PRE-CLINICAL TRIALS: IMPACT OF TACTILE STIMULATION ON THE DEVELOPMENT OF ALZHEIMER'S DISEASE PATHOLOGY IN APP $^{NL-G-F}$ MICE.

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DEDICATION

…….To My Parents……

Abstract

Alzheimer's Disease (AD) is a one of the largest health crises in the world. Noise stress is identified as one of the risk factors for AD development. A series of research demonstrates the adverse of effects of both pre- and post-natal auditory/noise stress on the brain development in adult offspring and adults APP^{NL-G-F} mice, a mouse model of AD. There are limited but expensive pharmaceutical interventions to treat AD and most of the treatment options are not for cure or prevention, but to slow down the progression of the disease. The aim of this thesis was to examine the effect of tactile stimulation (TS) on AD-like symptoms and pathology. The results from this study suggest that TS improves the AD-like symptoms of cognition, motor, and anxiety-like behaviours, and that these improvements are associated with reduced AD pathology in APP mice.

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prayers and love; My parents and parents in laws, whose smiles are my remedy for anything. I will forever be grateful to you all.

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LIST OF ABBREVIATIONS

ACA	Anterior Cingulate Area
APP	Amyloid Precursor Protein
Aβ plaque	Amyloid Beta Plaque
Ach	Acetylcholine
AS	Auditory Stress
APP-TS	APP-Tactile Stimulation
APP-GS	APP-Gestational Stress
BB	Balance Beam
CAA	Cortical Amygdalar Area
EPM	Elevated Plus Maze
ER	Entorhinal Region
GS	Gestational Stress
Hpc	Hippocampus
IC	Iso-Cortex
Mpfc	Medial Pre-Frontal Cortex
MWT	Morris Water Task
NOR	Novel Object Recogniton
NA	Nucleus Accumbens
OA	Olfactory Area
PPA	Posterior Parietal Area
RA	Rhinal Area
RR	RotaRod
TS	Tactile Stimulation

Chapter 1: GENERAL INTRODUCTION

1.1.Alzheimer 's Disease:

Alzheimer disease (AD), a neurodegenerative brain disorder causes cognitive and motor skills deficits, as well as lack of motivation and deteriorated emotional and social behaviour. AD is the most common type of dementia, and accounts for about 60-80 % of dementias. According to the World Health Organization (WHO), about 50 million people are suffering from dementia worldwide, making it the 7th leading cause of death and costing about $1 trillion dollars annually in the world (WHO, 2018). The Alzheimer Society Canada reported that in 2018 more than half a million individuals were diagnosed with dementia in Canada and the health care cost is about 10.4 billion dollars per year. In addition, the prevalence of AD is predicted to be about a million Canadians by 2031 as the incidence rate is 14.3 per 1000 of the senior population (Government of Canada, 2018). There are many risk factors that causes AD directly and indirectly. Among many risk factors, stress is one of the key factors that is associated with AD. For the purpose of this study, I focused on the association between noise stress and the development of AD in APP mice, a mouse model of AD.

1.2. Alzheimer Disease and Brain Pathology:

Alzheimer's disease (AD) is a neurodegenerative brain disorder that causes deficits in cognition, motor coordination, and emotional and social behaviour. These symptoms are correlated with reduced neuronal complexity, increased deposition of plaques and tangles in the brain, abnormality in neurotransmitters, shrinkage of brain regions, and disrupted gamma brain signals.

1.2.1. Aβ Plaques and Neurofibrillary Tangles (NFTs):

One of the hallmarks of AD is the accumulation of beta amyloid plaques, a small spherical shaped proteinaceous extracellular deposition of 10-160 μm in length and consisting of 40-42 amino acid residues in the brain (Wippold et al., 2008). The peptide fragments appear to be in soluble forms at first, but later transform into senile plaques, an insoluble form of amyloid beta (Aβ) plaque. The Aβ plaque is formed as a result of abnormal proteolytic processing of amyloid precursor protein (APP), a transmembrane protein that is thought to play a role in synaptic formation and repair (Thinakaran & Koo, 2008). There are two secretase pathways involved in the formation of Aβ: β and γ secretase pathways. In the β secretase pathway, the β secretase cleaves the APP fragment into two parts: a soluble APP fragment (sAPPβ) and the carboxyl terminal C99 fragment (see Figure 1.1). The C99 fragment is further cleaved into Aβ oligomer extracellularly and APP intracellular domain (AICD) fragment intracellularly. The extracellular Aβ oligomers polymerise becomes neurotoxic for the brain (Wippold et al., 2008).

Another pathologic hallmark of AD is called neurofibrillary tangles (NFTs), which consist of hyperphosphorylated tau proteins inside the neuron (Wippold et al., 2008). NFTs result in instability of neurons and lack of intra- and intercellular communications, and can result in cell death (see Figure 1.1).

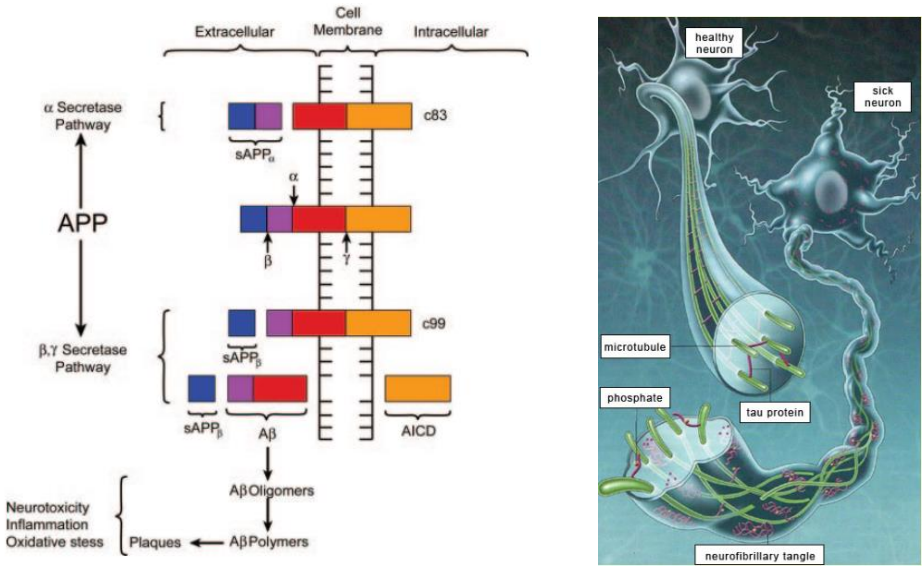

Fig 1.1: Proteolytic processing of amyloid precursor protein (left) (Wippold et al., 2008), and Neurofibrillary tangles in healthy and sick neurons (Right- University of McGill, The brain)

1.3. A Brief History of Alzheimer 's Disease:

In 1906, Dr. Alois Alzheimer observed shrinkage of brain size and abnormal deposition of extra- and intracellular plaques and tangles in his patient Auguste D, who was diagnosed with severe memory loss (Alzheimer Association, 2019). In 1910, Dr. Emil Kraepelin, one of Dr. Alzheimer's colleague, named the disease as Alzheimer Disease in the 8th edition of his book Psychiatrie (Alzheimer Association, 2019). Dr. Robert Katzman identified AD as the most common type of dementia in 1976 in his editorial published in Archives of Neurology (Katzman, 1976). In 1984, George Glenner and Cai'ne Wong discovered that beta amyloid protein is the key component of plaques (Glenner and Wong, 1984). Two years later in 1986, scientists discovered that tau protein is the chief component of tangles and that both plaques and tangles triggers the degeneration of nerve cells (Grundke-Iqbal et al., 1986). In 1987, researchers first identified the genetic link with AD, proving that chromosome 21 codes the amyloid

precursor protein (APP), a key molecule for beta-amyloid (St. George-Hyslop et al., 1987). In addition, apolipoprotein gene (APOE-e4) on chromosome 19 was identified as a risk factor for AD in 1993 (Corder et al., 1993). In 1995, scientists successfully developed a transgenic mouse model of AD called APP, by inserting a human APP gene (Mullan, 1995).

1.4. Knock-in-mouse, APP^{NL-G-F} :

A key component of the proposed studies is the use of a Knock-in mouse model, which is a second-generation mouse model of AD that was recently developed at the Riken Institute in Japan. It has a modified APP gene that has humanized Aβ sequence with three mutations (Swedish, Beyreuther/Iberian, and Arctic) in APP^{NL-G-F}. This model produces robust age-related spread of Aß aggregates and cognitive problems titrated to endogenous levels of APP. Since APP is not over-produced in human AD, this last point provides an important improvement over earlier Aβ-based mouse models of AD23. Earlier mouse models overexpressed APP or APP-presenilin1 (PS1), which led to accumulation of unusual fragments generated by α-secretase, such as C-terminal fragment-β (CTF-β). CTF-β is more toxic than Aβ and CTF-β does not accumulate in human AD brains. Indeed, the majority of neurologic features of earlier mouse models may be artifacts related to APP overexpression, which may explain the lack of translational success of candidate medications tested in clinical trials

1.5.Theory: Neuroplasticity

The term “neural plasticity” was first used by Santiago Ramón y Cajal (Fuchs and Flügge, 2014). It refers to the brain’s ability to reshape or rewire continuously as a result

of experiences throughout an individual's life. The example of neuroplasticity includes a change in brain signals, structures, synapses, and cell complexity. Throughout the lifetime there are two different types of plasticity: 1) experience-expectant plasticity (EEP); and, 2) experience-dependent plasticity (EDP). In EEP, the brain expects specific experiences to occur in order to develop healthy neural connections and it normally occurs during a critical period of brain development. As an example, in order to have the proper neural connections in the visual system, an individual is required to have visual experiences in his/her infancy. In contrast, according to the definition of EDP, the physiological and anatomical adaptations of the brain can occur from any experiences throughout an individual's life. One example of EDP is the pathological changes of the brain in AD and while others are the reparative changes in the brain related to the tactile stimulation (TS). In this thesis, I used TS to reshape or rewire the neural connectivity in the brain of APP mice. I predicted that TS would improve the AD-like behavioural symptoms and lead to a reduction in Aβ plaque formation and increased hippocampal volume in the APP mice.

Chapter 2: Early Tactile Stimulation Influences the Development of Alzheimer's Diseases in Gestationally Stress APP $^{NL-G-F}$ Adult Offspring.

2.1. Introduction:

The increasing incidence of dementia makes it a health threat in the world. Currently, about 50 million people are suffering from dementia worldwide and the cost of dementia was about US $1 trillion worldwide in 2018 (Alzheimer's Disease International, 2019). It is predicted that in 2050 the number of individuals with dementia will be about 150 million worldwide (WHO, 2019). Alzheimer's disease (AD) is the most common form of dementia, which consists of about 60-70% of all dementia cases (WHO, 2019). AD is a neurodegenerative brain disorder that causes cognitive and motor skill deficits combined with a lack of motivational, social, and emotional behaviours. These behavioural symptoms are associated with the neural symptoms such as: shrinkage of the cerebral cortex, hippocampus, and basal ganglia (Pini et al., 2016); formation of extracellular Aβ plaques and intracellular tau phosphorylated proteins (Marcello et al., 2015); synaptic loss (Hamos et al., 1989); and disrupted gamma oscillations (Iaccarino et al., 2016) in the brain. There are many identified risk factors for AD such as depression, auditory stress as a form of noise pollution, diabetes, anoxia, high blood pressure, low education, smoking, alcohol, obesity, brain injury, and physical inactivity to name few (Alzheimer's Society Canada, 2019).

Auditory/noise stress has been identified as strong risk factor of AD. Research on humans has demonstrated that night time noise levels, and distance from major roads are highly associated with AD (Carey et al., 2018). Research on mice has demonstrated that exposure to noise stress during the prenatal (Jafari et al., 2018, 2019) or postnatal periods (Jafari et al., 2017; Cheng et al., 2011) have a detrimental effect on brain structures,

neural networks, and related cognitive behavior in offspring and adult mice respectively (Jafari, Kolb & Mohajerani, 2019; Arnsten, 2015). Gestational auditory stress has proven to be harmful for infant brain and behaviour development and aggravates cognitive deficits and Aβ plaques formation in APP mice, a mouse model of AD (Jafari et al., 2019). In addition, prenatal stress has proven to alter the organization of neural circuits in the neocortex and hippocampus in rats (Mychasiuk, Gibb & Kolb, 2012). There is plenty of evidence from clinical research on humans showing that people living in major cities (Astell-Burt & Feng, 2018) and closer proximity to heavy traffic noise (Chen et al., 2019) have higher chance of being diagnosed with AD/dementia along with other cognitive impairment disorders (Tzivian et al., 2016). Previous research in our lab has demonstrated the adverse effects of auditory stress on the brain and behaviour in second generation of mouse model of AD (Jafari et al., 2017; Mehla et al., 2019; Karem, 2019). In this study, we aimed to explore the role of early tactile stimulation (TS) in mitigating the adverse effects of gestational auditory stress, as well as AD-like symptoms in adult APP offspring.

TS has beneficial impact on brain and behavioral development. Research in rodents has shown that TS aided recovery from brain injury (Gibb, Gonzalez, Wagenest, & Kolb, 2010). One mechanism may be that TS releases fibroblast growth factor-2 (FGF-2), which stimulates neurogenesis, cellular proliferation, survival, migration, and differentiation. Research on premature infants showed that TS improves the development of their cognitive and motor skills (Field et al., 1986) and may stimulate neuroplasticity later in life. Laboratory research on rodents has also well established that the improved cognitive and motor skills are associated with: increased FGF-2 (Comeau et al., 2007);

BDNF (Antoniazzi et, al., 2016); acetylcholine (Dudar et al., 1979); and synaptic plasticity (Kolb & Gibb, 2010, 2011) in the brain.

The effect of TS is strongest when it is applied during the early infantile period. Therefore, we designed the research project in which we applied TS from postnatal day 3-18 and observed the behavioural improvements at two months in one group of APP adult offspring and in another group at six months. Our predictions were: 1) early TS would mitigate the adverse effects of gestational auditory stress in adult APP offspring; and, 2) early TS would improve the cognitive, motor, and anxiety-like behaviours in adult APP offspring.

2.2. Methods and Materials:

2.2.1. Animals:

All the mice were housed in Canadian Center for Behavioral Neuroscience (CCBN) vivarium, and all the behavioral, brain anatomical and physiological tests and analyses were carried out according to protocols approved by the Animal Welfare Committee at the University of Lethbridge. $APP^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ (amyloid β-protein precursor), Alzheimer's disease transgenic mice carrying Swedish (NL), Arctic (G), and Beyreuther/Iberian (F) mutations were used in this research project. Twenty-four 8 weeks old female $APP^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice were individually mated with twenty-four male $APP^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice in standard shoe-box cages at 4:00 pm. For the recording of gestational length, a former protocol was followed (Jafari, Mehla, Kolb, et al. 2017). Upon the confirmation of ++ genotyping of the offspring using the tail snipping method, fifty five females $APP^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ offspring and sixty male $APP^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ offspring were used in this project. All mice were given access to food and water ad libitum by the animal care staff. The mice were maintained on a 12 hour light and 12 hours dark cycle in a 21ºC

temperature controlled room in the vivarium. All training and behavioral testing was performed by the same experimenter during the light phase.

2.2.2. Stress Paradigm:

Half of the pregnant mice were exposed to acute auditory sound (AS) stress for 24 hours every other day with an intensity of 90 dB during gestational day (GD) 12-16. The AS, consisted of an intermittent 3000 Hz frequency, was programmed by using MATLAB software and played the same repetitive sound for 1 second for 5400 times with an interval of 15 seconds between 2 sounds (Haque et al. 2004; Jafari, Faraji, et al. 2017). The reasons for using 3000 Hz intermittent frequency are that 3000 Hz is similar to traffic and environmental noise (Chang et al. 2014) and is easily audible to mice (Heffner and Heffner, 2007).

2.2.3. Experimental Design:

The offspring from both gestational stress (GS) and no gestational stress (NGS) groups of $APP^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ strain were randomly assigned to two groups: 2 months and 6 months. Offspring from each of 2 and 6 months cohorts were further assigned to four different groups consisting of APP, APP-Tactile Stimulation (APP-TS), APP-Gestational Stress (APP-GS), and APP-Gestational Stress-Tactile Stimulation (APP-GS-TS), as per the following table. Each group consists of a minimum 4 male and 4 female mice.

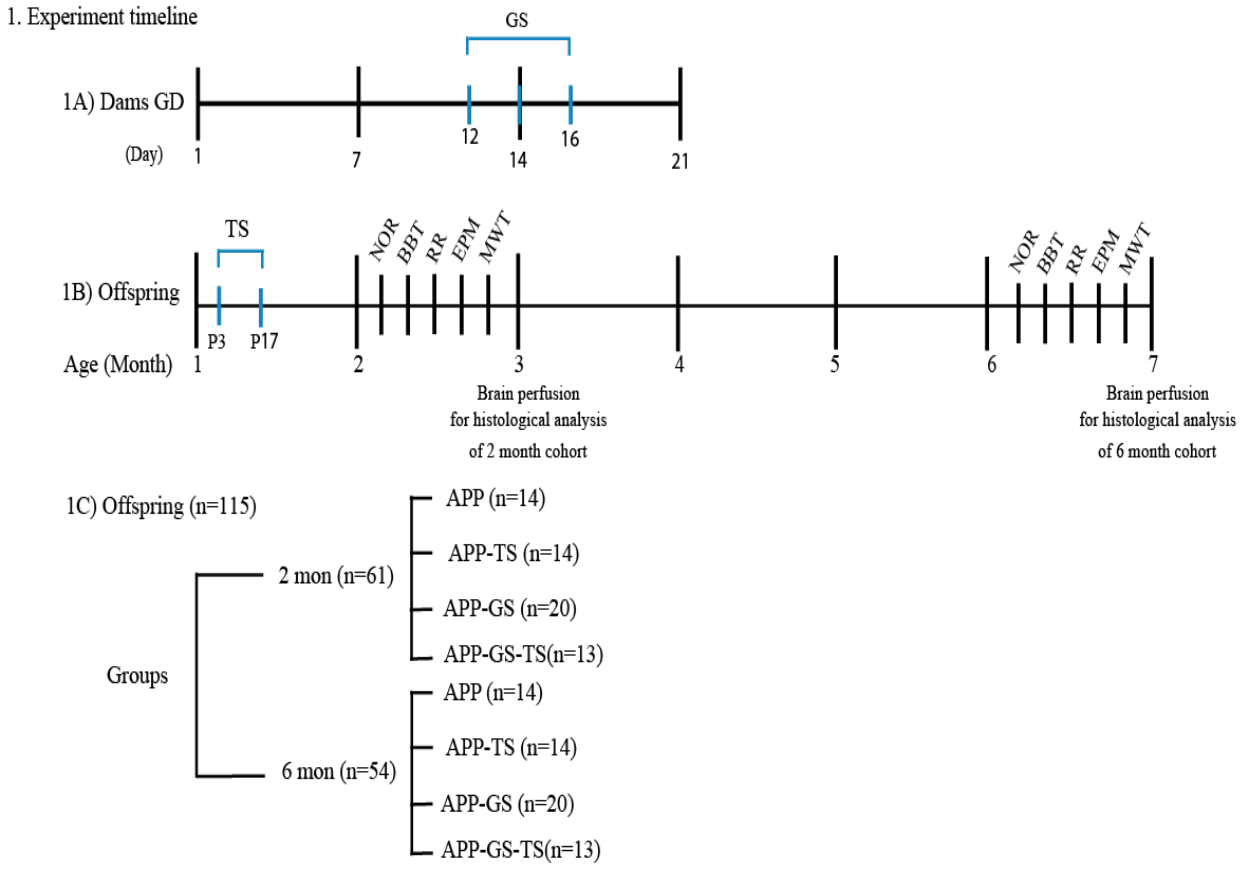

Fig 1: 1A) Timeline for gestational period and half of the pregnant mice received GS on GD 12, 14, and 16 for 24 hours per day. 1B) The timeline for offspring shows half of the pups received TS during Postnatal day 3-17, behavioural measurement at 2 and 6 months for 2 and 6 month cohorts respectively, and brain perfusion at 3 and 7 months for 2 and 6 month cohorts respectively. 1C) The experimental groups and total number of mice in each group consisting both male and female mice.

2.2.4. Tactile Stimulation Procedure:

Half of the offspring from both GS and NGS mice groups received TS for 15 minutes by gently brushing the body of the offspring using a soft sweeper broom at postnatal day (PD) 3-17, for 15 minutes with a frequency of 3 times a day for 15 days (8 am, 12 pm, and 4 pm). The offspring were weaned at PD 21 and housed with a group of 3-4 mice per cage until 3 and 7 months in the same animal room per 2 and 6 months cohorts respectively.

2.2.5. Behavioral Tests:

All the mice in both 2 and 6 month cohorts performed several behavioral tests at 2 and 6 months, respectively, to measure the effect of TS on cognitive and motor functions and anxiety-like behaviours. Balance beam (BB), rota-rod (RR), novel object recognition (NOR), activity box (AB), elevated plus maze (EPM), and the Morris water task (MWT) were conducted by the same examiner with an alternating order of animals. The mice in 2 and 6 months cohorts were perfused at 3 and 7 months respectively, at the completion of the behavioral testing.

2.2.5.1. Novel Object Recognition (NOR) Test:

The NOR test was conducted to observe and measure the short term memory in the mice. Each mouse was placed in the same open field arena of 47cm x 50cm x 30cm with 2 similar objects for 5 minutes. After a 3 minute break, each mouse was exposed to one old and a novel object and the activity was recorded for 3 minutes. The time (seconds) spent with each old and novel object was manually recorded for analysis (Jafari et al., 2017). The discrimination index (DI) was calculated by using the formula (time spent with novel object- time spent with old object)/total time spent with both novel and old objects) (Ennaceur & Delacour, 1988).

2.2.5.2. Morris Water Task (MWT):

The MWT task was performed to measure the spatial navigation abilities of the mice. Each mouse was placed in a 153cm diameter pool filled with water (23-25ºC). The pool was located in a room with distal cues and virtually subdivided into 4 quadrants with starting points at north, west, east, and south. A hidden platform was placed in one fixed quadrant and was submerged ~1.0 cm during all 8 training days. Non-toxic white tempura paint was added to the pool water to make the water opaque, so that the mice would not

have been able to see the platform. Each mouse was trained with 1 trial per day from each quadrant for 8 consecutive days (Water2100 Software vs.7, 2008). During each trial, the mouse was placed in the tank and each trial was stopped either once the mouse reached the platform, or if the mouse was unable to find the platform in 60 seconds. Data were recorded using an automated tracking system (HVS Image, Hampton, U.K.) and swim time (sec), swim speed (m/s), and swim distance (m) were calculated for analysis. On day 9, a probe triall was conducted, during which the platform was removed, and each mouse was allowed to swim freely for 60 seconds. For the analysis of the probe trial, the time spent in the quadrant where the platform was located during training days was measured. (Jafari et al., 2017).

2.2.5.3. Balance Beam (BB) Test:

The BB test was performed to measure the motor skills of each mouse. To conduct this test, the mice were trained to traverse across a 1 cm diameter, 100 cm long beam, which was 50 cm above a foam pad to cushion falling mice, to reach an escape box. On day 1, each mouse was trained for 3 successful trials. On day 2, the mouse's traverse activity was recorded for 3 trials and manually scored for the mean latency (sec), distance travelled (cm), and number of foot slips for analysis. (Jafari et al., 2018; Tamura et al., 2012).

2.2.5.4. Rotarod (RR) Test:

The RR test was performed to measure the motor skills and the strength of gait in each mouse. All the mice were trained to walk on an automated four lanes RR treadmill (ENV-575 M Mouse, Med Association Inc) on day 1. On day 2, each mouse was placed on the RR treadmill at 8rpm and 16rpm constant speed and at a 4-40 rpm alternating

speed and the time (sec) each mouse was able to stay on the RR treadmill was recorded for 3 trials (Brooks and Dunnett, 2009).

2.2.5.5. Elevated Plus Maze (EPM) Test:

The EPM is a measure of anxiety-like behaviour in mice. The EPM apparatus was constructed from black Plexi-glass and had two closed arms and two open arms. It was 40 cm high and two open arms were 5 cm wide and 27 cm long. The two closed arms were 10 cm wide, 40 cm long and had 40 cm high walls. Each mouse was placed in the center of the EPM facing the closed arms. A camera was set up above the maze to film each mouse for 5 minutes. Each mouse was manually scored for time spent in the open arms (seconds), time spent in closed arms (seconds), number of entries to open arms, and number of entries to closed arms. (Jafari et al., 2017). The EPM ratio was calculated by subtracting the number of entries to open arms from the number of entries to closed arms divided by the total number of entries to both open and closed arms (Jafari et al., 2018).

2.3. Results:

In both 2 and 6 month cohorts there were four different groups: APP, APP-TS, APP-GS, and APP-GS-TS in this experiment and the number of animals were used was consistently 115, in each behavioural test. None of the behavioural tests showed sex differences ($P \geq .05$). Two way ANOVA was done for 2 month and 6 month cohorts separately. The Bonferroni post-hoc test was used for each behavioural test, due to similar variance in each groups. Asterisks indicate $*P \leq 0.05$ or $**P \leq 0.01$ or $***P \leq 0.001$ and partial eta squared ($\eta 2$) indicates the effect size.

2.3.1. Novel Object Recognition (NOR) Test:

The APP-GS mice spent significantly less time with the novel object compared to all of the other experimental groups. TS significantly increased the novel object

exploration in both APP and APP-GS mice. The overall significant effects among all of the four groups of 2 months cohort were: novel object time (F (3, 57) = 10.623, P≤ .0001, Ƞ2= .376, power = .998) and discrimination index ratio (F (3, 57) = 13.718, P≤ .0001, Ƞ2= .437, power = 1.000). The overall significant effects among all of the four groups of 6 months cohort were: novel object time (F (3, 57) = 63.560, P≤ .0001, Ƞ2= .806, power = 1.000), old object time (F (3, 57) = 12.861, P≤ .0001, Ƞ2= .456, power = .998), and DI ratio (F (3, 57) = 14.429, P≤ .0001, Ƞ2= .485, power = 1.000).

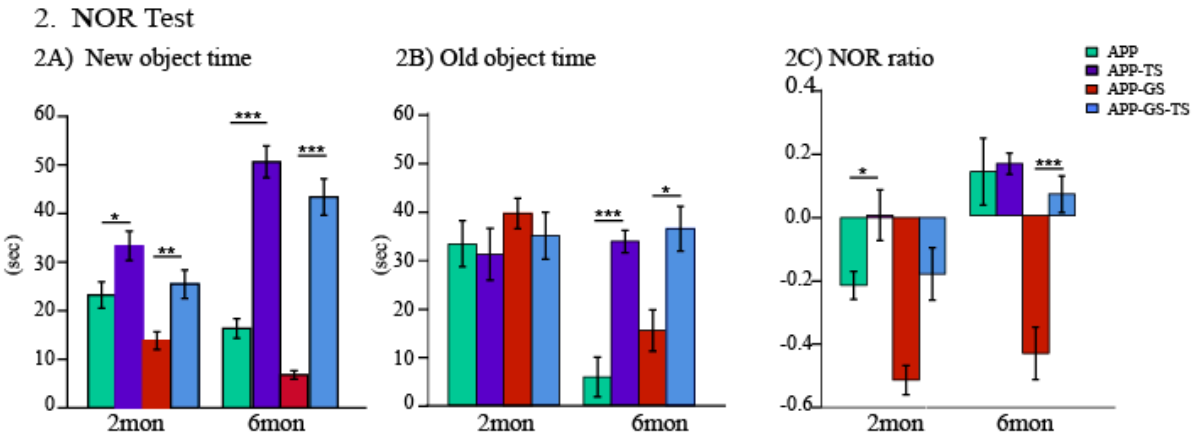

Fig 2: 2A. The APP-TS group in both 2 and 6 month cohorts spent the highest time (sec) with the new object. 2B. The time spent with the old object was not significantly different in the 2 month cohort, but the APP group spent significantly less time with old object in the 6 month cohort. 2C. In both 2 and 6 month cohorts APP-GS mice showed lower preference for new object.

In the 2 month cohort, the novel object time was significantly higher in APP-TS mice compared to APP (F (1, 26) = 5.972, P = .022, Ƞ2= .199, power = .650) and in APP_GS_TS mice compared to APP-GS (F (1, 26) = 12.209, P = .002, Ƞ2= .296, power = .922). Similarly, the DI ratio was significantly higher in APP-TS mice compared to APP (F (1, 26) = 5.849, P = .024, Ƞ2 = .196, power = 641) at 2 months. No significant differences were observed in the old object exploration time among all of the groups at 2

months. A Bonferroni post-hoc analysis revealed that the APP-TS group spent significantly more time with the novel object in comparison with APP-GS (P≤ .0001), and APP-GS group spent a significantly less time with novel object in comparison with APP-TS (P≤ .0001), and APP-GS-TS (P = .013) in the 2 month cohort. No significant difference was revealed from a Bonferoni post hoc analysis in the time spent with old object among the groups. In addition, the DI ratio was highest in the APP-TS mice in comparison with APP-GS (P≤ .0001) and was lowest in the APP_GS in comparison with APP (P = .005), APP-TS (P≤ .0001), and APP-GS-TS (P = .002) as per Bonferoni post hoc analysis in the 2 month cohort.

In the 6 month cohort, the novel object time was significantly higher in APP-TS mice compared to APP ($F(1, 25) = 85.778$, P≤ .0001, $\eta 2 = .789$, power = 1.000) and in APP-GS_TS mice compared to APP-GS ($F(1, 25) = 90.527$, P≤ .0001, $\eta 2 = .797$, power = 1.000). In contrast, the old object time was significantly lower in APP-TS mice compared to APP ($F(1, 25) = 33.046$, P≤ .0001, $\eta 2 = .590$, power = 1.000) and in APP-GS-TS mice compared to APP-GS ($F(1, 25) = 6.532$, P = .018, $\eta 2 = .221$, power = .687) in the 6 month cohort. In addition, the DI ratio was significantly higher in APP-GS-TS compared to APP-GS ($F(1, 25) = 25.005$, P≤ .0001, $\eta 2 = .521$, power = .998), but not significantly different between APP and APP-TS in the 6 month cohort. A Bonferoni post-hoc analysis revealed that the APP-TS group spent significantly more time with the novel object in comparison with APP (P≤ .0001) and the APP_GS (P≤ .0001) and APP-GS group spent significantly less time with the novel object in comparison with APP-TS (P≤ .0001) and APP-GS_TS (P≤ .0001) in 6 the month cohort. Surprisingly, APP-TS spent the highest amount of time exploring the old object in comparison with APP (P≤ .0001) and APP-GS (P = .006). The APP mice spent the lowest amount of time with old

object compared to APP-TS (P≤ .0001) and APP-GS-TS (P≤ .0001) as per Bonferoni post hoc analysis. In addition, the DI ratio was highest in the APP-TS mice in comparison with APP-GS (P≤ .0001) and was lowest in the APP-GS in comparison with APP (P≤ .0001), APP-TS (P≤ .0001), and APP-GS-TS (P≤ .0001) as per Bonferoni post hoc analysis in the 6 month cohort.

2.3.2. Morris Water Task (MWT):

The APP-GS mice were significantly slower to locate the sub-merged platform, demonstrated a higher swim distance, and a reduced probe time in the target quadrant compared to all of the other groups. TS significantly improved the performances on all three measures for both of the APP and APP-GS mice.

The overall significant effects among all of the four groups of 2 months cohort were: latency ($F(3, 57) = 17.648$, $P \leq .0001$, $\eta 2 = .500$, power = 1.000), swim distance ($F(3, 57) = 8.551$, $P \leq .0001$, $\eta 2 = .521$, power= 1.000), and probe time ($F(3, 23) = 6.532$, $P = .001$, $\eta 2 = .270$, power= .961). The overall significant effects among all of the four groups of 6 months cohort were: latency ($F(3, 50) = 32.647$, $P \leq .0001$, $\eta 2 = .680$, power = 1.000), swim distance ($F(3, 50) = 42.970$, $P \leq .0001$, $\eta 2 = .737$, power = 1.000), and probe time ($F(3, 50) = 10.198$, $P \leq .0001$, $\eta 2 = .399$, power = .997).

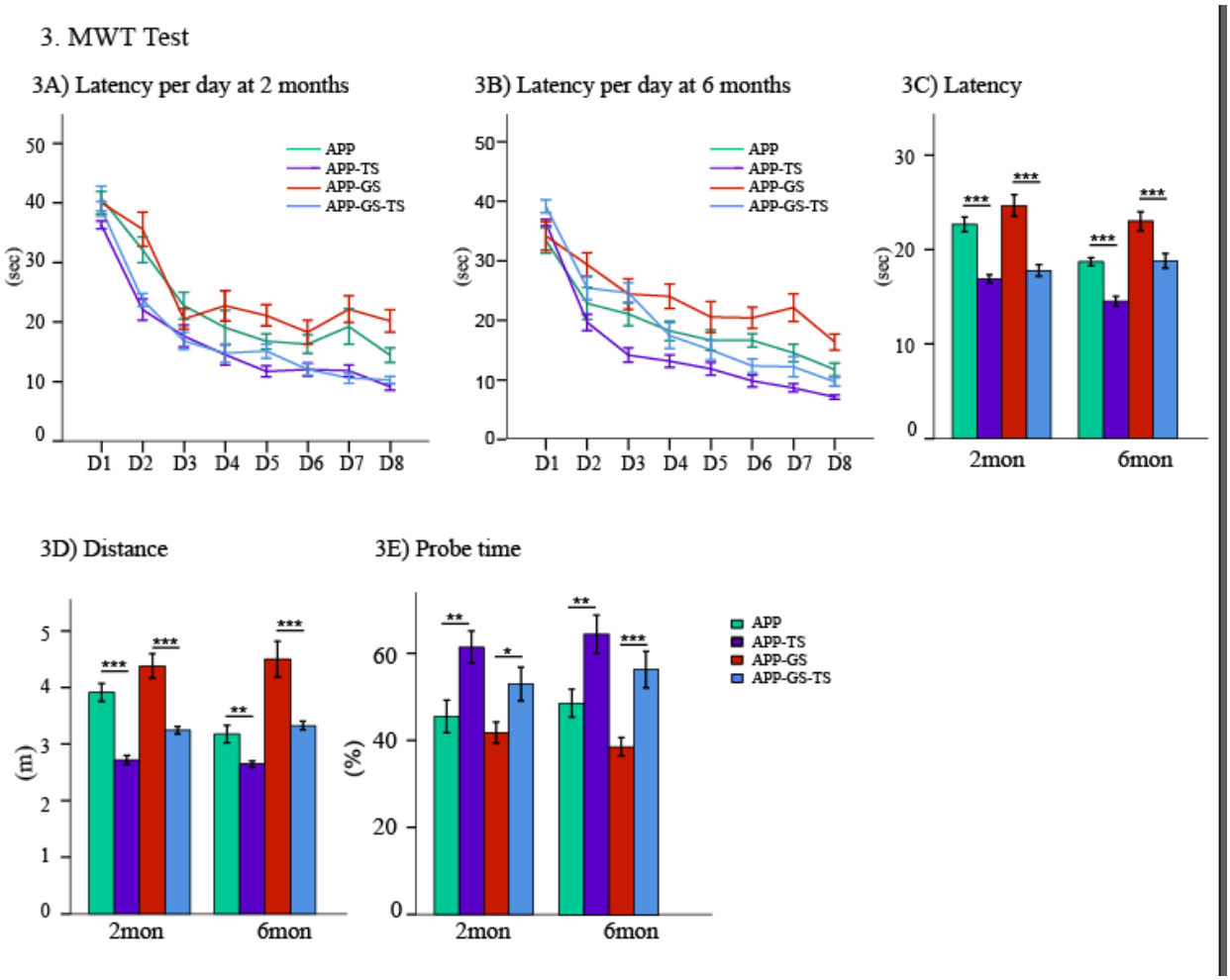

Fig 3: 3A & 3B. The progression of swim latency from Day 1-8 in both 2 and 6 months cohorts. 3C. Swim latency was longer in APP-GS mice in both 2 and 6 months cohorts. 3D. Swim distance was significantly higher in the APP-GS mice in both 2 and 6 months cohorts. 3E. Probe time was significantly longer in APP-TS in both the 2 and 6 month cohorts.

In the 2 month cohort, the swim latency during training days was significantly decreased in the APP-TS mice compared to APP ($F(1, 26) = 42.993$, $P \leq .0001$, $\eta 2 = .642$, power = 1.000) and in APP-GS-TS compared to APP-GS ($F(1, 31) = 19.526$, $P \leq .0001$, $\eta 2 = .402$, power = .990). Similarly, the swim distance during the training days also was decreased significantly in the APP-TS mice relative to APP ($F(1, 26) = 40.900$,

P≤ .0001, Ƞ2 = .630, power = 1.000) and in APP-GS-TS compared to APP-GS (F (1, 31) = 14.735, P≤ .001, Ƞ2 = .390, power = .957). In addition, during the probe day, the amount of time spent in the target quadrant was significantly higher in the APP-TS mice compared to the APP (F (1, 26) = 8.404, P =.008, Ƞ2 = .259, power = .794), in the APP-GS-TS compared to APP-GS (F (1, 31) = 6.365, P = .017, Ƞ2 = .337, power = .684). A Bonferroni post hoc analysis revealed that the APP-TS mice took significantly less time to locate the hidden platform during training days in comparison with APP (P = .001) and APP-GS (P≤ .0001) and APP-GS took significantly more time than the APP-TS (P≤ .0001) and APP-GS-TS (P≤ .0001); APP-TS mice swam the least distance compared to APP (P≤ .0001) and APP-GS (P≤ .0001) and APP-GS (P≤ .0001) swam the longest distance compared to APP-TS(P≤ .0001) and APP-GS-TS(P≤ .0001); APP-TS mice spent the highest amount of time in the target quadrant during the probe test compared to APP (P = .016) and APP-GS (P =.001) and APP-GS mice spent the least amount of time in the target quadrant compared to APP-TS (P = .001).

In the 6 month cohort, during training days the swim latency was significantly decreased in the APP-TS mice compared to APP (F (1, 25) = 39.476, P≤ .0001, Ƞ2 = .632, power = 1.000) and in APP-GS-TS compared to APP-GS (F (1, 25) = 21.243, P≤ .0001, Ƞ2 = .480, power = .993). Similarly, the swim distance during the training days was also significantly decreased in the APP-TS mice relative to APP (F (1, 25) = 14.735, P = .001, Ƞ2 = .390, power = .957) and in APP-GS-TS compared to APP-GS (F (1, 25) = 37.571, P≤ .0001, Ƞ2 = .620, power = 1.000). In addition, during the probe day, the amount of time spent in the target quadrant was significantly higher in the APP-TS mice compared to the APP (F (1, 25) = 8.222, P = .009, Ƞ2 = .263, power = .784), in the APP-GS-TS compared to APP-GS (F (1, 25) = 17.142, P≤ .0001, Ƞ2 = .427, power = .977).).

A Bonferoni post hoc analysis revealed that the APP_TS mice took significantly less time to locate the hidden platform during training days in comparison with APP (P≤ .0001), APP-GS (P≤ .0001), and APP-GS-TS (P≤ .0001) and APP-GS took significantly more time than the APP (P≤ .0001), APP-TS (P≤ .0001) and APP-GS-TS (P≤ .0001); the APP-TS mice swam the least distance compared to the APP-GS (P≤ .0001) and APP-GS-TS (P = .012) and APP-GS mice swam the longest distance compared to APP (P≤ .0001) APP-TS (P≤ .0001) and APP-GS-TS (P≤ .0001); APP-TS mice spent the highest amount of time in the target quadrant during the probe test compared to APP (P =.015) and APP-GS (P≤ .0001) and APP-GS mice spent the least amount of time in the target quadrant compared to APP-TS (P≤ .0001) and APP-GS-TS (P = .005).

2.3.3. Balance Beam (BB) Test:

The APP-GS mice were significantly slower to traverse the beam and made more slips than all of the other groups at both 2 and 6 months. TS significantly improved performance on both measures for both APP and APP-GS group. The overall significant differences among all the four groups in latency at 2 months is F (3,57)= 8.289, P≤ .0001, Ƞ2 = .319, power = .989; at 6 month is F (3,50)= 21.543, P≤ .0001, Ƞ2 = .584, power = 1.000 and in number of foot slips at 2 months is F (3,57)= 2.955, P = .041, Ƞ2 = .143, power = .668; at 6 months is F (3,50)= 31.003, P≤ .0001, Ƞ2 = .669, power = 1.000.

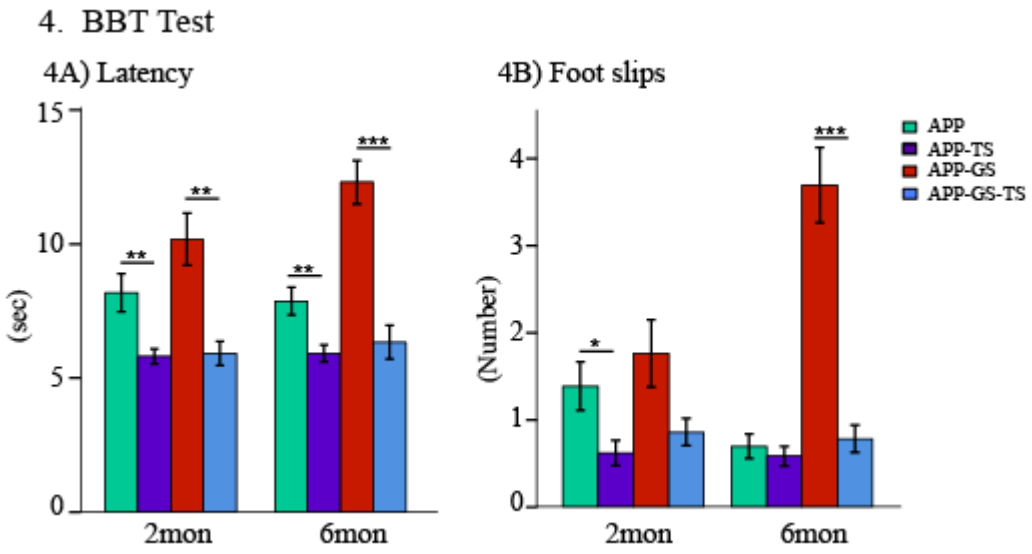

Fig 4: 4A. The APP-TS mice were the fastest and APP-GS mice were the slowest in latency in both 2 and 6 month cohorts. 4B. The APP-TS mice exhibited a decreased number of foot slips, whereas APP-GS exhibited an increased number of foot slips in both 2 and 6 month cohorts.

At 2 months, APP-TS mice took significantly less time to cross the beam ($F(1, 26) = 9.35$, $P = .005$, $\eta 2 = .280$, power = .835) and exhibited a reduced number of foot slips ($F(1, 26) = 5.240$, $P = .031$, $\eta 2 = .173$, power = .594) compared to APP group. Similarly, the APP-GS-TS group took significantly less time to cross the beam ($F(1, 31) = 11.427$, $P = .002$, $\eta 2 = .283$, power = .904), and exhibited a reduced but not significant reduced number of foot slips ($F(1, 31) = 3.296$, $P = .080$, $\eta 2 = .102$, power = .419) relative to APP-GS mice at 2 months. A Bonferroni post-hoc analysis revealed that the APP_GS group took the longest time to cross the beam relative to APP-GS-TS ($P = .001$), and APP-TS ($P \leq .0001$), and had the highest number of foot slips relative to APP-GS ($P = .046$) at 2 months.

At 6 months, APP-TS mice took significantly less time to cross the beam (F (1, 25) = 9.684, P = .005, Ƞ2 = .296, power = .846) compared to APP group, but there was no difference in the number of foot slips (F (1, 25) = 0.639, P = .432, Ƞ2 = .027, power = .119). Similarly, the APP-GS-TS group took significantly less time to cross the beam (F (1, 25) = 26.156, P≤ .0001, Ƞ2 = .532, power = .998), and exhibited a significantly reduced number of foot slips (F (1, 25) = 28.868, P≤ .0001, Ƞ2 = .557, power = .999) relative to APP-GS mice at 6 months. At 6 months the APP-GS group took the longest time to traverse the beam relative to APP (P≤ .0001), APP-TS (P≤ .0001), and APP-GS-TS (P≤ .0001), and had a significantly a lower number of foot slips compared to APP (P≤ .0001), APP-TS (P≤ .0001), and APP-GS-TS (P≤ .0001) mice as per Bonferroni post hoc analysis.

2.3.4. Rotarod (RR) test:

The APP-TS group showed significantly better performance among all of the groups in all 8 rpm, 16 rpm, and 4-40 rpm at both 2 and 6 months age, but the 4-40 rpm at 2 months. TS significantly improved the RR performances in both APP and APP-GS mice at both at 2 and 6 months.

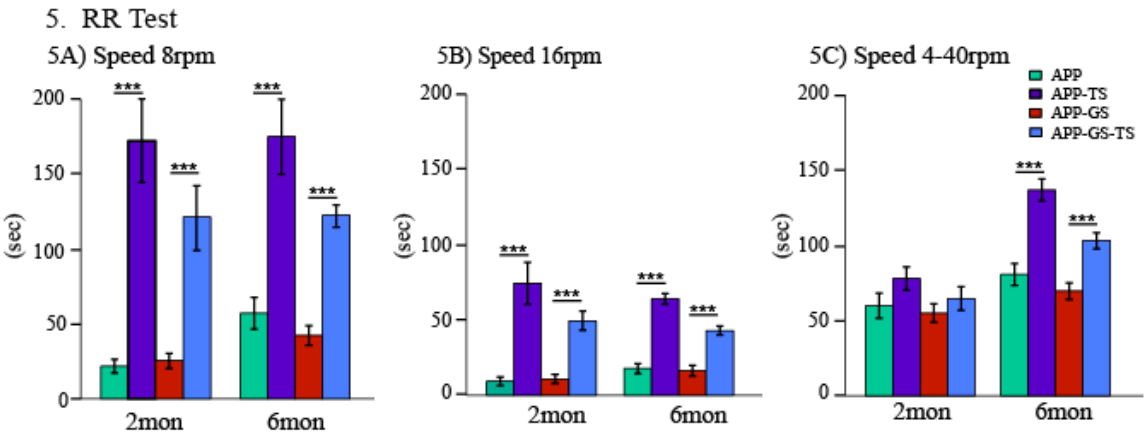

Fig 5: 5A. An increased time spent with at (8 rpm) speeds in APP-TS mice of both 2 and 6 months cohorts. 5B. An increased time spent at high (16 rpm) speeds in APP-TS mice of both 2 and 6 months cohorts. 5C. There was no significant difference in the time spent at alternating (4-40 rpm) speeds among the groups in 2 month cohort, whereas APP-TS spent highest time in 6 month cohort.

At 2 months, the overall statistically significant effects in all four groups are at 8 rpm: $F(3, 57) = 20.214$, $P \leq .0001$, $\eta 2 = .534$, power = 1.000; at 16 rpm: $F(3, 57) = 16.991$, $P \leq .0001$, $\eta 2 = .490$, power = 1.000; and at 4-40 rpm: $F(3, 57) = 1.688$, $P = .181$, $\eta 2 = .087$, power = .417. Similarly, at 6 months the overall statistically significant effects among the groups are at 8 rpm: $F(3, 50) = 17.069$, $P \leq .0001$, $\eta 2 = .527$, power = 1.000; at 16 rpm: $F(3, 50) = 41.693$, $P \leq .0001$, $\eta 2 = .731$, power = 1.000; and at 4-40 rpm: $F(3, 50) = 18.794$, $P \leq .0001$, $\eta 2 = .551$, power = 1.000.

In the 2 month cohort, compared to APP mice, APP-TS group showed significantly improved performance in RR speeds, i.e., 8 rpm: $F(1, 26) = 25.407$, $P \leq .0001$, $\eta 2 = .514$, power = .998; 16 rpm: $F(1, 26) = 19.238$, $P \leq .0001$, $\eta 2 = .445$, power = .998. Similarly a pairwise comparison revealed that APP-GS-TS mice exhibited improved performances in all three RR speeds, i.e., 8 rpm: $F(1, 31) = 31.917$, $P \leq .0001$, $\eta 2 = .524$, power = 1.000; 16 rpm: $F(1, 31) = 34.448$, $P \leq .0001$, $\eta 2 = .543$, power = 1.000 relative to APP-GS group. A Bonferroni post-hoc analysis revealed that the APP-TS group showed significantly improved performances among all the groups in two RR speeds, i.e., 8 rpm: APP ($P \leq .0001$) and APP-GS ($P \leq .0001$); 16 rpm: APP ($P \leq .0001$), APP-GS ($P \leq .0001$) at 2 months.

At 6 months, a pairwise comparisons revealed that APP-TS mice showed improved performances in all three RR speeds, i.e., 8 rpm: $F(1, 25) = 18.343$, $P \leq .0001$, $\eta 2 = .444$, power = .984; 16 rpm: $F(1, 25) = 92.328$, $P \leq .0001$, $\eta 2 = .801$, power =

1.000; and 4-40 rpm: F (1, 25) = 36.372, P≤ .0001, Ƞ2 = .534, power = .998 compared to APP. Similarly, APP-GS_TS mice exhibited improved performances in all three RR speeds, i.e., 8 rpm: F (1, 25) = 60.218, P≤ .0001, Ƞ2 = .724, power = 1.000; 16 rpm: F (1, 25) = 25.708, P≤ .0001, Ƞ2 = .528, power = .998; and 4-40 rpm: F (1, 25) = 18.377, P≤ .0001, Ƞ2 = .444, power = .984 relative to APP-GS at 6 months. At 6 months the APP-TS mice exhibited significantly improved performances in all three RR speeds, i.e., 8 rpm: APP (P≤ .0001) and APP-GS (P≤ .0001); 16 rpm: APP (P≤ .0001), APP-GS (P≤ .0001); 4-40 rpm: APP (P≤ .0001) and APP-GS (P≤ .0001) as per Bonferroni post-hoc analysis.

2.3.5. Elevated Plus Maze (EPM) test:

The APP-TS mice were significantly less anxious and spent significantly more time in the open arms, less time in the closed arms compared to the other experimental groups. TS reduced the anxiety like behavior in both APP and APP-GS mice. At 2 months, the overall significant effects among all of the four groups were: open arm time (F (3, 57) = 3.146, P = .033, Ƞ2 = .151, power = .699), closed arm time (F (3, 57) = 26.577, P≤ .0001, Ƞ2 = .601, power = 1.000), and EPM ratio (F (3, 57) = 1.731, P = .172, Ƞ2 = .089, power = .427). At 6 months, the overall effects among all of the groups were: open arm time (F (3, 50) = 54.430, P≤ .0001, Ƞ2 = .601, power = 1.000), closed arm time (F (3, 50) = 60.348, P≤ .0001, Ƞ2 = .797, power = 1.000), and EPM ratio (F (3, 50) = 38.645, P≤ .0001, Ƞ2 = .720, power = 1.000).

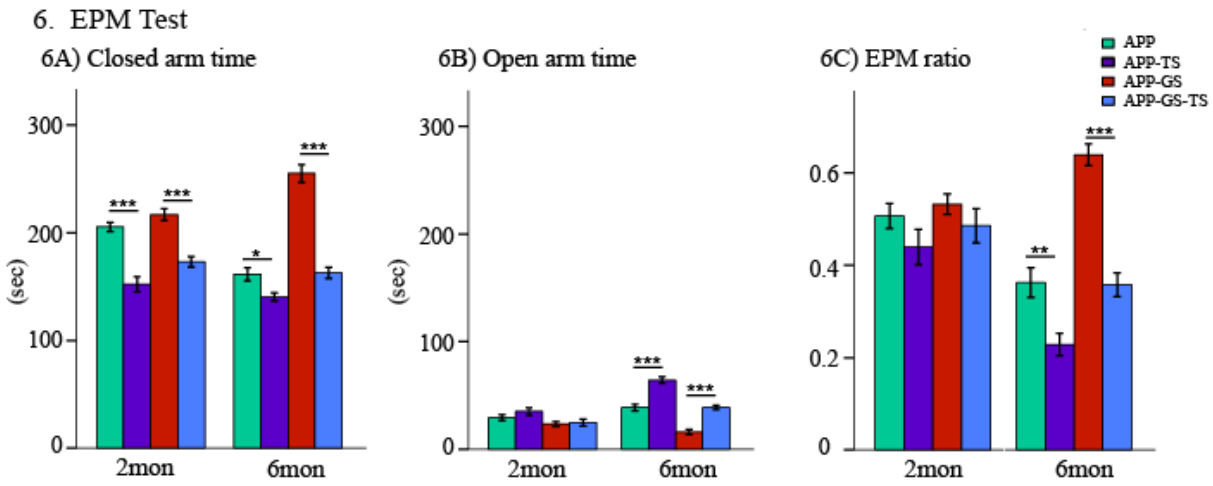

Fig 6: 6A. Shows significantly shorter closed arms time in APP-TS mice in both 2 and 6 months cohorts. 6B. Shows significantly longer open arms time in APP-TS mice only in 6 months cohorts. 6C. Shows highest EPM ratio for closed arms entries in APP-GS only in 6 months cohort.

A pairwise comparison of the 2 month cohort revealed that the closed arm time was significantly higher in APP mice compared to APP-TS ($F(1, 26) = 37.225$, $P \leq .0001$, $\eta 2 = .608$, power = 1.000), and in APP-GS mice relative to APP-GS-TS ($F(1, 26) = 29.931$, $P \leq .0001$, $\eta 2 = .508$, power = 1.000). No significant effects were observed among the groups in either open arm time or EPM ratio at 2 months. Similarly, in the 6 month cohort the open arm time was significantly higher in APP-TS mice compared to APP ($F(1, 25) = 39.539$, $P \leq .0001$, $\eta 2 = .632$, power = 1.000) and in APP-GS-TS mice relative to APP-GS ($F(1, 25) = 38.167$, $P \leq .0001$, $\eta 2 = .624$, power = 1.000). The closed arm time was significantly lower in APP-TS mice relative to APP ($F(1, 25) = 7.675$, $P = .011$, $\eta 2 = .250$, power = .756) and in APP-GS-TS mice compared to APP-GS ($F(1, 25) = 64.640$, $P \leq .0001$, $\eta 2 = .712$, power = 1.000) at 6 months. In addition, the EMP ratio was significantly lower in APP-TS mice compared to APP ($F(1, 25) = 9.791$, $P = .005$, $\eta 2 =$

.299, power = .850) and in APP-GS-TS relative to APP-GS (F (1, 25) = 54.353, P≤ .0001, Ƞ2 = .715, power = 1.000) group at 6 months.

A Bonferroni post-hoc analysis revealed that APP-TS mice spent significantly highest time in the open arms compared to APP-GS (P = .021) and significantly lowest time in the closed arms compared to APP (P≤ .0001) and APP-GS (P≤ .0001) in the 2 month cohort. However, in the 6 month cohort APP-TS mice spent significantly longer time in the open arms relative to APP (P≤ .0001), APP-GS (P≤ .0001), and APP-GS-TS (P≤ .0001). In contrast, at 6 months APP-GS spent the most amount of time in the closed arms relative to APP (P≤ .0001), APP-TS (P≤ .0001), and APP-GS-TS (P≤ .0001) as per Bonferroni post-hoc analysis. In addition, at 6 months the EPM ratio was significantly lowest in the APP-TS mice compared to APP (P = .007), APP-GS (P≤ .0001), and APP-GS-TS (P = .019) groups, but was significantly highest in the APP-GS mice relative to APP (P≤ .0001), APP-TS (P≤ .0001), and APP-GS-TS (P≤ .0001) group as per Bonferroni post-hoc analysis.

2.4. Discussion:

The key findings of this study were: 1) early TS mitigates the adverse effect of GS; and, 2) early TS improves the AD-like symptoms in adult offspring at 2 and 6 months. We consider each finding in turn.

2.4.1. Early TS mitigates the adverse effect of GS in the adult offspring at both 2 and 6 months:

GS has detrimental influence on brain and behavioural development. As per the previous publications on the effect of GS on adult offspring in our lab, it has been well demonstrated that GS accelerates the progression of Aβ plaque deposition in adult APP offspring mice and the impaired cognition, motor, anxiety-like behaviours are associated

with accumulation of Aβ plaque in the brain (Jafari et al., 2017; 2018; 2019). The behavioural findings from this study also demonstrate the similar deficits in cognition, motor, and anxiety-like behaviours as a result of GS in the adult offspring. In this project we applied early TS as a therapeutic intervention with a goal of reversing the effect of GS in adult APP offspring. Results suggest it was successful.

GS influences the HPA and HPG axes and these two axes also modify the function of each-other (Toufexis et al. 2014). GS elevates the corticosterone levels (Jafari, Mehla, Afrashteh, et al., 2017) in dams, which initiates a long-lasting dysfunction in the HPA negative feedback loop. Elevated corticosterone in the dams influences the level of corticosterone in the offspring and adversely impacts their brain and social stability (Saavedra-Rodriguez & Feig, 2013). Stress during pregnancy also affects the prefrontal cortex and hippocampus development of offspring and the alterations of these are associated with reduced spatial and cognitive functions (Mychasiuk, Gibb, & Kolb, 2011). As a result, the offspring also exhibit impaired cognition and motor skills and anxiety-like behaviors as adults. In our study, the APP mice that received GS spent less time in the open arms and had less number of entries to the open arms of the maze compared to the APP mice. In addition, APP-GS mice showed less exploratory behaviour towards the novel object. Both APP and APP-GS groups that received TS exhibited improved performances in both EMP and NOR tests. Mice that received TS showed less anxiety and more interest in novel object exploration. GS also impaired cognitive and motor skills in adult offspring. The results also demonstrated that TS improved the performances of adult offspring in the MWT, BB, and RR test. Research on rats demonstrated that TS early in life reduced the corticosterone level in infants (Jutapakdeegu et al., 2003). Studies in rats showed that TS released FGF-2 (Gibb, 2004),

which crosses the blood brain barrier and stimulated neurogenesis. TS also increases BDNF, which enhances the proliferation and protection of neurons in the Hpc, and perhaps other brain regions as well. Hence, TS during earlier life stages plays a critical role in neuroplasticity in later in life.

Surprisingly, we observed increased old object time in the NOR test in both of the APP and APP-GS offspring that received TS. It may be a result of enhanced exploration and activity in mice that received TS. Another important observation was that the effect of TS was stronger in the 6 month cohort than in the 2 month cohort because the earliest onset of AD-like symptoms and pathology is at about 3 months in this APP mouse model (Jafari et al., 2017).

The APP strain is a mouse model of AD, which will develop AD-like symptoms and pathology with an early onset of AD at the age of 2-3 months. The symptoms of AD in the APP mice will decline as the disease progresses with increased age. However, the application of TS during the earlier stages of their life plays such an important role, by prolonging the onset of AD and also improving exploratory, spatial, and anxiety-like behaviours in both 2 and 6 month adult offspring.

2.4.2. Early TS improves the AD-like symptoms in adult offspring at 2 and 6 months:

Impaired learning and memory along with deterioration in motor skills and elevated anxiety are common symptoms of AD in humans. A series of studies in our lab have shown the similar deficits in APP mice (Jafari, et al., 2017, 2018, 2019; Mehla et al., 2019; Karem et al., 2019). In addition, the declined cognitive and motor skills are associated with the formation of Aβ plaques and with age as the formation of Aβ plaques increases with age in these APP mice (Jafari et al., 2018; Mehla, et al., 2019). The goal of

this study was to see whether application of early TS would influence learning and memory, motor, and anxiety-like behaviours in adult APP offspring at 2 and 6 months. The results from the NOR and MWT tests suggest that TS improves the cognition in the mice that received TS. In the NOR test, APP mice that received TS demonstrated higher preference for the novel object. Similarly, in the MWT test the APP mice that received TS displayed significantly shorter latency and swim distance during the training days, and significantly longer probe time, suggesting improved spatial learning and memory (Angeles et al., 2016; Kolb & Gibb, 2010).

The positive impact of TS also observed in motor skills among APP adult offspring at both 2 and 6 months. In the BB test, the APP mice that received TS were faster to traverse the beam and had fewer slips. Similarly, in the RR test APP mice that received TS were able to stay longer time in the rotating rod in all three different speeds at both 2 and 6 months. The findings from both BB and RR tests suggesting the improvements in motor balance and coordination in APP mice as a result of early TS. Research in APP mice has also shown that age plays a vital factor in deteriorating AD-like symptoms in this APP mice and at 6 months the motor deficits are worse (Jafari et al., 2018). In this study, we showed that early TS prevents the motor skills even at 6 months of age in APP mice.

In addition, TS positively influenced anxiety-like behaviour and was also beneficial in this APP mice. In the EPM test, the mice that received TS spent the highest time in the open arms and exhibited the highest EPM ratio for the open arms. These results suggest that early TS helped to reduce the anxiety-like behaviour in the APP mice, and these findings were more significant at 6 months. The mechanisms of the effects of TS on both AD and stress are not firmly established and likely are multiple. Further investigation will

be performed on the brain anatomy such as hippocampal volume and size and numbers of Aβ plaques and corticosterone hormone level.

2.5. Conclusion:

The application of TS in adult APP mice has shown that TS improves AD-like symptoms and pathology in adult APP mice. In this study we successfully implemented the beneficial impact of early TS in APP adult offspring. In addition we were able to show that early TS helped to triumph the adverse effect of gestational auditory stress in APP adult offspring as well. These results suggest that TS has a preventative mechanism in this APP mice. Further research is required to discover the neural mechanism regarding change in the gene expression, electrophysiology, neurotransmitters, FGF-2, and synapses as a result of early TS.

Chapter 3: Tactile Stimulation Improves Cognition, Motor, and Anxiety-Like Behaviours and Attenuates the AD Pathology in Adult APP $^{NL-G-F}$ mice.

3.1. Introduction:

Dementia is a major current and future health threat in the world. According to Alzheimer's Disease International, every 3 seconds one more individual is diagnosed with dementia. If the incidence of dementia continues at the same rate, by 2050 there will be about 131.5 million cases worldwide. Dementia does not have just a negative impact on physical and psychological, social, and economic status in patients, but also in the life of patients, families, and caregivers also. The cost of dementia was more than US $1 trillion worldwide in 2018 (Alzheimer's Disease International, 2019). Alzheimer's disease (AD) is the most common form of dementia which consists of about 60-70% of dementia cases (WHO, 2019).

AD is a neurodegenerative brain disorder that causes cognitive and motor skills deficits. Along with these deficits, an AD patient's symptoms are preceded by lack of motivation and deteriorated emotional and social behaviour. These behavioural symptoms are associated with the formation of extracellular Aβ plaques and intracellular tau phosphorylated proteins (Marcello et al., 2015), shrinkage of the cerebral cortex, hippocampus (Hpc), and basal ganglia (Pini et al., 2016), reduction of acetylcholine (Fischer et al., 1989), synaptic loss (Hamos et al., 1989), and disrupted gamma oscillations (Iaccarino et al., 2016) in the brain. An extensive amount of research shows that the olfactory area is one of the first brain regions affected by AD (Thomann et al., 2009). Therefore, a patient with AD exhibits lack of appetite (Sheard, 2014), which may result in a lack of nutrients received by their brain and body and may play a role in neural atrophy (Lange, et al., 2019). In this study we emphasize the two hallmarks: 1) the size

and numbers of Aβ plaques and the hippocampal volume of AD pathology; and, 2) the cognitive, motor, and anxiety-like behavior.

Tactile, auditory, visual, and olfactory sensory stimulation has been proposed as a treatments for neurological disorders like AD, Parkinson, epilepsy, schizophrenia, and drug addiction. The benefits of these rehabilitation strategies are: 1) they are non-invasive; 2) they are cost effective; and, 3) they are easily translatable from preclinical studies to human clinical trials. For the purpose of this study, we focused on the beneficial effects of tactile stimulation (TS) in treating AD. Forms of TS ranging from skin-skin contact for new born infants, to gentle massage therapy for adults, have been proven to be beneficial for infant brain development and recovery from adult injury respectively (Gibb, Gonzalez, Wagenest, & Kolb, 2010). The receptors at the end of hair follicle, the dendrites in corpuscles in dermal and epidermal regions, produce action potentials as a haptic response from TS. In addition, application of TS may also influence the peripheral nervous system (PNS), activating many endogenous mechanisms. Although the mechanism of TS in brain plasticity is not yet well understood, research shows that TS releases fibroblasts growth factor-2 (FGF-2) (Comeau et al., 2007), which crosses the blood brain barrier (BBB), and helps with neurogenesis, repair of nerve cells, cellular proliferation, survival, migration, and differentiation. A large body of research on premature infants shows that application of TS accelerates physical growth and improves cognitive and motor functions (Field et al., 1986). Further studies in Romanian and Chilean orphans showed that lack of body contact between caregivers and infants was one of the vital reason for cognitive and motor deficits in those infants (Herreros, 2013).

It is well established that TS has the strongest positive impact on cognitive and motor functions when it is applied during the early infantile period, and these behavioral

improvements are associated with the increased FGF-2 (Comeau et al., 2007), acetylcholine (Dudar et al., 1979), and synaptic plasticity (Kolb & Gibb, 2010, 2011) in the brain. A key question from a health perspective is whether TS will have a similar effect when it is applied during adulthood? If it does, then TS could be a suitable treatment option for diseases like AD. In this study, we aimed to assess cognitive, motor, and anxiety-like behaviours, and AD-like pathology such as Aß plaques and hippocampal volume to determine the effect of TS on adult APPNL-G-F/NL-G-F mice, a mouse model of AD. Our prediction was that TS would enhance the cognitive, motor, and anxiety-like behaviours, and that these improvements would be associated with reduced Aß plaques and increased hippocampal volume.

3.2. Methods and Materials:

3.2.1. Animals:

Mice were housed in Canadian Center for Behavioral Neuroscience (CCBN) vivarium, and all the behavioral, brain anatomical and physiological tests and analyses were approved by the University of Lethbridge Animal Welfare Committee. APPNL-G-F/NL-G-F (amyloid β-protein precursor), AD transgenic mice carrying Swedish (NL), Arctic (G), and Beyreuther/Iberian (F) mutations (Saito et al., 2014) provided by RIKEN Brain Science Institute were used in this research project. Nine females and six male APPNL-G-F/NL-G-F (APP) adult transgenic mice, and six female and 6 male C57BL/6J (C57) were used in this project. All mice were given access to food and water ad libitum by the animal care staffs. The mice were maintained on a 12 hour light and 12 hours dark cycle in a 21ºC temperature controlled room in the vivarium. All training and behavioral testing were performed by the same experimenter during the light phase.

3.2.2. Experimental Design:

Mice from both APP and C57 strains were randomly assigned to four groups consisting of APP with tactile stimulation (APP-TS) group, APP without tactile stimulation (APP-NTS), C57BL/6J with tactile stimulation (C57-TS) group, and C57BL/6J without tactile stimulation (C57-NTS) group as per the following table. Each group consists of a minimum 3 male and 3 female mice.

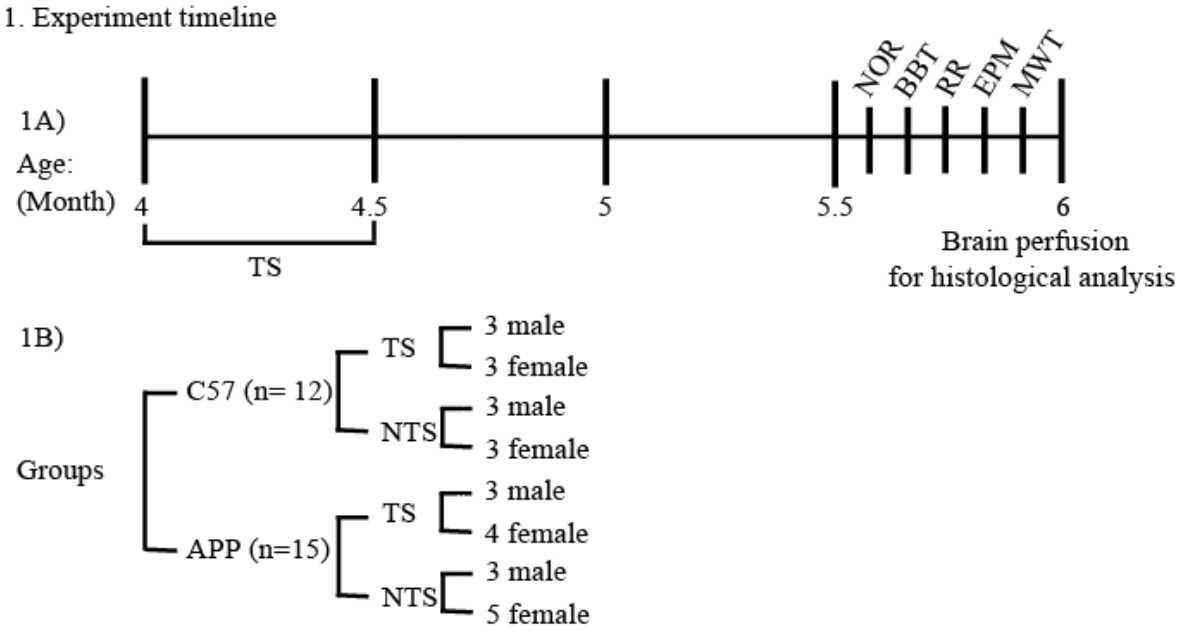

Fig 1: 1A. Shows the experiment timeline in months (age of mouse). 1B. Shows the number of groups and number of male and female APP mice in each group.

3.2.3. Tactile Stimulation Procedure:

All the mice were handled for 5 minutes twice a day for 5 days. Only the TS groups of APP and C57 mice received manual TS at 4 months of age by lightly massaging each mouse with an experimenter's fingers for 15 minutes with a frequency of 3 times a day for 15 days (8 am, 12 pm, and 4 pm). We applied TS when the APP mice

were 4 months old, because the earliest onset of Aβ plaque formation in APP mice is ~3 months of age (Jafari et al., 2017; Mehla et al., 2019). At 6 months the Aβ plaque formation is completely saturated in the brain and deficits in cognition, motor and anxiety-like behaviour are also associated with the Aβ plaque formation. Therefore, we decided to apply TS at 4 months to find out if TS improves cognition, motor, and anxiety-like behaviours at 5.5 months and Aβ plaque formation at 6 months of age.

3.2.4. Behavioral Tests:

Several behavioral tests were performed at 5.5 months to measure the effect of TS on cognitive and motor functions. The balance beam (BB), rota-rod (RR), novel object recognition (NOR), activity box (AB), elevated plus maze (EPM), and the Morris water task (MWT) were conducted respectively by the same examiner with an alternating order of animals.

3.2.4.1. Novel Object Recognition (NOR) Test:

The NOR test was conducted to observe and measure the short term memory in the mice. Each mouse was placed in the same open field arena of 47cm x 50cm x 30cm with 2 similar objects for 5 minutes. After a 3 minute break, each mouse was exposed to one old and one novel object and the activity was recorded for 3 minutes. The time (seconds) spent with each old and novel object was manually recorded for analysis (Jafari et al., 2017). The discrimination index (DI) was calculated by using the formula (time spent with novel object- time spent with old object)/total time spent with both novel and old objects) (Ennaceur & Delacour, 1988).

3.2.4.2. Morris Water Test (MWT):

The MWT task was performed to measure the spatial navigation abilities of the mice. Each mouse was placed in a 153cm diameter pool filled with water (23-25ºC). The

pool was located in a room with distal cues and virtually subdivided into 4 quadrants with starting points at north, west, east, and south. A hidden platform was placed in one fixed quadrant and was submerged ~1.0 cm during all 8 training days. Non-toxic white tempura paint was added to the pool water to make the water opaque, so that the mice would not have been able to see the platform. Each mouse was trained with 1 trial from each quadrant per day for 8 consecutive days (Water2100 Software vs.7, 2008). During each trial, the mouse was placed in the tank and each trial was stopped either once the mouse reached the platform, or if the mouse was unable to find the platform in 60 seconds. Data were recorded using an automated tracking system (HVS Image, Hampton, U.K.) and swim time (sec), swim speed (m/s), and swim distance (m) were calculated for analysis. On day 9, a probe trial was conducted, during which the platform was removed, and each mouse was allowed to swim freely for 60 seconds. For the analysis of probe trial, the time spent in the quadrant where the platform was located during training days was measured. (Jafari et al., 2017).

3.2.4.3. Balance Beam (BB) Test:

The BB test was performed to measure the motor skills of each mouse. To conduct this test, the mice were trained to traverse across a 1 cm diameter, 100 cm long beam, which was 50 cm above a foam pad to cushion falling mice, to reach an escape box. On day 1, each mouse was trained for 3 successful trials. On day2, the mice's traverse activity was recorded for 3 trials and manually scored for the mean latency (sec), distance travelled (cm), and number of foot slips for analysis. (Jafari et al., 2018; Tamura et al., 2012).

3.2.4.4. Rotarod (RR) Test:

The RR test was performed to measure the motor skills and the strength of gait in each mouse. All the mice were trained to walk on an automated 4 lane RR treadmill (ENV-575 M Mouse, Med Association Inc) on day 1. On day 2, each mouse was placed on the RR treadmill at 8rpm and 16rpm constant speed and at a 4-40 rpm alternating speed and recorded for 3 trials and the time (sec) each mouse was able to stay on the RR treadmill was recorded. (Brooks and Dunnett, 2009).

3.2.4.5. Elevated Plus Maze (EPM) Test:

The EPM is a measure of anxiety-like behaviour in mice. The EPM apparatus was constructed from black Plexi-glass, which had two closed arms and two open arms. It was 40 cm high and two open arms were 5 cm wide and 27 cm long. The two closed arms were 10 cm wide, 40 cm long and had 40 cm high walls. Each mouse was placed in the center of the EPM facing the closed arms. A camera was set up above the maze to film each mouse for 5 minutes. Each mouse was manually scored for time spent in the open arms (seconds), time spent in closed arms (seconds), number of entries to open arms, and number of entries to closed arms. (Jafari et al., 2017). The EPM ratio was calculated by subtracting the number of entries to open arms from the number of entries to closed arms, divided by the total number of entries to both open and closed arms (Jafari et al., 2018).

3.2.5. Quantification of Aβ plaque Area and Numbers:

The methoxy-04 solution was prepared by diluting methoxy-X04 into 10% dimethyl sulfoxide, 45% propylene glycol, and 45% sodium phosphate saline. A 5mg/ml prepared methoxy-X04 was placed on a rotator at 4ºC for 24 hours for better saturation, and stored the solution in 4ºC prior to the use. Methoxy-X04 was injected intraperitoneally at a dose of 10mg/kg using a 27 ½ G needle 24 hours before the

perfusion of each animal (Bisht et al, 2016). Methoxy-X04, a fluorescent dye that selectively binds to β-pleated sheets found in Aβ plaques, has stronger specificity in staining Aβ plaques (Hefendehl et al. 2011).

The mice were perfused after the completion of the behavioral tests at the age of 6 months. Each mouse was injected with .05mg/kg of pentobarbital intraperitoneally. Then each brain received trans-cardial perfusion with 1x PBS until the blood ran clear followed with 4% PFA and the brain was extracted and post fixed with 4% PFA at 4^{o}c for 24 hours. The brains were then transferred to 30% sucrose for solidification at least 48 hours before slicing with a cryostat machine with a thickness of 50µm. Nanozomer fluorescent machine was used to colour the plaques and tangles in each brain section for analysis.

Each brain section was imaged automatically by using the Hamamatsu Nanozoomer 2.0-HT Scan System (Hamamatsu Photonics. Hamamatsu Japan) with a .23 µm/pixel resolution for quantification of Aβ plaques. The Ilastik 1.1.7 software was used for the plaque quantification. There were six coronal sections (Bregma: ~ , +3.20, +2.96, +0.98, -2.06, -3.08, and -5.34 mm) that were selected corresponding to the mouse brain atlas (Paxinos and Franklin 2001) to quantify the total number of Aβ plaques and total plaque area (%) in each mouse brain (Saito et al. 2014). Twelve additional brain regions of interest (ROI's): isocortex (IC), olfactory area (OA), medial-prefrontal cortex (mPFC), anterior cingulate area (ACA), nucleus accumbens (NA), hippocampal area (HA), posterior parietal area (PPA), rhinal area (RA), entorhinal area (EA), cortical amygdalar area (CAA), midbrain (MB), and hindbrain (HB) from each brain were selected for Aβ plaque quantifications (Jafari et al., 2017, 2018).

3.3. Results:

There were four different groups: C57, C57-TS, APP, and APP-TS in this experiment and 27 animals were used consistently in each behavioural test. None of the behavioural tests showed sex differences (P≥.05) so these data were collapsed across sex. Two way ANOVA was done for each behavioural tests. The Bonferroni post-hoc test was used for each behavioural test, due to similar variance in each groups. The Bonferroni post-hoc analysis compares the means among multiple groups to determine significant differences between groups, while taking experiment errors into consideration. Asterisks indicate *P<0.05 or **P<0.01 or ***P<0.001 value and partial eta squared (η2) indicates the effect size.

3.3.1. NOR Test:

The APP mice spent significantly less time with the novel object compared to all of the other groups. TS significantly improved the performance on novel object exploration in both C57 and APP mice. The overall significant ANOVA results among all of the 4 groups were: novel object time (F (3, 23) = 33.054, P≤ .0001, Ƞ2 = .812, power = 1.000), discrimination index ratio (F (3, 23) = 27.209, P≤ .0001, Ƞ2 = .780, power = 1.000).

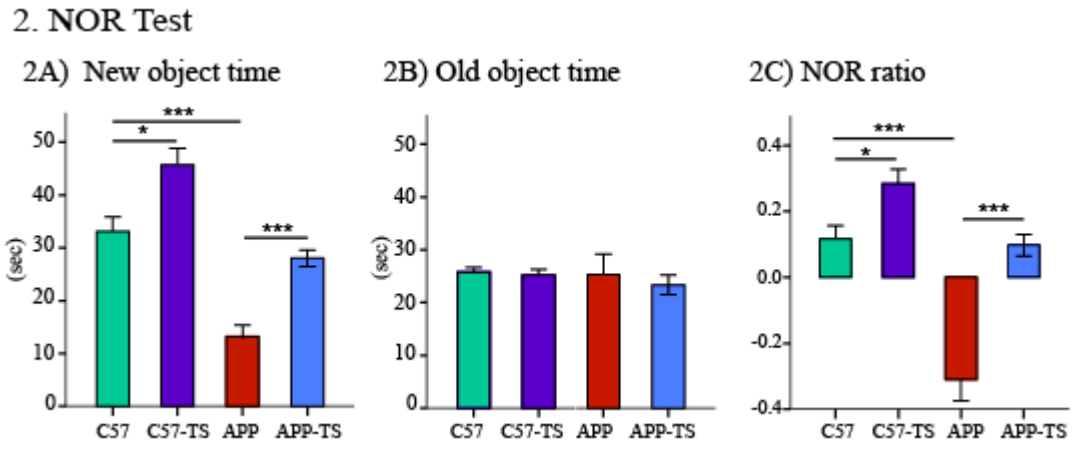

Fig 2: 2A. The APP group spent significantly less time (sec) with the new object compared to APP-TS, C57, and C57-TS groups. 2B. The time spent with old object was not significantly different among the groups. 2C. The discrimination index (DI) showed the APP group had a significantly higher preference for old object compared to APP-TS, C57, and C57-TS groups.

The novel object time was significantly higher in C57-TS compared to C57 ($F(1, 10) = 10.402$, $P \leq .012$, $\eta 2 = .565$, power= .806) groups, in C57 compared to APP ($F(1, 12) = 34.371$, $P \leq .0001$, $\eta 2 = .741$, power= 1.000), and in APP-TS related to APP ($F(1, 13) = 21.735$, $P \leq .0001$, $\eta 2 = .750$, power = .999) mice. The discrimination index ratio was higher in C57-TS mice relative to C57 ($F(1, 10) = 7.722$, $P = .024$, $\eta 2 = .491$, power = .683), in C57 mice relative to APP ($F(1, 12) = 25.974$, $P \leq .0001$, $\eta 2 = .684$, power = .997), and in APP-TS mice related to APP ($F(1, 13) = 24.509$, $P \leq .0001$, $\eta 2 = .690$, power = .994). No significant difference was observed in time spent with the old object among the groups ($F(3, 23) = .172$, $P = .914$, $\eta 2 = 0.22$, power= .077) (Figure 2). A Bonferroni post-hoc analysis revealed that the C57-TS group spent significantly more time with novel object in comparison with C57 ($P = .008$), APP ($P \leq .0001$), and APP-TS ($P \leq .0001$) and the APP group spent significantly reduced amount of time with novel object in comparison with APP-TS ($P = .001$), C57 ($P \leq .0001$), and C57-TS ($P \leq .0001$). The highest discrimination index ratio was observed in the C57-TS in comparison with APP ($P \leq .0001$) and the lowest discrimination index ratio was observed in the APP group relative to APP-TS ($P \leq .0001$), C57 ($P \leq .0001$), and C57-TS ($P \leq .0001$) as per Bonferroni post hoc analysis.

3.3.2. MWT test:

The APP mice were significantly slower to locate the sub-merged platform, showed a longer swim distance, and a reduced probe time in the target quadrant than each

of the other groups. TS significantly improved the performances on all three measures for both of the C57 and APP mice.

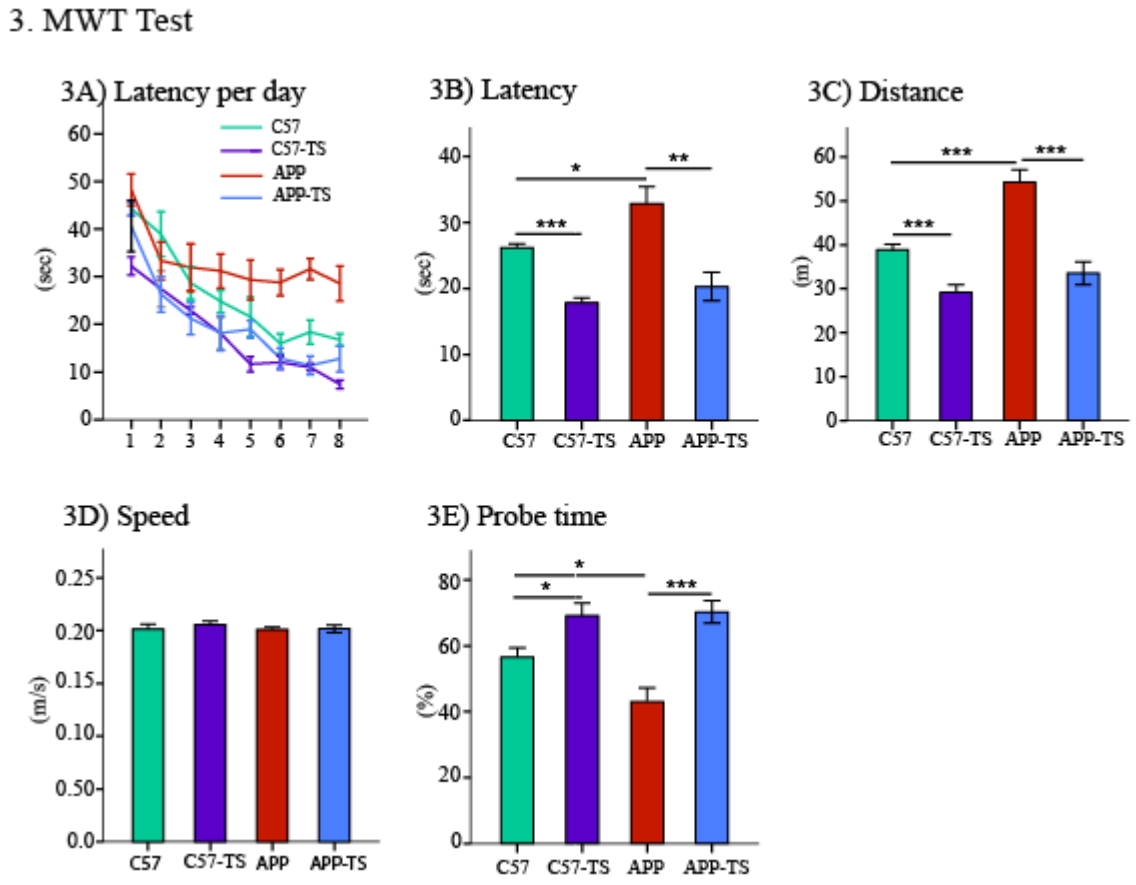

Fig 3: 3A. The progression of Swim latency from D1-D8. 3B. Swim latency was significantly longer in APP compared to APP-TS, C57, and C57-TS groups. 3C. Swim Distance was significantly higher in APP compared to APP-TS, C57, and C57-TS groups. 3D. No significant difference was observed in swim speed among the groups. 3E. Probe test: probe time was significantly longer in APP-TS compared to APP and C57 groups, but similar to C57-TS group.

The overall significant effects among all of the 4 groups are: latency ($F(3, 23) = 12.377$, $P \leq .0001$, $\eta 2 = .662$, power = .998), swim distance ($F(3, 23) = 24.008$, $P \leq .0001$, $\eta 2 = .791$, power = 1.000), and probe time ($F(3, 23) = 12.385$, $P \leq .0001$, $\eta 2 = .662$, power = .998). During training days, swim latency was significantly decreased in the

C57-TS mice compared to C57 ($F(1, 10) = 56.858$, $P \leq .0001$, $\eta 2 = .877$, power = 1.000), in the C57 mice compared to APP ($F(1, 12) = 4.859$, $P = .048$, $\eta 2 = .288$, power = .527), and in APP-TS mice compared to APP ($F(1, 13) = 14.642$, $P = .003$, $\eta 2 = .571$, power = .935). The swim distance during the training days was also significantly decreased in the C57-TS mice relative to C57 ($F(1, 10) = 19.843$, $P = .001$, $\eta 2 = .665$, power = .979), in the C57 mice relative to APP ($F(1, 12) = 19.746$, $P = .001$, $\eta 2 = .622$, power = .982), and in the APP-TS relative to ($F(1, 13) = 33.075$, $P \leq .0001$, $\eta 2 = .750$, power = .999). During the probe day, the amount of time spent in the target quadrant was significantly higher in the C57-TS mice compared to the C57 ($F(1,10) = 5.737$, $P = .043$, $\eta 2 = .418$, power = .557), in the C57 compared to APP ($F(1,12) = 6.087$, $P = .03$, $\eta 2 = .337$, power = .621), and in the APP_TS compared to the APP ($F(1,13) = 25.741$, $P \leq .0001$, $\eta 2 = .701$, power = .996). No significant differences were observed in terms of the swimming speeds among the groups ($F(3, 23) = .358$, $P = .784$, $\eta 2 = .053$, power = .107). A Bonferroni post hoc analysis revealed that the C57-TS mice took significantly less time to locate the hidden platform during training days in comparison with APP ($P \leq .0001$) and APP took significantly more time than the C57-TS ($P \leq .0001$) and APP-TS ($P = .001$). According to Bonferroni post hoc analysis the C57-TS mice swam the shortest distance during the training days compared to the APP ($P \leq .0001$) and APP mice swam the longest distance compared to the C57 ($P = .02$), C57-TS ($P \leq .0001$), and APP-TS ($P \leq .0001$). During the probe day, the C57-TS mice spent the highest amount of time in the target quadrant than the APP ($P = .001$) and the APP mice spent the least amount of time in the target quadrant than the C57-TS ($P = .001$), and the APP_TS ($P \leq .0001$).

3.3.3. BB Test:

The APP mice were significantly slower to traverse the beam and made more slips than each of the other groups. TS significantly improved performance on both measures for both the C57 and APP mice.

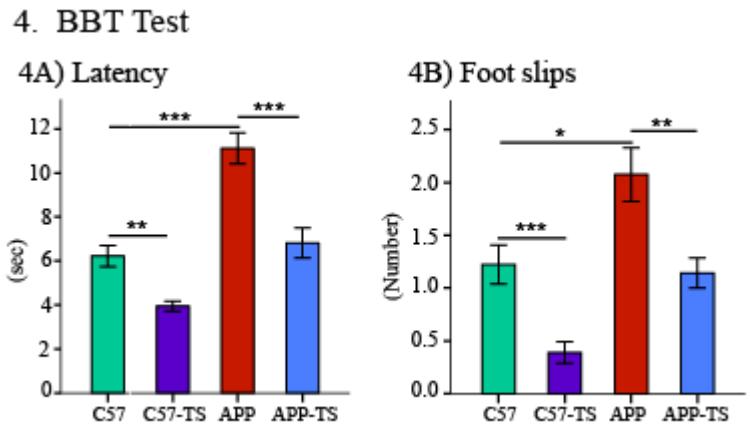

Fig 4: 4A. The C57-TS was the fastest group and APP was the slowest group in BB latency. 4B. The APP group exhibited increased number of foot slips, whereas C57-TS exhibited decreased number of foot slips relative to C57 control mice.

The overall significant differences among all four groups in latency is F (3, 23) = 25.420, P≤ .0001, Ƞ2 = .761, power = 1.000, and number of foot slips is F (3, 23) = 12.398, P≤ .0001, Ƞ2 = .608, power = .999. The C57-TS group took significantly less time to cross the beam (F (1, 10) = 15.142, P = .005, Ƞ2 = .654, power = .924) and exhibited a reduced number of foot slips in C57 (F (1, 10) = 25.005, P = .001, Ƞ2 = .758, power = .991) compared to C57 group. The C57 mice also took significantly less time to cross the beam (F (1, 10) = 25.857, P≤ .0001, Ƞ2 = .665, power = .997) and had a reduced number of foot slips F (1, 12) = 5.996, P = .029, Ƞ2 = .316, power = .620) in comparison to APP. In contrast, the APP group took significantly longer to cross the beam (F (1, 13) = 18.133, P = .001, Ƞ2 = .564, power = .977) and showed an increased number of foot

slips (F (1, 13) = 8.744, P = .01, Ƞ2 = .384, power = .785) compared to APP-TS. A Bonferroni post-hoc analysis revealed that the APP group took the longest time to cross the beam relative to C57 (P≤ .0001), C57-TS (P≤ .0001), and APP-TS (P≤ .0001) and had the highest number of foot slips relative to C57 (P = .05), C57-TS (P≤ .0001), and APP-TS (P = .019). The C57-TS group took significantly shorter time to traverse the beam relative to APP (P≤ .0001) and APP-TS (P = .044), and had significantly reduced number of foot slips compared to APP (P≤ .0001) as per Bonferroni post hoc analysis.

3.3.4. RR Test:

The APP group showed significantly impaired performance compared to APP-TS, C57, and C57-TS groups and C57-TS group exhibited the most improved performances in all RR speeds (8 rpm, 16 rpm, and 4-40 rpm) among the groups (Figure 5). TS significantly improved the RR performances in both C57-TS and APP-TS groups in relative to C57 and APP respectively.

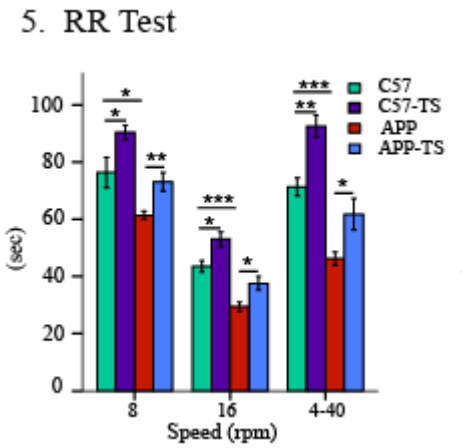

Fig 5: A gradual increase in time spent with low (8 rpm), high (16 rpm), and alternating (4-40 rpm) speeds in APP-TS, C57, and C57-TS groups compared to APP.

The overall significant differences between all four groups were at 8 rpm: F (3, 23) = 13.779, P≤ .0001, Ƞ2 = .646, power = 1.000; at 16 rpm: F (3, 23) = 21.735, P≤

.0001, ηp2 = .739, power = 1.000; and at 4-40 rpm: F (3, 23) = 25.446, P≤ .0001, ηp2 = .768, power = 1.000. Compared to C57 group, C57-TS mice showed significantly improved performance in all three RR speeds, i.e., 8 rpm: F (1, 10) = 5.661, P = .039, ηp2 = .361, power = .575; 16 rpm: F (1, 13) = 8.421, P = .016, ηp2 = .457, power = .744; and 4-40 rpm: F (1, 10) = 18.442, P = .002, ηp2 = .648, power = .971. Similarly, APP-TS group exhibited significantly improved performance in all three RR speeds, i.e., 8 rpm: F (1, 13) = 12.029, P = .004, ηp2 = .481, power = .893; 16 rpm: F (1, 13) = 8.155, P = .014, ηp2 = .385, power = .752; and 4-40 rpm: F (1, 13) = 7.388, P = .018, ηp2 = .362, power = .710 compared to APP mice. In contrast, the APP group exhibited impaired performances in all three RR speeds, i.e., 8 rpm: F (1, 12) = 8.865, P = .013, ηp2 = .446, power = .773; 16 rpm: F (1, 12) = 36.463, P≤ .0001, ηp2 = .768, power = 1.000; and 4-40rpm: F (1, 12) = 41.175, P≤ .0001, ηp2 = .789, power = 1.000 in relative to C57 mice. A Bonferroni post-hoc analysis revealed that the C57-TS group showed significantly improved performances compared to all other groups at all three speeds, i.e., 8 rpm: APP (P = .035), and APP-TS (P = .016); 16 rpm: APP (P≤ .0001), and APP-TS (P = .001), and 4-40 rpm: C57 (P = .016), APP (P≤ .0001), and APP-TS (P≤ .0001).

3.3.5. EPM Test:

The C57-TS mice were significantly less anxious, and spent significantly more time in the open arms of the maze and less time in the closed arms of the maze compared to the other experimental groups. TS reduced the anxiety like behavior in both C57 and APP mice. The overall significant effects noted between all of the four groups are: open arm time (F (3, 23) = 67.143, P≤ .0001, ηp2 = .914, power = 1.000), closed arm time (F (3, 23) = 16.092, P≤ .0001, ηp2 = .718, power = 1.000), and EPM ratio (F (3, 23) = 31.905, P≤ .0001, ηp2 = .834, power = 1.000).

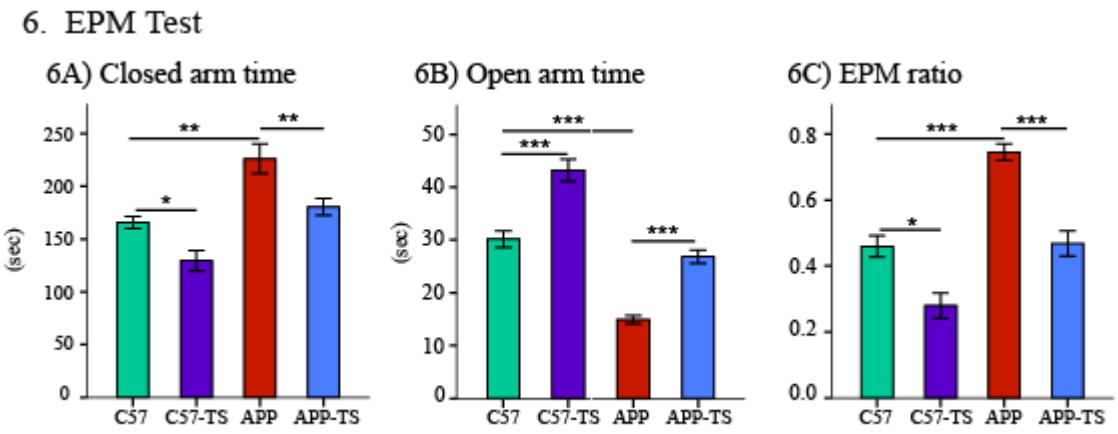

Fig 6: EPM test showed a significantly longer open arm time, shorter closed arm time, and lowest EPM ratio for closed arm entries in C57-TS compared to C57, APP, and APP-TS groups.

The open arm time was significantly higher in C57-TS mice compared to C57 ($F(1, 10) = 23.049$, $P = .001$, $\eta 2 = .742$, power = .986), in C57 compared to APP ($F(1, 12) = 88.735$, $P \leq .0001$, $\eta 2 = .881$, power = 1.000), and in APP-TS compared to APP ($F(1, 13) = 74.454$, $P \leq .0001$, $\eta 2 = .871$, power = 1.000). In contrast, the closed arm time was significantly lower in C57-TS mice compared to C57 ($F(1, 10) = 10.516$, $P = .012$, $\eta 2 = .568$, power = .810), in C57 compared to APP ($F(1, 12) = 12.492$, $P = .004$, $\eta 2 = .510$, power = .900), and in APP-TS compared to APP ($F(1, 13) = 9.675$, $P = .01$, $\eta 2 = .468$, power = .808). In addition, the EPM ratio was significantly lower in C57-TS mice relative to C57 ($F(1, 10) = 10.534$, $P = .012$, $\eta 2 = .568$, power = .811), in C57 related to APP ($F(1, 12) = 50.867$, $P \leq .0001$, $\eta 2 = .809$, power = 1.000), and APP-TS related to APP ($F(1, 13) = 41.357$, $P \leq .0001$, $\eta 2 = .790$, power = 1.000). A Bonferroni post-hoc analysis revealed that the C57-TS group had the longest open arms time in comparison with C57 ($P \leq .0001$), APP ($P \leq .0001$), and APP-TS ($P \leq .0001$) and the APP group had the shortest open arms time in comparison with APP-TS ($P \leq .0001$), C57 ($P \leq .0001$), and C57-TS ($P \leq$

.0001). In contrast, the C57-TS spent the lowest time in the closed arm related to APP (P≤ .0001) and APP-TS (P = .023), and APP spent the highest time in the closed arms relative to C57 (P = .003), C57-TS (P≤ .0001), and APP-TS (P = .023) as per Bonferroni post-hoc analysis. A Bonferroni post hoc analysis for EMP ratio discovered that the C57-TS showed lowest EMP ratio for closed arms compared to C57 (P = .014), APP (P≤ .0001), and APP-TS (P = .007) and highest EMP ratio for closed arms in the APP mice relative to C57 (P≤ .0001), C57-TS (P≤ .0001), and APP-TS (P≤ .0001).

3.3.6. Impact of TS on the amyloid- β (Aβ) plaque pathology:

The deposition of total number of Aβ plaques was higher in all 6 coronal sections and TS attenuated the formation of Aβ plaques in the APP mice. Although the pattern of increased number of Aβ deposition was observed in all 6 coronal positions of the APP mice compared to APP-TS, it was significantly higher in section + 3.20 ($F(1,10)= 5.885$, $P = .041$, $\eta^2 = .424$, power = .568), and + 0.98 ($F(1,10)= 6.529$, $P =.034$, $\eta^2 = .449$, power = .612). In addition, there was a trend for the total number of Aβ plaques to be higher in APP mice compared to APP-TS (P = .073).

TS also positively influenced the formation of Aβ by reducing the area of plaques (%) in APP mice. Again, the reduced pattern of the area of Aβ plaques (%) in all 6 coronal positions were observed; however, the area of Aβ plaques (%) was significantly smaller in + 3.20 ($F(1, 10) = 7.729$, $P = .024$, $\eta^2 = .491$, power = .684), and + 0.98 ($F(1, 10) = 8.455$, $P = .02$, $\eta^2 = .514$, power = .722). In addition, the total area of Aβ plaques (%) was significantly reduced in APP-TS mice compared to APP ($F(1, 10) = 9.991$, $P = .013$, $\eta^2 = .555$, power = .790).

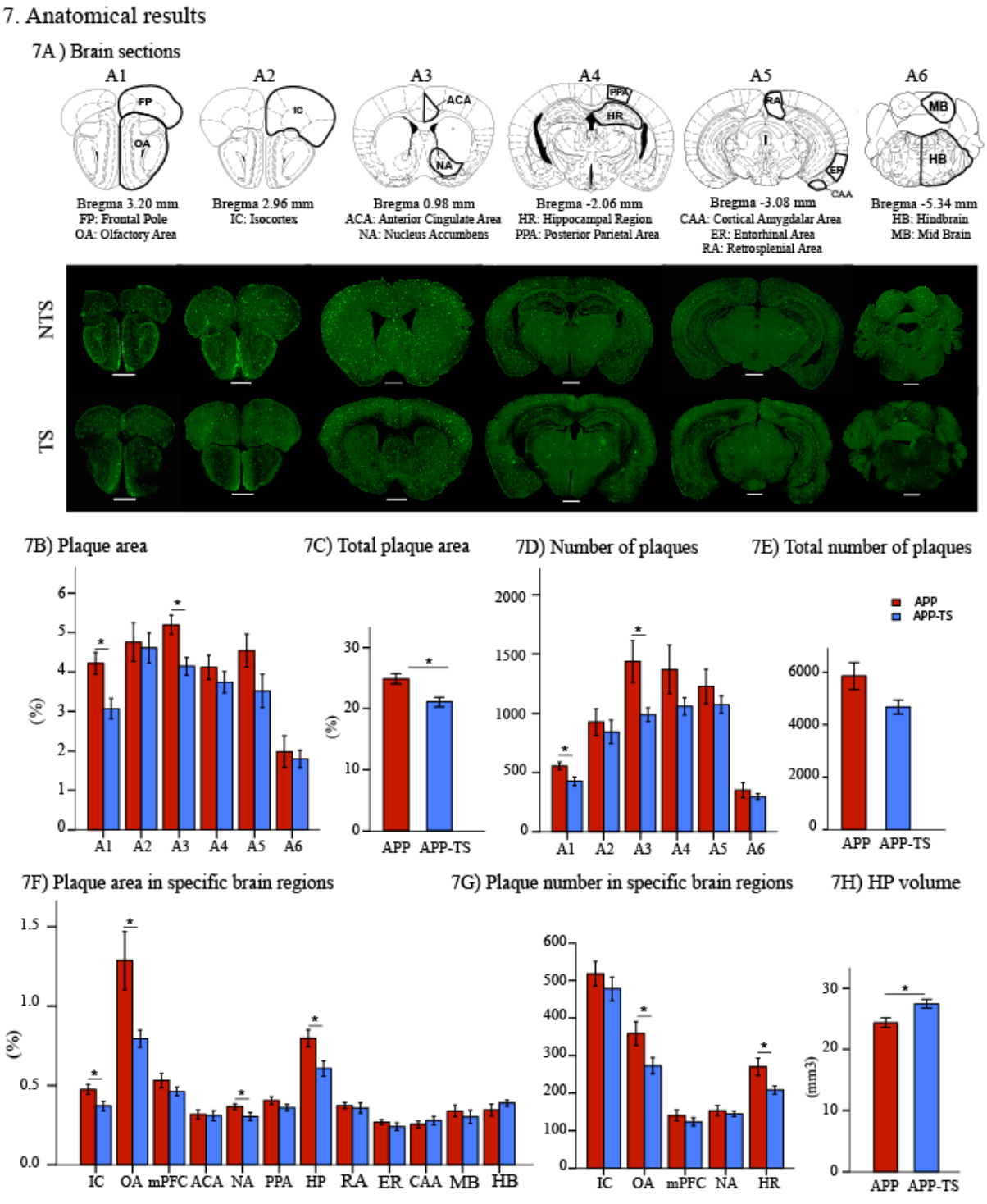

Fig 7. 7A. Shows the brain sections in six different bregma positions from both NTS and TS APP mice, 7B. Shows the Aβ plaques area (%) in six bregma positions, 7C. Shows the reduced total Aβ plaques (%) in APP-TS mice, 7D. Shows the number of Aβ plaques in

six bregma positions, 7E. Shows the reduction in total number of plaques in APP-TS mice, 7F. Shows the Aβ plaques area (%) in 12 different ROI's, 7G. Shows the number of Shows the Aβ plaques in 4 different ROI's, and 7H. Shows the larger hippocampal (HP) volume in APP-TS mice.

The positive influence of TS on Aβ plaque area (%) was also observed in ROI's. The reduced pattern of Aβ plaques area (%) was observed in all the ROI's but CAA and HB of APP-TS group compared to APP mice. However, APP-TS mice showed significantly reduced Aβ plaques (%) area in IC ($F(1, 10) = 6.148$, $P = .038$, $\eta 2 = .435$, power = .586), OA ($F(1, 10) = 6.183$, $P = .038$, $\eta 2 = .436$, power = .589), and HR ($F(1, 10) = 7.321$, $P = .027$, $\eta 2 = .478$, power = .660), compared to APP group. Furthermore, the number of Aβ plaques reduced in APP-TS group, but was significantly reduced in OA ($F(1, 10) = 5.044$, $P = .049$, $\eta 2 = .335$, power= .527) and HR ($F(1, 10) = 5.884$, $P = .036$, $\eta 2 = .370$, power = .591).

3.4. Discussion:

There are three main findings from this investigation: 1) TS ameliorated the cognitive and motor dysfunctions and reduced anxiety-like behavior; 2) TS attenuated the Aβ plaques size and numbers; and 3) TS enlarged the hippocampal volume in adult APP mice. We consider each finding in turn.

3.4.1. The impact of TS on cognition and motor learning and anxiety-like behavior

3.4.1.1. Cognition:

Impaired learning and memory is one of the common symptoms of AD in humans, and our findings from this study as well as the previous studies conducted in our lab (Karem, 2019; Jafari et al., 2018; 2019) demonstrate a similar impairment in APP mice. The goal of this study was to establish the influence of TS in improving the symptoms and pathology of AD in APP mice. Our findings from both MWT and NOR tests suggest

that TS improves cognition not only in APP mice, but also in C57 mice, which is the wild-type of APP mice. In the MWT test, both C57 and APP mice that received TS displayed significantly shorter latency and distance, and longer probe time, suggesting improvement in their spatial learning and memory (Angeles et al., 2016; Kolb & Gibb, 2010). Similarly, there was significantly increased time spent with the novel object and less time with old object in the NOR test showing that both groups that received TS demonstrated enhanced short-term memory (Richards et al., 2012). TS has been proven to be beneficial to treat depression-like symptoms in rats as TS positively influences the HPA axis (Angeles et al., 2016), increases the level of neurotrophic factors such as BDNF in the hippocampus, increases GFAP signaling (Antoniazzi et, al., 2016 and Roversi et al., 2019), prevents hippocampal damage due to neonatal hypoxia in rats (Rodrigues et al. 2004), and increases secretion of acetylcholine (ACh) in the hippocampus of rats (Dudar et al., 1979). TS in the form of maternal licking and grooming increases the brain-derived neurotrophic factor (BDNF) mRNA, NMDA receptors, improved spatial learning and memory in rats (Liu et al., 2000).

3.4.1.2. Motor Skills:

A deterioration in motor skills is a very common symptom of AD in humans. Our findings from this study also show similar motor deficits as shown in previous studies on APP mice (Jafari et al., 2018 & 2019). Our aim of this study was to reveal if TS improved the motor balance and coordination of APP mice. The results from both BB and RR test revealed that TS significantly improved the performances in both motor tests. In BB test both the C57 and APP mice that received TS traversed the balance beam faster, and had fewer foot slips, which indicates improved balance and motor coordination. Likewise, both groups that received TS markedly showed markedly improved performances on the

rotating wheel, suggesting enhancement of their motor coordination as well. Studies of TS on rats have previously shown improvements in a skilled reaching task (Kolb & Gibb, 2010; Gibb et al., 2010). Similarly, the application of TS has been proven to be beneficial in improving motor recovery in human stroke victims (Hunter et al., 2008) and motor development in preterm infants (Field et al., 1986). Numerous studies have shown that TS increased response to somatosensory stimulaton in the sensory motor cortex (Schaechter, 2011), dendritic length in frontal and sensorimotor cortex (Gibb et al., 2010), recovery of 20 Hz rebound in motor-cortical excitability (Parkkonen et al., 2018), and sensorimotor rhythm-based brain-computer interface performance (Shu et al., 2018). TS has also been shown to be beneficial in improving locomotion and exploratory behavior, as well as reducing protein carbonyl levels in the cortex, hippocampus, and sub-thalamic regions (Boufleur, et al., 2012). Application of gentle message therapy also increased urine dopamine by 31% (Field et al., 2009). These changes are important because enhanced neuro-synaptic plasticity in frontal and sensorimotor cortex, dopamine, and motor-cortical excitability plays very vital role in motor balance and coordination.

3.4.1.3. Anxiety-like behavior:

Anxiety-like behavior, due to stress and depression, has been identified as a risk factor for AD (Aznar and Knudsen, 2011). Anxiety may lead to frustration and possibly continue throughout the progress of AD. In this study, we aimed to determine the positive effect of TS on anxiety-like behaviour in APP mice. Our findings from the EPM test indicated that TS significantly reduced the anxiety in both C57 and APP mice, as these mice spent more time in the open arms and had an increased EPM ratio. Studies on rodents have shown that TS reduces anxiety-like behavior (Freitas et al., 2015; Boufleur, et al., 2012), increases the responsiveness to drugs such as benzodiazepine (Boufleur, et

al., 2012), and reduces the sensitization of psychostimulant drugs such as amphetamine (Mouhammad et al., 2010). Studies on either prenatal or postnatal TS have been shown to alter cortical thickness and striatum size (Muhammad and Kolb, 2011), increase plasma antioxidant compounds such as vitamin C and glutathione peroxidase in the cortex, hippocampus, and sub-thalamic region (Boufleur, et al., 2012), and lower plasma cortisol level (Zahra et al, 2018 and 2019). Field et al. (2009) reviewed the studies on the positive impacts of massage therapy on humans and concluded that massage therapy reduced saliva cortisol by 31%, and increased urine serotonin by 28%. Reduced cortisol and increased serotonin play a very essential role in improving anxiety-like behaviour.

3.4.2. The impact of TS on Aβ pathology in APP adult mice

The loss of cholinergic neurons, atrophy of hippocampal regions, the neocortex, and thalamus, and formation of tau-proteins, tangles, and Aβ plaques are a few of the neural symptoms of AD. In this study we investigated the effect of TS on Aβ pathology, and the hippocampal volume in APP mice. Although the formation of Aβ plaques was significantly reduced in some brain regions, but not all, a reduction pattern of Aβ plaques was observed thoughout the brains that received TS. One of the very first senses that begins to diminish is olfaction in early stages of AD patients (Kovács et al., 2001) and similar finding have been established in APP mice as well (Zahra et al., 2018). In our findings, the biggest significant anatomical difference observed was the reduced number and size of Aβ plaques in OA of the mice that received TS (Figure 6A). The formation of Aβ plaques is also dominant in most parts of the neocortex, and hippocampal regions (Zahra et al., 2018) of APP mice. In this study, we demonstrated that TS significantly reduced Aβ plaques numbers and sizes in the hippocampus and isocortex.

We also observed a significant reduction of the percentage of Aβ plaque areas and Aβ plaque numbers in bregma position + 3.2 mm and + 0.98 mm, and a pattern of decreased Aβ plaque numbers and in the percentage of Aβ plaque areas shown in all other coronal positions of the mouse of brains in the mice that received TS. A collapse across all the coronal planes revealed a significant reduction of the percentage of Aβ plaque areas in the mice that received TS. Further analysis of ROI's revealed a significant reduction of the percentage of Aβ plaque areas. A recent research by Martorell et al., (2019) shows that auditory and visual stimulation reduce Aβ plaque in the neocortex and hippocampus and improve spatial and recognition memory in 5XFAD mice.

3.4.3. The impact of TS on hippocampal volume (Hpc) in APP adult mice

Research on humans (Gosche et a., 2002) and rodents (Zahra et al., 2017 and 2018) has shown that one of the main hallmarks of AD is the shrinkage of hippocampal volume. We were able to show that application of TS in early stages of AD, prevents the Hpc volume from shrinking in APP mice. Similarly, along with the larger Hpc volume, there was a reduced Aβ plaque number, and reduced percentage of Aβ plaque area, which was associated with improved cognitive and motor skills in APP mice that received TS.

3.5. Conclusion:

Although TS has been successfully implemented in various clinical settings ranging from premature infants, institutionalized infants, work places, wound care, and treating HIV, this study the first to use this intervention in APP mice to counter the progression of AD pathology. Our findings demonstrate that TS improves cognitive and motor functions and anxiety-like behaviour in APP mice and these improved functions are associated with reduced Aβ plaque areas and numbers and increased hippocampal volume in their brain. These results suggest that TS, which is a non-invasive and cost-

effective interventions, could be applied to human AD patients, even after symptoms are obvious. These findings offer promise for the application of TS in patients with AD. However, further research is required to discover the brain mechanism regarding changes in the gene expression, electrophysiology, neurotransmitters, FGF-2, and synapses as a result of TS.

Chapter 4: General Discussion

4.1. Conclusion:

The first study (Chapter 2) showed that TS in early periods of life helped delay the onset of AD-like symptoms in early adulthood. In addition, the application of TS in the infantile period also helped to improve the AD-like symptoms in APP mice at 6 months of age, when the AD symptoms and pathology usually start to increase aggressively. In chapter three, we demonstrated that TS in adult APP mice at 4 months also has a beneficial impact on AD-like symptoms and pathology. These results provide a gateway for a possible cost effective non-invasive treatment and prevention in future clinical trials in humans with AD.

4.2. Anatomical Analysis to be Done:

In chapter two, no anatomical and physiological data were provided. In this study, we also collected plasma corticosteroids and brain from all the adult offspring. We expect to see a positive association between: 1) the improved AD-like behavioral symptoms and reduced corticosteroids; 2) the improved AD-like behavioral symptoms and reduced Aβ plaques in the brain; and, 3) improved AD-like behavioral symptoms and larger hippocampal volume.

4.3. Future Directions:

In the future, I plan to explore the molecular and physiological mechanisms of the brain induced by tactile, auditory, and/or visual stimulation therapy on APP^{NL-G-F} mice, a mouse model of AD. The following experiments will be a part of my future research studies.

4.3.1. Experiment 1: Assessing cognitive and motor behaviours to determine the effect of TS on AD pathology on the brain and behaviour of APP^{NL-G-F} mice. Hypothesis: a) TS

will reduce the AD associated cognitive and motor skills decline and b) these improvements will be correlated with: an increase of FGF-2, acetylcholine, spine density, and sharp ripples; a reduction of Aβ plaques; and a re-stabilization of gamma oscillations in the brain.

4.3.2. Experiment 2: Assessing cognitive and motor behaviours to determine the effect of 40 Hz of auditory stimulation (AS) on AD pathology on the brain and behaviour of APP^{NL-G-F} mice. One of the symptoms of AD in APP mice is that they exhibit disrupted gamma oscillations. Our theory is that the application of 40 Hz AS will balance the gamma oscillations in APP mice. The 40 Hz AS stimulation has proven to be beneficial in reducing Aβ plaques and improving the cognitive functions in APP mice (Martorell et. al 2019). Hypothesis: a) 40 Hz auditory tone will reduce the AD-associated cognitive and motor skills decline; and, b) these improvements will be correlated with: an increase of FGF-2, acetylcholine, spine density, and sharp ripples; a reduction of Aβ plaques; and, a re-stabilization of gamma oscillations in the brain.

4.3.3. Experiment 3: Assessing cognitive and motor behaviours to determine the effect of 40 Hz visual stimulation (VS) on AD pathology on the brain and behaviour of APP^{NL-G-F} mice. One of the symptoms of AD in APP mice is that they exhibit disrupted gamma oscillations. Our theory is that the application of 40 Hz VS will re-stabilize the gamma oscillations in APP mice. The 40 Hz VS stimulation has proven to be beneficial in reducing Aβ plaques and improving the cognitive functions in APP mice (Martorell et. al 2019). Hypothesis: a) 40 Hz flickering light will reduce the AD associated cognitive and motor skills decline; and, b) these improvements will be correlated with: an increase of FGF-2, acetylcholine, spine density, and sharp ripples; a reduction of Aβ plaques; and, a re-stabilization of gamma oscillations in the brain.

4.3.4. Experiment 4: Assessing cognitive and motor behaviours to determine the effect of TS, 40 Hz auditory, and 40 Hz visual stimulation together on AD pathology on the brain and behaviour of APP^{NL-G-F} mice. The theory of using all three sensory stimuli together to find out if the combination of three sensory stimuli has stronger impact on AD pathology in APP mice compared to single stimulus. Hypothesis: a) Application of Tactile, visual, and auditory stimulation combined will reduce the AD associated cognitive and motor skills decline and b) these improvements will be correlated with: an increase of FGF-2, acetylcholine, spine density, and sharp ripples; a reduction of Aβ plaques; and, a re-stabilization of gamma oscillations in the brain; and, c) the impact will be stronger than any single sensory stimulation.

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