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1. Zusammenfassung

Die Einschränkung der Ernährung wurde weitgehend mit positiven Auswirkungen auf die Alterung des Gehirns und altersbedingten neurodegenerativen Erkrankungen wie der Alzheimer-Krankheit (AD) in Verbindung gebracht. Diese irreversible, progressive Hirnstörung ist mit der Akkumulation von Amyloid beta in senile Plaques und dem daraus resultierenden neuronalen Verlust, synaptischer Dysfunktion und Veränderungen in der Aktivität neuronaler Netzwerke verbunden. Da inzwischen auch akzeptiert worden ist, dass AD durch eine jahrzehntelange, klinisch stille, prodromale Phase der Erkrankung gekennzeichnet ist, wurde die vorliegende Studie mit dem Ziel durchgeführt, die Auswirkungen eines präventiven intermittierenden, every-other-day (EOD) Ernährungsschemas im Gehirn der 5XFAD transgenen Mäuse zu untersuchen, einem häufig verwendeten transgenen Mausmodell der Alzheimer-Krankheit.

Weibliche 5xFAD-Mäuse und ihre nicht-transgenen Wurfgeschwister wurden ab einem Alter von 2 Monaten *ad libitum* (AL) oder der EOD Ernährungsschema ausgesetzt. Die allgemeinen Auswirkungen von EOD auf Plaquebildung und Gliose wurden analysiert, gefolgt von der quantitativen immunhistochemischen Analyse von Parvalbumin und Calbindin im dorsalen Hippocampus. Es wurde eine separate Analyse der Subregionen CA1, CA3 und DG Hippocampus durchgeführt.

Die immunhistochemische Analyse zeigte einen deutichen Anstieg der Entzündung bei 5XFAD Mäusen, gefüttert nach der EOD Ernährungsschema, was sich in der Expression von mikroglia- und astrozytären Markern widerspiegelt. Dieser Anstieg wurde begleitet von einer Zunahme des proinflammatorischen Zytokins TNF-α und darüber hinaus von einer Zunahme der Parvalbumin-Immunreaktivität in allen Subregionen des dorsalen Hippocampus, der in Tg-EOD Mäusen im Vergleich zu Tg-AL Tieren analysiert wurde. Die Anzahl der PV-exprimierenden Neuronen wurde jedoch nicht verändert. Der Calbindinanstieg wurde nur in der CA3-Subregion des Hippocampus nachgewiesen.

Die vorliegende Studie zeigt, dass das tägliche Fütterungsprogramm einen Einfluss auf kalziumbindende Proteine im Hippocampus von 5xFAD-Mäusen hat. Der EOD-induzierte Anstieg von PV und CB deutet auf eine spezifische Veränderung der Netzanregungsfähigkeit hin und könnte daher von therapeutischer Bedeutung sein.

2. Abstract

Food restriction has been widely associated with beneficial effects on brain aging and age-related neurodegenerative diseases such as Alzheimer's disease (AD). This irreversible, progressive brain disorder is associated with the accumulation of amyloid-beta into senile plaques and consequent synaptic dysfunction, neuronal loss, and changes in the activity of neural networks. As it is now also accepted that AD is characterized by decades-long, clinically silent prodromal phase of the disease, the present study was conducted with an aim to examine the effects of preventive intermittent, every-other-day (EOD) feeding regimen in the brain of the 5XFAD mice, a commonly used transgenic model of Alzheimer's disease.

Female 5xFAD transgenic (Tg) mice and their non-transgenic littermates were exposed to *ad libitum* (AL) or EOD feeding regimen, beginning at 2 months of age. The general effects of EOD on plaque formation and gliosis were analyzed first, followed by the quantitative immunohistochemical analysis of two calcium-binding proteins, parvalbumin (PV) and calbindin (CB), in the dorsal hippocampus. A separate analysis of CA1, CA3, and DG hippocampal subregions was performed.

Immunohistochemical analysis revealed a substantial increase of inflammation in the brain of 5XFAD mice following the EOD regimen, reflected by the increase in expression of microglial and astrocytic markers. This increase was accompanied by an increase of proinflammatory cytokine TNF-α in Tg-EOD mice in comparison to Tg-AL animals and furthermore, by the increase of parvalbumin-immunoreactivity in all subregions of dorsal hippocampus analyzed. The number of PV-expressing neurons was, however, not changed. Calbindin increase was detected only in CA3 subregion of the hippocampus.

The present study demonstrates that every-other-day feeding regimen worsens inflammation in 5xFAD mice and further affects hippocampal calcium-binding proteins. EOD-induced increase in PV- and CB-immunoreactivity points to specific alteration in network excitability contributing to pathology and thus could be of therapeutical importance.

3. Introduction

3.1 Alzheimer's Disease

Alzheimer's disease (AD) is an irreversible, progressive brain disorder with no known cure (*Kumar A, Sidhu J, Goyal A, et al., 2020*). It has been estimated to affect 35,6 million people worldwide, which makes AD the most common type of dementia among older adults (*Julien et al., 2017*). Currently, it is ranked as the sixth leading cause of death in the United States, but recent approximations rank AD as the third cause of death in the elderly following heart disease and cancer (*National Institute on Aging*). If breakthroughs are not discovered, rates could exceed a three-fold increase (152 million) and lead to the AD epidemic worldwide by 2050 (*Patterson, 2018*).

In most people with Alzheimer's disease, the onset of symptoms appears in their mid-60's. Symptoms in the early stages are mild and gradually worsen over time. Memory loss, the clinical symptom most commonly associated with AD, usually manifests first as an incapacity to form and store new memories, followed by progressive impairment in the recall of older memories. With further progression of neuronal dysfunction or neurodegeneration, numerous other behavioral disturbances occur. AD patients often present apathy, depression, eating and sleeping disorders, aggressive behavior, and other non-cognitive symptoms that finally affect a person's ability to perform everyday activities *(Apostolova, 2016).* In the most severe stage, the person depends completely on care of others.

On average, the life expectancy of a person with AD is four to eight years after the diagnosis. However, sometimes the person can exceed the given life expectancy and live up to

20 years after the initial diagnosis, depending on other factors (*Patterson, 2018*). Therefore, the care for AD patients represents a significant personal and economic burden for individuals and societies. At present, the cost of the disease has been estimated to be about a trillion US dollars a year, and it is predicted to double by 2030 (*Patterson, 2018*). Due to the worldwide surge of AD patients, there is an urgent need to better understand AD's etiology and to develop new treatments for this disease.

3.1.1 AD Pathology

Alzheimer's disease is named after Dr. Alois Alzheimer, who in 1906 reported the case of patient named Auguste Deter with unusual mental illness, characterized by strange behavioral symptoms and a loss of short-term memory *(Möller and Graeber, 1998).* After the patient's death, Dr. Alzheimer performed a histopathological study of the brain and found two types of lesions: extracellular senile plaques that are now known to be accumulations of amyloid-β (Aβ) peptide and intracellular neurofibrillary tangles made of hyperphosphorylated (phospho) tau protein. Plaques and tangles in the brain are still considered as the main histopathological hallmarks of Alzheimer's disease (*Wang et al., 2008*).

Plaques initially accumulate in the hippocampus and then cortex, brain regions that are involved in the storage of long-term and short-term memories. These structures are therefore particularly vulnerable in AD *(Selkoe, 2004).* Over time, accumulation of Aβ peptide further amplifies a pathogenic cascade and the entire brain becomes affected which leads to further development of the AD neurodegenerative phenotype.

Other brain changes include inflammation and atrophy. Although microglial cells were found to localize near senile plaques as early as at the beginning of the 20th century *(del Rio*

Hortega et al., 1927), it took several decades to recognize their importance in AD pathology. As residual immune cells of the central nervous system (CNS), microglial cells have the ability to repeatedly proliferate in order to preserve homeostasis throughout life (*Askew et al., 2017*). It is now well-known that microglia is activated by various changes in the CNS that threaten the immune system, such as systemic or localized inflammations, nerve injury, ischemia, mutations in genes involved in encoding innate immune components or the presence of pathological protein lumps (*Luo and Chen, 2012*). For toxic Aβ aggregates and tau proteins, it has been also shown that they activate the microglial cells and induce their specific, transformed morphology in AD (*Baik et al. 2016*). Furthermore, it is also recognized today that in the brains with ongoing neuroinflammation, microglia develop an increased sensitivity to inflammatory stimuli (*Perry and Teeling, 2013*). This occurrence has been named priming and has been also reported to cause microglial senescence, an irreversible dysfunctional state of these immune cells (*Prokop et al., 2013*) reflected in their reduced phagocytic ability, lowered motility and increased cytokine production *(Heneka et al., 2015).* Therefore, the activation and proliferation of microglia are significantly increased throughout neurodegenerative changes, including those that occur in AD *(Perry et al., 2010*).

Astrocytes react as well in AD, contributing further to reactive gliosis and pro-inflammatory signaling cascade that additionally worsen the neuroinflammation. Astrocytes adjust to all types of CNS insults through a process called reactive astrogliosis that became a pathological hallmark of structural lesions in CNS. Similar to activated microglial cells, reactive astrocytes in AD are found to cluster around dense-core plaques. Namely, prolonged neuroinflammation affects APP processing through beta-secretase and accelerate Aβ production, resulting in reduced Aβ clearance and phagocytosis by activated glial cells that further leads to an elevation in Aβ burden *(Sastre et al. 2003; 2006).*

Additional types of lesions in AD were found primarily in the hippocampal region and include Hirano bodies and granulovascular degenerations. Similarly to plaques found throughout the cortical tissue, and the tangles primarily localized in limbic and association cortices, these AD-specific lesions have specific distribution pattern and are particularly found in CA1 subregion of the hippocampus *(Serrano-Pozo et al., 2011).*

Finally, AD is characterized by the loss of neuropil and synaptic elements and ultimately by the neuronal loss. This loss of synapses and neurons has been hypothesized to generally follow the pattern of neurofibrillary tangle formation; however, it remains questionable whether plaques or tangles are causative of neuronal or synaptic loss *(Serrano-Pozo et al., 2011).*

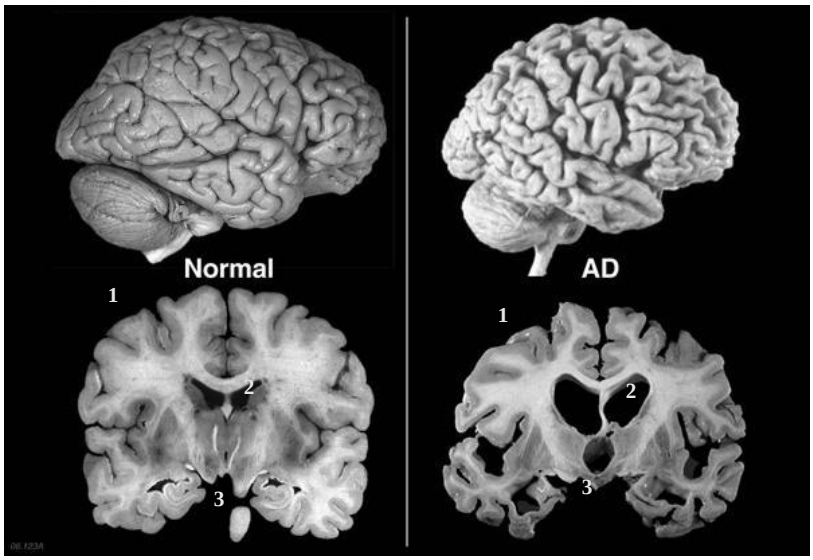

Figure 1: Brain atrophy in advanced Alzheimer's disease. (1) Extreme shrinkage of cerebral cortex; (2) Severely enlarged ventricles; (3) Extreme shrinkage of hippocampus (addapted from: https://alzheimersdiseasebiol2095.wordpress.com/alzheimers-effect-on-the-brain/)

As a result of neuronal loss, the brain of AD patients is also characterized by the shrinkage of the brain tissue. In contrast to aging, where neuronal loss is not associated to brain shrinkage, an overall atrophy of the cortex, with a 20-25% reduction in cortical volume, was observed in

AD patients compared to the brain of healthy aged individuals *(Mouton et al., 1998).* Beside the cortex, neurodegeneration is eventually found in the hippocampus, mainly in the CA1 region and hilus of the DG, but surprisingly not in the CA2, CA3 or the granular layer of the DG *(Terry et al., 1991).* Although an age-related decline in size of the hippocampus was also observed in the aging brain, patients with Alzheimer's disease show more prominent hippocampal atrophy in the medial temporal areas of both hemispheres *(Hayashi et al., 2009),* with an approximate loss of volume between 20% and 52*% (Mega et al., 2002).* Evidence assembled in several studies points in general that changes in hippocampus are an indicator for Alzheimer's disease. Hippocampal volume was therefore suggested as a means of grading cognitive decline and as a basis for early diagnosis of Alzheimer's disease *(Anand and Dhikav, 2012).*

3.1.2 Molecular mechanisms of AD

The amyloid cascade hypothesis

The amyloid cascade hypothesis was proposed for the first time around 30 years ago *(Hardy and Higgins, 1992).* It suggests that the disease itself involves a series of abnormalities in the process of generation and secretion of specific peptide, amyloid β, which is the cleavage product of the amyloid precursor protein (APP). An imbalance in production and clearance of amyloid β causes the accumulation of the protein and formation of the senile plaques, which becomes the cause of the disease over time *(Hardy and Selkoe, 2002).*

APP is a large, type-1 transmembrane glycoprotein with a long N-terminal domain and short cytoplasmic tail, which is expressed throughout the central nervous system. In humans, it is encoded by the APP gene on chromosome 21. The exact physiological function of the protein, however, is still not completely revealed. Experimental studies on hippocampal cell cultures

have shown that this protein plays an important role in the normal formation of synapses *(Priller et al., 2006),* and that its expression increases under stress *(Sanabria-Castro et al., 2017).* Mice deficient in this protein exhibit disruption of neuromuscular synapses *(Wang et al., 2005),* whereas overexpression increases synaptic plasticity and spatial memory in mice *(Ma et al., 2008).*

The proteolytic processing of APP can occur in two different ways, by the action of the membrane-associated enzymes α-secretase, β-secretase and γ-secretase (Figure 2) *(Wang et al., 2008).*

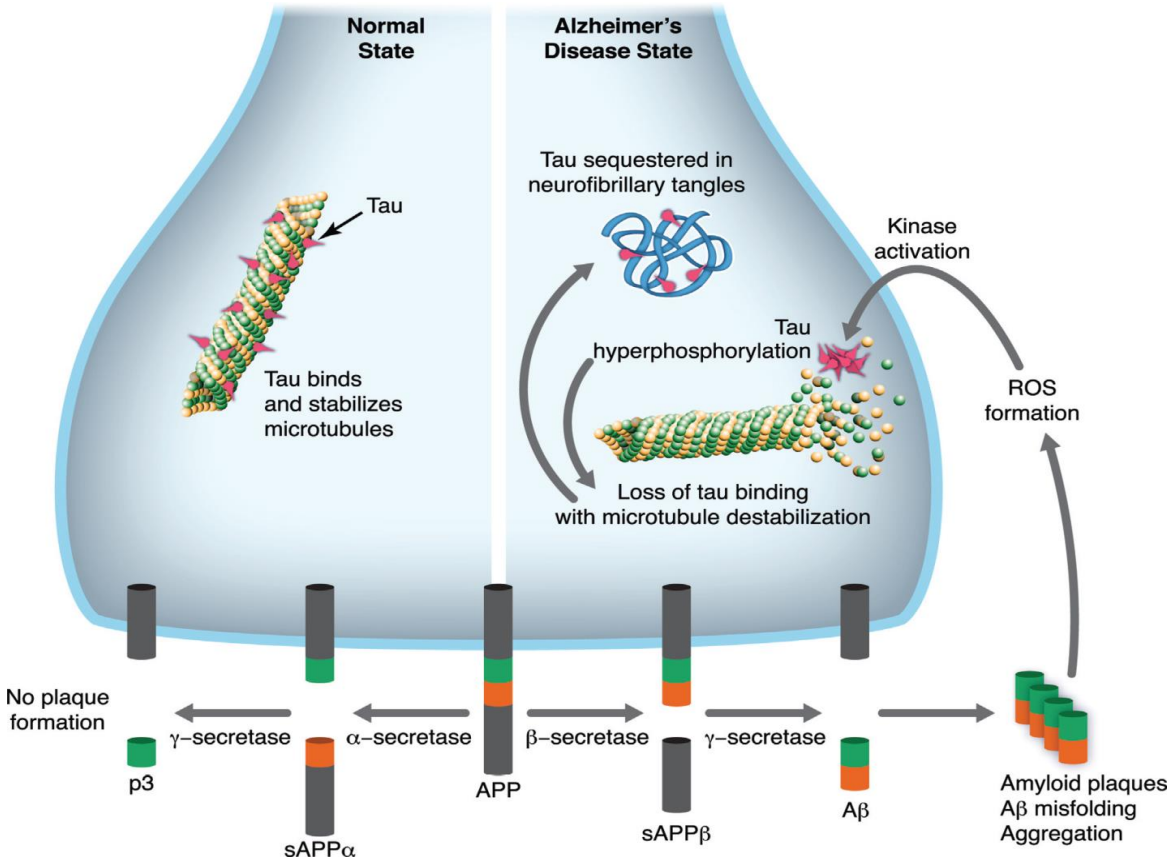

Figure 2: Senile plaques and neurofibrillary tangles are pathological hallmarks of Alzheimer's disease (from *Byrne, Heidelberger and Waxham, 2014*).

In the first, so called non-amyloidogenic pathway, APP is cleaved by α-secretase and soluble APPα (sAPPα) is formed. Under these normal conditions, the remaining membrane-associated C-terminal 83-amino acid fragment (C83) is further cleaved by γ-secretase and 3 kDa fragment (P3) and APP intracellular domain (AICD) are generated.

In the second, amyloidogenic pathway that is of pathological importance, the APP is initially cleaved by β-secretase and soluble i.e. sAPPβ forms. When the remaining membrane-associated fragment is then cleaved by γ-secretase, the Aβ peptides are produced *(Chasseigneaux and Allinquant, 2011).* Aβ peptides can be of different lengths, from 38 to 43 amino acids *(Dong et al., 2012),* but the most abundant are peptides of 40 or 42 amino acids ($A\beta_{40}$ and $A\beta_{42}$), with $A\beta_{42}$ being the dominant and the most toxic form of peptide that can promptly accumulate due to

its great insolubility *(Serrano-Pozo et al., 2011).* When mutations that are linked to the development of familial AD are present, the increase in the expression of $A\beta_{42}$ and the number of $A\beta_{42}$ aggregates is observed *(Palop and Mucke, 2010).* Therefore, in case of amyloidogenic pathway prevalence, the Aβ protein production is no longer regulated and it accumulates in large amount.

Tau protein

Another important protein implied to have major causative role in the etiology of AD is tau protein. In fact, it became obvious that the amyloid hypothesis alone is not sufficient to explain all the changes that occur within AD since the correlation between the amount of Aβ and senile plaques in the diseased brain and the severity of dementia in individuals diagnosed with AD remained questionable. Furthermore, all attempts to treat AD using Aβ-targeting drugs have ended in failure, implying the importance of recent findings indicating that the key factor for the development and progression of AD is most likely tau *(Kametani and Hasegawa, 2018).*

The neurofibrillary tangles (NFT) are intracellular inclusions, found in brain parenchyma extracellularly upon the death of nerve cells. They arise by the aggregation of hyperphosphorylated tau protein, a microtubule-associated protein that has a role in stabilizing microtubules *(Alonso et al., 1996).* Tau phosphorylation plays an important role in intracellular trafficking – tau is firstly removed from microtubules in order to allow the transport and dephosphorylation returns it to the microtubules *(Avila et al., 2004).* In AD, tau protein becomes defective by multiple phosphorylations, leading to the detachment of tau from the microtubule and causing microtubular structures to collapse and interrupt multiple cellular processes *(Guo et al., 2017).* Defective tau proteins are polymerized into paired helical filaments (PHF) and assemble with straight filaments (SF) forming NFTs in the neuron *(Iqbal et al., 2010).* Without

the skeleton, the neuron degenerates and the connections between the neurons are lost. The abnormal accumulation of the tau protein therefore eventually causes the death of the neuron and obstructs the brain function *(Gendron and Petrucelli, 2009).*

Numerous experimental models in cultured cells and mice have shown that abnormal tau affects normal tau and changes it to an abnormal type. Recent findings also suggest that APP, and not Aβ as thought earlier, may work as a receptor of irregular tau filaments and promote spreading of intracellular tau as well as its aggregation *(Takahashi et al., 2015).* Consequently, the hypothesis that tau aggregates firstly appear in a small number of brain cells, from where they spread to other regions, causing neurodegeneration and disease, has recently gained attention because it has been confirmed that tau proliferates and propagates between cells similarly to prion protein *(Goedert and Spillantini, 2017).*

Neuroinflammation

Over the past decade, another seemingly important feature of AD arose that may provide a better understanding of AD pathogenesis and further the explanation for the connection between the other two main AD pathological features. As mentioned above, in addition to Aβ plaques and NFT, the brains of patients with AD display evidence of a constant inflammatory response *(Tuppo and Arias, 2005; Walters et al., 2016)*

Brain inflammation has a double function, playing a protective role during an acute-phase response, but also becoming damaging when a chronic response is displayed *(Kim and Joh, 2006).* Acute brain inflammation is a well-known defense against infection, toxins, and injuries. The differences observed in neurodegenerative disorders including AD, are the disruptions in the

anti-inflammatory and pro-inflammatory signaling, and consequent occurrence of chronic inflammation (*Ferreira et al., 2014).* Neuroinflammation is not thought to typically appear on its own but rather as the result of the other pathologies or risk factors associated with AD that seems to increase the severity of the disease by intensifying β-amyloid and tau pathologies *(McGeer and Rogers, 1992).* This chronic neuroinflammation is assigned to activated microglia cells and the release a variety of proinflammatory and toxic products such as numerous cytokines, reactive oxygen species and nitric oxide. Therefore, microglia is now a central topic in the investigation of AD *(Kinney et al., 2018).*

3.1.3 Genetic and environmental risk factors for AD

The exact cause of AD remains elusive. It has been proposed that the disease results from multiple factors such as genetic, environmental and social *(Hersi et al., 2017).* Looking from the genetic aspect there are two types of AD: familial AD (FAD) and sporadic AD (SAD). They vary in the age of onset and the risk factors for development of the disease

Early onset AD is diagnosed in individuals under the age of 65 years and it accounts for a small percentage of all AD cases (5–10%). Of those cases, 13% are familial forms, with genetic predisposition leading to the disease *(Campion et al., 1999).* FAD has been shown to result from mutations in genes coding for APP and presenilin 1 and 2 (PS1 and PS2, respectively). In fact, mutations in PS1 were shown to be responsible for the majority of diagnosed FAD cases *(Kelleher and Shen, 2017).* Presenilins are transmembrane proteins primarily expressed in endoplasmic reticulum (ER) and membrane *(Benilova et al., 2012).* They are catalytic subunits of abovementioned intramembrane complex, gamma-secretase, that cleaves various other

substrates besides the APP. PS mutations cause gamma-secretase disruption, leading to abnormal APP cleavage and the formation of aggregate-prone $A\beta_{42}$ peptides.

Late onset AD is the most common form of dementia *(Tosto et al., 2019).* It shares the same clinical and pathological features of early onset AD but is diagnosed in individuals who are over the age of 65 years *(Efthymiou and Goate, 2017).* SAD was also associated with a combination of environmental and genetic factors. The strongest non-modifiable risk factor for developing SAD is age, and it has been estimated that after the age of 65, the risk of developing AD doubles roughly every five years *(Pase, Satizabal and Seshadri, 2017).*

APOE4, a variant of APOE gene, is the most significant genetic risk factor for sporadic early onset AD as well as late onset AD *(Chartier-Hariln et al., 1994).*

Epidemiological studies have also identified that a third of AD cases might be attributable to metabolic and environmental factors such as diabetes, hypertension, obesity, smoking, lack of exercise, and exposure to toxic metals (aluminium, copper), pesticides (organochlorine and organophosphate insecticides), industrial chemicals (flame retardants) and air pollutants *(Yegambaram et al., 2015).* The contribution of these factors to disease pathogenesis has been linked to epigenetic modifications of AD-linked genes *(Nicolia et al., 2015),* however, clear association is still not fully revealed.

3.1.4 Prodromal AD

With the recent discovery that various AD deposits, including β-amyloid and tau, may accumulate years before the beginning of active disease, there has been an alteration in the research focus from patients with active disease to the patients that are in so called “prodromal”

phase of the disease. The other reason for such different approach to AD was also the limited success of existing anti-amyloid drugs in patients with established AD symptoms *(Fischer and Agüera-Ortiz, 2017),* as well as the recognition of the importance of intervening before a major neuronal damage takes place.

Therefore, the refinement of diagnostic criteria and clear distinction between AD pathophysiological processes and clinically observable syndromes was made. The gradual decline in neurocognitive and behavioral function was introduced and it appears that the disease progression follows the path of neuropathological brain changes occurring over time in the disease *(Welsh-Bohmer, 2008).* In the early stages, also called the latent phase, the symptoms may not be visible at all. With the time passing, the pathology accumulates causing the early symptoms to emerge, and at that point the disease has reached the prodromal stage. The prodromal stage is then quickly followed by symptomatic stage, which is the fully manifested clinical disease.

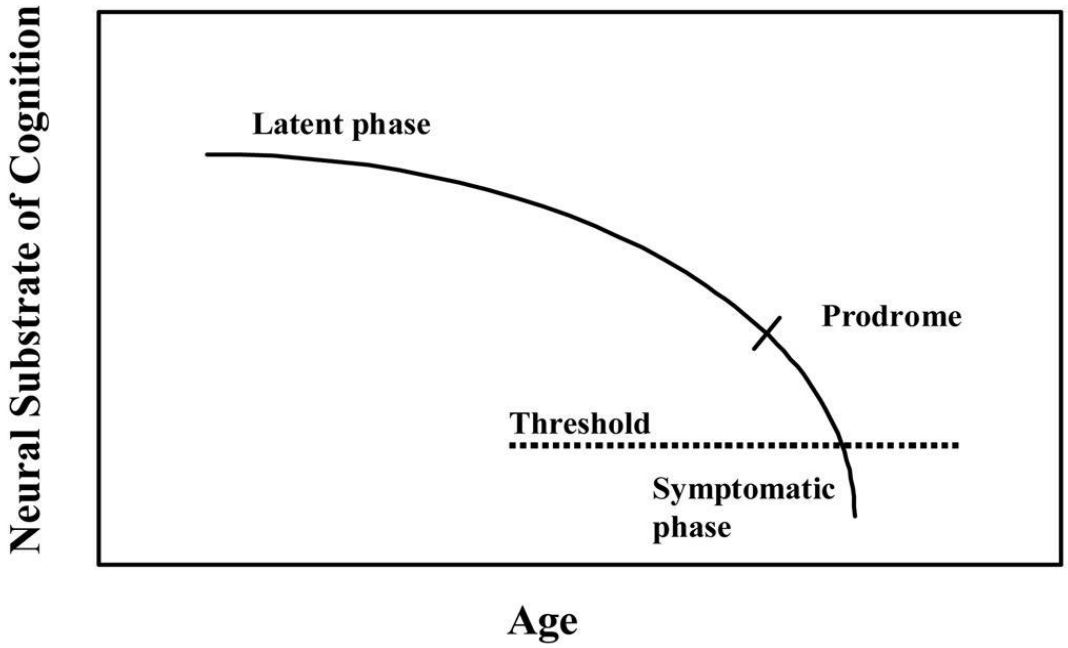

Figure 3: Chronic Disease Model of AD *(Welsh-Bohmer, 2008)*

3.1.5 Treatment

Currently approved Alzheimer's medications have been confirmed to help with memory loss symptoms and other cognitive changes for a short period of time. There are two essential types of drugs that are used to treat cognitive symptoms:

- Cholinesterase inhibitors (ChEIs) are the most common medicines prescribed to patients with mild-to-moderate AD. It is believed that these drugs work by increasing cell-to-cell communication levels and by preserving a chemical messenger depleted in the AD brain, i.e. the neurotransmitter acetylcholine *(Ma et al., 2019).*
- Memantine (Namenda) is usually prescribed for the treatment of moderate to severe AD. It works by a different mechanism than the cholinesterase enzyme inhibitors normally active in the management of AD *(Rogawski and Wenk, 2006).* It blocks the effects of glutamate, a neurotransmitter in the brain that leads to neuronal excitability and, therefore, it prevents excessive stimulation in AD.

At the moment, however, there is no fully effective cure or treatment for Alzheimer's disease that can reverse or stop the progression of pathology related to the disease. One of the reasons is that the most of drugs developed for AD focused on the hypothesis of amyloid cascade, which involves Aß plaques as the disease's causal factor *(Efthymiou and Goate, 2017).* Novel therapeutically strategies, therefore, take into account the complex nature of AD pathogenesis, and explore disease-modifying therapies and drugs targeting multiple molecular pathways in order to develop the treatment that would at least slow down the progression of AD. Studies in transgenic mice with altered development of amyloid-β plaques have also shown that therapies lowering amyloid-β production are most effective when administered before the plaque formation *(Chakrabarty et al., 2012).* Therefore, several drugs targeting prodromal AD with

diverse points of impact has been proposed as potential drugs that can be used as the therapy in AD patients and are already in the clinical testing and trial phases *(Cummings et al., 2018; Kodis et al., 2018).* There are also some indications that specific diets might be able to improve some of the symptoms caused by AD.

3.2 Food restriction

Food restriction (FR) is the most documented non-genetic and non-pharmacological approach for improving health and postponing age-linked disorders *(Fontana and Partridge, 2015).* It was first shown that reduced intake of all dietary elements except vitamins and minerals has an effect on extended lifespan in rats *(McCay, Crowell and Maynard, 1935).* Food restriction was further shown to improve most aspects of health during aging *(Maeda et al., 1985).* Numerous anti-oxidative and anti-inflammatory effects of food restriction were reported in animals, including the prevention or the delay in onset of many chronic diseases, such as obesity, diabetes, cancer, nephropathy, cardiomyopathy and neurodegeneration *(Fontana et al., 2010).* In humans, many of the same physiological, metabolic and molecular changes associated with food restrictions and avoided malnutrition were also observed. These include loss of age-associated myocardial stiffness and autonomic dysfunction, lower core body temperature and downregulation of the PI3K/AKT/FOXO and inflammatory pathways in skeletal muscle *(Fontana and Partridge, 2015).*

In the brain, beneficial effects of both daily calorie restriction (CR) or intermittent feeding (IF) such as every-other-day (EOD) feeding were also found, reflected by the reduced levels of proinflammatory cytokines, reactive oxygen species, and increased insulin sensitivity *(Martin et al., 2006).* Neuroprotective effects were also demonstrated during aging and in several animal models of injury. When appointed in the middle age, FR causes delay or prevention of brain functional impairments that are age-associated and benefits healthy aging by improving the motor coordination *(Mladenovic Djordjevic et al., 2010; Singh et al., 2015).* Rats that were put on CR feeding regimen for 2–4 months also showed resistance of hippocampal neurons to chemically induced degeneration. This decreased damage of hippocampal neurons was further associated with the preservation of learning and memory in a water maze spatial learning task *(Bruce-Keller et al., 1999).* Furthermore, IF improved age-related cognitive deficits in the triple-transgenic model of AD *(Halagappa et al., 2007).*

3.2.1 Types of food restriction regimens

Calorie restriction (CR) represents a 20%-40% reduction in average daily calorie intake, without causing malnutrition. Meal frequency is maintained in most cases *(Fontana and Partridge, 2015).* On the other hand, fasting characterizes more extreme version of FR, intermittent feeding, where nutrients are at some point completely eliminated. Intermittent feeding focuses on the eating frequency and may or may not include a limitation in the calorie intake during non-fasting periods.

There are a variety of feeding/fasting regimens:

- Intermittent feeding (IF) also known as alternate-day feeding (ADF) or every other day feeding (EOD) regimen includes unrestricted feeding every other day, and no or minimal calorie consumption on the days in between *(Raffaghello and Longo, 2017)*

- Periodic or prolonged fasting (PF) in which caloric intake is absent for two or more consecutive days during two or more weeks and unrestricted on all other days *(Raffaghello and Longo, 2017).* One of the most used patterns is 5:2 eating pattern that involves unrestricted eating for 5 straight days each week, followed by 2 days of restricted caloric intake.

- Time-restricted feeding where meals are consumed within a limited number of hours (*e.g.* 6-8 hours) every day, with nothing consumed during the other hours.

Lately, more attention was paid to the 5:2 eating pattern. This form of diet is being tested on obese population, aged between 55 and 70, with insulin resistance. The aim of the experiment was to find out how 8 weeks of the 5:2 diet, compared to a regular diet, affects insulin resistance and the brain chemicals and the results are expected to be reported at the beginning of the next year *(National Institute on Aging, 2018).*

3.2.2 Molecular mechanisms of neuroprotection by food restriction

Stress response

It is well known that the access to food forms one of the deepest behavioral sets. Therefore, the removal of satisfactory food sources triggers psychological and physiological stress in the organism, at least to a certain extent *(Martin et al., 2006).* Animals exposed to FR display metabolic and hormonal adaptations such as chronically elevated levels of glucocorticoids, paradoxically found to accompany various beneficial effects of FR *(Patel et al., 2002).* Furthermore, even on the cellular level stress-induced changes can be observed.

Numerous stress-related proteins like heat-shock proteins (HSPs) and glucose-regulated proteins (GRPs) have been measured in the brains of rats maintained on either *ad libitum* or CR diets. These proteins act as chaperones and interact with several down-stream proteins in cells, ensuring their proper folding, as well as the degradation of damaged proteins *(Shiber and Ravid, 2014).* Increasing levels of chaperone proteins may be a also protective response during the aging *(Lee et al., 1999).* For example, neuroprotective abilities of HSP-70 and GRP-78 were shown against injury and death in experimental models of neurodegenerative disorders *(Lowenstein et al., 1991).* Matching that discovery, levels of HSP-70 and GRP-78 were found to be increased in neurons of the CR rats in comparison to their age-matched *ad libitum* fed controls *(Lee et al., 1999).*

These findings might suggest that CR can induce a mild stress response in neurons, as a result of reduced energy availability, as well as to trigger increase in cellular stress resistance and the repair of damaged proteins and cells *(Martin et al., 2006).*

Antioxidant effects

The major source of reactive oxygen species are mitochondria, especially complex I in neuronal mitochondria *(Kausar, Wang and Cui, 2018).* The superoxide anion radical is usually generated at small concentrations during oxidative phosphorylation, but levels rise significantly after mitochondrial injury, for instance, due to intracellular calcium overload induced by excitotoxic injury *(Balaban et al., 2005).* Superoxide is then converted to hydrogen peroxide that serves as a source of hydroxyl radicals. The manifestations of neurological disease are therefore the result of oxidative damage of numerous biomolecules and DNA *(Nita and Grzybowski, 2016).*

A large number of the neurological deficits that occur subsequent to stroke, head trauma, anoxia or even Alzheimer's disease can be ascribed to secondary injury induced by glutamate excitotoxicity and, subsequently, intracellular calcium overload, mitochondrial dysfunction and oxidative stress *(Canevari et al., 2004).* Age-related oxidative damage to DNA is delayed by calorie restriction. Concentration of peroxidised lipids is also decreased, as well as the amount of damaged bases in nuclear and mitochondrial compartment *(Hunt et al., 2006).* Brain mitochondria isolated from CR rats show significantly less hydrogen peroxide production than those from age-like controls fed *ad libitum (Sanz et al., 2005).*

The answer of how the calorie restriction actually decreases mitochondrial production of reactive oxygen species is still unclear, yet uncoupling proteins (UCP) seem to be involved. They span the mitochondrial inner membrane and allow the moving of protons from the inside of the membrane space to the matrix, separating the electrochemical gradient from ATP generation. This uncoupling significantly reduces the mitochondrial membrane potential as well as the production of reactive oxygen species *(Harper et al., 2004).* Enhanced UCP activity has also

been associated with increased longevity and neuronal resistance to ischemic, toxic, traumatic and epileptic injury *(Liu et al., 2006).* Another theory suggests that CR does not increase lifespan due to reducing ROS levels but due to an increase in the expression of enzymes that protect against these highly reactive molecules that reduce net oxidative stress *(Gillespie et al., 2016).*

Neurotrophic factors

Since both IF and CR cause a mild stress response in brain cells, this can lead to the activation of compensating mechanisms, such as upregulation of neurotrophic factors like brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) as well as the heat shock proteins *(Duan et al., 2003).* IF regimens have been demonstrated to lessen neuronal damage and improve the functional outcome in animal models of seizure-induced damage due particularly to BDNF *(Duan et al., 2001).* Furthermore, the production of BDNF was associated with FR-induced increase in hippocampal neurogenesis in rats and mice *(Duan et al., 2001).*

Following all the indications above, dietary regimes could be of benefit in neurodegenerative disorders such as AD.

3.3 Hippocampus and parvalbumin interneurons

Hippocampus is one of the key brain structures for most vertebrates. It is located in the temporal lobe and has a structure similar to that of a seahorse *(Anand and Dhikav, 2012).* It is involved in the control of both short- and long-term memory, as well as in the emotional responses. Research has shown that neurogenesis also takes place in the hippocampus, even in adulthood. When hippocampus is affected due to a neurodegenerative illness such as AD or a

traumatic brain injury, the affected individual develops a short memory loss and further, the spatial memory loss *(Gilbert and Brushfield, 2009).* However, the long-term memory will remain less affected because it is stored in other parts of the brain.

One of the first brain areas affected by Alzheimer's disease is the hippocampus *(Frisoni et al., 2010).* Namely, an early sign of AD is when a person's short-term memory begins to deteriorate. Following directions might be hard as well. As the disease progresses, it becomes more difficult for the patient to function in daily life and this has been correlated with the loss of hippocampal volume. It was also observed that patients with mild cognitive impairment (MCI) have a 10-15% hippocampus volume loss, whereas the ones with AD are characterized by the hippocampal volume loss of 15-30% *(Frisoni et al., 2010).*

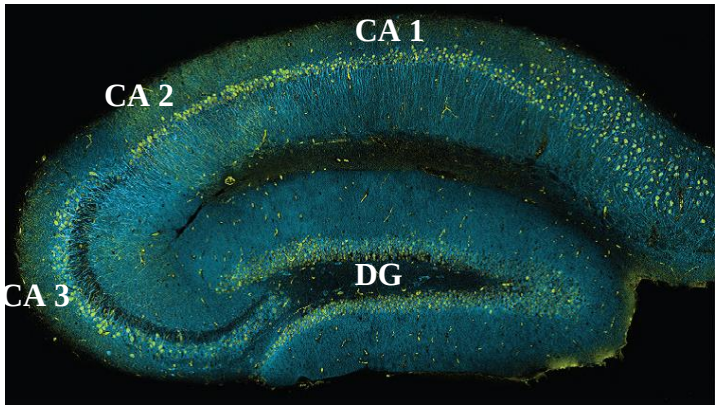

Figure 4: Mouse hippocampus (adapted from *Taguchi et al., The Journal of Neuroscience, 2017.*)

Structure of the hippocampus

The hippocampus consists of hippocampus proper and dentate gyrus (DG) that are separated by hippocampal sulcus and curve into each other *(Anand and Dhikav, 2012).* Hippocampus proper can be divided into two regions: a large-celled proximal region and a

smaller-celled distal region *(Watson et al., 2012).* Although there have been a few different terminologies concerning hippocampus regions, the one of *Lorente de Nó* has achieved more common usage *(Andersen et al., 2007).* He divided the hippocampus proper (Cornu Ammonis) into four CA areas: CA1, CA2, CA3 and CA4. There are remarkable differences between all three areas concerning intrinsic and extrinsic connectivity. In general, the laminar organization is similar for all three hippocampus fields. The main layer of cells is called the pyramidal cell layer. The narrow, relatively cell-free layer is located deep within the pyramidal cell layer and is called *stratum oriens,* and the fiber-containing *alveus* is deeper within that. One more layer, called *stratum lucidum,* is only to be found in CA3, and it is located just above the pyramidal cell layer. It contains the mossy fiber axons originating from the DG. The *stratum radiatum* is superficial to *stratum lucidum* in CA3 and directly above the pyramid cell layer in CA2 and CA1. *Stratum lacunosum-moleculare* is the most superficial layer. Perforant pathway fibers from the entorhinal cortex travel and terminate in this layer. The DG lies at the end of hippocampus proper and consists of a layer of tightly packed granule cells. It plays an important role in processing information from entorhinal cortex (EC) to CA3 *(Ohm, 2007).*

Throughout hippocampus, a vast diversity of interneurons can be found which contain gamma aminobutyric acid (GABA) as the principal inhibitory neurotransmitter of the CNS. In particular, 21 subtypes of these interneurons were identified and they display heterogeneous morphological and physiological features *(Somogyi and Klauseberger, 2005).* One approach to classify GABAergic interneurons is based on the expression of Ca^{2+}-binding proteins (CBPs) such as parvalbumin (PV), calbindin D-28k (CB) and calretinin (CR) *(Lawrence et al., 2010).* These CBPs are among the major fast cytoplasmatic calcium buffers in the central nervous system *(Baimbridge et al., 1992)* and the disturbance of neuronal calcium homeostasis and consequent molecular events affect neuronal viability and synaptic plasticity. Therefore,

alteration in CBPs may represent an early step in the development of neuronal degeneration *(Foster, 2007).*

PV interneurons have been shown to be involved in several brain functions, including synaptic plasticity *(Donato et al., 2013)* and the initiation of network oscillation *(Amilhon et al., 2015).* The loss of the PV neuron subpopulation was correlated with enhanced facilitation *(Caillard et al., 2000)* and transmitter release *(Muller et al., 2007)* and therefore, their role in the excitation/inhibition balance was proposed. There are also increasing evidence of disruption of inhibitory control and the hyperactivity of neuronal networks in AD brain that can underlie major deficits in cognitive function such as learning and memory that is accompanied by loss of PV-expressing neurons *(Brady and Mufson, 1997; Mikkonen et al., 1999).* The reduction in CB expression has been also observed in the brains of humans with AD, but it has not been demonstrated yet that these changes underlie AD-related dysfunction *(Kook et al., 2014).* Although studies further show a possible link between hippocampal PVs and social memory *(Klausberger and Somogyi, 2008),* the role of hippocampal interneurons as well as their specific acting mechanism in this process are still not fully understood.

3.4. Aims of the thesis

Numerous neuroprotective effects of food restriction are well-known in the literature. It is also known that neuronal degeneration associated with Alzheimer's disease correlates with impaired calcium homeostasis and changes in calcium-binding proteins. As AD is characterized by decades-long, clinically silent prodromal phase of the disease, the present study was conducted with an aim to examine the effects of preventive intermittent, every-other-day feeding regimen in the brain of 5XFAD mice, a commonly used transgenic animal model of Alzheimer's disease, particularly on parvalbumin- and calbindin-expressing neurons in the dorsal hippocampus.

4. Methods and Materials

4.1 Animal model

Transgenic 5XFAD mice and their background strain (B6SJLF1/J) were purchased from the Jackson Laboratory (Cat. No: 3484-JAX and 100012-JAX, Bar Harbor, Maine, USA). Animals used in this study were obtained by crossing 5XFAD transgenic male mice with B6SJLF1/J female mice (B6/SJL genetic background). Genotyping of the mice was performed by PCR and the use of specific set of primers, as recommended by the provider. Only female F1-offspring was used.

All animal procedures were in compliance with Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković"- National Institute of Republic of Serbia, University of Belgrade (#1-06/13).

5XFAD mice carry five familial Alzheimer's disease mutations: Swedish (K670N, M671L), Florida (I716V), and London (V717I) mutations in human amyloid precursor protein (APP695) and two mutations (M146L and L286V) in the human presenilin 1 protein under control of the Thy-1 promoter that is neuron-specific and expresses transgenes solely in neurons. As early as 2 months of age, 5XFAD mice start to develop amyloid deposits and exhibit neuroinflammation in hippocampal and cortical areas. Three months later, at the age of 5 months, the degeneration of synapses can be detected, while at the age of 4–6 months, the deficits in hippocampal memory start to develop *(Oakley et al., 2006; Ohno, 2009; Ohno et al.,*

2007). The robust neuronal loss in the subiculum, cortical layer V, and the medial septum can be observed in 9-month-old animals *(Eimer and Vassar, 2013).*

4.2 Feeding regimens

A total of 41 female mice were included in the study: 5XFAD transgenic mice (Tg), n=21 and their non-transgenic littermate controls (non-Tg), n=20. The animals were housed under standard conditions (23±2°C, 60–70% relative humidity, 12 hour light/dark cycle, free access to water) with food (standard laboratory chow pellets containing 8.34% water, 21.61% crude protein, 2.36% crude fat, 6.68% crude fiber, 6.55% crude ash, 1.95% minerals; Veterinarski zavod Subotica) provided *ad libitum* (AL) until the animals have reached 2 months of age. At that point, 5XFAD mice were randomly assigned to two different feeding groups: the 5XFAD-AL (Tg-AL) group that continued to receive food *ad libitum* (n=12), while the 5XFAD-EOD (Tg-EOD) group was fed *ad libitum* every other day (EOD) for the next 4 months (n=9) (Figure 5). The non-transgenic mice were divided the same way, in groups fed AL (non-Tg-AL; n=12) and EOD (non-Tg-EOD; n=8) that were used as the control groups.

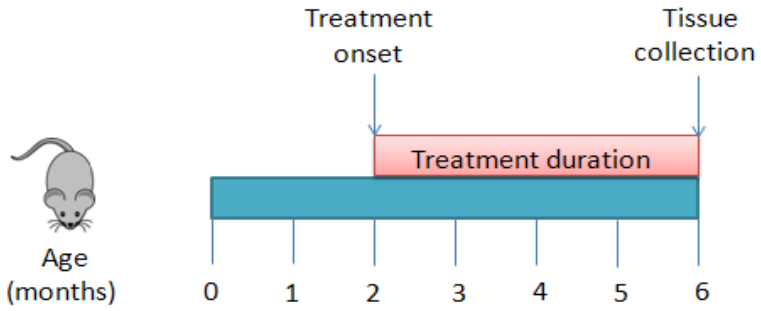

Figure 5: Experimental timeline. 2-month-old 5XFAD mice and their non-transgenic littermate controls were divided into two groups: one group was fed *ad libitum* (non-Tg-AL, 5XFAD-AL), while the other was fed every other day (non-Tg-EOD, 5XFAD-EOD) for a period of 4 months.

The body weight of the animals was measured every two weeks. It is well-known that EOD feeding regimen does not cause body weight reduction since animals have food available *ad libitum* on the feeding day and could compensate on the following day *(Anson et al., 2003)*. For the same reason, it is also unlikely that the EOD feeding regimen caused any vitamin and mineral deficiency.

4.3 Biochemical analyses

At the 6 months of age, mice were anesthetized by intraperitoneal injection (100 mg/kg ketamine and 10 mg/kg xylazine) and blood samples for serum preparation were taken via cardiac puncture between 10 and 12 AM. Fasting serum glucose concentration was determined by the glucose oxidase procedure using a commercial kit (Glucose Diagnostics #635; Sigma Chemical Co., St. Louis, MO). Insulin concentration was determined by commercial insulin ELISA kit (INEP, Belgrade, Serbia), with mouse insulin as a standard. Total cholesterol, triglycerides, AST, ALT, urea and total proteins in the serum were measured using standard spectroscopic methods and Biosystems A-35 analyzer (Biosystems, S.A., Barcelona, Spain), according to manufacturer's instructions.

4.4 Tissue collection

Following cardiac puncture, animals were transcardially perfused with 0.1 M phosphate-buffer saline (PBS, pH = 7.4). The brains were quickly removed and dissected on ice; from the right hemisphere, the entire cortex and hippocampus were isolated for further WB and ELISA

analyses, snap-frozen in liquid nitrogen and stored at −80 °C. For histological studies, the left hemispheres were fixed in freshly prepared 4% paraformaldehyde (PFA) in PBS for 24 hours and cryoprotected in 30% sucrose/PBS at 4°C until complete saturation. The hemispheres were then sectioned (coronal sections, 30 µm) using a cryostat (Leica, Wetzlar, Germany) at the levels approximately -1.656 to -2.255 from the bregma according to The Allen Mouse Brain Atlas *(2008; Allen Institute for Brain Science, Allen Mouse Brain Atlas, http://mouse.brain-map.org/static/atlas)* and further stored as free-float sections in a cryoprotective buffer (0.05 M phosphate buffer, 25% glycerol, and 25% ethylene glycol) at -20°C.

4.5 Enzyme-linked immunosorbent assay (ELISA)

To determine the concentration of Human $A\beta_{42}$, a commercially available $A\beta_{42}$ Human ELISA Kit (Invitrogen) was used according to manufacturer's instructions. In brief, cortical and hippocampal tissues were homogenized in 8 volumes of 5 M guanidine HCl/50 mM Tris HCl (pH 8.0) and further incubated at RT for 3 h with shaking. Samples were than diluted with cold PBS containing protease inhibitor cocktail (Roche), and centrifuged at 16 000 g / 4 °C for 20 min. The assessment of $A\beta_{42}$ concentrations in the supernatant was performed using concomitantly generated standard curve.

4.6 Western blot

For whole protein extracts, cortical tissue was homogenized and sonicated in 10 volumes of RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% Triton X-100, 1 mM EDTA, 1 mM EGTA) that contained protease and phosphatase inhibitors (Roche

Diagnostics, Basel, Switzerland). After the centrifugation at 20 000g / 4 °C for 30 min, the supernatants were collected and stored at −80 °C. Concentration of the proteins was determined using the Micro BCA Protein Assay Kit (Pierce Biotechnology, Massachusetts, USA) and the Bovine Serum Albumin (BSA) as a standard. SDS-polyacrylamide gel electrophoresis (10% or 12%) was used to separate identical amounts of proteins (5 or 10 μg per lane), that were further blotted onto PVDF membranes (GE Healthcare, Little Chalfont, UK). In order to block non-specific binding, PVDF membranes were incubated in 5% non-fat dry milk/TBST (150mM NaCl, 50mM Tris, pH 7.4, and 0.1% Tween20) for 1h at room temperature (RT). Following the blocking step, membranes were shortly rinsed with TBST and incubated overnight with primary antibodies at 4°C, in 2% blocking solution (Table 1 for details of primary and secondary antibodies used). The membranes were rinsed 3-5 times for 5 minutes in TBST before they were incubated for 1h on RT with appropriate Horse Radish Peroxidase (HRP)-conjugated secondary antibodies. Visualization of HRP-immunoreactive bands was achieved using enhanced chemiluminiscence (ECL, GE Healthcare, Little Chalfont, UK) and film exposure (Kodak Biomax). Each blot was re-probed with mouse anti-β-actin or goat anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody. Densitometric analysis and quantification of signals was performed using *Image Quant* software (v. 5.2, GE Healthcare). Results are expressed as relative values obtained by normalization, i.e. the use of corresponding signals of loading controls. The levels of the target protein in Tg-AL and Tg-EOD mice were calculated in relation to the suitable control value in non-Tg-AL mice (set to 100%).

Table 1. Primary and secondary antibodies used for WB

Primary antibody (manufacturer, catalog number)	Secondary antibody (manufacturer, catalog number)
Astrocytes	
Mouse anti-GFAP (Millipore, MAB360)	HRP-conjugated anti-mouse (Dako, P0260)
Tumor necrosis factor α	
Mouse anti-TNF-α (Abcam, ab1793)	HRP-conjugated anti-mouse (Dako, P0260)
Loading controls	
Mouse anti-β-actin (Sigma-Aldrich, A5316)	HRP-conjugated anti-mouse (Dako, P0260)
Goat anti-GAPDH antibody (Santa Cruz Biotechnology, sc-20357)	HRP-conjugated anti-goat (Santa Cruz Biotechnology, sc2350)

4.7 Histology

4.7.1 Thioflavin staining

For detection of Aβ plaques, the sections were incubated in 1% aqueous Thioflavin-S (Sigma, T1892) for 8 minutes and further rinsed in 80% ethanol, 95% ethanol and distilled water. All sections were subsequently mounted on slides and covered with Fluoroshield mounting medium (Abcam, ab104139).

4.7.2 Immunohistochemistry (IHC)

Sections were initially rinsed 3 times for 5 minutes with 0.1 M PBS, pH 7.4. For light microscopy, The Vectastain Universal Elite ABC kit (Vectastain, PK-6200) was used. To avoid high nonspecific background staining due to endogenous peroxidase, sections were incubated in 3% H_2O_2, 10% methanol in PBS for 15 minutes at RT and to avoid nonspecific binding, sections were incubated for 1hr at RT in Blocking serum (Normal serum) from the ABC kit, following the manufacturer's instructions. The sections were further incubated overnight at RT with

monoclonal mouse anti-parvalbumin antibody (PARV-19) and, after PBS wash (3 times for 5 minutes), with Biotinylated secondary antibody from the ABC kit for 1 hour at RT. After rinsing with PBS, 3 times for 5 minutes, sections were incubated for 45 min at RT in ABC Reagent, from the ABC kit prepared following the manufacturer's instructions. Following the last step, the sections were thoroughly rinsed in PBS. For detection of PV+ neurons, the sections were incubated in DAB (Vector, SK-4100) for 10 minutes and rinsed for 5 minutes in tap H_2O, distilled water and PBS, respectively. All sections were then mounted on slides and covered with Permount medium (Fisher Chemical) and cover slips (Menzel-Gläser).

Table 2. Primary and secondary antibodies used for IHC

Primary antibody (manufacturer, catalog number)	Secondary antibody (manufacturer, catalog number)
Parvalbumin positive neurons	
Monoclonal Anti-Parvalbumin antibody, clone PARV-19 (Sigma, P3088)	Biotinylated secondary antibody, ABC kit (Vectastain, ZE0829)

4.7.3 Immunofluorescence

For immunofluorescence (IF), sections were rinsed 3 times for 5 minutes with 0.1 M PBS, pH 7.4. To avoid nonspecific background staining, sections were incubated in 10% normal goat serum in PBS for 30 minutes at RT. The sections were further incubated overnight at RT with primary antibodies: rabbit anti-anti-ionized calcium binding adaptor molecule 1 (Iba-1) to detect microglia; mouse anti-glial fibrillary acidic protein (GFAP) for detection of astrocytes; monoclonal mouse anti-parvalbumin (PARV-19) and anti-calbindin D-28K antibody for detection of parvalbumin- and calbindin-positive neurons in hippocampus, respectively. After incubation in primary antibodies, and rinsing 3 times for 5 minutes with PBS, sections were

incubated with fluorophore-conjugated secondary antibodies for 1h at RT (Table 3. for detailed information on antibodies used). Following incubation in secondary antibody, the sections were rinsed once more in PBS (3 times for 5 minutes). All sections were finally mounted on slides and covered with Fluoroshield mounting medium containing DAPI (Abcam, ab104139) and cover slips (Menzel-Gläser).

Table 3. Primary and secondary antibodies used for IF

Primary antibody (manufacturer, catalog number)	Respective secondary antibody (manufacturer, catalog number)
Microglia	
Rabbit anti-Iba-1(Wako, NC9288364)	Alexa fluor-568-conjugated anti-rabbit (Invitrogen, A11036)
Astrocytes	
Mouse anti-GFAP (Millipore, MAB360)	Alexa fluor-568-conjugated anti-mouse (Invitrogen, A-11004)
Parvalbumin positive neurons	
Monoclonal anti-Parvalbumin antibody, clone PARV-19 (Sigma, P3088)	Alexa fluor 568-conjugated anti-mouse (Invitrogen, A-11004)
Calbindin positive neurons	
Rabbit anti-Calbindin D-28K antibody (Millipore, AB1778)	Alexa fluor 488-conjugated anti-rabbit (Invitrogen, A-11008)

4.7.4 Analysis of Thioflavin S-stained plaques

Evaluation of Thioflavin-S-positive plaques was done on images taken on AxioVision fluorescent microscope (Carl Zeiss) in a single plain (5x magnification). Threshold processing (Otsu) and analysis were done using *ImageJ* software (US National Institutes of Health). The total number of plaques as well as other parameters of plaque morphology were acquired using particle analysis. Altogether, 5 fields at 2-4 nonadjacent sections that included anterior, central and posterior part of both primary somatosensory cortex and posterior parietal cortex were

analyzed in each animal. Representative images were obtained from 15-20 overlapping images using the *Image Composite Editor* software (Microsoft).

4.7.5 Quantification of Iba1-positive microglia and GFAP-positive astrocytes

Quantification was performed on images taken on BZ9000 fluorescent microscope at 20x magnification and the use of threshold processing (Otsu) in *ImageJ* software (US National Institutes of Health). The percentage of the area that was covered by the Iba-1- and GFAP-positive signal was measured. Altogether, 5 fields at 2-4 nonadjacent sections, which included anterior, central and posterior part of both primary somatosensory cortex and posterior parietal cortex, were analyzed in each animal. Images of higher magnification were taken on Nikon confocal microscope with standardized gain, digital offset and laser intensity, and these conditions were kept identical for all groups.

4.7.6 Quantification of parvalbumin- and calbindin-positive interneurons

Counting of PV+ interneurons was performed from photomicrographs taken by light microscopy on Axio Observer Microscope Z1 (Carl Zeiss, Germany) at 20x magnification. In each animal, 2-4 nonadjacent sections from anterior, central and posterior part of dorsal hippocampus were analyzed, and separate analysis of total areas of hippocampal CA1, CA3 subregions and DG was performed. *ImageJ* software was used (US National Institutes of Health).

Integrated density of PV+ and CB+ interneurons was performed from the photographs taken by Leica TCS SP5 II confocal microscope. Scans were done by the use of 20x objective, 1.5 zoom at a resolution of 1024x1024 pixels and at a scan speed of 400 Hz. Z-stacks for two channels were made from 22 scans of 0,05 μm steps starting from the top. For each animal, layers from 18 to 4 were analyzed using *LAS AF Lite* sofware. The analyzed structure was contoured and the background density was subtracted from the density obtained.

4.8 Statistical analysis

All data are presented as mean ± SD. For statistical analyses of Aβ load (Figure 1B and 1C) and the variances between the two groups, F test and Student's t-test were used. One-way analyses of variance (ANOVA) followed by Tukey's post hoc test was applied for multiple comparisons between three experimental groups. For all analyses, normality test was initially conducted using GraphPad Prism 7.0 software. A p value of less than 0.05 was considered as significant.

5. Results

5.1 General health and body weights of the animals

5XFAD mouse model is characterized by an early onset of amyloid plaques deposition in cortical and hippocampal areas accompanied by the loss of appetite and body weight at the age of 9 months *(O'Leary et al., 2018).* Therefore, the appearance and general behavior of animals in all groups was analyzed first. At the age of 6 months, Tg-EOD mice did not phenotypically differ from Tg-AL or non-Tg-AL animals. The body weights did not differ among groups as well, although a trend toward decrease in Tg-AL mice was observed in comparison to non-Tg-AL animals (p=0.069, one-way ANOVA). Body weight gain was 2.355 ± 1.869, 0.163 ± 1.132, 1.763 ± 1.064, 0.27 ± 0.793 for non-Tg-AL, non-Tg-EOD, Tg-AL and for Tg-EOD animals, respectively. Therefore, a significant change in the body weight gain was neither observed.

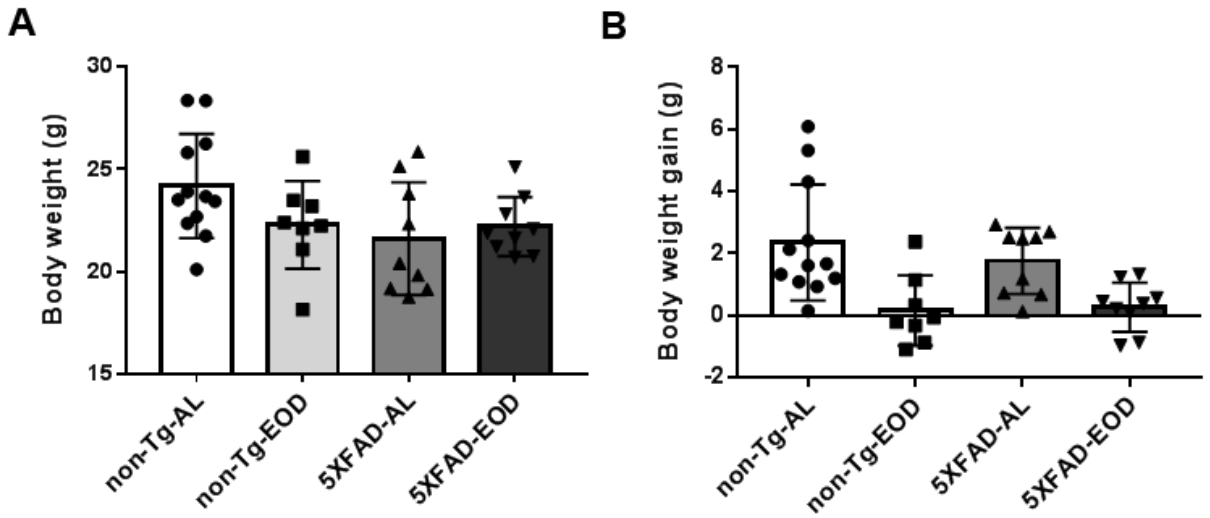

Figure 6: Body weight analysis. (A) Body weight measurements and (B) Body weight gain of non-Tg AL, non-Tg EOD, Tg-AL and Tg-EOD animals (one-way ANOVA).

5.2 Serum parameters

The biochemical profile of serum revealed expected decrease in the level of fasting glucose in non-Tg-EOD group in comparison to non-Tg-AL (p=0.0172, one-way ANOVA; Fig. 7A). Serum glucose levels were, however, similar in non-Tg-AL, Tg-AL and Tg-EOD experimental groups indicating normal glucose metabolism with this particular type of FR regimen in 5XFAD animals. Insulin levels were not changed as well (Fig. 7A). For all other serum parameters analyzed, including total cholesterol, triglycerides, AST, ALT, urea and total proteins no significant change was observed (Fig. 8). Adrenal glands were also of the same weight in all groups analyzed (Fig. 7B).

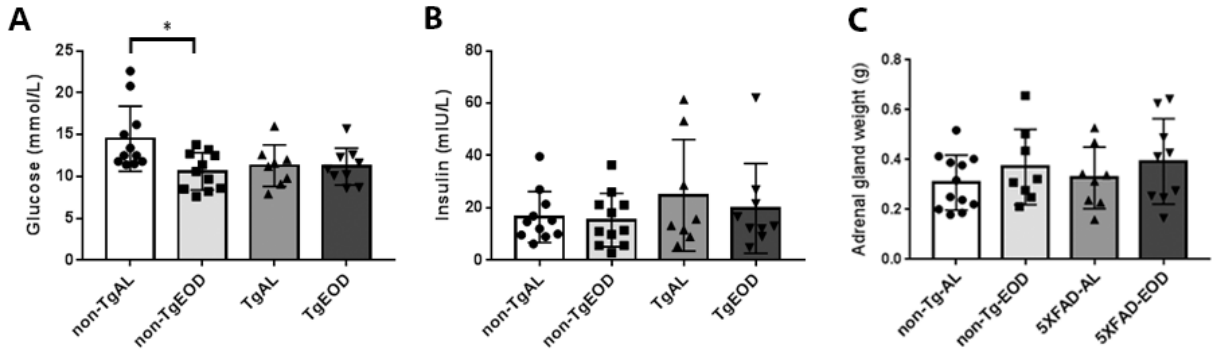

Figure 7: Biochemical analyses of fasting glucose and insulin in the mouse serum and adrenal gland weight. Only glucose levels between non-Tg-AL and non-Tg-EOD were significantly changed (p=0.0172, one-way ANOVA). Statistical significance is determined with one-way ANOVA followed by Tukey's post hoc test.

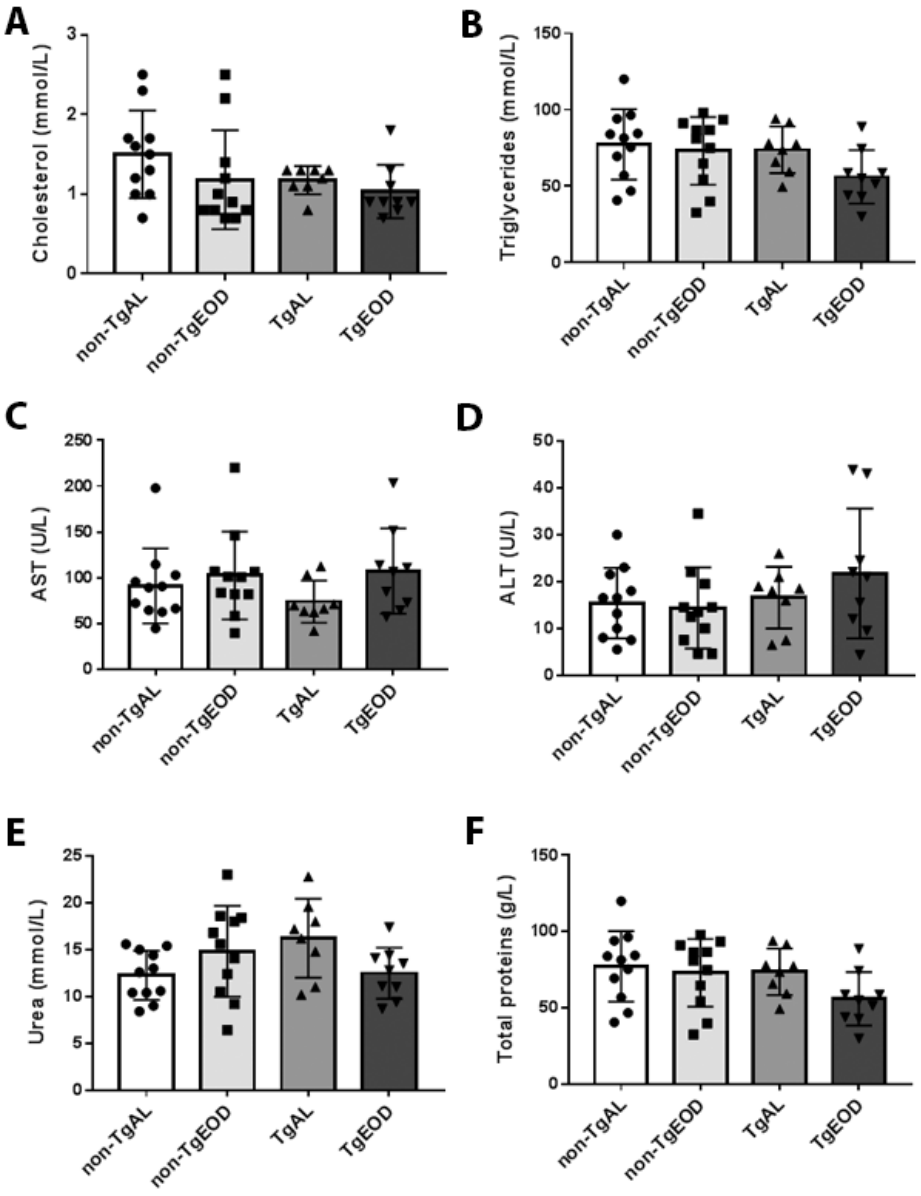

Figure 8: Biochemical analyses of mouse serum. No changes were observed in total cholesterol, triglycerides, alanine transaminase (ALT), aspartate transaminase (AST), urea and total proteins levels. Statistical significance is determined with one-way ANOVA followed by Tukey's post hoc test.

5.3 EOD feeding regimen caused no change in amyloid-β deposits in the cortex of 5XFAD female mice

Senile plaques, a major hallmark of AD-like pathology in 5XFAD mice, represent deposit of Aβ produced by enzymatic cleavage of its precursor, APP that is overexpressed in these particular mice. In order to evaluate the effect of EOD feeding regimen on plaque formation and deposition in the cortex of 5XFAD mice, histochemical staining of coronal brain sections at the end of the experiment was performed. Brain sections were stained with Thioflavin S (ThS), fluorescent dye that binds to polypeptides and proteins containing antiparallel β-pleated sheet secondary structure, and therefore interacts with Aβ-containing plaques (Figure 9).

Quantitative analysis of ThS-positive plaque load in the cortical tissue of Tg-EOD mice revealed no change in total number of plaques in comparison to Tg-AL group regardless of the size of plaques quantified (p=0.4643, two-tail Student's t-test; Figure 10A and Figure 11A).

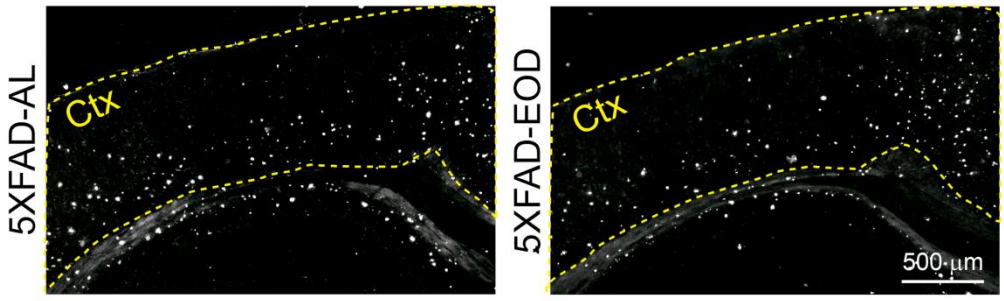

Figure 9: ThS-positive plaque load in the cortex of 5XFAD-EOD mice. Representative images of Thioflavin S-positive amyloid deposits in the cortex of 5XFAD-AL and 5XFAD-EOD mice. Scale bar = 500 µm;

Further detailed analysis by plaque size (more and less than 100 μm^2), average plaque size and percentage of total area covered by plaques also implied no changes in the distribution

of plaques within cortical tissue/layers of primary somatosensory cortex and posterior parietal cortex (Figure 10 and Figure 11).

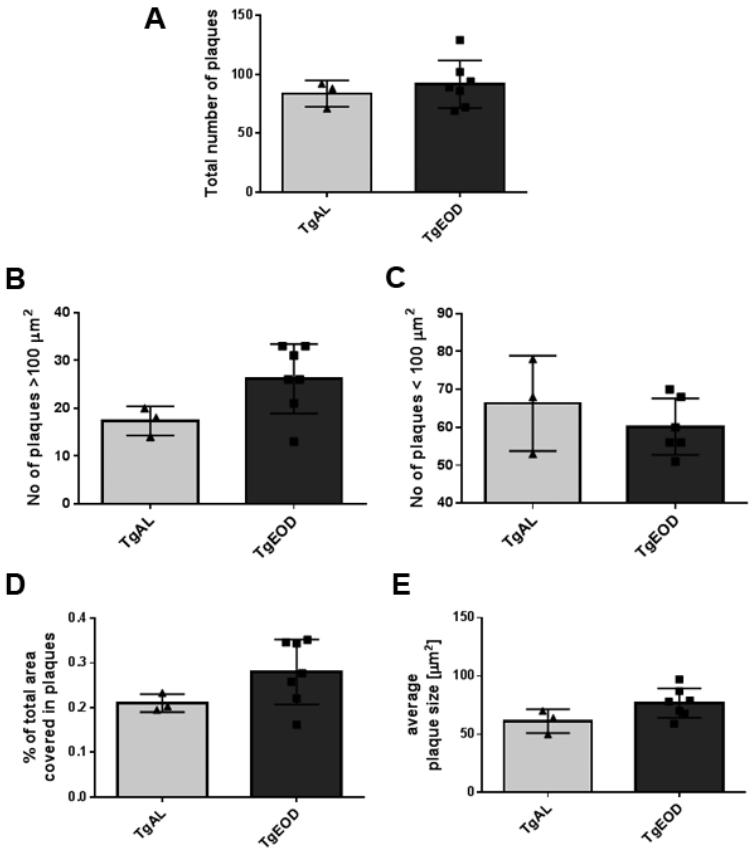

Figure 10: Quantitative analyses of Thioflavin S-positive amyloid plaques in the primary somatosensory cortex of 5XFAD mice. (A) Total numbers of plaques, (B) numbers of plaques > 100 µm^2, (C) numbers of plaques < 100 µm^2 (D) total area covered in plaques, (E) average size of plaques evaluated after Thioflavin S staining and the analysis using ImageJ software. Data are shown as single points per animal with bar graphs representing mean ± SD. *n* = 4-7 mice per group. No significant changes were observed in any one of the analyzed parameters (one-way ANOVA followed by Turkey's post hoc test).

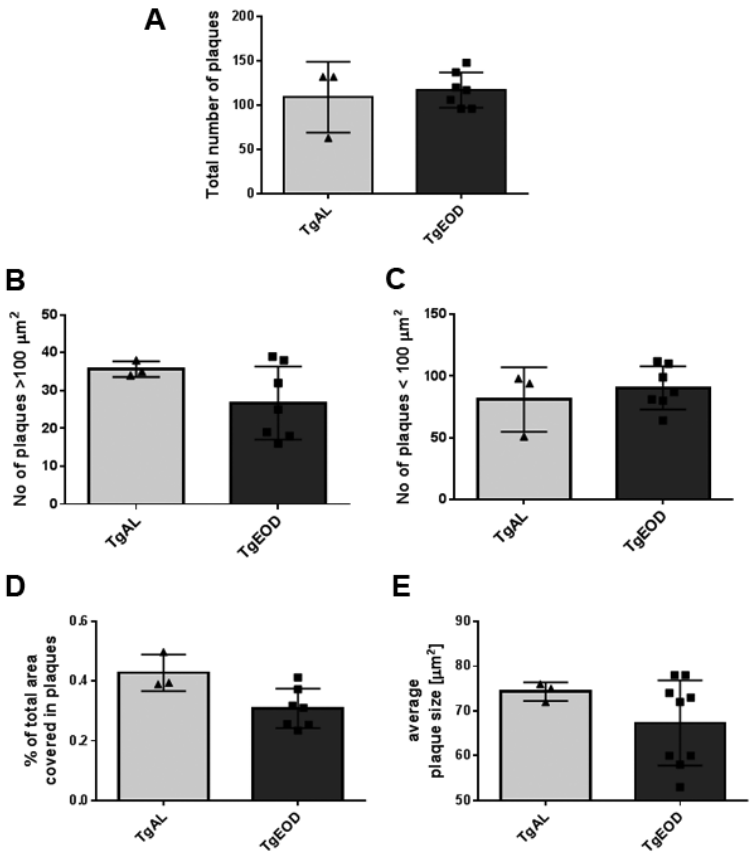

Figure 11: Quantitative analyses of Thioflavin S-positive amyloid plaques in the posterior parietal cortex of 5XFAD mice. (A) Total numbers of plaques, (B) numbers of plaques > 100 µm^2, (C) numbers of plaques < 100 µm^2 (D) total area covered in plaques, (E) average size of plaques evaluated after Thioflavin S staining and the analysis using ImageJ software. Data are shown as single points per animal with bar graphs representing mean ± SD. *n* = 4-7 mice per group. No significant changes were observed in any one of the analyzed parameters (one-way ANOVA followed by Turkey's post hoc test).

The total amount of $A\beta_{42}$ peptide was evaluated as well, in the cortical and hippocampal homogenates, by ELISA. Analysis revealed no changes in $A\beta_{42}$ levels in cortical or hippocampal tissue of Tg-EOD mice in comparison to Tg-AL animals (p=0.2782, two-tail Student's t-test, Figure 12).

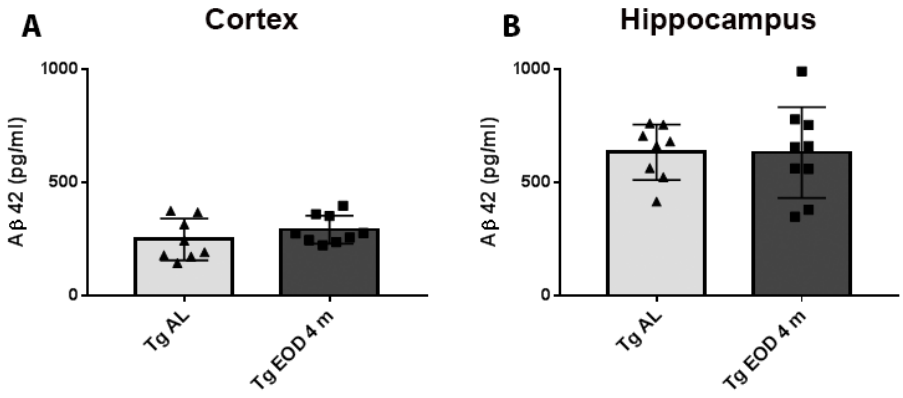

Figure 12: No changes in Aβ42 levels in the cortex and hippocampus of 5XFAD –EOD mice. Total amyloid-β 42 (Aβ42) levels in the cortical and hippocampal homogenates of Tg-AL and Tg-EOD mice, determined with ELISA. Data is shown as single points per animal with bar graphs representing mean ± SD. *n* = 4-7 mice per group. (two-tail Student's t-test).

5.4 EOD feeding regimen up-regulates Iba-1-positive staining in 6-month-old 5XFAD mice

Neuroinflammation is one of the first signs of pathology in 5XFAD mice that starts as early as 2 months of age *(Oakley et al., 2006).* Therefore, the neuroinflammatory status and gliosis in EOD-fed mice was further examined. Microglial activation was evaluated by fluorescent immunostaining (Figure 13) using antibody for microglial marker, ionized calcium-binding adapter molecule (Iba-1), and the measurement of total area percentage covered (Figure 14).

As expected, immunohistochemical analysis revealed the increase of Iba-1-immunoreactivity in cortical areas of 5XFAD-AL mice in comparison to their non-Tg-AL littermates (Figure 13). Quantitative analysis further revealed that this increase was by 4-fold

(p=0.0017, one-way ANOVA). Obvious activation of microglial cells was additionally increased in Tg-EOD mice by 2-fold in comparison to Tg-AL animals (p=0.0025, one-way ANOVA; Figure 14).

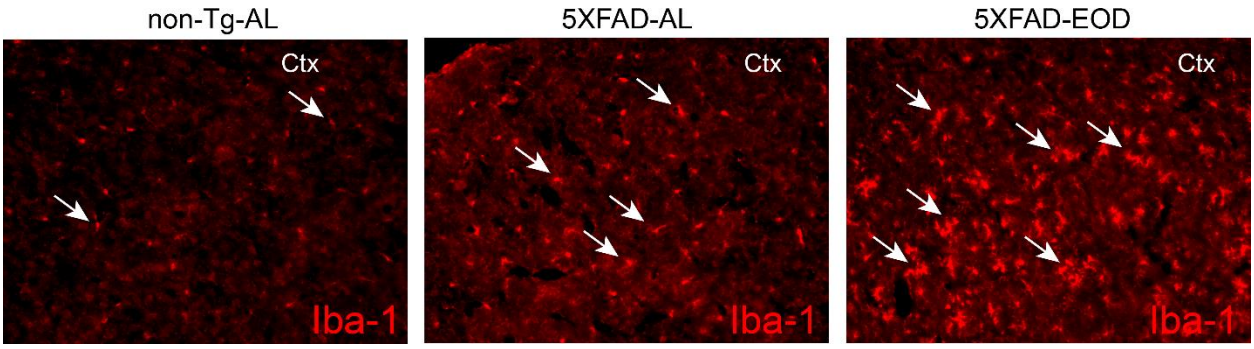

Figure 13: EOD feeding regimen induces neuroinflammation in 6-month-old 5XFAD female mice. Representative images (20x magnification) of Iba-1-positive staining in the cortex of non-Tg-AL, Tg-AL and Tg-EOD mice.

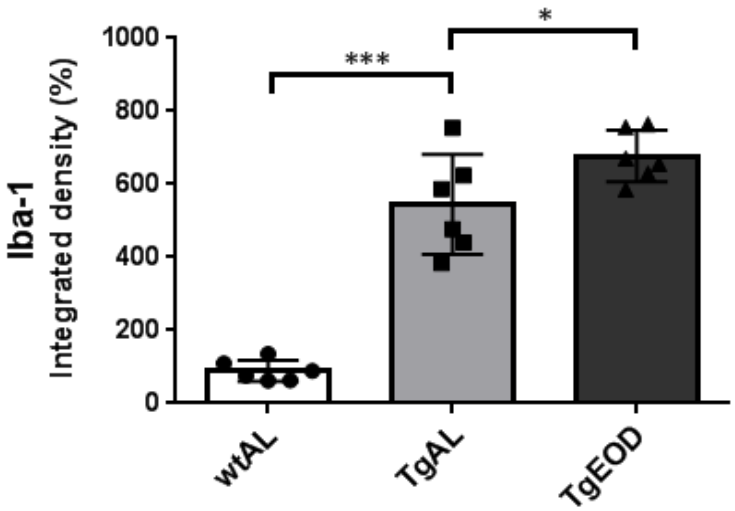

Figure 14: EOD feeding regimen induces neuroinflammation in 6-month-old 5XFAD female mice. Quantification of Iba-1-positive immunohistochemical staining in the cortex of non-Tg-AL, Tg-AL and Tg-EOD mice

5.5 EOD feeding induces astrogliosis in 6-month-old 5XFAD mice

Astrogliosis was initially evaluated by immunostaining and the measurement of total area percentage covered by the glial fibrillary acidic protein (GFAP)-positive signal (Figure 15 and 16, respectively). As presented at Figure 16, quantitative immunohistochemical analysis revealed a 3-fold increase in the GFAP immunoreactivity in Tg-AL mice in comparison to non-Tg-AL mice (Figure 16, p=0.0003, one-way ANOVA). More abundant immunoreactivity of the cortical area was further observed in Tg-EOD group than in Tg-AL group when GFAP marker was used (Figure 16).

Western blot analysis confirmed this increase of GFAP expression in total cortical tissue. Increased GFAP protein levels were detected in Tg-AL mice in comparison to non-Tg-AL mice by 2.6 fold (p=0.0079, one-way ANOVA), while the increase in Tg-EOD group in comparison to Tg-AL mice was for 35% (p=0.0001, one-way ANOVA) (Figure 17).

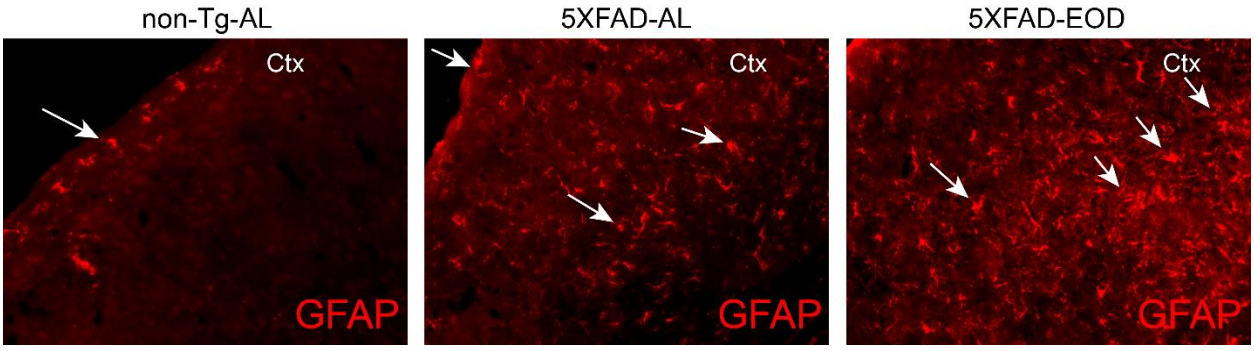

Figure 15: EOD feeding regimen induces astrogliosis in 6-month-old 5XFAD female mice. (A) Representative images of GFAP-positive staining in the cortex of non-Tg-AL, Tg-AL and Tg-EOD mice (20x magnification)

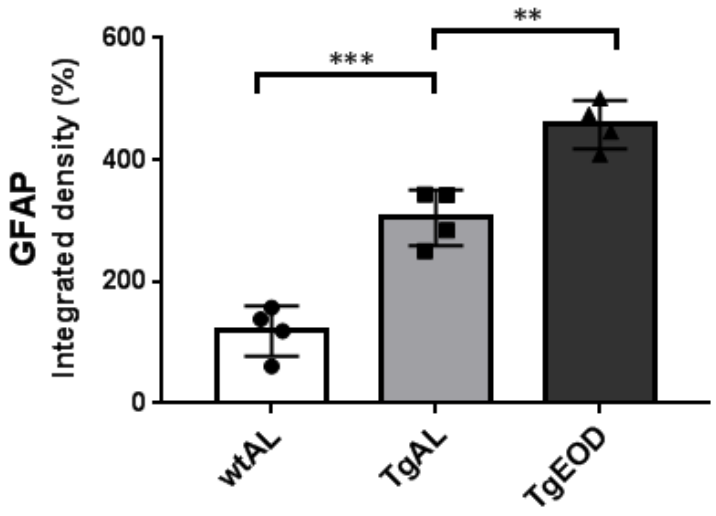

Figure 16: EOD feeding regimen induces astrogliosis in 6-month-old 5XFAD female mice. Quantification of GFAP-immunoreactivity in the cortex of non-Tg-AL, Tg-AL and Tg-EOD mice.

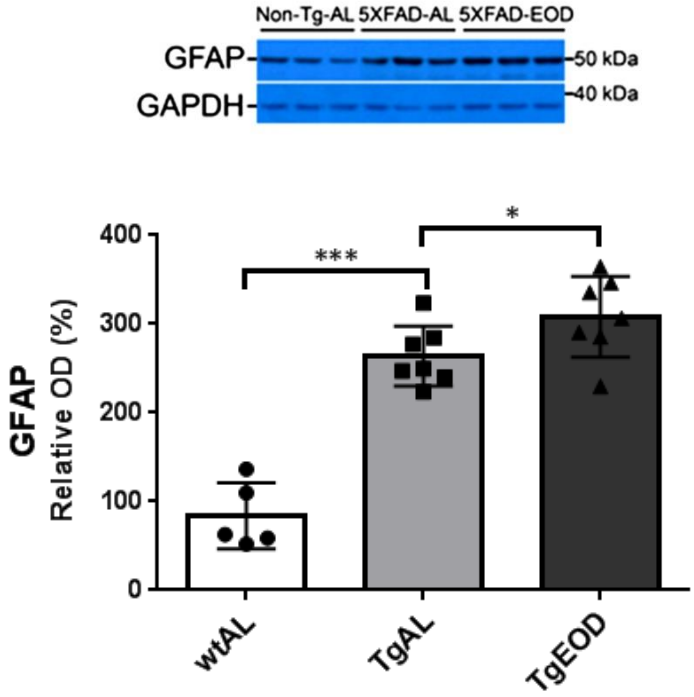

Figure 17: EOD feeding regimen induces astrogliosis in 6-month-old 5XFAD female mice. Relative abundance of GFAP normalized by GADPH in the cortex of non-Tg-AL, Tg-AL and Tg-EOD mice detected by Western blot analysis. Data are shown as single points per animal with bar graphs representing mean ± SD. Representative immunoblot is shown above the graph. *n* = 4-7 mice per group. Statistical significance by ANOVA.

5.6 EOD feeding regimen increases levels of TNF-α in 6-month-old 5XFAD

Additionally, levels of tumor necrosis factor alpha (TNF-α), a potent pro-inflammatory cytokine involved in neuronal damage and disease pathogenesis, were evaluated by WB. Consistent with the increased gliosis, when compared to non-Tg-AL controls, TNF-α protein levels were increased in Tg-AL by 25% (p=0.483, one-way ANOVA) and further increased in Tg-EOD mice by 50% (p=0.025, one-way ANOVA; Figure 18).

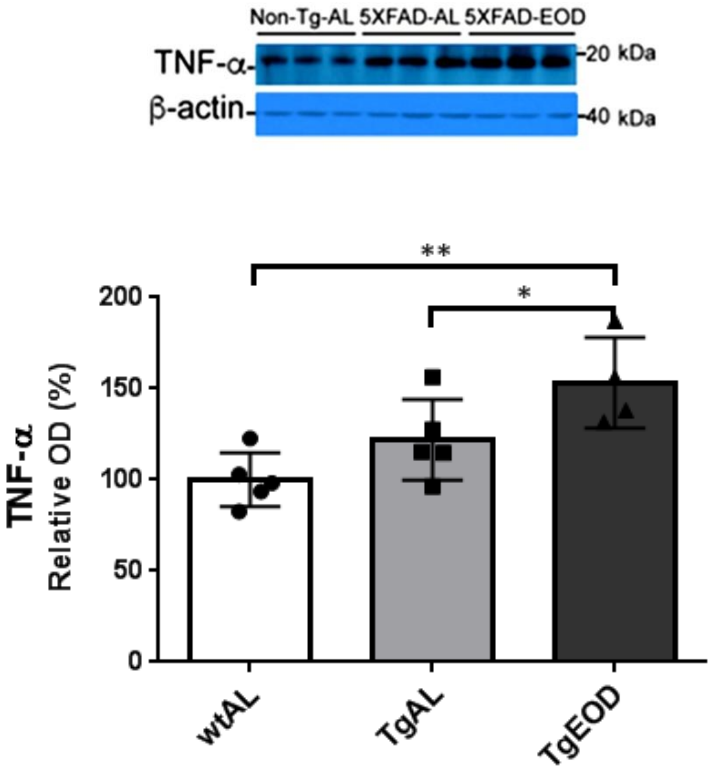

Figure 18: EOD feeding regimen increases levels of TNF- α. Relative abundance of TNF-α normalized by the β-actin protein level in the cortex of non-Tg-AL, Tg-AL and Tg-EOD mice. Data are shown as single points per animal with bar graphs representing mean ± SD. Representative immunoblot is shown above the graph. *n* = 4-7 mice per group. Statistical significance analyzed by one-way ANOVA.

5.7 EOD feeding regimen has no significant effect on PV+ interneurons in the hippocampus

PV interneurons are crucial for maintaining proper excitatory/inhibitory balance, and their loss in different AD models and AD patients was previously reported *(Zallo et al., 2018; Mikkonen et al., 1999).* In order to evaluate the effects of EOD feeding regimen on PV-expressing neurons in dorsal hippocampus of 5XFAD mice, quantitative analysis of PV-positive (PV+) interneurons in distinct hippocampal subregions, CA1, CA3 and DG was performed.

Overall, analysis revealed no significant difference in the number of PV+ interneurons between non-Tg and Tg animals, regardless of the feeding regimen applied and the region analyzed. Furthermore, representative photomicrographs did not imply gross changes in the structure of the hippocampus (Figure 19)

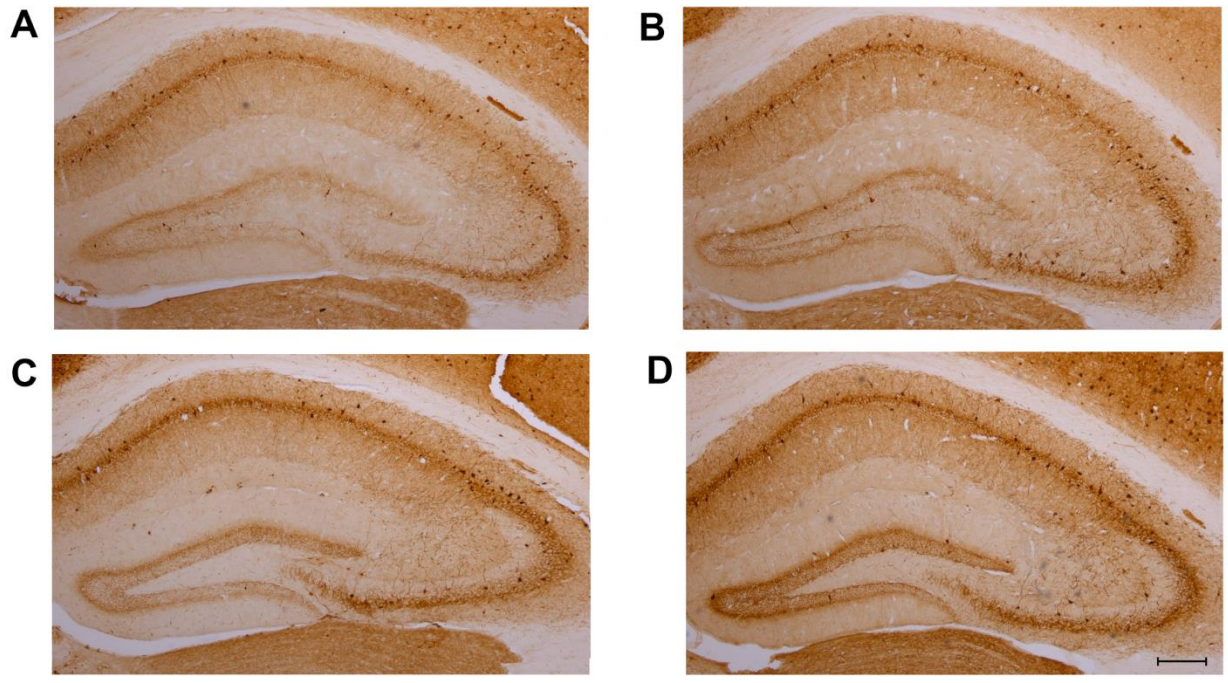

Figure 19: Representative photomicrographs of parvalbumin-positive staining in the hippocampus. (A) Dorsal hippocampus of non-Tg-AL, (B) non-Tg-EOD, (C) Tg-AL and (D) Tg-EOD animals. Scale bar = 400 μm.

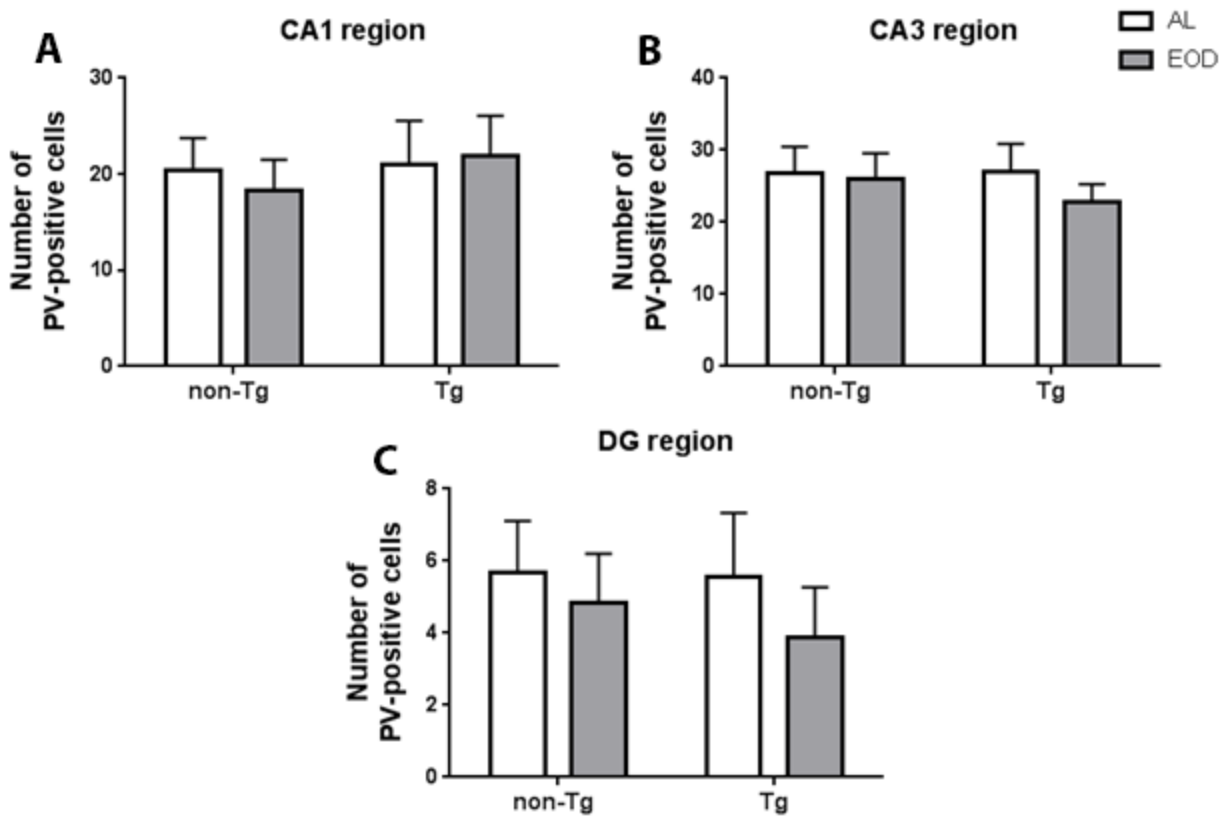

Figure 20: Quantitative analysis of the effects of EOD feeding regimen on PV+ interneurons in CA1, CA3 and DG regions of the hippocampus of non-Tg and Tg mice. Total number of PV neurons was determined by manual counting. Data are presented as mean ± SD.

Although no significant effect of treatment was acquired via 2-way ANOVA, a trend towards the decrease in number of PV+ neurons was revealed, especially in CA1 region of Tg-AL mice compared to non-Tg animals (Figure 20). On the other hand, a decrease in the number of PV expressing neurons in Tg-EOD mice compared to Tg-AL mice of the same age shows a trend in all three observed subregions of hippocampus.

5.8 The effects of EOD feeding regimen on PV- and CB-immunoreactivity in CA1 and CA3 regions of hippocampus

Quantification of PV- and CB-immunoreactivity was further performed in order to examine the effects of EOD feeding regimen in the dorsal hippocampus of 5XFAD mice in more details. Double immunolabeling in CA1 and CA3 regions was analyzed by confocal microscopy and as expected, distinct distribution of PV- and CB-immunoreactivity was observed in all regions analyzed. This discrepancy was most obvious in CA3 hippocampal region, with parvalbumin-immunoreactivity present in stratum pyramidale and the calbindin-immunoreactivity dominantly present in the area consisting mostly of mossy fibers. Representative photomicrographs are presented on Fig. 21 and Fig. 22.

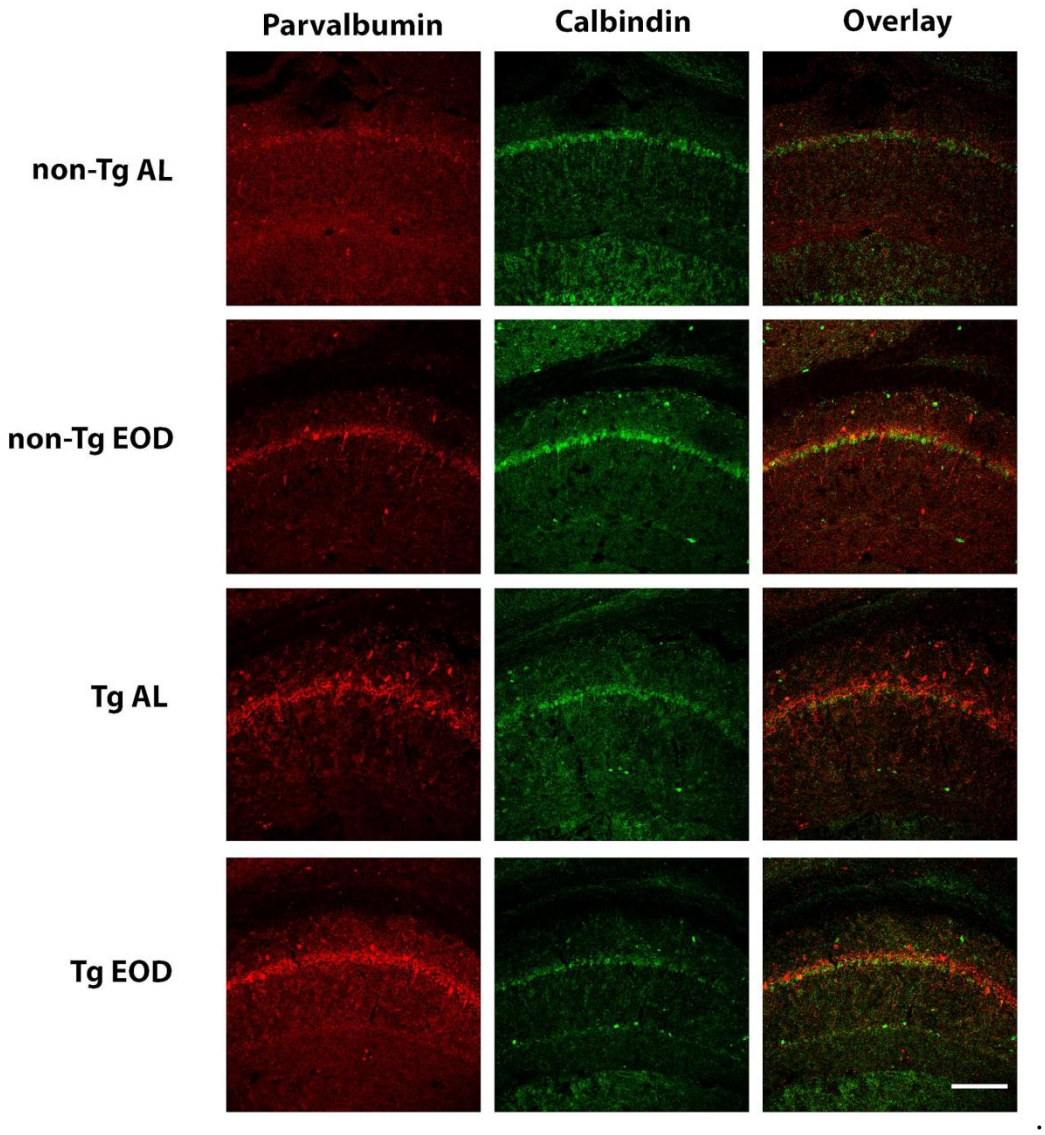

Figure 21: PV- and CB-immunoreactivity in the CA1 hippocampal subregion. Representative micrographs of CA1 region following double immunofluorescence labeling of PV and CB in the dorsal hippocampus of non-Tg AL, non-Tg-EOD, Tg-AL and Tg-EOD animals. Scale bar = 100 μm.

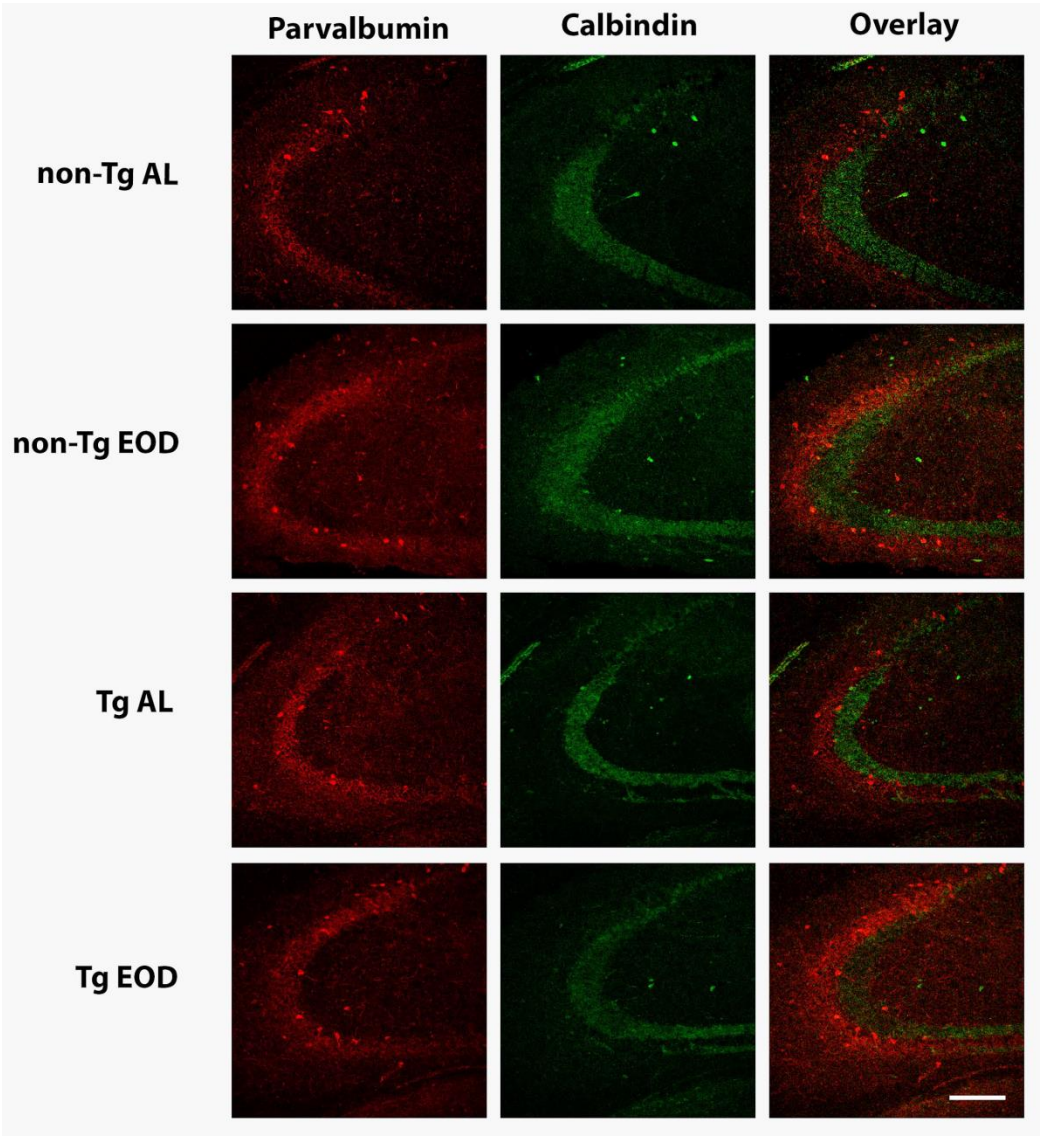

Figure 22: PV- and CB-immunoreactivity in the CA3 hippocampal subregion. Representative micrographs of CA3 region following double immunofluorescence labeling of PV and CB in the dorsal hippocampus of non-Tg AL, non-Tg-EOD, Tg-AL and Tg-EOD animals. Scale bar = 100 μm.

Overall, quantitative immunohistochemical analysis revealed no change in immunoreactivity of both PV and CB in Tg-AL animals in comparison to non-Tg animals (Fig. 23 and Fig. 24, respectively). However, higher signal intensity was observed in both non-Tg and Tg animals when EOD feeding regimen was applied.

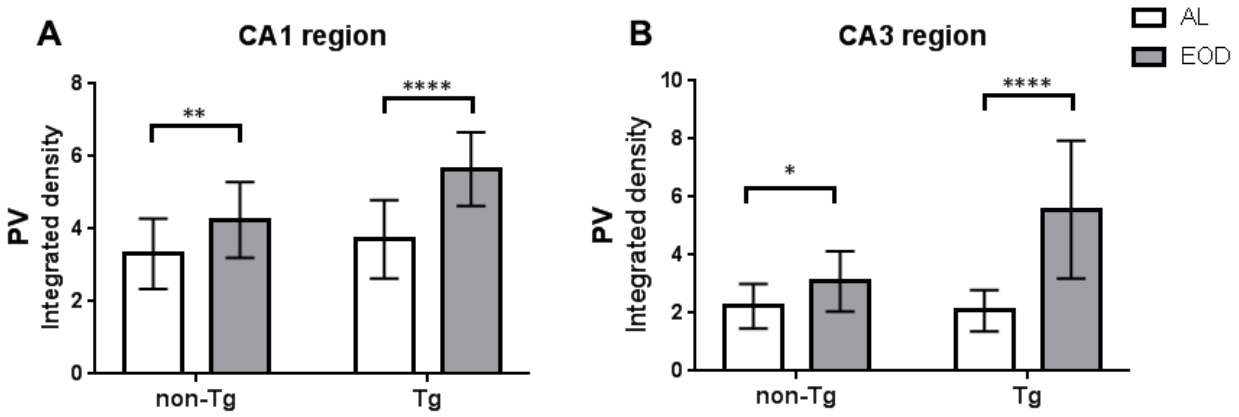

Figure 23: The effects of EOD feeding regimen on PV-immunoreactivity in the dorsal hippocampus of non-Tg and Tg mice. Integrated density was determined by quantitative immunohistochemistry. Data are presented as mean ± SD. *p < 0.05, **p < 0.005, ****p < 0.0001

In particular, 2-way ANOVA revealed a significant effect of diet on PV-immunoreactivity in the CA1 region of non-Tg ($p = 0.0013$) and Tg mice ($p < 0.0001$) (Figure 23A). A corresponding effect of EOD was also observed in CA3 region of the hippocampus, where significantly increase by 40% in non-Tg and by 250% in Tg mice was observed ($p = 0.0028$ and $p < 0.0001$, respectively; Figure 23B). Furthermore, total signal intensity was more prominent in CA1 in comparison to CA3 region of the dorsal hippocampus of all animals analyzed.

Significant differences in CB-immunoreactivity following EOD feeding regimen were observed in CA1 region of non-Tg mice ($p <0.0001$), as well as in the CA3 region of Tg mice ($p = 0.0107$) (Fig. 24A and Fig. 24B, respectively). In CA1 region of Tg mice, and CA3 region of non-Tg mice no noticeable effects of FR were detected.

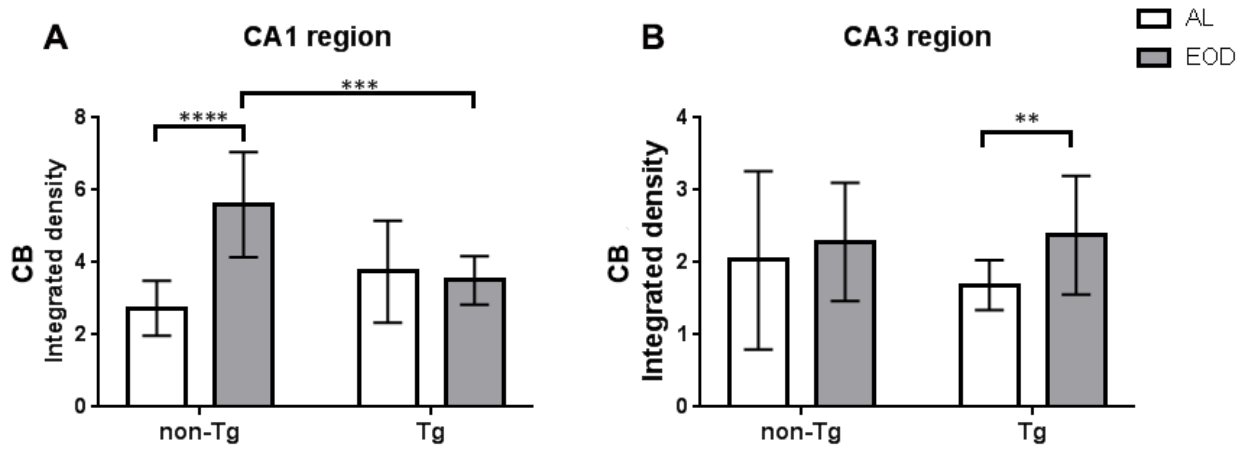

Figure 24: The effects of EOD feeding regimen on CB-immunoreactivity in the dorsal hippocampus of non-Tg and Tg mice. Integrated density was determined by quantitative immunohistochemistry. Data are presented as mean ± SD. **p < 0.005, ***p < 0.0005, ****p < 0.0001

6. Discussion

The earliest idea that ageing can be slowed down by altering the amount of food consumed was proposed by Loeb and Northrop at the beginning of the 20th century *(Loeb and Northrop, 1917).* About two decades later, first experiment regarding CR was conducted, providing evidences that restricted access to food have beneficial effects in rats and prolongs mean and maximal lifespan in comparison to *ad libitum* feeding *(McCay et al., 1935).* Calorie restriction was then found to prolong life-span in short-living organisms as well and to reduce the severity of risk factors for diabetes and cardiovascular diseases in rodents *(Fontana, et al., 2010).* Consequently, the focus on FR has increased, numerous studies were performed and in last decades, FR has been considered as the most potent non-genetic and non-pharmacological approach for improving health and postponing age-related disorders *(Fontana and Partridge, 2015).* Many reports have also confirmed neuroprotective effects of FR, both CR and intermittent feeding such as EOD, in animal models of epileptic seizures, stroke, traumatic brain injury and neurodegenerative diseases *(Arumugam et al., 2010; Bruce-Keller et al., 1999; Halagappa et al., 2007; Loncarevic-Vasiljkovic et al., 2012; Lončarević-Vasiljković et al., 2009).*

Therefore, the starting hypothesis of the present study was that the EOD feeding regimen could have beneficial effects in the 5XFAD animal model of AD. To our surprise, analysis revealed no significant change in amyloid burden and an increase in inflammation in 5XFAD-EOD mice accompanied by the increase in proinflammatory cytokine TNF-α, suggesting that 4 months of preventive EOD feeding regimen exacerbates AD-like pathology in this particular animal model. In addition, although the number of PV-positive cells in the hippocampus was not

changed, an increase in PV-immunoreactivity has been observed following EOD feeding regimen, both in CA1 and CA3 subregions of the dorsal hippocampus and both in non-Tg and Tg animals. CB-immunoreactivity was increased only in the CA3 region of 5XFAD-EOD mice in comparison to 5XFAD mice fed *ad libitum*.

5XFAD mice coexpress a total of 5 mutations associated with FAD - 3 in APP and 2 in PS1. The model is widely used as it recapitulates many of AD-related pathology and functional impairments with a quite early-onset and aggressive demonstration *(Oakley et al., 2006).* The most important advantage of this particular model is the appearance of neuronal death in cortical and hippocampal regions *(Eimer and Vassar, 2013),* the phenomenon that is similar to human pathology and absent in other animal models of AD. In line with the strategy that preventive treatments should start before irreversible cellular changes occur and before the onset of the symptoms, the 5XFAD animals and their non-transgenic littermates were fed AL until they reached the age of 2 months, as this represent the time point when plaques start forming in this animal model *(Oakley et al., 2006).* The duration of treatment was for 4 consecutive months, in order to examine the possible neuroprotective effects during both the prodromal and exponential phase of the pathology.

EOD feeding regimen used in this study, on the other hand, represents a milder form of intermittent feeding regimen that increasingly gains attention in the research of beneficial effects of food restriction *(Gotthardt et al., 2016).* Its specific advantage is that it intensifies the fasting effect and by this induces great metabolic fluctuations in comparison to other FR regimens. Sustained general food and mineral consumption are, however, preserved, due to increased food consumption above the energy needs on the feeding days. Our results concerning the body weights and weight gain of the treated animals are therefore expected and in line with previous

reports revealing no change during or at the end of EOD feeding regimen implementation *(Anson et al., 2003; Marinković et al., 2007; Smiljanic et al., 2018; Zhang et al., 2017).*

Similar to CR, EOD feeding regimen was also established as the FR regimen that prevents and attenuates various cellular dysfunction and degeneration and extends the lifespan *(Martin et al., 2006).* Its beneficial effects on age-related modifications in synaptic plasticity *(Mladenovic Djordjevic et al., 2010; Singh et al., 2012; Speakman and Mitchell, 2011)* and following excitotoxic and ischemic insults has also been clearly shown *(Anson et al., 2003; Fann et al., 2014; Kaur et al., 2008; Parinejad et al., 2009; Sharma and Kaur, 2005).* The mechanism behind its protective effects was hypothesized to be the same as for other FR regimens, including the action as a low-level stressor that induces basic protective mechanisms including reduced inflammation and oxidative stress and increased production of the neurotrophic factors like BDNF *(Martin et al., 2006).*

In AD animal models, on the other hand, beneficial effects of CR were mostly investigated *(Mouton et al., 2009; Patel et al., 2005; Schafer et al., 2015; Wang et al., 2005).* Concomitant amelioration of behavioral deficits was then reported for long-term CR and IF in triple transgenic (3xTgAD) mice, however, the accompanying decrease in the levels of Aβ and phospho-tau were not found in the hippocampus of mice subjected to IF suggesting that this dietary approach may protect neurons downstream of AD pathogenesis *(Halagappa et al., 2007).* In contrast, positive effect of the EOD feeding regimen on the clearance of amyloid-β from the brain was shown in APP/PS1 mice and was further associated with the change in the polarity of aquaporin 4 *(Zhang et al., 2017).* Research performed on tau-related AD models did not reveal consistent effects as well *(Brownlow et al., 2014).*

It is important to note that contradictory results for FR are well-known in the literature, especially regarding the effects on longevity and cognitive deficits *(Ingram and de Cabo, 2017;*

Sohal and Forster, 2014). FR has been reported to be deleterious when introduced at very young or old age *(Cardoso et al., 2016; Forster et al., 2003)* emphasizing the importance of the type, onset and duration of food restriction as major factors determining the final behavioral/molecular outcome. Greater survival rate of C57BL/6 in comparison to DBA/2 mice was explicitly shown to be due to lower basal metabolic rate, lower oxygen consumption, higher oxidative stress and higher body fat, i.e. the lower rate of energy expenditure *(Sohal et al., 2009).* Therefore, it was proposed that the beneficial effects of FR depend on the metabolic state of the organism and that proper body function can be limited by the imbalance between energy intake and dissipation, further causing predisposition towards diseases *(Dorighello et al., 2014).* Namely, FR can easily lead to malnutrition, and this, in combination with the loss of immunocompetence, was indeed identified to be directly associated with increased postoperative complication rates, risk of infection and higher mortality rates in patients *(Thomas et al., 2016; Waitzberg et al., 2001*). Furthermore, negative effects of food restriction were shown in wound healing *(Hunt et al., 2012),* ALS mouse model *(Hamadeh et al., 2005),* atherosclerosis model *(Dorighello et al., 2014),* and models of toxicity to some compounds *(Seki et al., 2000).* Diverse side effects that involve hypotension, hormonal irregularities, osteoporosis, altered cold sensitivity, loss of strength and the development of psychological conditions such as depression and dysphoria were reported in humans that practice FR as well *(Dirks and Leeuwenburgh, 2006).*

Chronic inflammation represents one of factors implicated in the control of energy expenditure. Inflammation is an essential immune response to many factors amongst which infection, trauma and disease that recruit the immune cells through pro-inflammatory signaling pathways to the area where the change occurred *(Newcombe et al., 2018).* The brain has its own resident immune cells – microglia, that are specialized for phagocytosis and get activated similarly to the above-mentioned immune cells. Furthermore, neuroinflammation is one of the

main steps regarding the pathogenesis of AD *(Heneka et al., 2015*) and it has been shown that inflammation may have a separate effect on different pathways, which in turn promote the loss of neurons and synaptic plasticity. As our data show, EOD caused an exacerbation in inflammation in the brain of 5XFAD mice and therefore, neuroinflammation could underlie the lack of beneficial effects in Aβ clearance observed in this particular animal model. Namely, previously conducted studies have shown that TNF-α, a potent pro-inflammatory cytokine involved in neuronal damage and disease pathogenesis, can activate p38 MAPK signal transduction in microglia, as well as astrocytes and neurons. This can further activate microglia to release p38MAPK and other pro-inflammatory cytokines, forming a never-ending circle adding to the progression and rigorousness of the disease *(Bachstetter and Van Eldik, 2010).*

In context of the present study, it is also important to note that 5XFAD mouse model also recapitulates various peripheral symptoms comparable to those frequently observed in AD patients *(Poehlman and Dvorak, 2000; Sergi et al., 2013)* among which are also important loss of appetite, body weight and weight of white adipose tissue at 9 months of age *(O'Leary et al., 2018; Gendron, 2015; Poehlman and Dvorak, 2000; Sergi et al., 2013).* The decrease in food intake and reduced energy expenditure could therefore be classified as an advantage of the model when examining the impact of FR. Nevertheless, taking into consideration that FR can act on several pathways, it is yet to be explained how EOD induces inflammation in this particular animal model of AD.

Neurodegenerative processes in AD can also induce alteration in different neurotransmitter systems, including the GABAergic. Deficits in GABA transmission were indeed found in AD patients and animal models *(Bai et al., 2014; Verret et al., 2012)* indicating the importance of interneurons involved in regulation of oscillatory network activities. Morphological analysis of human AD hippocampal tissue revealed a reduction in the

immunoreactivity and number of specific subpopulations of CBP-expressing GABAergic interneurons including those that express PV and CB *(Brady and Muffson, 1997; Solodkin et al., 1996; Mikkonen et al., 1999)* further implying their involvement in AD-related network dysfunction and memory deficits.

PV interneurons are profuse GABAergic inhibitory interneurons that provide input and output inhibition to excitatory pyramidal neurons in various brain areas, among which the hippocampus *(Klausberger and Somogyi, 2008; Klausberger, 2009).* Including other roles, they are important for assessing oscillatory network activity and managing plasticity subsequent to behavioral learning *(Donato et al., 2013; Hu et al., 2014; Tukker et al., 2007).* Atypical inhibitory synaptic transmission is now widely recognized as a key factor throughout early AD pathogenesis *(Palop et al., 2007; Busche et al., 2015; Kiss et al., 2016),* with PV interneurons as potential source of the inhibitory transmission disruption in mouse models of AD *(Iaccarino et al., 2016; Yang et al., 2016; Hollnagel et al., 2019).* Hyperexcitability of hippocampal inhibitory PV interneurons was also found to significantly contribute to the dysfunction of neuronal network as well as memory impairment in APP/PS1 mice (*Hijazi et al., 2019).*

A research conducted on rats, showed a change in the number of PV interneurons in DG when the subjects were deprived of proteins, but FR caused no change in the number or density of PV interneurons in the same region *(Cardoso et al., 2013).* Our findings also show no significant difference in the number of PV interneurons in any of the three hippocampus regions analyzed. However, the increase in PV-immunoreactivity could be in line with the role of GABAergic dysregulation in the pathogenesis AD. Namely, this outcome correlates with previously obtained findings that changes in inhibitory transmission in the hippocampus are associated with spatial memory deficiencies, predominantly through PV interneurons (*Busche et al., 2015; Royer et al., 2012; Ognjanovski et al., 2017*).

It is not, however, yet certain how PV interneurons and their functions are changed in AD, or how they contribute to the progression of the disease. The up-regulation of PV-immunoreactivity following EOD feeding may, however, represent compensatory mechanism to counteract the inflammation that was observed when EOD feeding regimen was implemented and to protect the remaining interneurons in hippocampus by increased axonal and dendritic sprouting and synaptogenesis.

In conclusion, the results of the present study provide evidence of detrimental effects of EOD in 5XFAD mouse model of AD, as opposed to widely accepted protective effects of FR on neurons and synapses during ageing *(Guo et al., 2000; Mladenovic-Djordjevic et al., 2010).* EOD-induced modifications of neuroinflammation in this Alzheimer's disease mouse model also had a substantial effect on PV-expressing cells in the hippocampus that may represent a compensatory mechanism regardless of Aβ pathology. Therefore, future research of the impacts of FR in AD pathology is required in order to extensively determine the degree, length and age of FR initiation required to obtain optimal health benefits and support neuronal stability.

7. List of abbreviations

Abbreviations	Explanations
AD	Alzheimer's disease
AICD	amyloid precursor protein intracellular domain
AL	ad libitum
ALT	alanine transaminase
ANOVA	Analysis of variance
APP	Amyloid precursor protein
AST	aspartate transaminase
Aβ	amyloid-β
Aβ40	amyloid beta 40
Aβ42	amyloid beta 42
BDNF	brain-derived neurotrophic factor
BSA	Bovine Serum Albumin
C83	C-terminal 83-amino acid fragment
CA	Cornu Ammonis
CB	calbindin
CBPS	Ca2+-binding proteins
CNS	central nervous sy tem
CR	calorie restriction
CR	calretinin
ChEIs	Cholinesterase inhibitors
DAB	3,3′-Diaminobenzidine
DAPI	4′,6-Diamidino-2-phenylindole dihydrochloride
DG	Dentate gyrus
EC	entorhinal cortex
ELISA	Enzyme-linked immunosorbent assay
EOD	every other-day feeding
ER	endoplasmic reticulum
FAD	familial AD
FR	Food restriction
GABA	gamma aminobutyric acid
GADPH	glyceraldehyde 3-phosphate dehydrogenase
GDNF	glial cell line-derived neurotropic factor
GFAP	Glial fibrillary acidic protein
GRP-78	glucose-regulated protein 78
HRP	Horse Radish Peroxidase

HSP-70	heat shock protein-70
IF	intermittent feeding
Iba-1	ionized calcium binding adaptor molecule 1
MAPK	mitogen-activated protein kinase
MCI	minimal cognitive impairment
NFT	The neurofibrillary tangles
PBS	Phosphate-buffered saline
PF	Periodic or prolonged fasting
PFA	paraformaldehyde
PHF	polymerized into paired helical filaments
PS	presenilin
PV	parvalbumin
RT	room temperature
SAD	sporadic AD
SF	straight filaments
TG	transgenic
TNF α	Tumor necrosis factor α
ThS	Thioflavin S
UCP	uncoupling proteins
WB	western blot
sAPPα	soluble APPα

8. References

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