# Gonadotropin-releasing hormone (GnRH) treatment in a mouse model of Alzheimer's disease: Reducing amyloid pathology and promoting neurogenesis

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# Gonadotropin-releasing hormone (GnRH) treatment in a mouse model of Alzheimer's disease: Reducing amyloid pathology and promoting neurogenesis

**A Master's Thesis Submitted**
**to the Department of Medical Science**
**and the Graduate School of Yonsei University**
**in partial fulfillment of the**
**requirements for the degree of**
**Master's of Medical Science**

**Yumi Son**

**January 2025**

**This certifies that the Master's Thesis of Yumi Son is approved**

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**January 2025**

# ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my thesis supervisor, Professor Eosu Kim, for his invaluable direction and mentorship. He has taught me what it means to think like a scientist and guided me with clear logic and reasoning. Every piece of advice he provided offered profound insight and invaluable lessons that have greatly enriched my academic journey.

I am deeply thankful to Professor Hyunjeong Kim for her meticulous guidance in conducting scientific experiments and interpreting data as a scientist. Her detailed instructions and support have been instrumental in refining my experimental approach and understanding of scientific analysis.

I would also like to thank Professor Ki Woo Kim for his thoughtful engagement with my work. His willingness to pose scientific questions has provided invaluable insights and clear direction, helping me analyze more broadly and deeply.

I deeply appreciate all members of my laboratory colleagues in the Laboratory for Alzheimer's Molecular Psychiatry (LAMP). I would like to particularly thank PhD So Yeon Cho for her extensive support and guidance on my dissertation topic.

I would also like to extend my deepest gratitude to my parents and my brother, who have always respected and supported every choice I have made. Their unwavering belief in me and encouragement to follow the path I desire have been the driving force behind my perseverance on this journey.

Finally, my experience in this lab has been one of the most meaningful and valuable periods of my life. It has been instrumental in guiding me toward the path I want to pursue. The memories and lessons I have gained will remain with me as a treasured part of my journey.

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## ABSTRACT

# Gonadotropin-releasing hormone (GnRH) treatment in a mouse model of Alzheimer's disease: Reducing amyloid pathology and promoting neurogenesis

**Purpose of Study**: Gonadotropin-releasing hormone (GnRH) is primarily known for its role in regulating sexual maturation and fertility. Both GnRH and its receptor, GnRHR, are observed in various regions of the central nervous system and peripheral tissues, suggesting that GnRH may have functions beyond reproduction. Recent studies indicate that GnRH may enhance memory and cognitive functions, as well as exhibit neurogenic effects. These previous findings suggest the potential of GnRH as a therapeutic option for Alzheimer's disease (AD). Therefore, I investigated the impact of long-term GnRH administration on amyloid pathology, neuroinflammation, neurogenesis, and cognitive function in 5xFAD mice, a model of AD.

**Method**: The expression of GnRH and GnRHR was characterized in various brain regions using immunofluorescence and western blot analysis. A dose of 2 ng/kg of GnRH was subcutaneously injected into both female and male 5xFAD mice over 8 weeks. This was followed by behavioral assessments, immunohistochemistry for amyloid and glial markers, and quantification of neurogenesis and neuroplasticity factor.

**Results**: GnRH treatment significantly reduced amyloid-beta (Aβ) levels, which are key contributors to AD, particularly in male mice. Furthermore, GnRH treatment significantly decreases neuroinflammation by reducing microglial activation, a major factor in the progression of AD. Additionally, GnRH treatment promotes neurogenesis and neuroplasticity, as evidenced by an increased number of doublecortin (DCX)-positive cells

and elevated levels of brain-derived neurotrophic factor (BDNF). However, significant behavioral effects were not observed on cognitive functions as assessed by the novel object recognition test, Y-maze, three-chamber test, and passive avoidance test.

**Conclusion**: These findings suggest that GnRH administration may serve as a promising therapeutic strategy for slowing the progression of AD and enhancing neurogenic effects. Furthermore, the study suggests that abnormalities in GnRH homeostasis may be associated with neurodegeneration.

**Keywords**: Gonadotropin-releasing hormone (GnRH), 5xFAD, anti-dementia, neurogenesis, neuroplasticity, neuroinflammation, cognitive function, neuroprotection

# I. Introduction

## 1. The novel role of GnRH

Gonadotropin-releasing hormone (GnRH) is primarily recognized for its role in regulating sexual maturation and fertility. GnRH is produced and secreted by the hypothalamus, where it acts on the anterior pituitary gland to stimulate the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Ultimately, it stimulates the secretion of sex hormones, including estrogen, progesterone, and testosterone, from the gonads.[1] GnRH levels are likely to correlate with those of estrogen[2], FSH[3], and LH[4] levels, all of which have already established associations with cognitive function. Interestingly, GnRH modulation in the hypothalamus is important for aging in male mice[5]. Research has demonstrated that restoration of GnRH pulsatility improves lifespan, recognition memory, locomotion, body coordination, and age-related physiology features[5]. This research aimed to indicate that GnRH may have functions beyond its well-characterized role in reproduction, such as anti-aging and cognitive regulation.

Rather than limiting its scope to the hypothalamus, it is essential to consider that GnRH may have roles in other regions, potentially serving various functions. Both GnRH and its receptor (GnRHR) are also expressed in several areas outside the hypothalamus within the central nervous system (CNS)[1], suggesting its role in these regions as well. Indeed, recent studies indicate that GnRH may enhance memory and cognitive functions.

The maintenance of the GnRH system appears to play a role in brain development and supporting cognitive functions[6]. Restoring the physiological GnRH secretion pattern in the Down syndrome (DS) mouse model and patients experiencing a deficiency of GnRH neurons has been shown to ameliorate cognitive deficits[6].

Another study demonstrated that GnRH prevented Aβ-induced memory deficits in rats while increasing neuronal excitability in the CA1 region[7]. However, these effects were

attributed to GnRH acting through increased levels of 17β-estradiol. In contrast, the study aims to highlight the direct role of GnRH by showing the expression of both GnRH and GnRHR in the regions associated with cognitive functions, particularly the hippocampus.

There have been cases where the intrinsic role of GnRHR has been indirectly demonstrated through other drugs. One study found that a drug candidate, GV1001, activates GnRHR and their downstream signaling pathways, which resulted in improvement in cognitive and memory abilities in 3xTg-AD mice by inhibiting neuroinflammation and lowering the levels of Aβ oligomers and phospho-tau[8]. It was found that GV1001 mitigates neuroinflammation by facilitating a neuroprotective phenotype in microglia and astrocytes, thereby decreasing the neurotoxic forms. This concept is noteworthy as GnRH could function as a crucial mediator independently of neuroprotective factors.

In a previous study, the administration of GnRH into the hypothalamic third ventricle of aged mice promoted adult neurogenesis in the dentate gyrus (DG) region[5]. The finding that GnRH exerts neurogenic activity within the hippocampus indicates that GnRH may travel within the brain to promote neurogenesis[5], or it may prompt a signal to distant regions.

Other studies indicate that GnRH enhances both the outgrowth and length of neurites accompanied by an increasing the expression of neurofilaments[9]. It is conceivable that GnRH may also be involved in neuronal plasticity parallel to its gonadal function[9].

Given the recently suggested role of GnRH in enhancing cognitive function and promoting neurogenesis, I hypothesize that GnRH may also possess therapeutic potential in AD. Therefore, I sought to investigate whether GnRH administration could reduce the pathological progression of AD and improve cognitive function. More importantly, I assessed whether GnRH could restore neurogenesis, which is typically diminished in AD.

## 2. Mouse model of Alzheimer's disease, 5xFAD

The 5xFAD transgenic mouse model, which overexpresses amyloid precursor protein

(APP) and presenilin 1 (PSEN1) and includes five familial AD variants, is a widely utilized model for studying AD. This model is characterized by early Aβ plaque deposition[10], neuronal and synaptic loss[10], and neuroinflammation[11], all of which contribute to cognitive deficits. By four months of age, 5xFAD mice exhibit increased amyloid pathology and decreased synaptic plasticity, along with subsequent increases in microglial density in the cortex and hippocampus[12]. LAMP1, a marker of dystrophic neurites, increases in proportion to amyloid plaque load[12]. Aβ and amyloid plaques impair long-term synaptic plasticity, learning, memory, and cognitive function[13]. Given these pathological features, this study aims to assess the potential neuroprotective effects of GnRH in 5xFAD mice, focusing on neurogenesis, inflammation, and cognitive function. Experiments were conducted with five male and five female mice per group, including both 5xFAD and control groups. I administered low-dose GnRH subcutaneously over eight weeks, based on previous literature indicating the effectiveness of short-term and low-dose treatment in promoting neuroprotection.

The 5xFAD mice exhibit memory impairments in the Y-Maze and novel object recognition (NOR) tests[11,14], making these models suitable for assessing cognitive improvements. Additionally, the medial septum (MS), which plays a role in social memory[15], shows high expression levels of GnRH and GnRHR, prompting us to conduct the three-chamber test. The passive avoidance test, which assesses learning mediated by the amygdala, hippocampus, and cortical regions[16], was also employed to evaluate cognitive function. Immunofluorescence staining was used to determine the expression of Aβ and beta-site amyloid precursor protein cleaving enzyme 1 (BACE1), a key enzyme involved in the cleavage of amyloid precursor protein (APP) to produce Aβ. The activity of BACE1 is directly linked to the formation of amyloid plaques, which are a hallmark of AD.

Moreover, neurogenesis and neuroplasticity markers were identified through staining. Doublecortin (DCX) has recently been recognized as a marker for neurogenesis, as it is specifically expressed in newly generated neurons[17]. Brain-derived neurotrophic factor

(BDNF), a neuroplasticity marker, plays a critical role in forming various types of memories and is essential for the long-term storage of information in the amygdala, hippocampus, and insular cortex[18]. BDNF may be significant in counteracting memory decline, which is common in aging and exacerbated in neurodegenerative disorders[18]. Therefore, it would be crucial to investigate DCX and BDNF to determine whether GnRH has neurogenic effects.

### 3. AD and GnRH

In 5xFAD mice, significant declines in GnRH-related gene transcription and hypothalamic GnRH mRNA levels were observed, mirroring patterns seen during the transition from youth to middle age[19]. Moreover, the expression of the functional GnRH decapeptide, GnRH variant 1, has shown highest expression in adult mice and has declined with age across various brain regions, including hypothalamus, cerebral cortex, olfactory bulb, striatum, brainstem, and cerebellum[20]. Based on these findings, we hypothesized that GnRH insufficiency in the CNS might contribute to the progression of AD. Furthermore, a recent study revealed that combined administration of GnRH and oxytocin improved cognitive function in 5xFAD mice, alongside a reduction in Aβ levels.[19]

Considering the previous studies, it can be suggested that a GnRH supplement may serve as a treatment option for AD. Therefore, I administered GnRH to 5xFAD mice to evaluate its potential anti-AD and neurogenic effects.

# II. Method

## 1. Laboratory animals

### 1.1. C57BL/6 mice

The mice were maintained in a specific pathogen-free (SPF) facility, free of microorganisms and parasites, and protected from external contamination. They were kept at a temperature of 20-26 ℃ with a humidity of 50±5% in a 12-hour light and dark cycle. The mice were housed in cages enriched with litter and nesting materials, with 3-5 mice per cage to create a natural environment essential for behavioral experiments.

### 1.2. 5xFAD mice

5xFAD mice were maintained under similar SPF conditions as the C57BL/6 mice. To maintain the transgenic line, two C57BL/6 females and one 5xFAD transgenic male were co-housed for mating. Pregnant females were separated for individual breeding once pregnancy was confirmed, and pups were genotyped at 2-3 weeks of age. Homozygous wild-type and heterozygous transgenic offspring were distinguished to ensure sufficient numbers of each genotype for parallel experimentation. The 5xFAD mice overexpress amyloid precursor protein (APP) and presenilin 1 (PSEN1), including five familial AD variants (PSEN1 L286V, PSEN1 M146L, APP KM670/671NL, APP V717I, APP I716V)[21], under the control of the Thy1 mini gene[21]. These mice are used as a model of AD, characterized by the production of Aβ and amyloid plaques in neurons.

### 1.3. Genotyping of 5xFAD mice

Genotyping was performed using standard polymerase chain reaction (PCR) with DNA isolated from ear biopsies. The presence of APP and PSEN1 transgenes was confirmed using specific primers. The primer sequences for APP were: forward (AGG ACT GAC

CAC TCG ACC AG) and reverse (CGG GGG TCT AGT TCT GCA T). For PSEN1, the primer sequences were: forward (AAT AGA GAA CGG CAG GAG CA) and reverse (GCC ATG AGG GCA CTA ATC AT). An internal positive control was used with forward (CTA GGC CAC AGA ATT GAA AGA TCT) and reverse (GTA GGT GGA AAT TCT AGC ATC ATC C) primers.

## 2. Experimental design

### 2.1. Overview

The 5xFAD mice were treated with either 2 ng/kg GnRH dissolved in saline or normal saline as a vehicle. GnRH was administered by subcutaneous injection once daily for eight weeks. The experiments were conducted in eight-month-old mice, considering the progression of dementia pathology in this model. From day 53 to day 58 of GnRH administration, cognitive behavioral assessments were conducted, followed by tissue collection.

### 2.2. Subcutaneous injection

The subcutaneous injection was chosen due to its low vascularization, resulting in slow and continuous absorption of the injected compound. This method is effective for hormones that require sustained delivery at low doses[22]. GnRH was administered at the same time each day, between 9:00 and 10:00 a.m., to maintain consistency.

## 3. Behavioral assessment

### 3.1. Novel object recognition test

The novel object recognition (NOR) test was conducted to evaluate recognition memory.

Mice were allowed to adapt to an open field chamber for 10 minutes in the habituation phase. After 24 hours, mice explored two identical objects for 5 minutes during the familiarization phase. Following 2- and 24-hour delays, the mice were exposed to one familiar and one novel object for 5 minutes each. The discrimination index (DI) was calculated using the formula: $DI = (T_N - T_F) / (T_N + T_F)$, where $T_N$ represents exploration time for the novel object and $T_F$ for the familiar object. This test leveraged the natural preference of mice for novel objects to assess episodic memory, which involves the transition of information from the perirhinal cortex to the hippocampus for long-term storage[23].

### 3.2. Y-maze test

The Y-maze test was used to evaluate spatial working memory. The apparatus consisted of a Y-shaped chamber with three arms forming 120-degree angles. An 'alternation' was defined as consecutive entries into all three arms. Mice were allowed to navigate the maze for 10 minutes freely, and the spontaneous alternation percentage was calculated with this formula: (number of alternations / [number of arm entries - 2]) × 100. The test measures spatial working memory, which is dependent on the prefrontal cortex and the hippocampus.

### 3.3. Three-chamber test

The three-chamber test assessed social behavior, including social preference and social recognition. Mice were initially habituated to the three-chamber apparatus for 10 minutes. In the social preference phase, the mice were presented with a stationary object in one chamber and a novel mouse in another chamber. Their exploration time was measured for 10 minutes. In the social recognition phase, the mice were presented with a familiar mouse and a novel mouse, and their exploration time was again measured for 10 minutes. This test was used to assess social preference and recognition indices, which are indicative of social cognition and can be affected in models with cognitive or social

impairments[15].

### 3.4. Passive avoidance test

The passive avoidance test was conducted to evaluate aversive memory. The apparatus consisted of two connected chambers: a light room and a dark room. During the habituation phase, mice were allowed to explore both chambers freely for 10 minutes, with the light turned on only in the bright room. After 24 hours, in the learning phase, mice were placed in the dark room, and upon crossing into the light room, they received a mild electric shock (0.5 mA for three seconds). The following day, in the test phase, mice were placed back into the dark room, and the latency to cross into the light room was recorded, with a maximum observation time of 300 seconds. Mice with impaired memory were expected to cross sooner, while normal mice would typically avoid the light room for the entire duration.

## 4. Immunohistochemistry staining and western blot

### 4.1. Tissue collection and preparation

Mice were euthanized in a $CO_2$ chamber for three minutes, followed by cardiac perfusion with saline to clear blood from the tissues. The brains were harvested, and the dissected hippocampus was homogenized for western blot analysis. The other side of the brain hemisphere intended for immunofluorescence staining was fixed in 4% paraformaldehyde (PFA) for 24 hours, followed by immersion in 30% sucrose solution for 48 hours. Brain tissues were stored at -80 °C and sectioned coronally at a thickness of 20 μm for subsequent staining procedures.

### 4.2. Immunohistochemistry staining

To evaluate neuroinflammation, sections were stained for the astrocyte marker GFAP (#PA1-10004, Invitrogen, Massachusetts, USA) and microglia marker Iba-1 (#019-19741,

Fujifilm Wako, Tokyo, Japan). Aβ staining was performed using the D54D2 (#D54D2, Cell signaling technology, Massachusetts, USA) antibody, following permeabilization with 0.3% Triton X-100 for 1 hour, blocking with 5% BSA for 1 hour, and subsequent antibody staining. GnRH (#ab281844, Abcam, Cambridge, England) staining involved antigen retrieval by heating in sodium citrate solution at 95°C for 20 minutes. Retrieval for GnRHR (#ab183079, Abcam, Cambridge, England) and DCX (#SC-8066, Santacruz, California, USA) was performed using Tris-EDTA. DCX-positive cells were counted in three sections of the hippocampus from the same anatomical location to estimate neurogenesis. Confocal images were captured using an LSM 700 confocal microscope, and fluorescence intensity was quantified using ZEN and ImageJ.

### 4.3. Western blot analysis

The proteins of homogenized hippocampus tissue were extracted via centrifugation. The samples of proteins were separated by SDS-PAGE and transferred to membranes for immunoblotting. After incubation with primary and secondary antibodies, fluorescence images were captured using the LI-COR Odyssey CLx imaging system. Fluorescence intensity was analyzed using Image Studio software. The following antibodies were used: Aβ (#SC-28365, Santacruz, California, USA), BACE1 (#5606, Cell signaling technology, Massachusetts, USA), APP(#2452, Cell signaling technology, Massachusetts, USA), and BDNF (#ab108319, Abcam, Cambridge, England).

### 4.4. Statistical analysis

Statistical analyses were conducted using GraphPad Prism 8 (San Diego, CA, USA). All data were statistically analyzed using an unpaired two-tailed *t*-test with Prism 8 to compare the differences between the GnRH-treated and control groups. Statistical significance was determined as P-values less than 0.05. All data are presented as mean ± standard error of the mean (SEM).

# III. Results

## 1. Distribution of GnRH and GnRHR in the brain

The immunohistochemical analysis revealed widespread distribution of both GnRH and GnRHR across various brain regions, including the hippocampus. As shown in Figure 1, GnRH expression was detected in the hippocampal regions CA1, CA2, and DG, as well as other brain areas such as the medial habenula (MHb), ventromedial preoptic area (VMPO), medial septum (MS), and olfactory bulb (OB), medial eminence (ME) and hypothalamus. These findings suggest the potential role of GnRH in addition to reproductive functions.

Co-staining of GnRHR with NeuN, a mature neuronal marker, further demonstrated the localization of GnRHR in mature neurons within the hippocampus and hypothalamus (Figure 2). The widespread expression of GnRHR indicates that GnRH may exert its effects through direct action on mature neurons. Additionally, in Figure 3, the western blot analysis of GnRH and GnRHR expression in the hippocampus, hypothalamus, and PFC of male and female C57BL/6 mice also confirmed the existence of GnRH and GnRHR in these areas, though not to the extent observed in the hypothalamus.

A

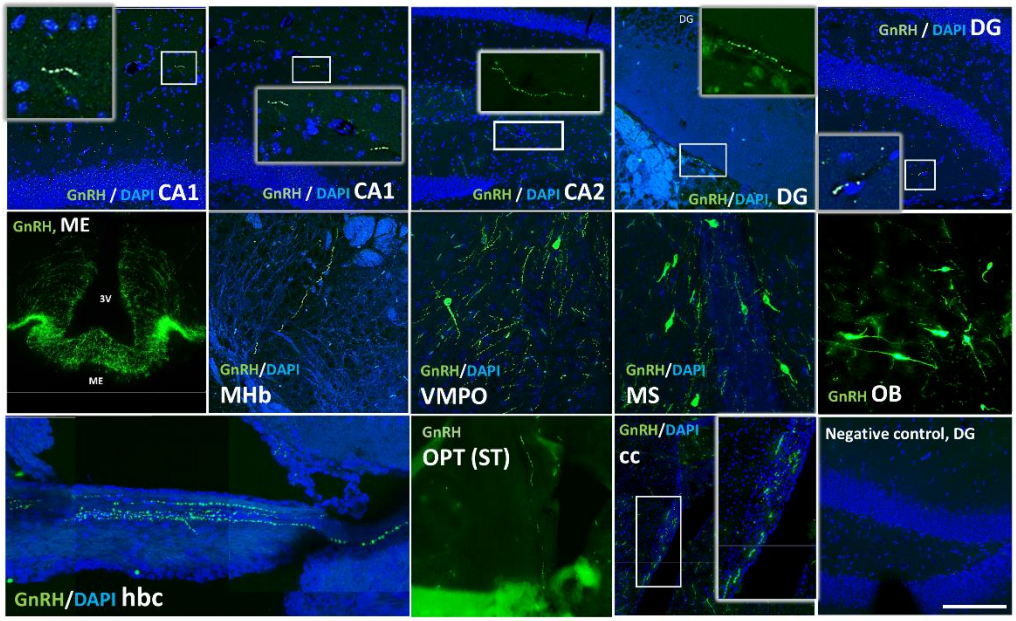


**Figure 1. The detection of GnRH in the brain**

**A**, Immunostaining image acquisition by confocal microscopy showing GnRH (green) expression in various brain regions, with DAPI (blue) as a nuclear counterstain. GnRH staining involved antigen retrieval by heating in sodium citrate solution at 95 °C for 20 minutes. GnRH was observed in the hippocampal regions CA1, CA2, and dentate gyrus, as well as in other brain regions, including the medial eminence, medial habenula, ventromedial preoptic area, medial septum, olfactory bulb, optic tract, and hippocampal commissure. Scale bar = 100 μm. DG, dentate gyrus; ME, medial eminence; MHb, medial habenula; VMPO, MS, medial septum; OB, olfactory bulb; ventromedial preoptic nucleus; hbc, hippocampal commissure; OPT, optic tract; cc, corpus callosum.

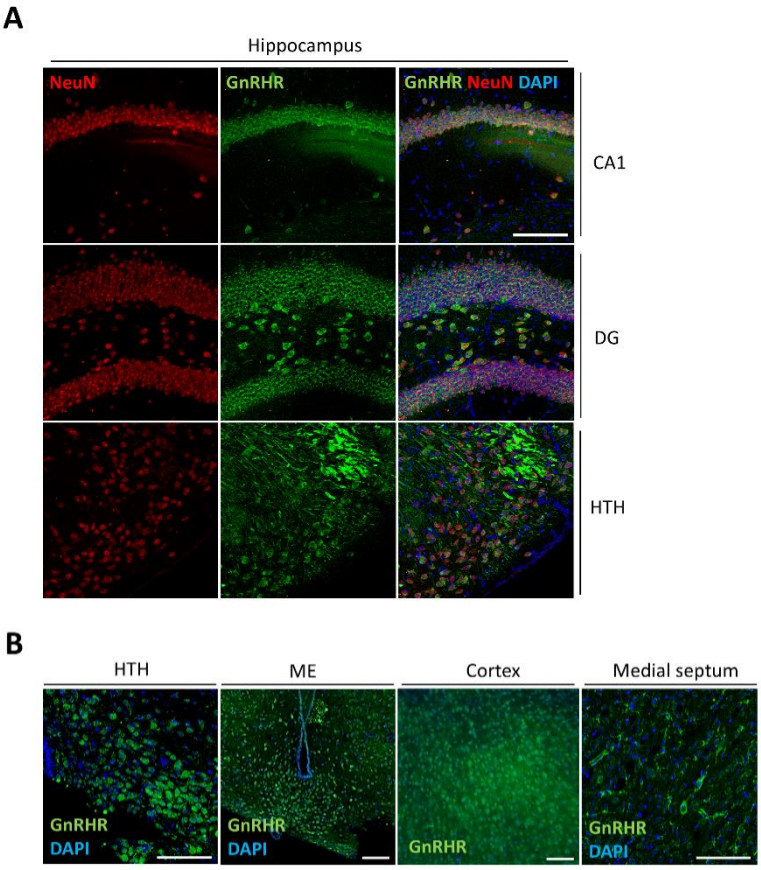


**Figure 2. GnRH receptor expression in the brain**

Immunostaining image acquisition via confocal microscopy showing GnRHR (green) expression, with DAPI (blue) as a nuclear counterstain. GnRHR staining involved antigen retrieval by heating in Tris-EDTA pH 9.0 solution at 95°C for 20 minutes. **A**, Co-staining of GnRHR (green) with a mature neuron marker NeuN (red) in the hippocampus, including regions CA1, DG, and hypothalamus, demonstrates the presence of GnRHR in mature neurons. **B**, GnRHR expression is shown in various regions, including the hypothalamus, medial eminence, cortex, and medial septum, indicating widespread distribution beyond the hypothalamus. Scale bar = 100 μm. GnRHR, GnRH receptor; NeuN, neuronal nuclei; DG, dentate gyrus; HTH, hypothalamus; ME, medial eminence.

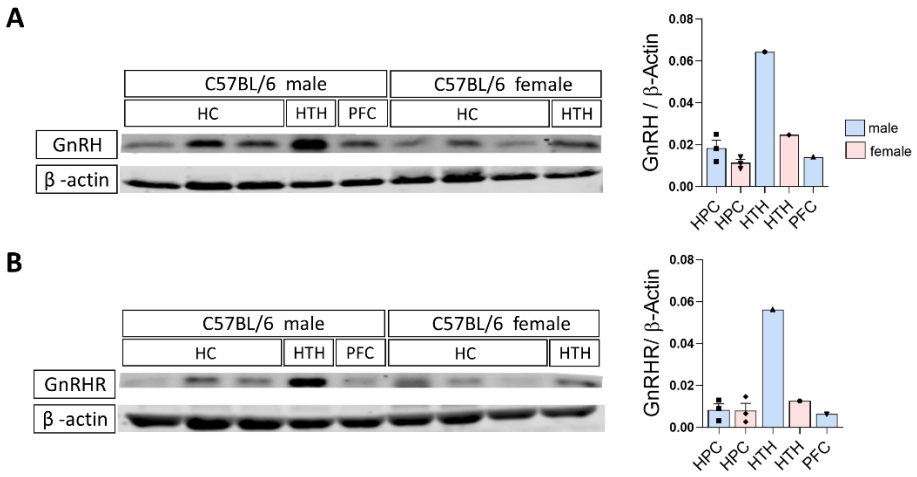


**Figure 3. Western blot analysis of GnRH and GnRHR in the brain**

**A-B**, Western blot analysis of GnRH and GnRHR expression in the hippocampus, hypothalamus, and prefrontal cortex of male and female C57BL/6 mice. Quantification of protein levels, using β-actin as a loading control, shows distinct expression patterns of GnRH **(A)** and GnRHR **(B)** across various brain regions. The data have been presented as mean ± SEM. GnRHR, GnRH receptor; HC, hippocampus; HTH, hypothalamus; PFC, prefrontal cortex.

I investigated the effects of long-term GnRH administration on amyloid pathology, neuroinflammation, neurogenesis, and cognitive function in 5xFAD mice, a model of AD.

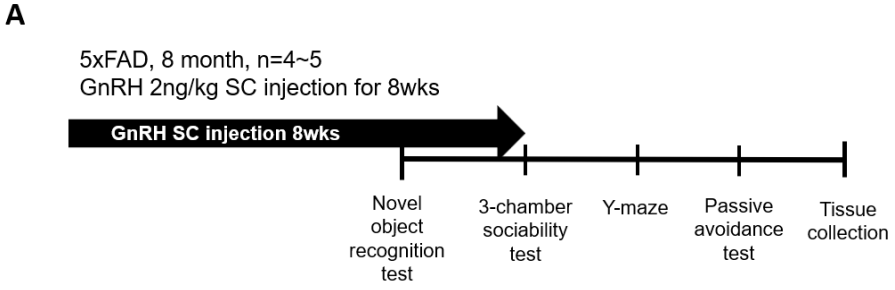


**Figure 4. Experimental scheme for GnRH treatment in 5xFAD mice**

**A**, Schematic representation of the experimental procedure. A dose of 2 ng/kg of GnRH was injected subcutaneously into both 8-month-old female and male 5xFAD mice over 8 weeks. Each group is comprised of 4~5 mice. Behavioral tests, including novel object recognition, three-chamber sociability, Y-maze, and passive avoidance, were performed during and after treatment, followed by tissue collection for further analysis. SC, subcutaneous.

## 2. Reduction of Aβ levels in 5xFAD mice

To first assess whether GnRH could modulate amyloid pathology, I first quantified the levels of Aβ and APP in the hippocampus of 5xFAD mice. Western blot analysis (Figure 5A) showed no significant changes in APP and BACE1 levels following GnRH treatment in female and male 5xFAD mice. Aβ levels, assessed using antibodies targeting oligomers and protofibrils, also showed no significant changes (Figure 5A). While no significant changes were observed in female mice, interestingly, immunofluorescence analysis of monomeric Aβ (5 kDa) levels in the dentate gyrus (Figure 5B) revealed a significant reduction in Aβ levels, specifically in the male 5xFAD mice compared to the control ($p = 0.0116$). These results indicate that while APP and BACE1 levels may not have shown statistically significant changes, GnRH treatment effectively reduces Aβ accumulation, particularly in male mice.

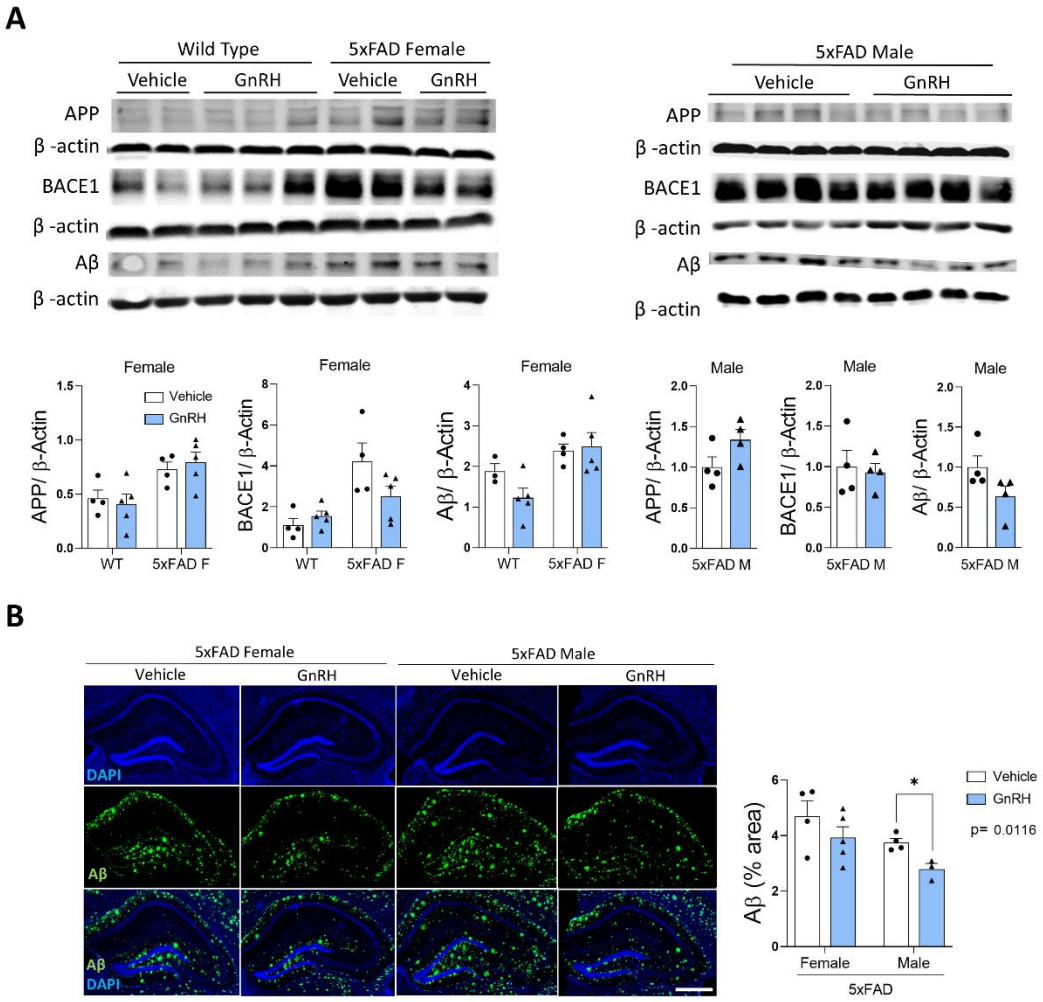


**Figure 5. Effects of GnRH treatment on amyloid pathology in 5xFAD mice**

**A**, The western blot analysis shows quantification of protein levels in the hippocampus of 5xFAD mice. The changes in the levels of amyloid precursor protein (APP), β-secretase enzyme (BACE1), and Aβ, with β-actin serving as a loading control, were not statistically significant. **B**, Aβ (green) immunofluorescence images and quantification of the fluorescence intensity in the DG area measured as % value were shown. DAPI (blue) was used for nuclear staining. The level of Aβ expression in the DG area shows a significant reduction in GnRH-treated male 5xFAD mice. The data have been presented as mean ± SEM, Scale bar = 500 μm, *p < 0.05. Amyloid precursor protein, APP; β-secretase enzyme, BACE1; Aβ, Amyloid-β; DG, dentate gyrus; WT, wild type.

### 3. Anti-inflammatory effects of GnRH

I evaluated the effect of GnRH treatment on glial cell activation by assessing the levels of microglia and astrocyte markers that reflect their morphological alterations. The immunofluorescence intensity of Iba-1, a microglial marker, was compared between the GnRH and control groups. In female mice, no differences were observed in the CA1 and CA3 regions, while the DG region showed reduced intensity in GnRH-treated 5xFAD male mice (Figure 6). These results indicate that GnRH decreases microglial activation in the DG region. In male mice, no differences were observed in all areas (Figure 6). However, GFAP, a marker of astrocyte activation, showed no difference between GnRH and vehicle-treated groups across CA1, CA3, and DG (Figure 7).

### 4. Neurogenic effects and neuroplasticity support by GnRH

I also investigated the effects of GnRH on neuroplasticity and neurogenesis. BDNF levels were increased in GnRH-treated 5xFAD mice (Figure 8A-C). Although the changes in the BDNF levels did not attain statistical significance when male and female data were analyzed separately (Figure 8B), the combined data showed significantly increased BDNF levels (Figure 8C), suggesting enhanced neuroprotection and synaptic function.

In 5xFAD mice, the number of DCX-positive cells, which serve as an indicator of newly formed neurons, was reduced to approximately 50% of the levels observed in wild-type control mice (Figure 9). Notably, in GnRH-treated female 5xFAD mice, the number of DCX+ cells was restored to comparable levels in wild-type mice. These results indicate that GnRH treatment enhances neurogenesis, demonstrating its potential to counteract the neurodegenerative effects in AD models.

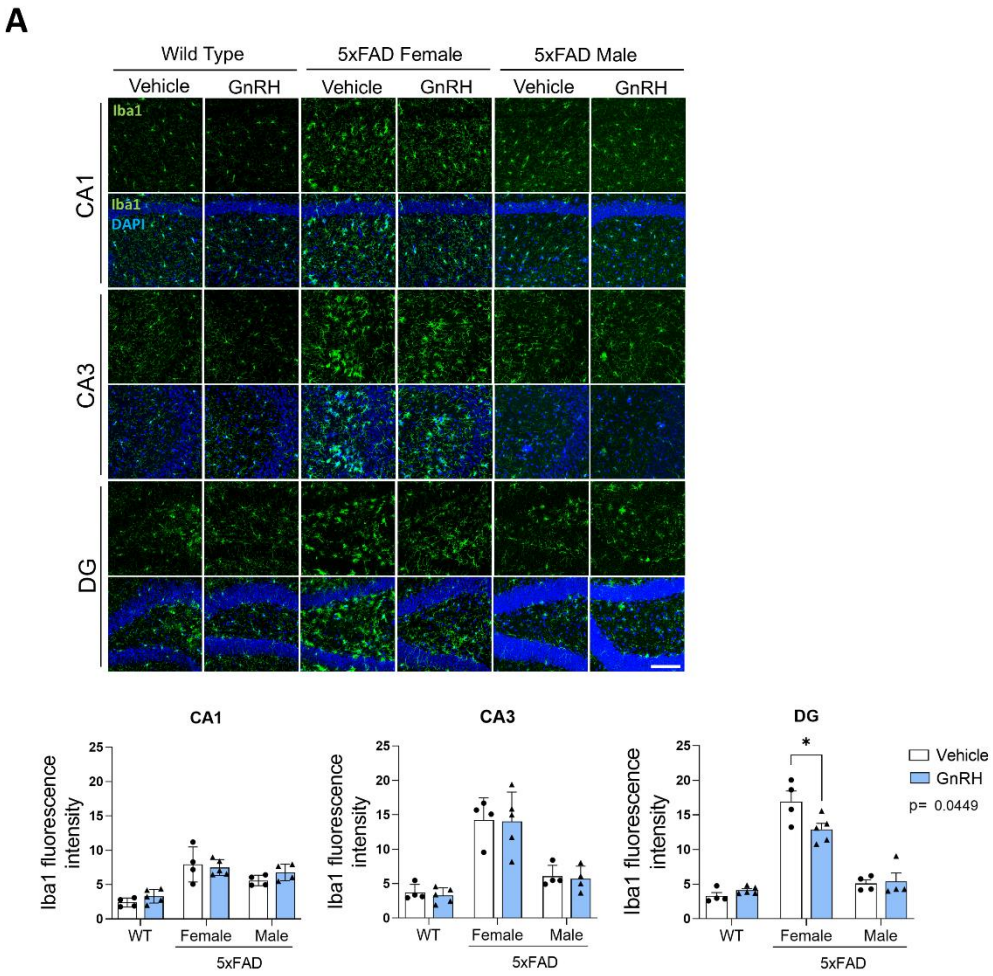


**Figure 6. GnRH reduced microglial activation in 5xFAD mice**

**A**, Iba-1 (green) immunofluorescence images show microglial activation in the CA1, CA3, and dentate gyrus regions of 5xFAD mice following GnRH treatment. DAPI (blue) was used for nuclear staining. Quantification of Iba-1 fluorescence intensity in female 5xFAD mice indicates that GnRH reduced microglial activation in the DG region. The data have been presented as mean ± SEM, Scale bar = 100 μm, *p < 0.05. DG, dentate gyrus.

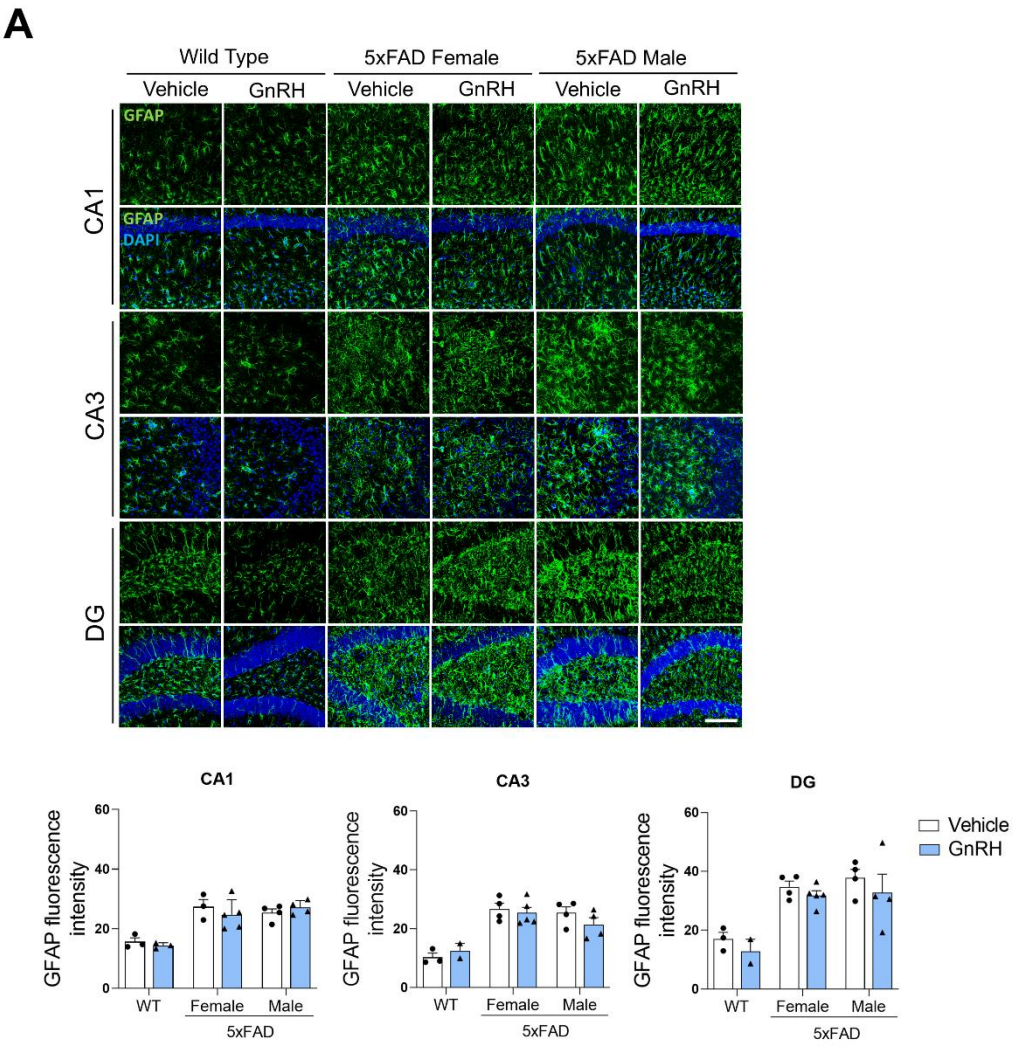


**Figure 7. Effects of GnRH treatment on astrocyte activation in 5xFAD mice**

**A**, Immunofluorescence images showing GFAP (green), a marker for astrocytes, in the CA1, CA3, and dentate gyrus regions in 5xFAD mice following GnRH treatment. DAPI (blue) was used for nuclear staining. Quantification of GFAP fluorescence intensity showed no significant changes between groups. The data have been presented as mean ± SEM. Scale bar = 100 μm. DG, dentate gyrus.

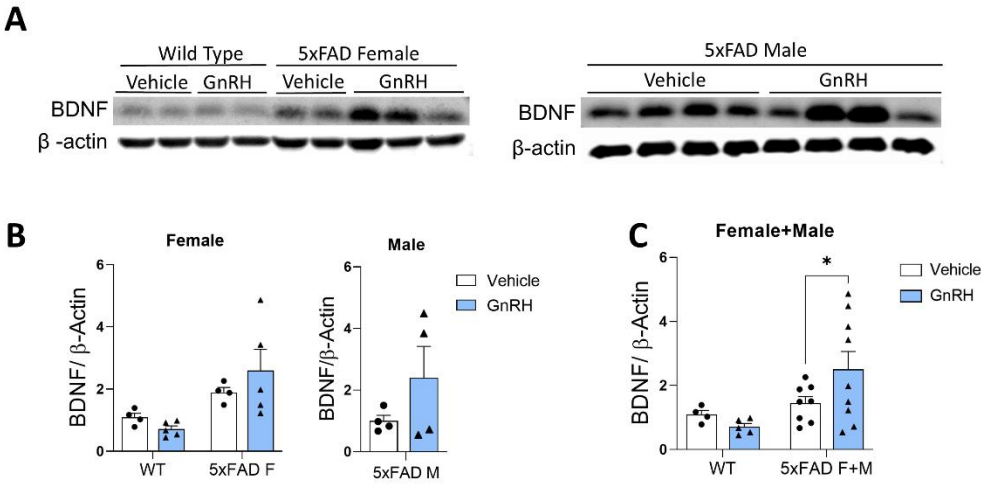


**Figure 8. GnRH enhances neuroplasticity in 5xFAD mice**

**A-C**, Western blot analysis shows BDNF levels in the hippocampus of 5xFAD mice. **B**, Intensities were quantified with β-actin as a loading control, and no statistically significant changes were observed. **C**, BDNF levels were increased in GnRH-treated 5xFAD mice when male and female data were combined. Data have been presented as mean ± SEM, *p < 0.05. BDNF, brain-derived neurotrophic factor.

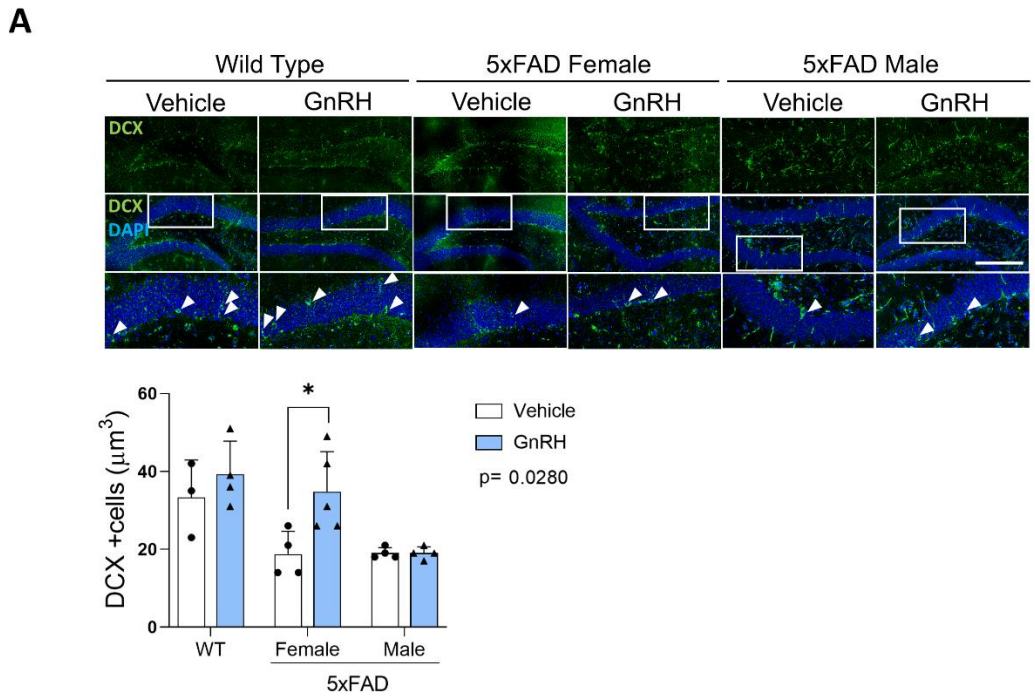


**Figure 9. Neurogenesis quantified by DCX expression in 5xFAD mice**

**A**, Neurogenesis quantified by immunofluorescence images of DCX (green) expression in 5xFAD mice following GnRH treatment. DAPI (blue) was used for nuclear staining. DCX-positive cells were counted in adjacent three tissues of mice, which serve as an indicator of newly formed neurons. Those in 5xFAD mice were reduced to approximately 50% of the levels observed in wild-type control mice. Notably, in GnRH-treated female 5xFAD mice, the number of DCX+ cells was restored to levels comparable to those in wild-type mice. The data have been presented as mean ± SEM, Scale bar = 100 μm, *p < 0.05. DCX, doublecortin; HT, hetero; WT, wild-type.

### 5. Effects of GnRH treatment on cognitive function

To assess the functional impact of GnRH treatment on cognition, I conducted a series of behavioral tests, including the novel object recognition test, Y-maze, three-chamber test, and passive avoidance test. No significant differences were observed in recognition memory (Figure 10A), working memory (Figure 10B), fear memory (Figure 10C), social preference memory, or social recognition memory (Figure 10D) across treatment groups. These findings indicate GnRH may not fully restore cognitive function in AD model mice.

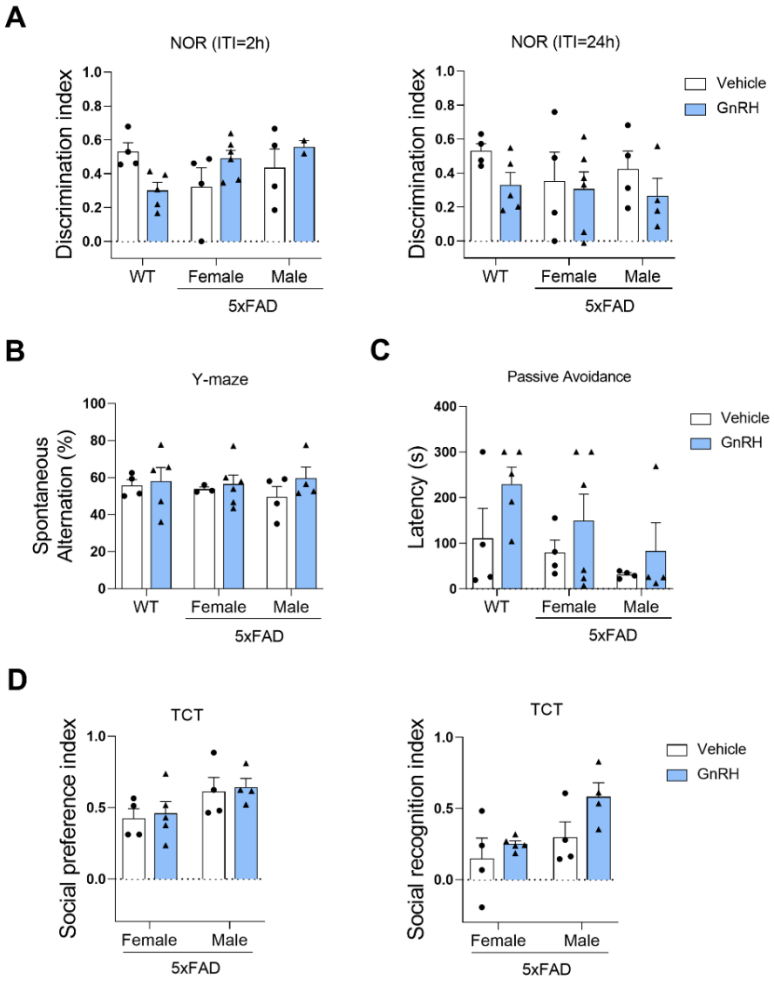

**Figure 10. Effects of GnRH treatment on cognitive function in 5xFAD mice**

**A-D**, Behavioral assessments show no significant differences in cognitive functions. **A**, NOR test with 2-hour and 24-hour intervals to assess recognition memory showed no significant differences in the discrimination index across treatment groups. **B**, Y-maze, the percentage of spontaneous alternation in the Y-maze test across groups, indicating no significant improvement in working memory. **C**, In the passive avoidance test, the index measures how well an animal remembers to avoid an unpleasant situation, calculated by the latency time (s) to enter a certain area. This result did not attain statistical significance. **D**, Social preference and social recognition tests in the three-chamber test (TCT) indicated no significant changes in social behavior between groups. Data have been presented as mean ± SEM. NOR, novel object recognition test; TCT, three-chamber test.

# IV. Discussions

This study aimed to examine the potential therapeutic effects of GnRH treatment in an AD mouse model. A substantial body of research has investigated other sex hormones in relation to AD, as well as other studies exploring the connection between GnRH and cognitive function. These previous findings suggest the potential of GnRH as a therapeutic option for AD. The findings of this study indicate a possibility that GnRH may have anti-AD and neurogenic effects. I found that GnRH contributes to the reduction of amyloid pathology and neuroinflammation while promoting neurogenesis and neuroplasticity support. Although these findings were inconsistent across animal sexes, they still suggest that GnRH may play a significant role in mitigating the pathogenesis of AD.

## 1. Extended roles of GnRH and GnRHR in the CNS

It is now recognized that GnRH and GnRHR are widely distributed throughout the brain[1]. The presence of GnRH and GnRHR in various regions of the CNS (Figure 1-3) suggests a broader functional role beyond reproductive regulation. The widespread expression of GnRHR, along with its co-expression with NeuN (Figure 3) throughout the brain, indicates that GnRH may exert its effects through direct action on mature neurons. GnRH may influence not only the hypothalamus but also potentially extend its effects to neurons in other regions of the brain.

Furthermore, recent research has identified another crucial player in the modulation of GnRH neurons. Historically, arcuate kisspeptin (KNDy) neurons, characterized by their expression of neurokinin B (NKB) and dynorphin A (Dyn), have been recognized as the primary pulse generators for GnRH within the hypothalamic arcuate nucleus (ARC) [24]. A recent study reveals that the cholinergic neurons that innervate GnRH neurons in the forebrain, particularly in the septal areas, directly influence GnRH neurons, highlighting their role in regulating the hypothalamic-pituitary-gonadal (HPG) axis regulation through ACh and ACh/GABA neurotransmission pathways in male mice[25,26]. Rather than being

solely regulated by the KNDy neurons, which monitor hormone levels and act as kisspeptin mediators when needed, a new regulator has been identified. This suggests that GnRH neurons may have an extended role beyond reproductive functions, which varies depending on the state of cholinergic neurons.

Interestingly, other studies have confirmed a decline in GnRH levels in aged and 5xFAD mice throughout the CNS[19,20]. The discovery of GnRH deficiency in these models suggests a hypothesis that the lack of GnRH may be related to the progression of aging and AD, potentially exacerbating the condition. As indicated by this study, restoration of GnRH in the AD model mouse reveals that GnRH contributes to neurogenesis and exhibits anti-AD properties. In addition to these findings, evidence from other studies demonstrating effects on cognitive function further suggests that the extended role of GnRH may be closely associated with AD.

## 2. GnRH reduces the Aβ levels in male mice

One of the study's major findings is the significant reduction in Aβ levels following GnRH treatment. The expression of Aβ in the DG area revealed a marked decrease in 5xFAD mice treated with GnRH, particularly in males (Figure 4B). However, the protein levels of BACE1 and APP did not change significantly. BACE1 is a key enzyme involved in the cleavage of APP to produce Aβ. Considering this, the findings suggest that the results may be interpreted as enhanced Aβ clearance rather than reduced Aβ production.

In addition, the lack of significant results in detecting Aβ (Figure 5A) larger than 50 kDa may be attributed to the stable interactions within fibrils, which are likely resistant to GnRH treatment. This suggests that the efficacy of GnRH may lie in promoting the clearance of newly formed Aβ rather than disassembling fibrillar aggregates.

The anti-AD effects of GnRH were observed exclusively in male mice, with no significant effects found in females. This difference may be due to two possible reasons. Firstly, variations in the hormone feedback loop mechanisms between males and females

can be proposed for the reason. Administration of GnRH can accelerate ovarian follicle depletion, thereby promoting aging in females. Secondly, it can be assumed that the difference in the progression rate of AD between 5xFAD males and females is a contributing factor. Further research should determine whether the effects observed in male 5xFAD mice can be replicated in female mice without the influence of the hypothalamic-pituitary-gonadal (HPG) axis feedback loop and the rate of AD progression. Different administration strategies may be required for female mice, including adjustments for administration duration, timing, dosage, and target regions. To complement these strategies, other experimental approaches could involve using viral vector-mediated gene transfer to implant a receptor that periodically mimics GnRHR activation signals, effectively minimizing the effects of the hormone feedback loop.

## 3. Anti-inflammatory effects of GnRH

Chronic neuroinflammation is a significant contributor to the progression of AD progression[27], as it exacerbates neuronal damage and degeneration through the activation of microglia and astrocytes. In their neurotoxic state, the overactivation of these glial cells produces excessive inflammatory cytokines, which further contribute to neuroinflammation and accelerate neuronal damage[27]. Previous in vitro studies revealed that the activation of GnRHR can shift glial cells from a neurotoxic to a neuroprotective phenotype[8]. I conducted GFAP and Iba-1 immunohistochemistry staining to determine whether the previous *in vitro* findings could be replicated in *in-vivo* research.

In this study, GnRH administration reduces microglia activation, as evidenced by decreased Iba-1 fluorescence intensity in the DG of GnRH-treated female 5xFAD mice (Figure 6). GnRH may exert its protective effects on AD through immune modulation, thereby preventing the acceleration of AD progression. Furthermore, the reduction in microglial activation suggests that GnRH may shift microglia from a neurotoxic phenotype to a neuroprotective one. Interestingly, no significant changes were observed in astrocyte

activation, as assessed by GFAP staining (Figure 7). This suggests that the observed decrease in Aβ levels could be mediated through the modulation of microglial activity rather than through astrocyte involvement. Microglia play a crucial role in the clearance of Aβ and the maintenance of homeostasis, while astrocytes are more involved in supporting neuronal function and maintaining the blood-brain barrier[28]. Taken together, these findings suggest that GnRH exerts its therapeutic effects in AD by modulating microglial activity to mitigate neuroinflammation and promote neuroprotection.

## 4. GnRH promotes neurogenesis

GnRH has been reported to have neurogenic effects following five weeks of subcutaneous injections in male C57BL/6 mice[29]. In this study, subcutaneous injections were extended to eight weeks to assess the effects in 5xFAD mice as AD progression was already advanced. Consistent with other studies, the investigation of this paper suggests a potential role for GnRH in promoting neurogenesis in the AD model. In 5xFAD mice, the number of DCX-positive cells, which serve as an indicator of newly formed neurons, was reduced to approximately 50% of the levels observed in wild-type control mice (Figure 9). Notably, in GnRH-treated female 5xFAD mice, the number of DCX+ cells was restored to comparable levels in wild-type controls. This recovery strongly suggests that GnRH treatment increases the formation of newly generated neurons. However, the fact that the results were significant only in female mice may be due to hormonal factors between males and females. In females, GnRH may promote neurogenesis by stimulating estrogen production, which has neuroprotective effects and plays a role in enhancing neurogenesis. Therefore, the effect may have been more pronounced in females[2,30].

I also investigated neuroprotective factors. BDNF, a key factor involved in synaptic plasticity and neuronal survival, was found to be elevated in GnRH-treated 5xFAD mice (Figure 8A,C). Although the increase in BDNF levels did not reach statistical significance when analyzed separately for male and female mice (Figure 8B), the combined data showed

a significant increase (Figure 8C). This suggests that GnRH enhances neuroplasticity.

These neuroprotective effects may be critical in counteracting the loss of neurons and synapses that drive cognitive decline in AD. Taken together, the findings suggest that GnRH promotes a neuroprotective environment even in the presence of AD pathology, offering a potential therapeutic strategy to mitigate neurodegeneration in the context of AD.

## 5. Effects of GnRH on cognitive function

In several behavioral tests assessing recognition memory, working memory, and social preference or recognition memory, no significant improvements were observed in the GnRH-treated groups compared to controls. However, in the passive avoidance test, some GnRH-treated female 5xFAD mice showed increased latency times comparable to those of wild-type controls, indicating partial recovery of cognitive function in these mice. Although this result did not reach statistical significance, it implies the possibility of improving cognitive function.

These results suggest that GnRH may not fully restore cognitive function in the AD mouse model under the current experimental conditions. Although statistically significant results were obtained in the molecular experiments, the effect did not consistently produce substantial changes across all metrics, and the limited effect size suggests that the influence was not strong. This suggests that the effect of GnRH was not substantial enough to be reflected in the behavioral experiments.

To better understand the potential cognitive benefits of GnRH in AD, future studies should focus on administering GnRH treatment at an earlier stage of the disease and for an extended duration. In this study, GnRH was administered from 8 to 10 months, when 5xFAD mice typically show significant progression of AD pathology, including extensive Aβ plaque accumulation and neuroinflammation. This period also corresponds to a time when GnRH levels are known to decrease in the brain. Identifying the point at which GnRH levels decline in the CNS of AD mice and administering treatment from that point onwards

could potentially lead to clearer effects.

In addition, given the normal secretion pattern of GnRH, methods such as using osmotic pumps for periodic secretion could be explored. Alternatively, it may be beneficial to administer GnRH locally to regions associated with cognitive function, such as the hippocampus or basal forebrain, rather than systemically to assess its effects more directly. This may provide a more favorable window for intervention and increase the likelihood of observing cognitive improvements in behavioral assessments.

## 6. Speculations on the reduction of GnRH in AD mice

To understand how GnRH contributes to AD progression, it is essential first to explore why GnRH levels are reduced in the aging or AD models. Under normal conditions, a decrease in estrogen or testosterone would typically increase GnRH levels through the hormone feedback loop. However, in aging or AD mouse models, GnRH levels exhibited an unexpected reduction contrary to the anticipated hormonal feedback response.

Interestingly, there is a system that regulates GnRH neurons and declines during AD progression, which is the cholinergic system. The cholinergic hypothesis, one of the earliest theories regarding the pathogenesis of AD, highlights the critical role of the cholinergic system in the development of the disease[31]. Acetylcholine (ACh), a key excitatory neurotransmitter crucial for learning, memory, and cognitive functions, is regulated by the central cholinergic nervous system through its synthesis and release[31,32]. It is well established that AD is characterized by a marked reduction in cholinergic neurons and a profound deficiency of acetylcholine in the brain[33]. I speculated that the decrease in ACh, a regulator of GnRH neurons, observed during early AD stages could potentially explain the reduction in GnRH levels and thereby contribute to AD progression. Future studies should aim to dissect the mechanisms of GnRH signaling and clarify which downstream pathways drive these effects. These approaches may help further elucidate the role of GnRH in neuroprotection and cognitive function. In conclusion, the study supports the

hypothesis that the disruptions in GnRH homeostasis may be associated with neurodegeneration.

# V. Conclusions

In conclusion, the study demonstrates that long-term administration of GnRH has anti-dementia and neurogenic effects in 5xFAD mice, characterized by reduced amyloid pathology, decreased neuroinflammation, and enhanced neurogenesis and neuroplasticity. These findings suggest that GnRH is a potential therapeutic strategy for slowing the pathology of AD. Furthermore, the study suggests that abnormalities in GnRH homeostasis may be associated with neurodegeneration. Future research should aim to elucidate how GnRH is involved in these effects and optimize treatment protocols.

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Abstract in Korean

# 알츠하이머병 마우스 모델에서 생식샘자극호르몬방출호르몬 처치: 아밀로이드 병리 감소 및 신경 발생 촉진

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손유미

생식샘자극호르몬방출호르몬 (GnRH)은 주로 성적 성숙과 생식력을 조절하는 역할로 잘 알려져 있다. 최근 시상하부 외에도 중추 신경계 다양한 부위에서 GnRH와 그 수용체인 GnRHR이 관찰되었고, 이는 GnRH가 생식 이외의 기능을 가지고 있을 수 있음을 시사한다. 최근 연구에 따르면 GnRH는 기억력과 인지 기능을 향상시킬 뿐만 아니라 신경발생 효과를 나타낸다고 한다. 이러한 이전 연구 결과는 알츠하이머병에 대한 치료제로써의 GnRH의 가능성을 시사한다. 따라서 본 연구에서는 알츠하이머병 모델인 5xFAD 마우스에서 장기간 GnRH 투여가 아밀로이드 병리, 신경염증, 신경발생 및 인지 기능에 미치는 영향을 연구하였다. 행동 평가와 형광 면역 조직 염색 및 웨스턴 블롯 염색법을 활용하였다.

다양한 뇌 영역에서 GnRH 및 GnRHR의 발현을 측정한 결과, 시상하부, 대뇌 피질, 해마 그리고 기저핵 등에서 발견되었다. 또한, GnRHR과 신경세포 마커가 함께 염색되었다. 이 결과를 기반으로, 다양한 뇌 영역의 신경세포 상에서 GnRHR가 기능할 가능성을 보여주었다.

암컷과 수컷 5xFAD 마우스에 8주 동안 피하 주입법으로 2 ng/kg의 GnRH를 처치하였다. 그 후, 인지 행동 평가를 통하여 새로운 물체 인식 능력, 작업 기억, 회피 기억 그리고 사회적 상호작용 및 기억력을 시험하였다. 또한, 아밀로이드 병리 및 신경 염증의 변화를 확인하였다. 더 나아가 신경 발생 및 신경 가소성 인자의 변화를

확인하였다. GnRH 처치는 아밀로이드 병리에 관련된 BACE1이나 APP의 수치에는 변화를 주지 못했지만, 알츠하이머병의 주요 원인인 아밀로이드 베타 (A$\beta$) 수치를 유의하게 감소시켰다. 또한 GnRH 처치는 알츠하이머병 진행의 주요 요인인 미세아교세포 활성화를 감소시켜 신경 염증을 감소시켰다. 또한, GnRH 처치 이후 신경발생 마커인 DCX 양성 세포의 수가 증가하였고, 신경가소성 마커인 뇌유래신경영양인자 (BDNF) 수치가 상승하였다. 그러나 행동 평가를 통한 인지 기능의 변화는 관찰되지 않았다.

이러한 연구 결과는 GnRH 투여가 알츠하이머병의 진행을 늦추고 신경발생 효과를 향상시키는 유망한 치료 전략이 될 수 있음을 시사한다. 더 나아가, 본 연구는 GnRH 항상성의 이상이 신경 퇴행과 관련이 있을 수 있음을 암시한다.

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**핵심되는 말** : 생식샘자극호르몬방출호르몬, 항치매, 신경발생, 신경가소성, 신경염증, 인지기능, 신경보호