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Alterations in protein phosphorylation in the amygdala of the 5XFamilial Alzheimer's disease animal model

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ABSTRACT

Alzheimer's disease is the most common disease underlying dementia in humans. Two major neuropathological hallmarks of AD are neuritic plaques primarily composed of amyloid beta peptide and neurofibrillary tangles primarily composed of hyperphosphorylated tau. In addition to impaired memory function, AD patients often display neuropsychiatric symptoms and abnormal emotional states such as confusion, delusion, manic/depressive episodes and altered fear status. Brains from AD patients show atrophy of the amygdala which is involved in fear expression and emotional processing as well as hippocampal atrophy. However, which molecular changes are responsible for the altered emotional states observed in AD remains to be elucidated. Here, we observed that the fear response as assessed by evaluating fear memory via a cued fear conditioning test was impaired in 5XFamilial AD (5XFAD) mice, an animal model of AD. Compared to wild-type mice, 5XFAD mice showed changes in the phosphorylation of twelve proteins in the amygdala. Thus, our study provides twelve potential protein targets in the amygdala that may be responsible for the impairment in fear memory in AD.

1. Introduction

Alzheimer's disease (AD) is the most common underlying disease for dementia in humans.[1] Two major neuropathological hallmarks of AD are neuritic plaques mainly composed of amyloid beta peptide (Aβ)[2–4] and neurofibrillary tangles mainly composed of hyperphosphorylated tau.[5–7] These hallmarks are often observed in the cerebral cortex and the hippocampus of AD patients.[8,9] In addition to memory loss and cognitive dysfunction, AD patients frequently show abnormal emotional states including confusion, delusion, manic and depressive episodes and altered fear status.[10,11] Despite the significance of understanding the neuropsychiatric symptoms including emotion-related learning in AD, it is still unclear which molecular changes are involved in AD patients' abnormal fear responses.

The amygdala, in addition to the hippocampus, is an important brain region that is affected by neuritic plaques and neurofibrillary tangles.[12] The amygdala is involved in emotional behaviors, such as depression, fear memory and anxiety.[13] It is a key structure in the formation of fear memory.[14] Several studies have found marked amygdala atrophy caused by Aβ and have reported that this atrophy is correlated with emotional changes such as conditioned fear in early AD.[15–23] However, the physiological function and molecular mechanism of the amygdala in AD remain poorly understood.

Protein phosphorylation is the most common post-translational modification of proteins. It causes activation, deactivation and modification of proteins and therefore influences signaling pathways in various cell types.[24,25] There are over 250 protein kinases in

Abbreviations: AD, Alzheimer's disease; 5XFAD, 5XFamilial AD.

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the brain[26] and many studies have reported that kinase-mediated phosphorylation of a few proteins plays a critical role in the amygdala which is associated with fear memory.[27,28] However, the role and mechanism of protein phosphorylation in AD are not yet fully understood.

There are several animal models used for AD research.[29] Among these models, the 5XFAD mouse is the most recently produced animal AD model. 5XFAD mice (APPSwFlLon, PSEN1*M146L*L286V) show impaired memory, neuronal death, astrogliosis and aggregation of Aβ in the brains.[30–32]

In this study, we investigated the emotional behavior changes in 6-month-old 5XFAD mice and also examined the changes in protein phosphorylation in the amygdala of 5XFAD mice compared to wild type (WT) mice by using phosphoantibody array.

2. Materials and methods

2.1. Animals

All of the experimental procedures were approved by the Animal Care Committee of Seoul National University (Approval number: SNUIBC-150421-4). We obtained 5XFAD mice from Jackson Laboratories (ME, USA) and maintained them by crossbreeding hemizygous transgenic mice with B6SJL F1 mice. We confirmed founder transgenic mice by PCR, and non-transgenic littermates served as controls. 5XFAD mice overexpress mutant human APP695 with the Swedish mutation (K670N, M671L), the Florida mutation (I716V), and the London mutation (V717I) as well as human PS1 containing two FAD mutations (M146L and L286V). These mice exhibit AD-related pathology earlier than other animal models, and accumulating Aβ is observed in the deep cortex and subiculum at 2 months of age. These mice have reduced level of synaptic marker proteins at 4–9 months and show neuronal loss and memory deficits at 4–6 months of age.[32] 5XFAD mice were housed with a 12 h light/dark cycle in a temperature-controlled environment. Behavioral tests were performed in the Animal Behavior Laboratory of the Seoul National University.

2.2. Cued fear conditioning test

Cued fear conditioning was tested as described previously.[33] WT (n = 13) and 5XFAD (n = 12) mice were used for cued fear conditioning test. Each scrambler was connected to an electronic constant-current shock source that was controlled via an interface connected to a Windows 7 computer running EthoVision XT 8 software (Noldus Information Technology, VA, USA). A digital camera was mounted on the steel ceiling of each chamber, and video signals were sent to the same computer for analysis. The experimental protocol for cued fear conditioning is shown in Fig. 1A.

2.2.1. Habituation

To exclude possible freezing due to the novel environment, we allowed the mice to habituate to the chamber. On day 1, we brought mice into the habituation room, and placed them into a training chamber (33 cm × 29 cm × 32 cm) individually for 5 min without any cue.

2.2.2. Training

On day 2, we placed each mouse in the conditioning chamber (33 cm × 29 cm × 32 cm) for 3 min (for pre-shock) without any shock or tone. Then, mice received two repetitions of a tone (70 dB) – foot-shock (0.7 mA) pairing for 2 s, at 58 s inter-trial intervals. After conditioning, we removed the mouse from the chamber and returned it to its cage.

2.2.3. Testing

On day 3, each mouse was placed in the test chamber with no cues, after which the tone was played for 2 s without foot-shock, and this process was repeated twice. We measured the freezing response time during the 58 s in each session after the tone was played.

2.3. Open field test

WT (n = 8) and 5XFAD (n = 8) mice were used for open field test. The apparatus is a 40 × 40 × 3 cm acrylic box. Each mouse was placed in the center of the box, and its movement was recorded for 30 min. The test was recorded using a digital camera attached to the ceiling and was analyzed using Ethovision 8.5 (Noldus, Wageningen, Netherlands). To analyze the locomotor activity of the mice, we measured the total distance moved in the open field test.

2.4. Rotarod test

WT (n = 13) and 5XFAD (n = 11) mice were used for rotarod test. The rotarod test was performed as described previously.[34] Mice were placed on a treadmill, and the speed accelerated from 4 revolutions/min to 30 revolutions/min over 3 min. We performed 3 training sessions per day and repeated the training for 3 consecutive days. To evaluate locomotor activity, we measured the latency to falling off the treadmill on each of the 3 days.

2.5. Phosphoantibody array

To confirm protein phosphorylation, we performed an experiment using a phosphoantibody array assay kit (Full Moon Biosystems, CA, USA) according to the manufacturer's instructions. We used whole tissue from the amygdala of the WT (n = 3) and 5XFAD (n = 3) mice which were not exposed to the fear conditioning test. Briefly, 50 μg of protein was biotinylated using the biotin reagent (Full Moon Biosystems, CA, USA) after lysing the amygdala removed from WT or 5XFAD mice. We used the coupling solution to bind the antibodies and samples, and then samples were incubated for 2 h on a shaker at room temperature. Finally, we added Cy3-streptavidin solution into the labeled samples to provide fluorescence, which was detected using a GenePix 4000b microarray scanner (Agilent, CA, USA). The antibodies are covalently immobilized on a high quality glass surface coated with proprietary 3-D polymer material. We analyzed the images using GenePix Pro 6.0 (Agilent, CA, USA) and organized the data using Genowiz 4.0 (Ocimum Biosolutions, A.P., India).

2.6. Bioinformatics analysis

To investigate the potential molecular function of the identified protein, we analyzed GO term enrichment and KEGG pathway enrichment using DAVID 6.7 (http://david.abcc.ncifcrf.gov/home.jsp) ($p < 0.05$).

2.7. Statistical analysis

All data are expressed as the means ± standard error of the mean (SEM) values. Non-parametric Mann–Whitney U test, independent-samples *t*-test and one-way ANOVAs using LSD post-hoc comparisons (IBM SPSS Statistics 20, IL, USA) were used to determine statistical significance. The results were considered to be statistically significant if $p < 0.05$.

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