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NEUR 4995: Effects of TCB-2 on Alzheimer's Neuropathology

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Abstract

Background: In recent years there's been a surging interest in the pharmacological benefits of serotonin receptor (5-HTR) agonists to treat psychiatric disorders, one of them being Alzheimer's Disease (AD). Research has hinted at the effects of these 5-HTR agonists on hippocampal (HPC) neurogenesis, Amyloid-b (Ab) plaque deposition and microglial activation. These are all hallmark biomarkers of AD. In light of the rise in dementia diagnoses, studies should examine different methods to moderate these symptoms.

Objective: This study shows the effects of the drug (4-Bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide (TCB-2) as a candidate to ameliorate AD biomarkers of neuropathology in the APP^{NL-G-F} mouse model at 6 and 10 months of age (early and late stages).

Methods: Fourteen APP^{NL-G-F} mice were fed 0.35g of Nutella®. The treatment group received 5 mg/kg of TCB-2 whereas the controls received the vehicle only. Feeding occurred every 3 days for the length of 29 days. Afterward, they were tested on the Novel Object Recognition (NOR) task for 6 days and subsequently perfused to collect their brains and apply immunohistochemistry analysis.

Results: The TCB-2 treatment was significantly more effective at decreasing Aβ plaque count in the younger cohort, resulting in a trend of cognitive enhancement as well. It provided anti-inflammatory aid and decreased HPC surface area across all ages.

Conclusion: Due to the ambiguous role of serotonin in learning and memory, our research hopes to expand its literature. The results prove that there's potential for 5-HTR agonists to be used as medical treatments to prevent the development of AD neuropathology.

Introduction

It has been estimated that by 2050 the number of people diagnosed with dementia will increase up to 114 million (Wimo et al., 2003). Among the most prevalent forms of senile dementia, Alzheimer's disease (AD) is characterized by the overexpression of the Amyloid Precursor Protein (APP) which encodes for Amyloid-β (Aβ) plaques (Jankowsky & Zheng, 2017).When Aβ plaques misfold, they aggregate and cause neurodegeneration throughout the brain, but the hippocampus (HPC) has been distinguished as the principal locus of investigation for the symptomatic memory impairments (Justice, 2018; Murphy & LeVine, 2010).

Neuroscientists have recently adopted the concept that the appearance of Aβ plaques throughout the connectome triggers microglial activation which begins a neurotoxic cascade, leading to apoptosis (Chuang et al., 2021). In healthy individuals there is a balance between the production of Aβ plaques and the subsequent immune response via microglia, phagocytes native of the central nervous system (CNS; Lee & Landreth, 2010; Sarlus & Heneka, 2017). However, when this homeostasis is disrupted by the overproduction of plaque, the inflammatory response due to microgliosis has been correlated with a worsening of AD pathology. This would entail HPC atrophy, increase in Aβ plaque deposition and cognitive impairments (Arends et al., 2000; Flanary et al., 2007). Therefore, it is clear why this pathway of neural inflammation is of interest to those who research AD.

Precedent research has hinted at a positive correlation between the rate of 5-HT_{2A}R loss and the cognitive decline in AD patients (Naughton et al., 2000); with the new developed technology and drugs we could test if this relationship is consistent with these claims as 5-HT_{2A}R are eminently spread throughout the hippocampus (Berumen et al., 2012).

Based on this background information we will observe the effects of the (4-Bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide (TCB-2) a serotonin (5-HT) receptor agonist on AD biomarkers such as the ones stated above. TCB-2 pertains to a class of hallucinogenic drugs with a high selectivity for the serotonin 2A sub-type receptor (5-Ht_{2A}R); it is specifically selective for the rat, mouse and human 5-HT_{2A} receptor (Di Giovanni & De Deurwaerdère, 2018; Fox et al., 2010; McLean et al., 2006). Additionally, it has been shown that microglia cells transcribe for 5-HT_{2A} mRNA meaning that they express the receptors that would be directly affected by this kind of agonist (Krabbe et al., 2012). In this same regard, it's known that through its ability to regulate inflammation, serotonin modulates many immune systems in the CNS (Herr et al., 2017; Roumier et al., 2019).

Our study will incorporate the use of the $APP^{NL\text{-}G\text{-}F}$ model of AD. This is a newer mouse strain in which the endogenous APP promoter has been knocked-in with the $^{NL\text{-}G\text{-}F}$ (Swedish, Arctic, Iberian) Aβ mutation (Nilsson et al., 2014; Saito et al., 2014). This allows for the typical overexpression of APP, but in a more controlled manner that parallels that of human clinical trials (Latif Hernandez et al., 2016; Sasaguri et al., 2022).

The paper by Mehla et al. (2019) compared plaques at ages 6 and 9 months, but stated that amyloidosis doesn't become aggressive until the latter age point in the $APP^{NL\text{-}G\text{-}F}$ model. Therefore, our study will include both stages, subsequently we will be able to compare the strength of the drug on both early and late-stage deposition of these Aβ plaques; both +/+ and -/- genotypes will be present to facilitate the comparison of neuropathology between the homozygous and knock-out model.

Preliminary evaluations were done to decide which dose of the drug would be used (refer to Appendix A). As such, it should be noted that this strain is derived from the C57BL/6J mouse

that we have done the dose evaluation study on, which means previous observations from that study will be applicable to this one as well.

In order to test whether the progression of AD in the HPC correlates with cognitive impairments, we ran the Novel Object Recognition (NOR) task (Vogel-Ciernia & Wood, 2014). The HPC specializes in episodic memory, a type of declarative memory which holds different attributes but most important for this task are the fact that it specializes in memorizing contextual details, and it can do so even after just one episode (Rudy, 2008).

The purpose of the NOR task is quite straightforward, we are testing whether the animal can discriminate between the old and new object; and if said discriminatory skills are still intact, then the animal should spend most of its exploration time on the novel object. It is expected that animals affected by AD will show a decrease in performance, which should worsen with age as there is evidence that Aβ plaque correlates with memory deficits in Alzheimer's mouse models (Grayson et al., 2015). The NOR study ran by Mehla et al. (2019) was able to observe memory impairment differences between the 6- and 9-months old mice. Since we will be using similar aged cohorts, we will be able to make comparisons between the memory impairments of two different stages of AD progression. Additionally, we will be able to observe whether the treatment with TCB-2 can combat these memory impairments as it has been shown in previous studies (Afshar, 2019; Zhang et al., 2016). Moreover, from a study ran by Bouet et al. (2018) it seems that the NOR task is sensitive to serotonergic receptors' activation, meaning that these can modulate for cognitive performance.

The proposition of the role of serotonin on AD pathology is somewhat novel and there have been little reports on the effects of TCB-2 as treatment drug for Alzheimer's neuropathology. Therefore, this research would be a great opportunity to expand the understanding on its effects.

Materials and Methods

Animals

Fourteen $APP^{NL\text{-}G\text{-}F}$ mice of each sex pertaining to the ages of 6M (+/+, n = 6), 10M (+/+, n = 6) and 11M (-/-, n = 2) were pair housed on a 12:12 light/dark cycle. Throughout the experiment the animals had food and water made available to them ad libitum.

Drugs

The 5-HT_{2A}R agonist drug known (TCB-2) was purchased from Tocris Biosciences ® (Bristol, UK). A stock solution was made following supplier's instructions to make a 5 mg /kg solution. It was then frozen at - 20 ºC until needed .Before the administration of the drug started, the mice were habituated with the vehicle for 6 days. The animals were fed 0.35 g of Nutella ®, the treatment group received 5 mg/kg of TCB-2 into their food whereas the control just received the vehicle, occurring every 3 days for the length of 29 days. A summary of the cohorts is shown below in Table 1.

Table 1. Summary of Experimental Cohorts

Strain	Genotype	Sex	Age When Perfused (months)	Dose of TCB-2 (mg/kg)
$APP^{NL\text{-}G\text{-}F}$	+/+	M	6	5.0
	+/+	M	6	5.0
	+/+	F	6	5.0
	+/+	F	6	5.0
	+/+	M	6	n/a
	+/+	F	6	n/a
	+/+	M	10	5.0
	+/+	M	10	5.0
	+/+	F	10	5.0
	+/+	F	10	5.0
	+/+	M	10	n/a
	+/+	F	10	n/a
	-/-	M	11	n/a
	-/-	F	11	n/a

NOR

After the 29 days of treatment, the mice were tested on the Novel Object Recognition (NOR) task for 6 days. During the first four days of the task the mice were handled and consecutively habituated to the same context they were going to be trained and tested in. This context consisted of a white square-shaped tub that was filled with enough bedding to be comfortable for the mice; each mouse was habituated in said context for 10 minutes. On the training day, two identical objects were placed inside the tub and the animal was allowed to explore them for 10 minutes. The following day the mice's memory skills were tested by replacing one of the familiar objects with a novel one (the position of the novel object was alternated within trials), they were allowed to explore them for 5 minutes. On both the training and testing day, the items were cleaned with 70% isopropyl alcohol to mask any previous odour cues and allowed to dry completely between trials. An example of the task's apparatus is shown below in Figure 1. Subsequently, the animals were sacrificed, and their brains were collected for analysis of AD pathology with the use of immunohistochemistry.

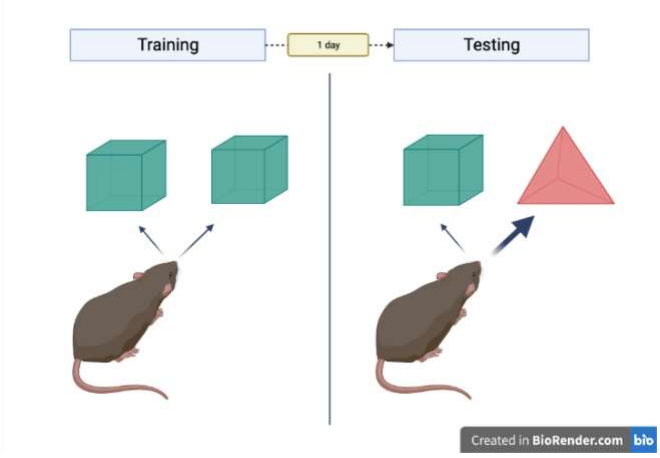

Figure 1. Model of the NOR task. After being habituated for 4 days in one context the animals are subsequently trained with two identical objects which they are allowed to explore for 10 mins. The following day one of the objects is alternatively replaced with a novel one, the animals

are allowed to explore for 5 minutes. It is predicted that healthy animals will spend more of their exploration time on the novel object whereas animals with memory impairments will have dampened discriminatory skills, resulting in ambiguous explorative focus.

Immunohistochemistry

The mice's brains were frozen and sectioned at 40μm using a sliding microtome; 1:6 series were collected. The sections from one series were mounted on Superfrost + slides from a PBS dish, air dried and processed for fluorescent immunohistochemistry. For Aβ plaques, slides were fixed in 4% buffered paraformaldehyde then processed for antigen retrieval using 70% formic acid. Slides were permeabilized in 0.1% Triton-X in Tris Buffered Saline (TBS) and blocked in 2% Bovine Serum Albumine (BSA) with 0.1% Triton-X in TBS. Primary antibodies were added to the blocking solution, 1ml/slides in a gently agitating sealed humid chamber for 2 days. Antibodies used were anti-β-Amyloid, 17-24, Clone 4G8 (mouse, 800701, Biolegend) 1:1000 and anti-Iba1 (Rabbit, SAF4318, 019-19741, Wako) 1:1000. Slides were rinsed and the secondary antibodies, goat-anti-rabbit-Alexa-594 (IgG (H+L), Invitrogen, A11037), 1:1000 and goat-anti-mouse-Alexa Fluor Plus 488 (IgG (H+L), A32723, Thermo (Fisher/ Invitrogen) 1:1000 were added to the blocking solution, 1ml/slide for 24h in a gently agitating dark sealed humid chamber. After rinses, slides were cover slipped with Vectashield or Vectasheild with DAPI (H-1000 or H-1200, Vector Laboratory), sealed with nail polished and imaged using a scanning microscope (NanoZoomer Digital Pathology RS (Hamamatu). An example of it is shown below in Fig.2.

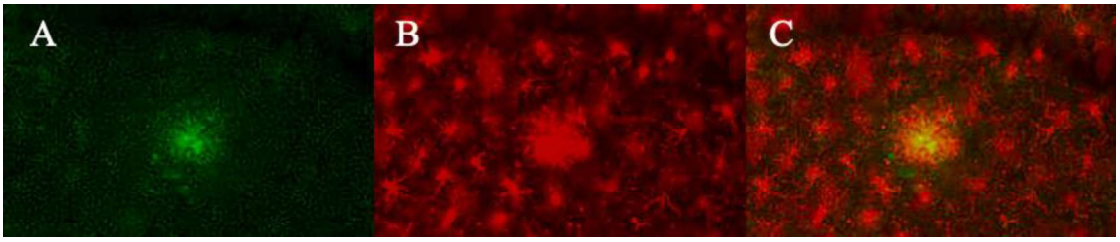

Figure 2. A) Aβ plaque in a 10M APP$^{NL\text{-}G\text{-}F}$ (+/+) mouse, stained with Anti-B-Amyloid, 17-24, Clone 4G8 (mouse, 800701, Biolegend). **B)** Activated microglia in 10M APP$^{NL\text{-}G\text{-}F}$ (+/+) stained with Anti-Iba1 (Rabbit, SAF4318, 019-19741, Wako). **C)** Illustration of microglia aggregation on an Aβ plaque as a neurological immune response.

Statistics

Hippocampal Surface Area

The image processing program known as ImageJ 1.4.3.67 was used to calculate the area of the HPC sections. This was done by segmenting six coronal HPC sections per animal and calculating their surface area based on the program's measuring function. The areas were then summed together in order to get an approximate HPC surface area, however these data points still had to be converted from pixels to mm^2. Pixels were converted to mm^2 using the following formula; two-tailed t-tests were then performed with Excel to test for statistical significance among variables. Data presented as mean ± SD.

$$\text{Area } (\text{mm}^2) = \frac{\text{Summed Area (pix)}}{(\text{Scale (pix/mm)})^2}$$

Aβ Plaque and Microglia Count

The Ilastik 1.1.7 software was used for the quantification of Aβ plaque and microglia count in each coronal HPC section. This application automatically generates plaque and microglia size estimates for each HPC section and because we used 6 HPC sections per brain,

this meant that we received 6 different count estimates (as csv files) per brain/animal. To facilitate the calculations for the sum, average and count for both Aβ plaque and microglia, an R-code was made (for further information check Appendix B). Two-tailed t-tests were then performed with Excel to test for statistical significance among variables. Data presented as mean ± SD.

NOR Scoring

The iMovie 10.3.2 video editing software was used to score behaviour. Any explorative behaviour towards the objects ≥0.3s was recorded to then measure the animal's discrimination index (DI). This unit of measure was calculated with the formula shown below, taken by Vogel-Ciernia and Wood's paper (2004, p.11). Two-tailed t-tests with Excel followed to test for statistical significance between variables. Data presented as mean ± SD.

$$\text{Discrimination Index (DI)} = \frac{\text{Time exploring the novel object} - \text{time exploring the familiar}}{\text{time exploring novel} + \text{familiar}} \text{ x } 100$$

Results

Hippocampal Surface Area

Figure 3 shows that overall, the treatment with 5mg/kg of TCB-2 slightly decreased the average HPC surface area of both 6M (n = 4, 44.0 ± 1.27) and 10M (n = 4, 47.3 ± 2.47) mice. The opposite is true in the age-matched controls which show bigger HPC surface areas in the older mice (n = 2, 48.5 ± 1.74) , showing slightly bigger values than the younger cohort (n = 2, 45.3 ± 2.23). The null 11M (n = 2, 47.7 ± 2.05) group to demonstrate healthy HPC surface area values.

Aβ Plaque Deposition

As seen in Figure 4, the 5-HT_{2A}R agonist had opposite effects on Aβ plaque count depending on the AD progression stage it was administered at. We can see that as predicted, the Aβ plaque count decreased in the treated 6M mice (n = 4, 3227 ± 59.7) compared to the age-matched controls (n = 2, 3532 ± 63.7). However, the treatment had the opposite effect on plaque deposition in the 10M mice (n = 4, 4705 ± 51.9) which show increased values compared to the age-matched controls (n = 2, 3975 ± 42.7). After applying two-tailed t-tests on all the variables, it resulted that the treatment significantly ($p < 0.05$) decreased the number of Aβ plaque more efficiently in the 6M mice compared to the 10M ones.

Microgliosis

Treatment with 5 mg/kg of TCB-2 had the same pattern of effects when administered at either stage of AD progression in the $APP^{NL\text{-}G\text{-}F}$ mouse (Fig 5.). Overall, the younger 6M mice show lower levels with (n = 4, 4825 ± 19.0) and without treatment (n = 2, 6440 ± 11.4) compared to the older 10M mice which show higher levels of microgliosis both before (n = 2, 6440 ± 11.4) and after treatment (n = 4, 6513 ± 23.8). We've also added the null 11M (n = 2, 7821 ± 11.4) group to demonstrate that microgliosis occurs even in healthy individuals and is an innate immune response.

NOR Performance

Figure 6 shows that as predicted, AD progression in the $APP^{NL\text{-}G\text{-}F}$ mouse results in worse discriminatory skills in the NOR task. Treatment with 5 mg/kg of TCB-2 seems to increase the DI in 6M mice (n = 4, 59.7 ± 18.5) compared to their age-matched controls (n = 2, 38.8 ± 5.0). The same is true for the older 10M mice, which show slightly higher DIs when treated (n = 4, 18.2 ± 27.7) compared to not being treated (n = 2, 17.4 ± 12.2). The null 11M (n = 2, 47.7 ± 16.0) group to healthy discriminatory levels.

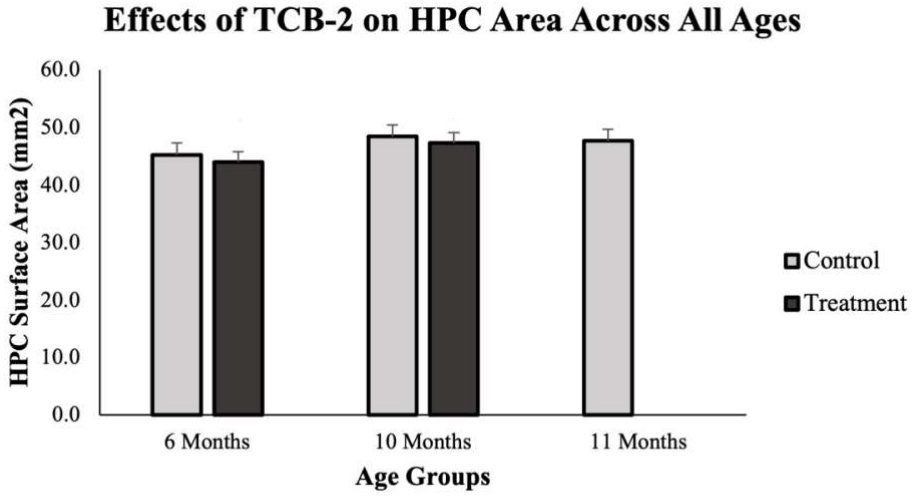

Figure 3. Comparison of treatment effects on HPC surface area across all age groups. Following the feeding of 5mg/kg of TCB-2 for 28 days, the 6M mice (n = 4) showed similar HPC size (44.0 ± 1.27) compared to the age-matched controls (n = 2, 45.3 ± 2.23). The treatment had a similar pattern of effect in the older group, in which the control's values (n = 2, 48.5 ± 1.74) are higher than when treated (n = 4, 47.3 ± 2.47) . The 11M (-/-) group (n = 2) was added to display healthy HPC area levels (47.7 ± 2.05).

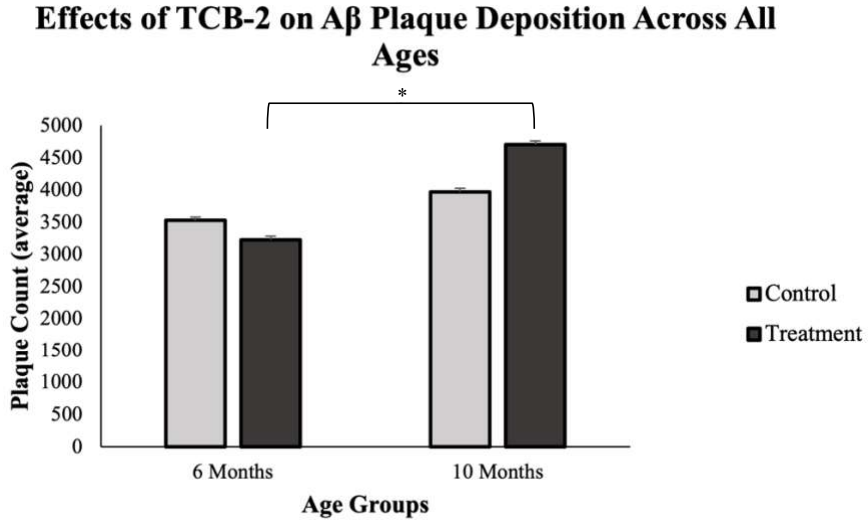

Figure 4. Comparison of treatment effects on plaque count across all age groups. Following the feeding of 5mg/kg of TCB-2 for 28 days, the 6M mice (n = 4) showed significantly smaller

numbers of plaque count (3227 ± 59.7) compared to the age-matched controls (n = 2, 3532 ± 63.7). The treatment had the opposite effect in the older group, in which the control's values (n = 2, 3975 ± 42.7) are lower than when treated (n = 4, 4705 ± 51.9) .* $p < 0.05$.

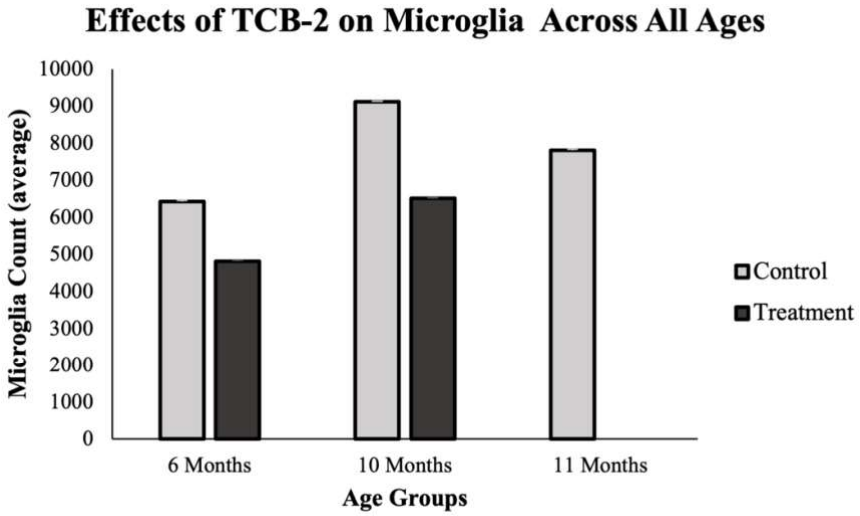

Figure 5. Comparison of treatment effects on microglia count across all age groups. Following the feeding of 5mg/kg of TCB-2 for 28 days, the 6M mice (n = 4) showed smaller numbers of microgliosis (4825 ± 19.0) compared to the controls (n = 2, 6440 ± 11.4). The same pattern showed in the control 10M (n = 2, 6513 ± 23.8) and treated (n = 4, 6513 ± 23.8) mice. The 11M (-/-) group (n = 2) was added to illustrate healthy levels of microglia (7821 ± 11.4).

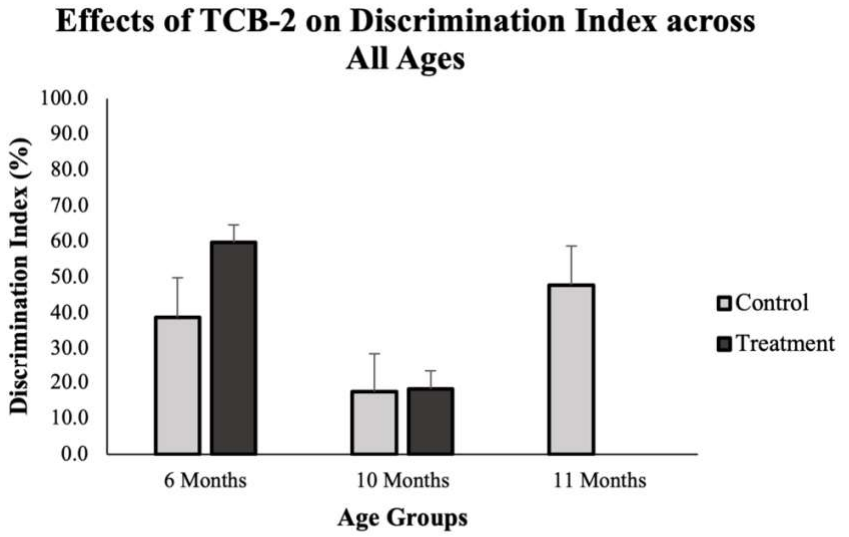

Figure 6. Comparison of Discrimination Indexes (DIs) in NOR performance across all ages. 6M controls (n = 2, 38.8 ± 5.0) showed worse discriminatory skills than their treated counterparts (n = 4, 59.7 ± 18.5). Performance drastically worsened at the 10M mark (n = 2, 17.4 ± 12.2) even after treatment (n = 4, 18.2 ± 27.7). The 11M (-/-) group (n = 2) was added to display healthy discrimination levels (47.7 ± 16.0) in the task.

Discussion

Precedent research investigated the effects of the 5-HT_{2A}R agonist known as TCB-2 in both rats and mice models of Alzheimer's disease (Afshar, 2019; Fox et al., 2010; Zhang et al., 2016); however, all of the studies listed beforehand chose to inject (either by i.p or i.c.v) their respective doses of TCB-2. Because to our knowledge no one has yet reported the effects of this drug on AD neuropathology when delivered through the GI tract, the study at hand chose this method which would hopefully prove a less inorganic and invasive administration while also expending the literature on this serotonin receptor agonist.

We observed the effects of 5 mg/kg of TCB-2 on different AD biomarkers in the $APP^{NL\text{-}G\text{-}F}$ mouse model at two different stages of disease progression, at 6 months representing the earlier

stages and at 10 months, representing the later stages of AD development. Specifically, we looked at whether TCB-2 would affect HPC surface area, Aβ plaque count/deposition, microgliosis and NOR performance.

Hippocampal Surface Area

The paper by Afshar et al. (2018) claims that after injecting TCB-2 via i.c.v, rats showed reduced neuronal loss in the hippocampus it is commonly known that this necrosis usually results in the atrophy of the organ and subsequent estimates of surface area (Pang et al., 2022). Other studies suggested that this same drug increased the levels of brain-derived neurotrophic factor (BDNF) mRNA, promoting cell growth (Tsybko et al., 2020). Therefore, we predicted that by feeding 5 mg/kg of TCB-2 we would observe higher HPC area values compared to non-treated animals; however, this was not the case. As can be seen in Fig.3, the treatment with the 5-HT_{2A}R agonist did not provide aid in maintaining healthy levels of HPC area compared to the controls. In fact, the treatment had the opposite effect that we predicted and seems to have slightly atrophied the HPC.

Whether these results are dependent on increased neuronal loss is not something we can conclude based on the methods used to calculate the HPCs surface areas. The software that was used merely measured the size of the HPC's coronal sections in pixels, this does not render for an appropriate analysis of the neurobiological changes that have occurred because of the applied treatment. Namely, we don't know if the decrease in surface area is specific to cell loss and not another factor. As such, past research claims that neurogenesis in the hippocampus is dependent on the serotonin 1A subtype receptor (5-HT_{1A}R) rather than the one we have investigated for (Carhart-Harris & Nutt, 2017).

Aβ Plaque Deposition

As can be seen from Fig.4, the administration of TCB-2 had opposite effects depending at which stage of the disease it was administered at. In the 6M group, the treatment had the predicted effect, decreasing the amount of plaque throughout the HPC. However, the 10M group shows elevated numbers compared to its age-matched control cohort.

It is often the case that certain treatments have no effect after a pathology has developed to a certain point, if it is true that TCB-2 is counterproductive regarding plaque proliferation when administered at 10M, then this report offers a precautionary note to not prolong the administration of this 5-$HT_{2A}R$ as it's more likely to worsen the situation rather than keep it unchanged. Nevertheless, these results remain puzzling, however, these trends might make more sense once we look at them in conjunction with the effects of TCB-2 on microgliosis.

Microgliosis

Overall, feeding 5 mg/kg of TCB-2 seems to have provided anti-inflammatory results across both stages of the disease. This is congruent with previous observations which state that serotonin modulates inflammatory responses due to these immune systems expressing 5-HTRs (Roumier et al., 2019). However, if it is true that the microglial response to Aβ plaque often causes the worsening of neuropathology, these interpretations with the ones above are counter-intuitive, especially regarding the 10M cohort.

Our interpretation of this is that with the current surge of research implying that microglia aggregation on Aβ plaque might be contributing to the worsening of AD neuropathology (Streit, 2004), we neglect the fact that this remains an innate immune response to fight off the disease. Although this statement leans on a naturalistic fallacy, it is still worth considering. By decreasing the number of microglia, and therefore their chances to breakdown the Aβ plaques, with the use

of TCB-2, we might be causing the increase in plaque number that we see in the 10M mice affected by treatment as well in Fig.4.

However, the same pattern is not present in the younger cohort. Fig.5 in conjunction with Fig.4 show that administering TCB-2 caused a decrease in microgliosis and Aβ plaque count respectively. The pattern of the treatment's effects is the same in the 6M group regarding both microgliosis and Aβ plaque count. This might hint at the fact that neurobiological processes underly the difference in effects of microgliosis on plaque between the ages of 6M to 10M.

It is known that by the age of 9M plaque peaks and plateaus in the $APP^{NL\text{-}G\text{-}F}$ strain (Mehla et al., 2019).The strength at which these plaques deposit in the HPC at this stage of the disease, might require the aid of microglia in addition to a treatment in order to be fought . Therefore, although the treatment does provide anti-inflammatory effects across all ages, it might be counterproductive when applied in AD's later stages. Although, this evaluation goes against the popular belief that microglia-mediated phagocytosis might be more detrimental later in the disease's progression, ongoing research hints at the possibility that neuronal loss is a result of microglia degeneration and thus said immune response might prove essential in later-onset dementia (Prokop et al., 2013; Streit et al., 2021).

NOR Performance

In terms of behaviour, the findings from this study corroborate our prediction; namely, that the administration of TCB-2 improves the performance of mice affected by AD in the NOR task (this difference is not significant).

As we can see from Figure 6, the treated 6M animals showed better discriminatory skills compared to their age-matched controls. Contemporarily, the drug seems to have decreased the

amount of hippocampal Aβ plaque (Fig 4). These results comply with the past research that has been done on how Aβ plaque deposition correlates with memory deficits (Guillozet et al., 2003).

What we can also tell from these results is that the younger treated animals perform better than the healthy 11M group, which themselves do not show extreme preference for the novel object. This is interesting because the healthy animals do not express Aβ plaque misfolding in their brains, yet the younger AD animals who do, show better cognitive skills in this task. This could be underlying evidence that the treatment when administered at the initial stages of the disease, enhances the memory of the individual enough to surpass the HPC's healthy conditions. Although this comparison is not age-matched and should be considered with caution.

The same pattern of effects cannot be observed in the older 10M group, in which the treatment on average increased the number of hippocampal Aβ plaque (as seen in Fig. 4) and yet they performed slightly better (although barely) on the NOR task (Fig.6). These results imply a few things:

- It could be possible that the NOR task is not a good assay of hippocampal function
- There are systems that overlap in function, compete and substitute for one another in case one is not fully operative.

In regard to the first point, although this task might provide one of the best methods to measure HPC function as explained in the introduction, other studies have claimed that it is not a suitable test, as memories can be consolidated without the help of the HPC (Gidyk et al., 2021; McDonald et al., 2018). This is based on past research which claims that familiarity-based recollection is sustained by the perirhinal cortex (Buffalo et al., 1998; Haskins et al., 2008).

To support the latter point, others have already proposed different theories for learning and memory mechanisms, one of the most popular being the Multiple Memory System (MMS)

perspective which states exactly that there is more than one apparatus in the brain which influence a person's memory (McDonald & White, 1993; Squire, 2004; White & McDonald, 2002). With this theory in mind, we are proposing that the reason as to why the treatment still improves NOR performance although the older mice show increased plaque numbers is because another system (i.e., the perirhinal cortex) could have substituted for the HPC.

Although it should be noted that no type of analysis was done on the perirhinal cortex itself, which means that we cannot deduce from these results and implications alone that this was the system which was substituting for a possibly malfunctioning hippocampus in the procedure of this task.

Overall, the treatment seemed to be efficient at decreasing HPC area, significantly decrease Aβ plaque deposition and bettering learning and memory skills when administered during the earlier stages of Alzheimer's. This hints at the possibility that 5-HT_{2A}R agonists such as TCB-2 might be more effective as a method to prevent Alzheimer's neuropathology, rather than to treat it if an individual were to develop it.

Limitations and Future Directions

First and foremost, this study was done on a very small sample of animals, this means that although some statistical analysis resulted in significance, the effect size of these findings is rather negligible. Nonetheless, it proves to be a good pilot in case we want to move forward with research that investigates the collaterals of serotonin receptor agonists on non-human animal models of Alzheimer's.

In the future it would be interesting to develop a dose-dependent study that looks at the effects of this or similar drugs at varying amounts and for different lengths of time. Although it's

due noting that at high amounts serotonin can have inhibitory effects on memory and learning (Morley & Farr, 2014). As indicated by previous research serotonin agonists can usually have long-term impacts even after just one dose (Aday et al., 2020). However, we recommend to also look into 5-HT_{1A}R agonists as this subtypes' mRNA is more commonly found in the HPC compared to the 2A subtype (Burnet et al., 1995). Moreover, we should include the analysis of Tau tangles, another infamous biomarker of AD pathology (Goedert, 1993).

Since these preliminary results showed that the treatment was most effective when administered in the younger cohort of mice, maybe we should consider TCB-2 to be more effective as a drug to *prevent* the development of Alzheimer's rather than be used as a reactive treatment. In this case, it could be interesting to see the effects of the treatment at 3M of age, when the disease is just starting to develop in the $APP^{NL\text{-}G\text{-}F}$ strain (Mehla et al., 2019).

In regard to behavioural assays to measure learning and memory performance, we previously mentioned that the NOR task might not be a suitable choice. In fact, there's proof that this task can be solved without HPC activation. However, we should not discredit the use of NOR in general, as more modern work is arguing the fact that spatial, contextual and configural tasks all sit on a continuum of hippocampus sensitivity, and that these tasks *can* be modified to be very or less sensitive to the role of the HPC in learning (Gidyk et al., 2021; McDonald et al., 2018).

A high-cue ambiguity context test could be used to evaluate whether said structure is present in learning, since one of its roles is to also separate very similar contexts from one another without creating interference between memories (Rudy, 2008).

We could also include a fear conditioning test since research by Di Giovanni and De Deurwaerdère (2018) has already alluded at the effects of TCB-2 on aiding in the acquisition of extinction of fear memory, which often involves the amygdala. McGaugh's work (Rudy, 2008)

on the role of the amygdala in the Memory Modulation Framework explains that said system has a crucial role in influencing the acquisition – but not storing – of memories, particularly those regarding aversive conditions.

Additionally, there's a high rate of comorbidity between AD and depression (Caraci et al., 2010; Rapp et al., 2008),and when we consider that the latter disorder is correlated to the modulation of serotonin throughout the brain and involves the amygdala (Hamilton et al., 2008), then it would be wise to investigate this relationship further. Alas, by expanding on the possibility that psychiatric disorders often influence the development of one another, we might offer more insight into the methods an individual could take to prevent their emergence.

Finally, it should be noted that although we don't know if this drug can be considered a psychoactive when administered in mice, of the previous research that has been currently done with this drug, it's categorized as having a hallucinogenic component (Dearnley et al., 2021; Di Giovanni & De Deurwaerdère, 2018; McLean et al., 2006; Zhang et al., 2017).

Hallucinogens such as this have been shown to be a favourable method for therapy as they seem to result in low levels of toxicity (depending on the drug used). Malcolm and Thomas (2021) have run and evaluation of multiple serotonergic psychedelics, of these Psilocybin was considered low risk in creating severe serotonin toxicity. Based on this information when we take into consideration Fox et al.'s (2010) values, TCB-2 (K_i= 0.73) has a lower binding affinity to the rat's 5-HT_{2A} receptor compared to the commonly used Psilocybin's (K_i= 13). We could then predict that its toxicity on the animals diminished, for the price of effectiveness.

With the resurgence of psychedelics as medical treatments for psychiatric disorders (Calvey & Howells, 2018; Murnane, 2018), it would be interesting to further investigate the effects of even more potent 5-HT_{2A}R agonists (e.g., psilocybin) as there's already extensive background

research on its beneficiary effects on Alzheimer's dementia and its correlated depressive symptoms (Forester et al., 2022; Vann Jones & O'Kelly, 2020).

Conclusion

The study presented hopes to have expanded the current literature on the effects of 5-HT_{2A}R agonists, most specifically showing that using a novel method for administering 5mg/kg of TCB-2 renders for interesting results on AD neuropathology while also not compromising the welfare of the animal when fed at a semi-chronic pace. Although the role of serotonin in learning and memory remains somewhat of a polarizing topic in the field, we have shown here that there's potential for 5-HTR agonists to be used as medical treatments to prevent the development of AD neuropathology. More research should be done on the effects of 5-HT_{2A}R agonists and their neuropharmacological effects, but not just regarding systems that are directly associated with memory but to also investigate their influence on memory modulators. Because of the complex and intertwined relationships between psychiatric disorders, it is recommended that one takes a more comprehensive approach while studying them, so as to contribute with methods to prevent at least one if not either's development.

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Appendix A

The current research has administered the TCB-2 drug only via intra-peritoneal (ip) or intra- cerebroventricular (icv) injection (Afshar, 2019; Zhang et al., 2017). Both methods pose an

inorganic invasion of the animal's body. These are also very stressful practices that could induce responses which might affect the results of this project; therefore, they will be avoided. Because there's no antecedent research on the administration of this drug via food, a pilot study was completed in order to test if the mice would successfully absorb the drug through their stomach and to test which dose was most effective. Fox et al. (2010) characterized two major side effects following the activation of 5-HT_{2A}R: head twitches (HTs), and hypothermia. These behaviours were also observed by Haberzettl et al. (2014). The focus will be on the observation of HTs after the ingestion of the drug as it will allow for a non-invasive interpretation that the drug is taking effect. This evaluation was conducted on a small cohort of C57BL/6 mice as seen in Table 2.

Table 2. *Dose Evaluation Study Mice Cohorts*

Strain	Sex	Dose (mg/kg)
C57BL/6	M	2.5
	F	2.5
	M	5.0
	F	5.0
	M	10.0
	F	10.0

Methods

After being habituated to the Nutella® vehicle (0.25g) for 5 days, each animal received their assigned dose of TCB-2 as seen in Table 2. In total, the administration of the drug lasted 7 consecutive days to test for its short-term effects on the welfare of the animals. These mice were communally housed throughout the investigation to decrease the effects of stress by social isolation. We kept track of weight changes, drug intake and reaction times (marked by the

appearance of HTs) across administration days to study which dose was most appropriate for the consecutive thesis work.

In conjunction, because no research has been done on the delivery of this drug through the gastrointestinal (GI) tract, we tested whether this would be a fitting method of delivery which would still result in Fox's *et al.* (2010) marker of behaviour for the activation of 5-HT_{2A}Rs, namely the appearance of head-twitches. Data presented as mean ± SD.

Results

The weights of the animals across the 7-day dose evaluation study remained constant and did not reach unhealthy levels, these trends can be observed in Figure 7 which represents the effects of 2.5 (n = 2, 30.0 ± 0.47), 5.0 (n = 2, 26.8 ± 0.50) and 10.0 (n = 2, 29.7 ± 0.69) mg/kg of TCB-2 on the previously specified dependent variable. Because the weights barely changed, the same consistent trend can be observed in Figure 8 regarding the drug intake comparison across the 2.5 (n = 2, 15.0 ± 0.23), 5.0 (n = 2, 26.8 ± 0.51) and 10.0 (n = 2, 59.5 ± 1.38) mg/kg groups.

The same cannot be said for the patterns of reaction times to the drugs seen in Figure 9 which show dose-dependent appearance of HTs. On average, it resulted that as the dose increases, the reaction times slightly slow down as seen across the 2.5 (n = 2, 12.5 ± 2.43), 5.0 (n = 2, 13.6 ± 4.20) and 10.0 (n = 2, 14.4 ± 3.82) mg/kg groups. On the first day, said HTs appear as soon as 14.5, 16 and 18 minutes since commencing feeding in the respective groups. The fastest reaction times appear on Day 4 with HTs beginning on average at 8.7 minutes across all groups. Conversely, Day 6 contains the slowest reaction times to the drug across groups, appearance of HTs averages at 16.7 minutes since the start of feeding.

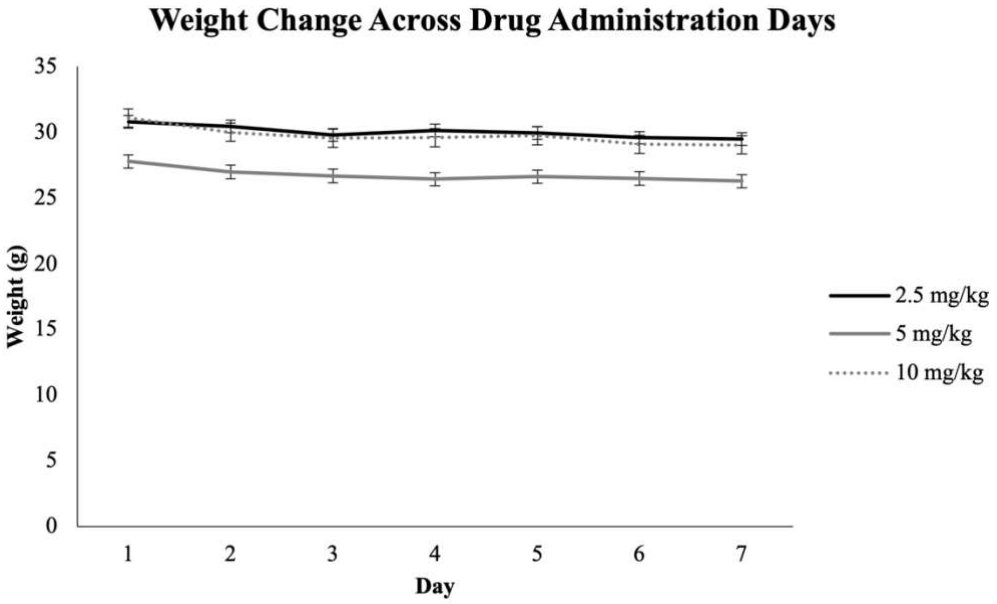

Figure 7. Comparison of weight change between groups across the TCB-2 dose evaluation study period on C57BL/6 mice. The animals' weights remained constant throughout the evaluation period showing that applying of 2.5 (n = 2, 30.0 ± 0.47), 5.0 (n = 2, 26.8 ± 0.50) and 10.0 (n = 2, 29.7 ± 0.69) mg/kg of TCB-2 for 7 consecutive days does not pose physiological threats to the well-being of the animals.

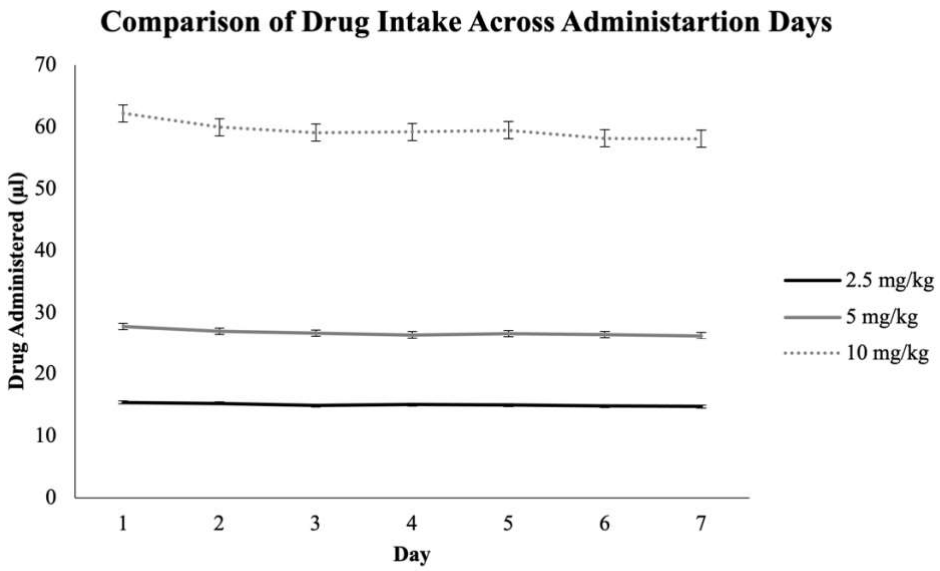

Figure 8. Comparison of drug intake between groups across the TCB-2 dose evaluation study period on C57BL/6 mice. The animals' drug intake amount didn't overly vary throughout the 7-day evaluation period. The amount of drug that is administered to each animal is dependent on their respective weights, so this variable as well showed consistent patterns in the 2.5 (n = 2, 15.0 ± 0.23), 5.0 (n = 2, 26.8 ± 0.51) and 10.0 (n = 2, 59.5 ± 1.38) mg/kg group.

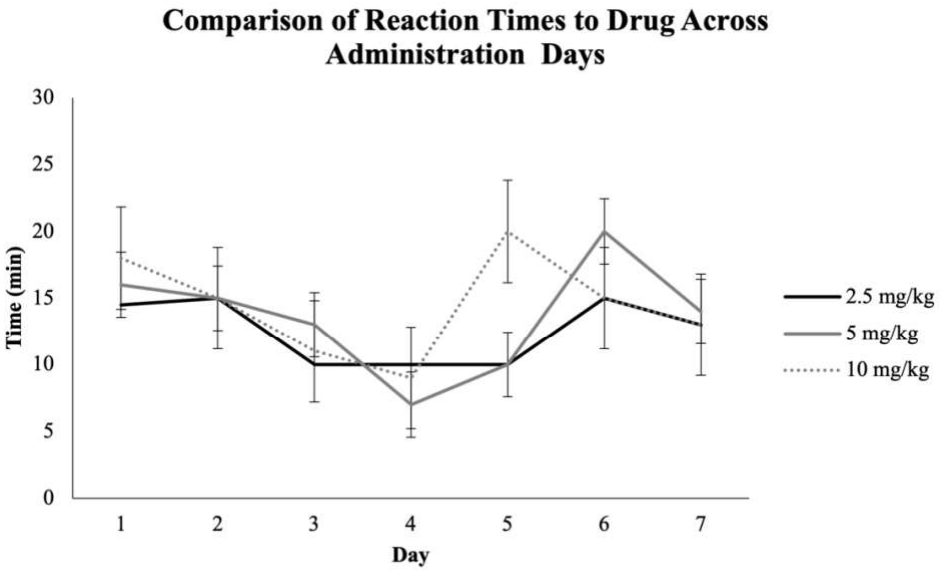

Figure 9. Comparison of reaction times (based on first appearance of HTs) between groups across the TCB-2 dose evaluation study period on C57BL/6 mice. On average, it resulted that as the dose increases, the reaction times slightly slow down as seen across the 2.5 (n = 2, 12.5 ± 2.43), 5.0 (n = 2, 13.6 ± 4.20) and 10.0 (n = 2, 14.4 ± 3.82) mg/kg groups. Day 4 results as the day with the fastest reaction times averaging at 8.7 minutes across all groups. Conversely, Day 6 contains the slowest reaction times across groups averaging at 16.7 minutes.

Discussion

This dose-dependent study was done to evaluate which dose would be most suitable to be used in the actual thesis' project. Additionally, it was run with the intention to test the absorption of the drug through the GI tract. To our knowledge, this is the first attempt at administering the drug through such a method due to the drug's novelty. Figure 7 in conjunction with Figure 8 show that the drug does not pose any life-threatening effects on the mice's physiology when the drug is administered for 7 consecutive days.

Regarding reaction times, in Fox et al.'s (2010) paper it was mentioned that by i.p injection head-twitches occur after 30 mins; this effect is proof that the drug is working with the investigated receptors (5-HT_{2A}). However, as can be seen in Figure 9, HTs appeared at 20 minutes at the latest since commencing feeding. This is definitely an unusual result when we take into consideration that most drugs act faster if injected into the bloodstream and are often degraded in the stomach (Bunonyo et al., 2022). Although because we used a 5-HTR agonist and based on Kim and Camilleri's (2000) work most of the production of serotonin is made in the gut, it is possible that this relationship might be the underlying reason as to why we got such fast reaction times.

Interestingly, Day 1 which represents the first time the animals were dosed and therefore thought of as most sensitive to the drug, did not result in the quickest appearance of HTs. Instead, this was achieved on Day 4, with an overall reaction time of 8.7 minutes following feeding. 5-HTR agonists can be quite potent and provide long-term effects even after one dose (Aday et al., 2020), meaning that these receptors can be easily over-saturated by continuous administration of drugs. When we consider the fact that we administered this through the GI tract, which offers a slower metabolic rate than via injection, then it's worth considering alternating the feeding in a semi-chronic manner to accommodate for all these conditions.

Therefore, when the actual thesis project took place, we decided to feed the animals every 3 days (for the length of 30 days) to avoid possible over-saturation of the 5-HTRs and decrease the chances of habituation to the drug's effects. This pace of delivery would allow the animals to recover from any additional stress to their bodies, allowing for weight maintenance throughout the actual project's timeframe. Regarding which dose amount might be most appropriate, we've decided to move forward with the administration of 5mg/kg. This is because there was no real difference between 5 and 10 mg/kg; however, as a precaution we've decided to go with the former. Because we didn't know the extent of the effects of these doses on AD neuropathology, in case the smaller dose was sufficient at fighting the biomarkers then this method would prove less stressful for the animals receiving the treatment. It would simply be wiser to start small and then move up as more results are gathered from the subsequent study.

Because we are going with this intermediate dose, we also decided to increase the vehicle's (Nutella ®) amount to 0.35g, this was done to maintain the mixture between the vehicle and drug to a less viscous consistency which is more appetizing to the mice. Furthermore, the actual project will be done on the $APP^{NL\text{-}G\text{-}F}$ mouse model of AD, meaning that there might be

some neurobiological differences between this former model and the C57BL/6 strain on which this dose evaluation study was done on. However, it's due noting that the $APP^{NL\text{-}G\text{-}F}$ mouse is derived from the C57BL/6J strain which means that the observations made during this evaluation period could still hold true in the future study.

Conclusion

This dose evaluation study proved successful in demonstrating that administration of the 5-HT_{2A}R agonist known as TCB-2 did not pose life-threatening consequences on C57BL/6 mice when fed at a variety of doses (2.5, 5.0 and 10.0 mg/kg) through the GI tract. Additionally, as a method of delivery it proved effective at exhibiting HTs, the behavioural marker representative of 5-HTR activation described by Fox et al. (2010). As a precaution we decided to use the 5.0 mg/kg dose and alternate the feeding to every 3 days to avoid the over-saturation of these receptors in the subsequent thesis study. However, once the effects of this dose and pace of delivery are recorded, it could be possible to change these conditions in future studies.

Appendix B

The Ilastik 1.1.7 software has proven to be an effective method to quantify Aβ plaque and Microglia. However, because we sample six coronal HPC sections from each brain/animal, the system automatically outputs "Size.in.pixels" estimates (as csv files) for each individual section rather than per brain/animal. Because of this caveat, we wrote an R-code that would take those six individual csv outputs, group them together to form one single csv file per brain/animal and subsequently render the calculations for sum, count and average simpler and faster than

without this code. In case someone in the future wanted to use this method and tailor it to their respective measurements, the R-code is as such:

```
library(dplyr)
library(dslabs)
library(readr)

HPC1 <- read.csv("~/Documents/Spring 2022/NEUR - 4995/CSV Files
Iba1/7807_APPNLGF_6MO_Iba1_s1_HPC1_table.csv")
HPC2 <- read.csv("~/Documents/Spring 2022/NEUR - 4995/CSV Files
Iba1/7807_APPNLGF_6MO_Iba1_s2_HPC2_table.csv")
HPC3 <- read.csv("~/Documents/Spring 2022/NEUR - 4995/CSV Files
Iba1/7807_APPNLGF_6MO_Iba1_s2_HPC3_table.csv")
HPC4 <- read.csv("~/Documents/Spring 2022/NEUR - 4995/CSV Files
Iba1/7807_APPNLGF_6MO_Iba1_s2_HPC4_table.csv")
HPC5 <- read.csv("~/Documents/Spring 2022/NEUR - 4995/CSV Files
Iba1/7807_APPNLGF_6MO_Iba1_s2_HPC5_table.csv")
HPC6 <- read.csv("~/Documents/Spring 2022/NEUR - 4995/CSV Files
Iba1/7807_APPNLGF_6MO_Iba1_s2_HPC6_table.csv")

HPC1_pixels<- HPC1 %>% select(Size.in.pixels)
HPC2_pixels<- HPC2 %>% select(Size.in.pixels)
HPC3_pixels<- HPC3 %>% select(Size.in.pixels)
HPC4_pixels<- HPC4 %>% select(Size.in.pixels)
HPC5_pixels<- HPC5 %>% select(Size.in.pixels)
HPC6_pixels<- HPC6 %>% select(Size.in.pixels)
```

```
Brain_7807 <- left_join (HPC1_pixels, HPC2_pixels, HPC3_pixels, HPC4_pixels, HPC5_pixels, HPC6_pixels, by
="Size.in.pixels", suffix = c(".x", ".y"))


Sum_7807 <- sum(Brain_7807)
Count_7807 <- count(Brain_7807)
Mean_7807 <- (Sum_7807/Count_7807)


HPC_7807 <- Brain_7807 %>% dplyr::summarise(Sum = sum(Brain_7807), Count = count(Brain_7807), Mean =
((Sum_7807/Count_7807)))
row.names(HPC_7807) <- "7807"


write.csv(HPC_7807,"~/Documents/Spring 2022/NEUR - 4995/CSV Files Iba1/HPC_7807_Iba1.csv")
write.csv(Brain_7807,"~/Documents/Spring 2022/NEUR - 4995/CSV Files Iba1/Brain_7807.csv")
```