Proceedings of the 4th International Conference on Biological Engineering and Medical Science
DOI: 10.54254/2753-8818/73/2024.19400

Investigating the Initiate and Onset Mechanisms of Alzheimer's Disease Through Natural and Chimeric Animal Models

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Abstract. Alzheimer's disease (AD) is a long process and its pathological pathways preceding diagnostic symptoms remain unclear. Therefore, this unclear situation has greatly hindered the research progress of AD-related treatment. We use Octodon degus to design experiments to screen out AD-related mRNA in early stage. Then, we designed and tested multiple molecular beacons and shRNAs to evaluate the correlation and specificity between AD and target mRNA. Next, we investigate whether mice with tissue transplantation would develop sporadic AD. This study aims to explore the onset mechanisms of Alzheimer's disease (AD) using a variety of animal models, including degus and chimeric mice. By identifying new biomarkers and studying gene expression profiles associated with AD, this research seeks to elucidate early-stage disease mechanisms and potential pathways for therapeutic intervention.

Keywords: Alzheimer's disease (AD), Octodon degus, RNA-seq, Molecular Beacon, Interspecies chimeras.

1. Introduction

Alzheimer's disease (AD) onset is a long process and is characterized by complex pathophysiological changes that precede symptomatic manifestations by decades [1]. Few AD clinical trials have succeeded in recent years, two of the main reasons are that the process of AD when diagnosed has been quite late, causing plenty of irreversible damage already, and also the mostly used mice model poorly represents sporadic AD [2,3], causing continuous failure to the drug design and test.

AD is naturally induced by gene expression changes. Figuring out which gene expression starts the AD pathway is of great importance. Recent studies used brain transcriptome sequencing to screen out differentially expressed genes (DEGs) associated with AD but missing the earlier stage [4]. Some also used molecular beacons to detect mRNA expression from living cancer cells [5].

Degus are rodents showing sporadic AD spontaneously and are potential model animals for AD research [6]. With the help of high-throughput RNA-seq, we could design an experiment aimed at

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screening the starting expressing gene of AD. However, the costs and challenges associated with long-term animal studies were significant, highlighting the need for refined methods and models or accelerated disease onset methodologies.

Molecular Beacons (MB) are developing tools for live-cell interrogation [5]. Using MB to detect mRNA expression could avoid killing animals each time. By using near-infrared (NIR) fluorophores, the MB are visualized in vivo using Fluorescence Molecular Tomography (FMT) and Multi-Spectral Optoacoustic Tomography (MSOT). Also, in case the skulls are impenetrable, a magnetic resonance beacon detecting miRNA [7] is potentially editable to detect mRNA expression as another option.

Xenotransplantation models are used for insight into the mechanism of AD [8,9]. So, a xenotransplantation between degus and mice is feasible, to observe the spread of AD pathology between degus and mice. Beyond that, introducing chimeric model can combine into one circuit which can help to observe the AD spread between degus and mice [9].

This study focuses on preclinical stage pathways of AD, identifies early biomarkers, and intends to monitor disease progression through innovative animal model experiments. Next, we will attempt to evaluate the characteristics of the gene through biosensor design.

2. Methods

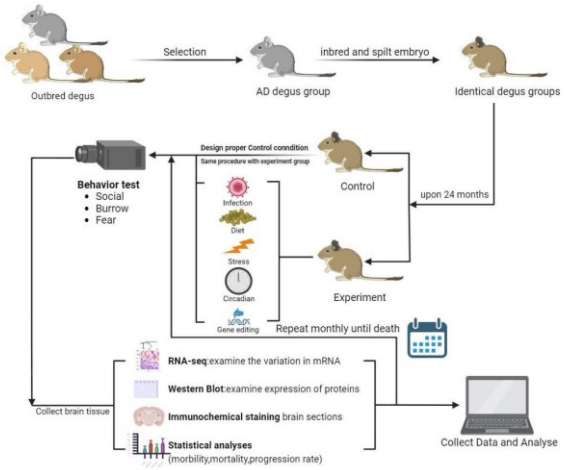

Figure 1. Design to study the effects of various factors on the changes in Degus expression over time and their impact on AD. Created on BioRender

2.1. Animal Preparation and Grouping

All degus (Octodon degus) used in this study were sourced from a single outbred colony. Outbred AD females had their inbred offspring. The embryo was separated from pregnant females and spilt into two. Each was injected into a pseudo- pregnant degu. After weaning, each degu was assigned a unique ID number and remained in the same colony for its entire life. The colony was expanded over a few generations.

They were housed in standard metal cages with wood shavings and a small metallic box with a single entrance, under a natural 12-hour light/dark cycle starting at 7 a.m., in an air-conditioned facility. They were given a commercial rodent diet with continuous access to water and food throughout the experiment. Each identical degu from embryo splitting is cataloged and equally separated into different groups (control and experimental), to ensure each time we could use genetically identical individuals.

The experimental group are separated into 5 groups. Each was conducted with a known risk factor [11]. The first group is inoculated with Herpes Simplex Virus Type1. The second group are fed with a high fat diet regularly. The third group was housed individually, exposed to acoustic and olfactory stimuli but with no visual or social contact with others, to induce isolation stress [10]. Fourth group are controlled to 14-14h cycle of light switch. Fifth group are gene-edited from the embryo, to change DNA

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sequence artificially to simulate mutation in the natural case. The control group are also divided into 5 groups. With all the other conditions and procedures, the same as the experimental group except for the experimental changes.

Monthly assessments of three-chamber test, burrowing test, novel-object recognition test, Barnes Maze test and so on are conducted to evaluate cognitive functions and memory retention of degus. Identical degus from the two groups will be selected and subjected to the same experiment to determine differences between them. The three-chamber test assesses cognitive traits, including overall sociability and responsiveness to social novelty, in rodent models by measuring their interactions with familiar and novel conspecifics. Utilizing an apparatus from San Diego Instruments, the test involves a 5-minute habituation period followed by a 10-minute 'sociability' session, where the subject interacts with a familiar intruder, and a 10-minute 'social novelty' session, where they encounter both the familiar and a novel intruder. The test measures the subject's social interaction preference by recording the time spent sniffing wire boxes with intruders and the time spent in the various test areas, thereby providing insight into their social preferences and recognition abilities.

The novel-object recognition test assesses object memory and preference for novelty in degus by following a structured protocol. After a 10-minute habituation to an empty testing arena, degus are introduced to two identical objects the next day, exploring them for 10 minutes. Following a 1-hour interval, degus are returned to the arena, now containing one familiar and one novel object, and allowed to explore for 5 minutes. The recognition index is determined by the proportion of time spent exploring the novel object compared to the total exploration time, providing a measure of the degus' memory and novelty preference.

The Barnes Maze test assesses spatial navigation, learning, and memory through a hippocampal-dependent task involving an elevated circular platform with 18 equidistant holes, where subjects must find a target escape hole using visual spatial cues. The test consists of three phases: habituation, training, and testing. In the habituation phase, subjects become familiar with the maze and escape box. During the training phase, they are trained over several days to find the escape hole. In the final test phase, subjects undergo a memory-retrieval session to assess their learning. Performance is measured by latency to the first visit of the escape hole, reference and working memory errors, and search strategies, categorized as random, serial, or spatial. The maze is cleaned between trials to control for variations in locomotor activity, and various metrics are recorded to ensure comprehensive analysis of spatial memory and navigation skills.

The burrowing test is a behavioral assessment used to evaluate cognitive performance in rodents by measuring their natural burrowing activity. In this test, a full burrow, consisting of a 200 mm long, 68 mm diameter tube filled with 1400 g of pellets, is placed in an individual test cage. The test measures the amount of pellets removed by the rodents after 2 and 6 hours. In a study involving 146 degus aged 4 to 5.5 years, the burrowing test was conducted across three sessions, each 48 hours apart. Degus that excavated less than 25% of the pellets were classified as 'AD-like' poor burrowers, enabling researchers to evaluate their cognitive abilities and potential age-related decline[6].

2.2. Biological Sample Collection

Upon euthanasia, dissecting brains were fixed in PBS, pH 7.4, then soaked in 30% sucrose in PBS prior to sectioning. Serial free-floating coronal sections were collected in 1x PBS and then transferred to a cryoprotective solution for storage at −20 °C[6].

2.3. Western blot

Extract target proteins from the cortex of degus by first dissolving the frozen tissue in a buffer containing 250 mM sucrose, 20 mM Tris-HCl (pH 7.4), 1 mM EGTA, 1 mM EDTA, and a Sigma-Aldrich protease inhibitor. Quantify membrane protein bands using ImageJ. Begin by extracting soluble target proteins with diethylamine, followed by extracting insoluble proteins with formic acid. This approach allows for the analysis of both soluble and insoluble protein expression levels[11].

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2.4. Immunohistochemical staining

Coronal brain sections underwent comprehensive immunohistochemical staining protocols. For immunofluorescence, sections were initially rinsed in 1xPBS, followed by blocking in 5% normal donkey serum and 0.1% Triton X-100. To mitigate autofluorescence, quencher was employed. Primary antibodies were applied for 72 hours at 4°C, followed by washing in 1xPBS and subsequent incubation with fluorescent secondary antibodies in PBS with DAPI for 30 minutes at room temperature. Sections were mounted using medium. Amyloid plaques were stained using Amyloid Plaque Stain Reagent after ethanol and water rinsing. Post-staining, the sections were washed and blocked according to the immunofluorescence protocol. Perineuronal nets were identified using biotinylated Wisteria floribunda agglutinin. Sections were prepared by rinsing in 1xPBS and blocked with 5% normal donkey serum and 0.075% Triton X-100, followed by incubation with biotinylated WFA for 72 hours at 4°C. Sections were sequentially incubated with primary antibodies, including 6E10, Aß40, Aß42, Aß43, pyroGlu3Aß (AßpE3), mOC23, mOC31, ubiquitin, smooth muscle α-actin (SMA), NeuN, PV, GFAP, IBA-1, and c-Fos, at appropriate dilutions for 72 hours at 4 °C, and then washed in 1x PBS, Alexa Fluor 488 conjugated streptavidin was applied for 2 hours, followed by DAPI counterstaining [6].

2.5. Total RNA and mRNA Isolation

Total RNA was extracted using an RNA kit following the manufacturer's instructions. Genomic DNA was removed with RNase-Free. RNA quantification was performed using a spectrophotometer, and integrity was assessed by agarose gel electrophoresis. mRNA was isolated from total RNA using a dedicated kit according to the manufacturer's guidelines. For sizing, quantitation and quality control of mRNA Bioanalyzer system was used, discarding samples with an RIN < 7[4].

2.6. RNA-sequencing

RNA-seq was conducted using strand-specific protocols for library preparation, fragmentation, and PCR enrichment of target mRNA. Two batches of paired-end library sequencing were done in an Applied Biosystems [4].

2.7. Imaging Techniques

2.7.1. Magnetic Resonance Imaging (MRI)

Using a magnetic resonance imaging (MRI) system, compare T2-weighted phantom images of isolated cells soaked in a molecular beacon mixture, both with and without mRNA present. Next, inject beacon-labeled viral particles into degus and perform MRI with the following parameters: slice thickness = 1.0 mm, TE = 114 ms, TR = 4000 ms [12,13].

2.7.2. Fluorescence Molecular Tomography (FMT)/Multispectral optoacoustic tomography (MSOT)

Use near-infrared imaging to determine the 3D quantitative fluorescence distribution in tissues. Carefully position anesthetized degus in a prone position in the FMT animal imaging box, with the brain facing sideways and placed on an imaging block to enhance skull area imaging. Data is acquired using a 750 nm laser (80 mW) through multiple point sources, with each scan lasting about seven minutes. Perform imaging every six hours post-induction using FMT to observe fluorescence distribution in the brain and identify the optimal imaging time [14].

MSOT (Multispectral Optoacoustic Tomography) was used for detailed imaging of 13- to 15-month-old female triple-transgenic AD degus and control degus. The study employed the MSOT system. This system uses a tunable laser source for light excitation and a 512-element transducer array for high-sensitivity detection of optoacoustic signals. Degus were injected with molecular beacon for imaging, and data acquisition was conducted at multiple wavelengths while the sample was moved through the transducer array within a controlled water bath. Raw signals were processed using a back-projection algorithm and linearly unmixed to differentiate tissue chromophores, creating pseudo-color images that

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represent the spatial distribution of deoxy-hemoglobin, oxy-hemoglobin, and the injected dye. This process allows for detailed analysis of biological tissues in the context of AD pathology[15].

2.7.3. Confocal fluorescence micrographs

Use near-infrared imaging to determine the 3D quantitative fluorescence distribution in tissues. Carefully position anesthetized degus in a prone position in the FMT animal imaging box, with the brain facing sideways and placed on an imaging block to enhance skull area imaging. Data is acquired using a 750 nm laser (80 mW) through multiple point sources, with each scan lasting about seven minutes. Perform imaging every six hours post-induction using FMT to observe fluorescence distribution in the brain and identify the optimal imaging time [6].

2.8. Statistical figure of morbidity

Record data of AD onset and individual death real time. Build a statistical figure between time and morbidity/mortality in each group after the experiment ends.

2.9. Molecular Beacon Design for RNA Detection

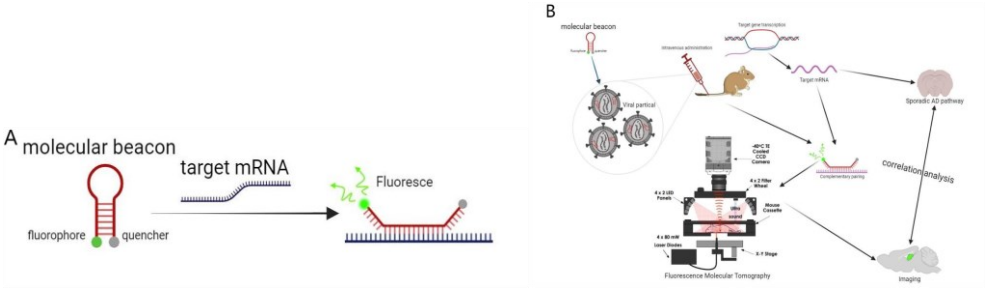

Figure 2. A) How molecular beacons work B) Application of molecular beacons in in vivo imaging of AD degus. Created on BioRender

Design a molecular beacon to indicate the target mRNA. The fluorophore separates from quencher after the beacon binds. Emitting fluorescence indicate the presence of target mRNA. Subsequently, developing multiple beacons to specifically detect AD-related RNA to confirm the timeline of relevant gene expression. After finding out the earliest expressed mRNA, injecting the beacons indicating target RNA into degu's brain by using lentivirus. Beyond that, using shRNA to interfere with the target gene and monitoring the variation of AD incidence or any sign of side effects on degus.

2.10. Chimeric Model Development

Due to the numerous advantages of mouse models, we sought to induce sporadic AD onset in a mice model. First, transplanting AD degu neurons into normal degu skulls to observe if it will show late-onset AD symptoms. If not observed, using Flow Cytometry to separate host cells for expression testing to determine whether the host cells were "infected" or not.

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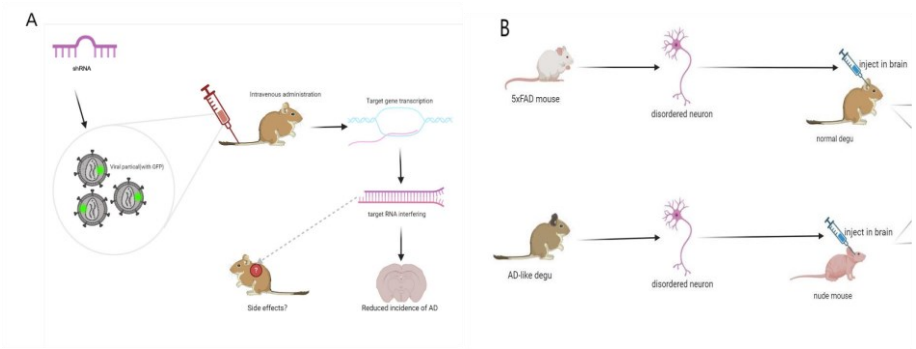

Figure 3. A) Knockdown of target mRNA and observation of its effects B) Neuronal xenotransplantation after donor exchange. Created on BioRender

In the same way, injecting 5xFAD mouse neurons into nude mouse skulls to see whether 5xFAD mouse cells would infect host cells and then swap the cell donors to repeat the experiments. At last, inducing 5xFAD mouse and AD degu neurons to transform into iPS cells and injecting cells into nude mouse blastocysts to cultivate chimeric mice and check if any sporadic AD symptoms are found. At the same time, AD degu iPS cells are injected into healthy degu as a control group. The cell expressions will be examined on necessary.

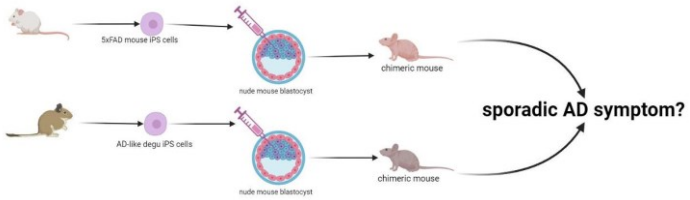

Figure 4. Design and observation of chimera model. Created on BioRender

3. Result

Use near-infrared imaging to determine the 3D quantitative fluorescence distribution in tissues. Carefully position anesthetized degus in a prone position in the FMT animal imaging box, with the brain facing sideways and placed on an imaging block to enhance skull area imaging. Data is acquired using a 750 nm laser (80 mW) through multiple point sources, with each scan lasting about seven minutes. Perform imaging every six hours post-induction using FMT to observe fluorescence distribution in the brain and identify the optimal imaging time.

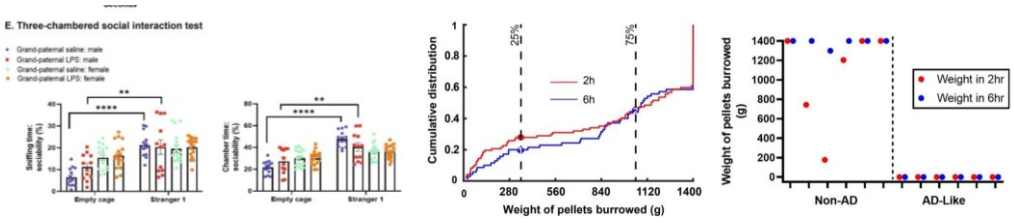

Figure 5. Sniffing time of degus was analyzed to assess changes in their responses to familiar and unfamiliar intruders, evaluating their social behavior [16]. By counting the amount of feed dug by degus and detecting its changes, the burrowing ability of degus can be tested to distinguish between AD and non-AD degus. In the study, elderly degus' digging performances were classified, with the top 25% categorized as the Non-AD group and the bottom 25% as the AD-like group [6]."

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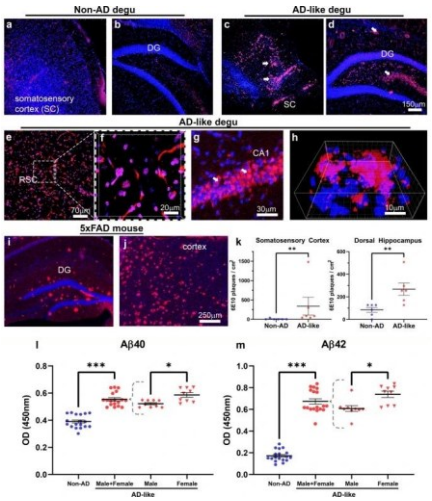

Figure 6. (a-j) Brain tissue sections were stained with the monoclonal antibody 6E10 to detect Aβ, and DAPI was used to counterstain nuclei. Imaging was performed using confocal fluorescence microscopy. (k) The spatial distribution and quantitative differences of plaques in the dorsal hippocampus and somatosensory cortex were observed. (l, m) The differences in the contents of Aβ40 and Aβ42 between the Non-AD and AD-like groups were detected by ELISA to further determine the differences between the two groups [6].

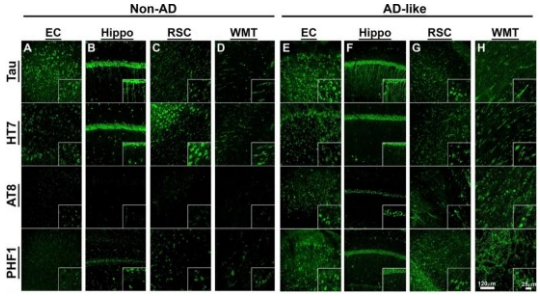

Figure 7. Brain tissue sections were taken and stained with monoclonal antibodies for tau and its pathological markers, and then imaged under a confocal fluorescence microscope. The figure illustrates that while the overall tau immunoreactivity is similar, the AD-like group shows higher reactivity for tau pathological markers [6].

Results show significant differences in 6E10+ plaques per cm² between AD-like and Non-AD groups (**$p < 0.01$). Panels l and m demonstrate that ELISA of formic acid extracts from brain slices of Non-AD and AD-like degus reveals higher levels of Aβ40 and Aβ42 in the AD-like group, with higher levels observed in AD-like females compared to males ($N = 3$ per group). Measurements, performed in triplicate, show significant differences between Non-AD and AD-like degus (***$p < 0.001$) and between AD-like males and females (*$p < 0.05$). The insets in each panel provide high-magnification views of staining signals within individual cells, including the hippocampus (Hippo), entorhinal cortex (EC), white matter tracts (WMT), and retrosplenial cortex (RSC). The graphs depict overall AD-correlated genes and those specific to the incipient AD stage.

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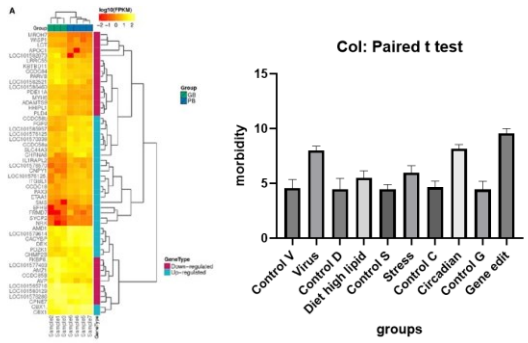

Figure 8. After determining gene expression via RNA-seq, heat map was generated to compare the gene expression differences between the experimental and the control group. The statistical data was systematically analyzed [4].

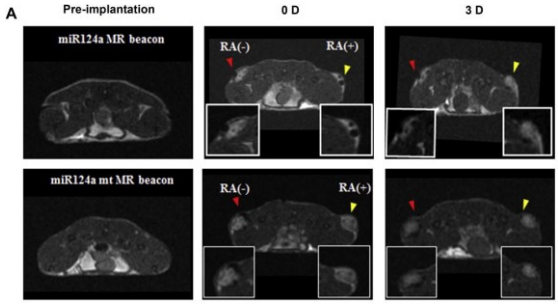

Figure 9. The figure shows MRI images of cells with endogenous expression of miR124a MR beacon implanted into nude mice. The distribution of the beacon can be observed to indicate the expression of miR124a. From left to right, the images are before transplantation, before differentiation, after transplantation, and three days after differentiation [7].

MRI findings revealed the structural difference and mRNA expression distribution. With FMT detection and imaging, high-resolution images show differential gene expression levels of target genes in vivo, establishing correlations between these expressions and degu AD incidence, and serving as basic evidence for regulating AD onset through target genes. The above imaging and analysis demonstrate a clear link between the genetic profiles and the early pathological changes. This multimodal approach provides a more nuanced understanding of the disease progression.

MSOT signals in the cortex were significantly stronger in AD brains than in healthy controls starting from 20 minutes after injection, with the greatest difference observed at 30 minutes. However, the signals at the superior sagittal sinus (SSS) may similar for both AD and control brains during the first hour post-injection

Based on the experiments above, we hypothesize that several genes that altered expression correlate with AD incidence at early stage have been found. Therefore, we intend to identify the earliest expression changes in the pathways that could specifically trigger AD onset. With molecular beacons and FMT, we can recognize the appearance order of different fluorescent dyes and determine the most AD-related gene. Additionally, if the process of shRNA interference does not induce any other side effects, it can be preliminarily concluded that the target gene regulates AD more specifically and involves fewer alternative pathways.

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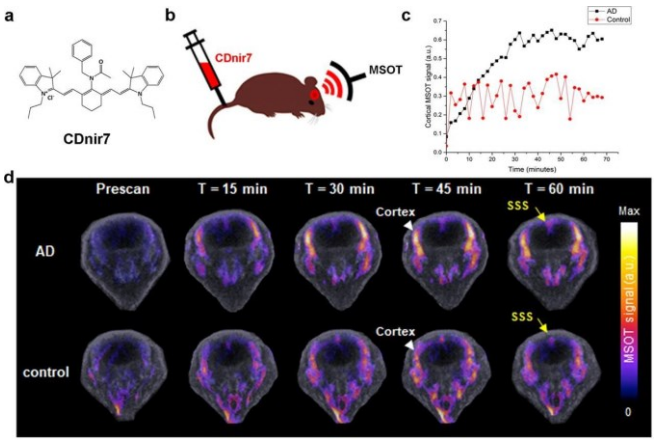

Figure 10. The figure shows the MSOT imaging of AD mice and control mice injected with a fluorescent dye CDnir7, which illustrates the differences in dye distribution over time between the two groups [15].

We found that the initial experiment was quite costly, potentially extending over three years or more due to the numerous procedures involved. This is largely due to the long onset process of AD. Despite their advantages in representing sporadic Alzheimer's disease (AD) onset, degus have limitations such as a long lifespan that increases costs, whereas mice, with, higher reproduction rates, remain the gold standard for research; thus, we aim to use findings from degus to modify mouse models to have sporadic AD onset to advance research. If we could accelerate the AD onset process, we would significantly reduce the cost. So, we put the AD degus neurons into the mouse brain to better investigate the neuron interaction of two animals.

Mice implanted with AD-stricken degus neurons may develop sporadic AD symptoms, or abnormal expression of AD-like features in normal neurons. If this happens, it proves that AD can be transmitted between heterogeneous neurons.

If normal mice implanted with 5xFAD mouse neurons also develop signs of sporadic AD, it may indicate that familial AD neurons can also induce the occurrence of sporadic AD. The interaction between the two types of neurons can be further studied.

By expressing different fluorescent proteins, we can observe the neural circuit composition of chimeric mice and observe whether chimeric mice have signs of disseminated AD. If symptoms or changes in expression occur, this is evidence that the stem cells formed by the induced AD neurons have indeed caused the spread of AD. The molecular mechanism should be investigated further in the future.

The chimeric models offer valuable insights into cell-to-cell interactions and disease propagation mechanisms. By using the Chimeric model, it is possible to further investigate the impacts of disordered cells on normal individuals. Moreover, by adding control experiments, the False positive activities in the experimental results can be avoided.

4. Discussion

However, some limitations still need to be noted. First, tests like RNA-seq are excessively used, leading to a large amount of degus being euthanized, causing possible ethical issues. It is also seen as an unsustainable act that could lead to experiment failure. To solve that, adjusting the frequency of testing and duration of experiment may be done in practice.

Second, the way molecules are delivered to the brain still needs to be assessed. In addition to viral vectors, exosomes injected directly into the brain may also be considered for beacon delivery. The major drawback is the inhomogeneous distribution of molecules in the tissue after injection. To better determine which is a more efficient method, a controlled experiment may be conducted using fluorescence dye to visualize the distribution. What's more, the lifespan of models, for chimeric models,

DOI: 10.54254/2753-8818/73/2024.19400

is short. This could stumble the observation of interaction between neurons. To reduce the effect, the proportion of foreign cells needs to be cautiously controlled.

We also envision a transgenic mouse model with multiple gene insertions. By introducing "longevity genes" and overexpressing the genes we screened, we want to confirm whether we can create a sporadic AD model with stable inheritance and high incidence. However, given that the complexity of this concept may surpass the capabilities of current gene editing technology, we plan to incorporate more advanced technologies into the design process in the future.

5. Conclusion

Our experiments aim to uncover the mechanisms behind Alzheimer's disease and pave the way for early diagnosis and targeted treatments. By combining fundamental research with practical applications, we aim to advance the design of model animals further. What's more, this study enhances our understanding of AD's early stages and provides a foundation for future research into targeted treatments. Discuss the implications of these findings for early diagnostic approaches and how they could inform the development of gene-specific therapies. Our findings also underscore the importance of developing more cost-effective and timely research strategies in AD model animals.

Acknowledgement

Bowen Liu and Songlin Liu contributed equally to this work and should be considered co-first authors.

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