Effect of Tff3 protein deficiency on expression of neurodegeneration-associated genes in hippocampi of aged mice

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Sveučilište u Zagrebu
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Ovaj rad je izrađen u Laboratoriju za istraživanje neurodegenerativnih bolesti na Zavodu za molekularnu medicinu Instituta Ruđer Bošković u Zagrebu, pod voditeljstvom dr.sc. Mirele Baus Lončar i suvoditeljstvom doc.dr.sc. Sofije Ane Blažević. Rad je predan na ocjenu Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu radi stjecanja zvanja magistar molekularne biologije.

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Porast incidencije neurodegerativnih bolesti odraz je kompleksnih kumulativnih procesa tijekom starenja te one uključuju interakcije brojnih organskih sustava. Smanjena razina Trefoil factor 3 (Tff3) proteina u likvoru Alzheimer pacijenata ukazuje na njegovu moguću ulogu u neurodegenerativnim procesima. Tff3 u gastrointestinalnoj mukozi sudjeluje u apoptozi, migraciji i angiogenezi, dok je njegova uloga u neuralnom tkivu gdje je prisutan još nepoznata. U ovom radu, postavili smo hipotezu da nedostatak proteina Tff3 dovodi do veće sklonosti miševa prema razvoju neurodegenerativnih procesa, koje smo mjerili putem relativne ekspresije gena *Bdnf*, *Epac2*, *Nfl*, *Egfr* i *L1cam*. U ovom radu korišteni su miševi stari dvije godine genotipa Tff3-/- C57Bl6/NCrl te odgovarajuće Wt kontrole. Naši rezultati pokazali su da nedostatak Tff3 proteina ne utječe na ekspresiju promatranih gena uključenih u neurodegenerativne procese.

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Master Thesis

Effect of Tff3 protein deficiency on expression of neurodegeneration-associated genes in hippocampi of aged mice

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The increase in the incidence of neurodegenerative diseases is a reflection of complex cumulative processes during aging which involve the interactions of numerous organ systems. Decreased levels of Trefoil factor 3 (Tff3) protein in the cerebrospinal fluid of patients with Alzheimer's disease indicate its possible role in neurodegenerative processes. Tff3 is involved in a variety of processes such as healing of mucosal surfaces in the gastrointestinal tract and apoptosis, migration, and angiogenesis, while its role in neural tissue is still unknown. In this paper, we hypothesized that Tff3 protein deficiency leads to a greater affinity of mice for the development of neurodegenerative processes, which we measured through the relative expression of the following genes:brain-derived neurotrophic factor (*Bdnf*), exchange protein directly activated by cAMP 2 (*Epac2*), epidermal growth factor receptor (*Egfr*), neurofilament light protein (*Nfl*) and L1 cell adhesion molecule (*L1cam*). Two - year - old mice of the *Tff3* -/- C57Bl6/NCrl genotype and appropriate Wt controls were used in this study. Our results showed that Tff3 protein deficiency does not affect the expression of the observed genes involved in neurodegenerative processes.

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LIST OF ABBREVIATIONS

AD - Alzheimer's disease

B2M - beta-2 microglobulin

Bdnf - brain-derived neurotrophic factor

CA1-CA3 - *cornu ammonis*

CAM - cell adhesion molecule

cAMP-GEF-ii - camp-guanine activated exchange factor

CNS - central nervous system

CSF - cerebrospinal fluid

DG - dentate gyrus

Egf - epidermal growth factor

Egfr - epidermal growth factor receptor

Epac2 - exchange protein directly activated by cAMP 2

GAPDH - glyceraldehyde 3-phosphate dehydrogenase

GI - gastrointestinal

GOI – gene of interest

HDAC2 - histone deacetylase 2

ITF - intestinal trefoil factor

L1cam - L1 cell adhesion molecule

LTD - long-term depression

LTP - long-term potentiation

MRI - magnetic resonance imaging

NDD - neurodegenerative disease

Nfl - neurofilament light protein

NFT - intracellular neurofibrillary tangles

NSC - neural stem cell

qPCR – quantitative polymerase chain reaction

RT – reverse transcription

SGZ - subgranular zone

TFF3 - trefoil factor 3 peptide

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1. INTRODUCTION

1.1. Trefoil factor 3 (TFF3)

The trefoil factor 3 (TFF3) peptide, also known as the intestinal trefoil factor (ITF), was discovered in humans in 1993 after the discovery of its homolog in rats in 1991 (Podolsky et al., 1993). TFF3 belongs to the trefoil factor (TFF) family, including TFF1 and TFF2 (Hoffman et al., 2001). Accepted nomenclature marks human proteins as TFFs and genes as *TFF 1-3* while rodent proteins are Tffs and genes *Tff 1-3*. TFF genes are located on the short arm of chromosome 21 in humans (Gött et al., 1996) and on chromosome 17 in mice (Ribieras et al., 1998). The TFF peptides contain one or two trefoil domains as their functional units. The domain consists of a sequence of 38/39 amino residues in which six cysteines form three intramolecular disulfide bonds (Thim, 1989; Thim, 1997). These bonds render the peptides resistant to proteolytic cleavage (Thim, 1994).

Human studies indicate the mucous epithelial cells, known as goblet cells, to be the main site of TFF3 production (Hoffmann et al., 2001; Hoffmann et al., 2002). A large amount of TFF3 can be found secreted in human intestinal goblet cells, the colon, salivary glands, conjunctival goblet cells, the uterus, and the cells of the respiratory tract (Thim, 1997). In all of these sites, TFF3 is co-secreted with mucin (Hoffmann et al., 2002). Also, it can be found in a variety of bodily fluids like breast milk, urine, sputum, cerebrospinal fluid, gastric juice, and tears, as well as in organs like the lungs, the pancreas, the mammary gland, the inner ear, lymphoid tissue, and the brain (Bernstein et al., 2015). Tff3 is also found expressed in the central nervous system (CNS) (Fu et al., 2014). Tff3 has an essential role in the healing of intestinal mucosa (Mashimo et al., 1996). It is also necessary for the promotion of cellular migration, apoptosis, and angiogenesis (Bossenmeyer-Pourié et al., 2002; Lubka et al., 2009; Mashimo et al., 1996; May et al., 1997). The Tff proteins on the epithelial surface operate in three principal ways: by the regulation of apoptosis (Lubka et al., 2019), by their involvement in migration processes, and by being part of mucous gels (Hoffmann et al., 2001). Tff3's expression is upregulated in cases of intestinal inflammation (Cook et al., 1999) and following a wound in the mucosa of the gastrointestinal (GI) tract (Lefebvre et al., 1996). Even though all peptides from the Tff family are able to promote cell migration, only

Tff3 is of paramount importance in the process of mucosal restitution (Mashimo et al., 1996; Podolsky, 1997).

There is an abundance of *TFF3* mRNA in most areas of the brain in both rodent and humans. More precisely, the transcripts can be found in the supraoptic and paraventricular nuclei of the rat and human hypothalamus (Bernstein et al., 2015; Probst et al., 1995; Probst et al., 1996), in the human cerebrospinal fluid (CSF) from the third ventricle, in the rat amygdala and the magnocellular neurons of the hypothalamus (Schwarzberg et al., 1999), as well as in the hippocampi and the cerebellum of mice (Hinz et al., 2004). Moreover, Tff3 is found and synthetized in oxytocinergic neurons of the hypothalamus which innervate the spinal cord, the pontine tegmentum, and the brain stem (Griepentrog et al., 2000; Jagla et al., 2000; Schwarzberg et al., 1999). Tff3 is probably co-accumulated with oxytocin in the neural lobe of the pituitary (Griepentrog et al., 2000; Hinz et al., 2004) and co-secreted with it into the bloodstream as a reaction to external stimuli (Hoffmann et al., 2001). Bernstein et al. systematically examined *TFF3* expression in the human brain and found that the TFF3 protein is present in neurons of various human brain regions. This wide cerebral distribution suggests that TFF3 has an important physiological role in the CNS (Bernstein et al., 2015). Nevertheless, this is still a novel research field and the protein's role has yet to be determined.

Altered *TFF3* expression in peripheral tissues has been associated with neurological disorders, such as schizophrenia (Gardiner et al., 2013) and Parkinson's disease (Zou et al., 2018). Moreover, pharmacological administration of TFF3 has been reported to facilitate learning, object recognition, and retention of memory (Schwarzberg et al., 1999), as well as evoke antidepressant-like behavior (Li et al., 2015; Shi et al., 2012). TFF3 was identified as a marker of neurodegeneration in amyloid-positive individuals expressed independently from tau and p-tau. Its low expression in the CSF strongly correlated with brain atrophy, ventricular expansion, and hippocampal atrophy (Paterson et al., 2014). As already mentioned, it has been shown that Tff3 is expressed during neurodevelopment (Belovari et al., 2015). Having a mitogenic effect, Tff3 has a role in postnatal cerebellar development where it is involved in the differentiation program of cells in the granule cell layer of the cerebellum (Hinz et al., 2004). Given the known Tff3 mechanism of action in intestinal epithelial cells where it acts as a motogen (Baus-Loncar and Giruad, 2005), we hypothesized that Tff3 could be exerting its role in the hippocampus by participating in similar cellular processes (cell migration, adhesion, mitogenic effect).

1.2. The hippocampus, adult neurogenesis, and neuroplasticity

1.2.1. Hippocampal structure and function

In 1957, the strange case of patient H.M. ignited interest in the neuroscientific community. The patient's hippocampus had been surgically removed as part of a treatment plan for epilepsy and was later found to be unable to produce any new memories (Scoville and Milner 1957). Following this discovery, the hippocampus was later found to be crucial in the processes of learning, spatial navigation, and the regulation of emotions. It is also the main site of memory processing, consolidation, and retrieval (Bartsch and Wulff, 2015; Tatu, 2014). The hippocampus is both structurally and functionally a heterogeneous structure. This heterogeneity of function is a reflection of the differential connectivity of its subregions (Moser and Moser, 1998).

Together with the cerebral cortex, the hippocampus is the brain area most affected in neurodegenerative diseases (NDDs), with regards to cognitive decline impairment (Arushanyan and Beier, 2008). Magnetic resonance imaging (MRI) studies of Alzheimer's disease (AD) patients have shown a strong connection between hippocampal structural change and its lesions (Wang et al., 2012), with Alzheimer's disease (Arushanyan and Beier, 2008; Paterson et al., 2014). Therefore, structural changes in the hippocampus are used as a biomarker for the diagnosis of pathological states (Lindberg et al., 2012).

The hippocampus is functionally divided into different subregions and subnetworks (Strange et al., 2014), with the main ones being the dentate gyrus (DG), the subiculum, and the *cornu ammonis* fields (CA1-CA3) (Schultz and Engelhardt, 2014). There are 3 layers of organization of the CA1-CA3 subfields' pyramidal cells: the stratum lucidum, the stratum radiatum, and the stratum lacunosum-moleculare (Bartsch and Wulff, 2015). In the human brain, the hippocampus is positioned ventrally in the medial temporal lobe (Figure 1) (Strange et al., 2014) and is shaped in the form of a crescent moon (Schultz and Engelhardt, 2014). In rodents, however, the hippocampus is situated right beneath the neocortex and is shaped like a cashew nut (Figure 2) Bartsch and Wulff, 2015). The longitudinal axis of the human hippocampus has an anterior-posterior orientation which corresponds to the ventral-dorsal orientation in rodents. The ventral hippocampus is associated with the regulation of emotion, while the dorsal hippocampus has a role in spatial memory and cognition (Moser and Moser, 1998). This finding may be further corroborated by the fact that the NMDA and AMPA receptor subunits are differentially expressed

in the dorsal and ventral rat hippocampi. As the center of an important brain network, it is connected to many other cortical and subcortical regions such as the neocortical association areas (Bartsch and Wulff, 2015).

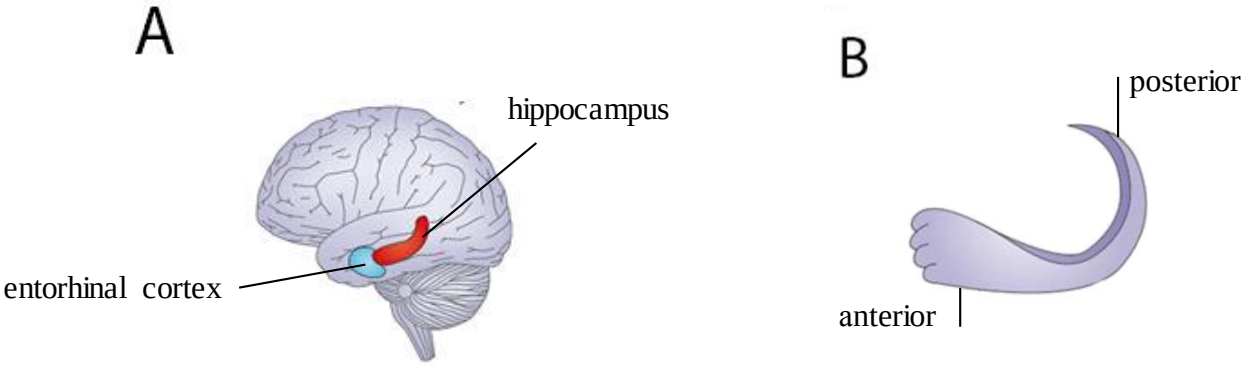

Figure 1. A) The hippocampus and entorhinal cortex in the human brain, B) The human hippocampus and its anterior-posterior orientation (Strange et al., 2014)

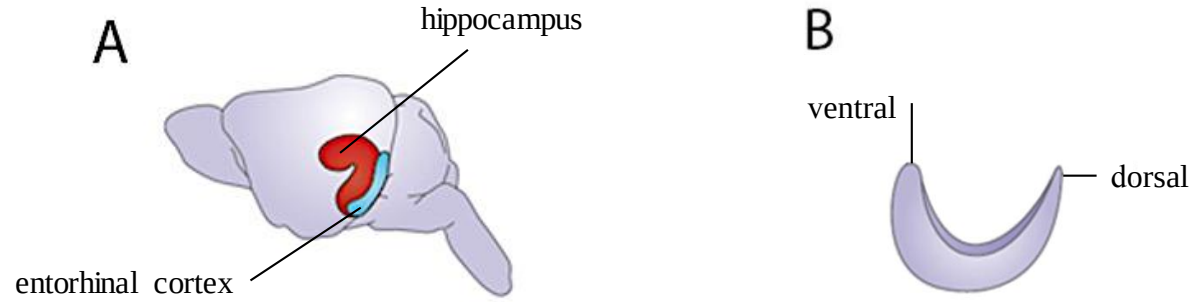

Figure 2. A) The hippocampus and entorhinal cortex in the rat brain, B) The rodent hippocampus and its ventral-dorsal orientiation (Strange et al., 2014)

All mammalian brains have a hippocampus that has what is essentially the same neuronal circuitry, making it an evolutionary old cerebral structure (Strange et al., 2014). Even though there are multiple interconnected pathways extant in the longitudinal axis of the hippocampus, this brain structure is anatomically connected primarily through a „trisynaptic loop" (Figure 3). Within this loop, the entorhinal cortex (EC) sends the main cortical input to the dentate gyrus (DG) via the perforant path consisting of layer II neurons and to CA1 via layer III neurons. The DG then signals to the CA3 region through the mossy fiber pathway. The next signal in the loop projects from the CA3 to the CA1 region via the Schaffer Collaterals. The pyramidal cells of the CA1 region then connect back to the EC (Knierim, 2015). Newer studies show that there are also projections coming from the EC to the CA3 and CA1 and signaling from CA3 back to DG and other CA3 neurons. Furthermore, the CA1 projects to the amygdala, the subiculum, and the prefrontal cortex. The subiculum projects back to polymodal association cortices in the neocortex (Schultz and Engelhardt, 2014). The CA3 hippocampal subfield and the DG have an essential role in memory encoding and early memory retrieval, while the CA1 hippocampal subfield is important in memory consolidation and late retrieval of autobiographical memory (Bartsch and Wulff, 2014).

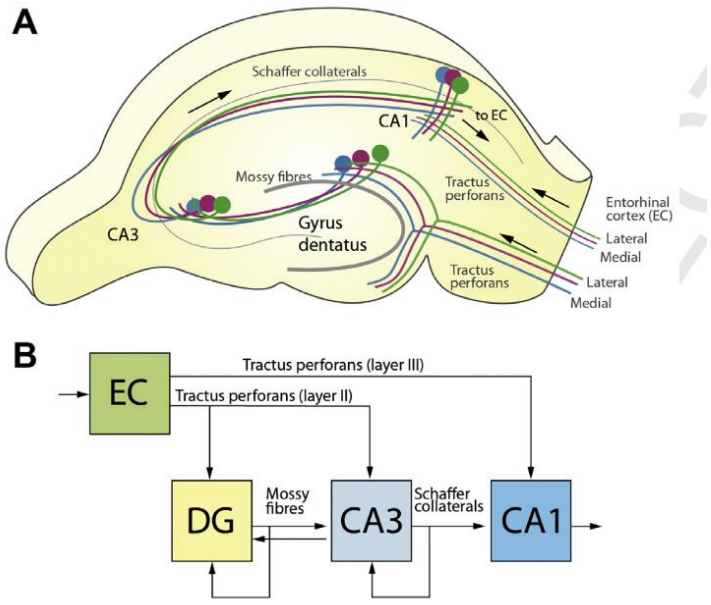

Figure 3. The „trisynaptic loop" as the primary neural circuit in information processing in the hippocampus – A) Structure of the loop, B) Pathways in the loop (Bartsch and Wulff, 2015)

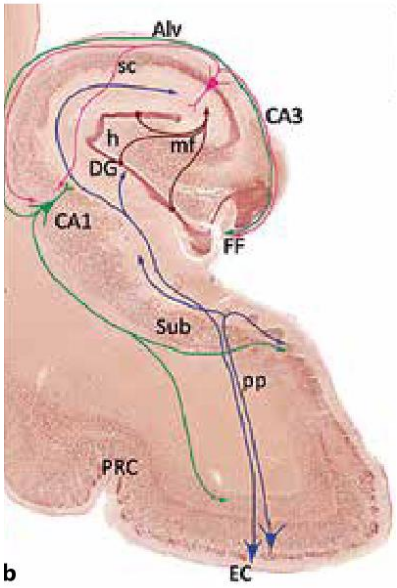

Figure 4. The "trisynaptic loop" shown inside the section of the human hippocampus (Schultz and Engelhardt, 2014)

1.2.2. Neuroplasticity and adult neurogenesis

The hippocampus shows a high degree of neuroplasticity, which includes structural dendritic and axonal modifications, changes in membrane excitability, synapse numbers, synapse connectivity, and the formation of new neurons (neurogenesis) (Leuner and Gould, 2010). Neuroplasticity is the ability to reorganize neuronal structure and function in response to internal and external stimuli and can be most appropriately seen in hippocampal neurons. This ability is one of the properties needed for memory formation (Spalding et al., 2013). Plasticity can occur at the presynaptic level by the regulation of neurotransmitter levels or postsynaptically by the modification of neurotransmitter receptors (Leal et al., 2017). Higher levels of neuroplasticity seen on the scale of neuronal networks are made possible by the basic levels of neuroplasticity that occur via molecular cascades. Protein kinases, phosphatases, and proteases all have important molecular roles in the regulation of synaptic plasticity, which is in turn reliant on the timely phosphorylation and dephosphorylation of synaptic proteins (Gulyaeva, 2017). Moreover, increased expression of plasticity-related genes such as *Bdnf*, *CREB*, as well as *AMPA* and *NMDA* receptors can lead to higher levels of neuroplasticity (Bartsch and Wulff, 2015).

The results of neuroplasticity can be seen in modified cerebral structure, brain activity and behavior. The processes of long-term potentiation (LTP) and long-term depression (LTD) are indispensable when discussing hippocampal neuroplasticity (Schultz and Engelhardt, 2015). Long-term potentiation is induced by a short-lived high-frequency stimulation of synapses (Baltaci et al., 2019). Characterized by the electrical activity of synaptic transmissions (Arushanyan and Beier, 2008) and an increase in synaptic response (Baltaci et al., 2019), long-term potentiation is the essential mechanism underlying learning and memory (Nicoll, 2017). There are two types of hippocampal LTP. The first type of LTP is postsynaptic, requires NMDA receptors, and involves excitatory synapses between the Schaffer collateral terminals and the CA1 subregion. The second type is presynaptic, does not require NMDA receptors, includes mossy fibers, and occurs between the DG and the CA3 pyramidal cells (Baltaci et al., 2019; Nicoll, 2017).

Adult neurogenesis is the main process required for neural plasticity and occurs in the olfactory bulb, the subventricular zone of the forebrain and the dentate gyrus of the mammalian hippocampus (Bartsch and Wulff, 2015, Spalding et al., 2013). The process of adult neurogenesis is shaped by a plethora of molecular stimuli, including various transcription and growth factors, neurotrophins, and microglia l inflammatory cytokines (Goncalves, 2016). Newborn adult neurons in the DG are needed for context-dependent memory, learning, and differentiation of similar memories (pattern separation) (Clelland et al., 2009). The dentate gyrus consists of 3 cell layers named the molecular, the polymorphic, and the granular (Amaral et al., 2007). Most of the DG is made of glutamatergic granular cells and it is thought that adult neurogenesis in humans is restricted to its subgranular zone (SGZ) (Bartsch and Wulff, 2015). The SGZ of the dentate gyrus is the place in the adult mammalian brain that contains neural stem cells (NSC) and allows their proliferation. The NSCs differentiate into intermediate progenitor cells (IPCs), which can then turn into immature neurons. The immature neurons can then finally differentiate into adult-born dentate granule neurons. In order to become adult neurons and part of the hippocampus, the dentate granule neurons go through several developmental stages (Figure 5) (Goncalves et al., 2016).

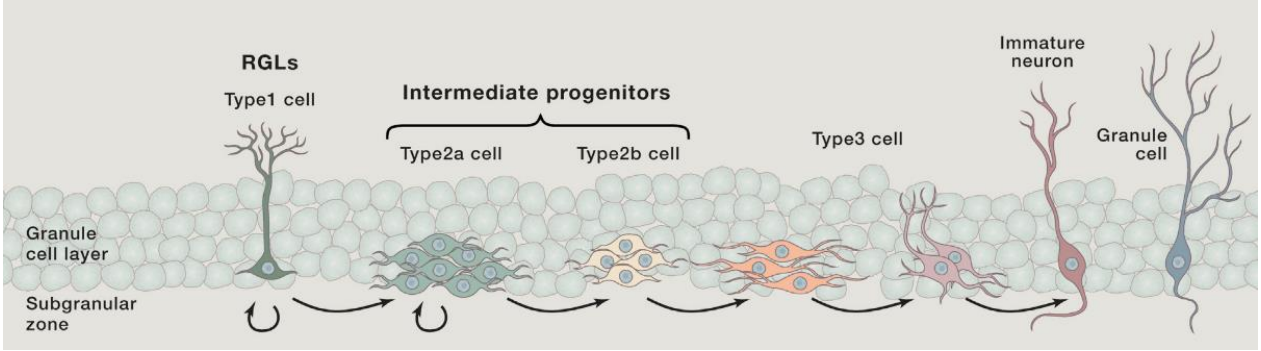

Figure 5. Adult hippocampal neurogenesis (Gonçalves et al., 2016)

Moreover, impairments in adult neurogenesis have been associated with neurodegenerative diseases like Alzheimer's, Parkinson's, and Huntington's disease (Gonçalves et al., 2016). In the hippocampi of AD patients, new neurons are found in low numbers (Moreno-Jiménez et al., 2019). Neuroplastic processes and mechanisms are more vulnerable in aged individuals, and neurogenesis in the dentate gyrus decreases with age. Even though beneficial in some aspects, neuroplasticity at the cellular level might be the cause of a higher dose of hippocampal vulnerability and, therefore, implicated in the formation of neurodegenerative diseases. In the early stages of AD, hippocampal vulnerability leads to destructive pathological changes in extracellular amyloid-beta protein and intraneuronal neurofibrillary tangles formation (Bartsch and Wulff, 2015). Advanced age is recognized as the number one cause of NDDs' onset in humans (Hou et al., 2019). Furthermore, neurogenesis levels decrease as a function of age in both humans and rodents (Gonçalves et al., 2016).

1.3. Neurodegeneration-related genes

1.3.1. Brain-derived neurotrophic factor (Bdnf)

Bdnf is a neurotrophic factor essential for neuronal survival, axonal growth, and synaptic plasticity, all of which are processes involved in learning and memory (Autry et al., 2012). Bdnf molecules in the hippocampus are localized at the presynaptic terminals of neurons, to where they are transported along the axons via large dense core vesicles (Leal et al., 2017). Bdnf controls excitatory and inhibitory synaptic transmission in the adult brain. Being a neurotrophin, it promotes downstream signaling through TrkB which leads to enhanced long-term potentiation of the hippocampal excitatory glutamatergic synapses (Miranda et al., 2019; Panja and Bramham,

2014). The activation of glutamatergic synapses depends on extracellular calcium levels (Leal et al., 2017). The Bdnf-TrkB complex induces mTORC1 activation and thereby regulates dendritic protein synthesis. Additionally, the ERK/MAPK cascade is set off by Bdnf and leads to the synthesis of translation-related proteins important for LTP (Panja and Bramham, 2014). However, when acting through p75, Bdnf produces long-term depression (Tanila, 2017).

1.3.2. Epidermal growth factor receptor (Egfr)

Egfr has so far been studied to a great extent. In adult rats, Egfr's presence has been described in layers IV and V of cerebral cortical neurons. Furthermore, the receptor has been found expressed in the subgranular zone of the adult hippocampus, which is the site of adult neurogenesis (Wong and Guillaud, 2004). Egfr ligands control the processes of maturation, cell survival, cellular proliferation, differentiation, and neurogenesis in both the developing and adult brain (Wang et al., 2012 8). Egfr regulates the proliferation of the following precursor cells: neural stem cells, neural progenitor cells, and glial progenitor cells (Wong and Guillaud, 2004). In the rat hippocampus, the activation of Egfr by the epidermal growth factor (Egf) has been proven to increase LTP in Schaffer/commissural-CA1 synapses *in vitro*, and to induce LTP in the perforate-path dentate granule cells in vivo, therefore having a role in synaptic plasticity (Yamada et al., 1997).

1.3.3. Exchange protein directly activated by cAMP 2 (Epac2)

Epac2, also known as the camp-guanine activated exchange factor (cAMP-GEF-ii) for the Rap and Ras-like GTPases, modulates neuronal excitability and promotes neurotransmitter release in glutamatergic neurons (Grandoch et al., 2010). cAMP binds to the two cAMP-binding domains on Epac2 and thereby improves the catalytic activity of Epac towards its GTP-ase target Rap (Jones et al., 2019). Having been found in great amounts in the mammalian cortex and the hippocampus, it was concluded that Epac2 promotes neurotransmission in the hippocampus (Fernandes et al., 2015). *Epac2* expression has so far been described in adrenal glands, the brain, the lungs, and vasculature (Grandoch et al., 2010; Zhang et al., 2018) and is especially highly expressed in the presynaptic terminals of axons (Fernandes et al., 2015). It is of note that *Epac2* mRNA expression

pattern is dependent on the developmental stage the tissue is in and is upregulated in adult brain tissue, as opposed to Epac1 that is active in fetal stages (Murray et al., 2008). Some of the processes that include Epac2 and mediation by cAMP are calcium handling, cardiac muscle contraction, cell proliferation and differentiation, apoptosis and inflammation, chromosomal integrity (Grandoch et al., 2010). Epac2 exerts its function on the ERK1/2 pathway, which leads to increased cell survival (Zhang et al., 2018). Hippocampus-related processes regulated by Epac2 and mediated by cAMP are neuronal plasticity (Fernandes et al., 2015), working and reference memory, and synaptogenesis (Jones et al., 2019).

1.3.4. L1 cell adhesion molecule (L1cam)

L1cam is a protein from the family of neural cell adhesion molecules (CAMs) which have an important role in mediating the interaction between the cell and its environment. CAMs have a role in cell migration, axonal and dendritic projection, and synaptic targeting (Fransen et al., 1997; Schmid and Maness, 2008). Neurons and glial cells in both the central and the peripheral nervous system (PNS) express L1cam on their surfaces during embryonic and postembryonic stages of development (Hortsch, 1996). Even though L1cam is important in the development of the brain because its activation of the ERK/MAPK pathway leads to cell migration, this signaling can also promote tumor progression (Schmid and Maness, 2008). Interactions of L1 peptides with other proteins in the brain lead to a calcium cascade activation resulting in the induction of neurite outgrowth. Additionally, L1cam molecules have been proposed to have a role in LTP in the hippocampus (Hortsch, 1996). L1cam is also responsible for the inhibition of a gene transcription blocker histone deacetylase 2 (HDAC2). HDAC2 inhibits the expression of synaptic plasticity-related genes and prevents normal cognitive functioning (Hu et al., 2020).

1.3.5. Neurofilament light protein (Nfl)

Nfl is one of the domains of neurofilaments (NFs) and has a role in neuronal structural stability. *Nfl* is expressed only in mature neurons in the CNS and the PNS (Gentil et al., 2012). It is an AD biomarker that detects neuroaxonal damage and is quite sensitive to neurodegenerative progression and changes throughout the stages of the disease (Hampel et al., 2018; Mattsson et al., 2019). It is usually found in the CSF, but it is as of recently possible to detect it in blood samples

as well (Hampel et al., 2018). Low levels of Nfls are expected in physiological conditions, while high levels tend to be present in aged individuals (Gaetani et al., 2019).

1.4. Aged mice model and hypothesis

In our study, we used an aged mice model to investigate the effect of Tff3 deficiency on the expression of genes involved in adult neurogenesis that are subsequently associated with the pathophysiology of AD in the murine hippocampus. Aged mice are susceptible to many health problems, some of them being neurodegenerative diseases such as Alzheimer's disease (AD). These mice are also more prone to major changes in physiology and behavior and may form lesions characteristic of aging (www.criver.com).

We monitored the expression of brain-derived neurotrophic factor (*Bdnf*), epidermal growth factor receptor (*Egfr*), exchange protein directly activated by cAMP 2 (*Epac2*), L1 cell adhesion molecule (*L1cam*), and neurofilament light protein (*Nfl*). We hypothesized that the depletion of the *Tff3* gene would lead to significantly altered levels of expression of analyzed genes in aged mice. Their altered levels would signify a potentially important role of Tff3 in neurogenesis processes and AD pathology and could be further explored as a potential biomarker of neurodegeneration.

2. STUDY AIM

The general aim of this study was to examine the role of the Tff3 protein deficiency on the expression of neurodegeneration-associated genes (*Bdnf*, *Epac2*, *Egfr*, *L1cam,* and *Nfl*) in the aging process. According to the described Tff3 protein function in apoptosis and inflammation, we have postulated that Tff3 deficiency would have an impact on the level of expression of neurodegeneration-relevant genes.

3. MATERIAL AND METHODS

3.1. Primer optimization

In order to verify the optimal conditions for adequate specificity and primer binding, we carried out primer optimization for five genes of interest (GOI) and two reference genes. The genes of interest investigated were the brain-derived neurotrophic factor (*Bdnf*), exchange protein directly activated by cAMP 2 (*Epac2*), epidermal growth factor receptor (*Egfr*), neurofilament light protein (*Nfl*), and L1 cell adhesion molecule (*L1cam*). The reference genes used were glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and beta-2 microglobulin (*B2M*).

One pair of primers was tested out for each gene (Table 1). The primers were created with the Primer Quest Tool software. The optimal temperatures for each primer were searched for within the binding range of temperatures for primers: 57°C – 65°C. The $MgCl_2$ concentrations used were: 2.5mM, 3mM and 3.5mM. SYBR Green was used as the coloring agent in the qPCR reaction. SYBR Green is a stain that binds to all double-stranded nucleic acids, including primer dimers. To be sure that we would get only one specific product in the PCR reaction (double-stranded DNA), primer optimization was necessary. We confirmed the qPCR reaction primer binding specificity upon the creation of a melting curve and polyacrylamide gel electrophoresis (PAGE). The primers whose melting curves had only one significantly more prominent peak were chosen for the experimental part of the study because they indicated only one major product present at the end of the reaction.

We analyzed the efficacy of the qPCR reaction. There were three dilutions made for which the Ct values were measured. Ct or cycle threshold is a value indicating the starting amount of nucleic acids in the sample. The obtained Ct values from the reactions were used by the StepOnePlus™ software to calculate the efficacy of the reaction. The software generates standard curves for each set of primers by plotting the log value of nucleic acids (the primary concentrations of samples used) versus Ct values from the PCR reactions. PCR efficacies for each gene of interest were then calculated from the slope of the standard curves by using the equation:

$$E = 10^{-1(\text{line slope})};\ \%E = E - 1(x100)$$

Table 1. Primers and optimised annealing temperatures

gene	primer	primer sequence	annealing temperature/^{o}C
Epac2	*Epac2* F	TGTCTCCAGTCCACAATCTTTC	58
	Epac2 R	GGATGTGTCTCTTGCTCATTCT	
Egfr	*Egfr* F	ACAGCGCTACCTTGTTATCC	59
	Egfr R	CATCCTCCATGTCCTCTTCATC	
Bdnf	*Bdnf* F	CAAGAGTCCCGTCTGTACTTTAC	59
	Bdnf R	GACTAGGGAAATGGGCTTAACA	
Nfl	*Nfl* F	GCTCTTTCCCAGCCTACTATAC	59
	Nfl R	TTCTCCTTCTCCTCCTCTTCT	
L1cam	*L1cam* F	AGCACAGCAAGAGGCATATC	65
	L1cam R	CCACATGGTAAGAGCTGTAAGG	

3.2. Animal experiments

3.2.1. Tissue isolation

The tissue samples I used were murine hippocampi of male, 2-year-old, *Tff3*-/-//C57Bl6/NCrl and Wt controls (C57Bl6/NCrl). The mice were bred at the Facility for laboratory animals of the Ruđer Bošković Institute from a mouse strain originally purchased from Charles River Laboratories. The mice were kept in small mice cages (dimensions: 235x135x130 mm) under the following conditions: 22^{o}C temperature, 12 hour light/dark cycle and 58% humidity. The mice were fed normal chow (Mucedola) and given water *ad libitum*. In total there were 16 samples, 8 Wt controls and 8 *Tff3*-/- mice. The experiment was approved by the Bioethical committee of the Ruđer Bošković Institute and the Ethics Committee of the Croatian Ministry of Agriculture on July 5th 2019. The mice were anesthetised using isoflurane, sacrificed by cervical dislocation and hippocampal tissue was isolated by certified scientists and stored on ice. All hippocampal tissue from the samples was homogenized.

3.2.2. RNA isolation and reverse transcription (RT)

The total RNA of the sample (NucleoSpin RNA; Applied Biosystems**)** was isolated according to the manufacturer's instructions. The RNA was purified (NucleoSpin R; Applied Biosystems) according to the manufacturer's instructions and the total RNA's concentration and purity was measured using the NanoPhotometer® N60. The RNA was reverse transcribed into cDNA (The High Capacity cDNA Reverse Transcription Kit; Applied Biosystems Step One) according to the manufacturer's instructions The contents of the reaction mix were as shown in Table 2. One sample contained 1.5 μg of RNA. The reaction was carried out in the Veriti 96 Thermal Cycler (Applied Biosystems) under the following conditions: 10min at 25°C, 120 min at 37°C, 5min at 85°C, and at the end stored at 4°C.

Table 2. RT reaction mix for 1 sample

10X RT buffer	4μl
25X dNTP Mix (100Mm)	1.6μL
10X radnom primer	4μL
MultiScribe Reverse Transcriptase (50U/Ul)	2μL
RNAse inhibitor	2μL
Nuclease-free H_2O	6.4μL

3.2.3. Quantitative polymerase chain reaction (qPCR)

The cDNA of each sample was amplified through a qPCR reaction using the StepOnePlus™ Real-Time PCR System. Each well contained concentrations and volumes according to Table 3 and 4. The reverse transcription and PCR steps were conducted separately, which allowed for optimized conditions to be used for each reaction (www.thermofisher.com). The experimental samples were done in triplicates, while the negative controls in duplicates. The dye we used for the detection was SYBR Green. There were 40 amplification cycles in each

reaction. The conditions were as follows: 3 min of preparatory denaturation at 95 ^{o}C, 1min of denaturation at 95 ^{o}C, 30 seconds of annealing at according primer annealing temperatures (Table 1), 30 seconds of DNA elongation at 72^{o}C. The machine measured the fluorescence levels during the elongation phase of the reaction.

Table 3. qPCR mix components

H2O	$MgCl_2$ (25 mM)	dNTP (5mM each)	M-buffer*	Taq_2	SYBR green (1000x)	ROX 100x reference dye	Total volume of mastermix
15.75µL	2.5µL						
15.25µL	3µL	1µL	2.5µL	1µL	0.0025µL	0.25µL	23µL
14.75µL	3.5µL						

*M-pufer: 800mM KCl, 100 mM Tris-HCl; pH=8,3

Table 4. Well contents for qPCR

	volume	concentration
mastermix	23µL	*
primer	1µL	5µM
cDNA	1µL	0.04µg/µL

*mastermix components are shown in Table 3

3.2.4. Polyacrylamide gel electrophoresis

The samples were ran on several polyacrylamide gels made as indicated in Table 5. The ratio of the DNA: loading buffer (10X bromophenol-blue) was 5:1. The electrophoresis conditions were: 100V, 400mA during 3h. We treated the gels with ethidium bromide and inspected them under UV light.

Table 5. 12%-gel components for PAGE

*mix AA:BIS	5.85 mL
10XTBE	4.875 mL
H2O	8.775 mL
10% APS	195 μL
TEMED	19.5 μL

*Rotiphorese Gel 40 (19:1)

3.2.5. Relative quantification of gene expression

I used the REST-MCS (c) version 2 (Relative Expression Software Tool – Multiple Condition Solver) software for the calculation of the relative expression of genes in real-time PCR. The used statistical model was the *Pair Wise Fixed Reallocation Randomisation Test* (c). The reference genes used were: *GAPDH* and *B2M*. The genes of interest were: *Bdnf*, *Egfr*, *Epac2*, *L1cam*, and *Nfl*. The expression of the GOI and reference genes was measured and then compared between the hippocampi of healthy Wt males and the males who were knockouts (KO) for the *Tff3* gene. The equation used by the software is shown in Figure 1.

$$R = \frac{(E_{target})^{\Delta CP_{target}\,(MEAN\ control\ -\ MEAN\ sample)}}{(E_{ref})^{\Delta CP_{ref}\,(MEAN\ control\ -\ MEAN\ sample)}}$$

Figure 1. Equation for the calculation of relative gene of interest expression

4. RESULTS

In this study, I first optimized the qPCR conditions for primers in order to be sure of the experiment's efficacy and specificity. The best conditions (Table 1) for primer performance were determined by analyzing PCR reactions at different annealing temperatures, melting curve analysis and final PAGE electrophoreses (Figure 1-2) check-up. It was important to obtain only one band for each gene of interest on the electrophoresis gel which signaled the correct specific bindings of the primers. The bands on the gels indicated the chosen primers to be appropriate for the experiment.

Table 1. Obtained optimal conditions for primers

Primers	Epac2	Egfr	BDNF	Nfl	L1cam
optimal temperature	58°C	59°C	59°C	59°C	65°C
optimal $MgCl_2$ concentration	3.5mM	3.5mM	2.5mM	3.5mM	3mM
Efficacy	103%	112%	112%	91%	108.21%

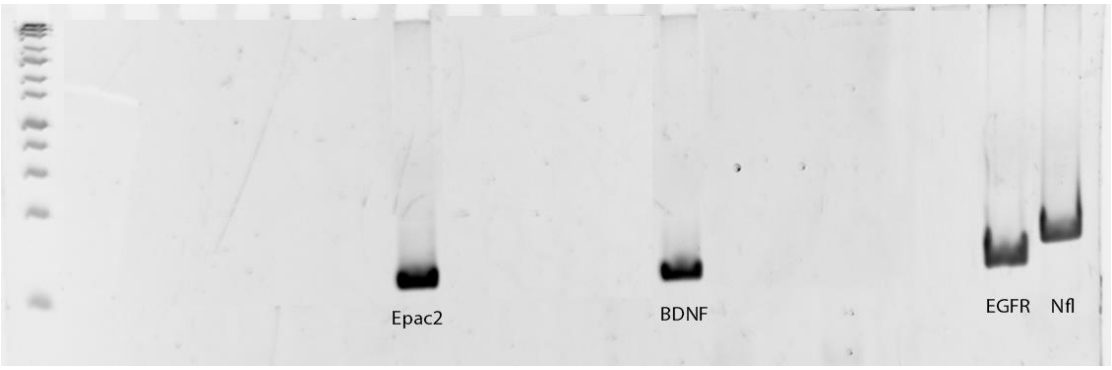

Figure 1. Gel electrophoresis – Epac2, BDNF, EGFR, Nfl

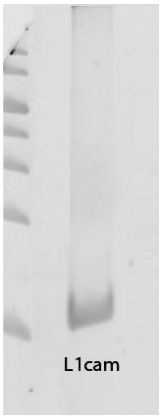

Figure 2. Gel electrophoresis – L1cam

Standard curves generated for each set of primers are presented in Figure 3-7. In ideal conditions in the qPCR reaction, the amount of the amplified DNA and the subsequent intensity of the SYBR GREEN dye should double each cycle, giving the efficacy of 100%. Given that the conditions were not ideal, the range of acceptable efficacy was approximately 95% to 110%. All slope values between -3.9 and -3.0 are acceptable.

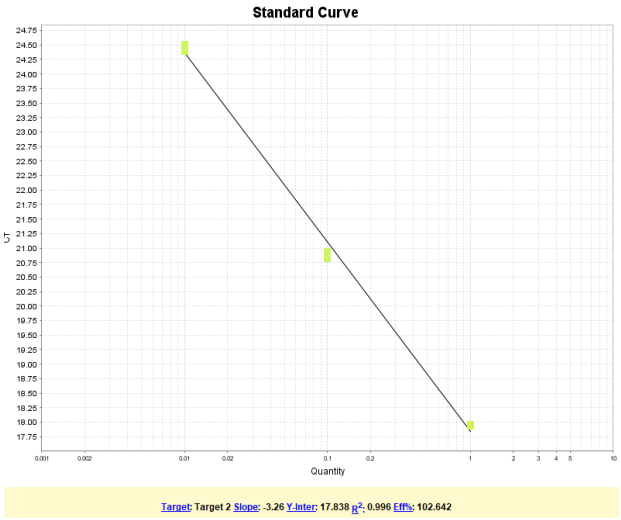

Figure 3. Epac2 F/R efficacy – 103%

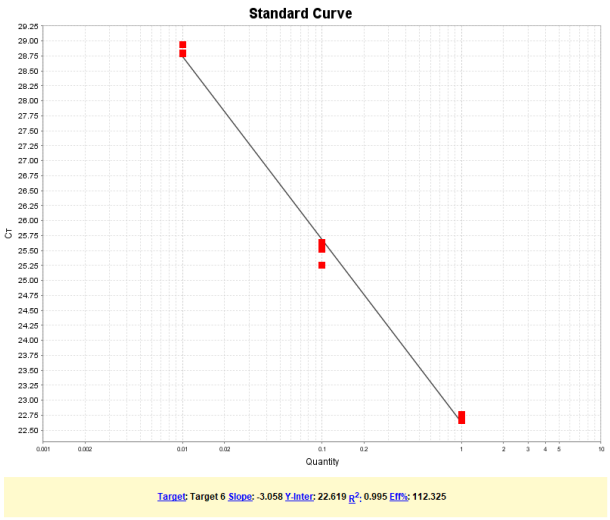

Figure 4. EGFR F/R efficacy – 112%

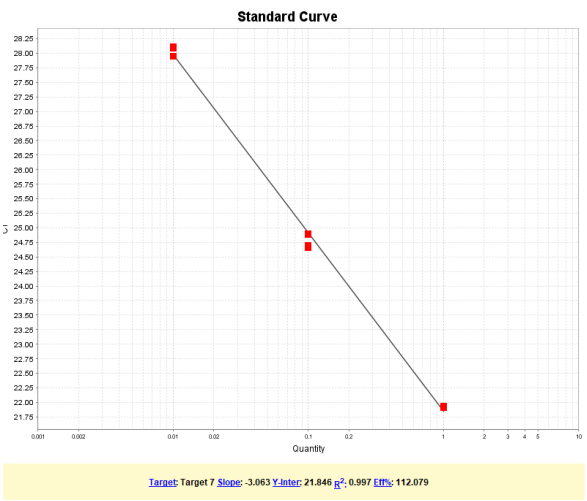

Figure 5. BDNF F/R efficacy – 112%

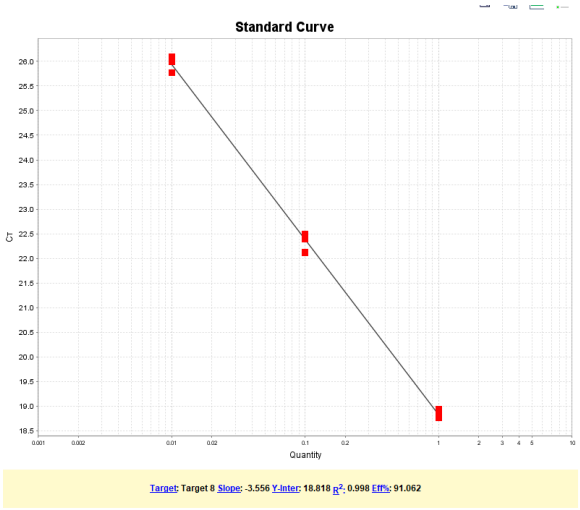

Figure 6. Nfl F/R efficacy - 91%

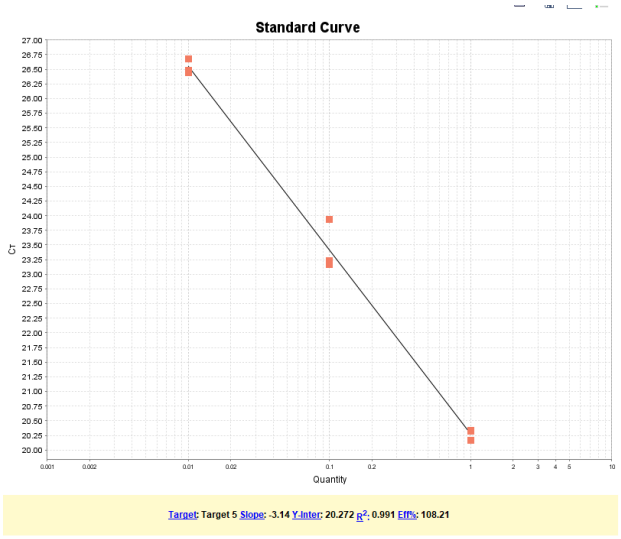

Figure 7. L1cam F/R efficacy – 108.21%

Using the *Pair Wise Fixed Reallocation Randomisation test* in the aforementioned REST-MCS software, I found no significant difference in expression ratios between the Wt and *Tff3-/-* samples. The expression levels of *Epac2*, *Egfr* and *Bdnf* were found to be slightly increased in *Tff3 -/-* mice and the expression levels of *Nfl* and *L1cam* were found to be slightly decreased in *Tff3 -/-* mice as presented in Table 2 and Figure 8.

Table 2. Expression ratios and absolute gene regulation of Epac2, Egfr, Bdnf, Nfl and L1cam

	Epac2	*Egfr*	*Bdnf*	*Nfl*	*L1cam*
expression ratio	1.061	1.060	1.177	0.882	0.996
gene UP-regulated by the factor	1.061	1.060	1.177		
gene DOWN-regulated by the factor				-1.133	-1.005
absolute gene regulation	1.061	1.06	1.177	0.882	0.996
standard error	0.1977	0.22046	0.33765	0.20789	0.23884
2log (bidirectional)	0.086	0.084	0.236	-0.181	-0.007
2log (standard error)	0.01593	0.01757	0.06754	0.03311	0.00155

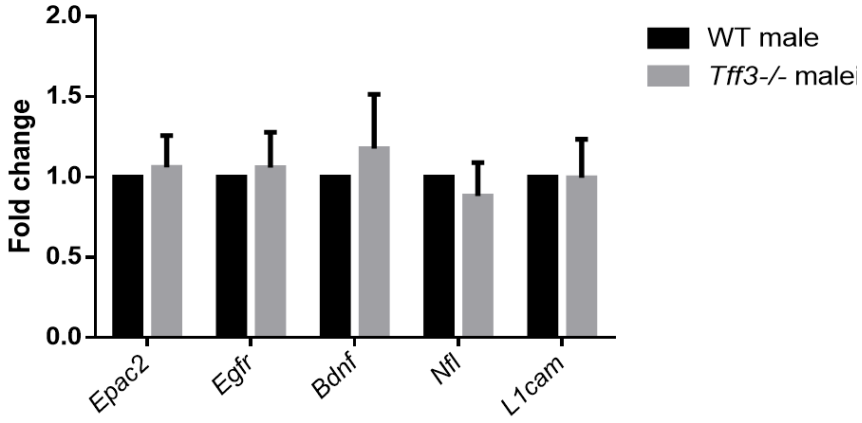

Figure 8. GOI expression ratios between *Tff3*-/- and Wt samples (standard errors indicated with standard bars)

5. DISCUSSION

Today, a major global health problem with a big impact on the quality of human life are different neurodegenerative diseases. As the human population is getting older and the individual's lifetime is expanding, cases of neurodegenerative diseases are rising in number (Hou et al., 2019). Thus, there is a need for better diagnostic techniques and new, more efficient therapeutic approaches for neurodegenerative diseases (Paterson et al., 2014; Wang et al., 2012). The most prominent among them is Alzheimer's disease, marked with cognitive decline, dementia, and difficulties in learning (Arushanyan and Beier, 2008; Breijyeh and Karaman, 2020; Gonçalves et al., 2016; Hou et al., 2019). Alzheimer's disease progresses through a reduction of synaptic proteins, neuronal loss, changes in synaptic morphology, and neuroinflammation (Breijyeh and Karaman, 2020). AD patients may possess various morphological changes in their brains such as a) intracellular neurofibrillary tangles (NFTs), which consist of p-tau aggregates; b) senile plaques, which consist of AB amyloid aggregates; or c) degenerated brain structures (Albert-Gascó et al., 2020). Most of the depositions of amyloid plaques and tau neurofibrillary tangles can be found in the medial temporal lobe and cortex of AD brains (Breijyeh and Karaman, 2020; De Paula et al., 2012; Price and Morris, 1999).

With age being the main risk factor in the development of neurodegenerative diseases (Fjell et al., 2015; Hou et al., 2019), I used 24-month-old (aged) male C57Bl/6NCrl mice in my study. Female mice were not used in the study so to avoid changes in sex hormones not exhibited in male mice (Belaya et al., 2018). The C57Bl/6NCrl aged mice show signs of cognitive decline as early as at 19-20 months of age (www.criver.com). For example, Bialuk et al. used 24-month-old C57BL/6J aged mice with a IL-6-deficiency in their study and found a significant decrease in spatial memory in older mice (Bialuk et al., 2018).

In order to identify Alzheimer's disease, biomarkers are regularly used (Paterson et al., 2014). The most common AD pathology biomarkers in use today are Aβ1-42, tau, and p-tau, which correlate to cognitive decline frequently observed in the disease (Blenow et al., 2010; Hou et al., 2019). These markers are obtained from the cerebrospinal fluid and reflect the levels of amyloidosis, neurodegeneration, and tau pathology (Paterson et al, 2014; Wang et al., 2012). Because of its reduced presence in the cerebrospinal fluid of individuals with pathological

Alzheimer's disease, Paterson et al. suggest a new potential CSF biomarker, the TFF3 peptide. Paterson et al. found lower levels of TFF3 to be associated with brain atrophy rates and ventricular expansion, as well as hippocampal atrophy (Paterson et al, 2014). Moreover, in 2014, Tong et al. found the levels of TFF3 in blood, urine, and feces to be useful in the assessment of side effects in the clinical trials of gamma-secretase inhibitors for the treatment of AD (Tong et al., 2012).

The *Tff3* - / - mice were chosen in this study because of their susceptibility to increased levels of neuronal cell death (Liu et al., 2013) and the known role of Tff3 in the normal functioning of the nervous system (Belovari et al., 2015; Bernstein et al., 2015; Gardiner et al., 2013). Additionally, *Tff3* -/- mice have been shown to be prone to decreased mucosal healing (Mashimo et al., 1996), cell apoptosis (Lubka et al., 2009) and impairments of the immune system (Fu et al., 2015).

The hippocampus is one of the brain structures most prominently altered in AD (Lindberg et al., 2012; Moodley and Chan, 2014) and also one of the brain areas where Tff3 can be found (Hinz et al, 2004; Wang et al., 2012). As mentioned in the introduction, the role of Tff3 in the CNS is a new intriguing field, which is why we wanted to analyze the expression of *Bdnf, Epac2, Egfr, L1cam*, and *Nfl* in the hippocampus of *Tff3*-/- mice and Wt controls with the aim to elucidate the possible role of Tff3 in adult neurogenesis, which is reduced in Alzheimers's disease.

Bdnf expression changes in the hippocampus can have a great impact on the state of the organism (Miranda et al., 2019; Autry et al., 2019), especially on cognitive decline in neurodegenerative diseases (Tanila, 2017). Belrose et al. found that postmortem human brains with non-Alzheimer tauopathies have reduced levels of Bdnf mRNA (Belrose et al., 2014). Patterson et al. found decreased levels of hippocampal long-term potentiation in mice lacking the *Bdnf* gene (Patterson et al., 1996). Li et al. showed that Tff3 increases the levels of Bdnf, pERK1/2, and CREB in the rat hippocampus, and Bdnf-ERK-CREB signaling in the hippocampus might be involved in Tff3-mediated antidepressant-like effects (Li et al., 2015). Given that finding, as well as altered Bdnf signaling in AD, we wanted to analyze the possible variation of its gene expression in our *Tff3*-/- mice model. The results did not show any significant change in *Bdnf* gene expression concerning Tff3 deficiency. This result could be attributed to the unpredictability of the effect of age on the organism.

It has been demonstrated that deficits of Egfr can lead to astrocyte degeneration in the murine cortex (Wagner et al., 2006). Chinery and Playford found that Tff3 and Egfr have remedial

synergistic effects on cell migration and gastric damage after Egfr phosphorylation (Chinery and Playford, 1995). Indeed, Tff3 has anti-apoptotic effects, but it can only exert this function through phosphorylating Egfr. This Egfr phosphorylation leads to the inhibition of p53-induced apoptosis (Kinoshita et al., 2000). Egfr activation also leads to the activation of the downstream MAPK/ERK pathway (Baus-Loncar and Giraud, 2005), ultimately leading to cell proliferation, which is one of the anti-neurodegenerative processes (Li et al., 2015). Egfr knockout mice develop neurodegenerative diseases in the first month of their life (Wong and Guillaud, 2004). The majority of Tff3 function studies were done mainly on intestinal epithelial cells and we wanted to analyze if there is an association between Egfr and Tff3 deficiency in the hippocampal tissue which could explain the findings that connect Tff3 and neurodegeneration. However, we did not find any significant difference in Egfr expression in *Tff3*-/- mice compared to Wt mice.

The expression pattern of Epacs is altered in chronic inflammatory diseases in which the cellular microenvironment is changed (Grandoch et al., 2010). Concerning pathological conditions, McPhee and co-workers found low *Epac2* mRNA levels and high *Epac1* mRNA levels in cerebral regions connected to Alzheimer's (McPhee et al., 2005). Defective responses to secondary messengers may bring about these types of disorders (Grandoch et al., 2010). We chose *Epac2* in our study as a potential gene involved in neurogenesis because of the ability of its protein to regulate synaptic plasticity in the CA3 region of the hippocampus (Fernandes et al., 2015) and because Epacs promote neurite outgrowth in the dorsal root ganglia and advance neurite regeneration in the spinal cord (Grandoch et al., 2010). Nevertheless, no significant change was found in *Epac2* expression in *Tff3*-/- mice compared to Wt.

Dysregulation of CAMs can lead to neuropsychiatric disorders (Schmid and Maness, 2008). Mutations in the L1 domain of the L1cam molecule can also cause the so-called L1 syndrome which includes four X-linked conditions: hydrocephalus, corpus callosum hypoplasia, MASA syndrome and spastic paraplegia (Fransen et al., 1997). Dahme et al. carried out a mutation on exon 8 of the *L1* gene and found a reduction in the size of the murine corticospinal tract (Dahme et al., 1997). In our study, however, no significant difference was found in *L1cam* expression in *Tff3*-/- mice.

Nfl gene mutations can lead to a pathologically significant accumulation of neurofilaments, which slow down neuronal transport. These mutations are one of the potential causes of Charcot-Marie-Tooth disease, a neurological disorder characterized by an impairment in the functioning of

Schwann cells of the peripheral nervous system (Gentil et al., 2012). Landau et al. found that increased levels of Nfl in plasma correspond to hypometabolism, future potential atrophy, and the decline of cognitive functions (Landau et al., 2012). Additionally, Mattsson et al. found increased Nfl levels in patients with AD dementia, mild cognitive impairment, preclinical AD, and prodromal AD and therefore concluded that Nfl can be used as a noninvasive biomarker of neurodegeneration in AD (Mattsson et al., 2019). Moreover, Disanto et al. reported that serum Nfl levels were significantly higher in individuals with multiple sclerosis and could therefore be used as relevant biomarkers of the disease (Disanto et al., 2017). Based on these results, we expected increased levels of Nfl in our *Tff3*-/- mice, but no significant difference was observed.

My study's results showed no significant difference in the analyzed genes between aged *Tff3*-/- mice and Wt controls. These results could be negative due to advanced mice age, for our models were 2 years old. The mice's old age might have created a loss of uniformity of physiological changes between the samples. This loss of uniformity could have rendered the animals more prone to having differing expression levels of the genes of interest and therefore lead to unexpected results. Furthermore, the expression levels of *Bdnf*, *Egfr*, *Epac2*, *L1cam* and *Nfl* are all subject to change throughout the lifecycles of both mice and humans. Perhaps it could be interesting to see the expression levels of the same genes in younger mice where the process of adult neurogenesis is more pronounced. Moreover, one strategy could be to experimentally induce neurodegeneration processes (pharmacologically, e.g streptozotocin induction or by nutritional excess, feeding mice high-fat diet) in mice and then to analyze the neurite growth processes and the neurodegenerative status concerning Tff3. Indicators of neurodegeneration such as certain behavioral changes could also be investigated. For example, mice could be subjected to learning and spatial memory tests such as the Morris water maze test in which the mice are evaluated on their ability and willingness to exit the maze (Morris, 1984). Nevertheless, it is clear that Tff3 has a role in the CNS and the development of the brain, and further research is needed to resolve the exact mechanism of its action. Tff3 could be used to provide new scientific insight and possibly new therapeutic interventions for neurodegenerative diseases.

6. CONCLUSIONS

- The optimal qPCR reaction conditions for the detection and quantification of *Bdnf*, *Egfr*, *Epac2*, *L1cam* and *Nfl* expression in hippocampus of mice were identified.

- There was no significant difference found in the expression levels of *Bdnf*, *Egfr*, *Epac2*, *L1cam* and *Nfl* between the hippocampi of *Tff3-/-* C57Bl6/NCrl and Wt C57Bl6/NCrl mice.

- Further research on aged mice with Tff3 deficiency is warranted.

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Curriculum Vitae

Born in 1996 in Zagreb (Croatia), I attended the 16th Gymnasium (High school with Extended Languages Curriculum). My tertiary education began in 2015 when I enrolled in the undergraduate program of Molecular biology at the Faculty of Science of the University of Zagreb. I obtained my Bachelor's degree in 2018 and the same year enrolled in the graduate program of Molecular biology. My first internship was conducted in the Laboratory of Plant Physiology at my faculty (under the mentorship of Željka Vidaković Cifrek, PhD, Prof). Besides my studies in Croatia, I also studied outside of my home country. During the summer of 2018, I did an Erasmus internship in the Laboratory of Molecular Bacteriology at the Medical University of Gdansk in Poland. As an Erasmus student in Spain in the summer semester of 2019, I was enrolled in biotechnological courses at the Technical University of Madrid.

There were a couple of projects which I volunteered on at my faculty: "Barcode of Life" (topic of molecular ecology under the mentorship of Lucija Šerić Jelaska, PhD) and research on the *Pontastacus leptodactylus* genome (topic of cytogenetics under the mentorship of Višnja Besendorfer, PhD, Prof), with the latter published as a scientific paper. Additionally, in June this year (2021) I was a student intern in Pliva (Teva) in the marketing and sales sector. Except from the accomplishments at my faculty, my academic development included acquiring foreign language speaking skills. During my education, I obtained a C1 IELTS certificate (English), a B2 DELE certificate (Spanish), and a B2 DELF certificate (French). Moreover, in the summer of 2020 I was awarded a scholarship by the French Government to spend a month in France studying French language and culture.

PUBLICATIONS:

Mlinarec, Jelena, et al. "The Pontastacus leptodactylus (Astacidae) repeatome provides insight into genome evolution and reveals a remarkable diversity of satellite DNA." ***Frontiers in Genetics*** 11 (2020): 1820.

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