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Effects of a THC-based Alzheimer Therapy on the Neuron Loss, Neurogenesis, and Inflammation

submitted by

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Data Sheet

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Abstract

The rise in an aging population worldwide will cause an increase in the prevalence of Alzheimer's disease among older people. To date, approved treatments used to slow down the progression of cognitive symptoms and to reduce problematic behaviours in AD. However, no pharmaceutical approach has been shown to have a real clinical benefit to treat Alzheimer's disease progression. Recently, there has been spreading interest in the modulation of the endocannabinoid system (ECS) and the medical use of the bioactive components of cannabis plant as a potential therapeutic approach to treat Alzheimer's disease. Several *in vitro* and *in vivo* studies showed that targeting the endocannabinoid system with the usage of cannabinoids can be beneficial to alleviate key hallmarks of AD, including Aβ and tau aberrant processing, neuroinflammation, excitotoxicity, mitochondrial dysfunction, and oxidative stress. In this project, the effects of Tetrahydrocannabinol (THC) administration were investigated on the neuron loss, neurogenesis, expression of endocannabinoid receptor 1 and neuroinflammation in two different age groups of the Tg4-42 mouse model.

THC is an active constituent of cannabis and a partial agonist to endocannabinoid receptors appeared as a prominent candidate for the modulation of ECS. Tg4-42 mice develop severe hippocampal neuron loss and memory deficits correlating with intraneuronal Aβ expression with no plaque and neurofibrillary tangle formation. Thus, Tg4-42 is a valuable AD mouse model displaying key hallmarks of the sporadic AD.

A preventative treatment group received the daily injection of THC for six weeks starting at the age of 3 months was compared to a therapeutic group receiving same treatment starting at the age of 5 months. For all steps, vehicle-treated control groups were maintained for both experimental groups. In this present work, it could be demonstrated that the number of immature neurons increased in the dentate gyrus of Tg4-42 mice in the therapeutic treatment group, whereas not in the preventative treatment group. Moreover, the stereological analysis revealed the significant increase in the number of neurons in the dentate gyrus upon THC administration in the therapeutic group. In order to assess the impact of THC treatment on the expression of cannabinoid receptor 1 and neuroinflammation markers in the therapeutic group, DAB immunohistochemistry was performed. The present findings provide evidence for desensitization or internalization of CB1 receptor, the significant decrease in microgliosis, and the constant level in astrogliosis in the dentate gyrus of Tg4-42 mice upon THC administration.

Zusammenfassung

Dadurch, dass die Menschen weltweit eine steigende Lebenserwartung haben, wird es zu einer ansteigenden Prävalenz der Alzheimer-Erkrankung kommen. Aktuell zugelassene Medikamente werden eingesetzt, um das Fortschreiten der kognitiven Dysfunktion und Verhaltensdefizite positiv zu beeinflussen. Derzeit existieren keine zugelassenen Behandlungsansätze, um dieses Fortschreiten aufzuhalten oder die Erkrankung zu heilen.

Seit einiger Zeit steigt das Interesse am Endocannabinoidsystem (ECS) in Forschung und medizinischem Einsatz von Inhaltsstoffen der Cannabispflanzen als potenzieller Behandlungsansatz in der Alzheimer-Erkrankung. Sowohl In-Vivo-, als auch In-Vitro-Studien zeigen, dass Endocannabinoide in die Pathologie der Alzheimer-Erkrankung in Bezug zu Abeta- und Tau-Pathologie, Neuroinflammation, Zelltoxizität, mitochondrialer Dysfunktion und oxidativem Stress eingreifen können.

In diesem Projekt wird der Einfluss von Tetrahydrocannabinol (THC) in einem Modell für die sporadische Alzheimer-Erkrankung (Tg4-42) in zwei Behandlungsansätzen untersucht. Dazu wurde der Nervenzellverlust und die Neurogenese, als auch die Cannabinoid-Rezeptor-1-Expression (CB1) im Gyrus dentatus analysiert. THC als Inhaltsstoff der Cannabispflanze ist ein partieller Agonist der Cannabinoid-Rezeptoren und beeinflusst das ECS.

Tg4-42-Mäuse exprimieren intraneuronal menschliches Abeta, weshalb sie einen signifikanten Nervenzellverlust in der CA1 Region des Hippocampus zeigen. Dieser wird sowohl immunhistochemisch, als auch in Verhaltensversuchen, wie dem Morris-Water-Maze sichtbar, wodurch diese Mauslinie als Modell für die sporadische Alzheimer-Erkrankung anerkannt ist. Das THC wurde den Mäusen über 6 Wochen täglich mit einer Konzentration von 20 mg/kg KG intraperitoneal verabreicht. In einem präventiven Behandlungsansatz beginnt die Behandlung mit 3 Monaten, in einem therapeutischen Behandlungsansatz mit 5 Monaten.

Um den Einfluss von THC auf die CB1-Expression zu beurteilen, wurde die DAB-Immunhistochemie durchgeführt. Aktuelle Erkenntnisse zeigen eine Desensibilisierung der CB1-Rezeptoren, eine signifikante Verringerung der Mikrogliose und eine konstante Astrogliose im Gyrus dentatus der Tg4-42 durch die THC-Behandlung.

List of Abbreviations

Abbreviation	**Description**
2-AG	2-arachidonoyl glycerol
ABC	Avidin-biotin complex
AD	Alzheimer's disease
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
Aβ	Amyloid beta
BrdU	Bromodeoxyuridine
CA1	Cornu Ammonis 1
CB1	Cannabinoid receptor type 1
CB2	Cannabinoid receptor type 2
CBD	Cannabidiol
CNS	Central Nervous System
DAB	Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DCX	Doublecortin
dH2O	Distilled water
DG	Dentate gyrus
ECS	Endocannabinoid System
EtOH	Ethanol
FAD	Familial Alzheimer's disease
FCS	Fetal calf serum
FDA	Food and Drug Administration
GFAP	Glial fibrillary acidic protein
Hom	Homozygous
Iba1	Ionized Calcium-Binding Adapter Molecule 1
NFT	Neurofibrillary tangle
NSC	Neural stem cells
PBS	Phosphate buffered saline
PSEN	Presenilin
ROS	Reactive oxygen species
RT	Room temperature

SEM	Standard error of the mean
SGZ	Subgranular zone
SVZ	Subventricular zone
THC	Tetrahydrocannabinol
TRH	Thyrotropin-releasing hormone
Veh	Vehicle

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1 INTRODUCTION

1.1 Alzheimer’s disease

Alzheimer’s disease discovered in 1906 by Alois Alzheimer, a German neurologist, and psychiatrist in his patient Auguste D (Ramirez, 2012). Auguste D had memory loss, paranoia, and psychological problems (Alzheimer's Association, 2018). After her death, Dr. Alzheimer performed an autopsy. He found severe shrinkage of the cerebral cortex, fatty deposits in blood vessels, and atrophied brain cells. Besides that, he discovered neurofibrillary tangles and senile plaques which have become indicative of Alzheimer’s disease (Alzheimer's Association, 2018).

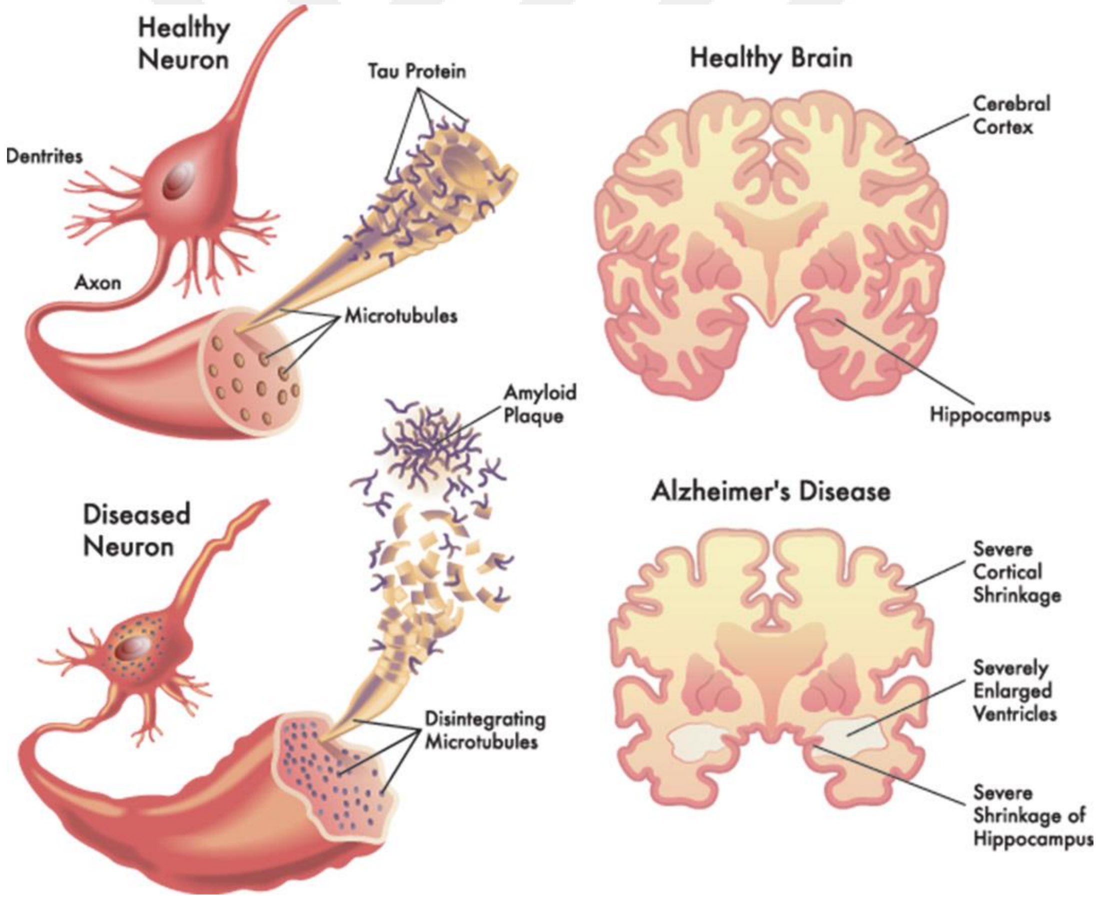


Figure 1: The progression of Alzheimer disease. Shrinkage of cortex and hippocampus, enlarged ventricles, amyloid plaque and tau protein deposition are clinical manifestations in AD brain. (Adapted from BrightFocus Foundation, 2018)

1.2 Clinical aspects of Alzheimer’s disease

1.2.1 Epidemiology

Alzheimer’s disease recognized by the World Health Organization as a global public health priority. It causes 60% to 70% of cases of dementia (Qiu, 2009). In the 2015 report, it was estimated that 46.8 million people worldwide were living with dementia. This number is estimated to double every 20 years, reaching 74.7 million in 2030 and 131.5 million in 2050 (World Alzheimer Report, 2015). By 2050, more than 13 million Americans are estimated to diagnose with Alzheimer's disease (AD), and health care costs are estimated approximately $1.2 trillion (Sibener, 2014).

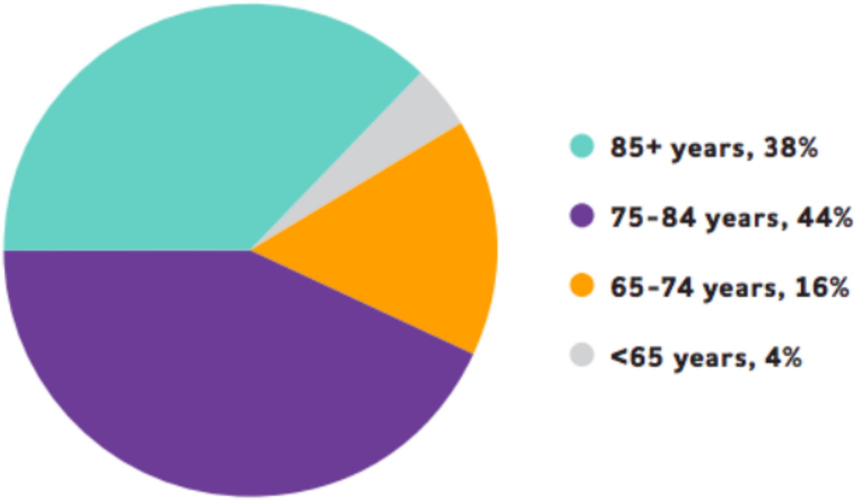


Figure 2: **Ages of people with Alzheimer’s Dementia in the United States, 2017.** (Adapted from Alzheimer's Association, 2017).

1.2.2 Risk Factors

Age is the biggest risk factor because the majority of people with Alzheimer’s disease are older than the age of 65 (Alzheimer's Association, 2018). Only 1-2% of Alzheimer’s cases occur due to known mutations. These mutations involve the gene for the amyloid precursor protein (APP) and the genes for the presenilin 1 and presenilin 2 proteins (PSEN1/2) (Bekris, 2010). A connection has been revealed between gene Apolipoprotein E (ApoE) and the occurrence of AD (Puglielli, 2003). ApoE is the principal cholesterol carrier in the brain.

One form of this gene, ApoE4, has been shown to increase the risk of developing AD (Holtzman, 2012). People who inherit one copy of the e4 form of the gene have a three times higher risk of developing Alzheimer’s compared with those with the e3 form. People who inherit two copies of the e4 form have an 8 to 12-fold risk (Michaelson, 2014). Cardiovascular disease risk factors are also related increasing the risk of AD (Luchsinger, 2004). These factors include smoking (Anstey, 2007), obesity in midlife (Loef, 2013), and diabetes type two (Vagelatos, 2013). There is an assumption that there may be a connection between the education level and the risk of developing AD. People with fewer years of education seem to have a higher risk than individuals with more years of formal education (Sando, 2008).

1.2.3 Disease Progression

The symptoms of Alzheimer's disease worsen over the time. However, the disease progression varies among individuals. An average patient lives four to eight years after their diagnosis (Alzheimer’s Association, 2018). Nonetheless, depending on other factors, survival time can be up to twenty years (Alzheimer’s Association, 2018). The stages of AD are separated into three different categories: mild Alzheimer's disease, moderate Alzheimer's disease, and severe Alzheimer's disease (Alzheimer’s Association, 2018). During the mild Alzheimer's disease stage, an individual can carry on independently. Despite that, an individual has mild but measurable changes in thinking abilities (Sperling, 2011). Moderate Alzheimer’s disease lasts two to ten years and this is the longest stage of AD. Patients often have a difficulty to remember, to express thoughts and to perform routine tasks (Mayo Clinic, 2018). During the final stage of disease, individuals have serious problems to carry on a conversation and to control their movement. Memory and cognitive skills are not enough to continue to a daily life. Significant personality changes may take place. Therefore, individuals need extensive help with daily activities. AD can cause complications such as immobility, swallowing disorders and malnutrition which increase the risk of death. Also, pneumonia is identified as a common cause of death among old people with AD. (Alzheimer’s Association, 2018).

1.2.4 Diagnosis

The only method to diagnose Alzheimer's disease is a brain autopsy. The current criteria for neuropathological confirmation of AD are based upon the presence of neurofibrillary tangle formation and the analysis of neuritic plaques (Braak and Braak, 1991). Without regard to that, physicians apply different approaches to make an accurate diagnosis. A medical and family history, recognizing cognitive and behavioural changes, making physical and neurological examinations, and utilizing brain imaging tools help physicians to make an accurate diagnosis of AD in 90% of all cases (BrightFocus Foundation, 2015). Structural imaging scans such as magnetic resonance imaging, computed tomography, and positron emission tomography can provide detailed information about the shape and volume of the brain (Braskie, 2014). In addition to imaging scans, there are different tests to assess mental status of a patient. Screening tests for AD include the Mini-Mental State Examination, Clock Drawing Test, and Cambridge Cognitive Examination (Woodford, 2007).

1.2.5 Treatment

Unfortunately, there is no cure for Alzheimer's disease but there are pharmaceutical treatments available to diminish symptoms. So far, there are two different types of drugs approved by the FDA: acetylcholinesterase inhibitors (rivastigmine, galantamine, donepezil) and N-methyl D-aspartate receptor antagonist (memantine). These medications have a small effect on symptoms of Alzheimer's disease (Matsunaga, 2015). No pharmaceutical treatment has been clearly shown to have real clinical benefit to prevent disease progression since these medications don't target pathogenic processes in AD (Kumar, 2015).

1.3 Neuropathological Hallmarks

1.3.1 Amyloid plaques

The deposition of neurotoxic amyloid-β (Aβ) peptides between neurons in the brain is one of the key hallmarks in Alzheimer's disease pathogenesis (BrightFocus Foundation, 2015). Despite the huge effort to identify molecular mechanisms of how Aβ involves disease progression remain unclear. Aβ peptides are produced by processing of the type I single transmembrane amyloid precursor protein (APP) by two proteases called β- and γ-secretase

(Wirths, 2107). Based on their morphology, amyloid plaques are divided into two groups: neurotic and diffuse plaques (Gouras, 2015).

1.3.2 Neurofibrillary tangles

Neurofibrillary tangles (NFTs) are another key histopathological hallmark of AD. Microtubule-associated protein tau was discovered as the main constituent of NFTs. Tau is aberrantly misfolded and abnormally hyperphosphorylated (Serrano, 2011). Various studies have shown that the amount and distribution of NFTs correlate with the severity and the duration of AD. (Gómez-Isla, 1997; Giannakopoulos, 2003; Ingelsson, 2004). Despite accumulated knowledge about tau biology, the molecular mechanisms of how tau involves AD pathogenesis remain to be elucidated.

1.3.3 Inflammation

Inflammation is observed pathologically in AD brain (Akiyama, 2000). Astrocytes, microglia, the complement system, cytokines and, chemokines are seen in the inflammatory process of the brain (Bouter, 2014). Activated microglia was observed in abundance in post-mortem AD brains (Frank-Cannon, 2009). Neuroinflammation was asserted as a mediator of neurodegeneration in AD which contributes to cell damage and neuron loss (Sardi, 2011). Damaged neurons, insoluble amyloid β peptide deposits, and neurofibrillary tangles may act as stimuli for inflammation in AD brain (Akiyama, 2000).

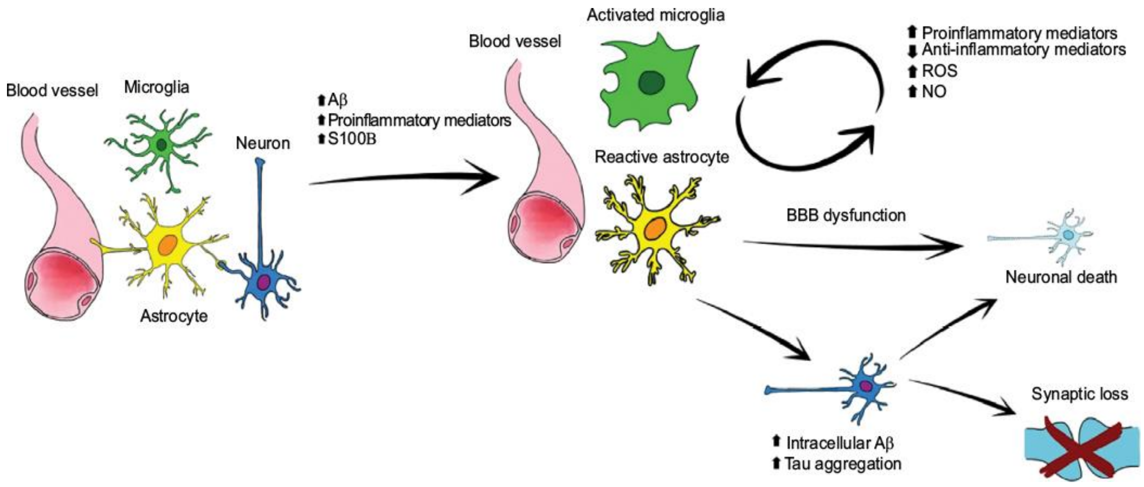


Figure 3: Illustration of the neuroinflammatory processes in AD. The presence of tau protein and amyloid plaques can induce activation of microglia and astrocytes. Activation of these cells leads to secretion of pro-inflammatory cytokines and reactive oxygen species which trigger synaptic loss and neuronal death. (Adapted from Bronzuoli, 2016)

1.3.4 Neuron Loss

Neuron loss is another major pathological hallmark observed in AD brain. The clinical signs of the disease are associated with the extent of neuronal loss in AD brain, notably in the hippocampus and cerebral neocortex (Niikura, 2006). Reduction in the number of neurons in AD brain, especially in hippocampal CA1 and the entorhinal cortex, relate to the severity of memory deterioration (Giannakopoulos, 2003). Stereology analysis demonstrated a significant neuron loss in the entorhinal cortex of patients with mild Alzheimer’s disease (Gómez-Isla, 1996). Neuron death is observed in normal aging. However, this process is more intense in Alzheimer’s disease particularly in the following regions of the brain: the precentral and postcentral gyri, parietal and temporal associative areas of the cortex, subiculum, and hippocampal field CA1 (Mukhin, 2017).

1.3.5 Neurogenesis

Neurogenesis is the process which new neurons emerge in the brain as a result of neural stem cell division (Kolos, 2016). It ends in most of the brain regions after birth. Per contra, there are two main sites in the adult mammalian brain where neural progenitor/stem cells present, the subventricular zone (SVZ) and the subgranular zone (SGZ) in the dentate gyrus of the hippocampus (Bond, 2015). Neural progenitor/stem cells present in the adult mammalian brain and contribute to brain plasticity (Kempermann, 1999). Data obtained from different transgenic mouse models of AD about postnatal neurogenesis in AD remain controversial. Some studies which used transgenic animals expressing the mutant amyloid precursor protein showed decreased neurogenesis either in the DG or in both the DG and the SVZ (Feng, 2001; Wang, 2004; Donovan, 2006; Zhang, 2007). On the other hand, some other studies showed increased neurogenesis in the adult hippocampus of APP-expressing transgenic mice (Jin, 2004; Chevallier, 2005; Toledano, 2007). Evidence from the rodents increased the knowledge of the neurogenesis biology. However, adult hippocampal neurogenesis in humans remains controversial. In 1998, Fred Gage demonstrated the existence of neurogenesis in the adult human brain (Gage, 1998). After that, this report was supported by neural stem cells isolation from the adult human brain (Palmer, 2001; Nunes, 2003). Currently, multiple studies have presented contradictory evidence about the alteration of postnatal hippocampal neurogenesis in humans. Boldrini and colleagues assessed the hippocampal neurogenesis in post-mortem brains from healthy human individuals ranging from 14 to 79 years of age. They found similar numbers of neural progenitor cells, immature neurons and mature neurons in the dentate gyrus of the hippocampus across ages (Boldrini, 2018). On the other hand, Sorrells and colleagues evaluated the extension of changes in hippocampal neurogenesis in post-mortem samples and surgical resection samples. They showed that the number of proliferating neural stem cells and young neurons in the dentate gyrus decreased sharply after 1 year from birth. During the age of 7 and 13, only a few young neurons were observed (Sorrells, 2018). The findings of alterations in postnatal neurogenesis correlated with AD are not in agreement. More systematic studies are required for further clarification.

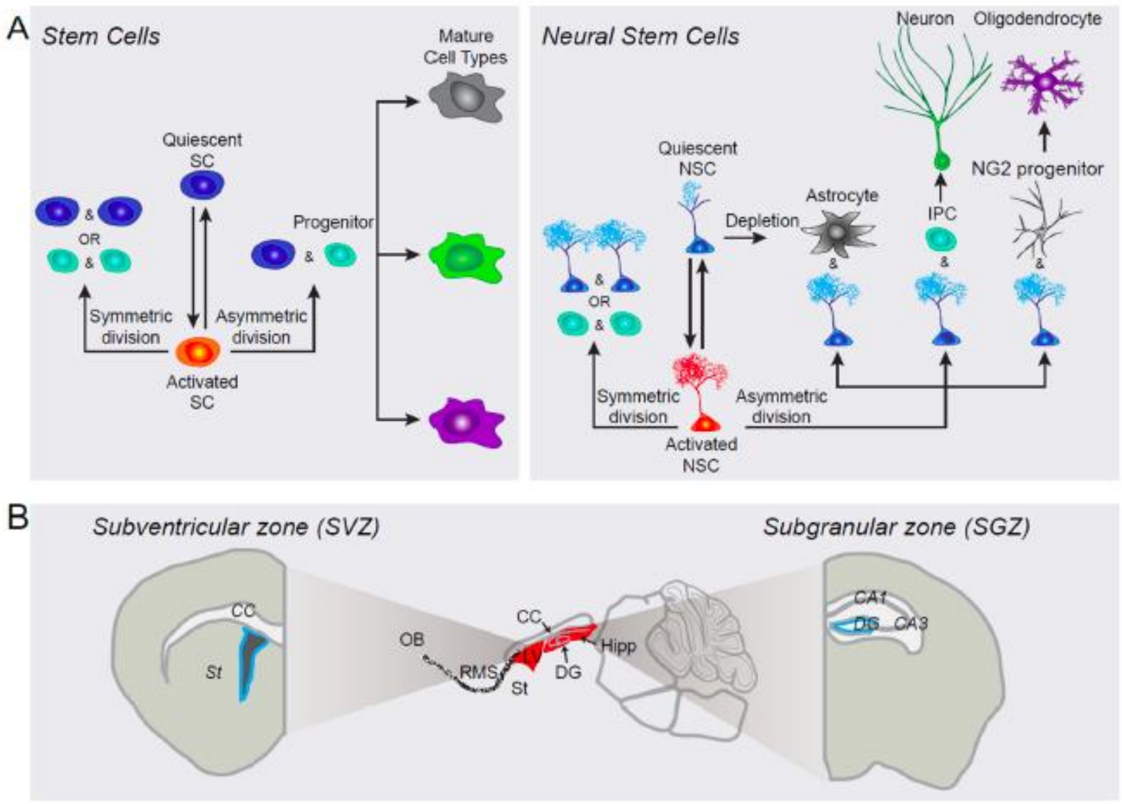


Figure 4: Behaviour of neural stem cells in adult niches. (A) A schematic diagram showing the potential life cycle of an adult stem cell, particularly adult neural stem cell (NSC). (B) A sagittal depiction of the adult rodent brain, focusing on two main niches where adult NSCs reside: the subventricular zone and the subgranular zone. The SVZ is located along the lateral ventricle in the forebrain, while the SGZ is located in the hippocampus along the dentate granule cell layer where it abuts the hilus. CC, corpus callosum; DG, dentate gyrus; Hipp, hippocampus; LV, lateral ventricle; NSC, neural stem cell; OB, olfactory bulb; RMS, rostral migratory stream; SC, stem cell; St, striatum. (Figure adapted from Bond, 2015), Copyright permission)

1.4 Hippocampus

Hippocampus is an extension of the temporal part of the cerebral cortex. It consists of two major parts: Cornu ammonis and dentate gyrus. Based on histology, Cornu ammonis is divided into four parts: CA1, CA2, CA3, and CA4 (Anand, 2012). The hippocampus is a part of the limbic system and is considered playing vital roles in the information processing from short-term memory to long-term memory, in learning and in the spatial memory (Morgado, 2011). The dentate gyrus contains three layers of neurons: molecular, granular, and polymorphic. The unique neuroanatomy of the dentate gyrus carries receives information from the entorhinal cortex and conveys to the CA3 field of the hippocampus (Amaral, 2007). The dentate gyrus is one of the special regions in the mammals' brain where the neurogenesis occurs during the adult life (Bonfanti, 2011). Atrophy of the hippocampal region is one of the characteristic features of AD. The hippocampus is vulnerable to Alzheimer's disease because it is the earliest brain region affected in the course of AD (Schuff, 2009).

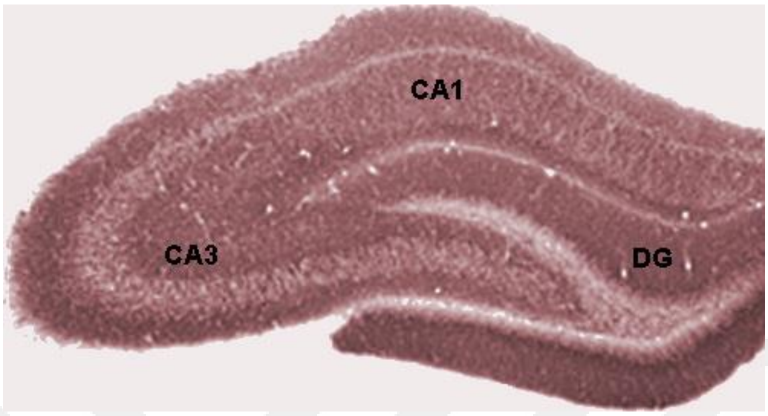


Figure 5: Diagram of the hippocampal regions in a rat brain.

1.5 Tg4-42 mice: the transgenic mouse model of Alzheimer's disease

Although existing models for AD cannot reproduce the complete spectrum of AD pathology, genetically modified animal models improve the understanding of the underlying mechanisms of the disease (LaFerla, 2012). Most AD cases are sporadic and the etiology remains unknown. The main criticism about conventional AD mouse models is the usage of genetic mutations related with the familial AD. APP and PS1 mutations are familial AD mutations seen in a minority of AD patients. In contrast, Tg4-42 model is the model to express N-truncated human Aβ4-42 without any other mutation with severe neuron loss and behavioural deficits. The Tg4-42 model produced the peptide Aβ4-42 which is one of the N-terminally truncated Aβ species found in the AD brain (Bouter, 2013). Also, it was demonstrated that Aβ4-42 is abundantly expressed among different Aβ species (Lewis, 2006). The Tg4–42 model does not develop extracellular amyloid plaques still the secreted Aβ4-42 forms soluble and neurotoxic aggregates (Bouter, 2013). Tg4-42 homozygous mice displayed intracellular Aβ beginning at two months of age (Bouter, 2014). In the line with this, Tg4-42 mice displayed elevated gliosis in the hippocampus starting from two months of age (Bouter, 2014). Accumulation of Aβ42 might induce neuron loss (Wirths & Bayer, 2012). Stereological characterization of Tg4-42 homozygous mice significantly showed neuron loss after four months of age. At the age of eight months, 65% neuron loss in homozygous Tg4-42 mice were observed (Bouter, 2014). Severe CA1 pyramidal neuron loss and reactive gliosis in the hippocampus of Tg4-42 mouse model correlated with intraneuronal Aβ4–42 expressions (Bouter, 2014).

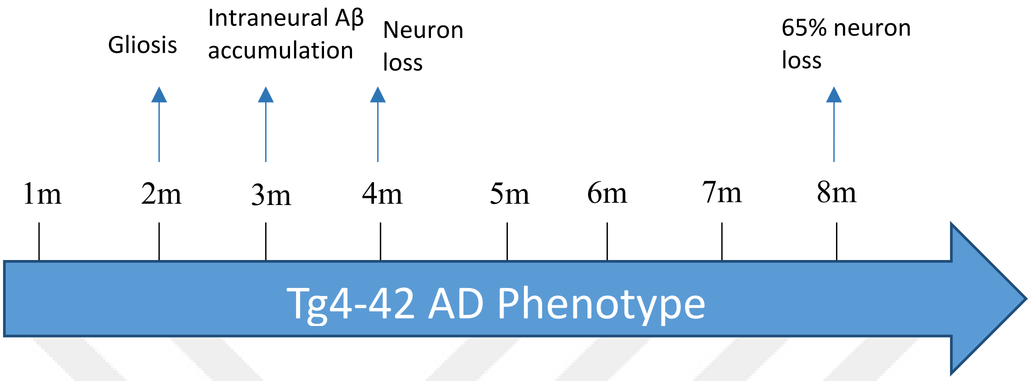


Figure 6: Schematic illustration of the AD-related phenotypes in homozygous Tg4-42 mice.

1.6 Endocannabinoid system

The ECS is composed of cannabinoid receptors, endocannabinoids, and the enzymes for the synthesis and degradation of the endocannabinoids. ECS is important neuromodulatory system that has impacts on central nervous system (CNS) development and synaptic plasticity (Lu, 2015). Endocannabinoids are lipophilic molecules synthesized from lipid membrane precursors (Piomelli, 2003). Endocannabinoids are generated naturally in the body. The best-studied ones are anandamide and 2-arachidonoyl glycerol (2-AG). The impacts of endocannabinoids are transmitted by CB1 and CB2 cannabinoid receptors expressed in the brain and immune system. Both CB1 and CB2 cannabinoid receptors are G protein-coupled receptors (Howlett, 2002). CB1 receptors are highly expressed in the central nervous system especially in the cortex, cerebellum, basal ganglia, and hippocampus (Mackie, 2005). CB1 receptors act as a regulator of excitatory and inhibitory neurotransmitter release and psychoactivity (Sanchez, 2012). Endocannabinoids are released by a depolarized neuron bind to CB1 receptors on pre-synaptic glutamatergic and GABAergic neurons resulting in a respective decrease in either glutamate or GABA release (Elphick, 2001). CB1 receptors involve regulation of brain functions such as cognition, memory, emotion and motor control (Howlett, 2005). In studies with CB1 receptor knockout mice, increases in anxiety, depression, and aggression were observed (Uriguen, 2004). CB2 receptors are expressed in the peripheral immune system and the central nervous system. However, CB2 receptors are expressed at lower levels in the CNS compared to CB1 receptors (Fagan &Campbell, 2014). This receptor is primarily found in microglia and astrocytes during neuroinflammation (Walter, 2003). Endocannabinoids are rapidly eliminated after their action. Degradation of anandamide is carried out by fatty acid amide hydrolase and 2-AG is metabolized by monoacylglycerol lipase (Fagan & Campbell, 2014).

1.7 Targeting Endocannabinoid system with THC in Alzheimer's disease

Targeting endogenous cannabinoid system is perceived as potentially promising option to treat neurodegenerative diseases. Accumulating evidence has shown that endocannabinoids may have a beneficial impact on neurodegenerative and neuroinflammatory diseases (Bedse, 2015). There are different exogenous ligands to CB receptors. These components are either derived from the Cannabis sativa plant or synthetically produced CB receptors' agonists and antagonists (Fagan &Campbell, 2014). Delta9-tetrahydrocannabinol (THC) is a well-known example of CB receptors' partial agonist which is the active constituent of cannabis. The therapeutic use of THC has gained serious attention during the last decade, providing a better understanding of its mechanism of action. Dronabinol and nabilone are synthetic forms of THC approved by FDA. They are used to treat nausea and vomiting related to cancer chemotherapy and anorexia related to weight loss and AIDS (Benyamina, 2014). Tetrahydrocannabinol's potential has been showed to control seizures in experimental animal models of epilepsy (Wallace, 2002).

1.8 Project objectives

Lack of effective treatments for Alzheimer's disease emphasizes the importance of finding new therapeutic approaches. In recent years, targeting the endocannabinoid system gained serious interest as a potential treatment approach for AD because the modulation of endocannabinoid system has been demonstrated to involve the reduced oxidative stress, neuroinflammation, and amyloid plaques (Aso, 2014; Ahmed, 2015).

The aim of this study is to investigate the potential of preventative and therapeutic approaches of THC treatment in the sporadic mouse model of Alzheimer's disease. In this project, the neuroprotective abilities of THC were investigated on the neuron loss, neurogenesis, and inflammation in the two different age groups of the Tg4-42 mouse model. This model develops severe hippocampal neuron loss and memory deficits correlating with intraneuronal Aβ expression without any plaque and neurofibrillary tangle formation (Bouter, 2013). A preventative approach was compared to a therapeutic approach in Tg4-42 mice. For this purpose, one experimental group mice received daily injected of Tetrahydrocannabinol (THC) for six weeks starting at the age of 3 months. Another experimental group received same treatment starting at the age of 5 months. At the age of two months, Tg4-42 mice displayed increased astrogliosis in the hippocampus, allowing us to evaluate the potential of THC to reduce neuroinflammation. Moreover, transgenic animals showed mild cognitive deficit due to neuron loss starting at the age of 4 months (Bouter, 2014). Therefore, this enables us to compare potential benefits of THC administration at different time intervals to find out best time point for the effectiveness of THC. For post-testing, animals were sacrificed at the age of 6,5 months and brain tissue was collected. Doublecortin and Ki67 markers were used in immunohistochemical staining to assess effects of THC on neurogenesis. GFAP and IBA1 markers were used to examine the impacts of THC treatment on inflammation in Tg4-42 mice. Unbiased stereology was applied to quantify neuron number to analyse the effect of THC based therapy in the dentate gyrus of Tg4-42 mice. For the duration, vehicle-treated control groups were maintained for both experimental age groups.

The purposes of the study can be summarized as follows:

Objectives of Project

- ✓ Examine the effects of THC based therapy on neurogenesis in Tg4-42 mice
- ✓ Verify the impacts of THC treatment on inflammation in Tg4-42 mice
- ✓ Determine the extent ofneuron loss in dentate gyrus with THC based therapy in Tg4-42 mice
- ✓ Test the changes of cannabinoid receptor type 1 in Tg4-42 mice with THC treatment

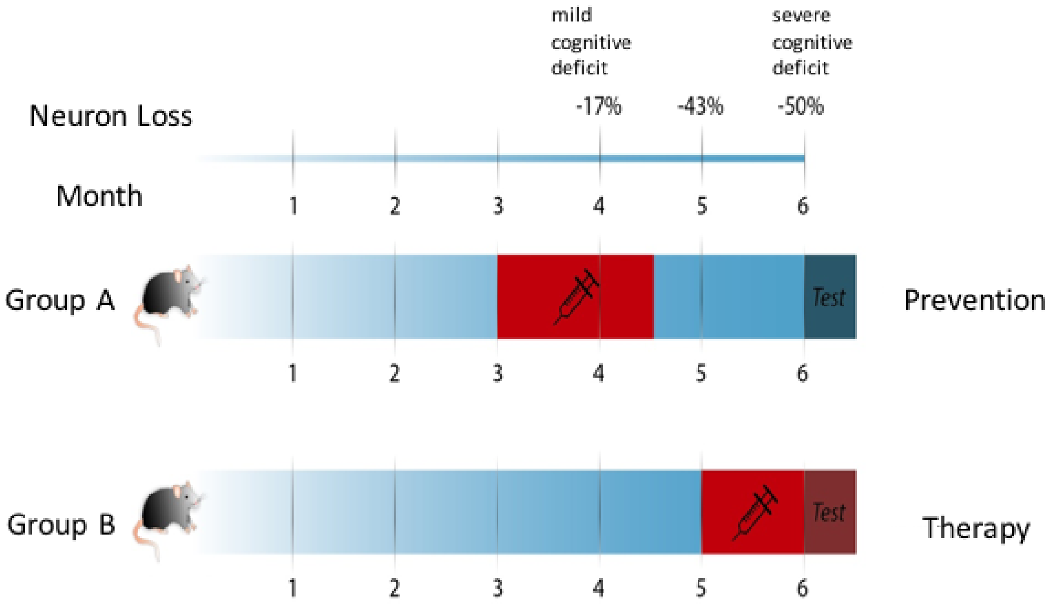


Figure 7: Illustration of animal's age in THC administration. (Generated by Yvonne Bouter)

2 MATERIAL AND METHODS

2.1 Chemicals, reagents

Table 1: **Chemicals and reagents**

Chemicals	**Manufacturer**
Acetic Acid	Merck, Darmstadt, Germany
Citric acid	Roth, Karlsruhe, Germany
Cresyl Violet	Merck, Darmstadt, Germany
DAB peroxidase substrate kit	Vector Laboratories, Burlingame, USA
Distilled water (ddH$_2$O)	B.Braun Melsungen, Germany
Ethanol	Roth, Karlsruhe, Germany
Fetal calf serum (FCS)	Roth, Karlsruhe, Germany
Fluorescence mounting medium	Dako, Agilent, Santa Clara, CA, USA
Formic acid	Roth, Karlsruhe, Germany
Hematoxylin	Roth, Karlsruhe, Germany
Hydrogen Peroxide	Roth, Karlsruhe, Germany
Isopropanol	Roth, Karlsruhe, Germany
Milk powder	Roth, Karlsruhe, Germany
Mounting medium	Roth, Karlsruhe, Germany
Natrium Acetate Trihydrate	Roth, Karlsruhe, Germany
Roti®Histokitt mounting medium	Roth, Karlsruhe, Germany
Tetrahydrocannabinol (THC)	THC Pharm, Frankfurt
Triton X-100	Roth, Karlsruhe, Germany
Tween 20	Roth, Karlsruhe, Germany
Vectastain Elite ABC Kit	Vector Laboratories, Burlingame, USA
Xylol	Roth, Karlsruhe, Germany

2.2 Technical Devices

Table 2: Technical devices

Device	Manufacturer
Centrifuge	Thermo Fisher Scientific, Waltham, USA
HM 335E Microtome	Microm, Germany
Microscope (Olympus Bx51)	OLYMPUS EUROPA, Hamburg, Germany
Microscope cover glasses	Thermo Fisher Scientific, Waltham, USA
Lipid Pen (Pap Pen)	Kisker Biotech, Steinfurt, Germany
Superfrost® glass slides	Thermo Fisher Scientific, Waltham, USA
Water bath for mounting of paraffin tissue	Medax, Olching, Germany

2.3 Antibodies

2.3.1 Primary antibodies

Table 3: Primary antibodies

Antiserum	Host	Isotope	Working dilution	Manufacturer
Doublecortin	rabbit	polyclonal	1:4000	Synaptic Systems
Ki67	rabbit	polyclonal	1:1000	Abcam
GFAP	guinea pig	polyclonal	1:1000	Synaptic Systems
CB1	rabbit	polyclonal	1:2000	Abcam
IBA1	guinea pig	polyclonal	1:1000	Synaptic Systems
Solanezumab biosimilar Aβ	human	monoclonal	1:2500	from Luke Miles, University of Melbourne

2.3.2 Secondary antibodies

Table 4: Secondary antibodies

Antiserum	Host	Isotope	Working dilution	Source
rabbit	goat	polyclonal	1:200	Dianova
guinea pig	goat	polyclonal	1:200	Dianova

2.3.3 Fluorescent antibodies

Table 5: Fluorescent antibodies

Antiserum	Host	Isotope	Working dilution	Fluorophore	Manufacturer
rabbit	goat	polyclonal	1:750	594 Dylight	Thermo
guinea pig	goat	polyclonal	1:750	488 Dylight	Thermo
human	donkey	polyclonal	1:750	594 Dylight	Thermo

2.4 Transgenic mice

Tg4-42 mice were generated in Prof. Bayer's lab. Tg4-42 mice express human Aβ4-42 fused to the murine thyrotropin-releasing hormone (TRH) signal peptide under the control of the neuronal Thy-1 promoter on a C57Bl/6J genetic background (Bouter, 2013). For this project, only homozygous Tg4-42 mice were used. To assess the hippocampal neurogenesis and the total number of neurons in the dentate gyrus, mix-gender and age-matched animals were used. In all immunohistochemistry experiments, only aged-matched female mice were used.

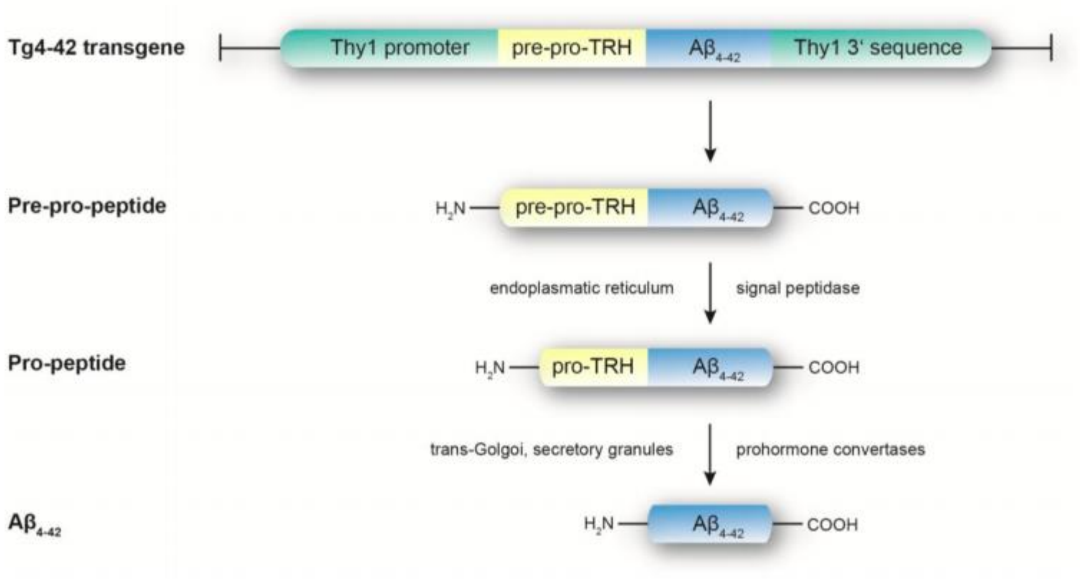


Figure 8: Diagram of Tg4-42 transgene. The murine Thy1 promoter makes the neuronal expression of the pre-pro-TRH-Aβ4-42 fusion peptide. Aβ4-42 is fused to pre-pro-TRH. An N-terminal signal sequence leads the pre-pro-TRH-Aβ4-42 fusion peptide into the endoplasmic reticulum. In the trans-Golgi network and secretory granules, the remaining of TRH signal peptide is cleaved by prohormone convertases. Aβ4-42 is formed and can be released from the cell. (Figure generated by Bouter, 2014)

Table 6: Number of animals per age and treatment group

Tg4-42 / 3 months / THC n=12	Tg4-42 / 5months / THC n= 10
Tg4-42 / 3 months / Vehicle n= 10	Tg4-42 / 5months / Vehicle n= 14

2.5 THC treatment

THC (20 mg/kg) and vehicle (5% ethanol, 5% Tween80) solutions were administered interperitoneally. Starting at 3 months and 5 months of age, Tg4-42 mice either received daily THC or vehicle treatment for 6 weeks. (performed by Marius Sichler)

2.6 Immunohistochemistry

2.6.1 Brain Slice Preparation

Brain tissues were embedded in paraffin. Paraffin-embedded brains were cut into 4 µm sagittal sections by using a HM 335E microtome (Leica). The sections were transferred into the cold-water bath to be placed onto Superfrost slides (Thermo Fisher Scientific). The glass slides were taken into a 45-50°C hot water bath. Then, slides were dried on a hot plate (Medax) for 20 minutes and overnight at 37 °C.

2.6.2 Immunohistochemistry on the paraffin section

For immunohistochemistry, sections were deparaffinized in xylene for 10 min. Then, sections are hydrated in a series of ethanol (100% EtOH for 10 min, 95% EtOH for 5 min, 70% EtOH for 1 min). After treatment with 30% H2O2 in PBS for 30 min to block peroxidases, antigen retrieval was done by boiling sections in 0.01 M citrate buffer pH 6.0 in a microwave for 10 min (800 W until boiling followed by 80 W for 8 min), followed by 15 min cool down. Afterward, sections were permeabilized with 0.1% Triton in 0.01 M PBS for 15 min. Sections were washed for 1 min in 0.01 M PBS, followed by 3 min treatment with 88% formic acid. 2 x 5 min washing steps with 0.01 M PBS were performed to remove formic acid. Sections were circled with a lipid pen (Pap Pen; Kisker Biotech) incubated for 1 hour at RT with a solution of 10% fetal cow serum and 4% milk powder in 0.01 M PBS to block unspecific antigens. Primary antibodies were diluted in 10% fetal calf serum in 0.01 PBS and were incubated overnight in a chamber at room temperature. Next day, sections were washed with 0.1% Triton in 0.01 M PBS three times for 5 min and with 0.01 M PBS for 1 min. After washing, the sections were incubated with biotinylated secondary antibodies at 37 °C for 1h in a chamber. Secondary antibodies were diluted with 0.01 PBS containing 10 % FCS. After washing sections with 0.01 MPBS for 15 min, Avidin-biotin complex (ABC) solution was prepared according to the instructions of the manufacturer using the Vectastain ABC Kit (Vector Laboratories). The ABC solution was incubated for 30 min at 4 °C before using. The sections were washed three times for 5 min with 0.01 M PBS. Then, each section was incubated for 1.5 h at 37 °C with 100 µl of ABC solution. After incubation, the slides were washed three times for 5 minutes with 0.01 M PBS to remove the ABC solution. Staining was visualized using DAB. The DAB solution was prepared by mixing 100 µl DAB stock solution (25 mg/ml DAB in 50 mM Tris/HCl) with 5 ml 50 mM Tris/HCl and 2.5 µl 30% H2O2. The sections were washed 3 x 5 min in 0.01 M PBS. Sections were placed under tap water and washed for 5 min. Then, sections were dehydrated in a series of ethanol and xylene (70% EtOH for 1 min, 95% EtOH for 5min, 100% EtOH for 10 min, 10 min xylol). Finally, stained slides were mounted with using Roti®-Histokitt mounting medium. Then, a coverslip was added to each slide. Bright field images of stained tissue were obtained using a BX-51 microscope equipped with a Camera (Olympus).

2.6.3 Free-floating immunohistochemistry

Frozen brain was cut on the cryostat (Leica) frontally in a series of 30 µm thick sections. Firstly, brain sections were hydrated for 10 min in cold 0.01 M PBS. Then, sections were transferred to 30% H2O2 in 0.01 M PBS for 30 min to block peroxidase activity. Sections were washed 3 x 10 min in 0.01 M PBS containing 0.1% TritonX-100 for membrane permeabilization. Unspecific blocking was done by treatment with 0.01 M PBS containing 10% FCS and 4% milk powder for 1 h at RT. Primary antibody was diluted in 0.01 M PBS with 10% FCS and was incubated overnight at RT. On the second day, sections were washed 3 x 10 min in 0.01 M PBS containing0.1% Triton X-100 followed by one washing step in 0.01 M PBS for 1 min. Then, sections were incubated with the secondary antibody in 0.01 M PBS containing 10%FCS for 2 h at RT followed by washing with 0.01 M PBS for 3 x 10 min. Afterward, sections were incubated in Avidin-Biotin complex (ABC) solution for 1.5 h at RT. After washing 3 x 10 min in 0.01 M PBS, staining was visualized using DAB solution. Sections were washed 3 x 10 min in 0.01 M PBS. After washing, sections were mounted in PBS onto Superfrost slides and left to dry overnight. The next day, sections were incubated in 0.01 M PBS for hydration. Then, they were counterstained with filtered hematoxylin for 40sec. After shortly being dipped in ddH2O, sections were washed under running tap water for 5 min. Then, sections were dehydrated in baths of the following EtOH concentrations: 1 min 70% EtOH; 5 min 95% EtOH; 10 min 100% EtOH followed by 2 x 5 min in xylol. Finally, sections were embedded using Roti® Histokitt mounting medium.

2.6.4 Fluorescent Immunohistochemistry on paraffin sections

The staining was done by using 4 µm paraffin sections. Firstly, sections were deparaffinized and hydrated in baths of the following xylol and EtOH concentrations: 2 x 5 min in xylol; 10 min in 100% EtOH; 5 min in 95% EtOH; 5 min in 70% EtOH. After 1 min washing with ddH2O, antigen retrieval was done by boiling sections in 0.01 M citrate buffer pH 6.0 in a microwave for 10 min (800 W until boiling followed by 80 W for 8 min), followed by 15 min cool down. Then, sections were permeabilized with 0.1% Triton in 0.01 M PBS for 15 min. Sections were washed with 0.01 M PBS for 1 min, followed by 3 min treatment with

88% formic acid and 1 min with 0.01 M PBS. Sections were circled with a lipid pen and incubated for 1 h at RT with a solution of 10% fetal cow serum and 4% milk powder in 0.01 M PBS to block unspecific antigens. The mixture of two primary antibodies was diluted in 10% fetal calf serum in 0.01 PBS. Sections were incubated overnight in a chamber at the room temperature. Next day, sections were washed with 0.1% Triton in 0.01 M PBS 3 x 5 min and then with 0.01 M PBS for 1 min. After washing, the sections were incubated with fluorophore-conjugated secondary antibodies (1:750) at 37 °C for1.5 h in a chamber. After that step, the sections were protected from light. After secondary antibody incubation, sections were washed 3 x 5 min in 0.01 M PBS. Counter-staining was done by dipping the sections for 1 min in a solution of 1.5 mg/l DAPI dissolved in ddH2O. Then, slides were washed two times for 1min in 0.01 M PBS. One drop of fluorescence mounting media was added to each section. Then, coverslips were added to each slide.

2.7 Quantification of neuron numbers

2.7.1 Cresyl violet staining

Frozen left brain hemispheres were cut frontally in series of 30 μm thick sections by using the cryostat (Leica). Sections were delipidated by performing following treatments: 2 x 10 min in work solution A followed by 20 minutes in work solution B and 2 x 10 min in work solution A. Afterwards, the sections were stained twice for 10 min with cresyl violet staining solution. Sections were washed three times with work solution A to remove cresyl violet. Then, sections were dehydrated using the following incubations: 3 x 10 min 100% ethanol, 10 min isopropanol and 2 x 5 min xylol. Slides were mounted using Roti-Histokit mounting medium. Then, a coverslip was added to each slide.

Work solution A: 13.61 g Natrium Acetate Trihydrate was diluted in 100 ml ddH2O. 40 ml of the generated 1 M Natrium Acetate solution was mixed with 9.6 ml 100% Acetic Acid.

Work solution B: 2.0 ml Triton X-100 were dissolved in 10 ml ddH2O. 2.5 ml of this stock solution were mixed with 50 ml ddH2O and 150 ml 100% ethanol.

Staining solution: 0.1 g cresyl violet was added to 1 l work solution A and stirred for 1 h.

2.7.2 Quantification of total neuron number in the dentate gyrus of the hippocampus

Stereological analysis was performed on cresyl violet stained brain sections to quantify the neuron numbers in the dentate gyrus with the unbiased design. BX51 stereology workstation (Olympus) with Stereo Investigator 7 software (MBF Bioscience) were used. The parameters used for stereological analysis of neurons in the dentate gyrus are listed in Table 7.

Table 7: Parameters for stereological analysis of dentate gyrus neuron numbers.

Parameter	Dentate gyrus
Sampling Grid (x) (µm)	133
Sampling Grid (y) (µm)	75
Sampling Grid Area (xy) (µm2)	9975
Counting Frame Width (X) (µm)	14
Counting Frame Height (Y) (µm)	14
Counting Frame Area (XY) (µm2)	196
asf	50.9
ssf	10
Z (µm)	5

The granular cell layer of the dentate gyrus was delineated at a low magnification (40x), respectively (DG: Bregma -1.30 to -3.80 mm). Neuronal nuclei were sampled randomly at a high magnification (100x) using optical dissector probes, and the total number of neurons was subsequently estimated by the optical fractionator method using a 2 µm top guard zone. On every grid site, the section thickness was measured with a 5 µm dissector height (Z). The number of neurons was estimated using the following formulas:

- $\mathrm{P} = asf\ x\ ssf\ x\ tsf$
- $\mathrm{N} = \sum_{i=1}^{n} (P\ x\ Q)i$

Where;
asf = area sampling fraction (xy/XY)
tsf = thickness sampling fraction (T/Z)

P = number of neurons
Q = total markers counted
ssf = section sampling fraction

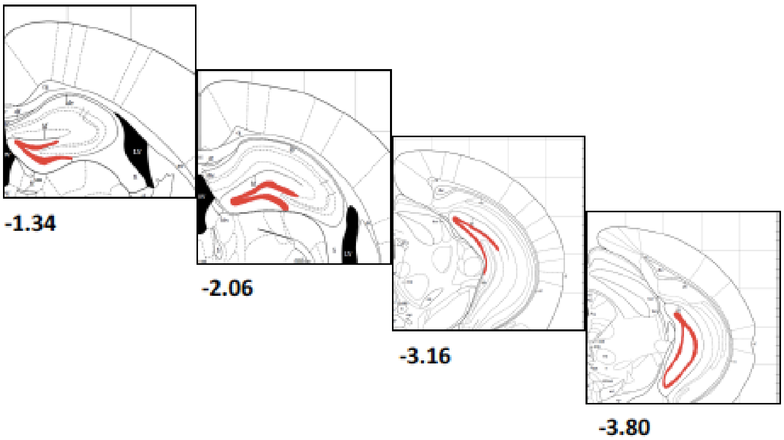


Figure 9: Schematic illustration of the counted areas in the hippocampus. The dentate gyrus was counted from Bregma 1.34 mm to -3.80 mm. (Adapted from Franklin & Paxinos, 2012)

2.7.3 Estimation of volume for the dentate gyrus

To estimate the volume of the granular cell layer of the dentate gyrus, the Cavalieri principle was used (Rosen & Harry, 1990). The formula used to calculate the volume is the following:

$$V = d\left(\sum_{i=1}^{n}(y_i)\right) - (t)\, y_{max}$$

Where:

V = Cavalieri's estimator of volume
d = distance between analysed sections (d = 300 µm)
yi = cross-sectional area of the i-th section
n = total number of sections
ymax = maximal value of y (maximum area)
t = thickness of ymax section

2.7.4 Quantification of DCX and Ki67 positive cells in the dentate gyrus of the hippocampus

The number of doublecortin (DCX) and Ki67 positive neurons in the dentate gyrus were counted using the Meander Scan platform of the Stereo Investigator 7 software. Sections were collected systematically by taking every 10th 30 µm thick coronal frozen section. Therefore, the adult neurogenesis rate was calculated by multiplying the counted number of DCX or Ki67 positive neurons by a factor 10.

2.8 Quantification of the neuroinflammation and CB1 receptor expression

3 slides were chosen for each animal, starting with the similar Bregma for the precise comparison. Representative images of 10× magnification were captured from the hippocampus of Tg4-42, and images of 20× magnification were captured from the dentate gyrus of Tg4-42. To capture images, Olympus BX-51 microscope equipped with an Olympus DP-50 camera was used. All images were captured with the same light and exposure settings.

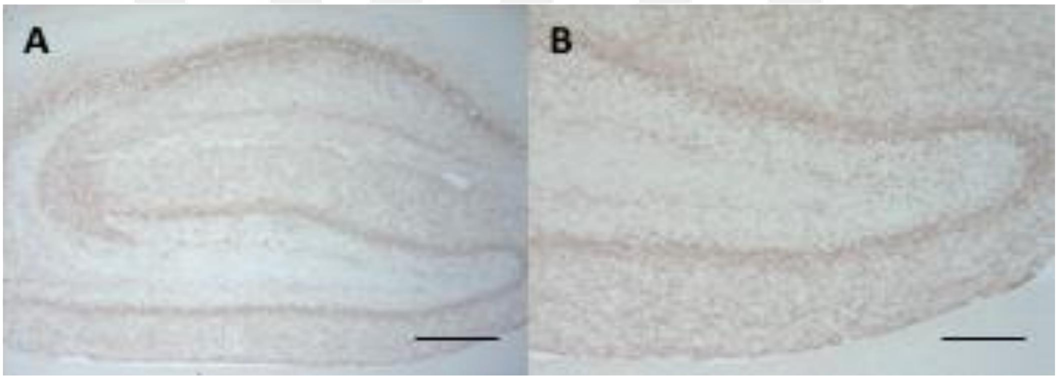


Figure 10: Sample image for DAB immunohistochemistry. Representative pictures of the hippocampus and the dentate gyrus stained with DAB immunohistochemistry to detect expression of CB1 receptors. Scale bar: (A) 200 µm; (B) 100 µm.

For quantification, the pictures were converted to 8-bit black and white pictures using ImageJ software. Afterward, the optimal intensity threshold was determined to detect only stained particles in the picture. Measurements were performed for a percentage of the area covered by the neuroinflammation and CB1 receptor expression with DAB immunohistochemistry.

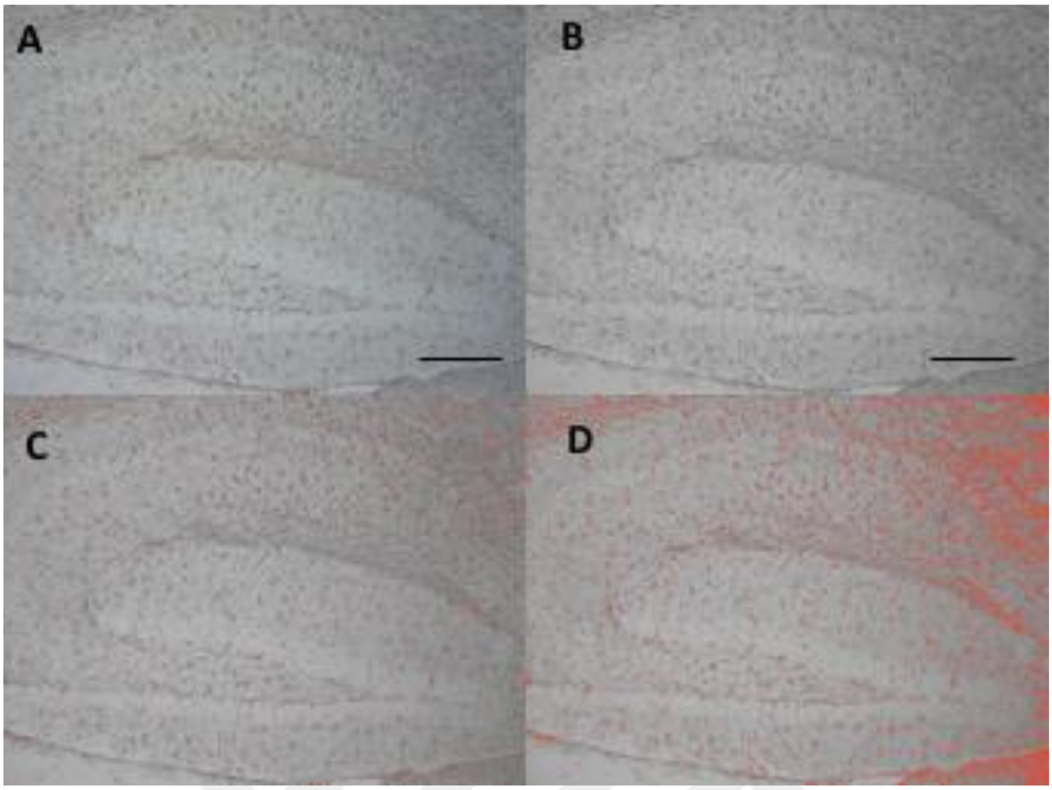


Figure 11: Images display subsequent grades of threshold adjustment in ImageJ software. (A) Initial picture of the hippocampus stained with GFAP to detect neuroinflammation. (B) The picture was converted to 8-bit black and white scale. (C) The picture with the optimal threshold value detected stained particles. (D) The picture with the high threshold value detected unspecific signals. Scale bar: (A, B, C, D) 200 µm.

2.9 Statistical analysis

Details of the statistical analysis are given in the respective results section and in the figure legends. Differences between groups were tested with unpaired t-test.

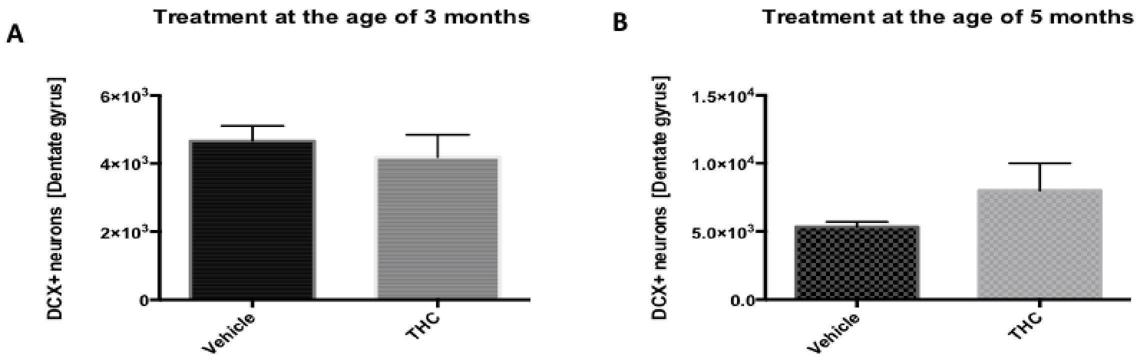
Significance levels were given as follows: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. The number of animals used for experiments is given in the figure legends (n). All data were given as means ± a standard error of the mean (SEM). All statistics were performed using GraphPad Prism version 6 for Windows (GraphPad Software, Inc., San Diego, CA, USA).

3 RESULTS

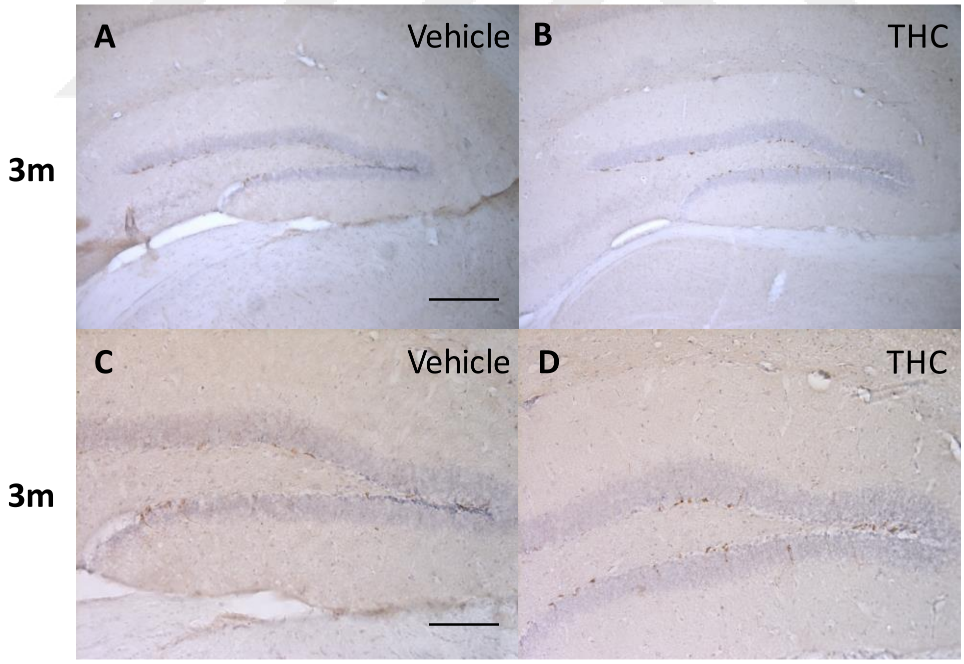
3.1 The assessment of the hippocampal neurogenesis upon THC treatment in Tg4-42 mice

3.1.1 Doublecortin quantification for the detection of immature neurons

Frozen brain sections were stained against Doublecortin (DCX) with DAB immunohistochemistry. Doublecortin is a microtubule associated protein expressed by neuronal progenitor cells and immature neurons. Thus, DCX is as a marker of new-born neurons in the adult dentate gyrus. DCX has been shown as a reliable marker to analyse an accurate number of newly generated neurons in the adult dentate gyrus (Rao, 2004). In order to investigate whether neurogenesis is affected by THC treatment, DCX immunohistochemical staining and quantification were performed in the dentate gyrus of Tg4-42 mice for preventative and therapeutic treatment groups (Fig. 12). The number of new-born neurons showed no significant alteration upon preventative THC treatment in the dentate gyrus of Tg4-42 mice compared to the vehicle group. (Graph 1A, veh: mean=4660, THC: mean= 4180). Besides that, DCX-positive immature neurons displayed elevation in the dentate gyrus upon THC treatment at the age of 5 months compared to vehicle-treated Tg4-42 mice. However, the result of unpaired t-test did not reach significance (Graph 1B, veh: mean=5359, THC: mean=8011).



Graph 1: The impact of THC treatment on the hippocampal neurogenesis in Tg4-42 mice. The number of DCX-positive immature neurons in the dentate gyrus of THC-treated and vehicle-treated Tg4-42 mice were quantified with unbiased stereological methods. (A) There was no significant difference in the number of DCX-positive neurons between THC-treated and vehicle-treated Tg4-42 mice, injected at the age of 3 months, analysed by unpaired t-test (veh n=9, THC n=11, p>0,05). (B) DCX-positive immature neurons increased in the dentate gyrus upon THC treatment in comparison to vehicle-treated Tg4-42 mice, injected at the age of 5 months but unpaired t test analysis showed no significance (veh n=11, THC n=8, p>0,05). Data is given as mean ± SEM (error bars).



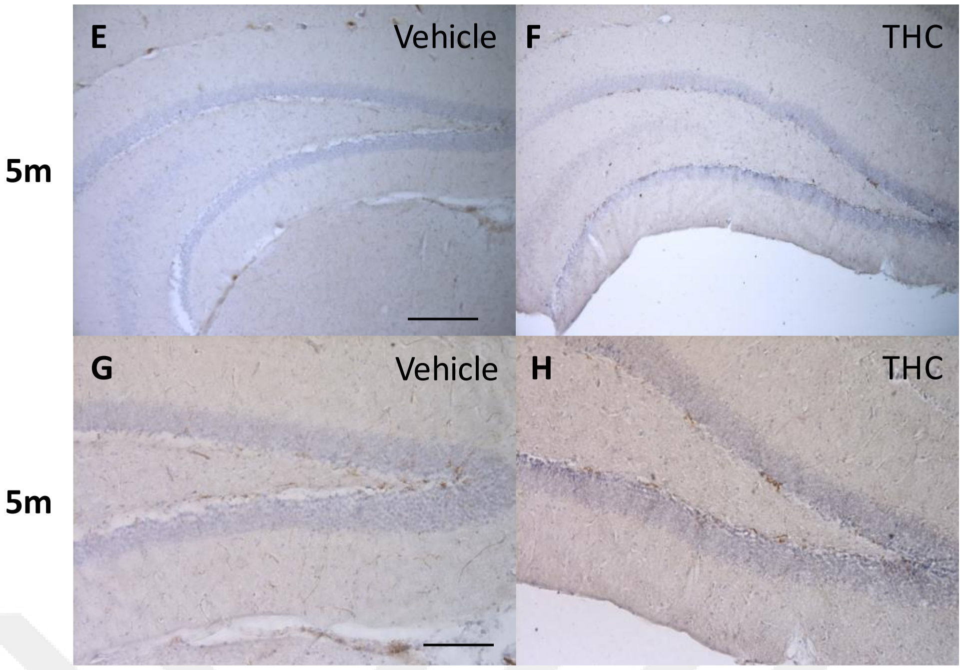
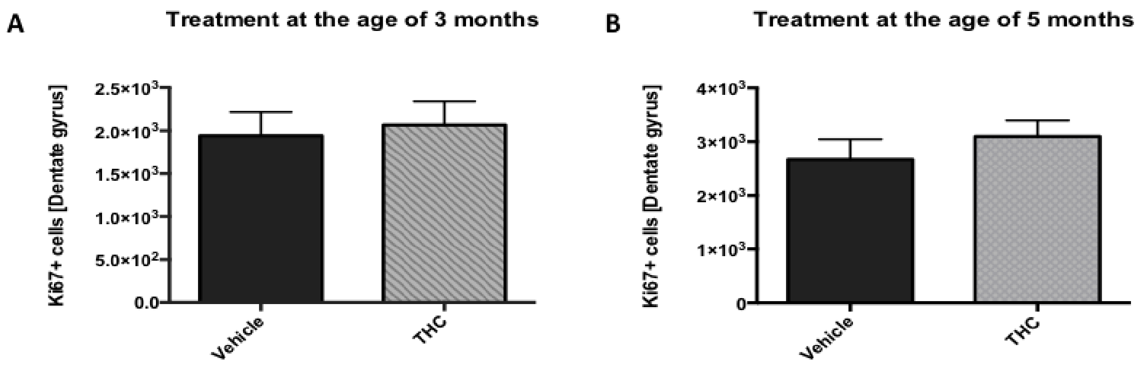


Figure 12: Hippocampal neurogenesis marked by DCX in Tg4-42 mice upon THC treatment. (A-D) Representative images of the dentate gyrus of Tg4-42 mice, displaying DCX-positive neurons for treatment groups of vehicle and THC, injection starting at the age of 3 months. (E-H) Representative images of the dentate gyrus of Tg4-42 mice, showing DCX-positive neurons for treatment groups of vehicle and THC, injected at the age of 5 months. Scale bar: (A, B, E, F) 200 µm; (C, D, G, H) 100 µm.

3.1.2 Ki67 quantification for the detection of proliferating cells

Frozen brain sections were stained with Ki67 using DAB immunohistochemistry. Ki-67 protein is the nuclear protein associated with the cellular division. In the fact that, Ki-67 protein is found during all active phases of the cell cycle but it is not present in resting cells (Scholzen, 2000). Therefore, Ki67 has been used as a reliable marker to assess the cell proliferation. To determine whether preventative or therapeutic THC treatment increase hippocampal neurogenesis, immunohistochemical staining and subsequent quantifications with Ki67 were performed (Fig. 13; Graph 2). The number of Ki67-positive cells remained constant upon THC treatment at the age of 3 months compared to vehicle group in the dentate gyrus of Tg4-42 mice (Graph 2A, veh: mean=1940, THC: mean=2065). Also, there was no significant change in the number of Ki67-positive cells in the dentate gyrus of THC-treated animals at the age of 5 months compared to vehicle-treated animals (Graph 2B, veh: mean=2672, THC: mean=3093).



Graph 2: The analysis of the hippocampal neurogenesis upon THC treatment in Tg4-42 mice. Ki67-positive cells were stained and quantified in the dentate gyrus of THC-treated and vehicle-treated Tg4-42 mice with unbiased stereological methods. (A) There was no significant difference in the number of Ki67-positive cells between THC-treated and vehicle-treated Tg4-42 mice, injected at the age of 3 months, assessed by unpaired t-test (veh n=7, THC n=11, p>0,05). (B) There was no significant alteration in the dentate gyrus of THC-treated Tg4-42 mice compared to vehicle-treated Tg4-42 mice, injected at the age of 5 months, assessed by unpaired t-test (veh n=11, THC n=9, p>0,05). Data is given as mean ± SEM (error bars).

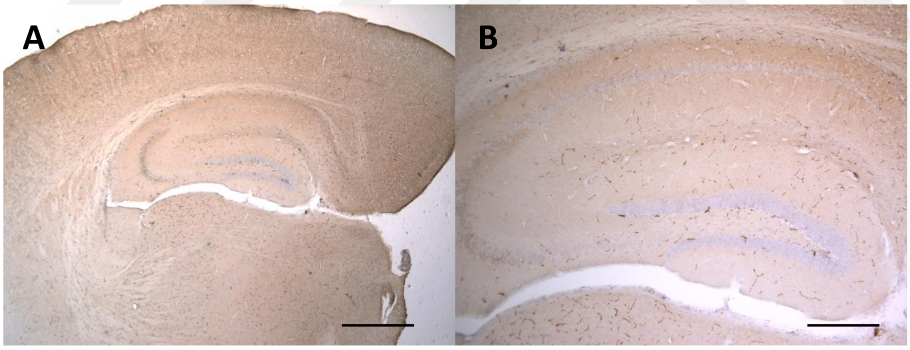
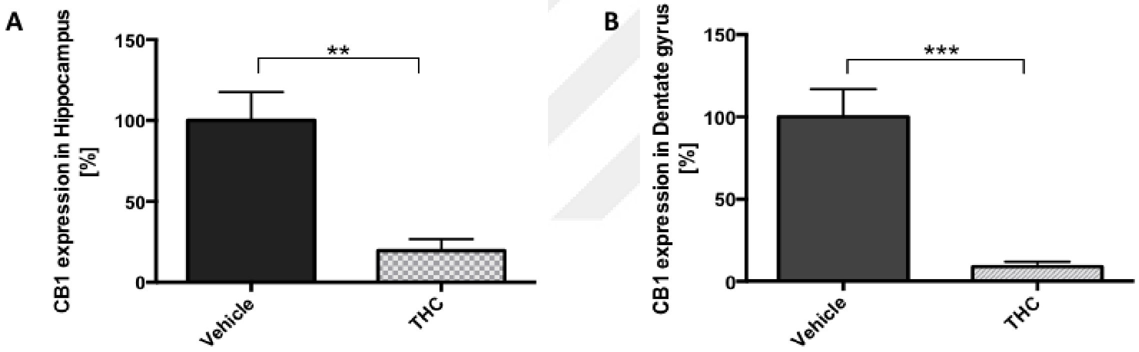


Figure 13: Hippocampal neurogenesis marked by Ki67 in Tg4-42 mice upon THC treatment. (A, B) Example images of the dentate gyrus of Tg4-42 mice, showing Ki67 stained cells in the dentate gyrus of Tg4-42 mice. Scale bar: (A) 500 µm; (B) 200 µm.

3.2 Immunohistochemistry in Tg4-42 mice upon THC treatment

3.2.1 CB1 receptor expression in THC-treated and vehicle-treated Tg4-42 mice

To evaluate the changes of cannabinoid receptor type 1 in Tg4-42 mice with THC treatment, DAB immunohistochemistry was performed for CB1 receptor expression. Unpaired t-test and immunohistochemical staining showed the strong decrease in CB1 expression in the hippocampus of THC-treated animals compared to vehicle-treated animals, injection started at the age of 5 months (Graph 3A; Figure 14 A and B). Furthermore, the result of the unpaired t-test revealed that CB1 expression in the dentate gyrus significantly abolished in THC-treated Tg4-42 mice compared to vehicle-treated Tg4-42 mice (Graph 3B). All data indicates that THC as a ligand induced desensitization or internalization of CB1 receptors.



Graph 3: The impacts of THC treatment on CB1 expression in Tg4-42 mice. (A) CB1 expression was significantly decreased in the hippocampus of THC-treated Tg4-42 mice compared to vehicle-treated Tg4-42 mice; injection started at the age of 5 months, assessed by unpaired t-test (vehicle n=5, THC n=4, p=0,0011). (B) CB1 expression was significantly reduced in the dentate gyrus of THC-treated Tg4-42 mice, compared to vehicle-treated Tg4-42 mice, analysed by unpaired t-test (vehicle n=5, THC n=4, p=0,0001). Data is given as mean ± SEM (error bars).

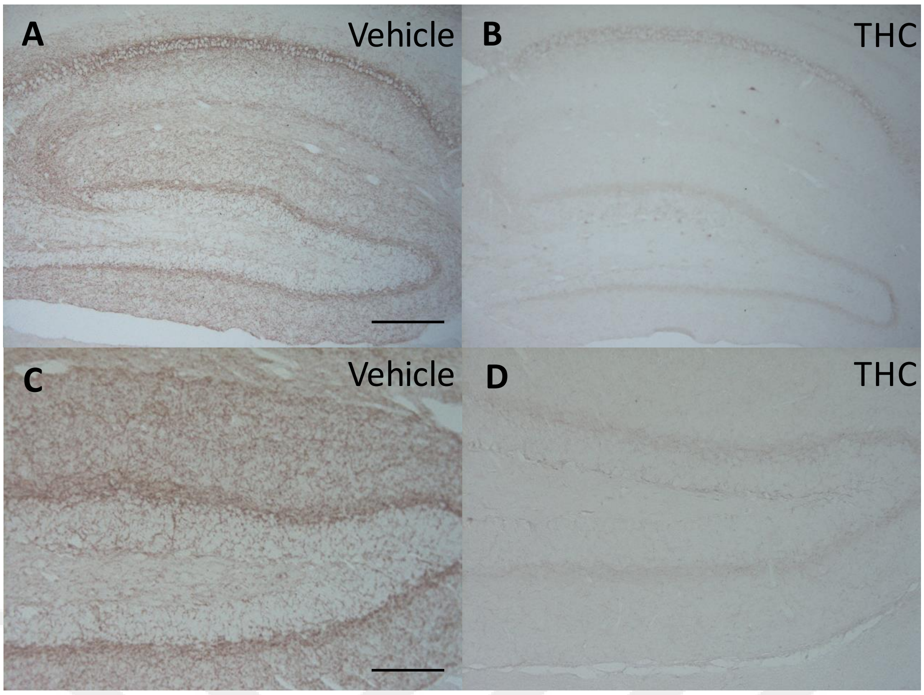
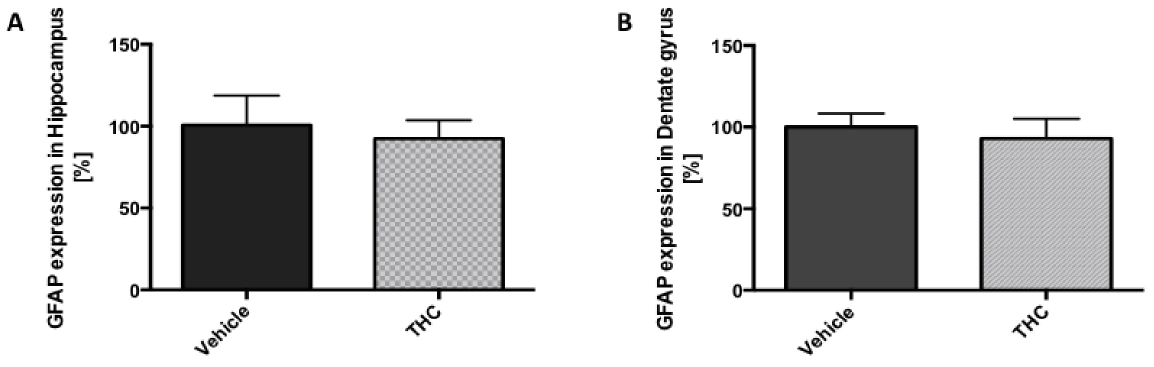


Figure 14: CB1 receptor expression in THC-injected and vehicle-injected Tg4-42 mice at the age of 5 months. (A, B) Representative images of the hippocampus of Tg4-42 mice, showing CB1 receptor staining for respective treatment groups of vehicle and THC. (C, D) Representative images of the dentate gyrus of Tg4-42 mice, displaying CB1 receptor staining for respective treatment groups of vehicle and THC. Scale bar: (A, B) 200 µm; (C, D) 100 µm.

3.2.2 Neuroinflammation in THC-treated and vehicle-treated Tg4-42 mice

To verify the impacts of THC treatment on the inflammation, DAB immunohistochemistry was performed against the inflammatory markers GFAP and IBA1. Astrogliosis was examined with GFAP staining so reactive astrocytes were observed in both experimental groups, starting the treatment at the age of 5 months. Unpaired t-test and immunohistochemistry staining images revealed no significant difference for GFAP positivity between THC-treated and vehicle-treated Tg4-42 mice in the hippocampus as well as in the dentate gyrus (Graph 4; Figure 15).



Graph 4: The assessment of neuroinflammation by GFAP marker in Tg4-42 mice upon THC treatment. (A) No significant difference in GFAP positivity was detected in the hippocampus of THC-treated animals compared to vehicle-treated Tg4-42 mice, starting age of 5 months, assessed by unpaired t-test (vehicle n=5, THC n=5, p>0,05). (B) No significant difference was detected between THC-treated Tg4-42 mice and vehicle-treated Tg4-42 mice in the dentate gyrus for GFAP positivity, assessed by unpaired t-test (vehicle n=5, THC n=5, p> 0,05). Data is given as mean ± SEM (error bars).

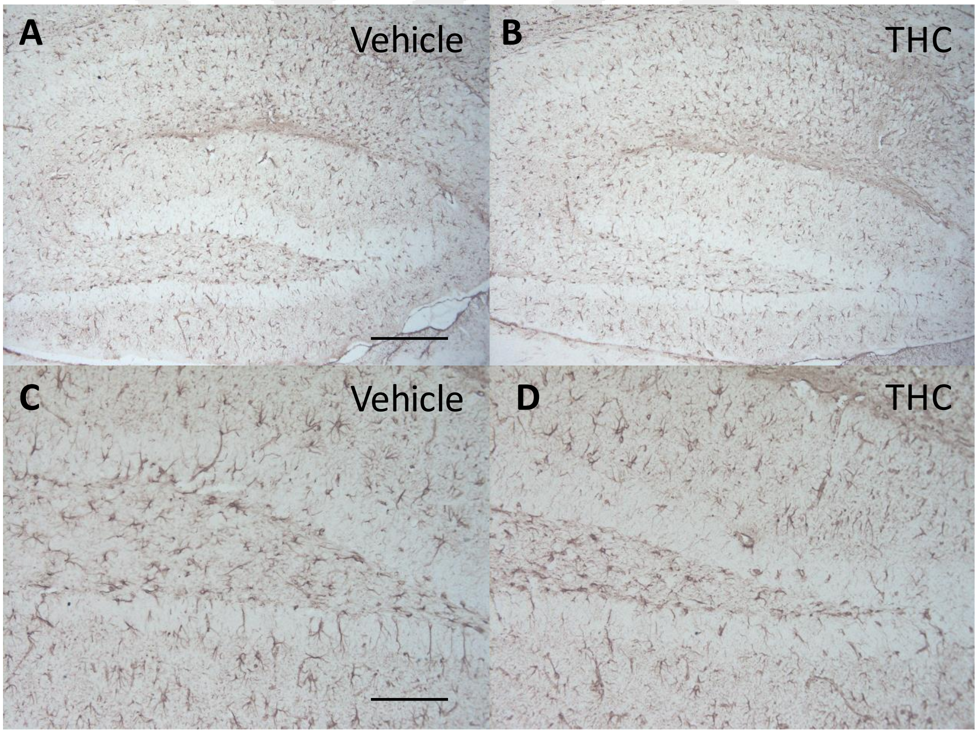
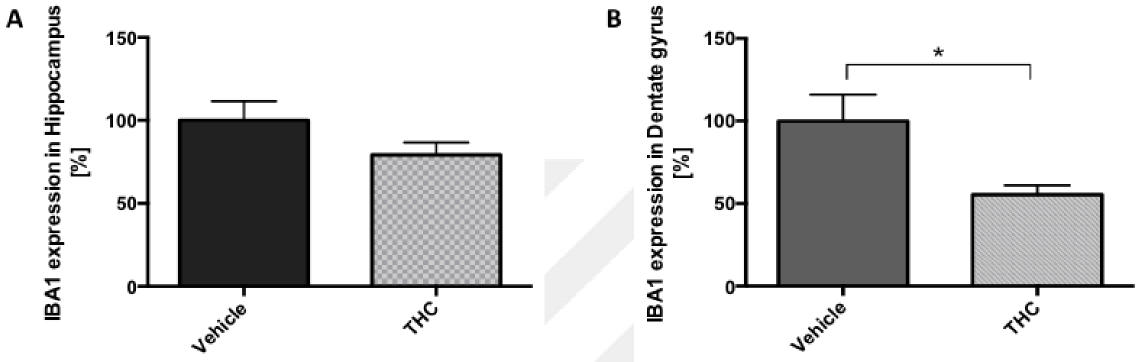


Figure 15: **Unchanged astrogliosis in THC-injected and vehicle-injected Tg4-42 mice at the age of 5 months.** (A, B) Representative images from the hippocampus of Tg4-42 mice, showing GFAP positivity for respective treatment groups of vehicle and THC. (C, D) Representative images of the dentate gyrus of Tg4-42 mice, displaying GFAP staining for respective treatment groups of vehicle and THC. Scale bar: (A, B) 200 µm; (C, D) 100 µm.

Microgliosis was detected with IBA1 staining by performing DAB immunohistochemistry. IBA1 expression was reduced with THC treatment in the hippocampus of Tg4-42 mice, however, without reaching statistical significance (Graph 5A). Moreover, the impact of THC treatment on the reduction of microgliosis was significantly detected in the dentate gyrus of Tg4-42 mice (Graph 5B). The IBA1 positivity reduction was also confirmed with immunohistochemistry staining images (Fig. 16).



Graph 5: Decreased microgliosis after THC treatment in Tg4-42 mice. (A) No significant difference for IBA1 positivity was detected between the THC-treated and vehicle-treated of Tg4-42 mice in the hippocampus, injection started at the age of 5 months, assessed by unpaired t-test (vehicle n=5, THC n=5, p>0,05). (B) The significant decrease in IBA1 expression was observed in THC-treated Tg4-42 mice in comparison to vehicle-treated Tg4-42 mice in the dentate gyrus, analysed by unpaired t test (vehicle n=5, THC n=5, p=0,0241). Data is given as mean ± SEM (error bars).

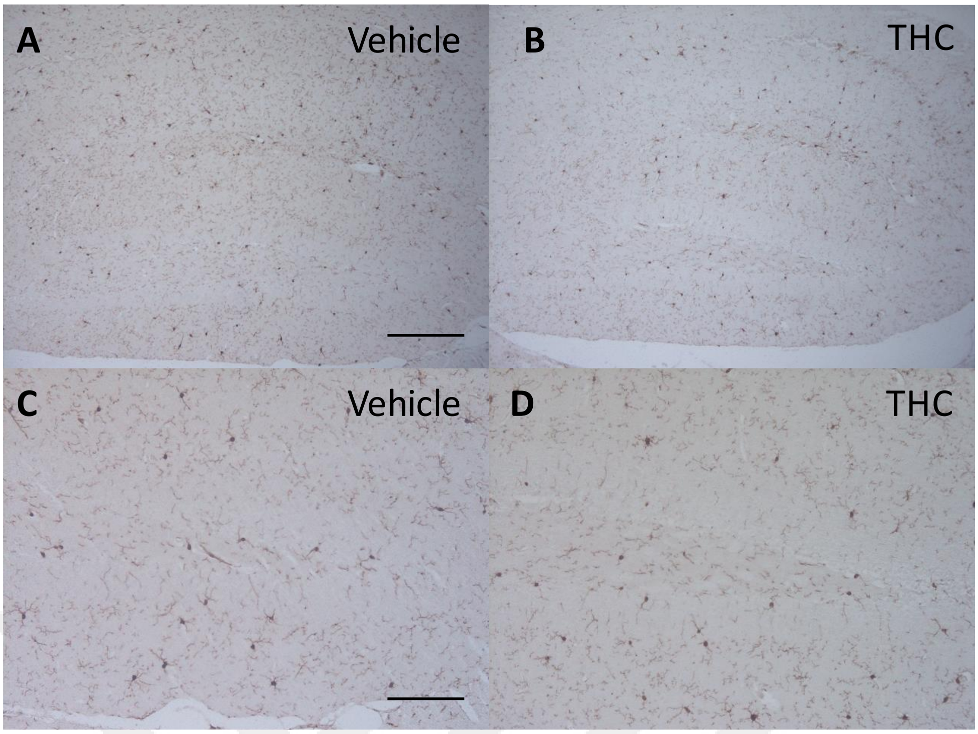
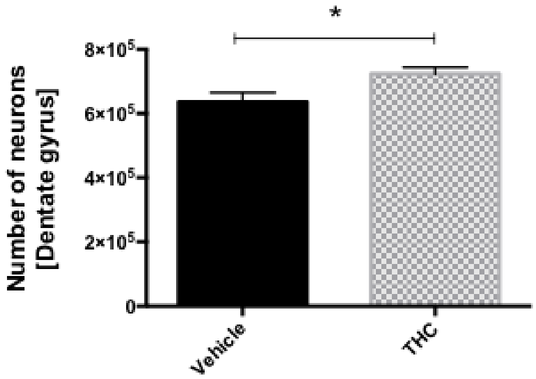


Figure 16: **IBA1 positivity in THC-treated and vehicle-treated Tg4-42 mice at the age of 5 months.** (A, B) Representative images from the hippocampus of Tg4-42 mice, showing IBA1 positivity for respective treatment groups of vehicle and THC. (C, D) Representative images of the dentate gyrus of Tg4-42 mice, displaying IBA1 staining for respective treatment groups of vehicle and THC. Scale bar: (A, B) 200 µm; (C, D) 100 µm.

3.3 The effect of THC treatment on the hippocampal neuron numbers in Tg4-42 mice

To evaluate the effects of THC treatment on the number of neurons in Tg4-42 mice, the number of neurons in the dentate gyrus were quantified using cresyl violet staining and unbiased stereological methods. Cresyl violet staining revealed an obvious neuron loss in the dentate gyrus of the vehicle mice in comparison to THC-treated mice (Fig. 17). To quantify the neuron numbers in the dentate gyrus in an unbiased way, design based stereology was performed. The dentate gyrus cell layer was quantified from Bregma -1.34 to -3.80 mm. Stereological analysis revealed that THC-treated animals displayed a significant increase of 14% in the number of neurons in comparison to vehicle-treated animals at the age of 5 months (Graph 6). (THC: mean = 636,787, SEM ± 28,568; vehicle: mean = 723,432, SEM ± 21,155, unpaired t-test, p = 0, 033)



Graph 6: The impact of THC treatment on the number of neurons in the dentate gyrus of Tg4-42 mice. The number of neurons was quantified in the dentate gyrus using unbiased stereology. The number of neurons was significantly increased in the dentate gyrus of THC-treated Tg4-42 mice, compared to the vehicle-treated Tg4-42 mice,at the age of 5 months, assessed by unpaired t test (vehicle n=10, THC n=8, p=0,033)

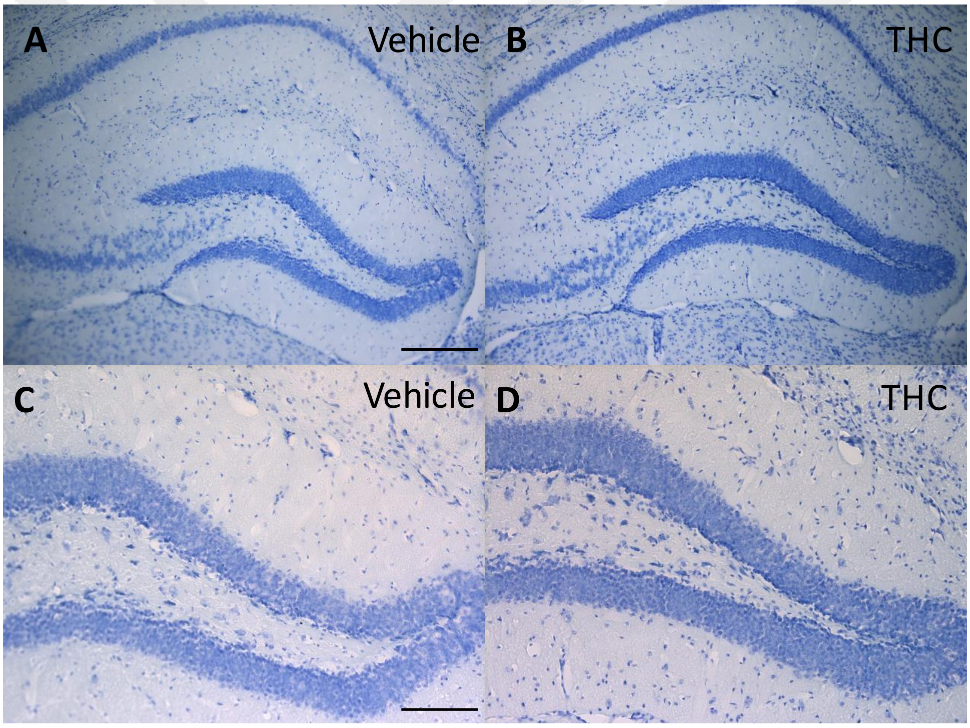


Figure 17: The analysis of the number of neurons in THC-injected and vehicle-injected Tg4-42 mice at the age of 5 months. (A, B) Representative images from the hippocampus of Tg4-42 mice, showing the cresyl violet staining for respective treatment groups of vehicle and THC. (C, D) Representative images of the dentate gyrus of Tg4-42 mice, displaying the cresyl violet staining for respective treatment groups of vehicle and THC. Scale bar: (A, B) 200 µm; (C, D) 100 µm.

3.4 Fluorescent immunohistochemistry in Tg4-42 mice upon THC treatment

To examine the effects of THC-based therapy on a broader scale, fluorescent immunohistochemistry was performed on paraffin sections. Fluorescent secondary antibodies were used against GFAP, IBA1, CB1 receptor, and intraneuronal Aβ to observe co-localization of pathological markers of Alzheimer's disease and the endocannabinoid receptor 1 upon THC treatment in Tg4-42 mice, treatment starting at the age of 5 months. Abundant GFAP positivity was observed in the hippocampus of both THC-treated and vehicle-treated animals. Also, CB1 receptor expression was down-regulated when stimulated with its ligand (Fig. 18). Triple-immunofluorescence analysis revealed that CB1 receptor expression and the positivity of IBA1 were reduced upon THC treatment, starting at the age of 5 months in Tg4-42 mice (Fig. 19).

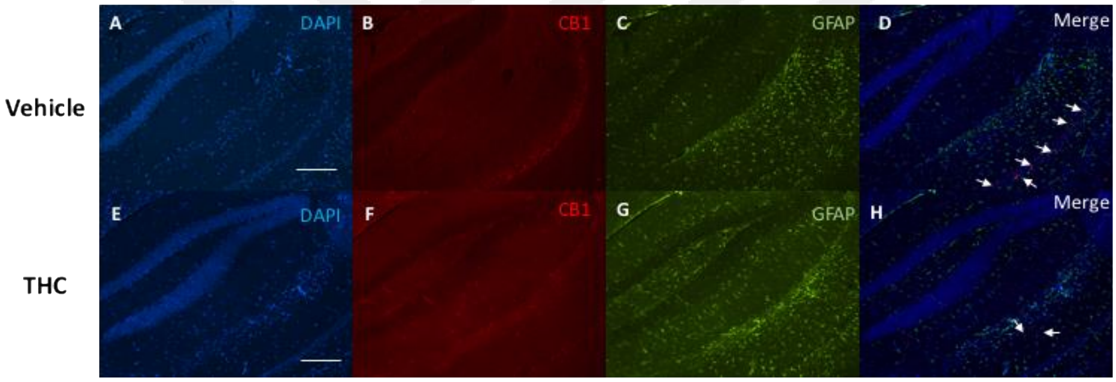


Figure 18: **Triple-immunofluorescence analysis for DAPI, CB1 receptor, and GFAP in Tg4-42 mice.** (A-H) Images from the hippocampus of Tg4-42 mice, showing immunofluorescence staining for respective treatment groups of vehicle and THC. (D, H) The overlay of staining patterns revealed that CB1 receptor expression was drastically reduced upon THC treatment at the age of 5 months, whereas GFAP was stained densely in both treatment groups. Scale bar: (A-H) 200 μm. Arrows showed CB1 expression.

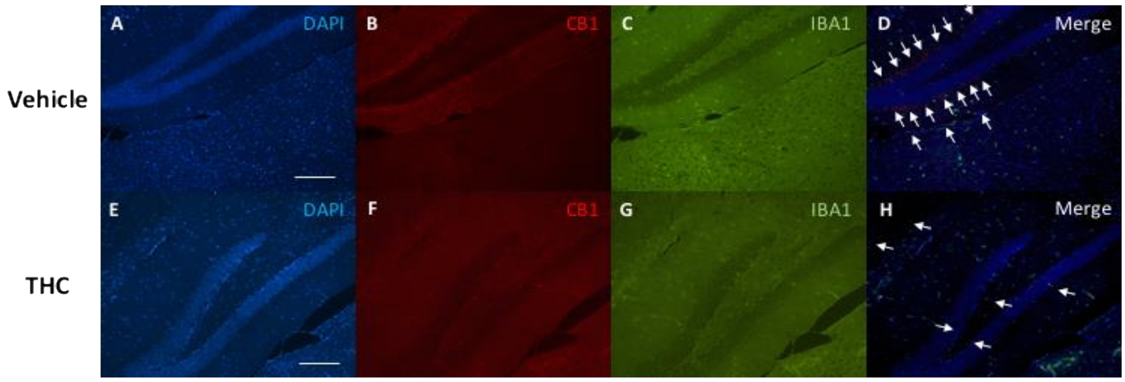


Figure 19: Triple-immunofluorescence analysis for DAPI, CB1 receptor, and IBA1 in Tg4-42 mice. (A-H) Images from the hippocampus of Tg4-42 mice, showing immunofluorescence staining for respective treatment groups of vehicle and THC. (D, H) The overlay of staining patterns displayed that CB1 receptor expression was severely reduced upon THC treatment at the age of 5 months as well as positivity of IBA1 reduced in THC treatment group. Scale bar: (A-H) 200 µm. Arrows showed CB1 expression.

Tg4-42 homozygous mice displayed intraneuronal Aβ expression starting at the age of two months. Tg4-42 mice displayed elevated gliosis in the hippocampus starting from two months of age (Bouter, 2014). In the line with this, GFAP-specific and Aβ-specific antibodies displayed the major co-localization of the neuroinflammatory marker and Aβ particles in the CA1 region of the hippocampus in Tg4-42 mice, whereas fluorescent signals for both antibodies showed no change with THC treatment, starting 5 months of age (Fig. 20). In addition, prominent Aβ and CB1 receptor immunoreactivity were detected, which were less frequent in the CA1 region of the hippocampus of THC-treated animals compared to vehicle-treated animals (Fig. 21).

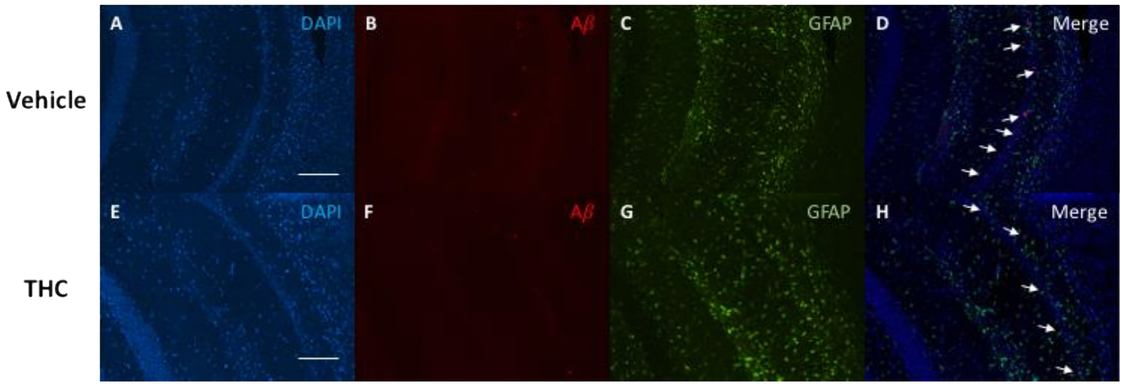


Figure 20: Triple-immunofluorescence analysis for DAPI, Aβ, and GFAP in Tg4-42 mice. (A-H) Images from the CA1 region in the hippocampus of Tg4-42 mice, showing immunofluorescence staining for respective treatment groups of vehicle and THC. (D, H) The co-localized staining patterns revealed that A**β** particles and GFAP positivity showed no vital alteration. Scale bar: (A-H) 200 µm. Arrows showed A**β** positivity.

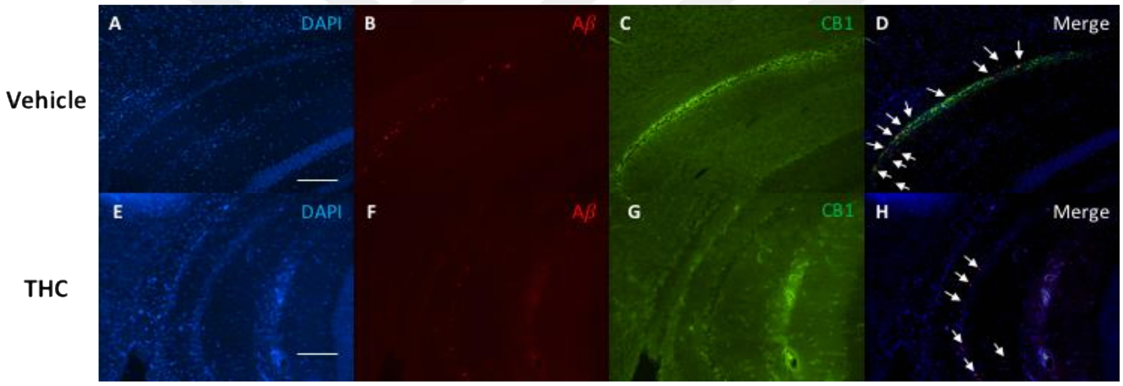


Figure 21: Triple-immunofluorescence analysis for DAPI, Aβ, and CB1 receptor in Tg4-42 mice. (A-H) Images from the CA1 region in the hippocampus of Tg4-42 mice, showing immunofluorescence staining for respective treatment groups of vehicle and THC. (D, H) The co-localized staining patterns demonstrated that CB1 receptor expression was profoundly reduced by THC treatment at the age of 5 months in Tg4-42 mice. Also, Aβ particles were reduced in CA1 region of the hippocampus in THC-treated animals compared to vehicle-treated animals. Scale bar: (A-H) 200 µm. Arrows showed A**β** positivity.

4 DISCUSSION

In this project, the potential effects of the preventative and therapeutic Tetrahydrocannabinol (THC) treatment were investigated on the neuron loss, neurogenesis, expression of endocannabinoid receptor 1, and neuroinflammation in two different age groups of the Tg4-42 mouse model.

4.1 The assessment of the hippocampal neurogenesis upon THC treatment in Tg4-42 mice

New neurons continue to be formed in the dentate gyrus of the adult mammalian hippocampus after the birth (Sorells, 2018). New neurons are generated from neural stem cells (NSC) which reside in the subventricular zone (SVZ) and the subgranular zone (SGZ) in the dentate gyrus of the hippocampus (Encinas, 2011). Accumulating evidence implies that the main function of adult NSCs is to provide an additional layer of plasticity to the brain (Christian, 2014). Behavioural studies suggest that adult neurogenesis might have an important role in neuroplasticity, neural network maintenance, and memory (Mu, 2011; Christian, 2014; Deng, 2010). The hippocampus, the critical region for learning and memory, is one of the most vulnerable regions in the brain to the damage of Alzheimer’s disease (AD). Once progressive neuronal loss takes place in the AD brain, regulating adult neurogenesis can be a promising to provide neuroprotection against AD. The status of adult hippocampal neurogenesis in Alzheimer’s disease remained to uncover. Only a few, and conflicting studies about the hippocampal neurogenesis in AD patients are present (Winner, 2015). The link between endocannabinoid system and neurogenesis has not been understood. However, the physiological role of endocannabinoid system in brain plasticity might play a vital role in regulating the hippocampal neurogenesis. Several studies showed that CB1 receptors activation might partially control adult neurogenesis. In CB1 lacking mouse model, hippocampal neurogenesis is deficient (Aguado, 2005). In the line with this, the reduction in adult neurogenesis was observed in CB2-knockout mice (Palazuelos, 2006). Moreover, a chronic treatment *in vivo* with HU210, a synthetic cannabinoid, promoted neurogenesis in the dentate gyrus of adult rats (Jiang, 2005). Although the exact association between endocannabinoid system and adult neurogenesis have not been elucidated, further studies might help uncover the potential of cannabinoids.

In the current study, the quantification of DCX+ immature neurons suggested that THC treatment can be efficient, applied therapeutically, to induce neurogenesis in the dentate gyrus of the Tg4-42 mice (Graph 1). However, the rise of hippocampal neurogenesis marked by DCX was not mirrored by the quantification of Ki67+ cells in the dentate gyrus of Tg4-42 mice neither with the preventative nor with the therapeutic approaches of THC treatment (Graph 2). The difference in the quantification of the hippocampal neurogenesis might depend on the methods used for the labelling of proliferating cells. Doublecortin labelling identifies new-born and immature neurons. However, Ki67 is a cellular marker for proliferation. Couillard-Despres *et al.* showed that quantification of DCX-positive cells provides the reliable measurement of modulations in the rate of adult neurogenesis (Couillard-Despres, 2005). Harris *et al.* suggested a new labelling method to identify the dividing adult hippocampal NSCs *in vivo*. They described that the labelling for the cell cycle marker Ki67 and selection against the intermediate progenitor cell marker TBR2 (Ki67^{+ve}; TBR2^{-ve} nuclei) made an accurate combination to trace dividing NSCs in the adult mouse hippocampus (Harris, 2017). In the future experiments, the new approach might give more accurate and fast results to identify and to quantify the adult hippocampal neurogenesis *in vivo*.

It can be hypothesized that THC might exert its neuroprotective effect by inducing the increase of the hippocampal neurogenesis in the dentate gyrus according to the findings of the present study. The literature about the effect of THC administration on the hippocampal neurogenesis is quite limited. Suliman *et al*. presented the evidence supporting the findings of the present study. They used different doses of THC and different markers (BrdU, GFAP, nestin, DCX) to label new-born cells. They showed that THC administration enhanced all markers in the hippocampus of rats (Suliman, 2018). On the other hand, the fate of immature neurons is not known, whether new-born neurons reside in the neurogenic niche or migrate and differentiate into mature neurons to incorporate into neuronal circuits. Little is known about how immature neurons integrated into circuits with diverse signals, and final choices such as quiescence versus activation, and maintenance versus fate commitment (Bond, 2016). To date, two publications contradictory to the present study could be found. Wolf *et al.* showed that chronic THC treatment decreased the precursor cell proliferation in DG of Nestin-GFP-reporter mice (Wolf, 2010). Furthermore, Steel *et al.* demonstrated that the chronic THC administration impairs hippocampal neuroplasticity and neurogenesis in trained, but not untrained Sprague-Dawley rats (Steel, 2014).

Another important point is the alteration of the hippocampal neurogenesis during the progression of AD. Some studies reported that the hippocampal neurogenesis was reduced in different AD mouse models (Mu, 2011; Wirths, 2017). Most of the reported transgenic AD mouse models carry APP, and PSEN1/2 mutations by expressing either single or multiple transgenes which involve rare, familial and early-onset autosomal dominant forms of AD. These transgenes negatively influence the production of new neurons (Mu, 2011; Wirths, 2017). On the other hand, we found out the number of DCX+ and Ki67+ cells in the dentate gyrus showed elevation in vehicle-treated Tg4-42 mice at the age of 5 months in comparison vehicle-treated Tg4-42 mice at the age of 3 months without the effect of THC administration (Graph 1 and 2). Tg4–42 mice overexpress mutant Aβ-42 peptides and represent the sporadic form of AD in the absence of mutant APP or PS1 overexpression (Bouter, 2013). Therefore, Tg4-42 mouse model might present more relevant findings of alteration of the hippocampal neurogenesis in comparison to APP, and PSEN1/2 expressing mouse models. However, differences in many parameters such as age, gender, the method of neurogenic analysis can lead drawing an uncertain conclusion. Therefore, in the investigation of different AD transgenic mouse models to clarify the relationship between the hippocampal neurogenesis and AD, similar parameters are required to apply draw the definite conclusions. To summarize, the findings of the present study suggest that the increase of neurogenesis upon therapeutic approach in THC treatment could be the promising approach for AD. Characterizing the process of adult neurogenesis and understanding human adult NSCs are essential for therapeutic applications against AD.

4.2 Immunohistochemistry in Tg4-42 mice upon THC treatment

4.2.1 CB1 receptor expression in Tg4-42 mice with THC treatment

CB1 receptors are expressed in the basal ganglia, substantia nigra, globus pallidus, cerebellum, and hippocampus in the rodent brain (Mechoulam, 2013). CB1 receptors are thought to be involved in the modulation of important brain functions such as cognition, memory, emotion and motor control (Howlett, 2005). Effects of THC are mediated by CB1 receptors in the central nervous system, which involves regulation of excitatory (glutamate) and inhibitory (gamma-aminobutyric acid) neurotransmitters (Syed, 2014; Selley, 2003). It was reported that CB1 receptor expression increased at the beginning of AD then declined during the course of the disease in AD patients (Farkas, 2012). Also, Ramirez *et al.* suggested that CB1 receptors were functionally impaired in AD (Ramirez, 2005).

It is fact that receptors are rapidly desensitized or internalized upon the chronic administration of their agonists, to reduce the effect of agonists (Mechoulam, 2013). In line with this, CB1 receptor expression in the dentate gyrus and the hippocampus were reduced drastically with the therapeutic approach of THC treatment in Tg4-42 mice (Graph 3). Furthermore, this observation was confirmed with the fluorescent immunohistochemistry in Tg4-42 mice (Fig.19 and 21). In addition, sufficient literature supports the findings of this present study. One example is that Tai *et al.* showed region-specific down-regulation and desensitization of CB1 receptor by applying the chronic high dose of THC administration to mice (Tai, 2015).

In future experiments, the results of the preventative approach of THC treatment in Tg4-42 mice should be revealed to deduce which treatment option will exert the desired effect better. The actions of cannabinoid receptor 1 are dose-dependently biphasic upon the agonist stimulation (Sulcova, 1998). A relatively high dose of THC administration was applied to Tg4-42 mice in the present study. To find the optimal dosage for THC treatment on the CB1 receptors, application of the low doses of THC can be noteworthy. Taken together, the chronic high dose of THC administration can induce desensitization or internalization of CB1 receptor in the present study.

4.2.2 Neuroinflammation in Tg4-42 mice upon THC administration

Neuroinflammation is one of the key manifested features in AD which may lead to cell damage and neuron loss (Akiyama, 2000; Sardi, 2011). Activated microglia and astrocytes were observed nearby neurons and Aβ plaques (Fagan, 2014). Notably, anti-inflammatory and antioxidant abilities of THC have been proved since the cannabidiol-THC combination has been used to alleviate neuropathic pain in multiple sclerosis patients (Smith, 2007). For the first time, we investigated impacts of therapeutic THC treatment on the neuroinflammation. Inflammatory markers, GFAP for astrogliosis and IBA1 for microgliosis, were used in Tg4-42 mice. Increased neurogenesis and the number of neurons upon THC treatment were accompanied by the decreased level of IBA1 in the dentate gyrus and hippocampus of Tg4-42 mice (Graph 5). Surprisingly, GFAP-positivity demonstrated no alteration in the dentate gyrus and hippocampus of Tg4-42 mice upon therapeutic THC treatment (Graph 4). Furthermore, these results were confirmed with fluorescent immunohistochemistry showing constant GFAP-positivity and reduction in IBA1 expression (Fig.18 and 19).

So far, only one paper was published about the effect of THC administration on neuroinflammation in the aspect of neurodegenerative diseases. Fishbein-Kaminietsky *et al.* tested whether the ultralow-dose of THC can protect against long-term cognitive damage caused by neuroinflammation. In their study, ICR mice received a single injection of THC (0.002 mg/kg) 48 hours before or 1-7 days after treatment with lipopolysaccharide (LPS; 10 mg/kg) and were examined with the object recognition test 3 weeks later. LPS caused long-lasting cognitive deficits and induced neuroinflammation, but they showed the administration of very low doses of THC before or after LPS protected the mice from this LPS-induced damage (Fishbein-Kaminietsky, 2014). However, the results of this publication were not quite comparable with the findings of the present study because of the high dose difference in THC administration, and the different causes for induction of neuroinflammation.

Tg4–42 mice expressed soluble Aβ4-42 forms mainly in the CA1 region of the hippocampus starting at the age of two months. In addition to that, mice displayed increased gliosis in the hippocampus starting from two months of age developed in the region where strong Aβ4-42 positivity was seen (Bouter, 2014). GFAP-specific and Aβ-specific antibodies revealed the co-localization of Aβ particles and activated astrocytes in the CA1 region of the hippocampus in Tg4-42 mice (Fig. 20). In line with this, Sastre *et al.* observed that Aβ molecules can induce the inflammatory response that activates microglia and astrocytes (Sastre, 2006). It is suggested that CB2 receptors can mediate neuroprotection by their anti-inflammatory activations but the underlying mechanism remained elusive (Fagan, 2014). The selective CB2 receptor agonists were shown to reduce the number of activated microglial cells around Aβ molecules and the levels of pro-inflammatory cytokines in APP transgenic mouse models (Martín-Moreno, 2012; Aso, 2015). Thus, it can be speculated that THC might exert its neuroprotective effect as a CB2 agonist, leading to clearance of Aβ molecules by inducing anti-inflammatory activations. In the present study, the reduction of microgliosis and Aβ immunoreactivity were observed in the CA1 region of the hippocampus in Tg4-42 mice upon THC administration therapeutically (Fig.19 and 21). In correlation with the findings, it has been shown that CB2 partial agonist (THC) significantly reduced Aβ molecules and aggregate formation in Aβ expressing mouse model of AD (Janefjord, 2014). Moreover, THC was shown to competitively inhibit the enzyme acetylcholinesterase and to prevent acetylcholinesterase-induced Aβ aggregation for Alzheimer’s disease (Eubanks, 2006). The effect of THC treatment in the preventative mice group should be revealed to compare the efficiency of THC administration on the prevention of inflammatory process. Furthermore, it can be suggested that applying further immunohistochemical staining against other markers of neuroinflammation might give a deeper understanding about the neuroprotective capacity of THC.

4.3 The effect of THC treatment on the hippocampal neuron numbers in Tg4-42 mice

One of the key hallmarks in the pathogenesis of Alzheimer's disease is the sharp decrease in the numbers of neurons in various regions of the brain (Mukhin, 2017). In particular, decreases in the number of neurons in CA1 region of the hippocampus and the entorhinal cortex, associated with the severity of memory impairments (Giannakopoulos, 2003). To study the extent of neuron loss in the AD animal model, the limited number of AD mouse models showed a reliable hippocampal neuron loss (Wirths & Bayer, 2010). Notably, Tg4-42 mice displayed 65% neuron loss in the CA1 layer of the hippocampus at the age of eight months (Bouter, 2014). Moreover, the stereological investigation showed that progressive neuron loss started with four months of age in the hippocampus of Tg4-42 mice correlates strongly with intraneuronal Aβ4-42 expression. (Bouter, 2014).

The decrease in neuron numbers can result from the impaired hippocampal neurogenesis and neuron death in AD (Mukhin, 2017). The number of neurons in the dentate gyrus depends on the equilibrium between the new neuron formation and death of present neurons. In the present study, the stereological analysis revealed that the number of neurons in the dentate gyrus of Tg4-42 mice demonstrated the significant increase with therapeutic THC treatment (Graph 6). In this present study, two claims can be argued to explain the significant increase of the number of neurons. The rise of the number of neurons can be directly correlated with the increased neurogenesis upon THC treatment. Strikingly, we observed that the animal brains which showed the highest neurogenesis upon therapeutic THC treatment are the ones which displayed the highest neuron numbers upon therapeutic THC treatment. Another possibility can be that THC treatment may keep the existing neurons alive in the neural circuits, inducing clearance of Aβ molecules and modulating neuroinflammation. All the findings of this study suggested that two arguments are within the bounds of possibility. The current literature research was not able to provide evidence to support or to refute the findings of the present study, whereas one study employed the similar approach. Scallet *et al.* used morphometric techniques to analyse the effects of chronic THC administration on the anatomical changes in the rat hippocampus. Rats received 10 to 60 mg/kg THC for 90 days and the results assessed by light and electron microscopy. They showed 44% reduction in the number of synapses per unit volume in THC-treated animals compared to vehicle-treated animals (Scallet, 1987). However, this publication should not be evaluated as a contra evidence for the present study because of the long duration time of THC administration and the absence of quantitative evidence using unbiased stereology.

In the future experiments, the number of neurons in the dentate gyrus of Tg4-42 mice upon preventative THC administration should be analysed to perceive the extent of neuron loss at the beginning of the disease. Furthermore, the critical neuron loss occurs in the CA1 layer of the hippocampus during the course of the AD. Therefore, the total neuron number should be investigated in the CA1 region in Tg4-42 mice for both preventative and therapeutic THC treatments.

4.4 Conclusions

For many years, it was accepted that THC impairs memory and cognitive function. On the other hand, the new paradigm has appeared in the recent years; THC regulates memory and cognition through endocannabinoid system in a biphasic manner depending on concentrations and the duration of the administration (Calabrese, 2018). Growing evidence proposes cannabinoids as promising therapeutics, which can target simultaneously neurodegeneration, neuroinflammation, oxidative damage, cognitive impairments, and clearance of Aβ in the AD brain (Bedse, 2015). The present study provided evidence for the increase of the hippocampal neurogenesis and the number of neurons; the reduction of neuroinflammation and Aβ molecules upon THC treatment. To conclude, our findings suggested that THC can be beneficial for modulating the endocannabinoid system to reduce the burden of Alzheimer’s disease.

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6 Statutory Declaration

I hereby assure that I have composed the present thesis entitled “Effects of a THC-based Alzheimer Therapy on the Neuron Loss, Neurogenesis, and Inflammation” independently and have used no other appliances than indicated. Parts being gathered from other works according to wording or meaning I have indicated in every single case by the declaration of a source.

I hereby state furthermore that I have produced my works according to the principles of good scientific practice in compliance with the valid “Richtlinen der Georg-August-Universität Göttingen zur Sicherung guter wissenschaftlicher Praxis”.

Date: 17 May 2018

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