Master's Thesis

석사 학위논문

Studies on Proteolytic Processing of Amyloid Precursor protein (APP) in Olfactory Epithelium using AD Transgenic Mice

Ameer Abu Bakr Rasheed (아미르)

Department of Brain Science

뇌과학전공

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by

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DGIST

A thesis submitted to the faculty of DGIST in partial fulfillment of the requirements for the degree of Master of Science in the Department of Brain Science. The study was conducted in accordance with Code of Research Ethics[1].

05/15/2014

Approved by

Professor Cheil Moon (Advisor)

Professor Keun-A Chang (Co-advisor)

1) Declaration of Ethical Conduct in Research; I, as a graduate student of DGIST, hereby declare that I have not committed any acts that damage the credibility of my research. These include, but are not limited to: falsification, thesis written by someone else, distortion or research findings or plagiarism. I affirm that my thesis contains honest conclusions based on my own careful research under the guidelines of my thesis advisor.

Studies on Proteolytic Processing of Amyloid Precursor protein (APP) in Olfactory Epithelium using AD Transgenic Mice

Ameer Abu Bakr Rasheed

Accepted in partial fulfillment of the requirements for the degree of Master of Science

05.15.2014

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MS/BS 아미르, **Ameer Abu Bakr Rasheed, Studies on Proteolytic Processing of Amyloid Precursor protein (APP) in Olfactory Epithelium using AD Transgenic Mice**

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Abstract

Olfactory impairment is a well-documented abnormality in Alzheimer's disease (AD). AD is known to begin with abnormal processing of amyloid precursor protein (APP), through sequential cleavages first by β-secretase and then by γ-secretase complex which leads to excess production of β-amyloid (Aβ) in the cortex. While olfactory dysfunction occurs in the incipient stages of AD even before Aβ deposition and plaque formation in the CNS, the functional correlation of olfactory deficit in relation to AD is not well understood. It may be critical to know the process underlying AD-related olfactory sensory loss to find some novel biomarkers. To this end, two different types of transgenic mice models were used including Tg2576, which overexpresses human APP and Tg6799 (also called 5xFAD), which expresses human APP and Presenilin1 both mutations together.

It was found unique APP processing in OE that has significance in providing not only possible biomarkers that can be used for screening and detection of AD before plaque formation but also for treatment purposes.

This data demonstrates that the abnormal processing of APP in the OE provides APP fragments including 25 kDa, 55kDa and 80 kDa that can be a potential biomarker in the very early and critical period in the stage of mild cognitive impairment, that is, the critical stage of AD occurrence (before Aβ plaque formation in the CNS). Such biomarkers can be accessed *via* biopsy and can be used for establishing improved early diagnostic procedure for the AD. Additionally, PS2 increased level was found in OE that possibly involved in unique APP processing and might also be crucial for understanding the γ-secretase role controlling AD through γ-secretase as a therapeutic target.

Keywords: Alzheimer's disease, olfactory system, Amyloid precursor protein, secretase and biomarker.

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INTRODUCTION

INTRODUCTION:

Alzheimer's disease is slowly progressive and irreversible brain disease which is one of the most common cause of dementia [1]. In AD patients not only suffer from cognitive but also motor and sensory loss [2]. Although the mechanism of AD is not well understood still AD pathology is characterized by extra cellular amyloid-β deposits and interacellular neurfibril tangles formation of hyperphosphorylation of tau protein. Being irreversible and neurological damaging disease, its very important to detect and diagonse at earlier or at some controlable time point. Some useful AD diagonstic biomarkers are required for this purpose. These biomarkers should also fullful the criteria of usefulness for AD detection. Biomarkers should become abnormal with the progression of disease in other words they should be dynamic and correlate with clinical symptom and severity of disease [3].

Recent research on use of specific AD biomarker for disease staging in vivo shows that Aβ dynamically correlates with disease at different stages of disease progression[4] [5]. But Aβ level varies in patients. Which suggests, through process of Aβ production starts earlier but Aβ as a biomarker only is not relibale , therefore an alternative biomarker must be found along with Aβ generation process.

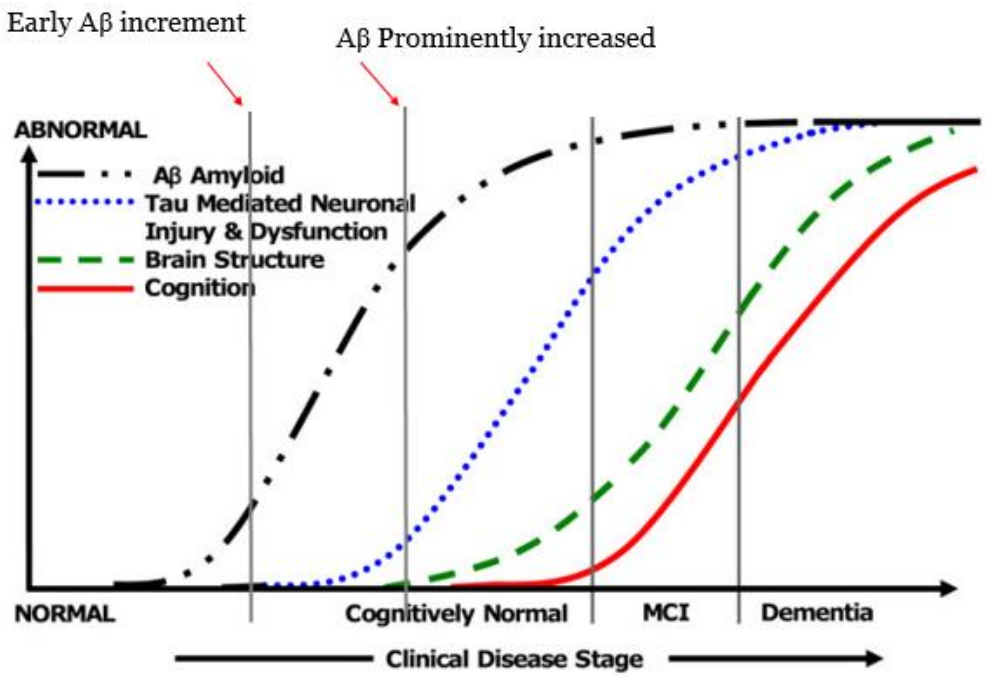


Figure1. Biomakers and AD early detection

Source; Modified from Ingelsson, M et al 2004. [4]

Aβ can be produced through proteolytic processing of APP (amyloid precursor protein), which takes central position in AD pathogenesis. APP is single-pass transmembrane protein with larger ectodomain. Although the physiological functions of APP are not well known but has neuroprotective function and positive effect on cell growth [6].

APP is mainly produced in neurons and rapidly metabolized by secretase enzymes through alternative splicing by two pathways [7] [8]. Nonamyloidogenic processing of APP involving two secretases α-secretase and γ-secretase while in amyloidogenic processing β-secretase (identified as transmembrane aspartase protease BACE1) and γ-secretase are involved. Product generated during both processings have soluble ectodomain (sAPP α and sAPP β) respectively along with identical product called AICD

(intracellular C-terminal fregmenets)[9]. More importantly amyloidogenic processing generates Aβ, a sequence contained by sAPP β part. In brain APP processing generates mainly Aβ40 and Aβ42 based on 40 and 42 amino acids residues depending on secretases (see secretase table 1) through alternative splicings [10]. Along with regulatory subunits of γ-secretase complex, catalytic subunits presenilin1 (PS1) and presenilin 2 (PS2) are involved mainly in deciding the length of these toxic form of Aβ. These toxic forms of Aβ can aggregate and form plauqe that has more toxic effect [11].

With the progression of AD, symptoms also spread along with it depending on the vlunerability of different party of nervous system. More vlunerable areas are suppose to be effected earlier to others, the reason AD smptoms appear in different regions in a sequential order and consistency, although meachnism is poorly understood. Out of these early symtoms, one is olfactory impairement [12], which suggest olfactory system is one of the early vlunerable region during AD progression. Therefore, finding the correlation between early phenomen of APP processing and one of ther earlier vlunerable area of nervous system might lead to valueable insights.

This research has focused on APP processing in peripheral structures, the olfactory epithelium(OE), as well as CNS structures responsible for processing of incoming olfactory signals such as olfactory bulb(OB). The present study found unique APP processing in OE that has significance in providing not only possible biomarkers (including 25kDa, 55kDa and 80kDa) that can be used for screening and detection of AD before plaque formation but also for treatment purpose. Additionally, PS2 increased level was found in OE that

possibly involved in unique APP processing and might also be crucial for understanding the γ-secretase role and controlling AD through γ-secretase as a therapeutic target.

Table 1. *Secretases responsible for APP processing.*

Secretase	Types/ subunits	Major function related to APP processing	References
α-secretase	Adam 9	Involve in regulation of α-cleavage, may promote sAPPα.	*[8]*
	Adam 10	APP processing enzyme with constitutive and regulated α-secretase activity.	*[13]* *[14]*
β-secretase	Bace1	BACE1 activity is thought to be the rate-limiting factor in Aβ generation from APP.	*[7]*
	Bace2	Cleaves APP near the α-secretase site much more efficiently than at the β-secretase site.	*[15]*
γ-secretase	Presenilin1	Presenilin-1 gene (PSEN1) mutations increase Aβ generation.	*[16]*
	Presenilin2	Presenilin 2 (PS2) mutations increase Aβ generation.	*[17]*
	Nicastrin	Nicastrin modulates presenilin mediated notch/glp-1signal transduction and APP processing.	*[18]*
	APH1	Aph-1 and pen-2 are required for Notch pathway signaling, gamma-secretase cleavage of βAPP, and presenilin protein accumulation.	*[19]*
	Presenilin Enhancer2	Transcriptional regulation of APH-1A and increased γ-secretase cleavage of APP.	[20]

MATERIAL AND METHODS

MATERIAL AND METHODS

1.1. Animal

1.1 .1. Transgenic Alzheimer's disease model Tg2576 mice

In this study, heterozygous Tg2576 mice were used, which express a human amyloid-β precursor protein (APP) variant linked to Alzheimer' disease, as developed and described previously [21]. Age-matched non-transgenic littermates were served as wild-type control. All animal experiments were approved and conducted in accordance with guidelines of Ethic Committee of Seoul National University & DGIST.

1.1.2. Transgenic Alzheimer's disease model Tg6799 mice

Another AD model used in this study was Tg6799 mice, which expresses human amyloid precursor protein (APP) with three familial Alzheimer's disease point mutations and two human presenilin1 mutations thus also known as 5x FAD mice. Both of these mutation types mainly contribute to increased production of Aβ42 [22]. Age-matched non-transgenic littermates were served as wild-type control. All animal experiments were approved and conducted in accordance with guidelines of Ethic Committee of Seoul National University & DGIST.

Table2. Transgenic models used for this study.

Transgenic Line	Strain	Approach	Mutation	Plaque for-mation	Mice used in this study(age)
Tg2576	**(APP sw)**	**cDNA (695)**	**Swedish APP**	**~12 Month**	**10 Month**
Tg6799	**APPSwFILo PSEN1*M146 L*L286V)6799 Vas/J**	**Pronuclear coinjection : APP and PS1 transgenes**	**Swedish, Florida and London APP & human PS1**	**~ 3 Month**	**2 Month**

1.2 Olfactory behavioral analysis

Food buried, behavior test was performed to measure the mice smell ability to find a buried food pellet using olfactory cues as previously described [23] [24] [25] [26]. Olfactory test was taken blindly without revealing any genotypic information of mice before and during the experiment. Mice were deprived of food around 35 hours with free access to water. Before starting the experiment, adaptation time was provided 5~10 min to let them adapt in new prepared cage with new bedding material. This step was important for mice to be adapted to the new environment so that they would able to focus on finding food in a new environment. Similar cage were prepared with bedding material depth approximately 5 cm and food pellet was buried 2.5 cm below the surface. Latency or cut-off time ~15 min maximum was provided to each mice to find buried food. Latency time was recorded, as time between mouse inserted into the cage and grasping the food pellet, precisely using video tracking software and system (EthoVision xt 9).

1.3 RT-PCR

Samples were quickly prepared right after head amputation of wild type and age-matched Tg2576 mice. Total RNA was extracted from the tissue using TRIzol reagent (Invitrogen, USA). RNA was dissolved in Nuclease free water, concentration and quality was measured through a spectrophotometer (NanoDrop 2000, Thermo Scientific, USA) at an absorbance of 260nm.

Using 1µg of total RNA with nuclease free water were denatured at~ 65°C for 5min and then immediately transfer on ice. cDNA was formed in two steps 1) using Nuclease free water, oligo (oligo dT15; TTT TTT) and RNA premix incubated for 10 min at 70°C. 2) Adding 5X transcriptase buffer, dNTP, Rnase inhibitor and in the end enzyme reverse transcriptase with final volume of 25µl altogether and then was incubated at 42°C for 1.5 hours and 10 min at 70°C in PCR machine (BIORAD, USA). cDNA was stored at -80°C for long term storage and for short term usage it was used on ice at 4°C. 1µ of cDNA was used for each primer's RT-PCR.

Real-time PCR reaction samples were made by using cDNA and primers (**Table 3.**) *via* QuantiTech® SYBR Green PCR Kit (cat#204141) with a final volume of 20µl. The reaction was run in real-time PCR machine (Rotor Gene Q, Qiagen, Germany) with following parameters: 95°C for 30 sec, 59°C for 30 sec and 72°C for 30 sec (35 cycles). Analysis and calculation was made through the ΔΔCt method. Cyclophilin A was used for normalization and to determine the ΔCt values and further results were relatively quantified as previously [27] using software (Rotor Gene Q series software). Significant was measured through student's t-test.

Table 3. *Primers and sequence.*

Gene	Accession #	Reverse primer sequence	Forward primer sequence
Cyclophilin A	NM_008907	GTCTCCTTCGAGCTG TTTGC	GATGCCAGGACCTGT ATGCT
Bace1	NM_001145947.1	TCG CTG TCT CAC AGT CATCC	AAC AAA CGG ACC TTC CACTG
Bace2	NM_019517.4	GAACTCCAGCAGCCTTGAAC	GGCAGTTTGGCATAACCAGT
Presenilin1	NM_008943.2	CTCGCCATTTTCAAG AAA GC	GGG CTT GCTCTC TGT TTT TG
Presenilin2	NM_001128605.1	GGAGGATGGAGA GAG CACTG	CCACCACGATCATAC ACA GC
Nicastrin	NM_021607.3	GCTTCAGCACCCTTGTCTTC	TAAGCAGGCCCAGAGACAGT
APH-1	NM_177583.4	CAATGGGGAGTTCGAGAAAA	GCTGGTGAAGGCTAGCAAAC
Presenilin Enhcancer2	NM_025498.2	CGTGATCTTGCGTCTGTCAT	AACGCCTCTCTGAAGAACCA

1.4 Western blot

Animals were deeply anesthetized and perfused transcardially using 0.9%saline. Samples were obtained and immediately stored at -20°C. For western analysis samples were homogenized in RIPA buffer (ThermoScientifc, USA) and 1% of protease/phosphatase inhibitor cocktail (Halt™, ThermoScientifc, USA) and centrifuged at 4°C with centrifuge speed of 13,000 rmp for 15 min to remove any insoluble material. After collecting proteins, concentrations were measured using BCA assay Kit cat# 23115 (Pierce, ThermoScientifc, USA). Subsequently, equal amounts of proteins (100 µg) were separated through gradient SDS-PAGE gels of 4-15% or 4-20%. Proteins were transferred to PVDF membranes (Millipore) and membranes were blocked by using 5% skim milk made in TBST (Tris-Buffered Saline Tween® 20) for 1 hour at room temperature. Primary antibodies, including 6E10 (Covance, SIG-39320, Princeton, NJ, USA), APP C-terminal (Millipore, AB5352, Billerica,

MA, USA) and others (shown in **Table.4**) having dilution of (1:1000) in the BSA (Bovine Serum Albumin)) were incubated for overnight at 4 °C. Blots were washed with TBST three times and then incubated with appropriate secondary antibodies for 1hr. Further blots were equilibrated with TBST three times and were developed with an ECL detection system (Super Signal®, ThermoScientifc, USA). Primary blots were re-probed with primary antibody GAPDH (Millipore, MAB374, Billerica, MA, USA) with dilution (1:1000). And the results were scanned and analyzed using an image J program.

Table 4. *Antibodies used for this study.*

Cat #	Antibody	Dilution	Supplier
SIG-39320	**Aβ (6E10)**	**1:1000**	**Covance, Princeton, NJ, USA**
SIG-39200	**Aβ (4G8)**	**1:1000**	**Covance, Princeton, NJ, USA**
AB5352	**APP-CT**	**1:1000**	**Millipore, Billerica, MA, USA**
MAB374	**GAPDH**	**1:1000**	**Millipore, Billerica, MA, USA**

RESULTS

Chapter 1: Olfactory dysfunction behavior

Behavior study of Alzheimer's disease mice models was essential for following reason.

1) To characterize the transgenic mice relevant to their genotype and also to observe behavior changes accordance with the disease, it was important to correlate the progression of disease with some prominent behavioral changes in it. On the basis of these behavior changes some underlying mechanism and processes can be understood. This could provide not only progression of disease but could also ensure the critical time window that might have therapeutic and diagnostic importance.

2) As previous study shows cognitive impairment occur in both Tg2576 mice in which disease progresses slowly as compared to Tg6799 which shows faster Alzheimer's disease progression (see more detail in table 5). In Tg2576 mice previous study also shows [28] that amyloid-β (Aβ) start appearing at age of 6 month and increases exponentially at age of 9~10month. Moreover, it is considered that Aβ is responsible for early decline in memory and later cognitive stability [29]. Studies also shows olfactory dysfunction occurs even before cognitive decline, although exact relation between Aβ and olfactory dysfunction is not well understood. Therefore, it was important to observe any olfactory related abnormal behavioral changes that might provide relationship of Aβ with olfactory dysfunction. Besides, it might also be fruitful understand the relationship of Aβ with the progression of disease by examining the behavioral changes at specific time point. In this study has focused the effect of Aβ on smell ability.

Table 5. *Transgenic Mice and Major events and differences in AD mice models used for this study.*

Major events	Tg2576 mice	Tg6799(5X FAD) mice	References
Plaque formation	Advanced age, slower, initiates plaque formation at ~12 months	Early age ,faster , initiates plaque formation at ~3 months	[22]
Neuronal loss	Cell death is minimal	Exhibit neuron loss; , large pyramidal neurons in cortical layer 5 and the subiculum are lost, activation of gliosis	[30]
Aβ-induction	Late Aβ-induced events	early Aβ-induced events	[31] [32]
BACE1 level elevation	BACE1 levels became elevated in parallel with amyloid burden , starting late	BACE1 levels became elevated in parallel with amyloid burden , starting early	[33]

Therefore, to investigate smell ability in relation Aβ, it was important to observe smell ability (behavioral change) at stage where Aβ exponentially increases so that prominent behavior changes could observed.

Thereby, food buried test was performed to check the mice general ability to smell. Food buried test was preferred being simple and reliable to confirm the initial assumption related to olfactory dysfunction in relation Aβ. Based on this, first, it was investigated the smell ability of Tg2576 mice at age of 10month (Aβ exponentially increases at this stage in these mice [21].

It was observed that at this stage mice showed abnormal olfactory behavior as compared to age-matched wild type mice. The average increased in latency time was found 165.75% as compared to age-matched wild type mice as shown below in **figure (2).**

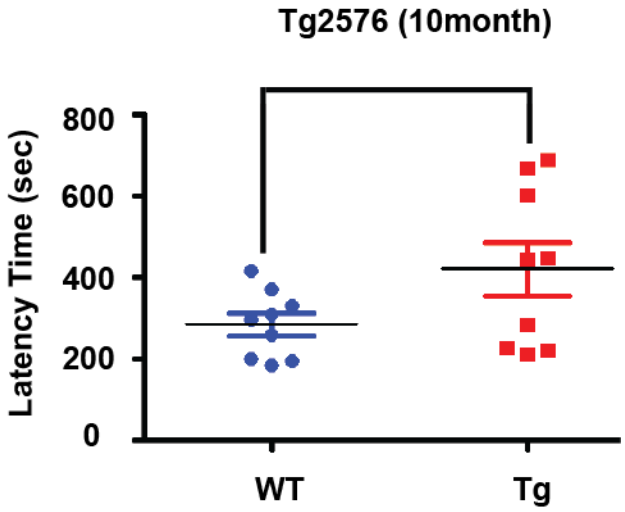


Figure2. Tg2576 10month mice food buried test performace; *perfomance of control mice (spheres), Tg2576 mice (squres). Data points represents latency time (mean±SED from n=9 animals for WT(control group) and n=9 for Tg2576 mice)*

Further, to verify, whether Aβ was involved in olfactory dysfunction and exponentially increase in Aβ might have vigorous effects on olfactory system or not, due to several reasons Tg6799 mice model was used. Tg6799 mice show intense AD pathology started quite earlier as compared to Tg2576 mice. Also Aβ generated in Tg6799 mice contain high Aβ42/40 ratio in this AD model that is quite toxic as compared to ratio of high Aβ40/42 produced in Tg2576 mice. Besides this, Aβ exponentially increased at early age of 2month

before plaque formation that reduces the aging factor involvement in smell ability in this study by using Tg6799 mice at 2month of age [22].

Tg6799 mice at age of 2month show increased latency time or in other words took more time to find food as compared to age-matched wild type mice. From which it was considered that with Aβ exponentially increase, behavioral abnormality was also increased as shown in **figure (3)**. Although difference was not found significant but still was important to make trend assessments and to consider relationship between Aβ and smelling ability. It was observed that Tg6799 mice at age 2month mice showed 225.93% increase in latency time compared to wild type. This observation indicate Aβ might play key role in smelling ability and might have potential for olfactory dysfunction.

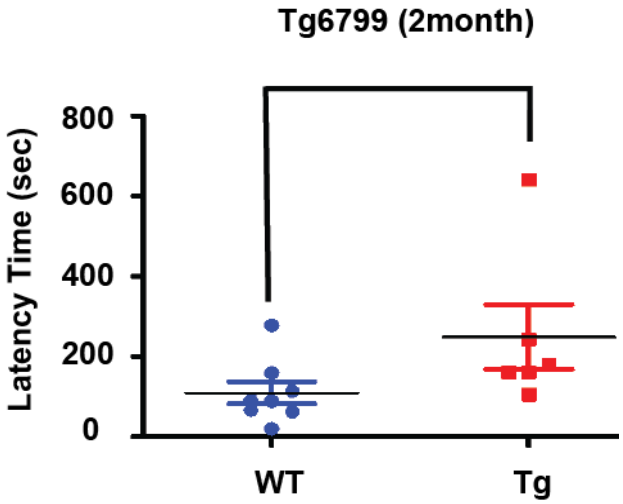


Figure 3. Tg6799, 2 month mice food buried test performace; *perfomance of control mice (spheres), Tg6799 mice (squres). Each data point represents latency time (mean±SED from n=8 animals for WT(control group) and n=6 for Tg mice)*

More precisly, observation was also made to find any apearantly abnormilty or injury other then Aβ related olfactory dysfunction. Except two mice (WT and Tg6799)

showed high latency time in there representive groups (figure 2) but still appreantly no other abnormailty/injury was found in these mice . These two mice might had potentially weak immunosystem against Aβ toxcity which had disrupt their smell ability more as compared to rest of group members.

Overall latency time differences can be seen below (shown in **table 6**) according to difference in age and type of mice used for these experiments.

Table.6 *Overall latency time differences of WT and TG mice; it can be seen in table below accordance with different age and type of mice used for these experiments.*

Genotype	Age	n(no. of mice)	Latency time(Mean±SED)
WT(Tg6799)	2month	8	109.9 ± 27.96
WT(Tg2576)	10month	9	285.6 ± 27.28
Tg6799	2month	6	248.3 ± 80.59
Tg2576	10month	9	473.4 ± 63.03

Similarly, observation was also made for Tg2576 mice along with wild type mice at age of 10month, mainly considering any injury, overweight and any other abnormality. However it was found that three mice showed abnormal behavior not mainly due to olfactory dysfunction but probably due to other possible reasons. One mice was injured, even more than two weeks were passed but still wound was found increased rather decreasing with time, probably due to poor healing process of wound. That indicates mice might have either diabetes mellitus that could effect this test [34] as wound was not healing with time or mice might had some other infectious disease. Probably, mice lazy behavior and less interest in food could be related to that's infection or any other internal disease. Beside this, size of one mice was found quite larger as compared to rest of its group members.

That mice was distinguished as fatty mice, although mice tried to sniff during the test but it was digging at different places and far away from hidden food in cage. Recent study also shows olfactory acuity alteration can occur in obese mice [35]. Lastly, one mice was sniffing in the air more than the floor of cage where food was hidden, trying to keep moving out of the cage. Possibly mice was not interested in finding the food rather going out. Possibly, mice was assuming that the source of food was outside compared to inside of case. Although mice found the food but it took much more time in sniffing outside of the cage. One possibility could be, mice need extra adaptation time in experimental cage, so that it could be used to new environment and took more interest in finding food. Based on these reasons, it was essential to exclude these three mice.

Although many factors are involved not only progression in of Alzheimer's disease but also in olfactory dysfunction. Out of them, one leading factor is aging [36]. To figure it out, aging and its effect in this study, it was found that the average latency time increased 292.62% at age of 10month wild type mice as compared to 2month wild type mice that also quite significant. In comparison of only wild type mice, it was examined that aging decreased the smell ability (as previously also described by [37], differences on the basis of aging was shown in **figure (4)** below.

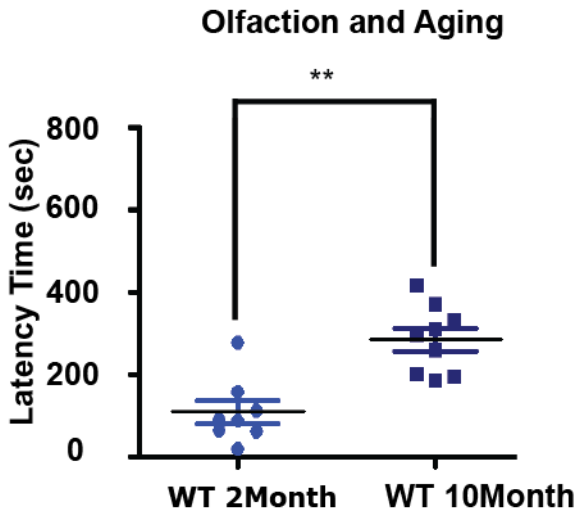


Figure 4. *Perfomance of wild type mice in relation to age (2 month & 10 month)*

Overall, results indicates olfactory dysfunction occurs with Aβ exponential increment. By using 2month Tg6799 mice, results also indicate that even at early age Aβ may disrupt smell ability more may be because of high Aβ42 / Aβ40 which is more toxic than Aβ40/ Aβ42 produced in Tg2576mice. However, still aging is also one of the key factor to contribute smell disability along with Aβ.

Chapter 2: Secretases in olfactory (peripheral) and central nervous system

Recent research revealed that Bace1 (β-sit amyloid precursor protein cleavage enzyme1) initiates Aβ formation and may also have vital role in early Alzheimer's disease pathology [38] [39]. It was also found that Bace1 elevated in postmortem of AD brain [40], suggesting with Bace1 increase involved in Aβ elevation and ultimately AD [41]. Therefore, secretases along with Bace1 play key role in AD and their expression along with AD decides the severity of AD [42].

To investigate key secretases involved in AD, cortex was used along with olfactory bulb(OB) as a part of CNS , also in both AD models used in this study (Tg2576 mice , Tg6799 mice) had Aβ pathology in cortex, especially layer 5 in case of Tg6799 mice. Besides this, cortex is well known representative of CNS[43]. Moreover, OB is also one of the CNS region behind nose and involved in smell information processing to other regions of the brain. OB is effected by Aβ and its accumulation prior to other brain regions along with abnormal nervous hyperactivity [44]. On the other hand OE is PNS area and if Aβ accumulation is associated with smell dysfunction in OB, suggesting even Aβ small quantity before plaque formation could be crucial to produce changes in OE. It might also be helpful to detect early biomarkers for Alzheimer's disease.

Therefore, to investigate the relationship of secretases and their changing expression with AD in PNS and CNS. First, study was focused the secretase expression in normal mice (without have AD, young and healthy mice).

Firstly, Bace1was analyzed, being a key secretase for AD. mRNA level was examined in normal young C57BL/6 mice age of ~2 weeks and analyzed. Based on this analysis Bace1 mRNA expression and its relation in CNS and PNS can be understood.

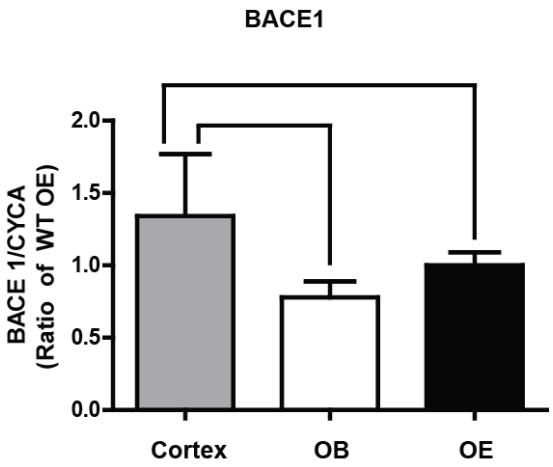


Figure 5. *Bace1 mRNA expression in C57BL/6 normal mice age of ~ 2 weeks*

It was observed that Bace1 mRNA expression was not significant between OB and OE being parts of CNS and PNS respectively comapred to OE as a control. Intrestingly, it was observed that Bace1 expression in OE was found more compared to OB as shown in **figure (5)**. That suggests, PNS may either have early to increase in Bace1 level compared to CNS and may be critical for early AD pathology in PNS. It was also revealed that in normal mice little difference in secretase mRNA expression in PNS, later might make PNS more vulunerable compared to CNS with the age and progress of disease.

Although, Bace1 normal function is related to axon guiding and sypase formation, but interestingly, it was found more in OE indicating some unkwnon role or function over there [7]. That provides clues for underlying functional differences in PNS compared to CNS based on secretases expression differences.

As Aβ formation through APP processing is not solitary performed by Bace1, rather other secretase called γ-secretase also participate in this processing. After initial cleavage

of Bace1, γ-secretase has role in making final cleavage for Aβ formation. Unfortunately, while considering APP processing and Aβ formation, γ-secretase is ignored due to its involvement in both amyloidogenic and non-amyloidogenic processing, producing APP fragments along with toxic Aβ and APP fragments without Aβ respectively Moreover, as γ-secretase also cleavages C-terminal of notch membrane fragments in similar way to APP processing [45]. However, through it has diversity in function but it key role in APP processing cannot be ignored, as recent research reveals that γ-secretase generated amyloid peptide from range of 38 to 43 amino acid [46], based on their length folding of peptides, toxicity is depending. Even though, understanding of mechanism of γ-secretase catalytic processing has been lacking but its therapeutic significance in both early and late onset of AD is notable.

To investigate γ-secretase mRNA expression in PNS compared to CNS, real time PCR data shown in **figure (6)**. As already mentioned about γ-secretase subunits (catalytic and regulatory) and their role in neurodegenerative diseases, especially AD. Accordingly, it's important to investigate their expression in normal young C57BL/6 mice age of ~2 weeks. Very interestingly, it is found that both catalytic subunits of γ-secretase (presenilin1 and presenilin2) are significantly increased and respectively in PNS compared to CNS. Not only this, regulatory subunits give also had similar pattern with increasing quantities in had PNS compared to CNS. Further, examining the results for regulatory subunits, nicastrin is although not significantly increased as compared to cortex. As nicastrin that promotes not only γ-secretase maturation but also regulator of a degrading an enzyme

which involves in degrading of Aβ fragments, thus indicating that in PNS it has effective in maturation γ-secretase and degradation Aβ fragments compared to CNS.

However, APH-1 increased mRNA expression level in OE while in OB decreased significantly as compared to cortex. APH-1, being regulator of γ-secretase maturation and essential for assembly of γ-secretase, it reveals that APH-1 has more vital role in assembly and maturation of γ-secretase in PNS compared to CNS.

Meanwhile, it was examined that PEN2 expression was significantly increased in PNS as compared to CNS. PEN2 is required for active γ-secretase [20], suggests γ-secretase has more active role in PNS compared to CNS. These results indicate in PNS of normal mice well assembled and mature γ-secretase with active and more effective role compared to CNS. Later with age and AD progression well assembled and mature form of γ-secretase may play striking role in APP processing in olfactory system by promoting some of product of APP fragments that can be involved in olfactory dysfunction.

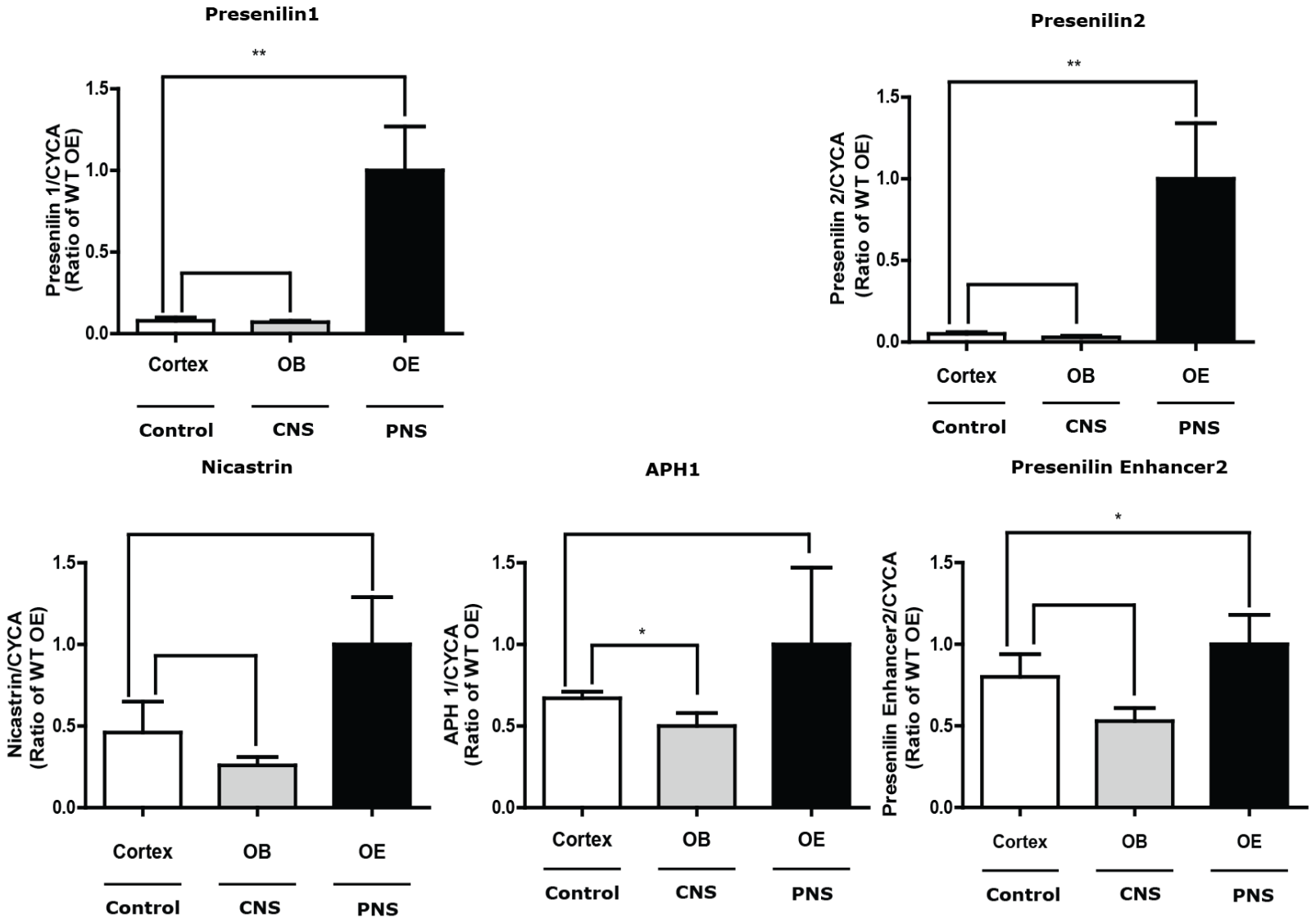


Figure 6.Gamma secretase mRNA expression in C57BL/6 normal mice age of ~ 2 weeks including catalytic subunits (pesenilin1 & Presenilin2) and regulatory subunits (Nicastrin, APH-1 and Presenilin Enhancer 2).

These results suggest OE as a part of PNS has distinct mRNA expression pattern of secretases as compared to OB which is part of CNS in relation to cortex.

However, APP protein is also processed by Bace2 protease, close homog of Bace1, that can participate in APP processing by making cleavage on three known sites of Aβ sequence including Phe19-Phe20, Phe20-Ala21, and Leu34-Met35[47]. Bace2, impairment might increase AD risk but contrary to Bace1action. Bace2 cleavage APP at different position compared to Bace1 and has ability to cut at Aβ sequences ultimately leads to Aβ destruction or degradation. This unique ability made it distinct from its homolog Bace1and make it worthwhile in ameliorating AD. Bace2 might change the end product by its cleavages, therefore its normal function and expression is important in enhancing the degradation of toxic peptide like Aβ.

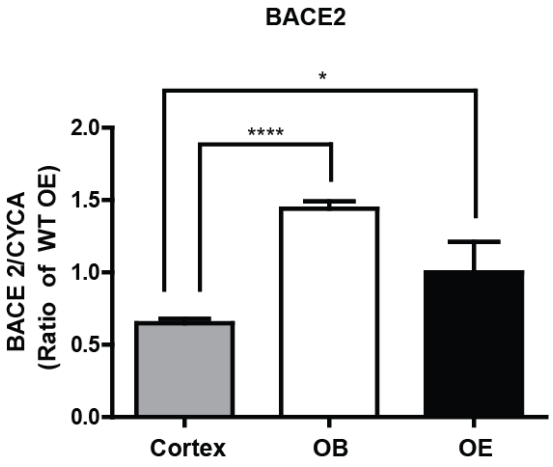


Figure 7. *Bace2 mRNA expression in C57BL/6 normal mice age of ~ 2 weeks*

To investigate Bace2 mRNA expression in normal young C57BL/6 mice age of ~2 weeks, based on OE as a control, PNS and CNS were compared. In OB, Bace2 expression is found significantly increased when compared with cortex. On the other hand, in OE, Bace2 expression also increased significantly but less significant than OB as shown in **figure (7)**. These results indicate that Bace2 high expression in OB and OE suggests Bace2 as a degrading enzyme might have might have more degrading role in OB and cortex and end product Aβ might be degraded faster as compared to cortex in these regions.

Additionally, both secretases (Bace1and γ-secretase) involved in AD pathology showed not much differences in pattern of mRNA while comparing between OB and cortex because they both were part of CNS. On the other hand OE being part of PNS showed clear difference as compared to OB and cortex. Based on this reason, while comparing wild type mice with transgenic mice, further study was made to use OB as a representative of CNS and OE as a representative of PNS.

Bace1,changes in AD postmortum as mentioned previously and reported in many studies [40]. Although, Bace1 plays key role in generating Aβ but still its physiological functions are not well understood.Recent research shows, its possible physiological roles including synapses regulation and cAMP/PKA/CREB pathway. Bace1 is known as rate limiting enzyme in Aβ production and contribution toward pathogenesis of AD pathalogy. It may also contribute AD through memory and cognetive deficits by regulating cAMP/PKA/CREB pathway independent of Aβ formation or its activity [48]. Consequently,

it's interesting to analyze its expression especially before Aβ plaque formation and to reveal its role accordingly during mild or onset of AD. To figure out its expression studies made on both 1) Tg2576mice-well characterized transgenic mice that expresses APP Swedish mutation. 2) Tg6799 mice-recently developed mice that expresses five familial mutations including APP mutations (known as Swedish, Florida and London) and Presenilin1 mutation (M146L and L286V) as mentioned in **table 2.**

In addition, Tg2576 mice develop Aβ plaque after ~10 months [28] while Tg6799mice shows Aβ plaque and elevation of Aβ42 form after age of ~2months [22]. However, Alzheimer's disease pathology can arise due to mutation in APP or Presenilin or both. Therefore, using this study on different transgenic mice might be helpful to analyze phenomena in single APP mutation mice or in mice having five mutations together. Also Tg6799 mice show Aβ42 toxic form that causes neuronal death which is was missing in tg2576 mice and while Aβ plaque can analyzed at early age of ~3month to minimizing aging factor. Therefore, at stage of before Aβ plaque formation in both Tg2576 and Tg6799 mice secretase expression can give proper direction whether mice are going towards Aβ plaque formation phenomena or some other way.

To figure out secretase expression and to reveal the pathology at critical stage before Aβ plaque formation in different transgenic mice is very crucial. This might be helpful for diagnostic and therapeutic both approaches.

Firstly, the study was made to analyze secretases expression in ~10months mice Tg2576 mice before Aβ plaque formation. Bace1 known as rate limiting enzyme, analyzed initially. It was observed that Bace1 mRNA expression was significantly increased in

Tg2576 mice OE as compared to age-matched wild type mice OE. In case of OB, Bace1 mRNA expression was found significantly increased in Tg2576 mice as compared age-matched to wild type mice as shown in **figure (8)**. Interestingly, these results indicate PNS had more increasing mRNA expression of Bace1 as compared to their control in Tg2576 mice. These results suggest OE could be more effected by during AD pathology and might be useful source for predicting AD biomarkers. If, in PNS AD pathology started more vigorously, then there might have a chance to find biomarkers over there although in CNS pathology could be at its early or mild stage.

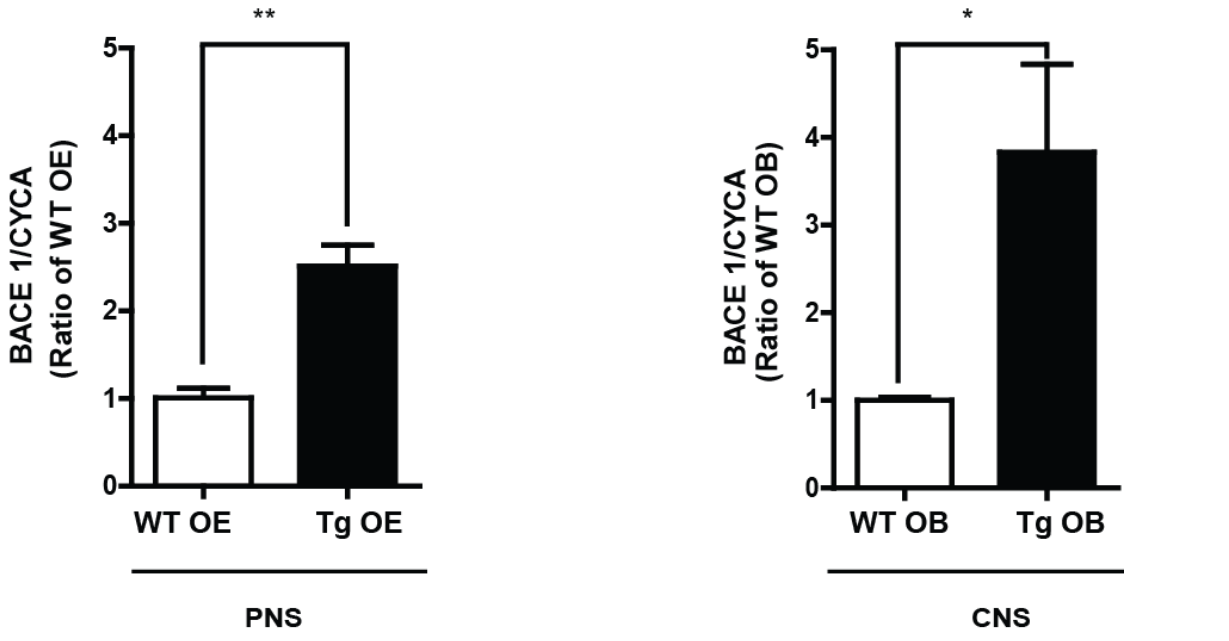


Figure 8. *Bace1 mRNA expression in Tg2576 mice at age of ~ 10month as compared to age-matched wild type both in pereipheral nerous system (OE) and central nervous system (OB).*

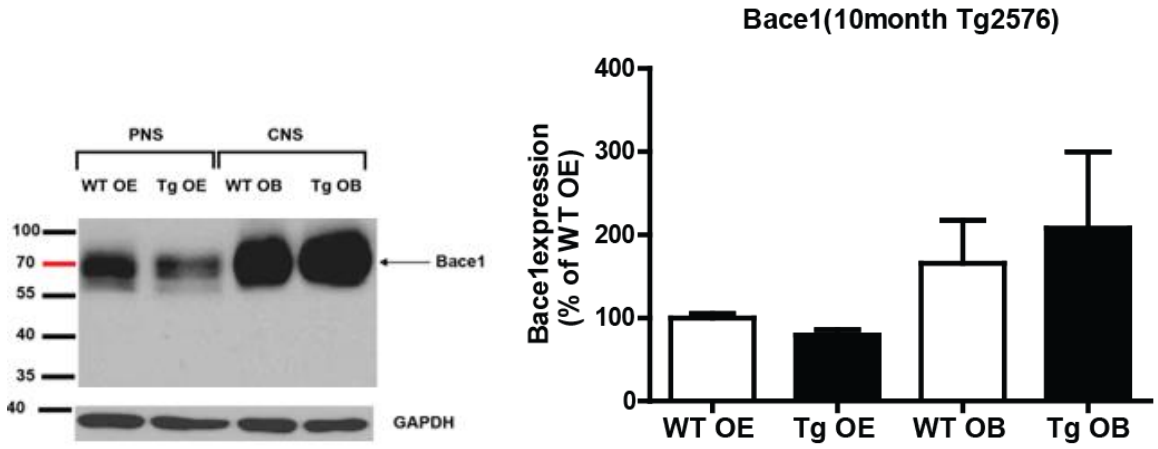


Figure 9. *Bace1 Protein expression in Tg2576 mice at age of ~ 10month as compared to age-matched wild type both in pereipheral nerous system (OE) and central nervous system (OB) and quantification.*

While protein expression of Bace1 secretase was found decreased in Tg OE compared to WT in contrast to CNS where Bace1 protein expression was found increased in Tg OB compared to WT OB at age of 10 month. As Bace1 has important role in neuronal surival and synapes formation and axonal targeting [48], therefore it may decreased in OE due to immaure OSN die due to apoptosis with overexpression of hAPP as shown by previous study [49] together with 10 month age contribute even more to this. While in OB more synapses and mature neurons are involed. Second reason could be Bace1 axonal transportation after formation in nucleus although Bace1 axonal transportation is not well understood yet.

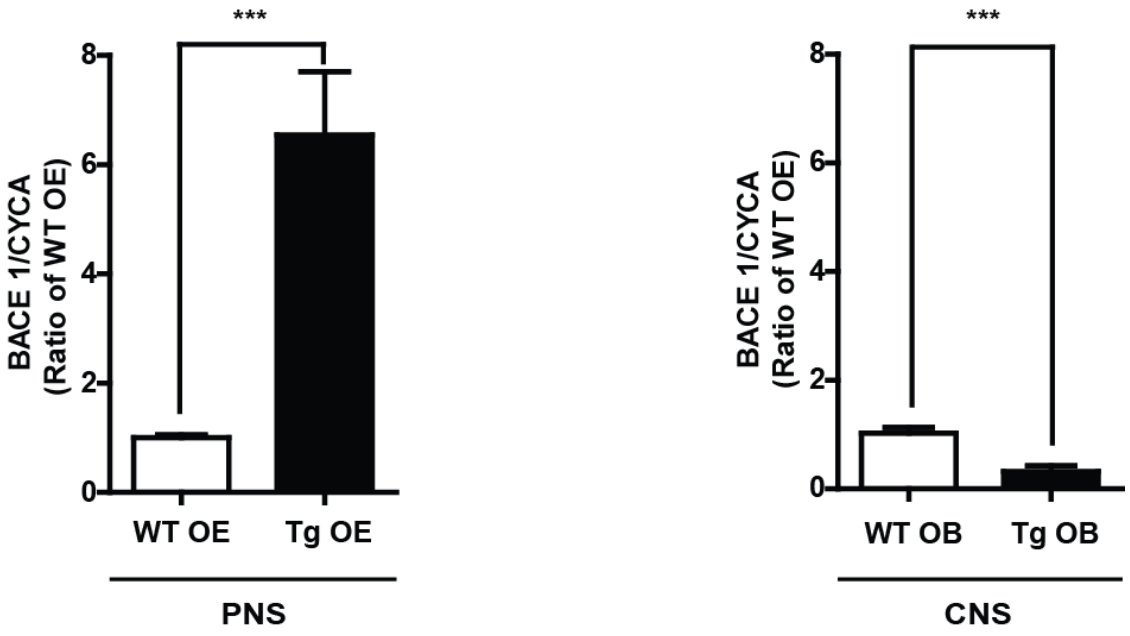


Figure 10. *Bace1 mRNA expression in Tg6799 mice at age of ~ 2month as compared to age-matched wild type both in pereipheral nerous system (OE) and central nervous system (OB).*

To confirm similar phenomenon further in ~2month age of Tg6799 mice was also observed, it was found that in OE of Tg6799 mice Bace1 expression was significantly high compared to age-matched wild type mice. And Bcae1expression was found significantly lowered in OB of Tg6799 mice as compared to wild type mice as shown in **figure (10)**. However, interestingly mRNA expression pattern of Bace1 was found in similar to Tg2576 mice (~ 10month), Bace1 increased in PNS and decreased in CNS.

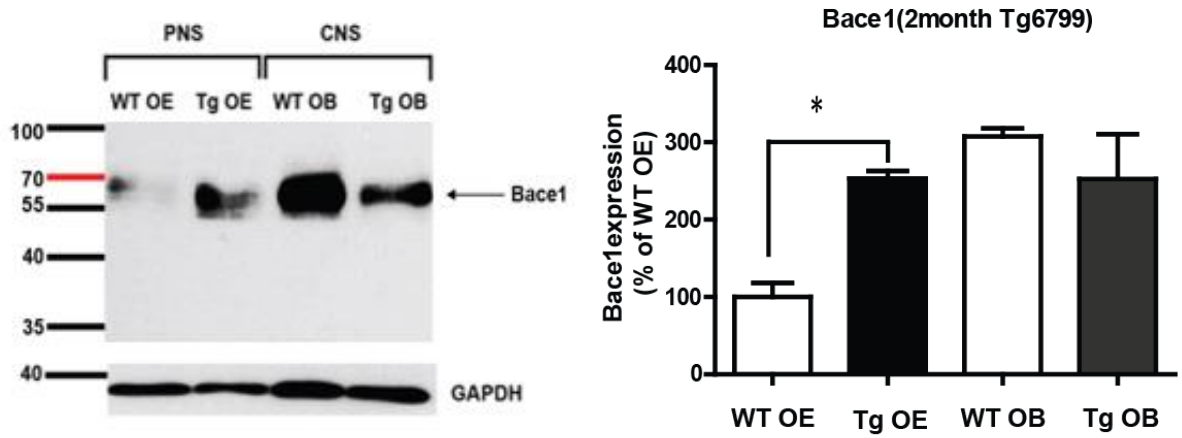


Figure 11. *Bace1 Protein expression in Tg6799 mice at age of ~ 2month as compared to age-matched wild type both in pereipheral nerous system (OE) and central nervous system (OB) and quantification.*

On other hand Bace1 protein expression was increased in Tg OE compared to WT OE, as previous study sugested Bace1 level become high at early age (as in this case of 2 month) due to Aβ42 formation in these mice that promotes Bace1 elevation[50]. While in OB it was found more due to involving in synapes formation and decreased in Tg OB due to lossing of synapases and plasicity due to more toxic form of Aβ that was Aβ42inovlved[51].

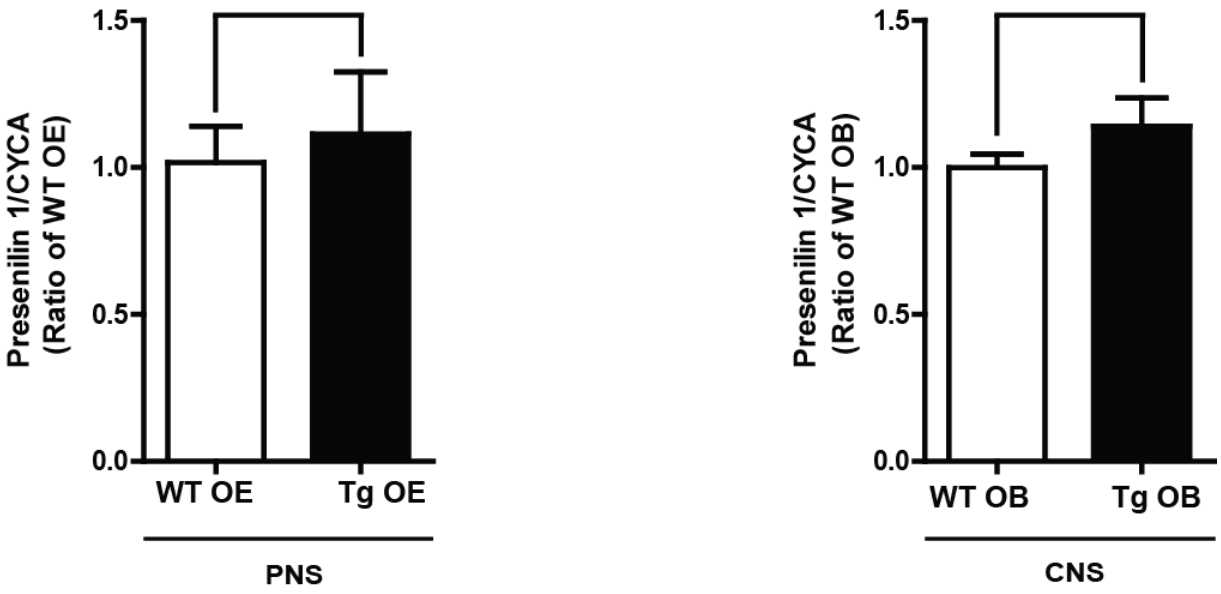


Figure 12. *Presenilin1 mRNA expression in Tg2576 mice at age of ~ 10month as compared to age-matched wild type both in pereipheral nerous system (OE) and central nervous system (OB).*

Secondly, γ-secretase was analyzed. γ-secretase catalytic subunits(presenilin1and presenilin2) were studied for comparison mainly. It was examined that presenlin1 expression increased 9.43% in OE of Tg2576 mice as compared age-matched wild type. Likewise, presenilin1 expression is increased 14% in OB of Tg2576 mice as compared to age-matched wild type mice as shown in **figure (12)**. These results indicate presenline1 mRNA expression pattern showed similarity in PNS and CNS but in increasing trend in both systems.

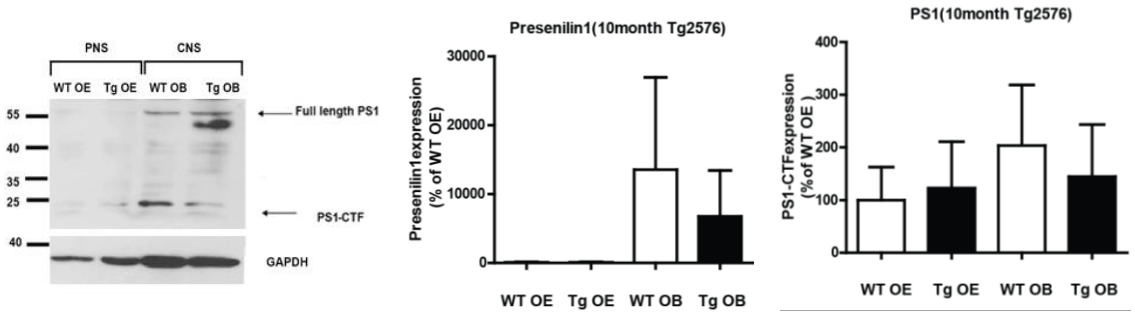
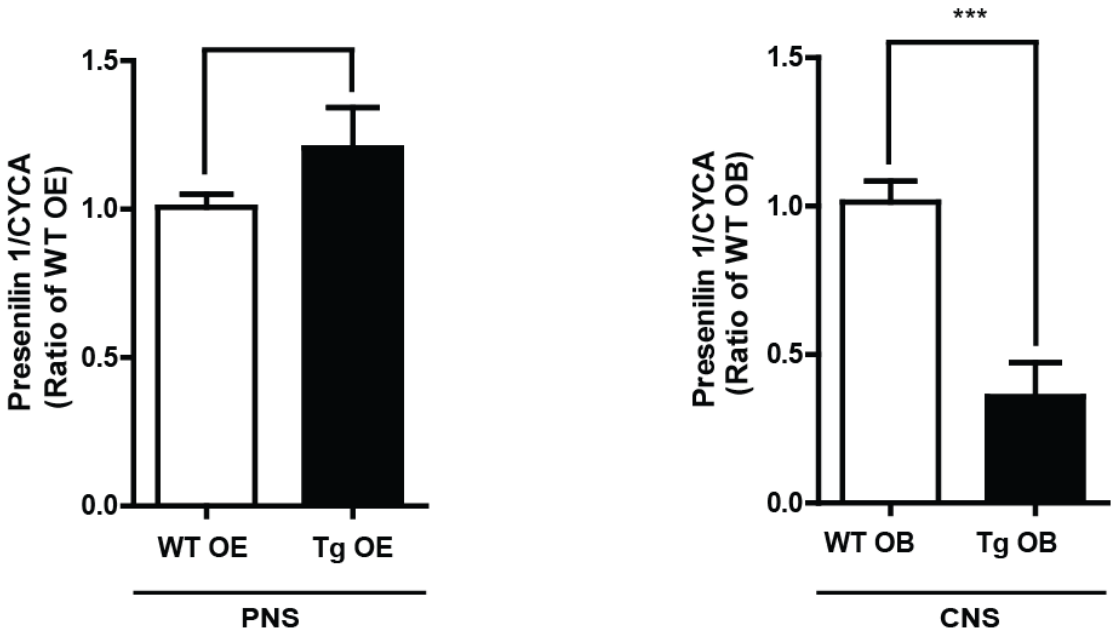


Figure 13. *Presenilin1 Protein expression in Tg2576 mice at age of ~ 10month as compared to age-matched wild type both in pereipheral nerous system (OE) and central nervous system (OB).*

Interstingly when protein expression of presenilin1 was analyzed, it was found that presenilin1 mainly involved in CNS compared to PNS. As full length presenilin1 or CTF were mainly found in OB. Quantified data indicate that compared to WT OE presenilin1 increases in both Tg OE and Tg OB that may be sufficient for promoting AD in both Tg OE and Tg OB.



Figure

14. *Presenilin1 mRNA expression in Tg6799 mice at age of ~ 2month as compared to age-matched wild type both in pereipheral nerous system (OE) and central nervous system (OB).*

Under similar scenario, it was also investigated presenilin1 in ~2month age Tg6799 mice. It was analyzed that presenilin1 mRNA expression was increased in Tg6799 mice as compared to age-matched wild type mice. However presenilin1 mRNA expression in OB was found significantly decreased as shown in **figure (14)**.

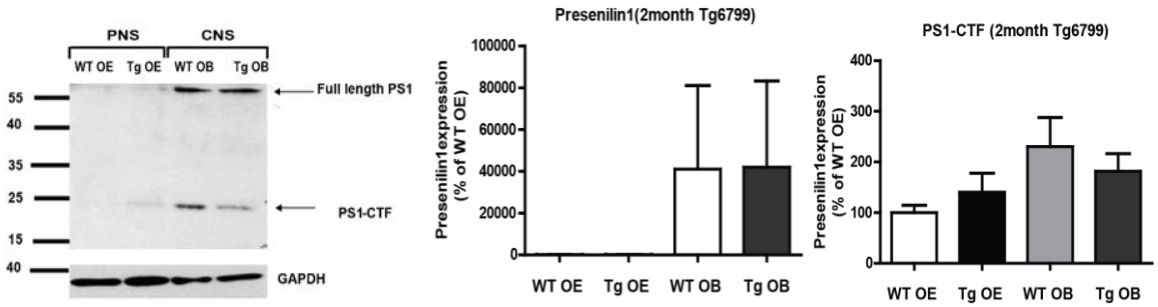


Figure 15. *Presenilin1 Protein expression in Tg6799 mice at age of ~ 2month as compared to age-matched wild type both in pereipheral nerous system (OE) and central nervous system (OB).*

Similar pattern was also found in Tg6799 mice that shows Presenlin1 mainly involved in CNS compared to PNS as full length and CTF of presenilin1 were mainly found in OB. These results indicate presenilin1 expression increased in PNS and CNS of Tg2576 mice ~10 month and Tg6799 mice 2 month age but overall quantification data show CNS expressed more presenilin1 also the C-terminal fragment expression in also found mainly in CNS has key role in activity of presenilin1 secretase [52].

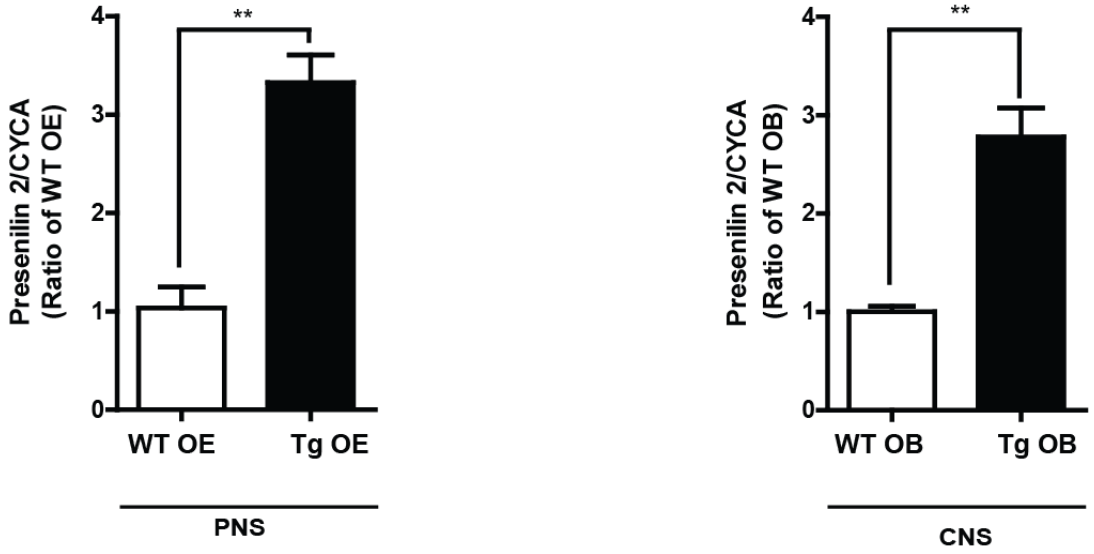


Figure 16. *Presenilin2 mRNA expression in Tg2576 mice at age of ~ 10month as compared to age-matched wild type both in pereipheral nerous system (OE) and central nervous system (OB).*

Furthermore, another catalytic subunit (presenilin2) of γ-secretase was analyzed. It was examined that in Tg2576 mice at age of ~10 month OE and OB both showed significantly increasing mRNA expression as compared to age-matched mice wild type as shown in **figure (16)**. These results indicate in absence of any mutation in γ-secretase subunit and with only one APP mutation (Swedish mutation) presenilin2 mRNA both in PNS and CNS increases significantly but higher in OE as can be seen through ratio compared to WT.

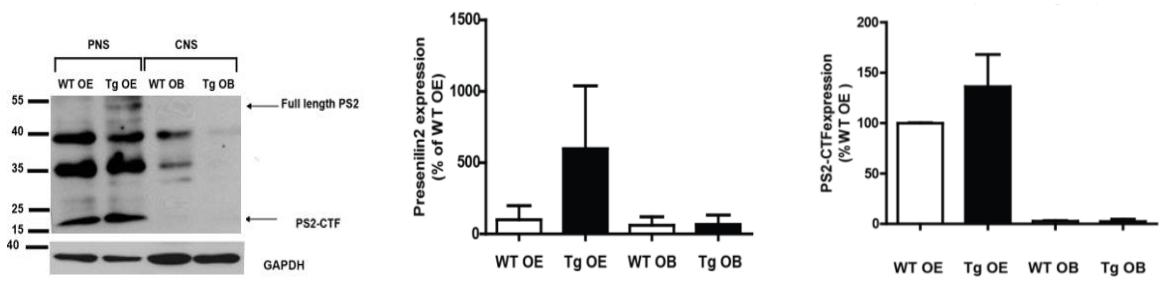


Figure 17. *Presenilin2 Protein expression in Tg2576 mice at age of ~ 10month as compared to age-matched wild type both in pereipheral nerous system (OE) and central nervous system (OB).*

Interestingly, as above results shown that presenilin1 was mainly found in CNS but presenilin2 was only found in OE. Additionally it was found that protein expression was increased in Tg OE compared to age-matched WT OE. While in OB no full length band was found. Similar pattern was found for C-terminal fragments of presenilin2 that can be seen dominantly in OE suggesting their possible role in PNS compared to CNS.

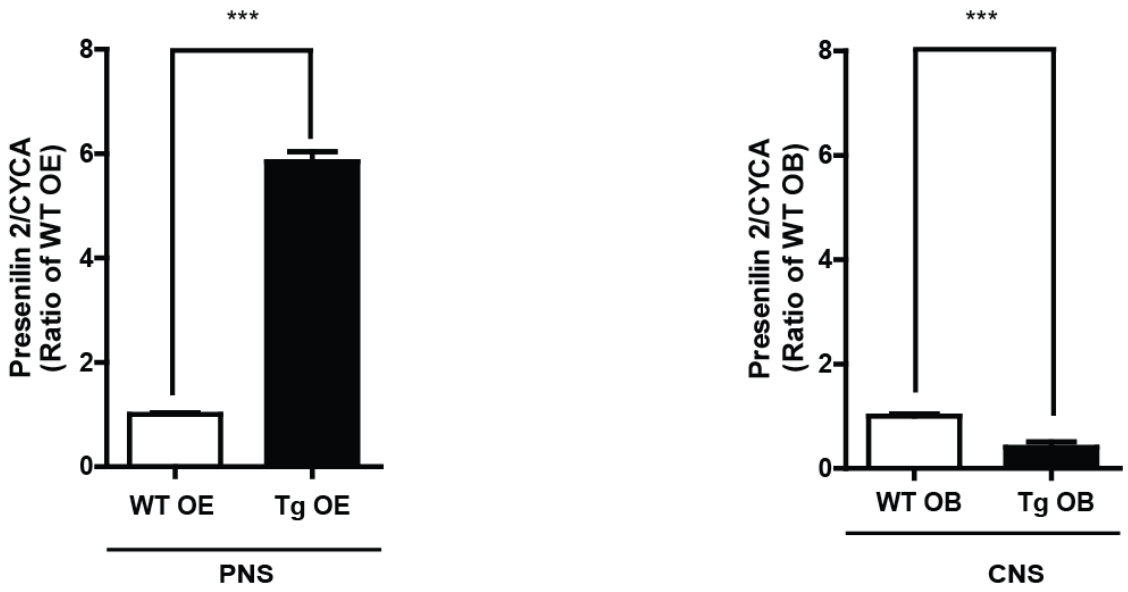


Figure 18. *Presenilin2 mRNA expression in Tg6799 mice at age of ~ 2month as compared to age-matched wild type both in pereipheral nerous system (OE) and central nervous system (OB).*

Subsequently, presenilin2 in ~2month age of Tg6799 mice was analyzed. It was examined that presenilin2 mRNA expression was significantly increased in OE of Tg6799 mice as compared to age-matched wild type mice. And presenilin2 mRNA expression was found significantly reduced in OB of Tg6799 mice as compared to age-matched wild type

mice as shown in **figure (18)**. These results indicate in presence of mutation in γ-secretase (mainly presenilin1 in these mice) subunit and with three APP mutation presenilin2 mRNA expression still can be found significantly increased in PNS as compared to CNS.

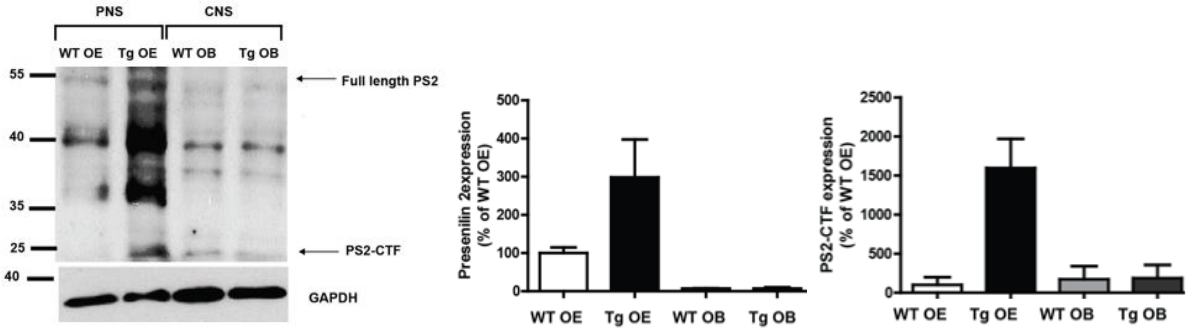


Figure 19. *Presenilin2 Protein expression in Tg6799 mice at age of ~ 2month as compared to age-matched wild type both in pereipheral nerous system (OE) and central nervous system (OB).*

Preseniline 2 protein CTF expression was similarly found higher in OE compared to OB, although full length presenline2 band was not found. Still it can be seen presenilin2 C-terminal are mainly found in OE that suggest it mainly involved in OE compared to OB . It was found no presenilin2 protein expression (full length) in Tg6799 mice which also suggested by promoting presinlin1 may dominate functionally also and may have supressing or alternatively role for γ-secretase [53].

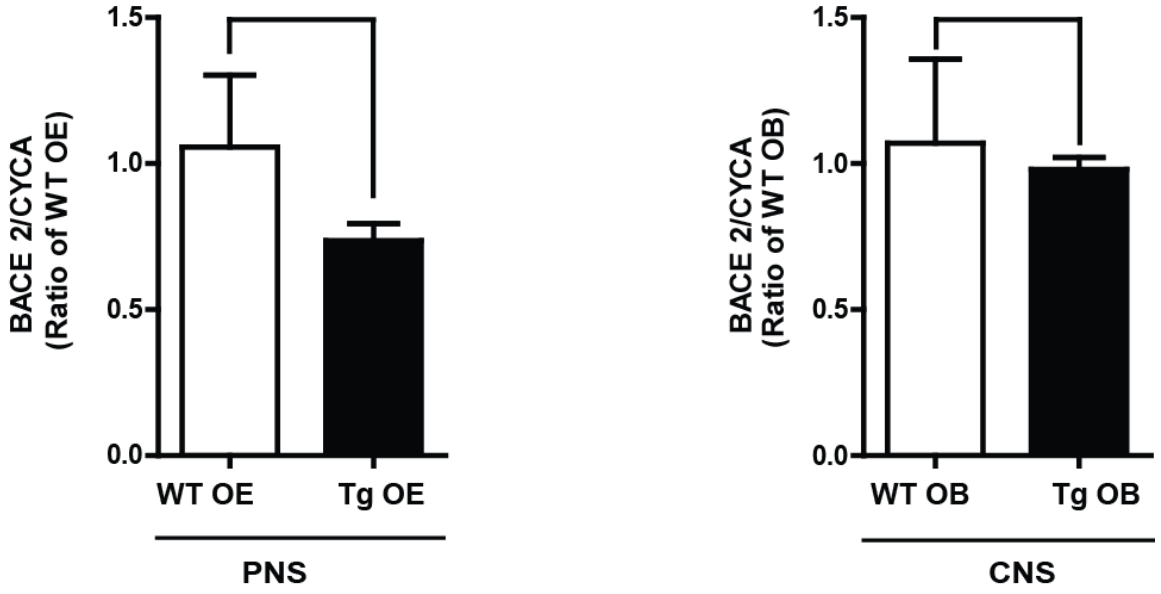


Figure 20. *Bace2 mRNA expression in Tg2576 mice at age of ~ 10month as compared to age-matched wild type both in pereipheral nerous system (OE) and central nervous system (OB).*

Additionaly, degrading enzyme Bace2 that can cleavage APP espacilly at sites of Aβ and causes destruction of Aβ , analyzed in Tg2576 mice at ~ age of 10month. It was found that both in OE and OB of Tg2576 mice showed bit reduced mRNA expression of Bace2 as compared to age-matched wild type mice. But both didn't showed significant reduction in mRNA expression level as compared to control as shown in **figure (20)**. These results indicate overall Bace1 level remains same in both PNS and CNS.

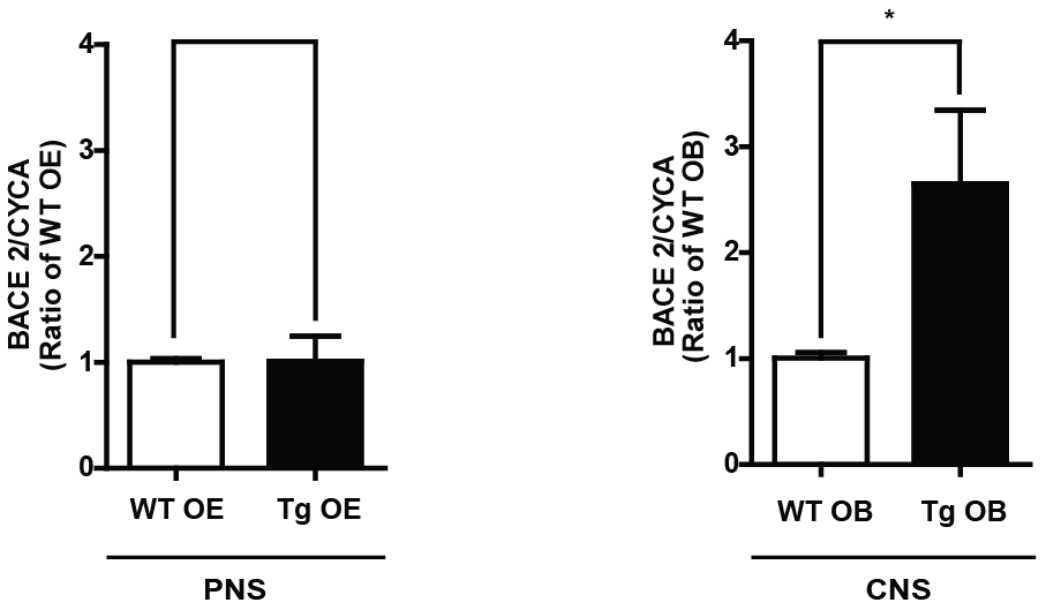


Figure 21. *Bace2 mRNA expression in Tg6799 mice at age of ~ 2month as compared to age-matched wild type both in pereipheral nerous system (OE) and central nervous system (OB).*

Subsequently, Bace2mRNA expression in ~2month age Tg6799 mice was analyzed. Bace2 was found no significantly changed in mRNA expression in OE of Tg6799 mice as compared to age-matched wild type mice. Interestingly, in OB mRNA expression of Bace2 was found significantly increased in Tg6799 mice age of ~2 month as compared wild type mice as shown in **figure (21)**. Together, these results suggest Bace2 can be found less in PNS as compared to CNS.

Overall, above results and secretases trend indicate that presenilin1 and presenilin2 mRNA can be found increased in PNS (OE) as compared to CNS (OB) although in OB presenilin1 possibly have role compared to PNS where presenilin2 expression was found high. While Bace2 secretase, which is also known as degrading enzyme that showed only

increasing pattern in OB of Tg6799 mice ~2moth age. It suggested that as in these mice and more toxic form of Aβ is formed known as Aβ42 that may activate Bace2 as degrading function. While OE is already in contact with outer environment that may have already stronger mechanism to deal with toxic substances and may not require Bace2 for this purpose.

Chapter 3: Biomarkers for early Alzheimer's detection through olfactory system

In Alzheimer's disease development one of the critical factors is generation of Aβ through sequential proteolytic processing of APP by β-secretase and γ-secretase enzymes[54]. APP is a transmembrane protein with large extracellular and small intracellular domain. Being transmembrane protein, APP can be processed as non-amyloidogenic processing, plays major role in numerous cellular functions including synaptogenesis, synaptic plasticity and also neuroprotective functions in nervous system [6]. On the contrary, amyloidogenic processing of APP (show in **figure 22**), at initial step β-secretase (Bace1) cleaves at N-terminal of large extracellular domain of APP, releasing APPβ ectodomain and concurrently membrane-bounded C-terminal fragment (CTF-99) [55]. Subsequently, C-terminal fragments are processed by γ-secretase from range of 38 ∼43 amino acids, producing potentially toxic fragments [45]. Moreover, Bace2 also can participate in APP processing by making cleavage on three known sites of Aβ sequence including Phe19-Phe20, Phe20-Ala21, and Leu34-Met35 [47].These secretase are involved in generating different potentially toxic fragments along with fragments that have neuroprotective function. However, accumulation of these potentially toxic fragments is not only one of the leading hallmark for AD but may also leads to olfactory dysfunction , abnormal behavior, memory impairment , neuronal death in AD models.

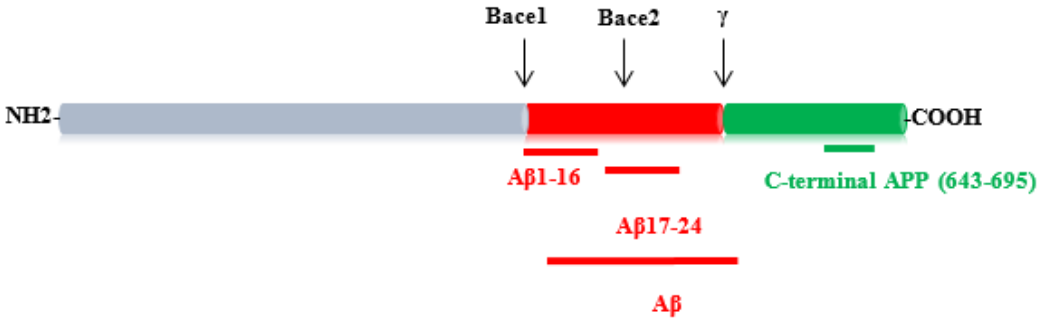


Figure 22. Amyloidogenic processing of APP: *Bace1 and γ-secretase involved in processing of APP and are responsible for generation of toxic Aβ. Bace2 may destruct Aβ and can work as degrading enzyme.*

It was hypothesized that different secretases expression in PNS as compared to CNS might involve in generation of APP fragments that could be detected prior to be produced in CNS. As olfactory dysfunction occur prior to memory impairment [44] suggesting with the progress of AD at early stages APP process differences might involve in generation of some potentially toxic fragments that could be only used as biomarker for early AD. Olfactory behavior tests and secretases mRNA expressions in this study, suggest there might be either unique APP processing in olfactory system or some unique APP fragments are formed during early AD. And these fragments might not only involved in AD but also olfactory dysfunction.

Therefore, it was important to figure out APP processing in OE (PNS) along with CNS area. To investigate that it was important to examine C-terminal fragments produced by Bace1, which is the secretase that initiate this processing and catalyzes APP into C-

terminal fragments which are subsequently catalyzed by other secretases as already mentioned above.

Thereupon, western blot was performed to examine the C-terminal fragment in PNS of a normal young C57 BL/6 mice at age of ~2 weeks as compared to CNS. As shown above in **figure (22)** C-terminal antibody (see also table for antibodies) detects all C-terminal fragments by detecting amino acid sequence from 643-695 on C-terminal of APP. It includes all C-terminals fragments of APP including (CTF-99) and (CTF-83) produced by β-secretase and α-secretase respectively [56]. Moreover, γ-secretase also catalyzes APP near Aβ sites far away from 643-695 amino acid sequences of APP, suggest all C-terminal and their pattern can be seen. Interestingly, it was found that C-terminal fragments in OE showed unique expression pattern as compared to other CNS areas including OB, cortex and hippocampus (cerebellum, hypothalamus not shown here)as shown in **figure (23)**. Nevertheless, all CNS brain area which were used in this study show similar C-terminal fragments pattern among them. This intriguing finding indicate PNS has unique APP processing as compared to CNS because the C-terminal fragments product between both systems can be characterized differently.

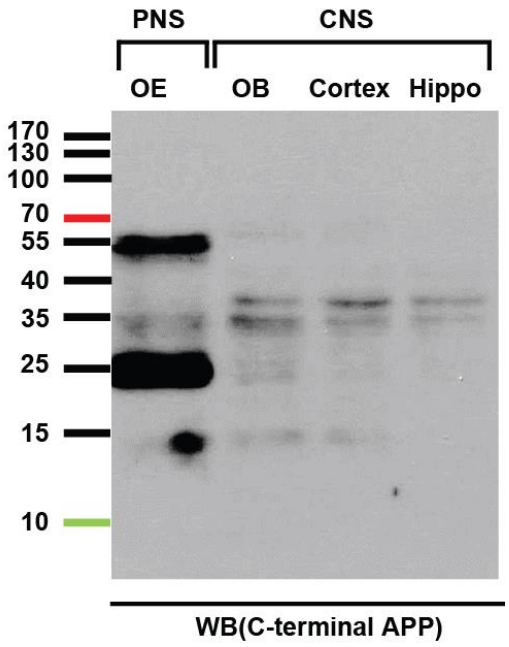


Figure 23. APP processing:*C-termianl fragments expression patteren in C57BL/6 normal mice age of ~ 2 weeks. Both in pereipheral nerous system (OE) and central nervous system (OB, cortex, hippocampus show distict C-terminal fregments pattern.*

But on the other hand, it was not sure whether these all distinct c-terminal found in OE are potentially toxic or not. Question is whether they have Aβ sequence also included in most of that C-terminal or not? If they have partially Aβ sequence or included whole Aβ sequence that might have potentially toxic C-terminal fragments and may aggregate differently too. To answer thsese questions, furhter analysis was made. To investigate APP fregments containg Aβ sequence (~ 1-42 amino acids), western blotting with well known antibody 6E10 as performed. 6E10 antibody is reactive to amino acid residue 1-16 of Aβ [57]. Interestingly, it was found that OE shows unique patteren compared to other CNS areas including OB, cortex hippocampus, hypothalamus and cerebellum shown in figure. Besides this, CNS shows quite similar pattern among them including full length APP

and α/βCTF shown in **figure (24).** While OE shows some unique bands also along with bands similar to CNS areas. These results indicate APP processing pattern in PNS is unique as compared to CNS and it might have some unique APP fragments along with other common peptides that are also found in CNS.

These results also suggest in AD mice model OE might express some exclusive peptides or APP fragments that can be helpful for detecting AD especially at early stages of AD progression.

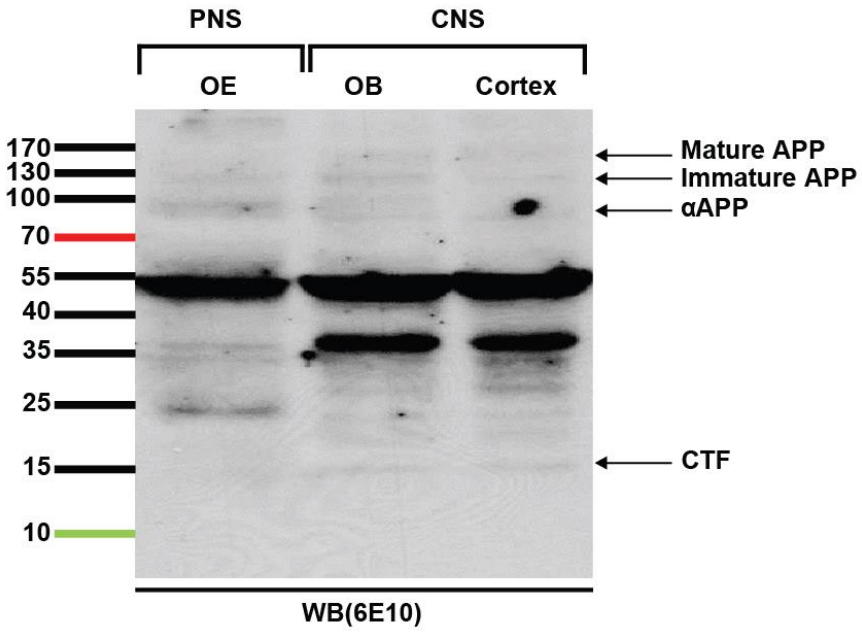


Figure 24. APP processing:*C-termianl framents expression patteren including* Aβ1-16 sequence *in C57BL/6 normal mice age of ~ 2 weeks.Pereipheral nerous system (OE)shows distinct APP fragments as compared to central nervous system (OB and cortex).*

Taking the advantage of these finding, first, well known mice model of AD Tg2576 mice were made as a first choice because of following reasons; Tg2576 mice express APP variant linked to AD and develop numerous neuropathological features of AD including Aβ plaque, dystrophic neurite and having inflamatory changes. On the contrary, this model lacks fibrill tangles , significant neuronal loss ,gross atrophy [22] and many other features mentioned in **table 5.** Based on these feature, they may be good model for studying pre-clinical stages of AD before onset of neuronal loss [58]. Previous research, also suggests these mice show Aβ from age of ~ 6 month of age and Aβ plaque are found distictly ~12 month. Therefore, it was important to find the time line in Tg2576 mice over there some phenmominal changes could be observed prominatly. Previous study also shows Aβ start to increase exponetially from 9~10 month age of Tg2576 [28]. This indicate at this stage the might be prominant changes in APP prcoessing are possible. Based on these facts, it was hypothesized that APP processing difference compared to wild type at this stage might be crucial.

Therefore, through western blotting experiments were performed using 6E10 primary antibody for APP processing in OE as compared OB in both age-matched wild type and Tg2576 mice age of ~ 10 month. Interestingly, it was found that whole APP was found increased. However remarkably, it was found one novel band that appears in Tg2576 mice OE at ~80 kDa as shown in **figure (25)** below. This novel APP fragment only appears at a stage when Aβ starts to increase exponetially at age of 9~10 month and more interestingly

before Aβ plaque formation. Along with that, other possible biomarker or APP fregments were also found of Tg including 25kDa and 55kD were almost disappeard in CNS (OB).

However, the novel band was not found at ~6 month age of Tg2576 (data not shown). Results suggest this novel fregment may start to form even at age of ~6 month due to differenes in seretase expression and APP processing in PNS as compared to CNS but these APP fregmants prominently appeared at age of 9 ~10 month.

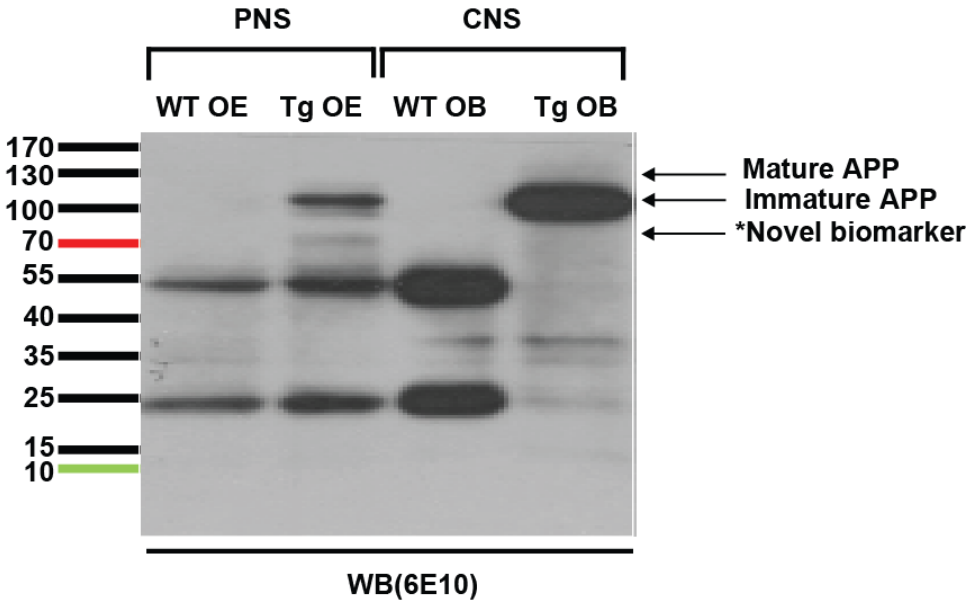


Figure 25. Novel biomarker

Accordingly, to confirm the novel APP fragment in OE of Tg2576 mice at age of ~10month, western bolt was performed using 4G8 primary antibody was used that is reactive with 17-24 residue of amino of Aβ (**antibodies table 4**).It was found that this antibody didn't detect novel APP fragment at ~80 kDa. These results indicate that novel

fragment didn't contain 17-24 amino acid residue of Aβ, fregment might be catalyzed Bace2 or some other unknown secretase.

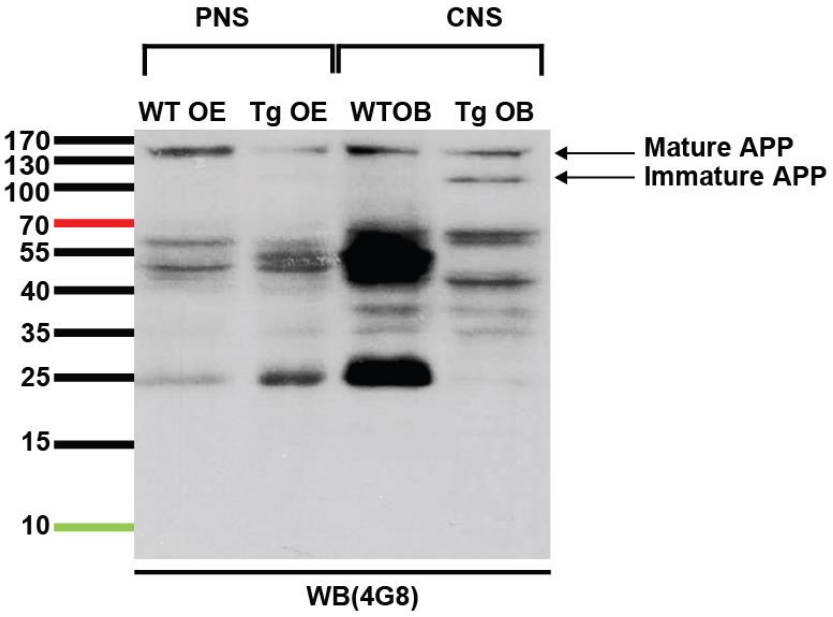


Figure 26. APP processing:*C-termianl framents expression patteren including* Aβ17-24 sequence

Accordingly, to confirm abrupt changes in APP processing parallel to Aβ exponentially increase, another AD mice model Tg6799 was used. As previously mentioned, these mice show plaque at ~age of 3 month and from age of 1.5~2 month Aβ increases exponentially before plaque formation.Although, two preneilin1 mutation and APP three mutation somehow enforced AD in these mices but still APP processing in PNS based on its distict pattern might provide any other type of APP fregments. And these

fregments might not appear prominantly otherwise but due to these five mutation some peptide may appear. As it was expected, interestingly,it was found ~37 a novel APP fregment appeared in Tg6799mice OE at age of ~2 month.These resutls suggest mutations in secretases or APP mutation as in Tg6799 mice not only interfer with APP processing in CNS but also with unique APP processing in PNS.Moreover, APP prcoessing in PNS may have numerou variety of APP fragments as compared to APP processing in CNS.Either single mutation or five mutation mice were tested in this study both provide novel APP fregments that can be used as biomarker for early detection of AD before plaque formation

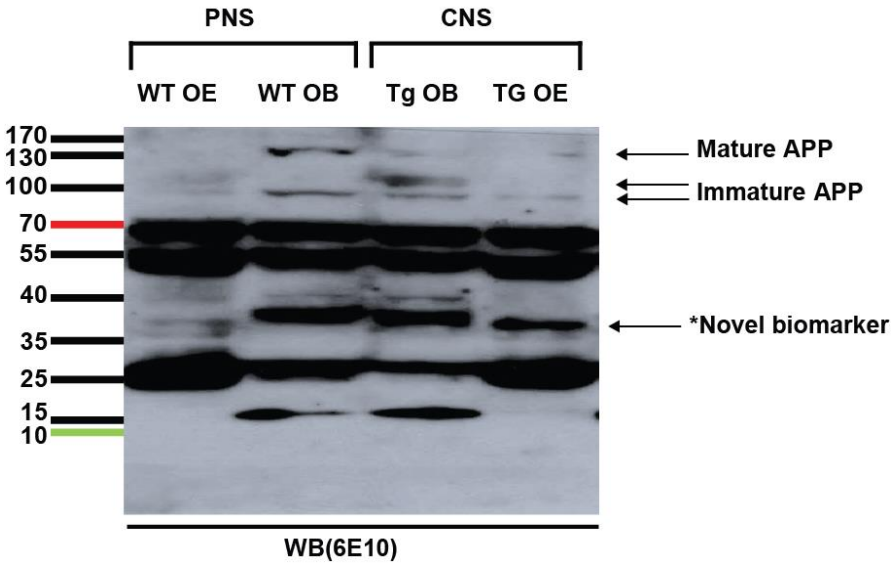


Figure 27. Biomarker in OE of tg6799:

. It was also examined that ~80kDa band didn't appear in these mice. There are several possible reasons that might involved behind it.

Mainly, in Tg6799 mice three APP mutation were genetically promoted, both Florida (I716V) and London(V717I) both promotes Aβ42 instead of Aβ40.On other hand, it has been seen major product formed due to Swedish mutation (K67N, M671L) Aβ40 [59] , same can be seen in Tg2576 which had high Aβ40/ Aβ42 ratio[21]. Similary presenilin1 mutation also endorse Aβ42 formation [16]. Therefore, Swedish mutation (K67N, M671L) proudct might not be seen prominantly. However, Tg2576 mice model is well known and studied, also include agaig factor and more likely to natural phenomen of AD. Therefore, this novel peptide might not only stricking finding for early detection of AD but also might be responsible for olfactory dysfuction.

DISCUSSION AND CONCLUSION

APP mainly produced in neurons and very quickly metabolized [60]. However there are different pathways through which it can be processed and can either form toxic Aβ or non-toxic fregments of APP[61]. This study was proceeded to not only understant APP processing and its functional relationship with olfactory dysfunction but also to figure out some of APP fregments that may be used as biomarker for detection of AD at critical stage before plaque formation. To achieve the ultimate goals, olfactory system was used that is very elegant system containg the neurons which are possibly accesible *via* biopsy. Along with critical stages of AD pathological changes are more observable, involving olfaction dsyfuction or odor processing. While in CNS memory imparement related problems can be observed at later stages of AD compared to olfactory imparement.

In this study, two different types of mice were used as mentioned earlier to examine APP products, enzyme expression, to figure out possible relation between Aβ and olfactory dysfuction and to find any possible biomarkers before plaque formation stage of AD. Therefore these experiments were performed on Tg2576 mice (age 9∼10 month) show Aβ exponentionally increased but no plaque formation was found at this stage in previous study[62], along with Tg6799 mice (age ∼2 month) early AD occurrence without any plaque formation and exponentially increasing level of Aβ [22] [30].

Using these transgenic mice and comparing with WT of age, olfactory dysfuction, secretase expression along with products were analyzed in this study.

Results indicate olfactory dysfction occur in both trangenic strains as Aβ exponentially increases. To eliminate the possibility of involment of aging factor in 10

month mice along with Aβ exponentionally increased, 2 month mice were examined and time delay in food buried test was found. That confirmend even at early age if Aβ is increased olfactory dysfuction can happened. It was observed that including aging factor and even in normal mice 2 month and 10 month mice show clearly signifcat difference in food buried test. Overal it can be derived that not only Aβ but also aging has key role in olfactory processing. To make experiment morevprecised, food buried test was taken with increaseing starvation time and more adaptation time. That provided equally chances to all mice to concentrate more food rather than wandering around and observing external enviormnet. It was ensured that time wasted by mice during experiment other than finding food was excluded. Moreover, any mice having injury or physically not fit for test was also exclude for sake of fair experimental results.

This study also show that secretases vary in their expression in PNS and CNS. And small change in these secretase may have critical role in progress of AD. To analyze secretase expression, young healthy mice age of 2 weeks C57BL/6 mice were used to minimize aging factor involvement during the study of secretase expression. Although Bace1 and Bace2 didn't show significant different in PNS and CNS but γ-secretase enzyme along with all its subunits including catalytic and regulatory subunits show clearly increased mRNA expression in PNS compared to CNS. This striking difference, provided chance to analyze further in AD case using these transgenic mice models.

The results carried out for Bace1 indicate its protein expression decreases in Tg2576 mice Tg OE suggested with loss of nuronal death of OSN may causes decrease in

Bace1 exprssion. This was further reinforced with 2month Tg6799 mice that indicate high expression of Bace1 in OE and possibe reasoned already mentioned in results section. However, γ-secretase catalytic subunits (PS1 and PS2), were interestingly protein expression observed oppositely in CNS and PNS respectively. Although the results of protein expression presenlin1 and presenilin2 showed little background rather than clean single band of these protein. Many studies showed similar results with background bands with these presenilin antibodies and they crop the band part mainly in their results. Same has been shown in case of presenilin2 case in results just to show clear difference that presenilin2 band was only found in Tg OE of Tg2576 10 month mice. These findings suggest PS2 has possible role in unique APP processing in OE compared to APP processing in OB or CNS. These results confirmed that APP processing in OE has unique processing and ultimately the unique product.

Based on earlier results APP processing was analyzed in PNS and CNS. It was analyzed through different antibodies including (C-terminal APP, 4G8 and 6E10) to have clear over view and in all cases OE product pattern was observed unique. That provided great chance to examine any possible biomarker that can be found. And interestingly three possible biomarker were observed based on highly reliable 6E10 antibody. However, biomarker /APP fragment observed at ~80 kDa was only found in Tg2576 mice OE compared to normal age-matched mice along with ~25 kDa and 55 kDa.

Along with these findings, APP process in Tg6799 was also analyzed, although in that mice mutation in APP promotes increasing level of product Aβ42/ Aβ40 with mutation in PS1. With extra changes still OE pattern was found unique compared to CNS.

And OE provided unique biomarker at ~37kDa only in Tg OE of Tg6799 mice compared to 2 month age-matched wild type. This study provide information about early detection of biomarkers in OE before plaque formation that could possibly significant for using biopsy techniques for screening of AD patients but also having great significance for treating drug at early stage before plaque formation. As recent study was unsuccesful due to treatment were made during and onward of plauqe formation in AD [63].

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Abstract in Korean

알츠하이머병(Alzheimer's disease, AD)에서의 후각 이상은 잘 알려져 있다. 알츠하이머병은 비정상적인 아밀로이드 전구단백질(APP)의 대사로 인해 발병한다고 알려져 있는데, 이런 아밀로이드 전구단백질의 공정(APP processing)은 먼저 베타-세크레타아제(β-secretase)로 절단되고 연이어 감마-세크레타아제(γ-secretase) 작용으로 절단되어 과량의 베타-아밀로이드(β-amyloid, Aβ)를 생성되는 과정으로 알려져 있다. 하지만 후각 상실이 알츠하이머병의 초기 단계, 특히 중추 신경계 안에서의 베타 아밀로이드 침착도 일어나기 전에 일어나지만 알츠하이머병과 어떤 연관관계를 가지는 지는 잘 알려져 있지 않다. 이런 연관관계에 대한 연구를 한다면 알츠하이머병 관련 후각 감각 상실에 대한 이해와 더불어 이를 통한 새로운 생체지표(biomarker)를 찾을 수 있는데 중요하게 작용 할 수도 있을 것이다. 따라서 이를 위해 형질전환마우스(transgenic mouse)인 인간 APP 를 과 발현 시킨 Tg2576 과 인간 아밀로이드 전구단백질, 프리세닐린 1(Presenilin1)를 모두 발현 시킨 Tg6799(5xFAD 라고도 불림)를 연구에 사용하였다. 또한 각각의 형질전환마우스에서 인지 감소가 나타난다고 알려진 10 개월(Tg2576)과 2 개월(Tg6799)시점에서 실험을 수행하였다.

정상마우스에서 중추 신경계로 대변되는 대뇌 피질이나 후구에 비해 말초 신경계인 후각 상피에서는 베타-세크레타아제, 감마-세크레타아제 1, 2 의 발현량과 그 작용에서 차이를 보였다. 더욱이 후각시스템은 말초 신경계로서 중추 신경계에서와 다른 형태의 아밀로이드 전구단백질의 공정이 나타남을 확인하였다. 알츠하이머병 모델인 두 가지 형질전환마우스에서 후각 시스템 내에서는 감마-세크레타아제 2 의 발현에서 정상과 유의성 있는 차이를 보였다. 아밀로이드 전구단백질의 공정에서도 차이가 나타났고 특이적으로 단백질 크기가 25KDa, 55KDa 와 80KDa 에서 특이적인 패턴이 보였고 이는 후각 상피에서 얻을 수 있는 알츠하이머병의 생체지표 후보가 될 수 있는 새로운 펩타이드 단편이 될 수 있다.

이러한 결과는 알츠하이머병 환자의 후각 상피에서 비정상적인 APP 공정이 진단 지표가 되는 새로운 펩타이드 단편을 생성함을 증명한다. 또한 AD 발병에 결정적이며 아주 초기에 해당되는 시기로 알려진 경도인지장애(Mild cognitive impairment, MCI) 시기에서 이 펩타이드 단편이 AD 를 진단 할 수 있는 생체지표 후보가 될 수 있음을 증명한다. 이러한 생체지표를 이용한다면 AD 조기 진단의 향상을 가져올 것이며 이를 위해 생검(biopsy)을 이용할 수 있을 것이다.

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With this, I hope through this work and similar further work in near future will make us enable to detect early detection of Alzheimer's disease. That will ensure our parents more secure and will not only make them able to live healthy and prosperous but also enable them to live out their lives with dignity and grace.

Curriculum Vitae

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PROFESSIONAL EXPERIENCE:

Sept.2009 – Dec.2009 Korea Research Institute of Biosciences and Biotechnology (KRIBB), -Daejon R.O.Korea

Visiting Student

EDUCATION:

Mar.2012 -	Dept. of Brain Science, DGIST -Daegu, R.O.Korea
	MSc. in Brain Science
	Thesis: Early detection of Alzheimer through olfactory dysfunction.
Feb.2007– Feb.2012	Department of Bio and Brain Engineering (KAIST),-Daejon, R.O.Korea
	Department of Biological Sciences (KAIST),-Daejon, R.O. Korea
	BSc. in Bio and Brain Engineering, BSc.in Biological Sciences (double Major)
Dec.2004 – Jan.2006	Government Post Graduate College, - Layyah, Punjab, Pakistan
	Faculty of Science (Pre-Medical) Student (Biology, *Physics, Chemistry)*
Dec.2002- Jan.2004	Government High School Serai, -Layyah, Punjab, Pakistan
	High School (Science) Student (*Mathematics, Physics,Chemistry,Biology)*

AWARDS:

Mar.2012-	Full scholarship for MS, DGIST, R.O.Korea
Feb2007 - Feb2012	Full scholarship for Bachelor of Science, KAIST, R.O.Korea.

PRESENTATION:

Nov, 2013.	2nd DGIST department of Brain Science Student Symposium, - Oral Presentation
	"Learning and decision making", Daejon,R.O. Korea
Title:	"Studies on proteolytic processing of APP in olfactory epithelium"

POSTER PRESENTATIONS:

Feb, 2013.	Nano-Bio Sensing, Imaging & Spectroscopy, Jeju, R.O.Korea (Feb, 2013)	-Poster Presentation
Jun, 2012	DGIST Brain Science Department Symposium "Neural Development", Daegu, South Korea	-Poster Presentation

PROFESSIONAL MEMBERSHIPS:

Society for Neuroscience

SPIE - The International Society for Optical Engineering

TECHNIQUES:

Behavior test (olfaction), RT-PCR, Brain sampling, Immunoblot, IHC, H&E staining etc.

PUBLICATIONS:

Article

1. Ameer Rasheed ; Ji Hye Lee ; Yoo-Hun Suh ; Cheil Moon; Studies on the correlation with olfactory dysfunction in a transgenic mice model of Alzheimer's disease. Proc. SPIE 8879, Nano-Bio Sensing, Imaging, and Spectroscopy, 88790U (May 20, 2013); doi:10.1117/12.2017726.