DEVELOPING HIPPOCAMPAL DELIVERY OF AAV-NEUROD1
AS A NOVEL THERAPY FOR ALZHEIMER’S DISEASE

A Thesis in
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by
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ABSTRACT

Alzheimer’s disease (AD) is the most common neurodegenerative disorder in the elderly and is the leading cause of most dementia cases. Patients with AD experience gradual loss of cognitive abilities. Reactive astrogliosis is one of the most well-known features of AD pathology. Here, we are using a widely used 5xFAD mouse model to study NeuroD1 effect in AD. After hippocampal delivery of AAV-NeuroD1, we observed a significant reduction of reactive astrogliosis in the hippocampus region by testing several markers such as GFAP, S100β, GLT-1, and Sox2. We also detected changes in calretinin+ neuronal projections and blood vessel distributions by CD31 staining. More excitingly, we were able to see an improvement of learning and memory by conducting a novel-object recognition test 1 month after NeuroD1 delivery. These results suggest a potential novel gene therapy for AD using NeuroD1 as a single factor. Besides, we also injected Aβ oligomers into the hippocampus as an alternative AD model which recapitulate neuronal loss in AD patients.
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<tr>
<td>AAV</td>
<td>adeno-associated virus</td>
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<tr>
<td>Aβ</td>
<td>beta amyloid</td>
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<tr>
<td>ACSF</td>
<td>artificial cerebrospinal fluid</td>
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<td>AD</td>
<td>Alzheimer's disease</td>
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<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
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<td>CR</td>
<td>calretinin</td>
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<td>Ctx</td>
<td>cortex</td>
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<td>DG</td>
<td>dentate gyrus</td>
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<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>IML</td>
<td>inner molecular layer</td>
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<td>NDS</td>
<td>normal donkey serum</td>
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<tr>
<td>NeuroD1</td>
<td>neuronal differentiation 1</td>
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<td>NGS</td>
<td>normal goat serum</td>
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<tr>
<td>NOR</td>
<td>Novel Object Recognition</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PEI</td>
<td>polyethylenimine</td>
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<td>PFA</td>
<td>paraformaldehyde</td>
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<td>SP</td>
<td>synaptophysin</td>
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<tr>
<td>SV2</td>
<td>vesicular glutamate transporter</td>
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I believe that the end of this chapter of my life is the start of a new one.
Chapter 1 Introduction

AD is the most common neurodegenerative disorder and it accounts for most of the dementia cases in the elderly. At behavioral level, the patients experience progressive loss of memory. At cellular level, the disease is characterized by progressive loss of neurons and synapses, accumulation of β-amyloid plaques and neurofibrillary tangles. It is also characterized by reactive gliosis, including reactive astroglisis in which astrocytes are activated. Besides, chronic inflammatory responses as well as blood brain barrier (BBB) breakdown are also known pathologies in AD.

Astrocyte is a type of glial cell in the brain and plays very important roles in neuronal development as well as supporting neurons with nutrients. Normal astrocytes are quiescent and have definite territories of their own. In acute injury or in disease conditions, astrocytes become reactive. They are characterized by increased proliferation abilities, hypertrophic morphology, increased expression of glial fibrillary acidic protein (GFAP), etc. In AD, Aβ plaques are most often surrounded by reactive astrocytes. These astrocytes have increased expression of GFAP and are considered to be triggered by accumulation of extracellular Aβ, neuronal or synaptic damages. These reactive astrocytes may be neuroprotective in early stages by secreting neurotrophic factors. But in the later stages of AD, they become neuroinhibitory by losing their normal function and also by secreting inhibitory factors such as CSPG.

Astrocytes provide neurons with nutrients by contacting blood vessels. The blood-brain barrier (BBB) is a barrier that limits neurotoxic plasma-derived proteins and others into the brain. Post-mortem studies showed AD patients have BBB damage and that blood-derived proteins are accumulated in the hippocampus and cortex. Studies have shown that BBB breakdown may contribute to cognitive impairment.

The hippocampus is a brain area critical for neurogenesis as well as in the process of learning and memory. It is especially vulnerable at early stages of AD and it is the brain region that suffers the most from Aβ plaque depositions and also other neuropathological damages in AD. As a result, dysfunctional neurogenesis may contribute to memory impairment in AD.
The AD model we use here is 5xFAD mouse model. There are many different AD models, each focus on different features of the disease pathology. 5xFAD mouse model is a widely used model because it can recapitulate major features of AD amyloid pathology. The cause of AD is not fully understood yet but the gene amyloid precursor protein (APP) and presenilin-1 (PS-1) are known to accounts for some of the familial cases of AD. 5xFAD mice overexpress three human APP mutations and two PS-1 mutations, which leads to early-age Aβ42 accumulation. In the hippocampus of 5xFAD mice, amyloid plaque deposition, reactive gliosis, chronic inflammation, and reduced synaptic protein levels have all been described.

However, 5xFAD mouse model cannot mimic neurodegeneration in AD patients. To recapitulate the neuronal loss in AD patients, we decide to establish another AD model in the lab - injecting Aβ oligomers (AβOs) into hippocampus. AβO is the most toxic version of Aβ and can cause severe neuronal loss and reactive gliosis within days. This method creates a model for sporadic Alzheimer’s disease, which is the most common form of AD.

Current therapies can only provide temporary relief from the symptoms, but eventually they cannot stop the loss of memory or other cognitive abilities. Stem cell therapy has been intensely explored for AD treatment. However, recent studies raised serious questions regarding the uncertainty of stem cell differentiation, long term survival of grafted cells, and unexpected tumor formation. Therefore, more effective and safer ways of treating AD are urgently needed.

Our recent work has demonstrated an innovative approach to convert reactive glial cells after brain injury into functional neurons in mouse brain. This was achieved by delivering retroviral vectors that carry a neural transcription factor NeuroD1 into the mouse cortex. More excitingly, we were also able to reprogram cortical reactive astrocytes into functional neurons in a 5xFAD mouse model. Compared with stem cell therapy, our work made an important breakthrough by directly converting reactive astrocytes into functional neurons without going through stem cell stage. What’s more, our astrocyte-converted neurons in AD mouse model were mature and functional. Two other groups have also succeeded in converting striatum astrocytes into neurons in vivo using a combination of transcription factors BAM (Brn2a, Ascl1, Myt1l) or Sox2 alone. However, no direct reprogramming from resident hippocampal reactive astrocytes to neurons has been achieved, especially in a neurodegenerative disease model.
Compared with retrovirus, adeno-associated virus (AAV) is smaller in size and is naturally defective for replication without helper virus. As a result, it has been a safer and more efficient way of gene delivery.

We demonstrate here that, by using AAV, the efficiency of NeuroD1 delivery is greatly enhanced. However, we found that the AAV-NeuroD1 infected cells are a mixture of converted neurons and endogenous neurons that were “leaked into”. Despite of the complication in hippocampus, our preliminary data indicates that, after injecting NeuroD1 AAV into the hippocampus of 5xFAD mice, reduction of reactive astrocyte markers (GFAP, S100β, Glt-1, Sox2) were observed. We also observed increased signal of calretinin nerve terminals in inner molecular layer (IML) and upregulation of blood vessel marker (CD31). All of these results indicate NeuroD1 in hippocampus may promote beneficial amelioration of AD pathologies. More importantly, the learning and memory of AD mice was greatly improved after AAV-NeuroD1 injection for 1 month in novel-object recognition (NOR) test. The cellular and behavioral improvements strongly indicate that AAV-NeuroD1 delivery into the AD hippocampus region could be a potential therapy for Alzheimer’s disease.
Chapter 2 Materials and Methods

2.1 AAV production

The AAV constructs were constructed by Dr. Zifei Pei. The vectors used in this thesis were: AAV5-hGFAP-Cre, AAV5-FLEX-NeuroD1-P2A-GFP, and AAV5-FLEX-GFP.

The hGFAP promoter was amplified by PCR. Later hGFAP promoter was inserted into AAV construct pAAV-MCS (Cell Biolab) vector. The Cre gene was then sub-cloned into AAV5-hGFAP to generate AAV5-hGFAP-Cre. NeuroD1 was obtained by PCR as well. Then NeuroD1 was fused with P2A-GFP and together they were sub-cloned into AAV-FLEX-GFP.

Recombinant AAV5 was produced in HEK-293 cells. pAAV expression vector, together with pAAV5-RC and pHelper plasmid transfected HEK-293 cells with PEI. 3 days after culturing, cells were scraped off and centrifuged. The cells were then frozen and thawed for 4 times in dry ice and 37 °C water bath. The lysate was then put through an iodixanol gradient buffer and centrifuged for about 1 hr with a speed of 45,000 rpm. The top layer containing the virus was collected and put through Millipore Amicon Ultra Centrifugal Filters. After centrifugation, purified virus was stored in - 80 °C.

2.2 Animals

All experiments were conducted on wildtype C57/BL6 and 5xFAD transgenic mice [Jackson Laboratory, B6SJL-Tg (APPSwF1lon,PSEN1*M146L*L286V) 6799Vas/Mmjax]. Mice were housed in a 12 hr light/dark cycle room and were supplied with sufficient food and water. All experimental protocols were approved by Penn State Institutional Animal Care and Use Committees (IACUC) and in accordance with National Institutes of Health (NIH) guidelines.
2.3 Preparation of AβOs

AβOs were prepared from synthetic human Aβ1-42 peptide (Biopeptide) as described previously. The solid peptide was first solubilized in hexafluoroisopropanol (HFIP) to a concentration of 1 mM (add 222.2 µl cold HFIP to 1 mg Aβ1-42) and then aliquot into microcentrifuge tubes (22 tubes, 10 µl = 45 µg). Then the solvent was evaporated to produce dried films, which could be stored in -80°C. The dried films were subsequently dissolved in sterile anhydrous dimethylsulfoxide (DMSO) to make 5 mM solution (add 2 µl DMSO to each film, 2 µl = 45 µg). This solution was diluted to 100 µM in ice-cold PBS (add 98 µl PBS to 2 µl solution, 100 µl = 45 µg) and incubated for 16 h at 4°C. The solution was then centrifuged (14,000 × g for 10 min) at 4°C to remove insoluble aggregates, and the supernatant containing soluble AβOs were used.

2.4 Stereotaxic injections

The mice were anesthetized using 2.5% Avertin (20ml/kg). The anesthesia effect was tested by toe-pinch. The mice were placed on the surgery setup. Artificial eye ointment was applied to protect the eyes. Then hair on the head was removed using a clipper. Ethanol and iodine solutions were applied three times. An incision was made in the midline and a hole was drilled according to the coordinates needed (hippocampus hilus region, AP-2.06, ML±1.4, DV-2.0). The injection volume was 1.0-2.0 ul and the flow rate was 0.15-0.2 ul/min. The needle was kept in the same site for 3 min and then was slowly withdrawn for another 3 min. The skin was glued back together using tissue glue and the mice put on heating pad for recovery. All mice was closely monitored for a week after the surgery.

2.5 Immunocytochemistry and data analysis

The animals were again anesthetized with 2.5% Avertin at the wanted time point. They were then perfused with cold ACSF and then exposed to post-perfusion fixation in 4% PFA in cold room. The brains were fixed for 24 hr and washed 3 times with 1xPBS solution. The brains were then sliced on vibratome to 40 um sections. The sections were then washed three times by PBS
and permeabilized in 0.3% Triton X-100 in PBS for 1 hr. Then the sections were blocked in 2.5% NGS, 2.5% NDS, and 0.1% Triton X-100 in PBS for another 1 hr. Then the sections were incubated in primary antibody solutions in 4°C for overnight. The second day, the sections were continuously incubated in primary antibody solution for 30 min and then washed with 0.05% Triton X-100 in PBS 5 min for 3 times. Secondary antibody was later added and the sections incubated for 1.5 hr. Again, the sections were washed in 0.3% Triton X-100 in PBS 10 min for 3 times. The sections were eventually mounted with anti-fading mounting solution with DAPI. All images were taken by confocal microscope (Olympus FV1000) with the Z-stacks feature.

The following primary antibodies were used: polyclonal anti-GFAP (rabbit, 1:1000, Abcam, Z0334), polyclonal anti-GFAP (chicken, 1:1000, Millipore, AB5542), polyclonal anti-GFP (chicken, 1:1000, Abcam AB13970), monoclonal anti-S100β (mouse, 1:600, Abcam, AB66028), polyclonal anti-SV2 (rabbit, 1:2000, DSHB), monoclonal anti-6E10 (mouse, 1:1000, Abcam, AB11132), polyclonal anti-NeuN (rabbit, 1:1000, Millipore, AB7878), polyclonal anti-Iba1 (rabbit, 1:1000, Wako, -19-19841), monoclonal anti-NeuroD1 (mouse, 1:1000, Abcam, AB60704), polyclonal anti-CR (goat, 1:1500, Millipore, AB1550), anti-Sox2 (rabbit, 1:1000, Millipore), anti-SP (guinea pig, 1:600, SYSY), monoclonal anti-iNOS (mouse, 1:500, BD transduction laboratories), anti-CD31 (rat, 1:800, BD Pharmingen), anti-Ki67 (rabbit, 1:800, Abcam, AB15580).

Images were taken randomly within the interested area and were analyzed by Image J software.

### 2.6 Novel-object recognition test

The animals were allowed 3 consecutive days for habituation. Each mice for 10 min. The box was cleaned up in between with 70% ethanol and wait until fully dry. Then D4 is the day for familiarization. Two same objects (flask or lego) were place in the box and the mice were allowed 10 min to become familiar with the object. Day 5 is the testing day when the box has one old object and one novel object. The mice were allowed 5 min for exploration and the process was recorded by a recording camera. We tested nine WT mice, five 5xFAD injected with AAV-GFP, and five 5xFAD mice injected with AAV-NeuroD1.
Chapter 3 Results

3.1 5xFAD mouse model as an early-onset Alzheimer's disease model

AD is characterized by β-amyloid deposition, intracellular tau tangles, synaptic dysfunctions, significant loss of neurons, reactive gliosis, chronic inflammation, and neurovascular dysfunctions. Among the many different AD animal models, the 5xFAD mouse model is a commonly used one due to recapitulating major features of AD amyloid pathology. 5xFAD mice overexpress three human amyloid precursor protein (APP) mutations and two human presenilin-1 (PS-1) mutations and β-amyloid (Aβ) 42 is accumulated at a very early age. In the hippocampus of 5xFAD, amyloid plaque deposition, reactive gliosis, chronic inflammation, and reduced synaptic protein levels have been described.

3.1.1 Increased level of Aβ deposition and reactive gliosis in 5xFAD mice

At 6 month of age, compared with their non-transgenic littermates, 5xFAD mice showed significantly increased level of Aβ deposition in the entire brain, especially deep layer cortex and hippocampus. In Figure 1A, anti-6E10 staining showed that, in the hippocampus, human Aβ expression is highly upregulated. Aβ plaques are located mostly in the molecular layer outside of the granular layer and sometimes also in dente gyrus region.

The abundancy of reactive astrocytes is very much correlated with the level of Aβ plaque load. This confirmed the possibility that reactive astrocytes could be triggered by extracellular toxin such as Aβ itself. Reactive astrocytes are characterized by upregulation of GFAP protein as well as increased proliferation abilities and elongated morphology. In Figure 1B, GFAP+ astrocytes are obviously increased in the hippocampus region. This increase is most significant in the hilus region and further spread to the entire dentate gyrus. Morphologically, reactive astrocytes are thicker in their processes. These reactive astrocytes are activated during development and they are quite different from normal quiescent astrocytes. Reactive astrocytes secrete inhibitory
factors to inhibit growth. One example is the secretion of inhibitory factor CSPG. CSPG is a type of proteoglycans is known to inhibit axonal regrowth after injury. Thus, in order to ameliorate the inhibitory disease condition, it would be helpful if the reactivity of these astrocytes can be reduced. In our work, the abundant hypertrophic reactive astrocytes in 5xFAD mice hippocampus could be used as the starting material to apply our in vivo reprogramming and be potentially converted to neurons.

In 5xFAD mouse model, another disease feature is the chronic inflammation. Microglia is the scavengers of the brain and is also activated by extracellular Aβ deposition. The activation of microglia indicates induced inflammation, which drives neurodegeneration. In normal brain, microglia has fine processes. In disease condition, Iba1 is significantly upregulated in the hippocampus (Figure 1C).
Figure 1. Aβ deposition and reactive gliosis in 5xFAD mice.

(A) 6E10 (red) immunofluorescence image shows 5xFAD mice has increased expression of human Aβ at hippocampus. (B) GFAP (cyan) staining shows reactive astrocytes in hippocampus. (C) Iba1 (green) staining shows chronic inflammation in 5xFAD mice hippocampus. All immunofluorescent staining was done on 6 month old male mice. Scale bars: 150 µm for (B) (C) 10x, 40 µm for (A) and (B) (C) 40x.
3.1.2 Neuronal network deficits in 5xFAD mice

During the progression of AD, subtle alterations of hippocampal synaptic efficacy happen long before evident neuronal degeneration. As a result, AD is also considered a “synaptic failure”\(^1\). In our 5xFAD mice, significant decrease and clustering of presynaptic markers SV2 and synaptophysin (SP) was observed (data not shown). This indicates that, in the AD disease condition, normal synaptic functions are compromised. In the future, more synaptic markers such as drebrin and PSD-95 should be tested.

Another interesting aspect of hippocampal neuronal network is the axon projections of hilar mossy cells. Hilar mossy cells send their projections both ipsilaterally and contralaterally to innervate the proximal dendrites of granule cells and inhibitory interneurons along the longitudinal axis of the inner molecular layer (IML) of the dentate gyrus (DG)\(^15,16\). Calretinin is a Ca\(^{2+}\) binding protein and is expressed in hilar mossy cells in normal mouse brain. Due to the innervation of mossy cells, the IML shows clear calretinin immunoreactivity. However, in some very old 5xFAD mice, we observed a significant decrease of calretinin signals in the IML, indicating either neuronal projection deficit or calretinin expression deficit (Figure 2A). To answer this question, we will perform axonal marker (SMI312) immunostaining on age matched 5xFAD and non-transgenic littermates in future experiments.

AAV itself is an anterograde tracer. Unilateral injection of AAV-GFP into dentate gyrus results in GFP\(^+\) nerve terminal in the contralateral side (Figure 2B). Note that GFP signal overlaps with calretinin signal at the contralateral IML, indicating GFP labeling calretinin\(^+\) mossy cells.
Figure 2. Calretinin* nerve terminal deficit in 5xFAD mice and mossy cell innervation to contralateral IML.

(A) Compared with WT littermates, 5xFAD mice showed significant reduction of calretinin signals in the IML of the DG. All tested mice are 12 months old. (Figure provided by Dr. Zheng Wu) (B) Using AAV-GFP as a anterograde tracer shows innervation of hilar mossy cell to contralateral IML in 5xFAD mice. Scale bar: 40 µm.

3.2 AAV is an efficient delivery system for NeuroD1 delivery into mouse brain

In order to target reactive astrocytes and maximize the expression of NeuroD1 for efficient conversion, we applied a novel AAV Cre-FLEX system. We built AAV plasmid with reversed “NeuroD1-P2A-GFP” cassette in between two pairs of lox sequences (lox2272 and loxP). The entire cassette is driven by a strong promoter (pCAG) and only in the presence of Cre recombinase, can the reversed cassette be flipped back to the correct direction (Figure 3A). To spatially control NeuroD1 overexpression only in astrocytes, we utilized astrocyte specific GFAP promoter to drive the expression of Cre recombinase. After co-injecting AAV5-pGFAP-Cre with either AAV5-pCAG-FLEX-NeuroD1-P2A-GFP (also named AAV5-FLEX-NeuroD1-GFP) or AAV5-pCAG-FLEX-GFP (AAV5-FLEX-GFP), NeuroD1 or GFP should be expressed strongly and specifically in astrocytes (Figure 3B).
We tested the efficacy of the AAV viral delivery of NeuroD1 in our 5xFAD model. Transgenic male mice aged 17 month were used for this experiment. 2 weeks post injection (2wpi), overexpression of NeuroD1 was detected in AAV5-NeuroD1 group but not in control AAV5-GFP group, indicating good AAV-mediated overexpression of NeuroD1 and low endogenous expression of NeuroD1 in adult hippocampus. Theoretically, only astrocytes could express pGFAP-Cre so that only in astrocytes, could GFP protein be detected. Surprisingly, we observed that, in the control group, GFP labels both astrocytes and neurons. This could partially be explained by the endogenous expression of GFAP in subgranular layer neural precursor cells, which means the precursors could be labeled by GFP. However, neurons in the dentate hilus should not be expressing GFAP thus are consider to be “leaked” into. When we inject AAV5-GFP in the cortex, some “leakage” was also observed, though at a much lower level. A potential explanation of this "leakage" is that, in response to inflammation, extracellular vesicles can mediate Cre recombinase mRNA to transfer into neurons\(^\text{17}\). Despite of the “leakage”, comparing with GFP control group, NeuroD1 group showed an increase in neuron number in dentate hilus, which indicates astrocyte-neuron conversion (Figure 3C and 3D). More repeats should be done to quantitatively evaluate conversion efficiency and increased neuron number.
Figure 3. AAV5 mediated delivery of NeuroD1 promotes astrocyte-neuron conversion in 5xFAD mice.

(A) Illustration of Cre-FLEX system. Note the blue and cyan triangle both indicate lox2272 but at different loci. Same with loxP. (B) Illustration of co-injecting AAV-Cre with AAV-FLEX-NeuroD1/GFP. Cre recombinase induces lox2272 and loxP sequence recombination and results in expression of genes. (C) GFP (green), NeuroD1 (red), and NeuN (cyan) immunofluorescence image of AAV5-GFP control group. GFP+ cells show both astrocyte and neuron morphology. No NeuroD1 signal detected. (D) GFP (green), NeuroD1 (red), and NeuN (cyan) immunofluorescence image of AAV5-NeuroD1 injection group. GFP+ cells show mostly neuron morphology and colocalized with NeuN signal. NeuroD1 overexpressed in hilus. All data acquired at 2wpi. Scale bars: 150 µm for 10x images, 40 µm for 40x images.
3.3 Beneficial effects of AAV-NeuroD1 injection into the hippocampus of 5xFAD mice

3.3.1 Reduction of reactive astrogliosis after AAV-NeuroD1 injection in 5xFAD mice

Upregulation of GFAP is a commonly used marker for reactive astrocytes. Reactive astrocytes tend to cluster surrounding Aβ plaques. As in Figure 4A, non-transgenic mice have a very low GFAP level in cortex (ctx) while AD mice have a significant increase of GFAP expression, indicating an increase of not only the level of reactive astrocytes but also the plaque load in 5xFAD mice. Besides, compared with non-transgenic littermates, 5xFAD also has an upregulation of GFAP in the DG of hippocampus region, although not as significant a difference as in cortex.

In order to test NeuroD1 effect on reactive astrocytes in the hippocampus, we stereotaxically injected AAV-NeuroD1 into the hippocampus. We waited 1 month after the surgery and perfused the mice. By comparing NeuroD1 and GFP groups, we observed that, 1 month after surgery, GFAP coverage area (%) reduced to 27.69% while the control group 38.74% in the dentate gyrus of the hippocampus region. At the same time, GFAP intensity (a.u.) was also reduced from 24.83 to 17.18 (Figure 4B and 4C). The reduction of both GFAP coverage area (%) and GFAP intensity (a.u.) indicates a reduction of reactive astrogliosis after NeuroD1 application.

Since we only injected into DG but not into cortex, the cortex should be less affected and more reflects the original disease severity of the mice. In Figure 4A, the AAV-NeuroD1 injected mouse showed more GFAP signal in the cortex compared to AAV-GFP injected mouse, indicating the NeuroD1 injected mice have more severe reactive astrogliosis to begin with. However, the NeuroD1 injected dentate gyrus region, which is more prone to NeuroD1 effect, showed less GFAP signal compared with GFP group. This further indicates that NeuroD1 can cause a reduction of reactive astrogliosis in the injection area.

As promising as it is, GFAP is only one marker for reactive astrocytes. We tested several more markers in the following experiments.
Figure 4. Reduction of GFAP coverage area and intensity in 5xFAD mice hippocampus at 1mpi.

(A) GFAP (white) immunofluorescence image of DG and cortex in non-transgenic littermates, 5xFAD mice, 5xFAD injected with AAV-GFP control virus, 5xFAD mice injected with AAV-NeuroD1 for 1 month. (B) Percentage of GFAP coverage area in four groups of mice at 1mpi. (C) Arbitrary units of GFAP intensity in four groups of mice. Scale bar: 40 µm.

Besides increased expression of GFAP, reactive astrocytes also show a higher level of S100β. S100β is a calcium binding protein and also neurotrophic signaling molecule expressed primarily by astrocytes. In AD patients, S100β level is elevated in the hippocampus and locates mainly in reactive astrocytes surrounding neuritic plaques18,19. Astrocyte overexpression of S100β correlates with the degree of neuritic pathology in Aβ plaques, indicating S100β as an alternative reactive astrocyte marker (the other one is GFAP).
Glutamate transporters are responsible for clearing extracellular glutamate and thus are very important for regulating synaptic network interactions and preventing neurotoxicity. GLT-1, expressed by astrocytes, is the dominant glutamate transporter in the cerebral cortex and hippocampus. It has also been reported that astrocyte-specific glutamate transporter GLT-1 is significantly reduced in AD brain.20 2 weeks after NeuroD1 AAV injection, we found a decrease of S100β and a substantial increase of GLT-1 in the hippocampus of 5xFAD mice (Figure 5A and 5B). This indicates a possible reduction of reactive gliosis and recovery of glutamate transporter on astrocytes. We hypothesized that AAV-NeuroD1 can push reactive astrocytes to become neurons and, at the same time, alleviate the reactive and dysfunctional state of the astrocytes.

Figure 5. Reduction of reactive astrogliosis after AAV5-NeuroD1 delivery in 5xFAD mice.

(A-B) GFP (green), S100beta (red), and Glt1 (cyan) immunofluorescence image of AAV5-NeuroD1 viral delivery in hippocampus at 2wpi. S100beta slightly decreased and Glt1 significantly increased. Scale bars: 150 µm for 10x images, 40 µm for 40x images.

Many astrocytes near plaques are found to be Sox2+.21 Thus, Sox2+GFAP+ cell number could also be one of the indicators that astrocytes are reactive. By comparing 7 month old 5xFAD and non-transgenic littermates, we demonstrate here that AD mice have a larger number of Sox2+GFAP+ cells. At the same time, the percentage of Sox2+GFAP+ cells in total GFAP+ cells is also higher. After injecting control AAV-GFP virus and AAV-NeuroD1 into mice hippocampus, we observed a reduction of Sox2+GFAP+ cell number and percentage in 5xFAD mice (Figure
The fact that, after NeuroD1 injection, there are less Sox2\(^+\) astrocytes indicates an amelioration of reactive astrocytes.

![Figure 6: Reduction of Sox2\(^+\) cell number in 5xFAD mice.](image)

(A) Sox2 (red) and GFAP (white) immunofluorescence image of AAV5-NeuroD1 viral delivery in hippocampus at 1mpi. (B) Quantification of Sox2\(^+\)GFAP\(^+\) and Sox2\(^+\)GFAP\(^-\) cell number. (C) Quantification of Sox2\(^+\)GFAP\(^+\) cell number percentage in all GFAP\(^+\) cells. Scale bar: 40 µm.

To conclude, the decrease of GFAP and S100\(\beta\), increase of GLT-1, and reduction of Sox2\(^+\)GFAP\(^+\) cell number all indicate a relief of reactive astrogliosis after NeuroD1 hippocampal
injection. This is very promising since reduction of reactive astrocytes indicates possible reprogramming to neurons (though we do not know the number yet) and also less neuroinhibitory factors in the local area.

### 3.3.2 Recovery of neuronal network deficit after AAV5-NeuroD1 injection in 5xFAD mice

In aged AD mice, the calretinin nerve terminal in inner molecular layer is compromised compared with non-transgenic littermates. After AAV-NeuroD1 injection, we performed calretinin immunostaining at 1mpi. In control group, the calretinin nerve terminals form a thin “band” that is comparable to non-injected AD mice. However, in AAV-NeuroD1 group, a recovery of calretinin “band” was noted, indicating a possible recovery of CR⁺ neuronal projections (Figure 7). However, some AD mice do not have the CR thin band deficit even when they age. As a result, we need to be more careful and should do more repeats in order to draw a conclusion.

![Figure 7](image)

**Figure 7. Rescue of calretinin⁺ nerve terminal in inner molecular layer of 5xFAD mice.**

Calretinin (red) and GFP (green) immunofluorescence image of AAV5-NeuroD1 viral delivery in DG at 1mpi. Scale bar: 40 µm.
3.3.3 Recovery of blood vessels in 5xFAD mice

It is known that neurovascular dysfunction is associated with AD. After AAV-NeuroD1 injection, we did observed an obvious increase of CD31\(^+\) blood vessel numbers in NeuroD1 group compared with GFP control group (Figure 8). This increase was observed mostly in the molecular region. Co-immunostaining of Thioflavin S (ThS; labeling senile plaques), GFAP, and CD31 showed abundant reactive astrocytes and reduced number of blood vessels surrounding ThS\(^+\) plaques. More repeats and further investigation need to be done to draw a conclusion of whether blood vessel number has been increased.

![Figure 8. Increase of blood vessels after AAV-NeuroD1 injection.](image)

GFAP (red) and CD31 (cyan) immunofluorescence image of 5xFAD hippocampus 2 weeks post injection. Scale bars: 150 \(\mu\)m for 10x images, 40 \(\mu\)m for 40x images, 40 \(\mu\)m for further enlarged images.
In this AD model, more repeats need to be done so as to quantify the number of newly generated neurons and the astrocyte repopulation. We will also perform immunostaining to examine whether the level of Aβ deposition, CSPG, and inflammation markers such as IL-1β, TNFα and CD68 will be reduced in the AD hippocampus after NeuroD1 mediated astrocyte-neuron conversion. We will further investigate neurovascular changes and blood vessel-astrocyte interaction by performing co-immunostaining of blood vessel markers (CD31, ZO-1) with astrocyte markers (GFAP, S100β) and astrocyte endfeet marker (AQP4).

3.4 Novel-object recognition test showed 5xFAD mice have significant improvements of memory after NeuroD1 delivery

Novel-object recognition test is used to assess recognition memory. This test is based on the spontaneous tendency of rodents to spend more time exploring a novel object than a familiar one. Percentage time spent with the familiar and novel object is compared and then a discrimination index will be generated. The bigger the discrimination index, the better the memory.

After injecting NeuroD1 and control AAV into AD mice, we performed the NOR test on three groups: WT littermates (n=9), 5xFAD injected with AAV-GFP (simplified as GFP group, n=5), and 5xFAD injected with AAV-NeuroD1 (simplified as NeuroD1 group, n=5). We tested the effect of hippocampal AAV-NeuroD1 delivery at 1 month post injection.

As in Figure 9, before injection, WT littermates have a much better memory compared with 5xFAD mice (WT before, GFP before, ND1 before). 1 month after surgery, NeuroD1 group had a significant improvement in terms of novel object recognition, indicating a great improvement of recognition memory (ND1 before vs. ND1 after). NeuroD1 group also was significantly different from GFP group (ND1 after vs. GFP after). Surprisingly, NeuroD1 group even exceeded WT group (ND1 after vs. WT after, WT did not experience any surgery but were tested together with GFP and NeuroD1 group). This result is very encouraging. However, future experiments will be needed to validate this result as the n number should be bigger for each group.
Figure 9. NOR test after NeuroD1 injection in 5xFAD mice.

The test was conducted 1 month after the surgery. WT n=9, GFP group n=5, NeuroD1 group n=5. * P < 0.05, ** P < 0.01, *** P < 0.001 (Student’s t-test).

3.5 AβO injection as an alternative mouse model to study NeuroD1 effect

3.5.1 Injection of AβOs as an alternative sporadic AD model

AD patient brains show severe neurodegeneration. Unfortunately, most of the AD animal models cannot mimic this remarkable deficit. To recapitulate the neuronal loss in AD patients, we decide to inject AβOs into hippocampus. AβO injection is known to cause severe neuronal loss and reactive gliosis within days. By conducting in vivo reprogramming in two distinctly different AD mouse models, we can make sure that our new technology is generally applicable to AD models and not limited to one particular mouse model.
Soluble AβOs are considered to be the most toxic form of β-amyloid, and are known to accumulate in the brains of AD patients. These soluble neurotoxins seem likely to account for the imperfect correlation between insoluble fibrillar amyloid deposits and disease-associated cognitive dysfunction. To create a model for sporadic Alzheimer’s disease, which is the most common form of AD, AβOs have been injected into animal brains. AβOs trigger abnormal signaling pathways that culminate with loss of memory and behavioral alterations that recapitulate clinical observations in AD patients. Reactive astrocytes, together with other cardinal features of AD pathology (including synapse loss, tau hyperphosphorylation and microglia activation), were observed in macaque brain after injecting AβOs.

AβOs were prepared from synthetic human Aβ1-42 peptide (Biopeptide) as described previously. Male and female mice aged 7 months were used. The mice received bilateral intrahippocampal injections (AP -2.06, ML ±1.4, DV -2.0) of 2 µg freshly prepared AβOs or DMSO control. After 3 days or 2 weeks, mice were perfused (Figure 1A and 1B). As expected, control injection of DMSO revealed no oligomer immunoreactivity detectable by the anti-human Aβ 6E10 (Figure 1C) and the oligomer-selective A11 antibodies (data not shown) at 2 weeks post-injection. However, mice injected with AβOs revealed intense 6E10 (Figure 1D) and A11 immunoreactivities (data not shown) in the hippocampus, which suggested successful injections of AβOs.
Figure 10. Stereotaxic injection of synthetic Aβ oligomers into adult mice brain.

(A) Chronogram representing the administration of AβOs in mice. Days post injection of AβOs or DMSO, dpa. (B) Stereotaxic injection (AP -2.06, ML ±1.4, DV -2.0) of AβOs. Red arrows illustrate injection site. (C) 6E10 immunofluorescence (red) image of DMSO injected hippocampus at 2wpa. No human Aβ was detected in DMSO control group. Weeks post injection of AβOs or DMSO, wpa. Yellow dot boxed area is enlarged on the right. (D) 6E10 immunofluorescence (red) image of AβOs injected hippocampus at 2wpa. Injected AβOs distributed along the needle track, into the granule cell layer and hilus region. Yellow dot boxed area is enlarged on the right. Scale bars: 150 µm for (C) left panel and (D) left panel, 40 µm for (C) right panel and (D) right panel.

3.5.2 Neuronal degeneration induced by AβOs injection

In order to investigate whether our AβO injection causes neuron degeneration, Fluoro-Jade C (FJC) staining (labeling degenerating neurons) was applied. At 3 days post AβO injection (dpa), AβOs injected mice exhibited massive FJC positive signals along the needle track and in the hippocampus (Figure 11A). Note that a clear boundary between degenerating neurons (FJC+) and healthy neurons (NeuN+) was observed in granule cell layer, which has densely packed neuronal somas. The loss of NeuN signals at FJC+ areas confirmed ongoing neuron
degeneration. AβOs injection causes neuronal degeneration up to 2 weeks post AβO injection (wpa), while the DMSO control has almost no FJC signals (Figure 11B and 11C). Therefore, we hypothesize that the neuron degeneration was caused by toxic AβOs, with the “boundary” possibly caused by the distribution of injected soluble AβOs.

![Image](image1.png)

**Figure 11. AβO injection into hippocampus causes neuron degeneration.**

(A) FJC (green) and NeuN (red) immunofluorescence image of AβO injected hippocampus at 3dpa. Massive neuron degeneration at granule cell layer and in hilus was detected. A clear boundary was showed between FJC⁺ degenerating neurons and NeuN⁺ neurons. White dot boxed area is enlarged on the right. (B) Continued FJC signal was detected at 2wpa. (C) DMSO control did not show visible FJC immunoreactivity. NeuN signal still intact along granule cell layer. Scale bars: 150 µm for low magnification images, 40 µm for high magnification images.

**3.5.3 Reactive gliosis induced by AβOs injection**

It is well known that both astrocytes and microglia are activated in AD patient brain. This activation is characterized by alterations in their morphology, such as hypertrophy of the cell
soma, increased branching, upregulation or de novo synthesis of cell surface or intracellular molecules, and proliferation. Similarly, in our AβO injection model, microglial cells are activated after 3 days. 6E10 immunofluorescence staining almost perfectly colocalized with Iba1 staining, indicating that AβOs are probably uptaken by, or bound to the surface of, activated microglia. It could be well explained by microglia’s immune defender’s role, including the uptake and clearance of Aβ\textsuperscript{30, 31}. Astrocytes, however, showed only modest increase of GFAP expression at 3dpa (Figure 12A). 2 weeks after AβO injection, astrocytes become reactive, consistent with previous knowledge of reactive gliosis that after injury, astrocytes start to proliferate from one week to two weeks. Microglia remains hypertrophic compared with DMSO control (Figure 12B and 12C). Higher resolution image shows that Aβ locates within the activated microglia at both 3dpa and 2wpa (Figure 12D). It could be well explained by microglia’s immune defenders role, including the uptake and clearance of Aβ\textsuperscript{32, 33}.

When subjected to CNS disease, microglia could be further characterized to two distinct functional phenotypes: M1 and M2. M1 is considered to be pro-inflammatory and “classically activated” phenotype. Inducible nitric oxide synthase (iNOS) is a marker for M1 phase microglia. M2, however, is considered “good” and anti-inflammatory, with arginase-1 (Arg-1) to be a marker\textsuperscript{34}. Compared with DMSO control, AβOs injection caused increased expression of iNOS at both 3dpa and 2wpa (Figure 12E-G). Arg-1 co-immunostaining is necessary in future experiments.
Figure 12. AβO injection into hippocampus causes reactive gliosis.

(A) GFAP (green), Iba1 (cyan), and 6E10 (red) immunofluorescence image of AβOs injected hippocampus at 3dpa. GFAP has modest increase. Microglia (Iba1) is activated and hypertrophic. Aβ (6E10) is colocalized with Iba1. (B) Reactive astrocytes are detected at 2wpa. Microglia marker Iba1 still colocalize with 6E10. (C) DMSO control did not show upregulation of GFAP or Iba1. No obvious 6E10 signal was detected. (D) 40x confocal images shows uptake of Aβ into activated microglia at both 3dpa and 2wpa. (E) Iba1 (red) and iNOS (green) immunofluorescence image of AβOs injected hippocampus at 3 dpa. iNOS colocalize with microglia processes. (F) Dramatic increase of iNOS expression level at 2wpa. iNOS colocalize with microglia processes. (G) DMSO control showed no upregulation of Iba1 or iNOS. Scale bars: 40 µm for (A) (B) (C) (E) (F) (G), 20 µm for (D).

To further confirm whether astrocytes and microglia are active and proliferative after the injection, a cellular marker for proliferation (Ki67) was used. At 3dpa, both astrocytes and microglia were proliferating, with microglia being the most proliferative population (Figure 13A and 13D-E). At 2wpa, Ki67+ cell number decreased significantly (Figure 13B and 13C). This indicates the two cell populations are most proliferative at early time point. After two weeks, they already are still hypertrophic but may not be as proliferative.
Figure 13. Both astrocytes and microglia are proliferative after AβO injection.

(A) GFAP (green), Iba1 (red), and Ki67 (cyan) immunofluorescence image of AβO injection at 3dpa. Majority of Ki67 signals colocalized with Iba1. (B) Microglia and astrocyte proliferation were decreased at 2wpa. (C) DMSO control showed no Ki67 immunoreactivity at 2wpa. (D) 40x confocal image showed microglia and astrocytes undergoing proliferation. Note bottom panel an astrocyte is dividing into two. Scale bars: 40 µm for (A) (B) (C), 20 µm for (D) (E).

Compared with the 5xFAD transgenic mouse model, this AβO injection model has the advantages of taking a shorter time to develop, generating severe neuronal loss locally, and induce severe reactive gliosis. In the future, we will inject AAV-NeuroD1 and control AAV-GFP virus into AβO injected hippocampus and test whether our in vivo astrocyte-neuron conversion can rescue the neurodegeneration and also bring back neuron-astrocyte balance. In addition, similar with the 5xFAD model, we will test via immunostaining CSPG, inflammation markers, and blood vessel markers, etc.
Chapter 4 Discussion

Our previous work showed an innovative and successful reprogramming of glial scar reactive astrocytes into functional neurons after brain injury using a single transcription factor NeuroD1. It was done via retroviral vectors. We were also able to convert reactive astrocytes in the cortex of 5xFAD mice into neurons. Here, we further tested the effect of NeuroD1 on the AD mouse model using AAV Cre-FLEX system. We targeted on hippocampus due to the fact that it is the area that suffers the most from Aβ deposition and thus affects the learning and memory. Our results showed that, after control virus infection, the infected cells were a mixture of GFAP expressing astrocytes and endogenous neurons. In the AAV-NeuroD1 injection group, the infected cells were also a mixture of converted neurons and endogenous neurons. This “leakage” could be partially explained by high GFAP expression in hippocampal neural progenitor cells in the sub-granular zone. However, the reason why the hilus region has so much “leakage” is still a mystery to us, which need to be further investigated. As a result, the converted neuron number and conversion efficiency was hard to quantify. Despite of the fact that hippocampus is a complicated region for in vivo reprogramming, we still observed that hippocampal delivery of NeuroD1 did have a great effect on improving the pathological conditions of 5xFAD mice hippocampus.

In AD hippocampus, astrocytes become reactive as the disease progress. After AAV-NeuroD1 injection, we observed reduction of both GFAP coverage area and intensity at 1 month post injection. This reduction was relatively consistent with each group having 2 mice. Since GFAP is a commonly used marker for reactive astrocytes, we are quite confident that reactive astrogliosis is reduced at certain level. However, this result needs to be repeated to confirm statistical significance. S100β, which is also elevated in the AD hippocampus, was reduced after NeuroD1 injection. But this staining was not quantified due to limited sample so it needs to be repeated in the future. GLT-1 is another staining we did and it showed substantial increase in NeuroD1 group. This indicates the re-gaining of normal astrocyte function in terms of uptaking excessive glutamate. Another indicator of reactive astrocytes is Sox2 signals. Our preliminary
results showed decrease of Sox2⁺GFAP⁺ cell number. However, what this means and whether it is consistent also needs to be further investigated in future experiments.

We also observed rescue of calretinin⁺ “band” after AAV-NeuroD1 injection. However, the problem with repeating this experiment is that the AD phenotype is sometimes not so severe that even aging AD does not have the calretinin “band” deficit all the time. In order to repeat this experiment, we need to have more consistent 5xFAD mouse line. If this rescue is the case, it will indicate that there’s conversion and the converted neurons are developing new neuronal networks. However, the subtypes of the converted neurons should be confirmed as well.

Blood vessel marker CD31 signal was greatly increased after AAV-NeuroD1 injection. We observed that the molecular layer has the most significant increase while hilus region remains the same. This experiment also needs to be repeated. At the same time, we should note that the increase of CD31 does not necessarily indicate the increase of blood vessel number. Since CD31 is a marker for endothelial cells, we should test whether this increase is caused by an increase of endothelial CD31 expression. Other blood vessel related markers, such as AQP4, ZO-1, should also be tested in order to make a full understanding of blood vessel changes and even blood vessel-astrocyte interaction.

Besides the mentioned immunofluorescence markers, it is also crucial to quantify whether Aβ deposition could be reduced in the long run. Furthermore, if reactive astrocytes could indeed be reduced, inhibitory protein such as CSPG should also show a decrease. Inflammation markers such as Iba1, IL-1β, TNFα, and CD68 should also be tested.

Behavior-wise, our NOR result showed significant improvement of 5xFAD mice after AAV-NeuroD1 injection. We have nine non-transgenic littermates, five 5xFAD mice injected with AAV-GFP, and five 5xFAD mice injected with AAV-NeuroD1. We need to test more mice in order to see if the results are consistent. Besides NOR test, other behavior tests such as radial arm maze and fear conditioning should also be used to confirm NeuroD1 effect.

In terms of mouse models to study AD, the 5xFAD mouse model has the advantages of accumulating Aβ globally and known cellular and behavioral deficits. However, due to the fact that the 5xFAD cannot recapitulate neuronal loss in AD, AβO hippocampal injection model was also applied to “kill” neurons. This model recapitulates neuronal loss and also takes much shorter time to develop. It will be used to confirm AAV-NeuroD1 effect in the future.
Meanwhile, when we are using the 5xFAD mouse model, we are aware that our hippocampal delivery methodology has its potential shortcomings. While Aβ42 is accumulated globally in almost the entire brain, our hippocampal delivery of AAV-NeuroD1 only targets on the hippocampus. Other brain areas such as prefrontal cortex are also impaired in 5xFAD mouse models but cannot be treated if the injection is local in hippocampus. Thus, an alternative approach to regenerate newborn neurons globally throughout the brain is needed in the long run.

To solve this potential problem (and also the “leakage” problem), we have generated a transgenic mouse line to conditionally express NeuroD1 in astrocytes using a Cre-LoxP system. We will first cross 5xFAD mouse line with GFAP-CreERT2 mice to obtain AD-CreER mice. We will then cross AD-CreER mice with our loxP-NeuroD1 mouse line to generate AD-CreER-NeuroD1 mice. CreER will not be expressed under normal condition, so the stop codon in front of NeuroD1 will stay and NeuroD1 will not be expressed. However, after administration of tamoxifen either through intraperitoneal injection or via food, CreER will enter the nucleus and mediate the cleavage of the stop codon so that NeuroD1 will be expressed in GFAP+ astrocytes in the entire brain.

By using mouse genetics, we will be able to conditionally express NeuroD1 in astrocytes to activate cell conversion globally in aging AD animals. Comparing to modulating hippocampal neurogenesis alone, this global cell conversion approach will gain insight into the function of global neurogenesis on cognitive functions, a novel approach that has never been explored before.

To conclude, NeuroD1 hippocampal delivery could be a potential therapy for the AD patients. The results in this thesis are still preliminary and more experiments should be done in the future to really establish this methodology as a therapy.
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