inhibited Aβ accumulation [77] and tau hyperphosphorylation and showed the effect of inhibiting neuronal apoptosis through the BDNF/CREB signaling pathway activity [78]. Currently, memantine is an NMDA receptor antagonist and has been approved as an AD treatment worldwide. In addition, DAOIB, an NMDA receptor antagonist, and triluzole with mechanisms that suppress glutamate, an NMDA agonist, are undergoing clinical trials [5].

1.8.2 L-type voltage-gated calcium channel (LTCC)

LTCC is a Ca^{2+}-permeable passage through which Ca^{2+} is introduced by the difference in membrane potential. Although nimodipine,

an early LTCC antagonist, is undergoing clinical trial as an AD treatment, it does not inhibit Aβ accumulation and is not effective in improving cognitive function [79]. Accordingly, amlodipine, an LTCC antagonist and hypertensive agent, is undergoing clinical trials as AD treatment in combination rather than through a single administration. In addition, clinical trials regarding the inhibition of surrounding factors involved in the LTCC activity, not a mechanism that directly inhibits the LTCC, are ongoing [5].

1.8.3 N-type calcium channel (NTCC)

NTCCs are mainly distributed at the postsynapse and are mainly involved in the development of dendrites and synaptic plasticity at the terminal site. Therefore, NTCCs primarily serve as protein receptors for the secretion of neurotransmitters and intersynaptic interaction. However, excessive Ca^{2+} concentration leads to the collapse of intersynaptic interactions and neural network formation [80]. Levetiracetam is an NTCC antagonist, which focuses on the recovery of neurotransmitters and neuroplasticity through the recovery of Ca^{2+} homeostasis in NTCCs [81]. Levetiracetam is originally used as a treatment for epilepsy, and it plays a

role in the development of synaptic formation and recovery of cognitive function in clinical trials for epilepsy [82]. Currently, levetiracetam is undergoing clinical trials for Alzheimer's disease.

1.9 TRPV1: Therapeutic Target for AD Treatment

Ca^{2+}-permeable receptors, which are currently the targets of new AD treatments, are targeted at the three receptors mentioned above. This review will address the potential of TRPV1, a Ca^{2+}-permeable pain receptor, as a treatment for Alzheimer's disease. TRPV1 is a member of TRP channel families. The TRP channel family mainly performs the function of recognizing external substances and is mainly distributed in the peripheral nervous system, and its function in the peripheral nervous system has also been studied. TRPV1 is a representative example of a TRP channel family. TRPV1 is a known capsaicin receptor and recognizes external fatal stimuli such as acid and heat [19]. Therefore, TRPV1 is expected to be more involved in Ca^{2+} homeostasis than in other Ca^{2+} receptors.

TRPV1 is very important for body temperature control. The administration of capsaicin, a TRPV1 agonist, in an animal model caused

an increase in the body temperature, so the animal tried to find a cooler place. Conversely, TRPV1 gene deficiency causes abnormalities in body temperature control, and the body temperature increased, showing symptoms of hyperthermia [83]. In addition, TRPV1 is a pain receptor, and many studies have focused on TRPV1 as a target receptor for painkillers. Currently, such painkillers have been developed and clinical trials are ongoing [84].

TRPV1 is expressed not only in the peripheral nervous system but also in the central nervous system, specifically in the hippocampus. Accordingly, studies have investigated the function of TRPV1 in the central nervous system [85]. TRPV1 is distributed throughout the brain through multiple experiments, including electrophysiological, polymerase chain reaction, and immunohistochemical techniques. It is also distributed from the presypnase to postsynapse in nerve cells [86].

Here, I would like to examine the function of TRPV1 in the hippocampus.

In humans, TRPV1 is distributed in CA1, CA3, and DG regions of the hippocampus and is distributed 6–7 times more than that in the spinal cord. Therefore, TRPV1 was expected to perform an important function in

the hippocampus [86]. Several studies have examined brain function by regulating TRPV1 activity. TRPV1 activation increased the severity of depression (concentration-dependent), which was suppressed by capsazepine, a TRPV1 antagonist [87]. Other studies have shown that TRPV1 inhibition increases synaptic plasticity and dendrite development [88].

TRPV1 directly affects other Ca^{2+} receptors. In an animal model of TRPV1 gene deficiency, the function of the NMDA receptor was degraded, and the administration of capsazepine, a TRPV1 antagonist, further degrades NMDA receptors [89]. Therefore, TRPV1 is involved in many brain functions and plays a major role in maintaining Ca^{2+} homeostasis.

However, TRPV1's function in Alzheimer's disease has not yet been revealed. Normal animal models but with TRPV1 gene deficiency showed improvement in memory and cognitive function [90], and a study reported this is caused by synaptic plasticity. However, this does not fully explain TRPV1's function in Alzheimer's disease.

Therefore, TRPV1 gene deficiency was examined in dementia animal models to observe changes in actual Ca^{2+} homeostasis, and thus

reveal the role of TRPV1 in Alzheimer's disease in terms of 1) Aβ accumulation, 2) inhibition of tau hyperphosphorylation, and 3) inhibition of neuronal apoptosis. In addition, I intend to identify practical improvements in memory and cognitive ability to reveal TRPV1 as a new target in the development of AD agents with Ca^{2+} homeostasis control. Furthermore, I would like to confirm the efficacy of AD treatment through α-spinasterol, a TRPV1 antagonist, and confirm its potential as a new AD treatment agent.

A triple-transgenic AD mouse model (3 × Tg-AD) was used to investigate the therapeutic efficacy of TRPV1 function loss in AD. The 3 × Tg-AD mouse model is a gene-mutated animal model in which three genes, namely, presenilin ($PS1_{M146V}$), human amyloid precursor protein (APP_{swe}), and tau (tau_{P301L}), have been introduced, which results in neuronal apoptosis and cognitive and memory impairments caused by Aβ and tau (pathological AD markers) overproduction. To investigate the role of TRPV1 in AD pathogenesis, I created a triple-transgenic AD mouse model (3 × Tg-$AD^{+/+}$) consisting of wild-type ($TRPV1^{+/+}$), knockdown ($TRPV1^{+/-}$), and knockout ($TRPV1^{-/-}$) TRPV1.

TRPV1 deficiency rescues cognitive and memory impairments via behavioral experiments, reduces Aβ and p-tau production, and inhibits neuronal apoptosis in the 3 × Tg-AD mouse model. Moreover, the mechanism underlying these beneficial effects is that TRPV1 deficiency reduces intracellular Ca^{2+} concentration and activates the BDNF/CREB signaling pathway.

1.9.1 TRPV1 deficiency rescues cognition and memory Impairments

To confirm the efficacy of TRPV1 deficiency in memory and cognitive function, behavioral experiments, novel object recognition, contextual fear conditioning tests, and the Morris water maze test were performed. TRPV1 knockdown (3 × Tg-$AD^{+/+}$/ $TRPV1^{+/-}$) and knockout (3 × Tg-$AD^{+/+}$/ $TRPV1^{-/-}$) restored learning in the 3 × Tg-AD mouse model, compared with the wild-type (3 × Tg-$AD^{+/+}$/ $TRPV1^{+/+}$), and the recovery of cognitive function in objects and spaces was confirmed.

1.9.2 TRPV1 deficiency reduces Aβ and p-tau production

TRPV1 deficiency reduces Aβ aggregation and p-tau production in the 3 × Tg-AD mouse model. The APP, polymeric Aβ, oligomeric Aβ,

and p-tau levels were increased in the wild-type (3 × Tg-AD$^{+/+}$/ TRPV1$^{+/+}$), but decreased in knockdown (3 × Tg-AD$^{+/+}$/ TRPV1$^{+/-}$) and knockout (3 × Tg-AD$^{+/+}$/ TRPV1$^{-/-}$) TRPV1. Primary cultured wild-type (3 × Tg-AD$^{+/+}$/ TRPV1$^{+/+}$) neurons were then used to confirm that these effects resulted from decreased intracellular Ca^{2+} concentration caused by TRPV1 deficiency. Aβ aggregation and p-tau expression levels were decreased with the administration of BAPTA (a Ca^{2+} chelator) and capsazepine (a TRPV1 antagonist). The decrease in Aβ aggregation and p-tau production was caused by the inhibition of intracellular Ca^{2+} flux due to TRPV1 deficiency.

1.9.3 TRPV1 deficiency mediates neuroprotective effects *via* the BDNF/CREB signaling pathway

Compared with 3 × Tg-AD, TRPV1 deficiency showed neuroprotective effects on the 3 × Tg-AD mouse model and lowered intracellular Ca^{2+} concentration, which activates the BDNF/CREB signaling pathway. Increased intracellular Ca^{2+} concentration by Aβ activates calpain activity and reduces BDNF levels [78, 91]. In the 3 × Tg-AD mouse model, the intracellular Ca^{2+} concentration was increased,

which inhibited the phosphorylation of CREB, a transcription factor of BDNF, and consequently decreased the expression of BDNF and its receptor, p-TrkB. TRPV1 deficiency normalized the intracellular Ca^{2+} concentration and activated BDNF/TrkB. These effects upregulate the Akt/ERK/CREB signaling pathway. The activated BDNF/CREB signaling pathway inhibits the action of various apoptosis-inducing markers. Accordingly, TRPV1 deficiency decreased intracellular Ca^{2+} concentration compared with 3 × Tg-AD, and the activated BDNF/CREB signaling pathway exhibited neuroprotective effects

1.9.4 α-Spinasterol (TRPV1 antagonist), an AD therapeutic agent

α-Spinasterol is a phytosterol first found in spinach [92]. Among phytosterols in spinach, α-spinasterol is present at a very high rate, 80%, and the LD50 of 479 mg/kg in a mouse model is not toxic [33]. Essentially, when α-spinasterol is administered orally to a mouse model, no damage to nerve cells or other organs was observed. As the development of AD treatments focuses on the prevention stage, the clinical trial period is prolonged, and side effects are a huge problem, depending on the toxicity of the treatment material. Regarding side effects, α-spinasterol is expected

to have great strengths. α-spinasterol is a TRPV1 antagonist that specifically binds to TRPV1 and degrades the function of TRPV1 [30, 31].

α-Spinasterol is a TRPV1 antagonist and appears to regulate various brain functions. It has pharmacological effects on the nervous system, such as anti-hyperalgesia, anti-nociceptive, anti-fibromyalgia, antidepressant, anti-convulsive, and anti-memory loss [93]. α-Spinasterol administered to mice was found to have an analgesic effect [94], antidepressant effect on behavioral experiments, and greater synergy effect when administered simultaneously with capsazepine, a TRPV1 antagonist [33]. In addition, α-spinasterol administered in mouse models showed no side effects such as neuronal apoptosis and movement, muscle strength, and body temperature changes [95]. As the brain is the target organ of AD treatment, treatment agents should reach the brain. Accordingly, the agent must penetrate the BBB to directly affect the brain. According to a pharmacokinetics study, oral administration of α-spinasterol passes through the BBB and reaches the brain [30]. Therefore, α-spinasterol's low toxicity and BBB permeability are very advantageous as an agent for AD treatment. In this study, I would like to confirm the potential of α-spinasterol as a new AD treatment agent.

Previously, I found that genetic TRPV1 deficiency in dementia animal models shows AD treatment efficacy. Thus, I hypothesized that α-spinasterol (TRPV1 antagonist) normalizes Ca^{2+} concentration, which leads to the inhibition of calpain activity, increase in BDNF levels, and inhibition of Aβ and p-tau production, thereby inhibiting neuronal apoptosis and cognitive function recovery. α-Spinasterol modulated Ca^{2+} influx, exhibited neuroprotective effects, and reduced Aβ and p-tau production against $Aβ_{1-42}$ in primary neurons. α-Spinasterol also decreased Aβ and p-tau production and promoted CREB phosphorylation, consequently increasing the expressions of BDNF and its receptor p-TrkB in the hippocampus of $Aβ_{1–42}$-treated mice. In addition, α-spinasterol rescued object and spatial cognitive function and memory deficits in mice. These results indicate that α-spinasterol alleviated AD by modulating Ca^{2+} homeostasis, which is fundamental to neuronal viability and functioning.

1.10 Summary

This review demonstrates that TRPV1 increases intracellular Ca^{2+} concentration, which leads to AD pathology. TRPV1-mediated Ca^{2+} influx impairs memory and cognitive functions and induces Aβ aggregation, p-

tau production, and apoptosis by deactivating the BDNF/CREB signaling pathway, which deactivates proapoptotic cascades. These findings also indicate that TRPV1 deficiency decreases intracellular Ca^{2+} concentration. Accordingly, it rescues memory deficits and reduces Aβ aggregation, p-tau production, and apoptosis by activating the BDNF/CREB signaling pathway in the 3 × Tg-AD mouse model. α-Spinasterol, a TRPV1 antagonist, rescues memory deficits and reduces Aβ aggregation and tau pathology by normalizing Ca^{2+} concentration. Overall, the results support the hypothesis that TRPV1 deficiency may have therapeutic effects on AD pathology. This study confirms TRPV1 as a therapeutic target for AD treatment and the potential of α-spinasterol as a new AD treatment agent (Fig. 1-1).

Figure 1-1

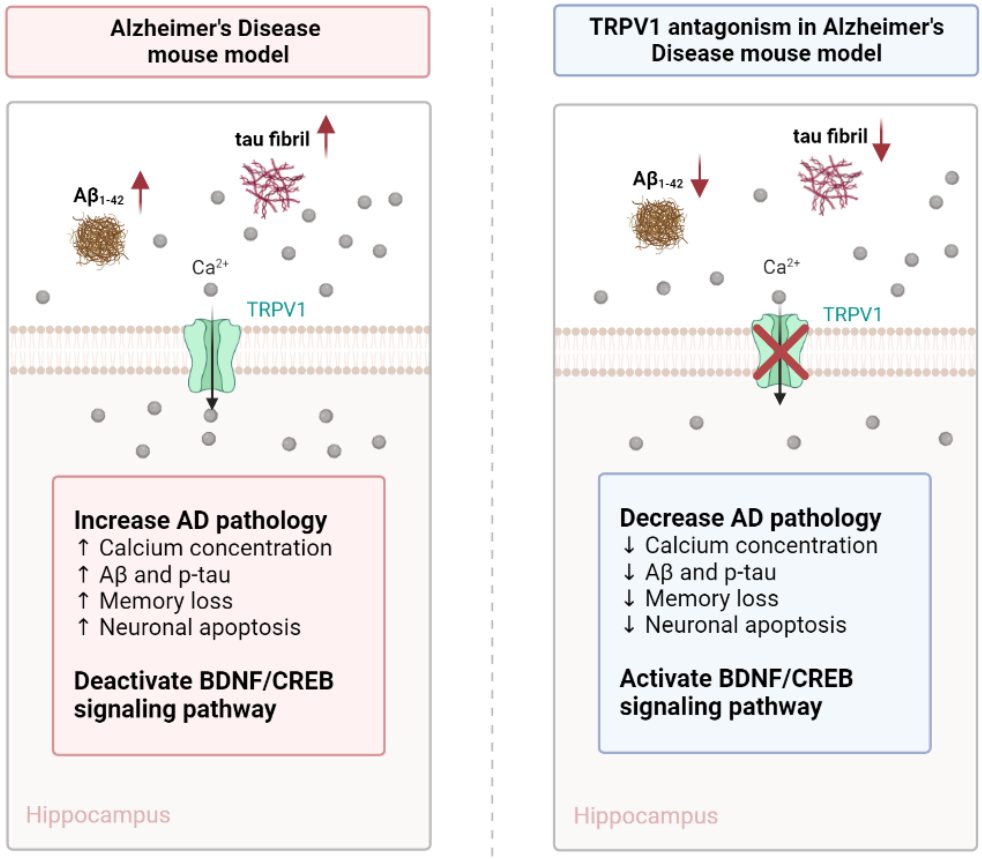

Fig. 1-1. Effects of TRPV1 antagonism in the pathogenesis of Alzheimer's disease. TRPV1 antagonism modulates Ca^{2+} influx, reduces Aβ and p-tau production, and exhibits neuroprotective effects. In addition, TRPV1 antagonism rescues object and spatial cognitive function and memory deficits via activating the BDNF/CREB signaling pathway. (Created with BioRender.com).

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Chapter 2.

Ca^{2+}-permeable TRPV1 pain receptor knockout rescues memory deficits and reduces amyloid-β and tau production in a mouse model of Alzheimer's disease

Abstract

In this chapter, I aimed to determine the therapeutic role of transient receptor potential vanilloid 1 (TRPV1) in Alzheimer's disease (AD) using an animal AD model. First, when TRPV1 is genetically deficient, cognitive behavioral experiments are conducted to determine whether memory and cognitive ability are improved. In addition, studies aimed to clarify whether TRPV1 brings recovery of calcium (Ca^{2+}) homeostasis in the hippocampus and whether TRPV1 alleviates the accumulation of amyloid-beta (Aβ) and tau, a pathological AD phenomenon. When an organism takes capsaicin from red chili peppers, the pain receptor TRPV1 protein provokes hot feeling. This Ca^{2+}-permeable cation channel is mostly expressed in the sensory neurons of the peripheral and central nervous systems (e.g., hippocampus and cortex). Preclinical studies have found that TRPV1 mediates behaviors associated with anxiety and depression. The functional loss of TRPV1 upregulates expression of genes involved in synaptic plasticity and neurogenesis. Thus, I hypothesized that TRPV1 deficiency may modulate AD. A triple-transgenic AD mouse model (3 × Tg-$AD^{+/+}$) with wild-type ($TRPV1^{+/+}$), hetero ($TRPV1^{+/-}$), and knockout ($TRPV1^{-/-}$) TRPV1 was generated to

investigate the role of TRPV1 in AD pathogenesis. The animals' memory function, hippocampal Ca^{2+} levels, and Aβ (Aβ and tau pathologies at 12 months old) were analyzed. Compared with 3 × Tg-$AD^{-/-}$/$TRPV1^{+/+}$ mice, 3 × Tg-$AD^{+/+}$/$TRPV1^{+/+}$ mice had memory impairment and increased levels of hippocampal Ca^{2+}, Aβ, and total and phosphorylated tau. However, 3 × Tg-$AD^{+/+}$/$TRPV1^{-/-}$ mice had better memory function and lower levels of hippocampal Ca^{2+}, Aβ, tau, and p-tau than 3 × Tg-$AD^{+/+}$/$TRPV1^{+/+}$ mice. The intracellular Ca^{2+} chelator BAPTA/AM and TRPV1 antagonist (capsazepine) were found to reduce the production of Aβ, tau, and p-tau in 3 × Tg-AD-derived primary neuronal cells. Taken together, these results suggested that TRPV1 deficiency promoted resistance to memory loss and had anti-AD effects. These findings imply that medications or food ingredients that modulate TRPV1 may be used therapeutically to prevent or treat AD.

2.1 Introduction

Transient receptor potential vanilloid 1 (TRPV1) is a calcium (Ca^{2+})-permeable nonselective cation channel and the receptor for capsaicin, the hot compound in chili peppers that induces searing pain [1]. TRPV1 is a molecular integrator of chemical and physical inputs that generate pain, and it is involved in thermogenesis and pain perception of noxious stimuli, such as heat and acid [1, 2]. The development of neuropathic pain and hyperalgesia is mediated by TRPV1, and some pain disorders are influenced by endogenous agonists that act on TRPV1 [3-5]. Thus, TRPV1 is a molecular target of pain control, and drugs that inhibit TRPV1 have been developed and sold as analgesics. According to study findings, TRPV1 is engaged in functions other than pain, and TRPV1 antagonists have a potential for use in the treatment of conditions like chronic cough, urge incontinence, and irritable bowel syndrome [5].

In addition to the peripheral nervous system, TRPV1 is found in parts of the brain (such as the cortex, hippocampus, cerebellum, mesencephalon, olfactory bulb, and hindbrain) where it may affect neuronal activity [6, 7]. TRPV1 has the capacity to influence symptoms of anxiety, depression, and schizophrenia [8-10]. The functional loss of

TRPV1 upregulates the expression of genes related to neurogenesis and synaptic plasticity [11]. Worm lifespan is limited by TRPV1 activation via Ca^{2+}-dependent activation of the calcineurin/CRTC1/CREB signaling pathway, but the disruption of a TRPV1 pathway promotes longevity [12, 13]. Ca^{2+} homeostasis control in neurons is impaired during aging, which leads to age-related hippocampal dysfunction and might increase aging-associated cognitive decline [12, 14, 15].

I hypothesized that TRPV1 deficiency modulates Ca^{2+} levels, hippocampal pathology, and cognitive decline in Alzheimer's disease (AD). To investigate the role of TRPV1 in AD pathogenesis, I generated a triple-transgenic AD mouse model (3 × Tg-$AD^{+/+}$) consisting of wild-type ($TRPV1^{+/+}$), hetero ($TRPV1^{+/-}$), and knockout ($TRPV1^{-/-}$) TRPV1 animals. The 3 × Tg-AD mice, created by introducing mutant forms of mouse presenilin ($PS1_{M146V}$) and human amyloid precursor protein (APP_{swe}) and tau (tau_{P301L}), gradually develop Aβ plaques and tau tangles. Long-term potentiation losses with aging are caused by synaptic dysfunction [16]. The generated 3 × Tg-$AD^{+/+}$ with $TRPV1^{+/+}$, $TRPV1^{+/-}$, and $TRPV1^{-/-}$ were analyzed and compared for the genotypes' memory function, hippocampal Ca^{2+} levels, and Aβ and tau pathologies. Additional

studies investigated the effects of the intracellular Ca^{2+} chelator BAPTA/AM and the TRPV1 antagonist capsazepine on the production of Aβ, tau, and p-tau.

2.2 Materials and Methods

2.2.1 Transgenic animal models

3×Tg-AD mice (kindly given by Professor Frank M. Laferla, UC-Irvine, Irvine, CA, USA) [16] and $TRPV1^{-/-}$ mice (Jackson Laboratories, Bar Harbor, ME, USA, stock #003770) were mated and used to examine the effect of TRPV1 on the development of AD. 3×Tg-AD mice are homozygous for APP_{swe}, tau_{P301L}, and $PS1_{M146V}$ ($3\times Tg\text{-}AD^{+/+}$). $3\times Tg\text{-}AD^{+/-}/TRPV1^{+/-}$ offspring were generated from a cross of $3\times Tg\text{-}AD^{+/+}$ and $TRPV1^{-/-}$ animals. The resulting $3\times Tg\text{-}AD^{+/-}/TRPV1^{+/-}$ offspring were crossed and nine genotypes of mice were generated (i.e., $3\times Tg\text{-}AD^{-/-}/TRPV1^{+/+}$, $3\times Tg\text{-}AD^{-/-}/TRPV1^{+/-}$, $3\times Tg\text{-}AD^{-/-}/TRPV1^{-/-}$, $3\times Tg\text{-}AD^{+/-}/TRPV1^{+/+}$, $3\times Tg\text{-}AD^{+/-}/TRPV1^{+/-}$, $3\times Tg\text{-}AD^{+/-}/TRPV1^{-/-}$, $3\times Tg\text{-}AD^{+/+}/TRPV1^{+/+}$, $3\times Tg\text{-}AD^{+/+}/TRPV1^{+/-}$, $3\times Tg\text{-}AD^{+/+}/TRPV1^{-/-}$). The $3\times Tg\text{-}AD^{-/-}/TRPV1^{+/+}$ mice were crossed to generate $3\times Tg\text{-}AD^{-/-}/TRPV1^{+/+}$ mice (wild-type). The $3\times Tg\text{-}AD^{+/+}/TRPV1^{+/+}$ mice were

crossed to generate 3×Tg-AD$^{+/+}$/TRPV1$^{+/+}$ mice (3×Tg-AD/ TRPV1$^{+/+}$). 3×Tg-AD$^{+/+}$/TRPV1$^{-/-}$ mice were crossed to generate 3×Tg-AD$^{+/+}$/TRPV1$^{-/-}$ mice (3×Tg-AD/ TRPV1$^{-/-}$). 3×Tg-AD$^{+/+}$/TRPV1$^{+/-}$ mice (3×Tg-AD/TRPV1$^{+/-}$) were generated by crossing 3×Tg-AD$^{+/+}$/TRPV1$^{+/+}$ mice with 3×Tg-AD$^{+/+}$/TRPV1$^{-/-}$ mice. Four groups each of wild-type, 3×Tg-AD/TRPV1$^{+/+}$, 3×Tg-AD/TRPV1$^{+/-}$, and 3×Tg-AD/TRPV1$^{-/-}$ animals were matched by age and sex. The mouse models (12 months of age) were kept at 21 ± 2 ℃, 12 h light: 12 h dark photoperiod (light period beginning at 0800 h) specific pathogen-free conditions in ventilated cages. Mice were provided with free choice to consume food and water. All animal testing was performed following protocols approval by the Institutional Animal Care and Use Committee of Seoul National University (SNU-140410-12, SNU-140410-14, SNU-140625-2).

2.2.2 Primary neuronal culture

Cerebral cortical tissue was dissected from day 15 embryonic 3×Tg-AD and non-transgenic mice, as previously described [49]. The Institutional Animal Care and Use Committee of Seoul National University (SNU-150625-1-6) approved the acquisition of the cortical

neurons. BAPTA/AM (Thermo Scientific, Waltham, MA, USA) and capsazepine (Selleckchem, Houston, TX, USA) were dissolved in DMSO (Sigma, St. Louis, MO, USA). Cultures at 6 days *in vitro* were treated with BAPTA/AM or capsazepine for 4 days, and the neurons were harvested for Western blot analysis to evaluate APP/Aβ, tau, and p-tau levels.

2.2.3 Novel object recognition test

I used a modified novel object recognition test. This test incorporates the mouse's natural tendency to explore novel stimuli [50]. During a habituation session performed 2 days before testing, the mice were allowed to explore (for 10 min) a test environment consisting of an empty opaque, custom-made Plexiglas box (35 cm × 45 cm × 25 cm). The sample object phase was performed 24 h later. Two identical white circular cylinders (the sample objects) were placed on the facing edge in the test environment, and the mice were given access to the objects for 10 min. After 24 h (the novel object phase), one of the sample objects in the test environment was replaced with a similar-size novel object (a colored miniature animal), and the mice were given 5 min of contact with this new arrangement. The amount of time that a mouse's nose was <1 cm from an

object was considered as the time that the mouse navigated the object. The amount of time that the mouse stood on the object was excluded. The discrimination ratio was the time used to navigate the novel object over the time used to navigate both objects.

2.2.4 Contextual fear conditioning test

During the contextual fear conditioning test, each mouse was exposed to three sessions: (1) habituation (adaptation to the training chamber), (2) training (exposure to foot shocks), and (3) testing (response to a context). The hippocampus-based contextual fear conditioning test was modified [19] and freezing behaviors were recorded. The freezing time was the time spent freezing over the total time. On day 1 of habituation, each mouse was kept in the chamber for 12 min without exposure to a foot shock. On the training day, each mouse was returned to the same chamber and allowed to adapt for 4 min. After 4 min, the mouse received a 2-s, 0.5 mA foot shock (100 s each trial, consisting of a 58 s baseline period, 2-s, 0.5 mA foot-shock period, and a 40 s post-shock period) for four trials. On the testing day, each mouse was returned to the training chamber and allowed to move freely for 4.5 min. Freezing

behavior was recorded and used as an indicator of fear memory.

2.2.5 Morris water maze test

A modification of the water maze procedure described by Morris was used to examine cognitive function [22]. A circular tank (diameter 90 cm, height 50 cm; 22 ± 2 ℃ water temperature) was used for the test. The tank consisted of four quadrants filled with water. An escape platform (6 cm diameter and 29 cm height) was submerged 1 cm below the water surface at the center of one of the four quadrants. Each mouse was trained for 4 days to learn and memorize visual cues placed outside the tank, which indicated the platform location. The swimming paths used by each mouse were recorded with a camera connected to a video recorder and a path tracking device SMART VIDEO TRACKING Software (Panlab, Barcelona, Spain). Four trials were performed each day during the 4-day training period. During each trial, each mouse was allowed 60 s to find the hidden platform and another 30 s to stay on the platform. If the mouse was unable to find the platform within 60 s, it was guided to it and allowed to remain on it for 30 s. The mean time (mean escape latency) that each mouse took to find the platform was recorded.

2.2.6 Fluorescence Ca^{2+} imaging

A microtome was used to take sections of the frozen brains of the mice. Coronal sections containing the hippocampus (anterior-posterior relative to bregma: 3.12–4.56 mm) were prepared to evaluate Ca^{2+}. The sections were placed onto silane-coated glass. They were then washed using PBS and loaded with 10 μM Ca^{2+} indicator fluo-4 AM (Thermo Scientific, Waltham, MA, USA) for 24 h. The stained sections were cleaned with PBS, cover-slipped with Prolong Gold Antifide Reagent containing DAPI nuclear stain (Invitrogen, Carlsbad, CA, USA), and examined using a confocal microscope (Carl Zeiss, Oberkochen, Germany). The fluo-4 AM fluorescence signal intensity was measured using the image J analysis program (National Institute of Health, Bethesda, MA, USA). Regions of interest that were 103,629 μm^2 and 62,177 μm^2 were selected from the hippocampus DG and CA1 areas, respectively.

2.2.7 Immunohistochemistry

A microtome was used to take sections from the frozen brains of the mice for immunohistochemistry assay. Coronal sections containing the hippocampus (anterior-posterior relative to bregma: 3.12–4.56 mm) were

prepared to evaluate APP/Aβ, tau, and p-tau. The sections were placed onto silane-coated glass and blocked for 1 h using 10% fetal horse serum (Thermo Scientific, Waltham, MA, USA) with 3% Triton X-100 in PBS. The sections were then incubated in primary antibodies, anti-APP/Aβ (6E10) antibody (Biolegend, San Diego, CA, USA; 1:1000), anti-tau (HT7) antibody (Thermo; 1:500), and anti-p-tau (AT8) antibody (Thermo; 1:200) for 24 h at 4 ℃. The sections were then washed and incubated in secondary antibodies, Alexa 488-conjugated donkey anti-mouse antibody for APP/Aβ (6E10) (Biolegend; 1:200), and Alexa 488-conjugated donkey anti-mouse antibody for tau (HT7) and p-tau (AT8) (Cell Signaling Technology, Danvers, MA, USA; 1:200). PBS was used to clean the stained sections. They were then cover-slipped with Prolong Gold Antifide Reagent containing DAPI nuclear stain and examined using a confocal microscope (Carl Zeiss). Fluorescence signal intensity was measured using the image J analysis program (National Institute of Health). Regions of interest that were 103,629 μm^2 and 62,177 μm^2 were selected from the hippocampus DG and CA1 areas, respectively.

2.2.8 Western blot analysis

Tissue lysates were prepared by homogenizing the tissues in radioimmunoprecipitation assay buffer (Cell Signaling). Tissue protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Western blot analyses were performed on 80–120 μg protein. Briefly, samples were separated using 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred to a polyvinylidene fluoride membrane (Merck Millipore, Burlington, MA, USA; 0.4 μm). The membranes were blocked in 5% bovine serum albumin (Bovogen Biologicals, Keilor East, Australia) in tris-buffered saline and Tween-20 (Junsei Chemical, Tokyo, Japan) and incubated overnight at 4°C with primary antibodies against APP/Aβ (6E10, Biolegend; 1:1000), tau (HT7) (Thermo; 1:1000), tau (46) (Cell Signaling; 1:1000), p-tau (s396) (Cell Signaling; 1:1000), p-tau (s202) (Cell Signaling; 1:1000), and β-actin (Sigma; 1:5000). After incubation with horseradish peroxidase-conjugated secondary antibodies (Sigma; 1:5000) for 1 h at room temperature, immunodetection was performed using an enhanced chemiluminescence detection kit (GE Healthcare, Chicago, IL, USA). Rosen RF et al.'s protocol was used for the detection of Aβ

multimers [51].

2.2.9 Statistical analysis

SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. The results were presented as mean ± standard error of the mean (SEM) values. The Morris water maze data were analyzed using two-way repeated measures ANOVA, followed by Tukey's test. Other data were analyzed using one-way ANOVA followed by Tukey's test.

2.3 Results

2.3.1 TRPV1 deficiency improved memory and learning deficits

Hippocampus-dependent novel object recognition, contextual fear conditioning, and Morris water maze tests were performed in 3 × Tg-AD mice to evaluate the role of TRPV1 in hippocampus-associated learning and memory deficits (Fig. 2-1). Rodents naturally prefer unfamiliar objects to those they are familiar with, and the capacity to distinguish between unfamiliar and familiar objects is a measure of recognition memory [17]. Fig. 2-1A presents the experimental schedule used in the

novel object recognition test. The novel object part of the novel object recognition test revealed that 3 × Tg-AD$^{+/+}$/TRPV1$^{+/+}$ animals spent less time investigating the novel object than wild-type 3 × Tg-AD$^{-/-}$/TRPV1$^{+/+}$ animals. In comparison to 3 × Tg-AD animals expressing TRPV1$^{+/+}$, the TRPV1-deficient 3 × Tg-AD animals required more time to investigate the novel object and had discrimination ratios that were much higher [$F(3,22) = 9.169$, $p < 0.01$] (Fig. 2-1B). According to this finding, recognition memory was impaired in 3 × Tg-AD$^{+/+}$/TRPV1$^{+/+}$ animals compared to wild-type 3 × Tg-AD$^{-/-}$/TRPV1$^{+/+}$ animals. TRPV1-deficient 3 × Tg-AD animals had less impairment than the 3 × Tg-AD animals expressing TRPV1$^{+/+}$.

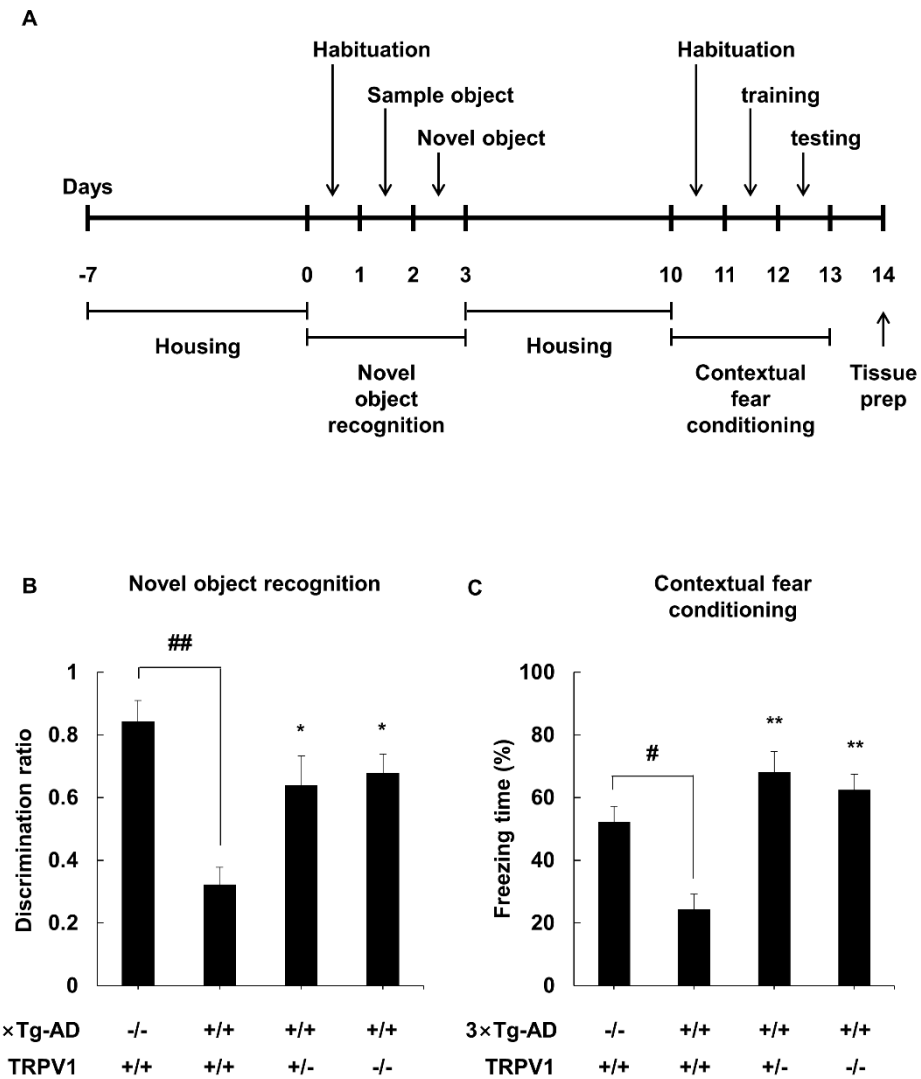
Hippocampal integrity affects the results the contextual fear conditioning test [18-20]. Fig. 2-1A displays the experimental timetable for the contextual fear conditioning test. Even in the absence of the aversive stimulus, animals that have learned to predict a negative outcome by associating a neutral context, such as a test chamber, with an unpleasant stimulus, such as an electrical shock, will react to that neutral context with a fear response, such as freezing behavior [21]，[18, 20]. The contextual fear conditioning test revealed that 3 × Tg-AD$^{+/+}$/TRPV1$^{+/+}$

animals had significantly lower freezing responses than wild-type 3 × Tg-AD$^{-/-}$/TRPV1$^{+/+}$ animals. Significantly more freezing behaviors were observed in TRPV1-deficient mice than in TRPV1-expressing animals [$F(3,22) = 13.448$, $p < 0.01$] (Fig. 2-1C). This result suggested that 3 × Tg-AD$^{+/+}$/TRPV1$^{+/+}$ animals were not as effective in learning to predict an aversive event compared with wild-type 3 × Tg-AD$^{-/-}$/TRPV1$^{+/+}$ animals. The TRPV1-deficient animals learned to anticipate an unpleasant experience more effectively than the animals expressing TRPV1$^{+/+}$.

The Morris water maze test's experimental schedule, which was used to evaluate spatial learning capacity, is shown in Fig. 2-1D [22]. The assumption of sphericity in the two-way repeated measures analysis of variance was not met ($p = 0.009$). Therefore, the degrees of freedom were adjusted using the Greenhouse-Geisser method. The analysis of the mean time used to locate the escape platform (i.e., escape latency time during the Morris water maze test) revealed statistically significant between-group effects (wild-type 3 × Tg-AD$^{-/-}$/TRPV1$^{+/+}$, 3 × Tg-AD$^{+/+}$/TRPV1$^{+/+}$, 3 × Tg-AD$^{+/+}$/TRPV1$^{+/-}$, 3 × Tg-AD$^{+/+}$/TRPV1$^{-/-}$) ($F(3,36) = 9.065$, $p < 0.001$) and training session effects ($F(2.38, 85.64) = 66.481$, $p < 0.001$) (Fig. 2-1E). A statistically significant interaction was also found between

genotype and training condition ($F(7.14,85.64) = 208.773$, $p = 0.022$). The wild-type 3 × Tg-AD$^{-/-}$/TRPV1$^{+/+}$ animals learned to identify the submerged platform more quickly throughout training sessions than 3 × Tg-AD$^{+/+}$/TRPV1$^{+/+}$ animals, which showed less improvement during training (Fig. 2-1E). According to the post hoc analysis's findings, wild-type 3 × Tg-AD$^{-/-}$/TRPV1$^{+/+}$ animals had a significantly lower mean escape delay time than 3 × Tg-AD$^{+/+}$/TRPV1$^{+/+}$ animals ($p < 0.01$). However, in comparison to 3 × Tg-AD animals expressing TRPV1$^{+/+}$, TRPV1-deficient 3 × Tg-AD animals (3 × Tg-AD$^{+/+}$/TRPV1$^{+/-}$ and 3 × Tg-AD$^{+/+}$/TRPV1$^{-/-}$) performed significantly better ($p = 0.019$ and $p = 0.003$, respectively) (Fig. 2-1E).

Figure 2-1


A
Habituation
Sample object
Novel object
Habituation
training
testing
Days
-7
0
1
2
3
10
11
12
13
14
Housing
Housing
Novel object recognition
Contextual fear conditioning
Tissue prep
B
Novel object recognition
##
Discrimination ratio
1
0.8
0.6
0.4
0.2
0
*
*
3×Tg-AD -/- +/+ +/+ +/+
TRPV1 +/+ +/+ +/- -/-
C
Contextual fear conditioning
Freezing time (%)
100
80
60
40
20
0
#
**
**
3×Tg-AD -/- +/+ +/+ +/+
TRPV1 +/+ +/+ +/- -/-

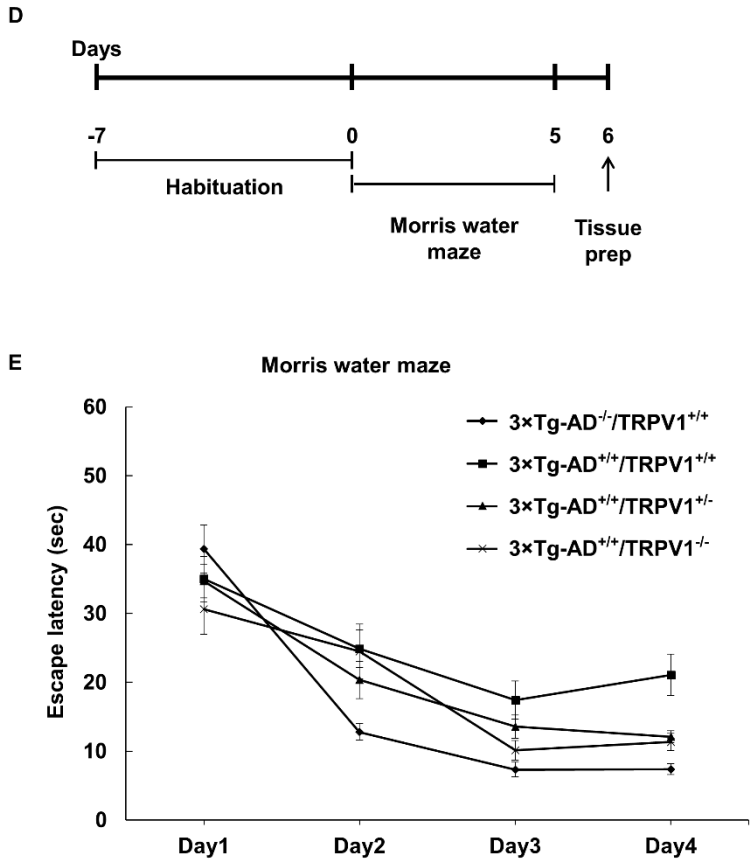


Fig. 2-1. Effects of TRPV1 deficiency on learning and memory in 3×Tg-AD mice. (**A**) Schedule used for novel object recognition and contextual fear conditioning behavioral testing. (**B**) Discrimination ratio results for novel object recognition test. (**C**) Freezing time (%) results for contextual fear conditioning test. (Mean ± SEM, #$p < 0.05$ and ##$p < 0.01$ vs. 3×Tg-AD$^{-/-}$/TRPV1$^{+/+}$, *$p < 0.05$ and **$p < 0.01$ vs. 3×Tg-AD$^{+/+}$/TRPV1$^{+/+}$, one-way ANOVA followed by Tukey's post hoc test; n = 5-6). (**D**) Experimental

timeline for Morris water maze behavioral testing. (**E**) Mean escape latency results for Morris water maze test. (Mean ± SEM, two-way repeated measures ANOVA, followed by Tukey's post hoc test; $n = 10$).

2.3.2 TRPV1 deficiency decreased the hippocampal levels of Ca^{2+}

To investigate the impact of TRPV1 deficiency on hippocampal Ca^{2+} levels, I used fluorescence Ca^{2+} imaging. Hippocampus sections were stained with Fluo-4AM, which indicated the presence of Ca^{2+}. Compared with 3 × Tg-$AD^{-/-}$/$TRPV1^{+/+}$ animals, Fluo-4AM-positive Ca^{2+} levels were higher in the hippocampal CA1 region (especially the soma of pyramidal neurons) and dentate gyrus (DG) (especially the dendrites of granule neurons) of 3 × Tg-$AD^{+/+}$/$TRPV1^{+/+}$ animals. Ca^{2+} levels increased in the hippocampal CA1 and DG of 3 × Tg-$AD^{+/+}$/$TRPV1^{+/+}$ animals, which were significantly decreased by TRPV1 deficiency in 3 × Tg-$AD^{+/+}$/$TRPV1^{+/-}$ and 3 × Tg-$AD^{+/+}$/$TRPV1^{-/-}$ animals (Fig. 2-2A–C). A strong DNA-binding fluorescent stain, 4′,6-diamidino-2-phenylindole (DAPI), was used to identify cell nuclei in the CA1 region and DG of the hippocampus.

Figure 2-2

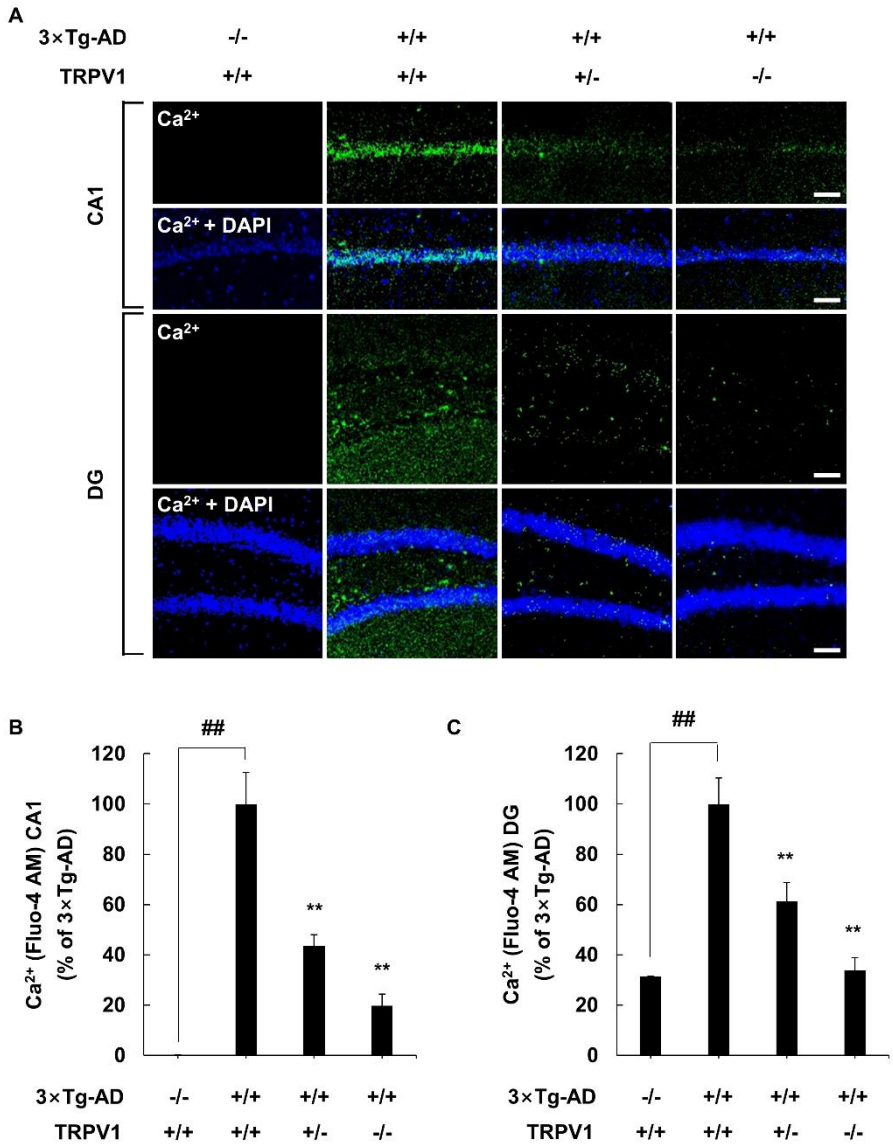


Fig. 2-2. Effects of TRPV1 deficiency on hippocampal Ca^{2+} levels in 3×Tg-AD mice. (**A**) Representative fluorescence confocal images of Fluo-4 AM-positive Ca^{2+} in the hippocampal CA1 and DG subregions. Nuclei stained

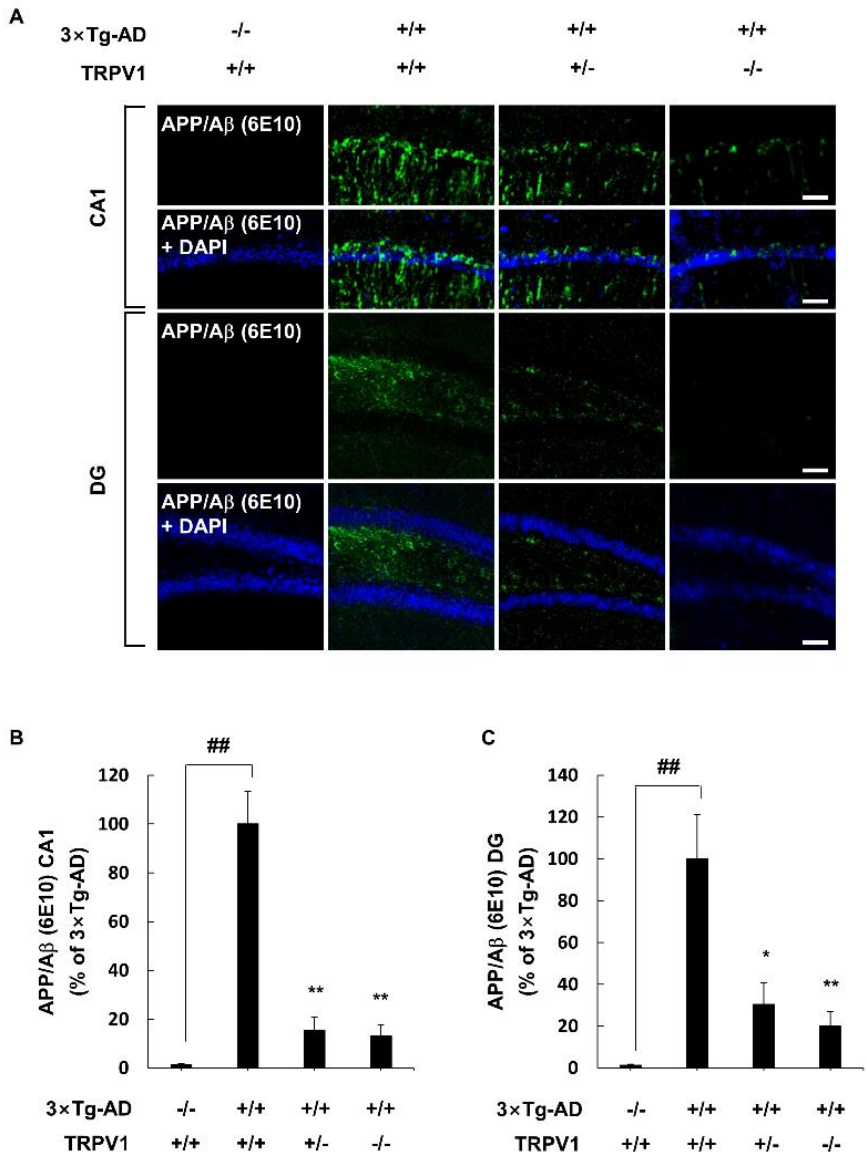
using DAPI. Scale bar = 50 μm. (**B-C**) Fluorescence Ca^{2+} intensities in the hippocampal (**B**) CA1 and (**C**) DG subregions (mean ± SEM, ##$p < 0.01$ vs. 3×Tg-AD$^{-/-}$/TRPV1$^{+/+}$, **$p < 0.01$ vs. 3×Tg-AD$^{+/+}$/TRPV1$^{+/+}$, one-way ANOVA followed by Tukey's post hoc test; $n = 4$ per group). CA, cornu ammonis; DG, dentate gyrus.

2.3.3 TRPV1 deficiency decreased hippocampal APP/Aβ levels

Immunohistochemistry analysis was conducted to measure the effect of TRPV1 deficiency on hippocampal APP/Aβ levels in 3 × Tg-AD animals. The levels of 6E10-positive APP/Aβ in the pyramidal neurons of the CA1 region and dendrites of granule neurons of the DG were higher in 3 × Tg-AD$^{+/+}$/TRPV1$^{+/+}$ animals than in 3 × Tg-AD$^{-/-}$/TRPV1$^{+/+}$ animals. They were significantly decreased by TRPV1 deficiency in 3 × Tg-AD$^{+/+}$/TRPV1$^{+/-}$ and 3 × Tg-AD$^{+/+}$/TRPV1$^{-/-}$ animals (Fig. 2-3A–C). DAPI was used to identify cell nuclei in the CA1 region and DG.

I also used Western blot analysis with Aβ (6E10) antibody to examine the effect of TRPV1 deficiency on hippocampal APP and Aβ production in 3 × Tg-AD animals. The levels of oligomeric Aβ were significantly greater in 3 × Tg-AD$^{+/+}$/TRPV1$^{+/+}$ animals than in 3 × Tg-AD$^{-/-}$/TRPV1$^{+/+}$ animals. TRPV1 deficiency significantly decreased the levels of oligomeric Aβ in 3 × Tg-AD$^{+/+}$/TRPV1$^{+/-}$ and 3 × Tg-AD$^{+/+}$/TRPV1$^{-/-}$ animals (Fig. 2-3D and E). Compared with 3 × Tg-AD$^{-/-}$/TRPV1$^{+/+}$ animals, hippocampal levels of polymeric Aβ and APP were not increased in 3 × Tg-AD$^{+/+}$/TRPV1$^{+/+}$ animals (Fig. 2-3D, F, and G).

Figure 2-3


A
3×Tg-AD -/- +/+ +/+ +/+
TRPV1 +/+ +/+ +/- -/-
CA1
DG
APP/Aβ (6E10)
APP/Aβ (6E10) + DAPI
B
APP/Aβ (6E10) CA1 (% of 3×Tg-AD)
##
**
C
APP/Aβ (6E10) DG (% of 3×Tg-AD)
*

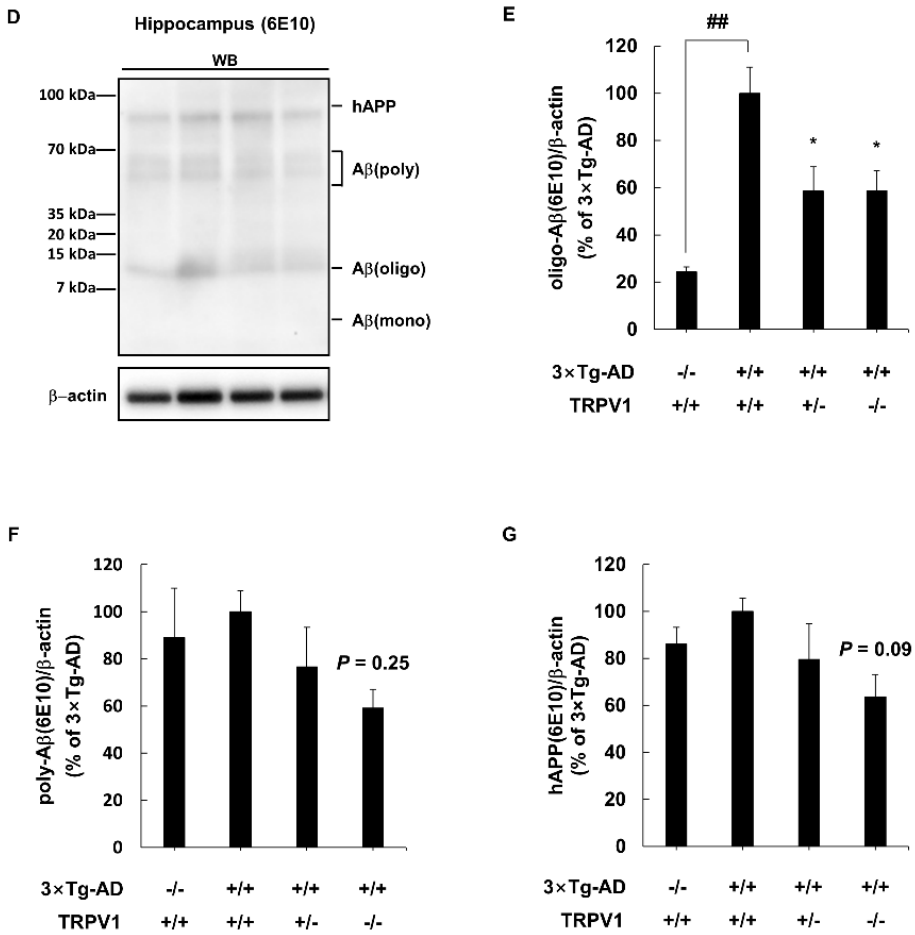


Fig. 2-3. Effects of TRPV1 deficiency on hippocampal levels of APP and Aβ in 3×Tg-AD mice. (**A**) Representative immunohistochemical confocal images of 6E10-positive APP/Aβ protein in the hippocampal CA1 and DG subregions. Nuclei stained using DAPI. Scale bar = 50 μm. (**B-C**) Immunohistochemical signal intensities of 6E10-positive APP/Aβ protein in the hippocampal (**B**) CA1 and (**C**) DG subregions. (**D**) Representative Western blots (n = 1 per lane) of 6E10-positive APP/Aβ proteins. (**E-G**)

Western blot densitometry results for 6E10-positive (**E**) oligomeric Aβ, (**F**) polymeric Aβ, and (**G**) human APP in the hippocampus (mean ± SEM, ##$p < 0.01$ vs. 3×Tg-AD$^{-/-}$/TRPV1$^{+/+}$, *$p < 0.05$ and **$p < 0.01$ vs. 3×Tg-AD$^{+/+}$/TRPV1$^{+/+}$, one-way ANOVA followed by Tukey's post hoc test; $n = 4$ per group). CA, cornu ammonis; DG, dentate gyrus.

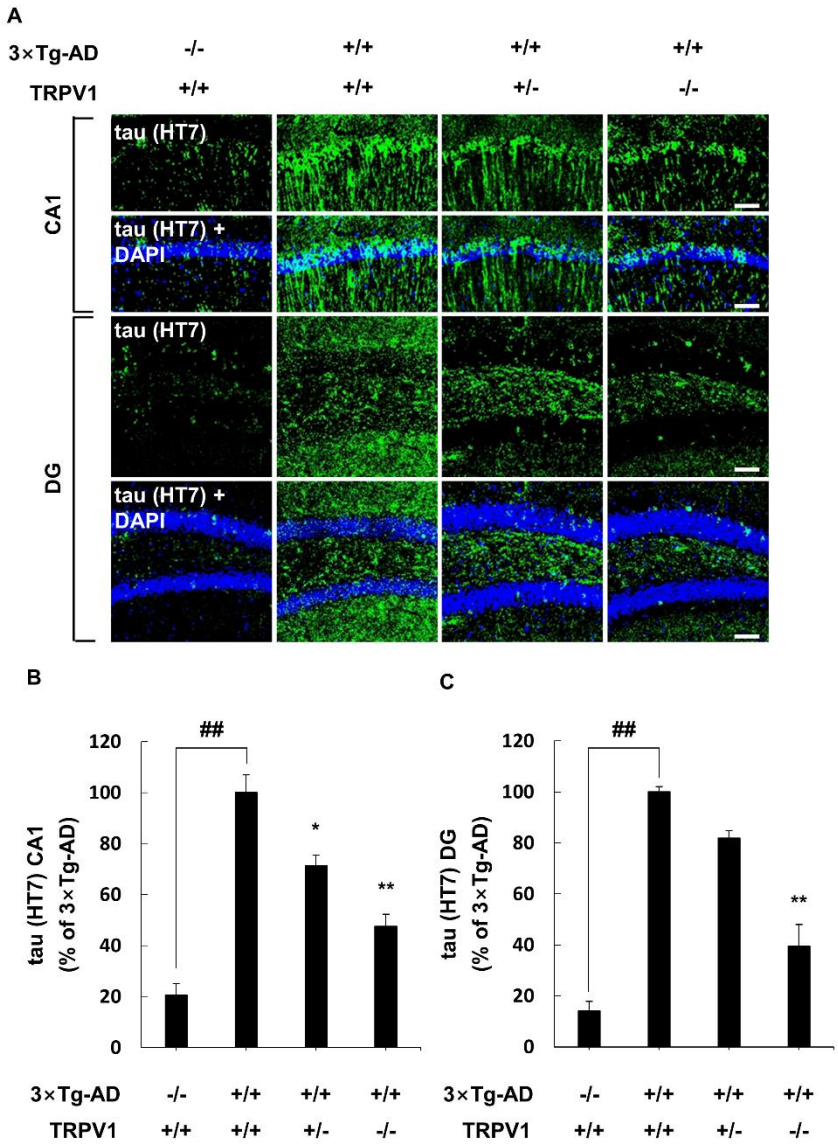
2.3.4 TRPV1 deficiency decreased hippocampal tau and p-tau levels

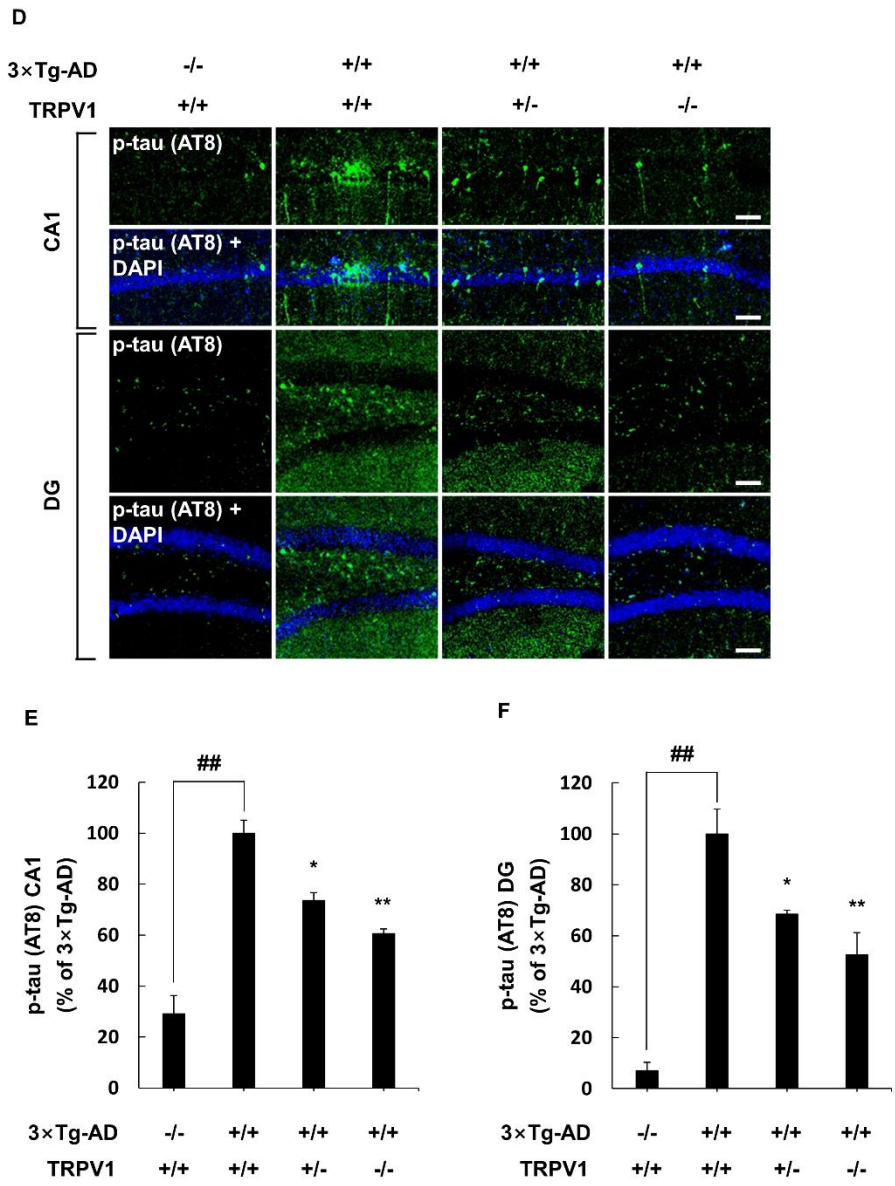
I employed an immunohistochemistry assay using a human-specific monoclonal antibody against tau, HT7, to assess the impact of TRPV1 deficiency on hippocampus tau and p-tau levels in 3 × Tg-AD$^{+/+}$/TRPV1$^{+/+}$ mice. In CA1 and DG regions of the stained sections of the hippocampus, HT7-positive tau levels were increased in 3 × Tg-AD$^{+/+}$/TRPV1$^{+/+}$ animals compared with those in 3 × Tg-AD$^{-/-}$/TRPV1$^{+/+}$ animals; the levels were significantly decreased by TRPV1 deficiency (Fig. 2-4A–C). Hippocampus sections were also stained with AT8, a human-specific p-tau antibody. In CA1 and DG regions, AT8-positive p-tau levels were increased in 3 × Tg-AD$^{+/+}$/TRPV1$^{+/+}$ mice compared with those in 3 × Tg-AD$^{-/-}$/TRPV1$^{+/+}$ mice. The levels were significantly decreased by TRPV1 deficiency in 3 × Tg-AD$^{+/+}$/TRPV1$^{+/-}$ and 3 × Tg-AD$^{+/+}$/TRPV1$^{-/-}$ animals (Fig. 2-4D–F). DAPI was used to identify cell nuclei in CA1 and DG regions.

I also examined the levels of tau and p-tau in TRPV1-deficient animals using Western blot analysis. Levels of tau (HT7 and tau 46) and p-tau (s396 and s202) in 3 × Tg-AD mice were significantly higher than in those in non-transgenic mice; TRPV1 deficiency decreased tau (HT7 and

tau 46) and p-tau (s396 and s202) levels (Fig. 2-4G–K).

Figure 2-4


A
3×Tg-AD
TRPV1
-/-
+/+
+/+
+/+
+/+
+/+
+/-
-/-
CA1
DG
tau (HT7)
tau (HT7) + DAPI
B
C
tau (HT7) CA1 (% of 3×Tg-AD)
tau (HT7) DG (% of 3×Tg-AD)
##
*
**
0
20
40
60
80
100
120


D
3×Tg-AD -/- +/+ +/+ +/+
TRPV1 +/+ +/+ +/- -/-
CA1
DG
p-tau (AT8)
p-tau (AT8) + DAPI
E
F
p-tau (AT8) CA1 (% of 3×Tg-AD)
p-tau (AT8) DG (% of 3×Tg-AD)
##
*
**
0
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40
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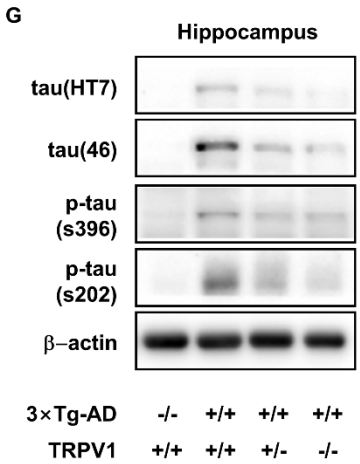
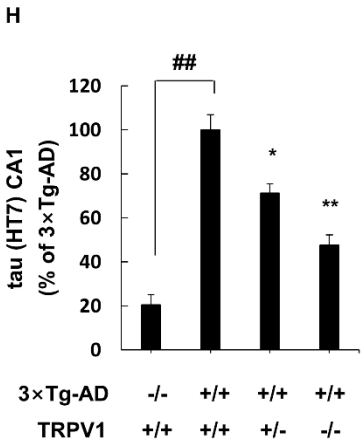
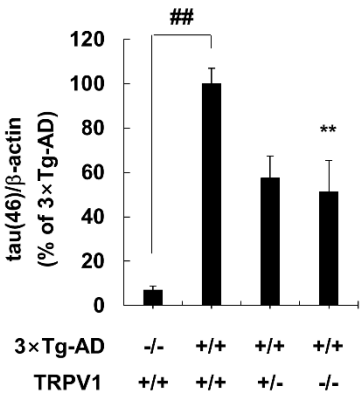
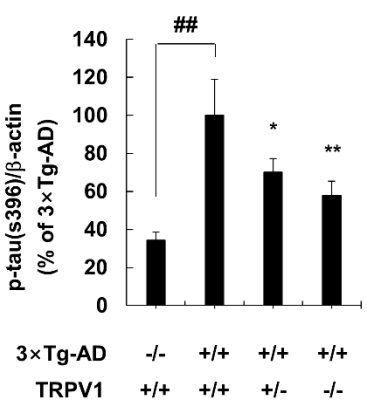
80
100
120


G
Hippocampus
tau(HT7)
tau(46)
p-tau (s396)
p-tau (s202)
β–actin
3×Tg-AD -/- +/+ +/+ +/+
TRPV1 +/+ +/+ +/- -/-


H
tau (HT7) CA1 (% of 3×Tg-AD)
##
*
**
3×Tg-AD -/- +/+ +/+ +/+
TRPV1 +/+ +/+ +/- -/-


I
tau(46)/β-actin (% of 3×Tg-AD)
##
**
3×Tg-AD -/- +/+ +/+ +/+
TRPV1 +/+ +/+ +/- -/-


J
p-tau(s396)/β-actin (% of 3×Tg-AD)
##
*
**
3×Tg-AD -/- +/+ +/+ +/+
TRPV1 +/+ +/+ +/- -/-

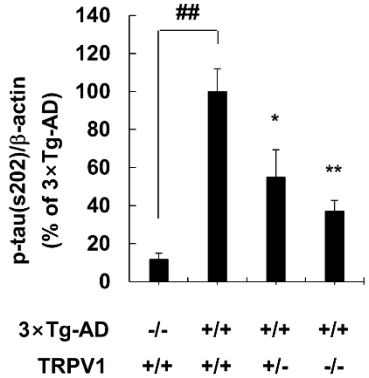
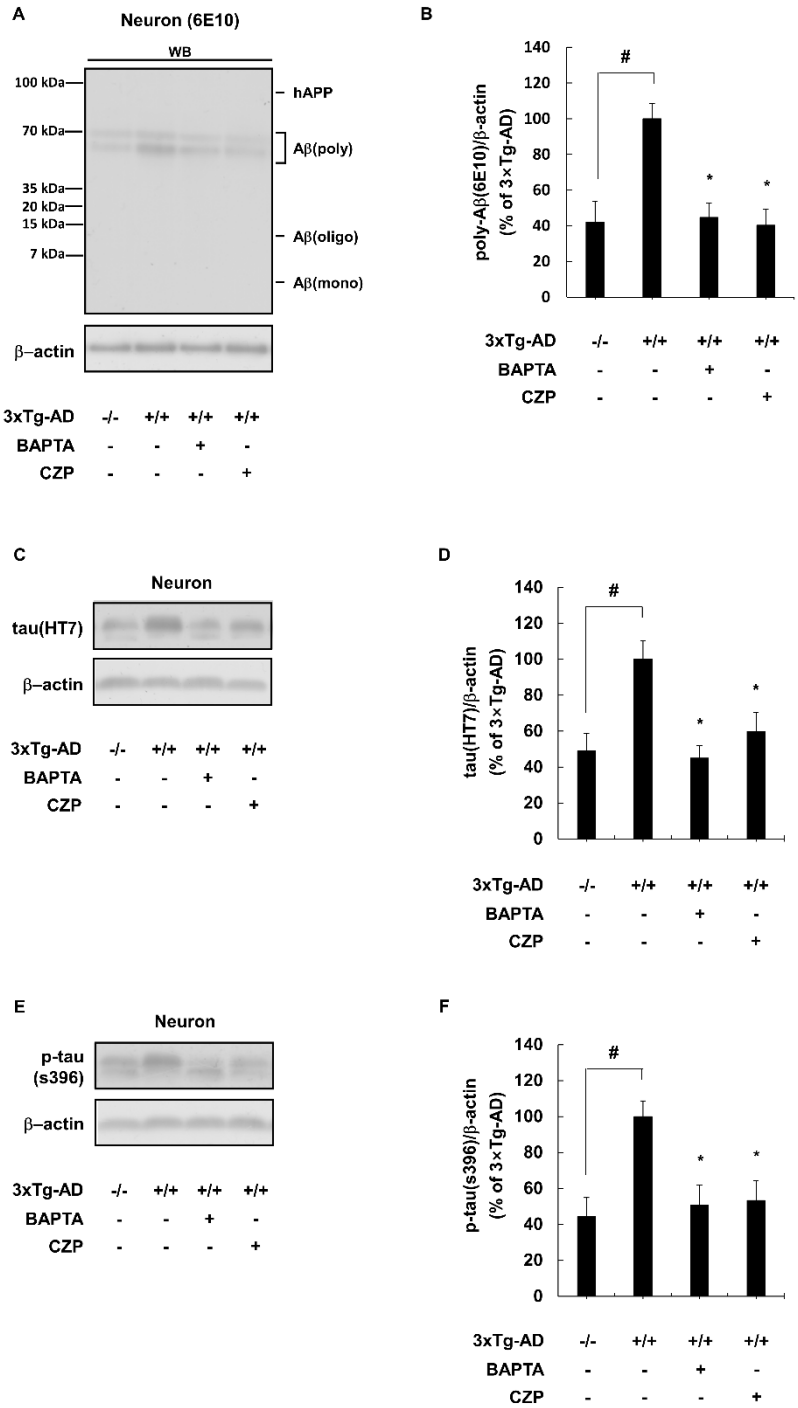

K
p-tau(s202)/β-actin (% of 3×Tg-AD)
##
*
**
3×Tg-AD -/- +/+ +/+ +/+
TRPV1 +/+ +/+ +/- -/-

Fig. 2-4. Effects of TRPV1 deficiency on hippocampal levels of tau and p-tau in 3×Tg-AD mice. (**A**) Representative immunohistochemical confocal images of HT7-positive tau protein in the CA1 and DG subregions. Nuclei were stained using DAPI. Scale bar = 50 μm. (**B-C**) Immunohistochemical signal intensities for HT7-positive tau protein in the (**B**) CA1 and (**C**) DG subregions (mean ± SEM, ##p < 0.01 vs. 3×Tg-AD$^{-/-}$/TRPV1$^{+/+}$, *p < 0.05 and **p < 0.01 vs. 3×Tg-AD$^{+/+}$/TRPV1$^{+/+}$, one-way ANOVA followed by Tukey's post hoc test; n = 4 per group). (**D**) Representative immunohistochemical confocal images of AT-8-positive p-tau protein in the CA1 and DG subregions. Scale bar = 50 μm. (**E-F**) Results for immunohistochemical signal intensity analysis of AT8-positive p-tau protein in the (**E**) CA1 and (**F**) DG subregions. (**G**) Representative Western blots (n = 1 per lane) of tau (HT7, tau 46) protein. (**H-K**) Western blot densitometry results for (**H**) tau (HT7), (**I**) tau (tau 46), (**J**) p-tau (s396), and (**K**) p-tau (s202) (mean ± SEM, #p < 0.05 and ##p < 0.01 vs. 3×Tg-AD$^{-/-}$/TRPV1$^{+/+}$, *p < 0.05 and **p < 0.01 vs. 3×Tg-AD$^{+/+}$/TRPV1$^{+/+}$, one-way ANOVA followed by Tukey's post hoc test; n = 4 per group). CA, cornu ammonis; DG, dentate gyrus.

2.3.5 Ca^{2+} chelator BAPTA/AM and TRPV1 antagonist capsazepine decreased Aβ, tau, and p-tau levels in primary neurons

Primary cortical neurons derived from wild-type and 3 × Tg-AD mice were cultured for 6 days and treated with the Ca^{2+} chelator BAPTA/AM (0.5 μM) or TRPV1 antagonist capsazepine (0.5 μM) for 4 days. According to the results of the Western blot analysis, the neurons from 3 × Tg-AD mice showed higher levels of polymeric Aβ (6E10), tau (HT7), and p-tau (s396) than the neurons from non-transgenic animals. The elevated levels of polymeric Aβ (6E10), tau (HT7), and p-tau (s396) were significantly reduced by BAPTA/AM or capsazepine treatment. (Fig. 2-5A-5F). This finding revealed that the production of Aβ, tau, and p-tau in 3 × Tg-AD mouse-derived primary neurons inhibited by Ca^{2+} chelation using BAPTA/AM or TRPV1 antagonism using capsazepine.

Figure 2-5


A
Neuron (6E10)
WB
100 kDa
70 kDa
35 kDa
20 kDa
15 kDa
7 kDa
hAPP
Aβ(poly)
Aβ(oligo)
Aβ(mono)
β–actin
3xTg-AD -/- +/+ +/+ +/+
BAPTA - - + -
CZP - - - +
B
poly-Aβ(6E10)/β-actin (% of 3×Tg-AD)
140
120
100
80
60
40
20
0
#
*
*
3xTg-AD -/- +/+ +/+ +/+
BAPTA - - + -
CZP - - - +
C
Neuron
tau(HT7)
β–actin
3xTg-AD -/- +/+ +/+ +/+
BAPTA - - + -
CZP - - - +
D
tau(HT7)/β-actin (% of 3×Tg-AD)
140
120
100
80
60
40
20
0
#
*
*
3xTg-AD -/- +/+ +/+ +/+
BAPTA - - + -
CZP - - - +
E
Neuron
p-tau (s396)
β–actin
3xTg-AD -/- +/+ +/+ +/+
BAPTA - - + -
CZP - - - +
F
p-tau(s396)/β-actin (% of 3×Tg-AD)
140
120
100
80
60
40
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*
3xTg-AD -/- +/+ +/+ +/+
BAPTA - - + -
CZP - - - +

Fig. 2-5. Effects of BAPTA and capsazepine (CZP) on levels of Aβs, tau and p-tau in primary neurons derived from 3×Tg-AD mice. (**A**) Representative Western blots (n = 1 per lane) of 6E10-positive APP/Aβ proteins in primary neurons derived from wild-type and 3×Tg-AD mice. (**B**) Western blot densitometry results for 6E10-positive polymeric Aβ in primary neurons derived from wild-type and 3×Tg-AD mice (**C, E**) Representative Western blots (n = 1 per lane) for tau (HT7) and p-tau (s396) protein in primary neurons derived from wild-type and 3×Tg-AD mice. (**D, F**) Western blot densitometry results for (**D**) tau (HT7) and (**F**) p-tau (s396) in primary neurons derived from wild-type and 3×Tg-AD mice (mean ± SEM, #p < 0.05 vs. 3×Tg-AD$^{-/-}$, *p < 0.05 vs. 3×Tg-AD$^{+/+}$, one-way ANOVA followed by Tukey's post hoc test; n = 4 per group).

2.4 Discussion

Sustained intracellular Ca^{2+} signaling disruption may have an early and significant role in AD pathogenesis [23-25]. Ca^{2+} is involved in many aspects of neurophysiology (e.g., synaptic plasticity, learning and memory, activity, and growth and differentiation) and pathophysiology (e.g., degeneration, necrosis, and apoptosis) [26]. Brain aging and AD-related neuronal dysfunction and degeneration are attributed to abnormalities in intracellular Ca^{2+} levels caused by impaired intra-/extracellular cation exchange, endoplasmic reticulum (ER), and mitochondria [15, 27]. In fibroblasts from asymptomatic AD patients at risk, increased inositol triphosphate receptor (IP_3R)-mediated Ca^{2+} signaling has been discovered [28]. Significant changes in the expression of Ca^{2+}-handling genes, a G protein signaling 4 regulator, and type B inositol 1,4,5-triphosphate 3-kinase B have been found in brain tissue from AD patients [29]. Expression of genes that regulate intracellular Ca^{2+} homeostasis was upregulated in 3-month-old 3 × Tg-AD mice compared to same-age wild-type (WT) mice. These genes include ATP2B1, which encodes the plasma membrane Ca^{2+}-ATPase, CACNB4, which encodes the voltage-dependent L-type Ca^{2+} channel subunit b-4, and RYR2 [30].

RyR-mediated Ca^{2+} release and L-type voltage-gated Ca^{2+} current (L-VGCC) density are significantly higher in the CA1 neurons of 3 × Tg-AD mice (aged 1-26 months) compared to age-matched WT animals [31, 32]. My study also revealed that among 12-month-old mice, 3 × Tg-$AD^{-/-}$/$TRPV1^{+/+}$ animals had markedly increased intracellular hippocampal CA1 and DG Ca^{2+} levels than 3 × Tg-$AD^{-/-}$/$TRPV1^{+/+}$ mice.

Ca^{2+} homeostasis in neurons can be disturbed by an external Ca^{2+} infusion produced by Aβ [24, 33, 34]. In vitro research shows that Aβ can open up ion channels on the surface of cells and improve lipid membrane permeability [33]. For example, exposure to Aβ activates glutamate receptors in cultured human cortical neurons, which results in aberrant increases of intracellular Ca^{2+} [35]. Aβ impairs neuronal Ca^{2+} homeostasis by causing cell membrane-associated oxidative stress, which interferes with the functioning of the glucose transporter glutamate transporter 3 (GLUT3), Ca^{2+}-ATPase, and Na^{+}/K^{+}-ATPase [36, 37]. Tau pathology is also connected to changes in intracellular Ca^{2+} homeostasis despite the exact mechanism of action is uncertain [24]; when the tau mutation is expressed in cultured neurons, it destabilizes intracellular Ca^{2+} homeostasis [38].

While Aβ and tau disrupt intracellular Ca^{2+} homeostasis, increased intracellular Ca^{2+} induces the accumulation of Aβ and p-tau and neuronal apoptosis that are characteristic of AD [24]. Increased Ca^{2+} release from the ER due to the particular interaction of PS1-M146L and IP_3R stimulates APP processing (Aβ generation), and mutations in PS are related with abnormal Ca^{2+} signaling [25]. However, in cultured neurons, Ca^{2+} influx induces tau levels that are comparable to those found in neurofibrillary tangles associated with AD [24, 39, 40]. It has been proposed that altered L-VGCC significantly contributes to the selective sensitivity of CA1 neurons to tau lesions [32], which could explain my observation that 3 × Tg-AD mice form tau and hyperphosphorylated tau lesions specifically in hippocampal CA1 neurons (Fig. 2-4A-B and 2-4D-E).

Because increased intracellular Ca^{2+} levels induce the characteristic features of AD, Ca^{2+} channel blocking has been investigated as a method to reduce Aβ-induced neuronal damage in vitro and exhibit neuroprotection in vivo [41]. Memantine, which antagonizes the Ca^{2+}-permeable NMDA receptor, has been approved by the US FDA to treat moderate-to-severe AD [34, 42]. Nimodipine, a Ca^{2+} channel blocker, has positive effects on AD patients, including slowing the rate of deterioration

[34, 43]. Drugs that reduce Ca^{2+} influx, including agents that block glutamate receptors [35], voltage-dependent Ca^{2+} channels [44, 45], and ryanodine receptor channels [46, 47], can protect neurons from Aβ damage. Moreover, the intracellular Ca^{2+} chelator BAPTA/AM and the TRPV1 antagonist capsazepine modulated Aβ, tau, and p-tau production in the neurons derived from 3 × Tg-AD animals. These results suggest that Ca^{2+}-permeable TRPV1 pain receptors might be a potent target for AD prevention and treatment by modulating Ca^{2+} homeostasis fundamental to neuron viability and function.

TRPV1 is involved in the pathogenesis of illnesses such diabetes-associated peripheral neuropathy, asthma, cystitis, chronic inflammatory pain, and hearing loss in addition to the AD neuropathies studied in this work [48]. Drugs that modulate TRPV1 channel activity have potential as effective treatments for such conditions. To the best of my knowledge, this study using an AD mouse model was the first to find that knocking-out Ca^{2+}-permeable TRPV1 pain receptors rescued memory deficits and production of Aβ, tau, and p-tau. TRPV1 antagonists may have therapeutic potential for AD since they can regulate the disruption of Ca^{2+} homeostasis in AD-affected neurons.

Previously, studies have examined synaptic plasticity and dendrite development caused by TRPV1 deficiency in normal mouse models. Through the AD animal models, this chapter identified the role of TRPV1 in the mechanism of AD onset and looked at the possibility of AD therapeutic targets of TRPV1. Studies have targeted various Ca^{2+}-permeable receptors for Ca^{2+} homeostasis regulation. Although clinical trials are currently underway, this study shows the potential for TRPV1 as a target for new Ca^{2+} homeostasis regulation.

In the next chapter, I would like to see the efficacy of TRPV1 in inhibiting neuronal apoptosis, which is very important in AD diagnosis.

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Chapter 3.

Ca^{2+}-permeable TRPV1 receptor deficiency mediates neuroprotective effects in a mouse model of Alzheimer's disease *via* the BDNF/CREB signaling pathway

Abstract

In Chapter 2, Ca^{2+} homeostasis recovery through genetic deficiency of TRPV1 was confirmed, resulting in improvement in memory and cognitive ability and reduction in Aβ accumulation and tau pathology. The three criteria currently used for AD diagnosis include: (1) Aβ accumulation, (2) tau hyperphosphorylation, and (3) neuronal apoptosis. Among them, Aβ accumulation and tau hyperphosphorylation were confirmed. In this chapter, I would like to confirm the third criterion, the effectiveness of inhibiting neuronal apoptosis through TRPV1 deficiency, and I want to reveal its mechanism of action.

As presented in Chapter 2, a study found that TRPV1 deficiency has anti-Alzheimer's disease (AD) effects on triple-transgenic AD mouse model (3 × Tg-$AD^{+/+}$). I looked at the expression of proteins in the brain-derived neurotrophic factor (BDNF)/cyclic AMP response element binding protein (CREB) pathway in order to better understand how TRPV1 deficiency regulates AD in a 3 × Tg-AD/TRPV1 transgenic mouse model. The findings demonstrate that CREB activation is caused by TRPV1 deficiency, which raises BDNF levels and stimulates the phosphorylation of CREB, protein kinase B, extracellular signal regulatory kinase, and

tyrosine receptor kinase B in the hippocampus. Furthermore, CREB activation due to TRPV1 deficiency upregulates antiapoptotic factor B-cell lymphoma 2 (Bcl-2), which consequently downregulates Bcl-2-associated X expression and decreases cleaved caspase-3 and cleaved poly (ADP ribose) polymerase, which leads to the prevention of neuronal apoptosis. In conclusion, TRPV1 deficiency exhibits neuroprotective effects by preventing neuronal apoptosis through the BDNF/CREB signaling transduction pathway in the hippocampus of 3 × Tg-AD mice.

3.1 Introduction

Alzheimer's disease (AD) is one of the most representative age-related neurodegenerative diseases accompanied by memory and cognitive deficits [1]. Although it has been over 100 years since AD was reported by Alois Alzheimer, the adequate AD treatment is not yet established. AD has several causes. The most notable hypotheses for AD development are the amyloid hypothesis and the hyperphosphorylated tau hypothesis [2, 3]. The deposition of both Aβ and tau neurofibrillary tangles causes several pathological symptoms including synaptic degradation, neuroinflammation, and neuronal cell loss, which induce memory and cognition impairment and finally severe dementia [4].

Besides amyloid and hyperphosphorylated tau, Ca^{2+} dyshomeostasis is also heavily associated with the etiology of AD [5-7]. As an intracellular messenger, Ca^{2+} takes part in a number of signal-transmission activities, including the activation of enzymes by phosphorylation or dephosphorylation, exocytosis, gene transcription, and apoptosis [8]. These functions of intracellular Ca^{2+} signaling in neurons are very crucial because they affect neural growth, memory formation, or

synaptic plasticity. In other words, Ca^{2+} dyshomeostasis causes neuronal damage and eventually neuronal apoptosis.

Soluble Aβ aggregates and increases neuronal Ca^{2+} influx in patients with AD, resulting in Ca^{2+} overload in neurons [9]. As a result, Pathogenic characteristics of AD brought on by Ca^{2+} dysregulation, such as neuronal apoptosis and degeneration. [10, 11].

In Chapter 2, I focused on these aspects and tried to mediate AD pathogenesis by regulating Ca^{2+} levels through transient receptor potential vanilloid 1 (TRPV1) and found that TRPV1 deficiency alleviated Aβ and tau deposition and consequently improved memory and learning deficits in 3 × Tg-$AD^{+/+}$ animals. Ca^{2+} was overloaded in 3 × Tg-$AD^{+/+}$ mice; as a result, memory and learning deficits occurred. However, in TRPV1 receptor knockout 3 × Tg-AD mice (3 × Tg-$AD^{+/+}$ /$TRPV1^{-/-}$), after the normalization of Ca^{2+} levels, memory and learning abilities were restored.

A curved area of the brain in the center called the hippocampus is crucial for memory and learning. Therefore, serious memory problems, including memory formation and consolidation, can result from hippocampal loss. Patients with AD had decreased hippocampal synaptic density, which is highly correlated to cognitive deficits [12]. Neuronal

apoptosis is linked to a several factors such as Aβ accumulation, increased oxidative stress, or DNA fragmentation in the brain of patients with AD [13]. These previous studies demonstrate a strong correlation between hippocampus neuronal apoptosis and AD patients' cognitive function..

Brain-derived neurotrophic factor (BDNF) is a major neurotrophic factor that regulates neuronal survival, differentiation, and plasticity [14]. The expressions of BDNF and its receptor, tyrosine receptor kinase (TrkB), are reduced in patients with AD [15, 16]. Evidence also revealed that Aβ directly or indirectly reduced BDNF levels, leading to memory and cognition deficits [17]. Therefore, the expression levels of BDNF and TrkB are important for evaluating the neuroprotective effect of TRPV1 deficiency on AD models.

The activation of TrkB initiates the activation of several downstream pathways, including the phosphorylation of extracellular signal-regulated kinase (ERK) and protein kinase B (Akt), which in turn phosphorylates and activates cAMP-response element binding protein (CREB) [18, 19]. ERK/mitogen-activated protein kinase is related to memory formation [20] and neuropretective effects [21]. Akt also plays an important role in neuroprotection [22]. CREB is a transcription factor that

regulates the proliferation or survival of neurons by binding to cAMP-responsive elements, thereby modulating gene transcription [23].

The B-cell lymphoma-2 (Bcl-2) family of proteins plays a critical role in apoptotic activities. The Bcl-2 family includes both the antiapoptotic protein Bcl-2 and the pro-apoptotic protein Bcl-2-associated X (Bax) or Bcl-2 antagonist/killer (Bak) [24]. When CREB is phosphorylated, it directly binds to Bcl-2 family promoter, activating the signaling cascade [25]. Bcl-2 forms a heterodimer with the apoptotic protein Bax and inhibits Bax activation, which regulates the activation of caspase-3 [26, 27]. Cleaved caspase-3 is involved in neuronal apoptosis in mammals [28]. Activated caspase-3 cleaves cellular proteins including poly (ADP ribose) polymerase (PARP). As PARP is an enzyme that plays an important role in DNA fragmentation repair, consequently, cleavage of PARP induces neuronal apoptosis [29].

In this study, I aimed to understand how neurological parameters are related to neuroprotective effects by preventing neuronal apoptosis in the hippocampus using TRPV1 receptor knockout 3 × Tg-AD mice to explore the effect of TRPV1 deficiency on neuronal loss in the hippocampus. I found that TRPV1 deficiency inhibits neuronal apoptosis

by promoting the BDNF/TrkB signaling pathway. TrkB activation triggers CREB activation, which in turn prevents caspase-3 activation, which enforces apoptosis.

3.2 Materials and Methods

3.2.1 Transgenic animal models

3xTg-AD mice (kindly given by Professor Frank M. Laferla, UC-Irvine, Irvine, CA, USA) is homozygous for APP_{swe}, Tau_{P301L} and $PS1_{M146V}$. I mated these 3xTg-AD mice with $TRPV1^{-/-}$ mice (provided from Jackson Laboratories, Bar Harbor, ME, USA, stock #003770) and generated offspring with nine genotypes. I selected four groups with or without AD pathology and TRPV1 depletion. Selected groups were: 3xTg-$AD^{-/-}$/$TRPV1^{+/+}$ mice (wild type, WT), 3xTg-$AD^{+/+}$ /$TRPV1^{+/+}$ mice, 3xTg-$AD^{+/+}$/$TRPV1^{+/-}$ mice and 3xTg-$AD^{+/+}$/$TRPV1^{-/-}$ mice. Mice in each group were raised until 12 months. Mice were kept at 21 ± 2 ˚C, 12 h light: 12 h dark photoperiod with specific pathogen-free conditions in ventilated cages and provided with free choice to consume food and water. All animal testing was performed following protocols approved by the Institutional Animal Care and Use Committee of Seoul National

University (SNU-140410-12, SNU-140410-14, SNU-140625-2).

3.2.2 Animal Dissection and Tissue Collection

Mice (n=5) in each group were anesthetized with Zoletil (50 mg/kg) by intraperitoneal administration and then transcardially perfused with normal saline followed by 4% paraformaldehyde solution. The brains were removed and post-fixed in 4% paraformaldehyde overnight at 4 ℃, and routine frozen sections (10 μm) were prepared for TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining and immunohistochemistry analysis. The other half of the mice in each group were sacrificed by decapitation, and the brains were rapidly collected and cut on an ice-cooled board. The hippocampus was dissected out and stored at -80 ℃ for TUNEL staining and western blotting analysis.

3.2.3 TUNEL Staining

TUNEL staining was performed on the frozen-embedded sections using the in-situ cell death detection kit (Promega, Madison, USA) according to the standard protocol provided by manufacturer. Apoptotic nuclei were visualized using the peroxidase-DAB reaction. The sections

were counterstained with hematoxylin. TUNEL-positive neurons in hippocampus were analyzed in each 3 regions of Cornu Ammonis 3 (CA3), Cornu Ammonis 1 (CA1) (especially the soma of pyramidal neurons), and dentate gyrus (DG) sections per high-power field (×40). TUNEL staining fluorescence signal intensity was measured using the image J analysis program (National Institute of Health, Bethesda, MD, USA). Regions of interest were selected from the CA3, CA1 and DG regions of the hippocampus.

3.2.4 Western blot analysis

Each hippocampal tissue was taken from five mice and analyzed one by one. There was no replication. Sample were prepared by homogenizing the hippocampus tissues. The protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Western blot analyses were performed on 50 μg protein. Briefly, samples were separated using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride membrane (Merck Millipore, Burlington, MA, USA; 0.4 μm). The membranes were blocked in 5% bovine serum albumin

(Bovogen Biologicals, Keilor East, Australia) in tris-buffered saline and Tween-20 (Junsei Chemical, Tokyo, Japan). Membranes were incubated overnight at 4 ℃ with primary antibodies against BDNF (Santa Cruz, California, USA, 1:1000), p-TrkB, (Cell Signaling, Danvers, MA, USA, 1:1000), TrkB (80E3, Cell Signaling, 1:1000), p-Akt (Ser473, Cell Signaling, 1:1000), Akt (Cell Signaling, 1:1000), p-ERK (E-4, Santa Cruz, 1:1000), ERK (Santa Cruz, 1:1000), p-CREB (Ser133, D1G6, Cell Signaling, 1:1000), CREB (48H2, Cell Signaling, 1:1000), Bax (Santa Cruz, 1:1000), Bcl-2 (Santa Cruz, 1:1000), cleaved caspase-3 (Santa Cruz, 1:1000), caspase-3 (Santa Cruz, 1:1000), cleaved PARP (Thermo, Waltham, MA, USA 1:1000), GAPDH (Cell signaling, 1:1000) and β-actin (Sigma, Burlington, MA, USA, 1:5000). After incubation with horseradish peroxidase-conjugated secondary antibodies (Sigma, 1:5000) for 1 h at room temperature, immunodetection was performed using an enhanced chemiluminescence detection kit (GE Healthcare, Chicago, IL, USA). The relative density of each band was measured using the image J analysis program (National Institute of Health, Bethesda, MD, USA).

3.2.5 Statistical Analysis

SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. The results were presented as mean ± standard error of the mean (SEM) values. All the data were analyzed using by Students' t-test.

3.3 Results

3.3.1 TRPV1 deficiency reduces neuronal apoptosis in the hippocampus 3 × Tg-AD mice

To determine the neuroprotective effect of TRPV1 deficiency, I performed TUNEL assay to detect neuronal apoptosis in wild-type (WT), 3 × Tg-AD$^{+/+}$ /TRPV1$^{+/+}$, 3 × Tg-AD$^{+/+}$ /TRPV1$^{+/-}$, and 3 × Tg-AD$^{+/+}$ /TRPV1$^{-/-}$ mice. CA3, CA1, and DG regions of the hippocampus were subjected to this assay. The nuclei of apoptotic cells in each region are shown in green fluorescence. The immunofluorescence results show that TUNEL-positive apoptotic cells were remarkably increased in all regions of the hippocampus of 3 × Tg-AD$^{+/+}$ /TRPV1$^{+/+}$ mice compared with the WT mice. However, apoptotic cells were tremendously decreased in all three regions of 3 × Tg-AD$^{+/+}$ /TRPV1$^{-/-}$ mice compared with 3 × Tg-

$AD^{+/+}$ /$TRPV1^{+/+}$ mice (Fig. 3-1A). Quantitative analysis also showed that TRPV1 deficiency significantly reduced apoptotic cells in CA3 (Fig. 3-1B), CA1 (Fig. 3-1C), and DG (Fig. 3-1D) regions by 67%, 67%, and 62%, respectively, compared with the same region in 3 × Tg-$AD^{+/+}$ /$TRPV1^{+/+}$ mice. These results indicate that TRPV1 deficiency protects hippocampal neurons from neuronal apoptosis caused by AD pathogenesis.

Figure 3-1

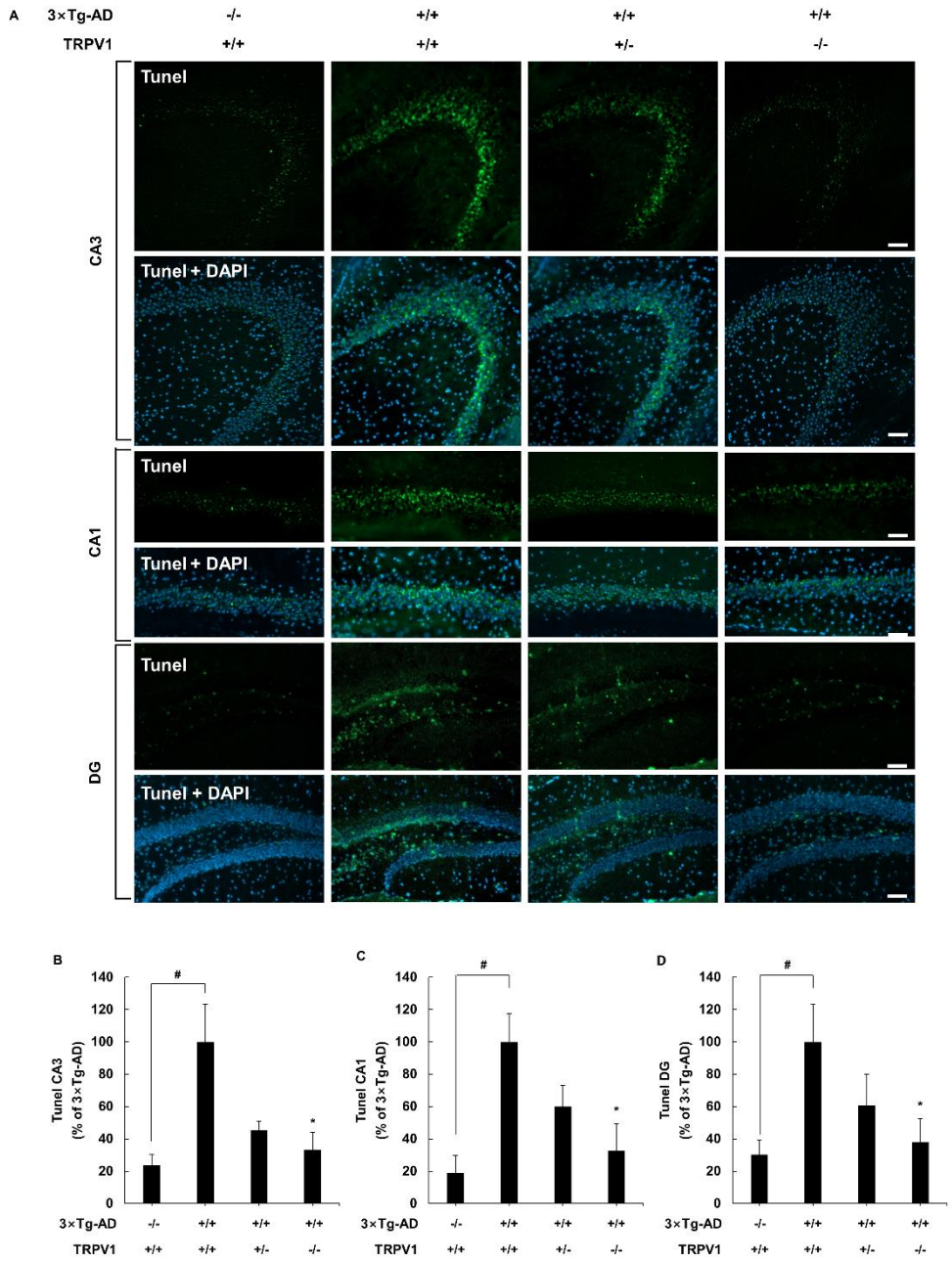

Fig. 3-1. Effect of TRPV1 deficiency on neuronal apoptosis in the hippocampus of WT, 3xTg-AD$^{+/+}$/TRPV1$^{+/+}$, 3xTg-AD$^{+/+}$/TRPV1$^{+/-}$ and

3xTg-AD$^{+/+}$/TRPV1$^{-/-}$ mice. (A) Representative photographs of TUNEL staining in the CA3, CA1 and DG region. Scale bar = 2.5 μm. (B-D) Quantification of TUNEL staining fluorescence intensity in the (B) CA3, (C) CA1 and (D) DG region. The results are presented as means ± SEM, # $p < 0.05$ compared to the WT, * $p < 0.05$ in compared to the 3xTg-AD$^{+/+}$, one-way ANOVA followed by Students' t-test; n = 5 per group.

3.3.2 TRPV1 deficiency increases BDNF expression levels and promotes TrkB receptor phosphorylation in the hippocampus of 3 × Tg-AD mice

To examine whether TRPV1 deficiency increases the levels of BDNF and activation of its receptor protein TrkB, Western blot analyses were conducted. BDNF expression levels significantly decreased in 3 × Tg-AD$^{+/+}$/TRPV1$^{+/+}$ mice by 26% compared with 3 × Tg-AD$^{-/-}$/TRPV1$^{+/+}$ mice. However, BDNF expression levels significantly increased in 3 × Tg-AD$^{+/+}$/TRPV1$^{-/-}$ mice by 625% compared with 3 × Tg-AD$^{+/+}$/TRPV1$^{+/+}$ mice (Fig. 3-2A and 3-2B). Similarly, p-TrkB expression levels proportional to TrkB and β-actin significantly decreased in 3 × Tg-AD$^{+/+}$/TRPV1$^{+/+}$ mice by 40% and 29%, respectively, compared to 3 × Tg-AD$^{-/-}$/TRPV1$^{+/+}$ mice. p-TrkB expression levels also significantly increased in 3 × Tg-AD$^{+/+}$/TRPV1$^{-/-}$ mice by 268% and 310%, respectively, compared with 3 × Tg-AD$^{+/+}$/TRPV1$^{+/+}$ mice (Fig. 3-2C and 3-2D). These results indicate that TRPV1 deficiency increases the levels of BDNF expression and promotes TrkB phosphorylation in the hippocampus of 3 × Tg-AD mice. As previously mentioned, it has been observed that BDNF, which is necessary for synaptic plasticity and neuronal survival, is diminished in

patients with AD. In this respect, the recovery of BDNF signaling pathway is considered a promising therapeutic approach to AD treatment [30]. Memantine, a well-known AD drug, has also been shown to improve preclinical AD pathology by raising BDNF protein levels, demonstrating the value of my findings [31].

Figure 3-2

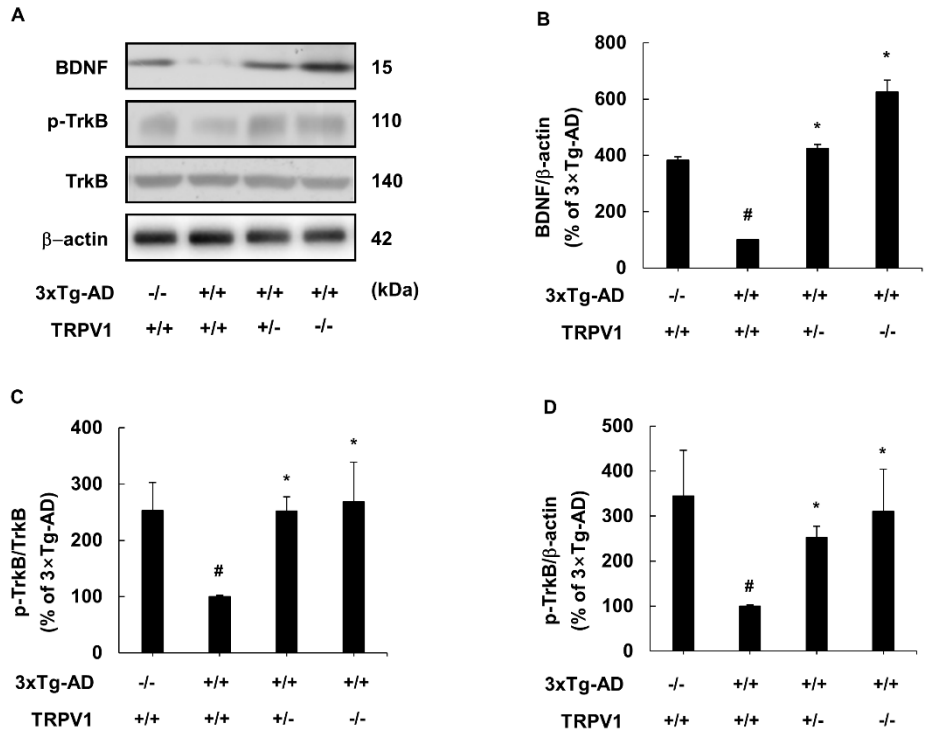

Fig. 3-2. Effect of TRPV1 deficiency on BDNF and p-TrkB protein expression levels in the hippocampus of WT, 3xTg-AD$^{+/+}$/TRPV1$^{+/+}$, 3xTg-AD$^{+/+}$/TRPV1$^{+/-}$ and 3xTg-AD$^{+/+}$/TRPV1$^{-/-}$ mice. (A) Representative Western blot bands showing expression levels of BDNF, TrkB and p-TrkB proteins. (B-D) Western blot densitometry results for (B) BDNF /β-actin, (C) p-TrkB /TrkB and (D) p-TrkB /β-actin protein expression level analysis. The results are presented as means ± SEM, $^{\#}$ $p < 0.05$ compared to the WT, * $p < 0.05$ compared to the 3xTg-AD$^{+/+}$, one-way ANOVA followed by Students' t-test; n = 5 per group.

3.3.3 TRPV1 deficiency increases the phosphorylation of ERK, Akt and CREB

To investigate whether TrkB activation leads to the activation of the downstream signaling of BDNF/TrkB, I measured the phosphorylation levels of Akt and ERK. Both Akt and ERK independently mediated the BDNF/CREB signaling pathway, where the downstream protein CREB plays an important role in memory formation by regulating BDNF [32]. Western blot analyses revealed that the levels of p-Akt and p-ERK were decreased in the hippocampus of 3 × Tg-AD$^{+/+}$/TRPV1$^{+/+}$ mice compared with that of 3 × Tg-AD$^{-/-}$/TRPV1$^{+/+}$ mice and the difference disappeared in 3 × Tg-AD$^{+/+}$/TRPV1$^{+/-}$ and 3 × Tg-AD$^{+/+}$/TRPV1$^{-/-}$ mice (Fig. 3-3A). The densitometry results show that TRPV1 deficiency induces the phosphorylation of Akt (Fig. 3-3B) and ERK (Fig. 3-3C), whereas baseline levels of Akt and ERK were unchanged. I then investigated the expression and phosphorylation level of CREB, which plays an important role in mediating the apoptosis pathway. In 3 × Tg-AD$^{+/+}$/TRPV1$^{+/+}$ mice, CREB phosphorylation levels decreased compared with WT mice. However, CREB phosphorylation levels in 3 × Tg-AD$^{+/+}$/TRPV1$^{-/-}$ mice were significantly increased, similar to those in WT mice. Previous studies

have reported that Akt and ERK mediate signal transduction in various neurodegenerative diseases, including AD [33]. Similarly, my results indicate that TRPV1 deficiency in the hippocampus of 3 × Tg-AD mice has neuroprotective effect by phosphorylating and activating Akt and ERK, thereby activating the CREB pathway (Fig. 3-3D–3F).

Figure 3-3

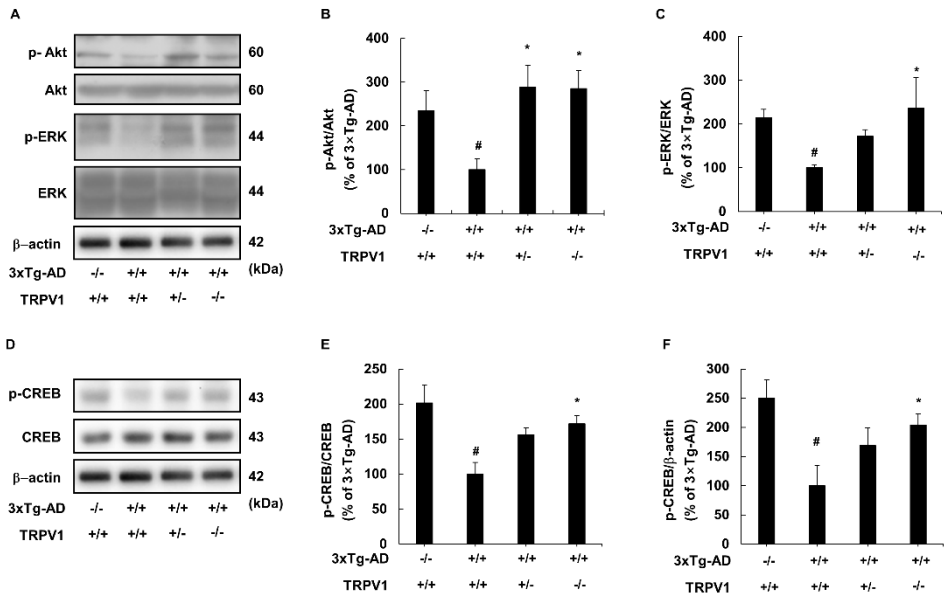

Fig. 3-3. Effect of TRPV1 deficiency on p-Akt, p-ERK and p-CREB protein expression level in the hippocampus of WT, 3xTg-AD$^{+/+}$/TRPV1$^{+/+}$, 3xTg-AD$^{+/+}$/TRPV1$^{+/-}$ and 3xTg-AD$^{+/+}$/TRPV1$^{-/-}$ mice. (A) Representative Western blot bands showing the expression level of p-Akt, Akt, p-ERK and ERK. (B-C) Western blot densitometry results for (B) p-Akt/Akt and (C) p-ERK/ERK. (D) Representative Western blot bands showing the expression level of p-CREB and CREB. (E-F) Western blot densitometry results for (E) p-CREB/CREB and (F) p-CREB/β-actin. The results are presented as means ± SEM, $^{\#}$ $p < 0.05$ compared to the WT, * $p < 0.05$ compared to the 3xTg-AD$^{+/+}$, one-way ANOVA followed by Students' t-test; n = 5 per group.

mean ± SEM values of three independent experiments. Mean values with different letters over the bars indicate significant difference ($p < 0.05$).

3.3.4 TRPV1 deficiency reduces the cleavage of caspase-3 and PARP in 3 × Tg-AD mice

TRPV1 deficiency lowers neuronal apoptosis in the hippocampus of 3 × Tg-AD mice, as shown in Figure 3-1. In this study, I studied how TRPV1 deficiency lowers neuronal apoptosis by controlling caspase-3 in the hippocampus of 3 × Tg-AD mice because caspase-3 is a crucial regulator in the course of AD [34]. Western blot analyses revealed that the Bcl-2 levels decreased, whereas those of Bax, cleaved caspase-3, and cleaved PARP increased in the hippocampus of 3 × Tg-AD$^{+/+}$ /TRPV1$^{+/+}$ mice compared with those of 3 × Tg-AD$^{-/-}$ /TRPV1$^{+/+}$ mice. These differences disappeared in 3 × Tg-AD$^{+/+}$ /TRPV1$^{+/-}$ and 3 × Tg-AD$^{+/+}$ /TRPV1$^{-/-}$ mice (Fig. 3-4A).

The densitometry results show that Bcl-2 expression levels were significantly increased in 3 × Tg-AD$^{+/+}$ /TRPV1$^{-/-}$ mice compared with 3 × Tg-AD$^{+/+}$ /TRPV1$^{+/+}$ mice. Consequently, the expression levels of apoptotic factors Bax (Fig. 3-4B), cleaved caspase-3 (Fig. 3-4C), and cleaved PARP (Fig. 3-4D) decreased in 3 × Tg-AD$^{+/+}$ /TRPV1$^{-/-}$ mice. These findings demonstrate that TRPV1 deficiency prevents neuronal apoptosis in the hippocampus by upregulating Bcl-2 and downregulating

the levels of Bax, cleaved caspase-3, and cleaved PARP.

Figure 3-4

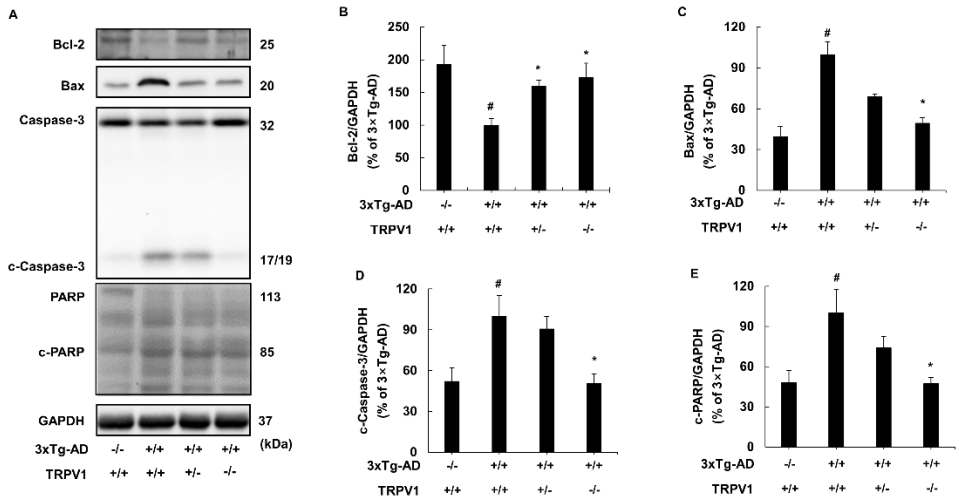

Fig. 3-4. Effect of TRPV1 deficiency on the expression of apoptosis-related proteins in the hippocampus of WT, 3xTg-AD$^{+/+}$/TRPV1$^{+/+}$, 3xTg-AD$^{+/+}$ /TRPV1$^{+/-}$ and 3xTg-AD$^{+/+}$/TRPV1$^{-/-}$ mice. (A) Representative Western blot bands showing expression levels of Bcl-2, Bax, cleaved caspase-3 and cleaved PARP (B-E) Western blot densitometry results for (B) Bcl-2/GAPDH, (C) Bax/GAPDH, (D) cleaved caspase-3/GAPDH and (E) cleaved PARP/GAPDH. The results are presented as means ± SEM, $^{\#}$ $p < 0.05$ compared to the WT, * $p < 0.05$ compared to the 3xTg-AD$^{+/+}$, one-way ANOVA followed by Students' t-test; n = 5 per group.

3.4. Discussion

Memory and cognition deficits are well-known symptoms of AD, and neuronal apoptosis contributes to most aspects of AD-related deficits. Memory and learning deficits are significantly impacted by Aβ and tau neurofibrillary tangles, but Ca^{2+} dyshomeostasis is also reported to play a significant role in the pathophysiology of AD [35]. Ca^{2+} plays a critical role as a signal carrier regulating synaptic signaling and neurotransmission. A study reported that Ca^{2+} is reported to be overloaded in patients with AD [36] and 3 × Tg-AD mice model [11], leading to memory and behavior disorders.

In a previous study, TRPV1 deficiency effectively restored Ca^{2+} levels in the hippocampus and consequently decreased Aβ and tau deposition, inducing anti-AD effects. Memantine, an NMDA receptor antagonist used to treat Alzheimer's disease, has been shown to have neuroprotective effects by preventing excessive Ca^{2+} influx into cells [37]. Subsequent studies have shown memantine's neuroprotective role in lowering hippocampus apoptosis [38]. These findings demonstrated that memantine inhibited the activity of caspase-3 [39] via preserving BDNF function [40] and stimulating the BDNF/TrkB/Akt/ERK/CREB signaling

pathway [41-43].

Apoptosis is a form of cell death that occurs through a series of molecular steps within the cell [44]. Results of the TUNEL assay show that TUNEL-positive cells were significantly increased in CA3, CA1, and DG regions of the hippocampus in 3 × Tg-AD$^{+/+}$ /TRPV1$^{+/+}$ mice. This increase was nearly completely prevented by TRPV1 deficiency in all three regions of the hippocampus (Fig. 3-1). These results indicate that TRPV1 deficiency exerts neuroprotective effects by preventing neuronal apoptosis.

A study reported that Aβ induces N-methyl-D-aspartate receptor activation, leading to intracellular Ca^{2+} overload, which in turn activates calpain and reduces BDNF levels [40, 45]. In this study, I hypothesized that TRPV1 deficiency protects neurons by inhibiting neuronal apoptosis in the hippocampus through Ca^{2+} normalization. Western blot analyses revealed that TRPV1 deficiency restores the protein expression of BDNF and the phosphorylation level of its membrane receptor TrkB (Fig. 3-2), suggesting that BDNF is involved in TRPV1 deficiency-mediated neuroprotective effects. Subsequent Western blot analyses showed that TRPV1 deficiency increases the phosphorylation of BDNF downstream

intracellular proteins Akt and ERK, which in turn activates CREB (Fig. 3-3).

When CREB is phosphorylated, p-CREB binds to Bcl-2 promoter and increases Bcl-2 expression [46]. Bcl-2 then binds directly to Bax and consequently inhibits the activation and oligomerization of Bax [47]. As a result, the apoptotic effect of Bax is blocked by pore formation in the mitochondria [48]. Additionally, Bax plays an essential role in activating caspase-3 by releasing cytochrome c from the mitochondria, which interacts with the apoptotic protease activating factor-1 and activates caspase-3 [13, 49-51]. When caspase-3 is cleaved and activated, it cleaves cellular proteins, including PARP, that can repair damaged DNA [52]. Thus, CREB phosphorylation upregulates Bcl-2 and downregulates Bax expression, which in turn prevents neuronal apoptosis [53, 54]. In this study, the expression levels of Bcl-2 were increased, whereas those of apoptotic factors Bax, cleaved caspase-3, and cleaved PARP decreased in 3 × Tg-AD$^{+/+}$ /TRPV1$^{-/-}$ mice compared with those in 3 × Tg-AD$^{+/+}$ /TRPV1$^{+/+}$ mice (Fig. 3-4).

Taken together, these findings suggest that TRPV1 deficiency has neuroprotective effects on neuronal apoptosis through the BDNF/CREB

signaling pathway that mediates the Bcl-2 family-related antiapoptotic pathway in the hippocampus (Fig. 3-5).

A proposed molecular mechanism of neuroprotection by TRPV1 deficiency in 3 × Tg-AD mice is shown in Figure 3-5. TRPV1 deficiency normalizes Ca^{2+} levels in the 3 × Tg-AD mouse model. Consequently, TRPV1 deficiency upregulates the BDNF/TrkB and downstream Akt/ERK/CREB signaling pathways. This signaling upregulates the antiapoptotic factor Bcl-2, which results in the downregulation of the apoptosis factor Bax, cleaved caspase-3, and cleaved PARP levels.

In conclusion, TRPV1 deficiency protects hippocampal neurons from neuronal apoptosis caused by AD pathogenesis in the 3 × Tg-AD mouse model via the BDNF/CREB signaling pathway. In addition, the normalization of Ca^{2+} levels by TRPV1 deficiency may be a therapeutic target to suppress memory loss and learning deficits in AD pathogenesis. This study confirmed that the genetic deficiency of TRPV1 inhibits three criteria currently used for AD diagnosis: (1) Aβ accumulation, (2) tau hyperphosphorylation, and (3) neuronal apoptosis. Through this, the potential of TRPV1 was confirmed in the Ca^{2+} homeostasis regulation mechanism, which is the target of AD treatment development.

In the next chapter, I would like to confirm the possibility of a new AD treatment agent through α-spinasterol, an antagonist targeting TRPV1.

Figure 3-5

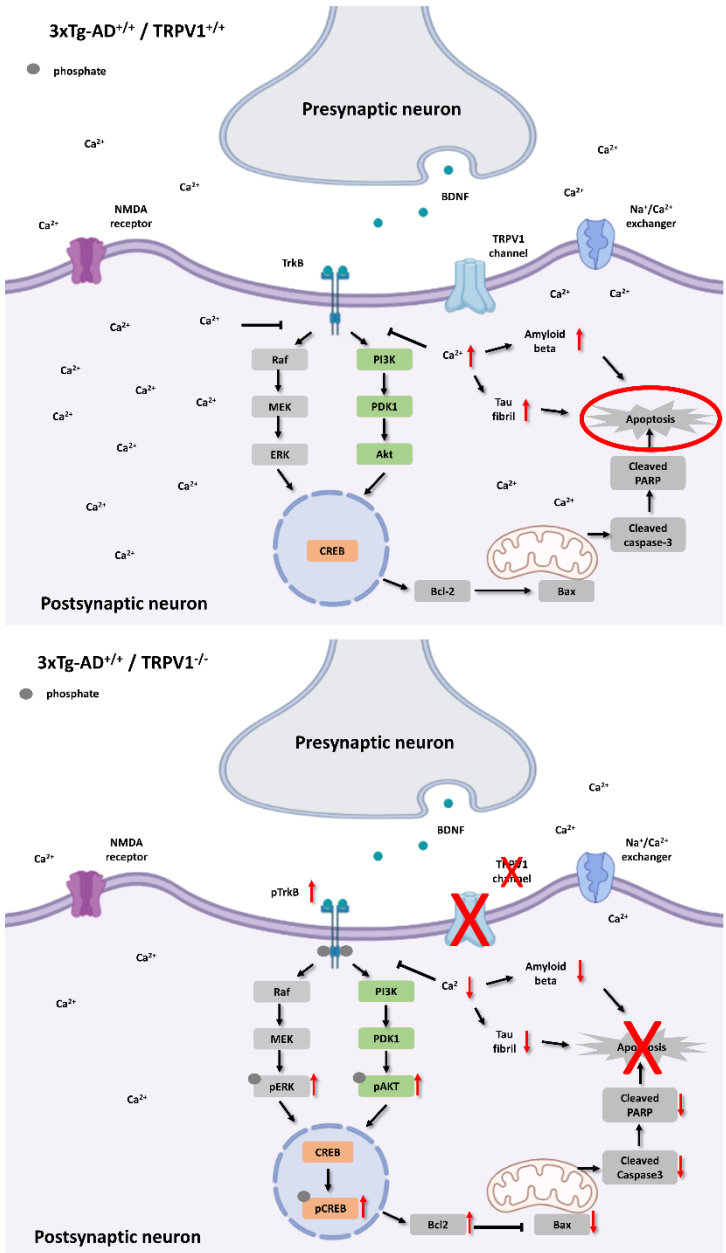

Fig. 3-5. Molecular pathway of neuroprotection by TRPV1 deficiency in 3xTg-AD mice. Ca^{2+} is overloaded in 3xTg-$AD^{+/+}$/$TRPV1^{+/+}$ and as a result,

neuronal apoptosis occurs (up). In 3xTg-AD$^{+/+}$/TRPV1$^{-/-}$ mice, Ca^{2+} level is normalized and neuronal apoptosis is prevented by activation of BDNF/CREB pathway (down). (Created with BioRender.com).

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Chapter 4

α-Spinasterol, Ca^{2+}-permeable TRPV1 receptor antagonist, rescues memory deficits and reduces amyloid-β and tau pathology

Abstract

I previously found that TRPV1 deficiency has anti-Alzheimer's disease (AD) effects on triple-transgenic AD mouse model (3 × Tg-AD$^{+/+}$), and I identified the potential of a target for new AD treatment. α-Spinasterol was first discovered in spinach and accounts for the largest proportion of phytosterol in spinach, accounting for approximately 80%. According to recent studies, α-spinasterol showed anti-inflammatory and anti-oxidant effects, which are neuroprotective effects beneficial for treating neurodegenerative diseases. α-Spinasterol could penetrate the blood–brain barrier (BBB) and affect the central nervous system.

This study aimed to identify whether α-spinasterol, a TRPV1 antagonist, may mitigate Alzheimer's disease (AD) in vivo and in vitro. The effect of α-spinasterol was investigated in Aβ_{1-42}-treated mouse models. I observed that Aβ_{1-42}-treated mice had memory impairment and increased levels of hippocampal Aβ and tau compared with PBS-treated mice. However, α-spinasterol-treated mice showed better memory function and lower levels of hippocampal Aβ and tau compared than Aβ_{1-42}-treated mice. α-Spinasterol decreased Aβ and p-tau levels and mitigated Ca^{2+} dyshomeostasis and neurotoxicity in primary neurons. My results suggest

that α-spinasterol may have anti-AD effects and promote resilience to memory loss and increased hippocampal Ca^{2+}, Aβ, and tau levels. Taken together, α-spinasterol modulates TRPV1, which can be used as a therapeutic agent for preventing AD.

4.1 Introduction

Previously, I investigated the therapeutic effects of TRPV1 deficiency in the pathogenesis of Alzheimer's disease (AD) using a triple-transgenic AD mouse model (3 × Tg-AD) with wild-type ($TRPV1^{+/+}$), hetero ($TRPV1^{+/-}$), and knockout ($TRPV1^{-/-}$). TRPV1 deficiency modulated Ca^{2+} levels, hippocampal pathology, and cognitive decline in AD. Furthermore, the BDNF/CREB signaling pathway was activated to suppress neuronal apoptosis. In this study, I would like to confirm the AD treatment efficacy of α-spinasterol, a TRPV1 antagonist, and confirm its potential as a new AD treatment agent.

α-spinasterol inhibits specific binding of resiniferatoxinin (RTX), TRPV1 agonist, to TRPV1. In addition, α-spinasterol is known to inhibit the activity of capsaicin, a representative agonist of TRPV1. Based on this, spinasterol is known as an antagonist that specifically binds to TRPV1. As a TRPV1 antagonist, α-spinasterol [1] has functional effects on antidepressant, antianxiety, anti-insomnia, anti-inflammatory, and neuroprotective activities [2, 3]. TRPV1 reduces mechanical allodynia, immobility time, and depression in a mouse model of fibromyalgia [4]. α-Spinasterol is a phytosterol found in various plants such as spinach [5]. It

accounts for the largest proportion (approximately 80%) of the phytosterol components in spinach [6]. α-Spinasterol functions directly on the brain because of its ability to cross the blood–brain barrier [7]. Despite exhibiting various beneficial properties, no studies have reported the anti-AD effect of α-spinasterol. In this study, I hypothesized that the TRPV1 antagonist α-spinasterol could alleviate AD by modulating neuronal Ca^{2+} levels, hippocampal pathology, and cognitive decline.

In this study, I investigated the effect of α-spinasterol on the AD pathogenesis in an AD mouse model ($A\beta_{1-42}$-treated mice) by unilateral injection of $A\beta_{1-42}$ into the hippocampus [8]. Intracellular Ca^{2+} levels and Aβ, tau, and p-tau production were examined in primary neuronal cell cultures for AD development.

4.2 Materials and Methods

4.2.1 Animals

Female ICR mice (8 weeks) were purchased from the Koatech company (Pyeongtaek, Korea). The mice were housed in the animal care facility (temperature 22 ± 2 ℃; humidity 40–60%, and a 12-h light/dark cycle) in Korea Institute of Science and Technology (KIST). Mice were

provided with free choice to consume food and water. All animal testing was performed the following protocols approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-140625-2) and the Animal Ethics Committee of KIST (KIST-5088-2022-08-107).

4.2.2 Drug and reagents

α-Spinasterol was purchased from Cayman (Ann Arbor, MI, USA) and dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions. Lyophilized 1 mg vial of $A\beta_{1-42}$ was purchased from Cayman (Ann Arbor, MI, USA). For treatment to primary neurons, $A\beta_{1-42}$ was prepared in 400 μL DMSO and incubated for 1 h at room temperature (RT) with rotation. Oligo-$A\beta_{1-42}$ was prepared by diluting $A\beta_{1-42}$ with neurobasal medium (Gibco, Carlsbad, CA, USA) at concentration of 100 μM. The oligo-$A\beta_{1-42}$ was incubated for 24 h at 4 ℃ with rotation. For stereotaxic surgery, $A\beta_{1-42}$ was prepared in 20 μL DMSO and incubated for 1 h at RT with rotation. Oligo-$A\beta_{1-42}$ was prepared by diluting $A\beta_{1-42}$ with PBS at concentration of 1 μg/μL and then incubated for 24 h at 4 ℃ with rotation.

4.2.3 Primary neuronal culture

Cerebral cortical tissue was dissected from day 15 embryonic ICR mice, as previously described. Cells were isolated by digestion with 0.05% trypsin and are re-suspended in minimal essential medium containing 10% heat-inactivated horse serum, 10% fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. The isolated cortical neurons were allowed to adhere on 0.2 mg/mL poly-D-lysine-coated culture dishes for 45 min and cultured in neurobasal medium supplemented with B27 (Gibco, Waltham, MA, USA), 1 mM glutamine, and 100 units/mL penicillin, 100 μg/mL streptomycin. Cultures at 6 days were treated with $A\beta_{1-42}$ and/or α-spinasterol for 24 h, and the neurons were harvested for Western blot analysis to evaluate APP/Aβ, tau, and p-tau levels.

4.2.4 Cell viability assay

Primary neurons, 5×10^5 cells per well, were cultured in 96-well for 6 days. $A\beta_{1-42}$ and/or α-spinasterol were applied to the cells for 24 h. MTT solution (Thermo Scientific, Waltham, MA, USA) was added to the media and incubated for 2 h. Cytotoxicity was measured using a

microplate spectrophotometer (Bio-Tek Power Wave XS, Winooski, VT, USA). The absorbance was measured at 490 nm.

4.2.5 Fluorescence Ca^{2+} imaging

Primary neurons were cultured on glass coverslips in a 24-well plate to evaluate intracellular Ca^{2+}. Cultures at 6 days were treated with $A\beta_{1–42}$ and/or α-spinasterol for 24 h. They were then washed using PBS and loaded with 10 μM Ca^{2+} indicator fluo-4 AM (Thermo Scientific, Waltham, MA, USA) for 24 h. The stained sections were cleaned with PBS, cover-slipped with Prolong Gold Antifide Reagent containing DAPI nuclear stain (Thermo Scientific, Waltham, MA, USA), and examined using a microscope (Carl Zeiss, Oberkochen, Germany). The fluo-4 AM fluorescence signal intensity was measured using the image J analysis program (National Institute of Health, Bethesda, MD, USA).

4.2.6 $A\beta_{1–42}$ stereotaxic surgeries

Eight-week-old mice were acclimatized to laboratory conditions for one week and divided into three groups: PBS-treated mice (PBS + PBS), $A\beta_{1–42}$-treated mice [$A\beta_{1–42}$ (1 μg/μL, 3 μL/mouse) + PBS], α-

spinasterol-treated mice [$A\beta_{1-42}$ + α-spinasterol (1.25 μM, 3 μL/mouse)]. After being anesthetized with an intraperitoneal injection of avertin (250 mg/kg), the mice were immobilized on a stereotaxic instrument. $A\beta_{1-42}$ (day 0 and 1), α-spinasterol (day 3 and 4), or PBS (day 0, 1, 3, and 4) was delivered (3 μl/15 min/mouse) into the hippocampus (coordinates from bregma: mediolateral (ML) = 1.30 mm, anteroposterior (AP) = −2.00 mm, and dorsoventral = −2.20 mm) gradually using a Hamilton syringe.

4.2.7 Morris water maze test

A modification of the water maze procedure described by Morris was used to examine cognitive function. A circular tank (diameter 90 cm, height 50 cm; 22 ± 2 ℃ water temperature) was used for the test. The tank consisted of four quadrants filled with water. An escape platform (6 cm diameter and 29 cm height) was submerged 1 cm below the water surface at the center of one of the four quadrants. Each mouse was trained for 4 days to learn and memorize visual cues placed outside the tank, which indicated the platform location. The swimming paths used by each mouse were recorded with a camera connected to a video recorder and a path tracking software (EthoVision; Noldus Information Technology,

Wageningen, The Netherlands). Four trials were performed each day during the 4-day training period. During each trial, each mouse was allowed 60 seconds to find the hidden platform and another 30 seconds to stay on the platform. If the mouse was unable to find the platform within 60 seconds, it was guided to it and allowed to remain on it for 30 seconds. The mean time (mean escape latency) that each mouse took to find the platform was recorded. The probe test was conducted after 4 days in same ways without platform. Each mouse was allowed 60 seconds freely and recorded. The video was analyzed using tracking software (EthoVision; Noldus Information Technology, Wageningen) to count the time spent in target quadrant area and platform area and the number of crossing.

4.2.8 Novel object recognition test

I used a modified novel object recognition test. This test incorporates the mouse's natural tendency to explore novel stimuli. During a habituation session performed 2 days before testing, the mice were allowed to explore (for 10 min) a test environment consisting of an empty opaque, custom-made Plexiglas box (35 cm × 45 cm × 25 cm). The sample object phase was performed 24 h later. Two identical white circular

cylinders (the sample objects) were placed on the facing edge in the test environment, and the mice were given access to the objects for 10 minutes. After 24 h (the novel object phase), one of the sample objects in the test environment was replaced with a similar-size novel object (a colored miniature animal), and the mice were given 5 minutes of contact with this new arrangement. The amount of time that a mouse's nose was <1 cm from an object was considered as the time that the mouse navigated the object. The amount of time that the mouse stood on the object was excluded. The discrimination ratio was the time used to navigate the novel object over the time used to navigate both objects.

4.2.9 Object location test

Object location test was conducted in same manner as described novel object recognition test. Last day of object location test, one of the two sample objects was moved to a different location.

4.2.10 Western blot analysis

Tissue lysates were prepared by homogenizing the tissues in radioimmunoprecipitation assay buffer (Cell Signaling, Danvers, MA,

USA). Tissue protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Western blot analyses were performed on 40 μg protein. Briefly, samples were separated using 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred to a polyvinylidene fluoride membrane (Merck Millipore, Burlington, MA, USA; 0.4 μm). The membranes were blocked in 5% bovine serum albumin (Bovogen Biologicals, Keilor East, Australia) in tris-buffered saline and Tween-20 (Junsei Chemical, Tokyo, Japan) and incubated overnight at 4 °C with primary antibodies against APP/Aβ (6E10, Biolegend, San Diego, CA, USA; 1:1000), tau (HT7) (Thermo, Waltham, MA, USA; 1:1000), p-tau (AT8) (Thermo, 1:1000), BDNF (Thermo, 1:1000), TrkB (Thermo, 1:1000), p-TrkB (Thermo, 1:1000), CREB (Cell Signaling, 1:1000), p-CREB (Cell Signaling, 1:1000), Calpain (Thermo, 1:1000), p-GSK3 (Thermo, 1:1000) and GAPDH (Cell Signaling, 1:1000). After incubation with horseradish peroxidase-conjugated secondary antibodies (Sigma, Burlington, MA, USA; 1:5000) for 1 h at room temperature, immunodetection was performed using an enhanced chemiluminescence detection kit (GE Healthcare, Chicago, IL, USA). Rosen RF et al.'s protocol was used for the detection of Aβ

multimers.

4.2.11 Statistical analysis

SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. The results were presented as mean ± standard error of the mean (SEM) values. All the data were analyzed using by Student's t-test.

4.3 Results

4.3.1 α-Spinasterol treatment decreases neuronal apoptosis and Ca^{2+} levels in primary neurons

Primary cortical neurons derived from ICR mice were cultured for 6 days and treated with $A\beta_{1-42}$ (5 μM) and/or α-spinasterol (0.3–5 μM) for 24 h. The results of MTT are shown in Fig. 4-1A. Cell viability was markedly reduced in primary neurons treated with $A\beta_{1-42}$ (5 μM), and α-spinasterol (0.3–5 μM) significantly mitigated the cell viability (Fig. 4-1A). I used fluorescence Ca^{2+} imaging to examine the effect of α-spinasterol on intracellular Ca^{2+} levels with Fluo-4 AM. Fluo-4-positive Ca^{2+} levels were significantly increased in $A\beta_{1-42}$-treated neurons compared with PBS-

treated neurons, and this increase was significantly mitigated by 1.2 μM α-spinasterol treatment (Fig. 4-1B – 1C).

Figure 4-1

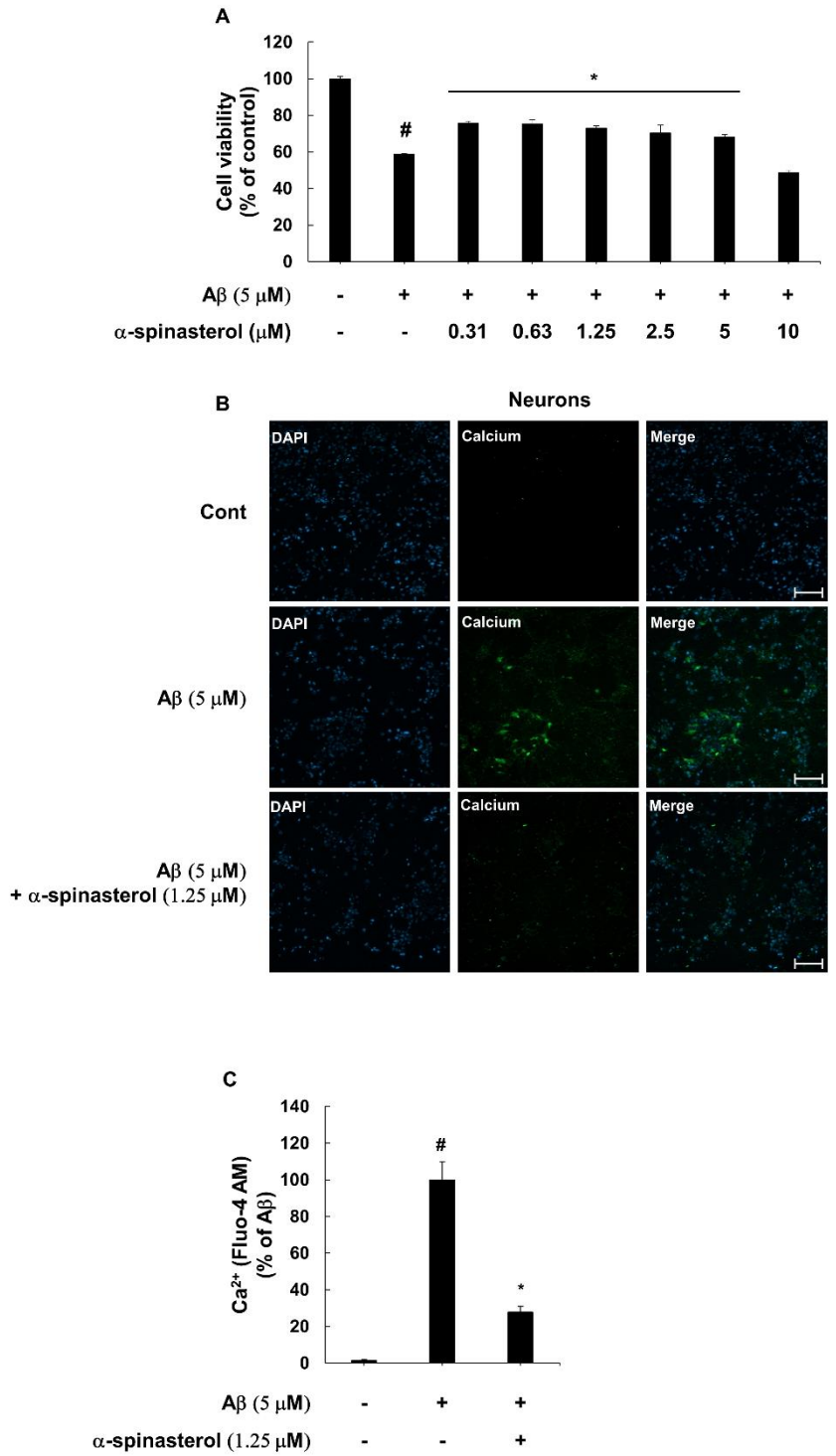
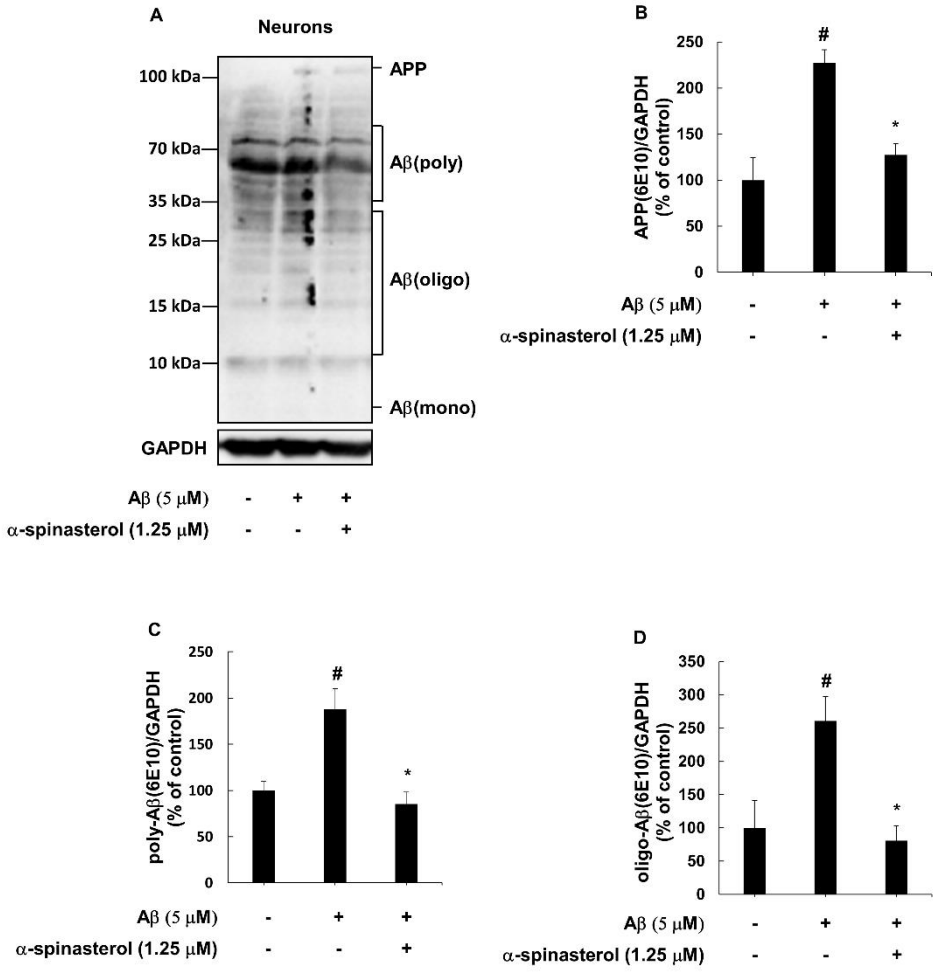

A
Cell viability (% of control)
120
100
80
60
40
20
0
#
*
Aβ (5 μM) - + + + + + + +
α-spinasterol (μM) - - 0.31 0.63 1.25 2.5 5 10
B
Neurons
DAPI
Calcium
Merge
Cont
Aβ (5 μM)
Aβ (5 μM)
+ α-spinasterol (1.25 μM)
C
Ca2+ (Fluo-4 AM) (% of Aβ)
140
120
100
80
60
40
20
0
#
*
Aβ (5 μM) - + +
α-spinasterol (1.25 μM) - - +

Fig. 4-1. Effects of α-spinasterol on neuroprotection and Ca^{2+} levels in primary neurons. (**A**) Neuroprotective effect of α-spinasterol and (**B**) representative fluorescence images of Fluo-4 AM-positive Ca^{2+}. Nuclei were stained using DAPI. Scale bar = 100 μm. (**C**) Fluorescence Ca^{2+} intensities. (mean ± SEM, #$p < 0.05$ vs. PBS-treated, *$p < 0.05$ vs. $A\beta_{1-42}$-treated, one-way ANOVA followed by Student's t-test; $n = 3$ per group).

4.3.2 α-Spinasterol treatment decreases Aβ and p-tau levels in primary neurons

The effect of α-spinasterol on Aβ aggregation and tau hyperphosphorylation was investigated in primary cortical neurons with Western blot analysis. The levels of APP, polymeric Aβ, and oligomeric Aβ were significantly increased in $A\beta_{1-42}$-treated neurons compared with PBS-treated neurons, and these increases were significantly reduced by treatment with α-spinasterol (Fig. 4-2A). The p-tau levels in $A\beta_{1-42}$-treated neurons were also significantly higher than those in PBS-treated neurons, and this increase was significantly reduced by α-spinasterol treatment (Fig. 4-2B).

Figure 4-2


A
Neurons
100 kDa
70 kDa
35 kDa
25 kDa
15 kDa
10 kDa
APP
Aβ(poly)
Aβ(oligo)
Aβ(mono)
GAPDH
Aβ (5 μM) - + +
α-spinasterol (1.25 μM) - - +
B
APP(6E10)/GAPDH (% of control)
250
200
150
100
50
0
#
*
Aβ (5 μM) - + +
α-spinasterol (1.25 μM) - - +
C
poly-Aβ(6E10)/GAPDH (% of control)
250
200
150
100
50
0
#
*
Aβ (5 μM) - + +
α-spinasterol (1.25 μM) - - +
D
oligo-Aβ(6E10)/GAPDH (% of control)
350
300
250
200
150
100
50
0
#
*
Aβ (5 μM) - + +
α-spinasterol (1.25 μM) - - +

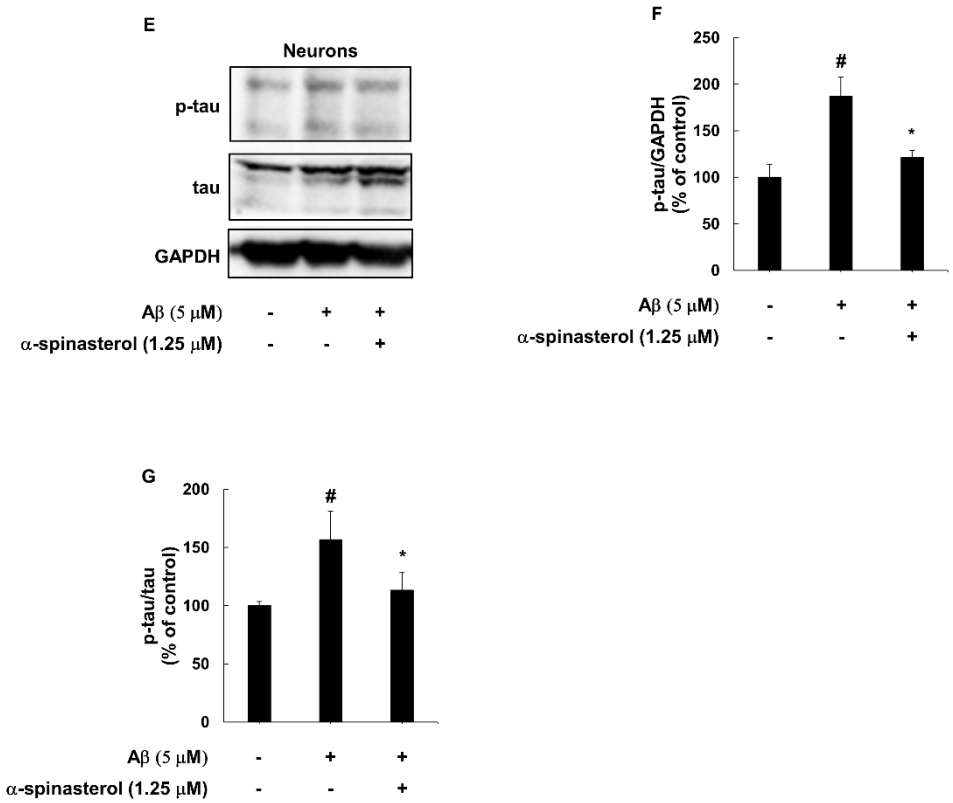


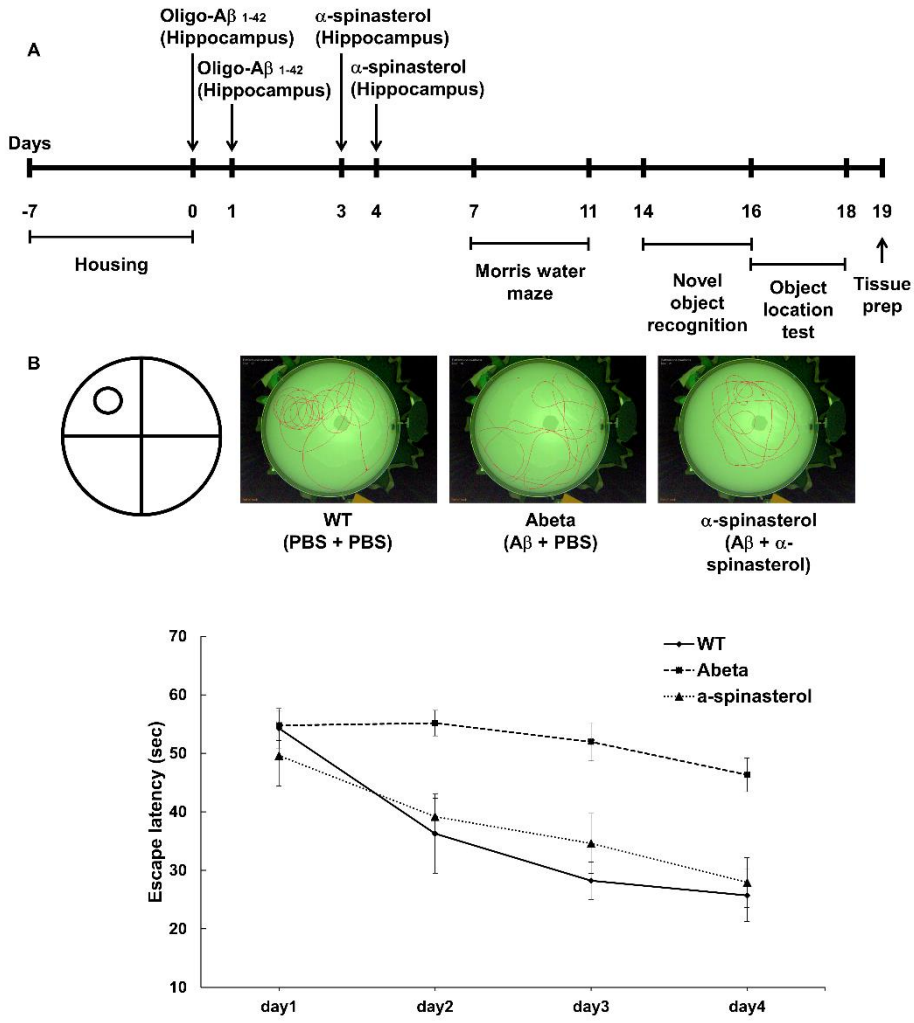
Fig. 4-2. Effects of α-spinasterol on Aβ and p-tau levels in primary neurons. (**A**) Representative Western blots of APP/Aβ proteins. **(B-D)** Western blot densitometry results for (**B**) APP, (**C**) polymeric Aβ, and (**D**) oligomeric Aβ. (**E**) Representative Western blots of tau and p-tau protein. **(F-G)** Western blot densitometry results for (**F**) p-tau, (**G**) p-tau/tau. (mean ± SEM, #p < 0.05 vs. PBS-treated, *p < 0.05 vs. $A\beta_{1\text{-}42}$-treated, one-way ANOVA followed by Student's t-test; n = 5 per group)

4.3.3 α-Spinasterol treatment ameliorates learning and memory loss in $A\beta_{1-42}$-treated mice

To investigate the effect of α-spinasterol on AD pathogenesis, mice were unilaterally infused with $A\beta_{1-42}$ (3 μg/mouse) or PBS in the hippocampus for 2 days. After $A\beta_{1-42}$ injection, α-spinasterol (3.75 μmols/mouse) or PBS was injected into in the hippocampus for learning and memory function improvement. The experimental schedule for behavioral tests is summarized in Figure 4-3A. $A\beta_{1-42}$-treated mice learned to locate the submerged platform more slowly during training sessions compared with PBS-treated mice, which was improved less throughout the training. However, mice treated with α-spinasterol + $A\beta_{1-42}$ demonstrated significant improvement than mice treated with $A\beta_{1-42}$ alone (Fig. 4-3B). The probe test at day 5 showed that $A\beta_{1-42}$-treated mice remained to have significantly shorter durations in the target quadrant and platform area than PBS-treated mice. In $A\beta_{1-42}$-treated mice, α-spinasterol treatment significantly increased the time spent in the target quadrant and platform area (Fig. 4-3C and 4-D). The number of crossings of the platform area was significantly reduced in $A\beta_{1-42}$-treated mice compared with that in PBS-treated mice. α-Spinasterol treatment prevented the decrease in the

number of crossings by $A\beta_{1-42}$-treated mice (Fig. 4-3E). During the novel object phase, $A\beta_{1-42}$ + α-spinasterol-treated mice spent more time examining the novel object and had significantly higher discrimination ratios than $A\beta_{1-42}$-treated mice (Fig. 4-3F). At the object location test, $A\beta_{1-42}$ + α-spinasterol-treated mice spent more time investigating displaced objects and exhibited significantly higher discrimination ratios than $A\beta_{1-42}$-treated mice (Fig. 4-3G).

Figure 4-3


A
Oligo-Aβ 1-42 (Hippocampus)
α-spinasterol (Hippocampus)
Oligo-Aβ 1-42 (Hippocampus)
α-spinasterol (Hippocampus)
Days
-7
0
1
3
4
7
11
14
16
18
19
Housing
Morris water maze
Novel object recognition
Object location test
Tissue prep
B
WT (PBS + PBS)
Abeta (Aβ + PBS)
α-spinasterol (Aβ + α-spinasterol)
Escape latency (sec)
70
60
50
40
30
20
10
day1
day2
day3
day4
WT
Abeta
a-spinasterol

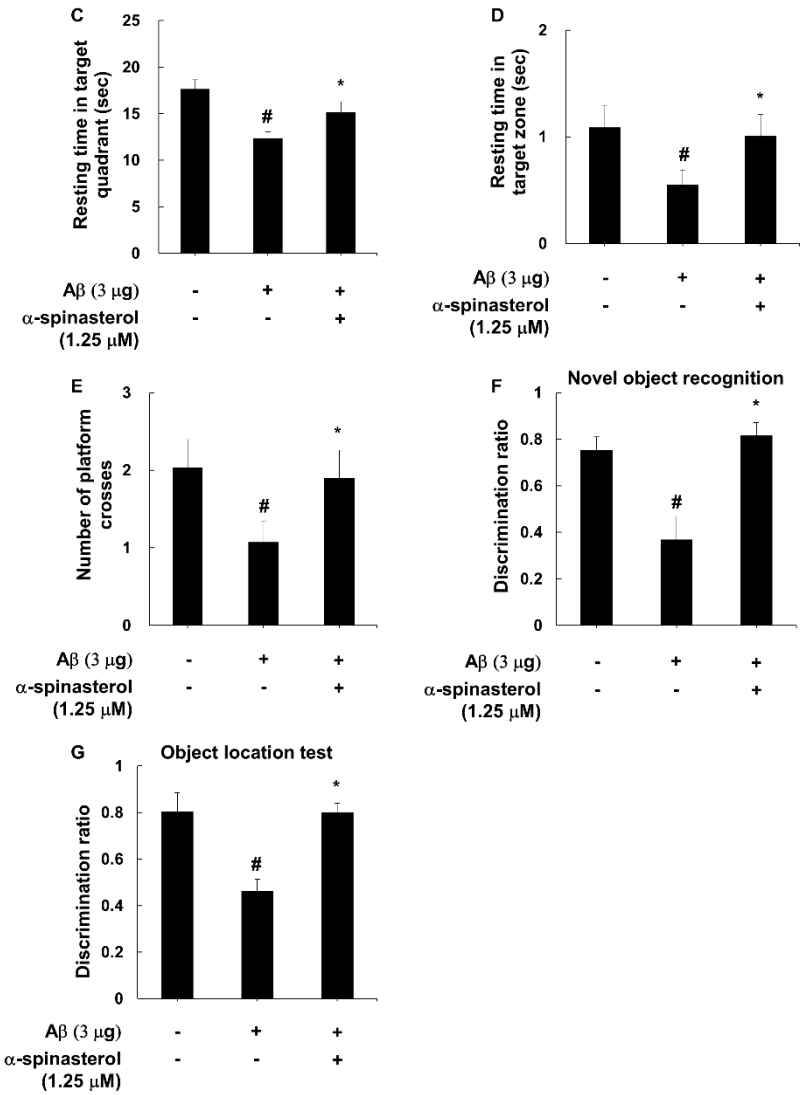


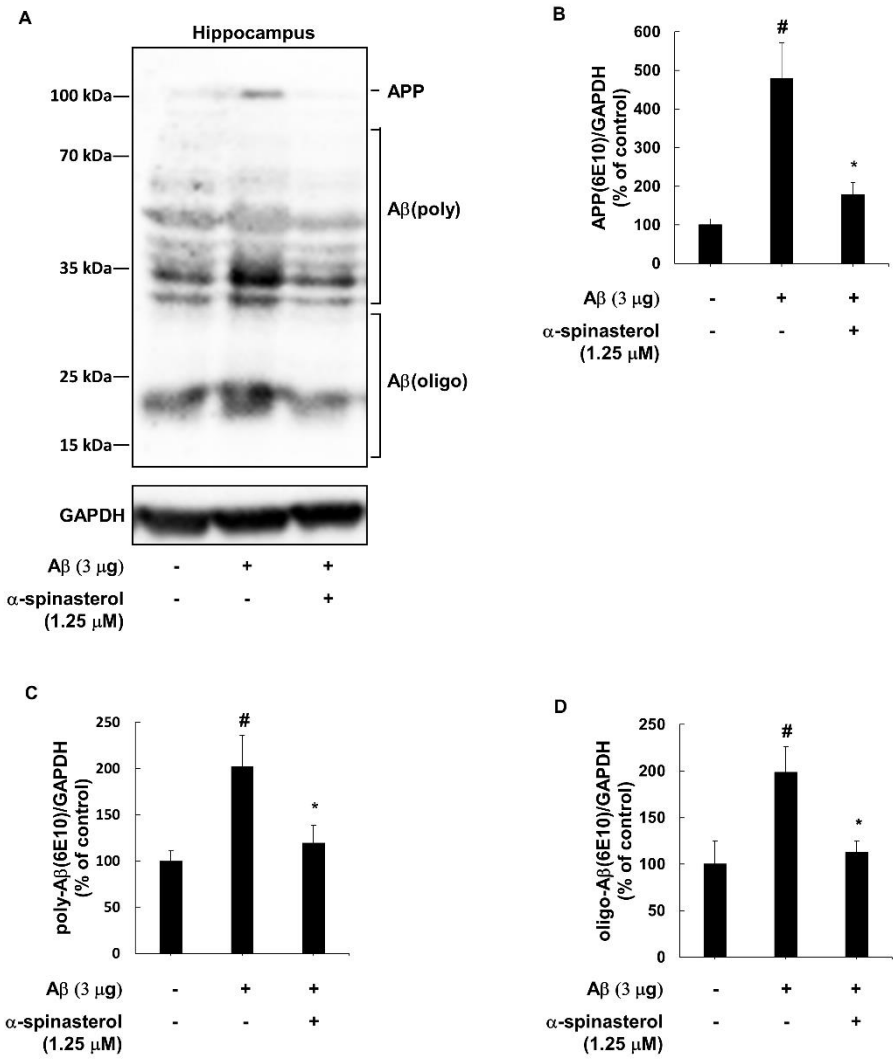
Fig. 4-3. Effects of α-spinasterol on learning and memory in $A\beta_{1-42}$-treated mice. (**A**) Schedule used for behavioral tests. (**B**) Mean escape latency results for Morris water maze test. Time spent in (**C**) target quadrant area and (**D**) platform area. (**E**) Number of cross in platform area. (**F**) Discrimination ratio results for novel object recognition test. (**G**)

Discrimination ratio results for object location test. (mean ± SEM, #$p < 0.05$ vs. PBS-treated, *$p < 0.05$ vs. $A\beta_{1\text{-}42}$-treated, one-way ANOVA followed by Student's t-test; $n = 10$ per group)

4.3.4 α-Spinasterol decreases Aβ and p-tau levels in the hippocampus of $A\beta_{1-42}$-treated mice

From five mice of each group, hippocampal tissues were taken 19 days after the first $A\beta_{1-42}$ infusion. Then, the effect of α-spinasterol on hippocampal Aβ formation and tau hyperphosphorylation was investigated in an AD mouse model using Western blot analysis. The results of the Western blot analysis indicated that compared with PBS-treated mice, $A\beta_{1-42}$-treated mice had significantly increased hippocampal levels of APP, polymeric Aβ, and oligomeric Aβ, and these increases were significantly mitigated by α-spinasterol (Fig. 4-4A–4D). Hippocampal p-tau levels in $A\beta_{1-42}$-treated mice were significantly higher than those in PBS-treated mice, and this increase was significantly reduced by α-spinasterol treatment (Fig. 4-4E–4G).

Figure 4-4


A
Hippocampus
100 kDa
70 kDa
35 kDa
25 kDa
15 kDa
APP
Aβ(poly)
Aβ(oligo)
GAPDH
Aβ (3 μg) - + +
α-spinasterol (1.25 μM) - - +
B
APP(6E10)/GAPDH (% of control)
600
500
400
300
200
100
0
#
*
Aβ (3 μg) - + +
α-spinasterol (1.25 μM) - - +
C
poly-Aβ(6E10)/GAPDH (% of control)
250
200
150
100
50
0
#
*
Aβ (3 μg) - + +
α-spinasterol (1.25 μM) - - +
D
oligo-Aβ(6E10)/GAPDH (% of control)
250
200
150
100
50
0
#
*
Aβ (3 μg) - + +
α-spinasterol (1.25 μM) - - +

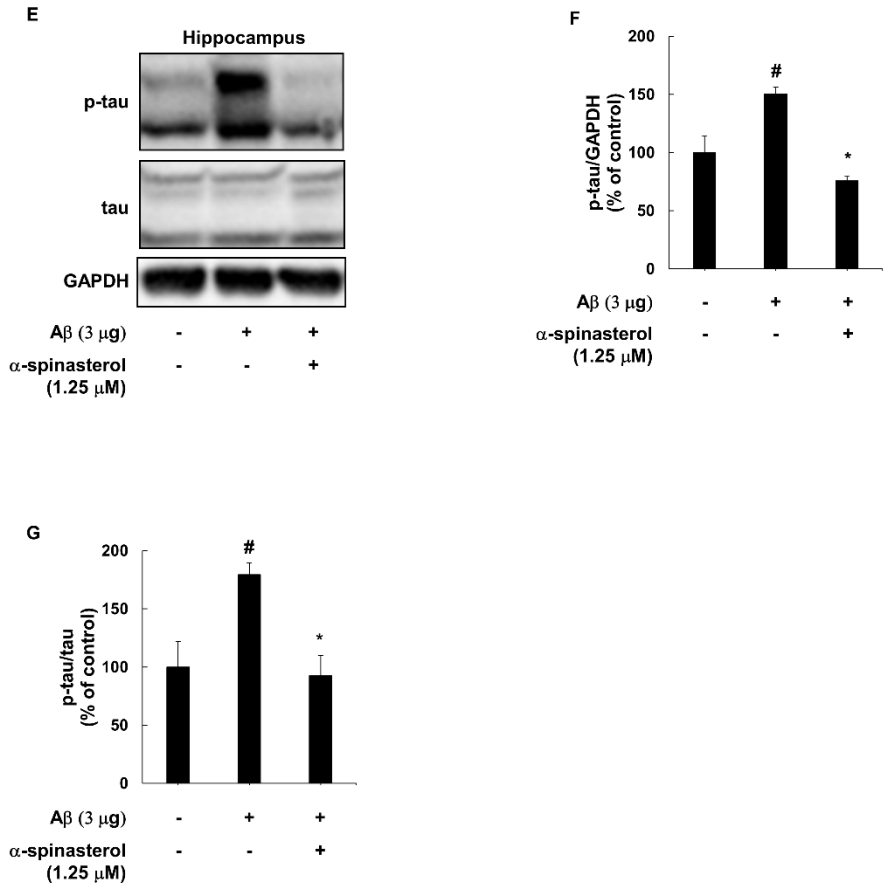


Fig. 4-4. Effects of α-spinasterol on hippocampal Aβ and p-tau levels in $A\beta_{1\text{-}42}$-treated mice. (**A**) Representative Western blots of APP/Aβ proteins. **(B-D)** Western blot densitometry results for (**B**) APP, (**C**) polymeric Aβ, and (**D**) oligomeric Aβ. (**E**) Representative Western blots of tau and p-tau protein. **(F-G)** Western blot densitometry results for (**F**) p-tau, (**I**) p-tau/tau. (mean ± SEM, #$p < 0.05$ vs. PBS-treated, *$p < 0.05$ vs. $A\beta_{1\text{-}42}$-treated, one-way ANOVA followed by Student's t-test; $n = 5$ per group)

4.3.5 α-Spinasterol treatment promotes the BDNF/CREB signaling pathway in the hippocampus of $A\beta_{1-42}$-treated mice

Learning, memory development, synaptic plasticity, and neuroprotection are all significantly influenced by brain-derived neurotrophic factor (BDNF) and its receptor protein p-TrkB [9, 10]. Because CREB is required for the regulation of BDNF, it is crucial for the development of memories [11]. Changes in Ca^{2+} concentration regulate CREB phosphorylation, but excessive Ca^{2+} concentration causes p-CREB to become dephosphorylated and stop functioning [12]. I investigated the effect of α-spinasterol on BDNF, p-TrkB, and p-CREB levels in the hippocampus of the AD mice model by Western blot analysis. $A\beta_{1-42}$-treated mice had significantly decreased hippocampal BDNF, p-TrkB, and p-CREB levels compared with PBS-treated mice. However, hippocampal BDNF, p-TrkB, and p-CREB levels were significantly increased by α-spinasterol treatment (Fig. 4-5A – 5D).

Figure 4-5

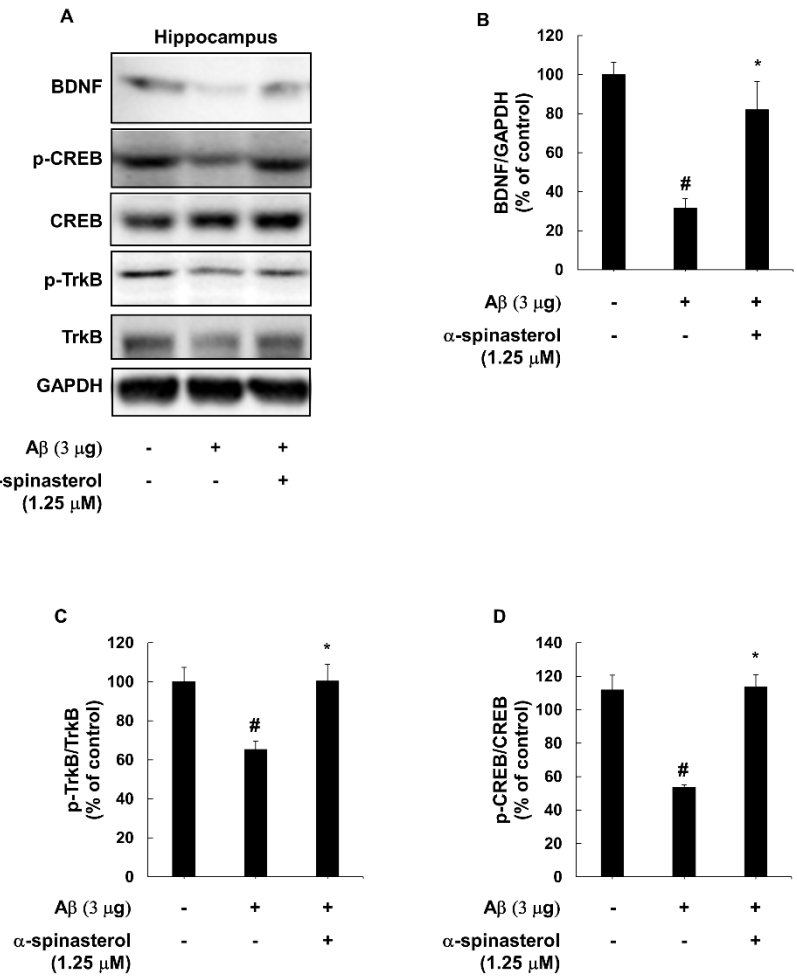


Fig. 4-5. Effects α-spinasterol on the BDNF/CREB signaling in $A\beta_{1-42}$-treated mice. (**A**) Representative Western blots of BDNF, p-CREB and p-TrkB proteins. **(B-D)** Western blot densitometry results for **(B)** BDNF, (**C**) p-TrkB/TrkB and (**D**) p-CREB/CREB. (mean ± SEM, #p < 0.05 vs. PBS-treated, *p < 0.05 vs. $A\beta_{1-42}$-treated, one-way ANOVA followed by Student's t-test; n = 5 per group)

4.4. Discussion

Previously, I found that TRPV1 deficiency influences memory function and levels of Aβ and tau, which is a distinctive feature of AD progression. TRPV1 deficiency decreased the levels of Ca^{2+}, Aβ and tau in the hippocampus of 3 × Tg-AD, and reduced memory loss is shown in 3 × Tg-AD. In addition, the effect of protecting nerve cells is observed through BDNF/CREB signaling pathway activation. I investigated whether the TRPV1 antagonist α-spinasterol, reduces the symptoms of AD in both in vivo and in vitro using an AD model. α-Spinasterol decreases Aβ and p-tau levels and increases BDNF and p-TrkB levels in the hippocampus of $A\beta_{1-42}$-treated mice and reduced memory loss in $A\beta_{1-42}$-treated mice. In addition, α-spinasterol decreases the level of Ca^{2+} and shows neuroprotective effects against $A\beta_{1-42}$ in primary neurons.

Sustained disruption of intracellular Ca^{2+} signaling appears to play an early proximal and possibly central role in AD pathogenesis [13-15]. Neuronal dysfunction and degeneration in brain aging and AD are caused by changes in intracellular Ca^{2+} handling by the endoplasmic reticulum (ER) and mitochondria, as well as impaired intra-/extracellular cation exchange [16, 17].

An Aβ-induced inflow of Ca^{2+} elicits the characteristic AD lesions, including neuronal apoptosis and neurofibrillary tangles [18-20]. Brain tissues from patients with AD showed significantly increased levels of Ca^{2+} [21]. Recently, antagonists targeting various Ca^{2+} receptors as AD treatments are undergoing clinical trials [22]. These findings imply that pharmacological inhibition of Ca^{2+}-permeable receptors may help to prevent or treat AD by modulating Ca^{2+} homeostasis, which is essential for neuronal viability and function.

The activation of TRPV1 induces the accumulation of intracellular Ca^{2+}, and TRPV1 agonists cause excessive mitochondrial Ca^{2+} loading in neurons, resulting in apoptosis [23, 24]. TRPV1 mediates neurotoxicity in mesencephalic dopaminergic neurons in vivo and in vitro, and neurotoxicity was accompanied by increased intracellular Ca^{2+} via influx through TRPV1, mitochondrial disruption, and cytochrome c release and caspase-3 activation [25]. On the contrary, acute restraint stress increases the expression of TRPV1 in the hippocampus, and TRPV1 antagonism reverses the behavioral deficits induced by stress in rats [26]. TRPV1 deficiency also has behavioral anxiolytic and antidepressant-like effects [27]. Thus, it was proposed that the pharmacological suppression of TRPV1 might offer a novel strategy for

the treatment of psychiatric illnesses and diseases associated with aging [26-29].

α-Spinasterol, a Ca^{2+}-permeable TRPV1 receptor antagonist, lowers the production of Aβ and p-tau and promoted the expressions of BDNF and p-TrkB in the hippocampus of $A\beta_{1-42}$-treated mice. In addition, α-spinasterol rescues memory deficits in $A\beta_{1-42}$-treated mice. Moreover, α-spinasterol modulates Ca^{2+} influx and exhibits neuroprotective effect against $A\beta_{1-42}$ in primary neurons. These results suggest that α-spinasterol alleviates AD by modulating Ca^{2+} homeostasis, which is fundamental to neuronal viability and functioning.

Through the genetic deficiency of TRPV1, not only the potential of AD as a treatment target, but also the possibility of a new AD treatment material, was confirmed using α-spinasterol, which is a known TRPV1 antagonist and a major component of spinach. Through this study, the role of TRPV1 in the mechanism of AD onset was investigated, and its mechanism of action was revealed. In addition, I demonstrated the potential of antagonist targeting TRPV1 as a target for the development of new AD treatments. In the future, I intend to develop a new AD treatment through research from derivatives to formulations using α-spinasterol.

Figure 4-6

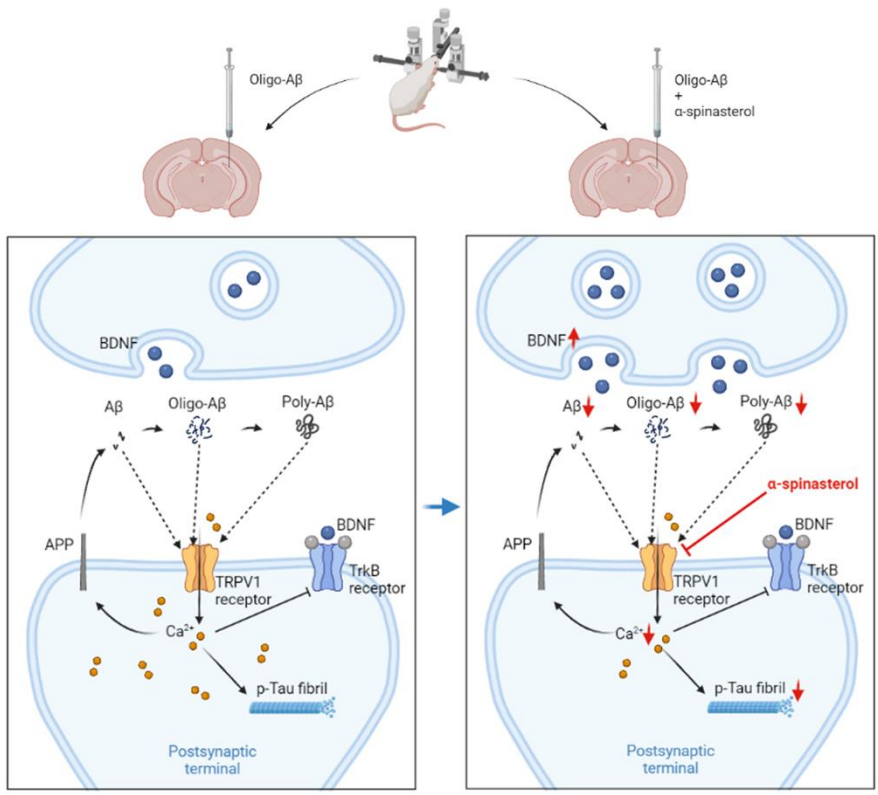


Fig. 4-6. Molecular pathway of anti-AD effect through α-spinasterol in $A\beta_{1-42}$-treated mice. Ca^{2+} is overloaded in $A\beta_{1-42}$-treated mice and as a result, the formation of amyloid beta and phosphorylation of tau are promoted (left). In α-spinasterol-treated mice, Ca^{2+} level is normalized and the formation of amyloid beta and phosphorylation of tau are prevented by activation of BDNF/CREB pathway (right). (Created with BioRender.com).

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국문초록

세계 보건기구 (WHO)에 따르면 전 세계적으로 5 천 5 백만 명 이상의 사람들이 치매를 앓고 있으며, 매년 1 천만 명 이상의 치매환자가 발생한다. 치매는 고령의 사람들에게서 주로 발병하며 전 세계적으로 고령화가 진행됨에 따라 치매의 발병률은 더 커질 것으로 예상된다. 치매의 가장 큰 증상은 인지기능 및 기억력 손상으로 치매환자가 주변 사람에게 의존하게 되게 된다. 이 때문에 치매는 치료뿐만 아니라 관리가 필요한 질병으로 이의 사회적 비용이 매우 큰 질병이다. 따라서 치매는 치매환자뿐만 아니라 주변 사람들에게도 영향을 미쳐 사회 전반적으로 큰 문제점으로 작용한다.

알츠하이머성 치매 (Alzheimer's disease)는 치매에서 가장 많이 발병되는 질병으로 치매환자 중 70-80%가 알츠하이머성 치매이다. 알츠하이머성 치매는 1901 년 Alois Alzheimer 에 의해 발견된 이후 약 100 년이 지났지만 적절한 치료법이 있지 않다. 알츠하이머성 치매환자의 뇌에서 Amyloid-β 와 p-tau 의 양이

정상사람보다 많다는 것이 알려짐에 따라 Amyloid-β 와 p-tau 는 알츠하이머성 치매의 진단 기준으로 사용되어왔다.

현재 알츠하이머성 치매의 발병 이유는 명확하지는 않지만 2 가지 유력한 가설이 있다. Amyloid-β 와 p-tau 가설로서 이 2 가지 가설은 Amyloid-β 와 p-tau 가 쌓여감에 따라 독성을 유발하고 이에 뇌에서 신경세포 사멸과 염증을 일으켜 인지기능 및 기억력의 저하를 가져온다는 것이다. 이 가설에 따라 Amyloid-β 와 p-tau 의 항체를 개발하여 Amyloid-β 와 p-tau 를 제거하는 기전으로 치료제 개발 연구가 이루어졌다. Aducanumab (Aduhelm^{tm})은 Amyloid-β 항체 치료제로 2022 년 FDA 에서 최초로 알츠하이머성 치매 치료제 승인을 받은 치료제이다. Aducanumab 에 의하여 Amyloid-β 가 제거되었지만 기억력 및 인지능력 개선의 효능은 보이지 않았다.

최근 칼슘 항상성 이상이 알츠하이머성 치매의 가장 큰 원인이다는 연구가 보고되었다. 알츠하이머성 치매환자의 뇌의 칼슘 농도가 정상인 보다 높아져 있다고 알려져 있다. 칼슘은

신경 세포에서 다양한 신호와 효소 활성 등에 영향을 주며, 특히 신경세포에서 칼슘은 신경전달물질로 작용하여 기억력 형성에 매우 중요한 역할을 수행한다. 알츠하이머성 치매의 치료제로 승인 받은 memantine 의 경우 칼슘 이온 통로인 N-methyl-D-aspartate (NMDA)를 저해하여 칼슘 농도를 낮추는 기전이다. 따라서 칼슘의 농도를 낮추는 것이 알츠하이머성 치매의 치료 효과를 보여준다.

The transient receptor potential vanilloid 1 (TRPV1)은 칼슘이온 통로로 말초신경계 뿐만 아니라 해마와 대뇌 등 중추신경계에도 존재한다. TRPV1 은 우울증과 불안 등 신경계통에 관여하며, TRPV1 기능이 저하될 시 신경과 시냅스 가소성이 발달된다는 연구결과가 있다. 본 연구 결과에 따르면 치매 동물 모델 (3 × Tg-AD)에서 TRPV1 을 유전적으로 결핍시켰을 때, 뇌의 칼슘 농도가 낮아지고 Amyloid-β 와 p-tau 의 생성과 신경세포사멸이 억제됨을 확인하였다. 또한 인지행동학적 실험에서 기억력과 공간 및 사물의 인지능력이 향상됨을 확인하였으며, 이의 원인이

칼슘 항상성 회복에 의한 BDNF/CREB signaling 의 활성화에 의한 것임을 확인하였다.

이를 통하여 TRPV1 의 길항제를 통해 칼슘 농도를 저하시키면 알츠하이머성 치매에 치료효과를 거둘 것이라 가설을 세울 수 있었다. 새로운 알츠하이머성 치매의 치료 소재를 개발하기 위하여 TRPV1 의 길항제이자 시금치의 주요 성분으로 알려진 α-spinasterol 를 활용하여 알츠하이머성 치매 치료 효능을 확인하고자 하였다. 그 결과 α-spinasterol 을 뇌에 투여 시 기억력과 공간 및 사물의 인지능력이 향상됨을 확인하였으며, 칼슘 농도의 저하에 따른 Amyloid-β 와 p-tau 의 생성 억제와 BDNF/CREB signaling 의 활성에 따른 신경세포 사멸 억제를 확인하였다.

본 연구는 칼슘 이온 통로인 TRPV1 이 유전적으로 결핍되면 인지 및 기억력 향상과 Amyloid-β 와 p-tau 의 생성이 억제되는 것을 확인하였으며, 이의 작용 기전이 칼슘 농도 저하에 의한 BDNF/CREB signaling 의 활성화에 의한 것임을 밝혀내었다. 또한

TRPV1 길항제이자 시금치의 주요 성분인 α-spinasterol 을 활용하여 알츠하이머성 치매 치료 효능을 확인하여 새로운 알츠하이머성 치매 치료 소재의 가능성을 확인하였다. 알츠하이머성 치매 동물 모델에서 TRPV1 의 유전적 결핍을 통해 이의 역할을 규명하였으며, 길항제인 α-spinasterol 를 활용하여 알츠하이머성 치매 치료 효능을 밝혀내는 통합적인 연구를 수행하였다.