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Investigating the Potential Impact of LH on the Progression of Alzheimer's Disease via Microglia Activation and C/EBPβ pathway

Yuanming Shi [1,a,+], Caixi Liu [2,b,*,+], and Yuanying Cui [3,c,+]

[1] Amberson high school, Markham, L3R 4H2, Canada
[2] Gower College Swansea, Swansea, SA4 6RD, United Kingdom
[3] Kent College Pembury, Tunbridge Wells, Kent, TN2 4AX, United Kingdom
a. info@ambersoncollege.ca, b. liuwinnie973@gmail.com, c. chloecuicui@icloud.com
*corresponding author
+ co-first authors

Abstract:

Background: Alzheimer's disease (AD) is a neurodegenerative disease that causes irreversible cognitive decline and memory loss. The disproportionate effect of AD on postmenopausal women prompts investigation into the role of hormonal changes in disease pathogenesis. However, previous studies on estrogen replacement therapies have shown inconsistent results. Therefore, further confirmation is needed to ensure the independent effects of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) on AD pathology. Our study will 1) focus on microglial activation pathway 2) focus on the LH-C/EBPβ-AEP pathway and its potential influence on AD progression.

Methods: Using cell lines to investigate whether microglial activation is present. HMC3 cells will be treated with LH or control phosphate buffered saline (PBS). Carry out Western Blot (WB) and qPCR to confirm presence of cytokines, which is the product of microglial activation. Using 3xTg-AD mice treated with LH or control PBS. To identify AD progression, immunofluorescent (IF) micrograph will be used to quantify amyloid-beta (Aβ) load. WB and qPCR will be used to identify cytokines. Morris water maze (MWM) will be carried out for cognitive testing. Using an ovariectomized mice model to investigate the LH-C/EBPβ-AEP pathway. Mice will be treated with LH antibodies or control IgG, followed by assessments using IVIS imaging and MWM test to measure spatial memory. IF, WB and qPCR will be used to quantify Aβ and Tau protein levels and gene expression changes. Furthermore, the study will explore the precise relationship between LH and C/EBPβ expression through inhibition experiments targeting AKT and ERK1/2 signaling pathways.

Expected results: Our hypothesis is that LH activates microglia, with both pro-inflammatory and anti-inflammatory cytokine released. This change in microglia function suggests LH has an effect on microglia and hence removal of Aβ plaques. Another hypothesis is that LH antibody manages to cross the blood-brain barrier. The cognitive ability will be improved in LH-Ab treated mice with reduced Aβ and Tau protein levels and downregulation of the C/EBPβ–AEP/δ-secretase pathway. Simultaneously, we expect that there will be a positive correlation between LH levels and expression of C/EBPβ-related pathways, suggesting the involvement of AKT and ERK1/2 signaling pathways in AD progression.

Conclusion: This study aims to provide evidence for the link between LH activation of the C/EBPβ pathway and AEP production, promoting our understanding of AD pathogenesis. The insights gained may implicate therapeutic strategies targeting LH mechanisms in postmenopausal women at risk of AD.

Keywords: Alzheimer's disease, luteinizing hormone, microglial function, C/EBPβ, AEP/δ-secretase.

1. Introduction

1.1 The Impact of Hormonal Changes on AD Risk in Postmenopausal Women

According to existing evidence, AD disproportionately affects women, with two-thirds of the patients being female. [1] At the same time, post-menopausal women frequently experience cognitive impairment and memory loss. [2] This can be due to the fact that after menopause, women undergo significant hormonal and gonadotropin alterations. [3] The reduced ovarian function results in diminished follicular production and lower estrogen levels, giving negative feedback on gonadotropin-releasing hormone (GnRH), thereby increasing the secretion of LH and FSH. [4]

Given the significantly higher incidence of AD in postmenopausal women, our investigations stem from whether decreased estrogen or elevated LH and FSH levels could potentially trigger Alzheimer's disease.

1.2 Limitations of previous studies

In our literature review, we explored the impact of hormone replacement therapy (HRT) using estrogen on AD pathology. However, it turned out to be variable outcomes, the Women's Health Initiative Memory Study (WHIMS) suggested increased risk of AD with HRT [5] while Multi-Institutional Research in Alzheimer's Genetic Epidemiology (MIRAGE) study showed that HRT in younger women was associated with reduced risk of AD. [6] Together, they suggest that the protective role of estrogen in the development of AD remains uncertain.

However, apart from estradiol, there are emerging research investigating the influence of FSH, revealing its role in activating CCAAT/enhancer binding protein beta (C/EBPβ) pathway and subsequently enhancing asparagine endopeptidase (δ-secretase/AEP) production, contributing to AD-like pathology. [7] [8]

Despite these findings, independent influence of FSH on modifying microglial function and the specific impact of LH on AD pathology remain inadequately studied. Existing literature highlights LH's potential role to contribute to AD [9] [10], yet comprehensive underlying mechanisms need to be further explored.

1.3 Addressing the limitations

To investigate the acute relationship between LH and Alzheimer's disease, as well as the underlying mechanism, our research is structured into two primary parts. One focuses on explaining LH's influence on microglial function. With reduced cleaning capacity of microglia, LH could potentially lead to increased amyloid-beta plaque accumulation. The other part examines whether LH activates the C/EBPβ-δ-secretase pathway, which could increase amyloid-beta production and precipitate irreversible Alzheimer's pathology.

1.4 The Impact of Microglial Activation on AD Risk

Microglia activation determines if Aβ plaques are being removed effectively.

To investigate whether LH has an impact on activation of microglia and therefore AD, we would like to see whether cytokines are released as the product of activation. If cytokines are identified, it is verified that LH does have effect on microglia.

Increased rate of AD progression is characterised by a build-up of amyloid β plaques. This is mainly caused by stimulations activating the pro-inflammatory M1 microglia. Traces of proteins released by M1 will therefore be present, for example, inflammatory cytokines and chemokines, such as tumur necrosis factor alpha (TNF-α), interleukin (IL)-6, IL-1β, IL-12, and CC chemokine ligand (CCL) 2. [11] It is likely that LH is associated with activating M1 microglia.

As LH receptors (LHR) are present on microglia [12], the aim of our investigation would be to confirm whether LHR becomes activated through a particular pathway.

1.5 The function of C/EBPβ in cellular biology

C/EBPβ is a known multifaceted transcription factor that binds to specific DNA sequences, such as those in the promoter region of genes like Lgmn. [13] The Lgmn gene encodes for legumain, an enzyme that processes inactive pro-AEP into its active form, AEP.[14]

In addition, C/EBPβ influences a variety of processes including cell differentiation (e.g., adipocytes, hepatocytes, immune cells), metabolic regulation (e.g., gluconeogenesis, lipid metabolism), and immune responses through cytokine modulation and inflammation pathways.[15] In addition, C/EBPβ plays a crucial role in embryonic development, tissue-specific differentiation and stress response, contributing to cellular adaptation and survival mechanisms.

1.6 AEP/δ-secretase and Alzheimer's disease

AEP is a type of endopeptidase within cells, which processes key proteins involved in AD pathology, including amyloid precursor protein (APP) and Tau protein.[16] By cleaving them into smaller, potentially neurotoxic fragments, plaques and tangles may be formed in the brain and inhibit normal activity of neurons. This process contributes to the neurodegenerative cascade observed in AD with declined cognitive abilities. Additionally, AEP's involvement in activating pro-inflammatory cytokines [17]

may exacerbate neuroinflammation, which further leads to neuronal damage.

Consequently, AEP emerges as a potential therapeutic treatment in AD, where targeting its activity could mitigate the accumulation of toxic protein and prevent dampened neuroinflammatory responses.

1.7 Signaling ways between LH and C/EBPβ

A series of intracellular events is triggered by the binding of LH to its receptor LHR. Activation of LHR leads to the production of cyclic adenosine monophosphate (cAMP), which in turn activates protein kinase A(PKA). Upon phosphorylation by PKA, CREB is then activated and able to bind to CRE sequences in the promoters of target genes [18], including C/EBPβ. This binding stimulates the transcription of C/EBPβ mRNA, which is then translated into C/EBPβ protein. The activity and nuclear translocation of C/EBPβ can be influenced by post-translational modification. [19] Once modified, C/EBPβ binds to CCAAT/enhancer elements on DNA within the nucleus, thereby controlling the expression of genes involved in cellular processes. Simultaneously, this process interacts with other signaling pathways, such as MAPK/ERK [20], to enhance cellular responses to LH stimulation, ensuring coordinated and effective cellular functions.

2. Objective 1

To confirm whether microglia is activated with the presence of LH.

2.1 Cell line experiment

Experiment groups: 4 groups

- human microglial cell line HMC3 [21] with/without LH treatment (N=4)
- mouse microglial cell line EOC 13.31 with/without LH treatment (N=4)

After preparing the cell culture, treat microglia cells with LH (2, 5, 10 IU) and PBS (5 IU). Collect the supernatant after centrifugation and identify cytokine released. Then carry out SDS-PAGE gel electrophoresis. Proteins will then be transferred to a membrane, incubated with primary and secondary antibodies, and analyzed for protein expression levels using chemiluminescence. Cytokines that will be tested include tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, IL-1β, IL-12, and CC chemokine ligand (CCL).

To confirm the presence of the cytokines, qPCR can be carried out.

Expected outcomes

plot results as a fold change in a bar graph. Cytokines are predicted to be present and demonstrate significant fold change when compared to PBS group. As IL-6 is known to be involved in ovulation with the presence of LH, this cytokine can further activate microglia.

2.2 Mice model experiment

Experiment groups: 4 main groups, each containing identical sub-groups for mice to be tested at different ages 2-4, 6-8, 12-14 months (in a sub-group N=7)

1.3xTG-AD female mice with LH injection, without C/EBPβ knockout

2.3xTG-AD female mice with LH injection, with C/EBPβ knockout

3.3xTG-AD female mice without LH injection, without C/EBPβ knockout (control)

4.3xTG-AD female mice without LH injection, with C/EBPβ knockout

Establish a dosing regimen daily for 3.5 months for newborn female transgenic mice. To knockout C/EBPβ pathway, CRISPR/Cas9 techniques will be employed.

To test for AD progression, 3 different types of experiment will be carried out.

2.3 Testing for cytokine presence

Similar to the experiment in 2.1, WB will be carried out followed by qPCR. Prepare brain sections by homogenizing in lysis buffer after euthanizing the mice (overnight, 4°C), followed by protein extraction and SDS-PAGE gel electrophoresis. qPCR can be carried out with extracted RNA from the brain tissue.

Expected outcomes

similar to experiment 2.1

2.4 Testing for Aβ load

With IF staining, all brain sections will be removed and incubated with primary anti-Aβ (1:400) antibodies once the mice have been euthanized. After washing with Tris-buffered saline, the sections will be incubated with a mixture of labelled secondary antibodies for detection. DAPI (1 μg ml−1) will be used for staining nuclei. The cleavage of mouse APP will be shown in the IF micrographs. Calculate Aβ load and plot in a bar graph.

Expected outcomes

Compare experiment 2 and 4, LH will have an effect on microglia function, where 2 > 4.

Compare experiment 1 and 3, LH will have an overall effect on transgenic mice, where Aβ load in 1+3 > 2+4, as C/EBPβ exists as an additional pathway.

Compare mice that sacrificed at different ages, LH will accelerate Aβ load accumulation over the time frames.

2.5 Testing for cognitive decline

When mice reach different points in age, the Morris water maze test will be carried out, indicating the spatial memory and cognitive ability of mice. Mice will be trained in a

round water-filled tub with extra maze cues before probe trials, receiving 4 trials daily for 5 days. Each trial lasts a maximum of 60 seconds. Those mice unable to reach the platform in time will be guided manually. After the training, a probe trial will remove the platform, measuring the percentage of time spent in the previously platform-containing quadrant over 60 seconds. Record distance travelled by mice and time spent to locate platform.

Expected outcomes

Groups treated with LH will spend longer time and distance locating the platforms, with a reduced percentage of time spent in the 'correct' quadrat, suggesting a worse AD symptom and progression.

Additionally, groups without C/EBPβ pathway will perform better.

Overall, declining cognitive abilities will be observed as mice ages. Groups treated with LH will show a faster rate of cognitive decline through the time frames.

3. Objective 2

To confirm the existence of the LH-C/EBPβ-AEP-AD pathway.

3.1 Ovariectomized mice model establishment

Carry out ovariectomy or sham operations on female 3x-Tg mice aged 3.5 months (N=15) that will be depleted of endogenous estrogen and will therefore have a higher expression of LH. After ovariectomy or sham operations, all the female 3xTg-AD mice will then be weaned at 3 weeks of age and housed with littermates of the same-sex in conventional cages at 20–25℃ condition with free access to food and water until sacrifice at age 10 mo.

3.2 Grouping of mice model

4 days after the ovariectomy or sham operation. Each mouse will receive either LH antibodies or goat IgG (200 μg) every 2 days, intraperitoneally for 2 months.

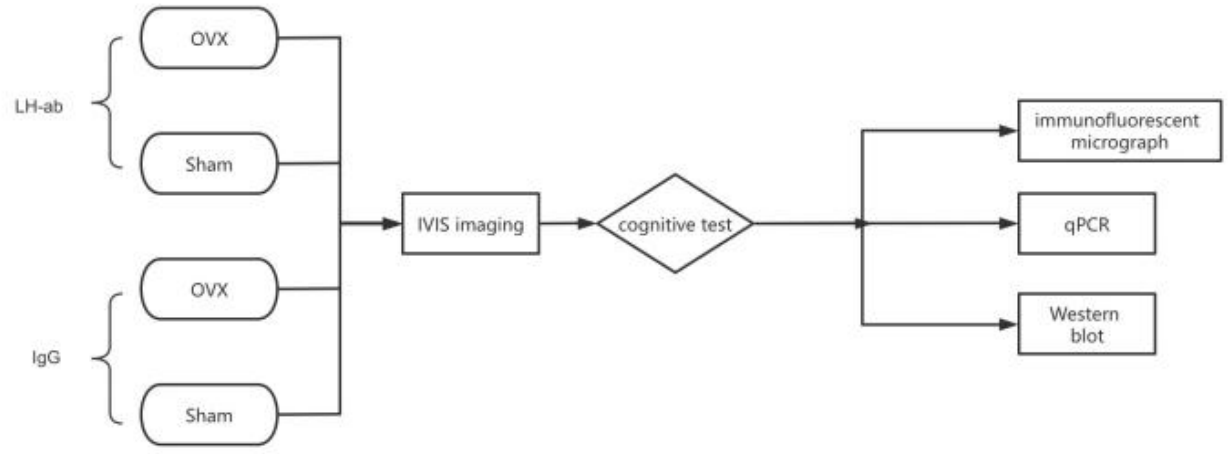

Figure 1: Specific experimental process of the models

(Figure 1) A group shows the setup of 4 groups of 3x-Tg mice with different treatments: (1) ovariectomized mice with IgG injection; (2) ovariectomized mice with LH antibodies injection ;(3) sham-operated mice with IgG injection ;(4) ovariectomized mice with LH-Ab injection.

3.3 IVIS imaging of LH-Ab injected mice

24 hours after the initial LH-Ab or IgG injections, the mice (N=3 per group) will then be injected with AlexaFluor750- labelled monoclonal LH-Ab (200 μg) or AlexaFluor750- labelled IgG intraperitoneally. The IVIS imaging will be performed 24 hours after the AlexaFluor750- labelled monoclonal LH-Ab or AlexaFluor750-labelled IgG injection. Typically, mice are fasted for a short period (4-6 hours) before imaging to reduce gut autofluorescence. The fluorescent signal following the injection of AlexaFluor750- labelled monoclonal LH-Ab or AlexaFluor750-labelled IgG will be quantified in dissected tissues using the IVIS Spectrum In Vivo Imaging System, showing localization of LH-Ab and IgG in the brain.

3.4 AD phenotype in LH-Ab treated mice

When mice are 5.5 months old, the Morris water maze test will be carried out, implicating the spatial memory and cognitive ability of mice. The cleavage of mouse APP and Tau will be shown in the Immunofluorescent micrographs.

3.5 Confirming the existence of the LH-C/EBPβ-AEP-AD pathway

When mice are 10-months old, use WB to compare the concentration of C/EBPβ, AEP, APP, Tau and Aβ fragments inside the brain of mice. Using qPCR to quantify the gene expression levels (C/EBPβ, Lgmn, APP and Mapt) inside the mice brain.

Expected outcomes

IVIS imaging of LH-Ab injected mice

We expect that the Epifluorescence graph can show labelling in the brain, implicating that AlexaFluor750-labelled

monoclonal LH-Ab or AlexaFluor750-labelled IgG have entered the blood-brain barrier, thus providing the prerequisite for the following further experiments. (Figure 2)

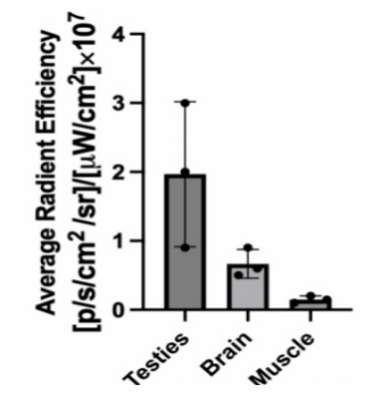

Figure 2: IVIS imaging of tissues from mice injected with AlexaFluor750-labelled monoclonal LH-Ab

AD phenotype in LH-Ab treated mice

Water maze

Knowing that ovariectomy will induce a marked deficit in spatial learning and memory retrieval, we expect that the percentage of time spent in the water maze for those ovariectomized mice will be higher than that spent by those sham-operated mice. Furthermore, ovariectomized LH-Ab treated mice will show significantly lower percentage of time compared to those ovariectomized mice with IgG, implicating that LH-Ab are able to reverse the cognitive decline in ovariectomized 3xTg mice. (Figure 3)

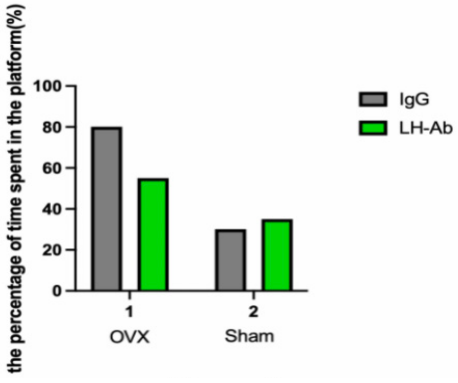

Figure 3: Different percentages of time spent in the platform(%) by (1) ovariectomized mice with IgG injection; (2) ovariectomized mice with LH antibodies injection ;(3) sham-operated mice with IgG injection ;(4) ovariectomized mice with LH-Ab injection.

For Immunofluorescent micrographs

We expect that the immunofluorescent labelling of Aβ and Tau$^{1\text{-}368}$ of those ovariectomized mice will be enhanced compared to sham-operated mice. Furthermore, ovariectomized LH-Ab treated mice will show reduced labelling compared to those ovariectomized mice with IgG, implicating that LH-Ab are able to inhibit plaque and neurofibrillary tangle formation in ovariectomized 3xTg mice.
Confirming the existence of the LH- C/EBPβ-AEP-AD pathway

Western Blot and qPCR

Same as the results in water maze and immunofluorescent micrographs, we expect that treatment with LH-Ab will cause an impressive reduction in ovariectomy-induced increases in gene expression and activation of the C/EBPβ–AEP/δ-secretase pathway, APP, Aβ and Tau cleavages in Western Blot. Also, we expect that in qPCR, C/EBPβ, Lgmn, APP and Mapt gene levels will be reduced with LH-Ab treatment. (Figure 4)

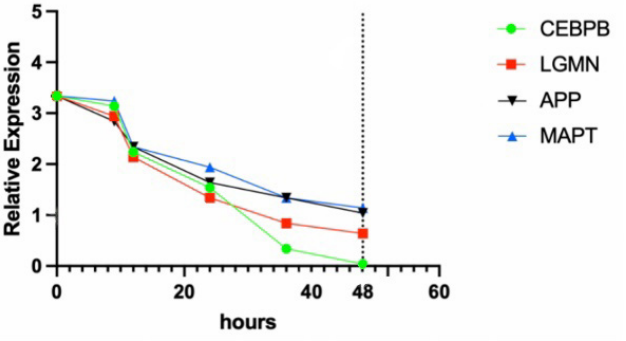

Figure 4: The relative expression of CEBPB, LGMN, APP and MAPT gene in LH-Ab treated mice

Together, these results can provide clear evidence for a role of LH in activating C/EBPβ and through production of AEP, leading to AD pathogenesis.

4. Objective 3

To identify the potential signaling pathway between LH and C/EBPβ with LH injection
Experiment 3.1
To identify whether LH can cross the blood brain barrier.
Samples
Ovariectomized female 3xTg mice aged 2.5 months (N=4, 8 group). After 4 days, inject LH (N=4, 4 groups) and PBS (N=4, 4 groups) respectively to mentioned mice 5 IU per mouse daily for 3 months. 24 hours after injection, use AlexaFluor 750-labelled LH injection and AlexaFluor 750-labelled PBS injection.

Methods

Use IVIS imaging to observe fluorescent signal to determine whether LH crosses the blood brain barrier.

Expected results

There is likely to be a significantly higher Average Radiant Efficiency in the brain in the LH injection group than PBS injection group which means LH can cross blood brain barrier.

Experiment 3.2

To identify that there is a close relationship between LH and C/EBPβ

Sample

Ovariectomized female 3xTg mice aged 2.5 months (N=4, 8 groups). After 4 days, inject LH (N=4, 4 groups) and PBS (N=4, 4 groups) respectively to mentioned mice 5 IU per mouse daily for 3 months.

Methods

Western blot

Rat cells will be washed with PBS and lysed in Radioimmunoprecipitation Assay buffer (RIPA), the resultant lysate will be centrifuged to get supernatant which will experience SDS-PAGE. After that, samples will be transferred to blotting membrane which will develop using chemiluminescent detection system. It will be used to confirm expression of C/EBPβ, AEP, APP, and Tau.

qPCR

mRNA levels of C/EBPβ, AEP, APP, and Tau will be analyzed using qPCR. Briefly, RNA will be isolated and relative quantification of gene expression will be calculated using the ΔΔCt method.

Expected results

It will indicate an increased level of C/EBPβ, AEP, APP, and Tau in LH injection group than PBS injection group (Figure 5), which means there is a positive correlation between LH and C/EBPβ as increases in LH level is associated with higher level of C/EBPβ expression. At the same time, we expect to see some key proteins in different signaling ways increase their concentration verses time, which will be used in the next experiment, such as AKT, ERK1/2, and PERK1/2, etc.

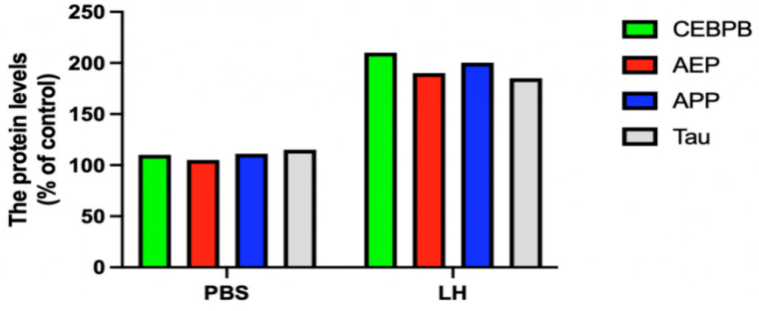

Figure 5: The protein levels of CEBPB, AEP, APP and Tau in PBS treated mice and LH treated ovariectomized mice

Experiment 3.3

To identify the exact signaling pathway between LH and C/EBPβ

Samples

Ovariectomized female 3xTg mice aged 2.5 months (N=8, 4 groups). After 4 days, inject LH (N=8, 4 groups) to mentioned mice 5 IU per mouse daily for 3 months. For the first group, inject 50ng/ml PTX as control group; for second group, inject 10μM AKTi-1/2 which inhibits AKT; for the third group, inject 10μM MEK1 which inhibits ERK1/2, for the last group, inject both 5μM AKTi-1/2 and 5μM MEK1.

Methods

Western blot and qPCR will also be employed.

Expected results: As for the first group, it will show the highest expression of C/EBPβ, AEP, APP, and Tau. However, as for the second and the third group, there will be similar decreases in the expression. And the last group will show greater decrease in expression than the second and the third group. (Figure 6) These data will indicate there are two exact signaling ways between LH and C/EBPβ through ATK and ERK1/2 respectively.

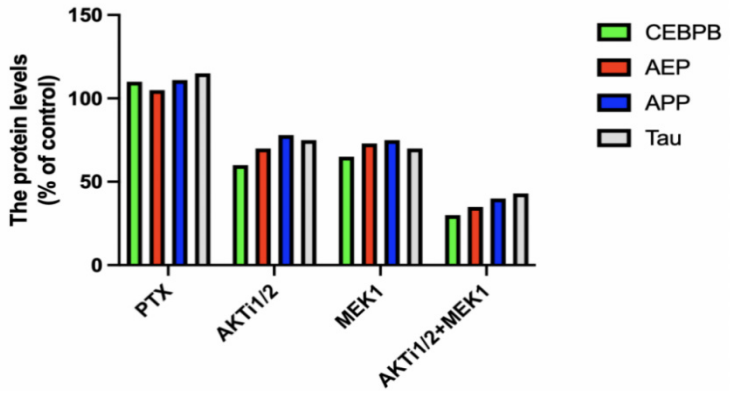

Figure 6: The protein levels of CEBPB, AEP, APP and Tau in LH treated ovariectomized mice with injection of (1) PTX;(2) AKTi1/2;(3) MEK1;(4) AKTi1/2 and MEK1

5. Conclusion

Objective 1: An elevated level of LH will result in reduced microglial clearance of amyloid-β.

Objective 2: The existence of the LH - C/EBPβ - AEP - AD pathway will be confirmed.

Objective 3: There will be two specific signaling ways between LH and C/EBPβ apart from the fastest cAMP pathway. (Figure 7)

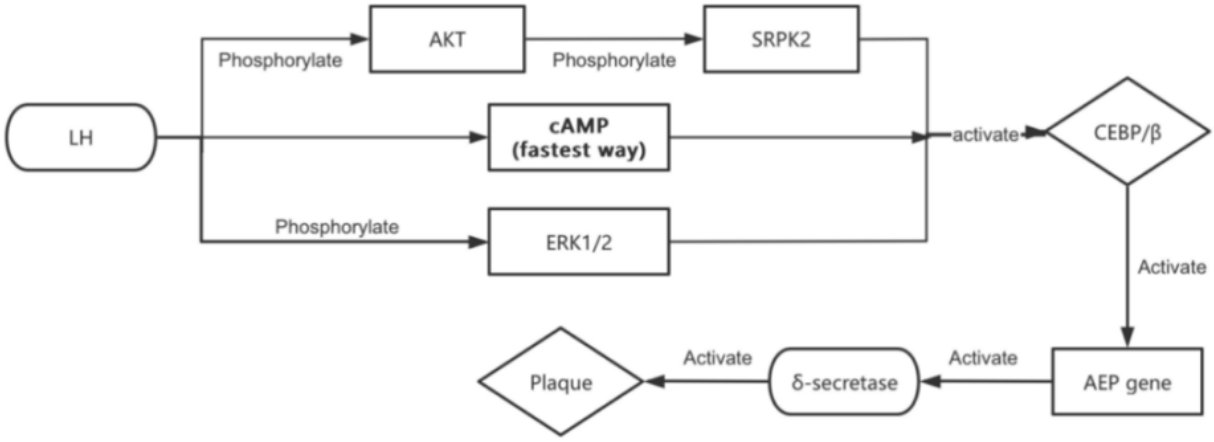

Figure 7: Potential signaling pathways between LH and C/EBPβ

6. Discussion

Reasonability of experiments

The level of LH will be investigated in wild type and transgenic mice to confirm transgenesis will not affect level of LH. As a result, there will be a small difference between wild type and transgenic mice. What's more, in order to confirm dosage of LH injection, a single dose of 2 IU, 5 IU OR 10 IU of recombinant human LH will be injected into female 3xTG mice. The elevation of LH at 5 IU will be similar to observed post-ovariectomy, as well as during the human menopausal transition.

Limitations

For objective 1

Cell lines are often treated with tumour cells to allow them to gain longer preservation time. This can alter the cell structure of microglia and reduce its credibility to represent normal human brain microglia cells. In addition, this cellular level experiment can be seen as inadequate as it does not take into account the complexity of human or mice brains. However, the benefit of cell line would be that its result directly shows how LH impacts microglia without the interference of other pathways. Therefore, once the results indicate activation of microglia cells, further experiments with mice model are carried out.

CRISPR/Cas9 can also be used to knock out LH receptors to further confirm the correct pathway is taking place in experiment 1.1.

For objective 2 and 3

C/EBPβ will only be focused on objective two and three,

yet other transcription factor with the C/EBP family will also influence AD progression through some unknown pathways [22]. Moreover, C/EBPβ will also regulate cytokine expression [23]. Therefore, distinguishing whether IL-6 is produced by microglia or by C/EBPβ in experiment one will be difficult. And it will also be hard to judge is it microglial removal function or C/EBPβ production function make the increased accumulation of plaques.

Future trajectories

It has already been known that women with one copy of APOE ε4 allele show a 4-fold increased risk. Moreover, women with two copies of APOE ε4 allele exhibit a 15-fold increased risk [24]. Therefore, the next step should be taken is to explore the relationship between LH and APOE ε4. Specifically, we can investigate whether there is an additive influence when both APOE ε4 and LH are present compared to participants with only LH by using APOE ε4-targeted replacement. What's more, the effect of LH on microglia will be confirmed through objective one. However, the exact pathway between LH and CatC which will lead to activation of microglia can be explored in the future.

Acknowledgements

Yuanming Shi, Caixi Liu and Yuanying Cui contributed equally to the article and should be considered co-first authors. Specifically, Yuanying Cui is the author of objective 1 and related details in the introduction, Caixi Liu is the author of objective 2 and related introduction and Yuanming Shi is the author of objective 3 and the discussion.

Statistical analysis

All the statistical data are performed using Prism software, version 10.3.0.

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