Restoring mGluR-LTD Failure with the Induction of Arc Protein in the CA1 Region of APP/PS1 Mice

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Abstract. Alzheimer's Disease (AD) has become a severe problem across the world. Besides the major two hypotheses (β-amyloid protein and tau protein), recent studies have also found that metabotropic glutamate receptor 5-dependent LTD (mGluR-LTD) failure is also one significant symptom of AD in APP/PS1 mice (AD mice model). Suppression of the inhibitor of de novo protein synthesis during the mGluR-LTD has successfully reversed the mGluR-LTD failure. Arc protein is an essential protein that coordinates the mGluR, and the level of Arc protein in the CA1 region of APP/PS1 mice is reduced. Therefore, an experiment is designed to increase the Arc level in APP/PS1 exogenously and endogenously to restore the mGluR-LTD by measuring post-synaptic responses.

1 INTRODUCTION

1.1 Background

Scientists have long been working to find a possible explanation for how Alzheimer's Disease (AD) is developed and methods for curing or mitigating the AD symptoms. There are several hypotheses about how it originated. The first one is the Tau and Amyloid-beta hypothesis. It asserts that tau, which accounts for the neuron tangles, and amyloid-beta, which clumps into plaques, act together to cause synaptic failure, resulting in AD (Förstl, Kurz 1999). The second one is about vascular problems, which may lead to a malfunction of the blood barrier. This failure inhibits glucose from reaching the brain as well as preventing the clearing away of toxic beta-amyloid and tau proteins (Förstl, Kurz 1999). Scientists have also studied microglia cells, a form of glial cells, as they fail to remove waste and beta-amyloid plaques in AD (Förstl, Kurz 1999). The initial symptoms of AD are sometimes mistaken as aging or stress, with the most serious deficit being the loss of short-term memory, along with other subtle vestigial deficits in executive functions of attractiveness, planning, flexibility, and abstract thinking (Bäckman, Jones, Berger, Laukka, Small 2004, Waldemar, Dubois, Emre, Georges, McKeith, Rossor, Scheltens, Tariska, Winblad 2007). With the development of AD, the early stages of AD are characterized by linguistic difficulties and impaired executive functions, deteriorating perception (agnosia), or degenerating execution of movements (apraxia) other than memory impairments (Förstl, Kurz 1999). Patients at the middle stages of AD show degeneration in speech, reading and writing skills; more importantly, the long-term memory starts to be impaired and behavioral and neuropsychiatric change become more prevalent (Förstl, Kurz 1999, Frank 1994). During the final stage of AD, patients completely lose speech ability, show extreme apathy and exhaustion, and eventually die of external factors like infection of pressure ulcers or pneumonia (Förstl, Kurz 1999, Frank 1994). The occurrence of AD greatly influences the society, such as the life of patients' family and high financial cost (Abreu, Forlenza, Barros, de. 2005). Moreover, during covid-19, the number of AD patients has increased by 16 percent, and one-third of the over-85 population is estimated to suffer from it by 2031((Bäckman, Jones, Berger, Laukka, Small 2004). As no one has ever been cured from AD, finding a therapy to avoid becoming its victim is essential.

Beside β-amyloid hypothesis and tau protein hypothesis being accepted as 2 major possible causes

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of Alzheimer's disease. Yang and co-workers found that in APP/PS1 mice (AD mice model), mGluR-LTD (Long-term Depression) failure occurs, and assumed this phenomenon as a potential cause of AD (Yang, Zhou, Zimmermann, Cavener, Klann, Ma 2016). Therefore, they tried to restore mGluR-LTD to mitigate the progress of AD in APP/PS1 mice. PERK is one of the four kinases of elF2 (eukaryotic initiative factor 2), and it is triggered to phosphorylate eIF2 on the α subunit which inhibits general synthesis of protein and impairs memory if the reduction in protein synthesis is persistent (Ma, Klann 2014, Trinh, Klann 2013, Wek, Jiang, Anthony 2006, Wek, Cavener, 2007). Yang and co-workers pharmacologically and genetically repressed the expression of PERK (Yang, Zhou, Zimmermann, Cavener, Klann, Ma 2016), in order to restore the *de novo* protein synthesis during the mGluR-LTD, and they successfully reversed the failure of mGluR-LTD in APP/PS1 mice. Another research by Yang and co-workers demonstrated that via pharmacological and genetic suppression of eEf2K (a kinase for elongation factor 2) (Yang, Zhou, Ryazanov, Ma. 2021), the hippocampal mGluR-LTD impairments in APP/PS1 mice had been alleviated.

1.2 Project Design

It has been known that mGluR-LTD failure occurred in APP/PS1 mice (Yang, Zhou, Zimmermann, Cavener, Klann, Ma 2016), and unregulated LTD is associated with dementia and AD (Wilkerson, Julia, Albanesi, Huber 2018). Based on this, it is assumed that if mGluR-LTD failure in APP/PS1 mice is reversed, the mice's AD symptoms will be improved.

Both these 2 studies focus on the restoration of the *de novo* protein synthesis, which is involved in process of mGluR-LTD, to alleviate the impairments of mGluR-LTD. Therefore, since Arc protein synthesis is included in de novo protein synthesis during mGluR-LTD, this project aims to investigate whether a great amount of Arc expression will reverse mGluR-LTD failure, in order to mitigate AD symptoms.

2 EXPERIMENTAL APPROACH

Whether the induction of a great amount of Arc protein will reverse mGluR-LTD impairments in the APP/PS1 mice.

3 METHODS AND MATERIALS

3.1 Mice

All mice were kept in a barrier rearing system committed to transgenic mice, which accords with the standards and policies of the US Department of Agriculture's Animal Welfare Information Center and the NIH Guide for Care and Use of Laboratory Animals. A 12-hour-light/dark cycle is maintained in the system, with a regular feeding and cage-cleaning schedule. male and female mice are equally selected for this research. APP/PS1 transgenic mice (APPswe +PSEN1/Δ9) and wild type mice were purchased. FMR1 hippocampus conditional knock-out (FMR1 cKO) were bred, and the method will be described below. The genotype of all mice was verified using PCR. Mice around 12-15 months old were used for this experiment.

3.2 Hippocampal Slice Preparation and Electrophysiology

The hippocampi were removed from the brains of mice of 12-15 months, then vibratome was used to make hippocampal slices at 400 μm(Hu, Serrano, Oury, Klann 2006). Slices were kept at room temperature and submerged in artificial CSF (ACSF), which contains the following (in mM:125 NaCl, 2.5 KCl, 1.25 NaH2PO4, 25 NaHCO3, 25 D-glucose, 2 CaCl2, and 1 MgCl2), and incubated for at least 2 h to be removed for electrophysiology.

For electrophysiology, slices were transferred to recording chambers (preheated at 32°C) which were superfused with oxygenated ACSF. Extracellular field and whole-cell patch-clamp recording in CA1 stratum radiatum using pipettes are performed (Schmeisser et al 2012). fEPSPs (field Excitatory PostSynaptic Potentials) and fEPSP slopes are recorded using the same pipettes. Long-term potentiation will be induced by a single tetanus of 100 pulses at 100 Hz (Malenka, Bear 2004). For long-term depression, CA1 is isolated by a microcut set at the edge of CA2/CA3 before recording synaptic response, and LTD is induced by 15 min paired-pulse stimulation at 1 Hz with 50 ms between single pulses in the presence of 1ml gabazine (Weiler et al 1997). For NMDA/AMPA ratio, NMDA and AMPA are separately recorded by compound EPSC (Excitatory PostSynaptic Currents) of -60mv and +40mv, and ten consecutive EPSCs for each holding potential will be averaged.

3.3 Drug Treatment

DHPG (Abcam, Cambridge, MA) will be prepared within a week and diluted before the experiment.

3.4 Endogenous Application

The Cre-LoxP system will be used to breed conditional knock-out mice suitable for endogenous expression of Arc protein. The translation of Arc protein is strongly related to a gene named FMR1. FMR1 gene codes Fragile X Retardation Protein (FMRP), which serves as a negative regulator of translation and binds to the mRNA (Weiler et al 1997). As shown in Figure 1, The Arc protein translation is induced by the mGluR activated by DHPG (mGluR agonist). As the FMRP binding to the mRNA. FMRP inhibits the process of *de novo* protein synthesis, so that the amount of protein translated is maintained at a normal level, as well as the intensity of mGluR-LTD (Hou, et al 2006, Laggerbauer 2001). Therefore, the FMR1 gene will be conditionally knocked out in the CA1 region of APP/PS1 mice, so that the Arc protein level in that region will increase, and the mGluR-LTD is likely to be restored. shows the mechanism of FMR1 inhibiting the translation of Arc protein. The occurrence of mGluR-LTD is closely related to the FMRP inhibition.

First, after finding a tissue specific promoter in the CA1 region of APP/PS1 mice, and then a Cre-recombinase gene will be introduced downstream after the promoter (Ray, Fagan, Brunicardi 2000). Following the treatment, the FMR1 gene in CA1 region of APP/PS1 will be genetically modified with 2 sets of LoxP sites. As shown in Figure 2, each set of LoxP sites contains 2 loxP sites flanking the FMR1, and one set of Loxp site will react with the Cre-recombinase, so that the FMR1 gene can be inverted and shut down. Afterwards, those 2 types of genetically modified mice (APP/PS1 Cre mice and APP/PS1 lox P) will mate with each other. After mating, their offspring will be APP/PS1/FMR1_ (CA1 region conditional knock-out) mice that can be used for further treatments.

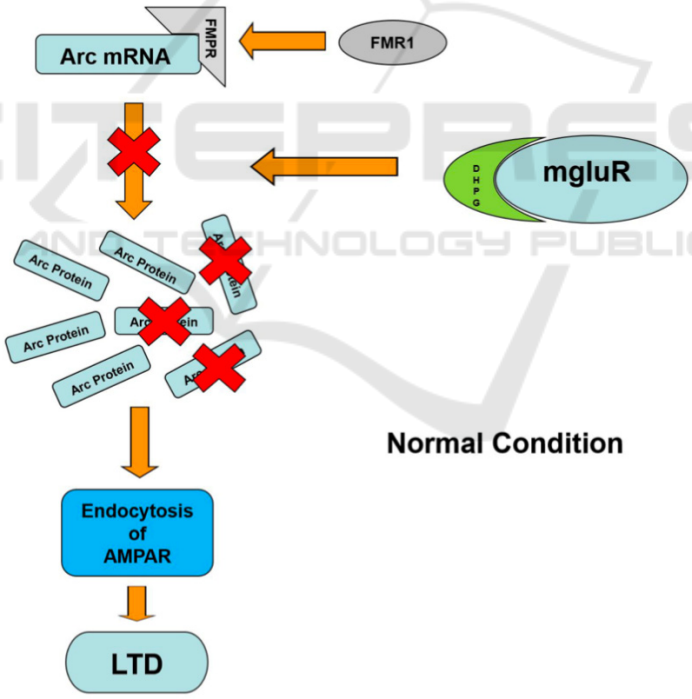

Figure 1: Mechanism of how FMR1 regulates the interaction between Arc mRNA and mGluR-LTD.

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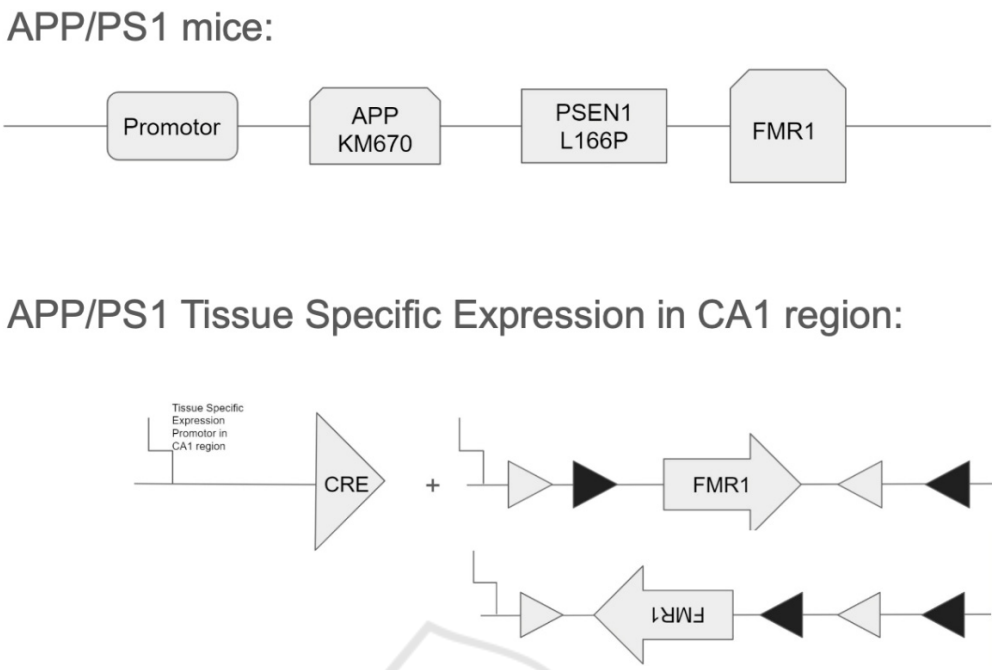

Figure 2: A sketch of how FMR1 conditional knock-out in CA1 region of APP/PS1 mice.

3.5 Exogenous Application

3.5.1 Peptide

A type of peptide called the Arg9 peptide will be used, which is the most efficient peptide (Ray, Fagan, Brunicardi 2000). The peptide gets into the cell in a non-endocytosis transmembrane process. This eliminates the need of entering the cell by endocytosis of proteins, which will send the endosome to the lysosome for degradation. However, if it's a non-endocytosis transmembrane process, it means that the peptide will first attach to the cell membrane and make a hole on the membrane. The hole allows the peptide to go into the cell. However, scientists are still investigating the detailed process of how peptide gets into the cell as the peptide can repair the hole so swiftly that scientists can't observe it (Jiawan, 2015).

3.5.2 Create Fusion Protein

The Arg9 peptide and arc protein will be fused together to get the Arg9-Arc protein gene through genetic engineering.

3.5.3 Construct Prokaryotic Expression Plasmid

After the fusion protein gene is obtained, it will be loaded into the plasmid for better transfecting cells. This recombinant plasmid includes both the target gene and the plasmid vector.

To get the target gene, the primers are designed first. The primers will allow DNA to be inserted into the plasmid. The design can be done by using Primer Blast or Primer Premier 5.0. The 5 primer's end will contain the his-tag, which allows for easy purification and detection of the recombinant protein. The 3 primer's end will contain the green fluorescence protein, which is useful for identifying the protein localization and analyzing the gene expression. Then the primers will clone the Arg9-Arc fragment, which is the target gene fragment wanted. Restriction enzyme digestion is performed to get this clonal fragment and the cohesive ends of the plasmid vector. The recombinant plasmid is then obtained via enzyme-linking of the fragment and the plasmid vector. This prokaryotic expression plasmid will then be sent to the sequence analysis company to confirm that it is constructed successfully.

3.5.4 Transfection of the Plasmid

A. Expression of the recombinant fusion protein
After DNA is loaded into the plasmid, it will be transfected into Escherichia coli (E. coli). In E. coli, the recombinant fusion protein will be expressed. As adding the inducer will increase the amount of expression, an inducer will be added when the bacterial growth reaches the exponential phase of growth. Herein, the amount of the inducer is being

added and the time it's going to induce will need to be taken into consideration. To optimize the expression conditions, tests will be set up to investigate under which conditions the protein will express the most.

Investigation of time for inducing

(1) Take bacterial fluid which has been induced 0 hour (h), 1h, 2h, 3h, 6h, 8h respectively.

(2) Extract lysates and run SDS-PAGE gel.

(3) Stain the protein gel and elute protein.

(4) Observe the protein band that is at the expected molecular mass of Arg9-Arc fusion protein.

(5) With time of inducing increases, the concentration of the target band increases, which means the brightness of the band increases. Observe after which hour, the brightness stops increasing. This means that with this hour's time of inducing, protein expression amount is maximized.

B. Purification of the recombinant fusion protein

Then the recombinant fusion protein will be purified by using the His-tag Ni purification system. This system can result in the protein of high concentration. The SDS-PAGE gel electrophoresis is the used to assess the protein purity. If the target protein band at the expected molecular mass of Arg9-Arc is distinct, it means that the purity of protein is high, and the protein is prepared to be sent into the cells.

4 GROUP SET-UP

1. 10 APP/PS1 mice + DHPG vs. 10 Wild type mice + DHPG (LTP, LTD and NMDA/AMPA measurements)

2. 10 APP/PS1 mice + exogenous Arc protein in dendrites + DHPG (in CA1 Region) vs. 1(LTP, LTD and NMDA/AMPA measurements)

3. 10 APP/PS1 FMR1 cKO mice + DHPG (in CA1 region) vs. 1 (LTP, LTD and NMDA/AMPA measurements)

5 DATA ANALYSIS

Data are presented as mean ± SEM. For comparison of the 2 groups, if the data are normally distributed, then a 2-tailed student t-test was used, otherwise, a Mann-Whitney test was used. For comparison between multiple groups, if the data are normally distributed, then an ANOVA was used, followed by individual post hoc tests when applicable, otherwise, a Kruskal-Wallis test was used.

6 DISCUSSIONS

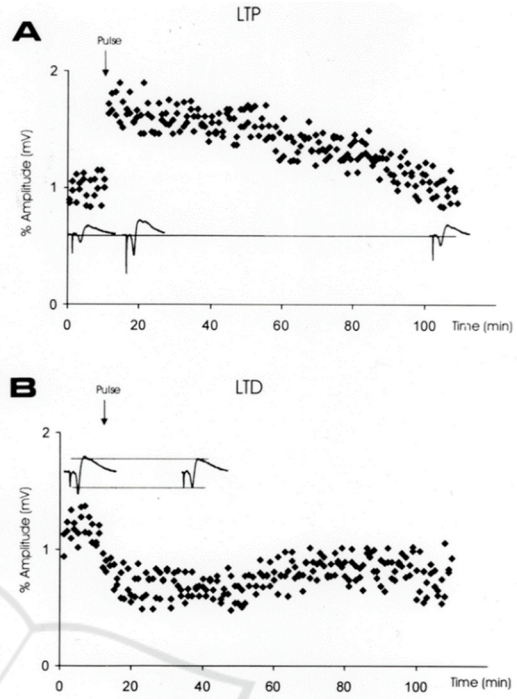

Figure 3: Expected result from electrophysiology showing LTP and LTD (Leff 2002).

If the results are in accord with the hypothesis, then the LTP and LTD is likely to be observed in the recorded diagram similar to Figure 3 below:

For AMPA ratio, a significant reduction of AMPARs on the synaptic surface will be detected. Based on these two phenomena, it can be concluded that the induction of Arc protein reverses the mGluR-LTD failure, and future study can be performed to investigate whether the AD symptoms have been mitigated with the induction of Arc protein.

7 CONCLUSIONS

In the experiment, the mGluR-LTD invalidation in APP/PS1 mice is reversed through inducing Arc protein through two approaches and observe whether the cognitive deficits in the mice have been mitigated. If the cognitive deficits are improved when mGluR-LTD failure is reversed, it means that the part of cognitive abilities is restored.

Meanwhile, if a significant reversal of mGluR-LTD invalidation in APP/PS1 mice can be observed, then the inducement of Arc protein can be a potential

cure for AD. Although, future investigation of Arc protein inducing mGluR-LTD failure is still required to ensure the multi-relationship among Arc protein, mGluR-LTD and the exact does of Ar protein that needs to maximize the efficiency.

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