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Mentor: Associate Professor Ivana Munitić, M.D., Ph.D.

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Marta Kolarić

Analiza potencijalne neuroinflamatorne patologije u leđnim moždinama miševa s insuficijencijom optineurina

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Abstract

Amyotrophic lateral sclerosis (ALS) is a complex neurodegenerative disease characterised by motor neuron loss in the brain, brainstem, and spinal cord. ALS is caused by mutations in more than 50 genes, including *OPTN*, encoding for optineurin, a ubiquitin-binding protein that regulates inflammatory signalling. It has been hypothesized that ALS-linked optineurin mutations cause protein loss-of-function, leading to dysregulated immune response that could trigger ALS. However, the exact mechanism of disease pathogenesis is still unclear given that optineurin participates in various other cellular functions including autophagy, cell death and others. Since most ALS cases display spinal onset, we characterised the potential neuroinflammatory pathology in the lumbar spinal cord of young adult mice carrying ALS-like optineurin truncation ($Optn^{470T}$). We assessed if this optineurin mutation is by itself sufficient to induce ALS-like neuropathology, or whether the latter could be provoked by lipopolysaccharide (LPS). We demonstrated an absence of exaggerated neuroinflammation, TDP-43 aggregation and neurodegeneration in the lumbar spinal cords of $Optn^{470T}$ mice for both basal and LPS-stimulated conditions. Our results indicate that optineurin truncation in mice does not induce ALS-like neuropathology in these settings, suggesting that a short-term inflammatory stimulus is insufficient to precipitate the disease. It is still an open question if chronic inflammation or mutation in other ALS-affected genes together with optineurin truncation could uncover the role of optineurin in neuropathology.

Key words: amyotrophic lateral sclerosis, optineurin, neuroinflammation, neurodegeneration

Sažetak

Amiotrofična lateralna skleroza (ALS) je kompleksna neurodegenerativna bolest karakterizirana gubitkom motoričkih neurona u mozgu, moždanom deblu i leđnoj moždini. ALS je uzrokovan mutacijama u više od 50 gena, uključujući i OPTN, koji kodira optineurin, ubikvitin vezujući protein uključen u regulaciju upalne signalizacije. Pretpostavlja se da mutacije optineurina u ALS-u uzrokuju gubitak funkcije proteina koje mogu izazvati narušeni imunosni odgovor te dovesti do razvoja ALS-a. Međutim, točan mehanizam patogeneze bolesti i dalje nije poznat budući da optineurin sudjeluje u raznim drugim staničnim funkcijama kao što je autofagija, stanična smrt i drugi. S obzirom na to da ALS slučajevi pokazuju spinalni oblik bolesti, okarakterizirali smo potencijalnu neuroinflamatornu patologiju u lumbalnim dijelovima leđne moždine mladih miševa s trunkacijom optineurina nalik ALS-u ($Optn^{470T}$). Ispitali smo ukoliko je mutacija optineurina sama po sebi dovoljna da izazove neuropatologiju nalik ALS-u ili ukoliko ju se može izazvati lipopolisaharidom (LPS). Demonstrirali smo odsustvo pretjerane upale, TDP-43 agregata te neurodegeneracije u lumbalnom dijelu leđne moždine $Optn^{470T}$ miševa pri bazalnim i LPS stimuliranim uvjetima. Naši rezultati ukazuju na to da trunkacija optineurina ne dovodi do ALS-slične neuropatologije u ovim uvjetima te sugeriraju da je kratkotrajni upalni podražaj nedovoljan kako bi uzrokovao bolest. I dalje je otvoreno pitanje mogu li kronična upala ili mutacije u drugim ALS-zahvaćenim genima zajedno s trunkacijom optineurina objasniti ulogu optineurina u neuropatologiji.

Ključne riječi: amiotrofična lateralna skleroza, optineurin, upala, neurodegeneracija

Table of Contents

1. Introduction

1.1. Amyotrophic lateral sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS) is a rapidly progressive adult-onset neurodegenerative disease with a complex genetic and environmental etiology (1). It was first described in the lectures of the French neurologist Jean-Martin Charcot during 1870s. ALS is marked by the progressive degeneration of upper motor neurons in the brain and the lower motor neurons in the brain stem and spinal cord (SC), which results in muscle weakness, atrophy, paralysis, and finally a fatal outcome within as little as 3-5 years upon diagnosis. The condition displays diverse initial symptoms depending on whether it primarily affects spinal or bulbar regions (2). ALS is difficult to diagnose since there are no reliable biomarkers available and the initial clinical picture overlaps with several other neurological or muscular disorders (1). Therefore, it is usually diagnosed rather late, with a diagnostic delay up to one year from the symptom onset.

The estimated global incidence of ALS is around 1 to 2.6 cases per 100,000 individuals per year, with a somewhat higher prevalence in men (3). Aging is the main risk factor in ALS, and the disease typically starts between 45 and 75 years of age, with an average of 55 (1,2). Additionally, there are variations in the mean age of onset when considering the two main types of ALS cases: sporadic (sALS), which account for approximately 90% of cases, and familial (fALS), which account for about 10% of cases (1). FALS cases are generally subjected to earlier age of onset. However, the genetic landscape of fALS and sALS is not strikingly different since similar mutations have been reported in both subtypes (2,4). Mutations in more than 50 genes have been implicated in ALS pathogenesis (2). The genes with a definitive link to ALS include superoxide dismutase 1 (*SOD1*), TAR DNA-binding protein 43 (*TARDBP,* encoding for TDP-43 protein), chromosome 9 open reading frame 72 (*C9ORF72*), fused in sarcoma (*FUS*), optineurin (*OPTN*), TANK-binding kinase 1 (*TBK1*), sequestosome-1/p62 (*SQSTM1*) and others

(2,4). They have been reported to contribute to disease by gain-of-function, loss-of-function, or both.

Mutations in *SOD1* are linked to gain-of-function mechanism (5). This mechanism of pathogenesis was proposed since SOD1 deficiency failed to cause ALS symptoms in mice, in contrast to the transgenic mice carrying the patient G93A mutation in SOD1 (mSOD1), which developed ALS-like pathology (6). *SOD1* mutations lead to the formation of cytoplasmic protein inclusions, which are a typical pathological hallmark of ALS (4). They impair proper protein folding by disturbing the chaperone system which regulates post-translational modification processes (5). Similar to *SOD1*, mutations in *TARDBP* may also lead to the formation of TDP-43 cytoplasmic protein aggregates (4). However, these mutations are rare in ALS patients, and in most, except in SOD1 and FUS mutation carriers, protein aggregates contain wild-type (WT) TDP-43 proteins. In these aggregates TDP-43 is ubiquitinated, phosphorylated, degraded, and found in the SC motor neurons of nearly all ALS patients (7–9). This makes TDP-43 aggregates one of the main hallmarks of ALS. Cytoplasmic TDP-43 acquisition correlates with its nuclear loss and can be attributed to the mutations or deletions in its primary structure (10). Since TDP-43 is involved in the regulation of RNA processes in homeostatic conditions, the mutations and/or aggregation can impair gene transcription and translation, microRNA biogenesis, RNA transport and RNA binding (4).

The pathogenesis of ALS is still incompletely understood, and various mutations can affect a wide range of cellular functions. SOD1 and TDP-43 aggregates share downstream molecular pathology (4). They further promote their aggregation by disturbing the endoplasmic reticulum (ER) quality control, specifically ER-associated degradation machinery. Subsequently, ER stress is induced, and this ultimately causes cytotoxicity. Other proposed mechanisms of ALS pathogenesis include dysregulation of glutamate homeostasis, cytoskeletal disbalance due to aggregates, impairment of mitochondrial Ca^{2+} tolerance and respiratory chain activity

and prion-like propagation. These changes can cause excitotoxicity, ROS upregulation, disturbance of intracellular trafficking and aberrant protein propagation to other neurons, all of which possibly provoke neuroinflammation. In contrast, neuroinflammation has been suggested to be an early event in a subset of ALS-linked mutations involving *OPTN* and *TBK1*, which are implicated in the regulation of inflammatory signalling (11,12). For these genes, the proposed mechanism of pathogenesis are loss-of-function mutations, which can possibly lead to ALS development. However, given that the proteins encoded by *OPTN* and *TBK1* regulate multiple cellular mechanism (e.g. autophagy and necroptosis etc., summarised in Table 1.), it is difficult to determine the exact pathway in which their deficiency would cause neurodegeneration onset, if any.

Altogether, ALS-linked genes exert a broad array of functions. It is likely an oversimplification to state that they contribute to disease either by gain-of-function or loss-of-function mechanisms, given that the pathogenesis of ALS is multifaceted and frequently involves a combination of both mechanisms. Due to the clinical, genetic and pathological complexity in ALS, it remains a major medical challenge, necessitating further research.

Table 1. Normal and pathological function of selected ALS-causing genes

Gene	Protein	Homeostatic functions	Suggested primary pathological feature
SOD1	Superoxide dismutase 1 (SOD1)	Inactivation of superoxide anions	Cytoplasmic inclusions
TARDBP	TAR DNA-Binding Protein, 43-Kd (TDP-43)	Regulation of gene transcription and translation, microRNA biogenesis, RNA transport and RNA binding	Cytoplasmic and nuclear inclusions, impaired gene transcription, gene translation, microRNA biogenesis, RNA transport and RNA binding
OPTN	Optineurin	Regulation of inflammatory signalling, membrane trafficking, necroptosis, autophagy	Disrupted inflammatory signalling, membrane trafficking, necroptosis, autophagy
TBK1	TANK-binding Kinase 1 (TBK1)	Regulation of inflammatory signalling, autophagy	Disrupted inflammatory signalling and autophagy

1.2. Neuroinflammation as a hallmark of ALS

ALS is characterized by dysregulated inflammatory signalling, similar to other neurodegenerative disorders such as Alzheimer's disease, frontotemporal dementia and Parkinson's disease (13). In ALS animal models and patients, inflammation is present early and advances as the disease progresses (14,15). In the central nervous system (CNS), it is characterised by activation of microglia and astrocytes. These glial cells activate in response to pathogen invasion, injury, or degenerative processes, all of which stimulate cell proliferation, morphological changes, and cytokine production (13). Activation of microglia and astrocytes can be assessed by immunofluorescence staining for specific markers, ionized calcium-binding adapter molecule 1 (Iba1) for microglia and glial fibrillary acidic protein (GFAP) for astrocytes. These markers show increased expression levels during glial activation. The intensity of fluorescence correlates with the progression of ALS. Mild microgliosis and astrocytosis are present at the early stages of disease and show gradual increase in intensity as ALS advances (14,15).

Microglia belong to the myeloid cell lineage and are the only innate immune cells in the CNS parenchyma, which they inhabit from early embryonic development (13). Microglia are most similar to peripheral macrophages. Both cell types maintain tissue stability by continuously sampling interstitial space by phagocytosis (16,17). Microglia and macrophages can also rapidly respond to cytokine stimulation (e.g. TNF-α and IL-1β) and pathogen- or damage-associated molecular pattern (PAMP and DAMP) recognition. They comprise the first line of defence as they direct immune responses in response to these stimuli (13,18). Activated microglia and macrophages change shape, transitioning from resting cells of smaller cell bodies and long processes to activated ameboid cells that have upregulated Iba1, larger cell body areas and shortened processes (16,17).

Similar to pro-inflammatory and anti-inflammatory phenotypes of macrophages, microglial activation phenotypes are usually separated into

classical (neurotoxic) M1, and alternative (neuroprotective) M2 phenotype (Fig. 1) (17,18). M1 and M2 phenotypes vary in gene expression leading to different cytokine and signalling molecule production (18). However, such clear-cut distinctions are rarely found *in vivo,* as glial activation is a spectrum that includes differences in both morphology and function, depending on the CNS region, type, and severity of insult (19). Astrocytes are another type glial cells with important roles in the CNS homeostasis, such as in regulation of neuronal metabolism and synapses (17). Additionally, although they are not immune system cells *per se*, they can contribute to CNS immune responses and neurodegeneration by loss of homeostatic function and cytokine secretion. Like microglia, astrocytes also have analogous A1/A2 phenotypes and have been implicated in the pathology of ALS (Fig. 1) (17,18). Proinflammatory M1 phenotype can be obtained through proinflammatory cytokine interferon (IFN-γ) or bacterial cell-wall products like lipopolysaccharide (LPS). As a result, M1 microglia then release pro-inflammatory factors IL-1β, TNF-α, IL-6 and prostaglandin E2 (PGE2). Similarly, A1 phenotype is obtained with IL-1β, IFN-γ, LPS, TNF-α, and IL-6 stimulation, after which A1 astrocytes release IL-1β, TNF-α, reactive oxygen species (ROS) and nitric oxide (NO). Long exposure of neurons to proinflammatory environment can cause neurodegeneration. In contrast, neuroprotective phenotypes of both glial cell subsets are obtained with IL-13, IL-10, TGF-β, and IL-4 stimulation (18). Activated M2 microglia then secrete anti-inflammatory factors IL-4, TNF-β, IL-10 and IGF-1, whereas A2 astrocytes secrete neurotrophic factors and thrombospondins (18). These anti-inflammatory factors are linked to promotion of neuro-regeneration and healing. Overall, the activation of glial cells can be both neurotoxic and/or neuroprotective depending on the specific environmental stimulus.

Microglia and astrocytes crosstalk in ALS (17). For instance, blocking proliferation of mutated SOD1 (mSOD1) microglia in transgenic mSOD1 mouse can result in decreased astrocyte activation (20). Similarly, transfer

of mSOD1 astrocytes to a healthy mouse can result in an activated microglial phenotype (21). M1 microglia facilitate induction of A1 astrocyte phenotype through IL-1, TNF-α, C1q production. In contrast, it is still not clear which astrocyte-derived factors directly drive M1 and M2 microglial changes, and how M2 microglia affect A2 astrocytes (18). It is notable that early upon the disease onset, microglia and astrocytes are thought to be neuroprotective as they secrete cytokines which promote regeneration and healing. However, in the presence of a chronic stimulus such as TDP-43 protein aggregates or neuronal debris, which represent most common ALS DAMPs, they eventually transform, adopting a neurotoxic phenotype (21,22). Adjacent neurons are harmed due to continuous proinflammatory cytokine secretion as microglia and astrocytes both fail to eradicate the DAMPs (Fig. 1) (21,23). In conclusion, during neurodegeneration glial cells display both harmful and protective behaviour, giving rise to the question if neuroinflammation is a consequence or cause of neurodegeneration.

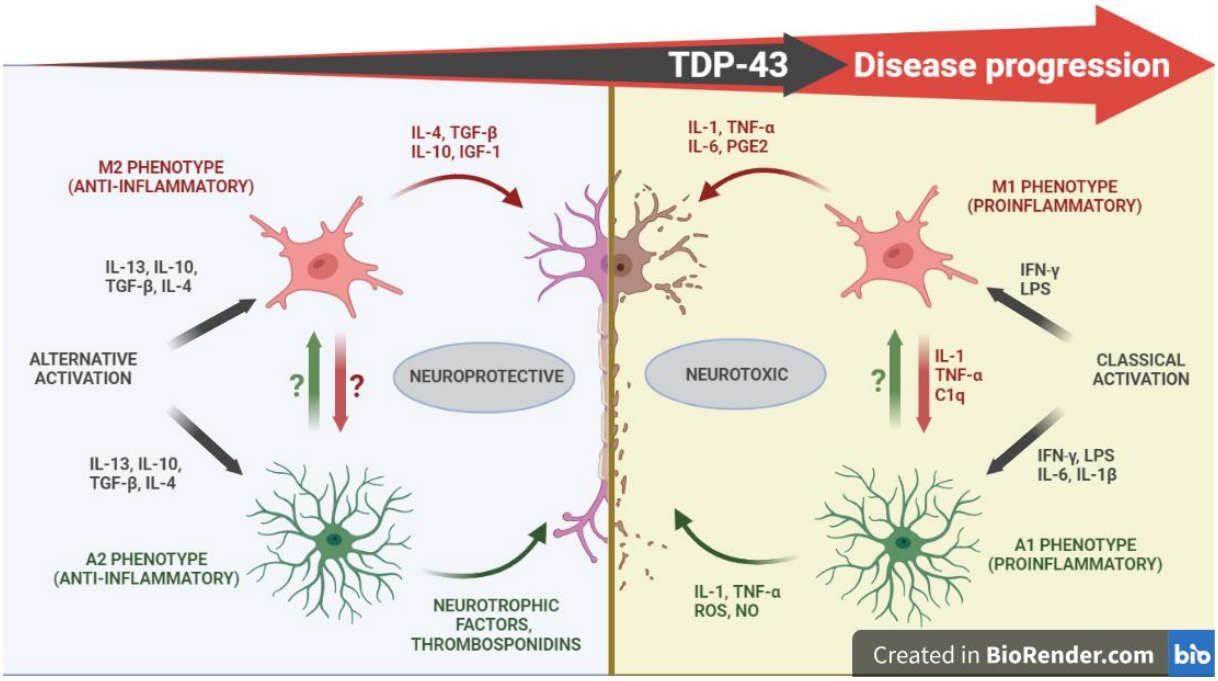

Figure 1. The neuroprotective and neurotoxic roles of microglia and astrocytes in ALS disease progression. This picture represents the proposed glial cell phenotype change and their effect on motor neurons during ALS progression. At the disease onset the anti-inflammatory signals

are predominantly present (i.e. IL-13, IL-10, TGF-β and IL-4) and induce M2 and A2 neuroprotective phenotypes. The glial cells promote healing and regeneration of neurons by anti-inflammatory factor production. However, upon continuous stimulation of glial cells by DAMPs (TDP-43) and an increase in pro-inflammatory signal intensity (i.e. IFN-γ and LPS for microglia and IFN-γ, LPS, IL-6 and IL-1β for astrocytes), they shift to neurotoxic phenotypes, M1 and A1. M1 microglia can also induce astrocytes transition to an A1 phenotype by the release of IL-1, TNF-α and C1q proinflammatory factors. At the end, motor neurons are unable to withstand the long exposure to inflammatory environment and degenerate. Question marks indicate lack of direct evidence in glial cell crosstalk. The figure was drawn in BioRender.

1.3. Optineurin

Optineurin is a multifunctional ubiquitin binding adaptor protein, which has been reported to regulate several cellular processes like neuroinflammation, autophagy, necroptosis, and vesicular trafficking (11,12). Its name stands for "optic neuropathy inducing", originating from the finding that *OPTN* mutations cause primary open angle glaucoma. The *OPTN* gene is found on human chromosome 10p13 and translates to a ubiquitously expressed cytoplasmic protein of 577 amino acids (24). Optineurin harbours two coiled coil domains (CC), microtubule-associated protein 1A/1B-light chain 3-interacting region (LIR), ubiquitin-binding domain in ABIN proteins and NEMO (UBAN) and zinc finger (ZF). UBAN and ZF compromise the ubiquitin binding (Ub-binding) region of optineurin. These domains are important for its interactions with numerous cellular proteins including receptor interacting protein kinase 1 (RIPK1; with function in necroptosis and inflammation), myosin VI (an exocytosis related protein) and CYLD (deubiquitinase). Moreover, at the N-terminus, optineurin binds TBK1, which is a protein implicated in inflammatory signalling and autophagy (Fig. 2) (11,12,24). By interacting with these and other proteins optineurin acts as an adaptor protein in inflammatory

signalling pathways, autophagy, cell trafficking and necroptosis. An interesting hypothesis is that these pathways interconnect via optineurin, and that optineurin deficiency in either of them is sufficient to trigger neurodegeneration (11,12). Given that this thesis is focused on the role of optineurin in neuroinflammation, only optineurin interacting proteins that are a part of inflammatory signal transduction will be further discussed.

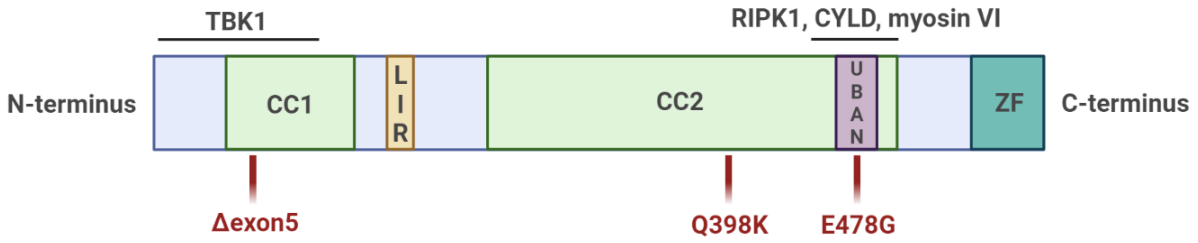

Figure 2. Optineurin protein domains, mutations and interacting partners. Simplified version of optineurin protein domains along with their corresponding interacting protein partners. Only optineurin partner proteins and mutations indicated in the main text are depicted (24). The figure is adapted from Slowicka et al, 2016 and drawn in BioRender.

1.4. Implications of optineurin in inflammatory pathways

Approximately 40 optineurin variants have been found in ALS patients (12). Mutations are enriched in the C-terminus and proposed to result in optineurin loss-of-function by limiting protein interactions via its UBAN (11,12). Confirmed pathogenic mutations of optineurin are: an exon 5 deletion (Δexon5), Q398X and E478G (Fig. 2) (25). The exon5 deletion results in altered mRNA processing, which generates a premature stop codon at the N-terminus. It is predominantly found in a homozygous state among ALS patients. Only a smaller subset of patients carries it in a heterozygous state (26). Q398X is a nonsense mutation of the UBAN region of exon 12 and causes a premature stop at the C-terminus (25). The disease has been shown in a homozygous state thus far. E478G is a missense point mutation that affects the UBAN region. Unlike the Q398X and most Δexon5 cases, E478G is found in a heterozygous state. Both Q398X and E478G preclude ubiquitin binding. In overexpression studies of Q398X and Δexon5,

optineurin levels were decreased which proposed a loss-of-function mechanism. As a result, Δexon5 and Q398X mutations were thought to trigger nonsense-mediated-mRNA decay. In contrast, overexpression of heterozygous E478G was reported to induce gain-of-function mechanisms by forming protein inclusions. However, since no mutated optineurin protein aggregates were found in ALS patients so far, it is suggested that the E478G mediated protein aggregation might be a consequence of an artefact in the tested cell line (12).

OPTN mutations have been reported to affect two inflammatory signalling pathways: TBK1/interferon regulatory transcription factor (IRF3) and nuclear factor kappa B (NF-κB) pathways (Fig. 3) (11,12). Optineurin was first implicated as a potential negative regulator of NF-κB inflammatory pathway due to high homology of the UBAN region of optineurin to that of NF-κB essential molecule (NEMO) (25). NEMO is an important positive regulator of the NF-κB signalling pathway (11,12). It was hypothesised that optineurin competes with NEMO, and that optineurin mutations could fail to suppress the NF-κB activation and potentially lead to hyperinflammation and motor neuron death (25). Normally, upon Toll like receptor (TLR) stimulation with PAMPs or DAMPs, RIPK1 gets ubiquitinated and docks NEMO (11,12). NEMO is a component of the inhibitor of nuclear factor-κB (IκB) kinase (IKK) complex. IKK complex activation leads to phosphorylation and ubiquitination of IκB, which is consequently degraded. IκB releases NF-κB, which then translocates from the cytoplasm to the nucleus and induces expression of proinflammatory factors.

In the initial study done in NSC-34 cell line (mouse neuroblastoma and spinal-cord hybrid), overexpression of optineurin E478G and Q398X mutations was reported to preclude inhibition of the NF-κB activation, unlike WT optineurin (25). The same was replicated in a study overexpressing WT optineurin and E478G in neuroblastoma Neuro2a cells (27). However, conflicting results were later obtained after generation of optineurin mouse models. Mouse models $Optn^{470T}$, $Optn^{D477N}$, $Optn^{-/-}$ and $Optn^{\Delta 157}$ mimic

pathogenic optineurin mutations (12). $Optn^{470T}$ and $Optn^{D477N}$ lack Ub-binding ability making them interesting to study given the abundance of mutations in the C-terminus (28,29). $Optn^{470T}$ model carries a truncation of the C-terminus and is correspondent to Q398X mutation (28). $Optn^{D477N}$ model carries a point mutation in the UBAN mimicking E478G (29). $Optn^{\Delta 157}$ carries a N-terminal deletion mimicking Δexon5 (30). Generation of optineurin deficient mouse models offered a better insight into optineurin mediated mechanisms of pathogenesis given that cell line studies are deprived of complex physiological and pathological processes that occur *in vivo*.

Studies utilising bone marrow-derived macrophages and bone marrow dendritic cells from $Optn^{470T}$ and $Optn^{D477N}$ showed that NF-kB function is not impaired upon TLR4 stimulation, suggesting that optineurin is dispensable for NF-kB activation (28,29). Additionally, mouse embryonic fibroblasts (MEFs) from $Optn^{-/-}$ presented the same phenotype (31). Optineurin dispensability in NF-κB activation was also demonstrated in primary microglia from $Optn^{470T}$, which did not show any NF-kB function impairments (32). However, it was recently demonstrated that E478G mutation leads to excessive NF-κB pathway activation in $Optn^{-/-}$ MEFs (33). The effect was retained upon E478G lentiviral delivery to mouse CNS causing neurodegeneration. Therefore, further studies regarding optineurin function in NF-κB activation need to be conducted. It is also of note that the relevance of optineurin mouse models for studying ALS pathogenesis is still unclear since they do not display typical motoric symptoms observed in ALS patients (12). Until recently, axonal degeneration and disfunction of ventral rearing activity in $Optn^{-/-}$ mice were the only significant functional defects reported (34). A recent study which was conducted on older $Optn^{-/-}$ mice demonstrated a significant motor neuron loss in the murine lumbar spinal cord (LSC). However, the mice did not exhibit motor deficits. The same authors also reported TDP-43 cytoplasmic mislocalisation, which is especially interesting given that no TDP-43 pathology was documented in

optineurin mouse models before (35). Moreover, TDP-43 aggregates are also present in ALS patients that carry optineurin mutations (36). Therefore, optineurin mouse models might replicate some aspects of ALS, necessitating further studies.

The role of optineurin in TBK1 signalling pathway is somewhat better understood. Stimulation of TLR4 and similar receptors activates TBK1 signalling pathway (Fig. 3) (11,12). Adaptor and anchor proteins are assembled to further recruit TBK1 which then undergoes ubiquitination and transautophosphorylation, which lead to activation of its kinase activity. Binding of ubiquitinated TBK1 by UBAN region and N-terminus of optineurin generates a positive feedback loop in which optineurin gets phosphorylated by TBK1. Subsequently, activated TBK1 phosphorylates IRF3, allowing its dimerization and nuclear translocation, resulting in type I IFN (IFN-β and IFN-α) production. Therefore, UBAN region of optineurin is needed for TBK1 activation. The latter was confirmed on primary peripheral myeloid cells from $Optn^{470T}$, $Optn^{D477N}$, $Optn^{-/-}$ and $Optn^{\Delta 157}$ mouse models (28,29,31,32). The studies observed reduced production of IFN-β, meaning that deficiencies in the optineurin Ub-binding region impair proper TBK1 function. The significance of optineurin in TBK1 activation was also confirmed in the CNS, as IFN-β levels were significantly reduced in the primary microglia cells from young $Optn^{470T}$ mice upon acute TLR4 stimulation (32). Additionally, alterations in IFN-β levels affect IRF7, NOS2, CXCL10, CXCL1, and IL-10 gene expression, but the significance is still unclear. Even though the primary microglia of $Optn^{470T}$ mice had altered IFN-β levels and presented an activated phenotype based on their morphology, no motor neuron degeneration occurred. Therefore, further research of neuroinflammatory pathology conducted on mice with optineurin deficiency is necessary to uncover the potential neuroprotective role of optineurin in inflammatory pathway.

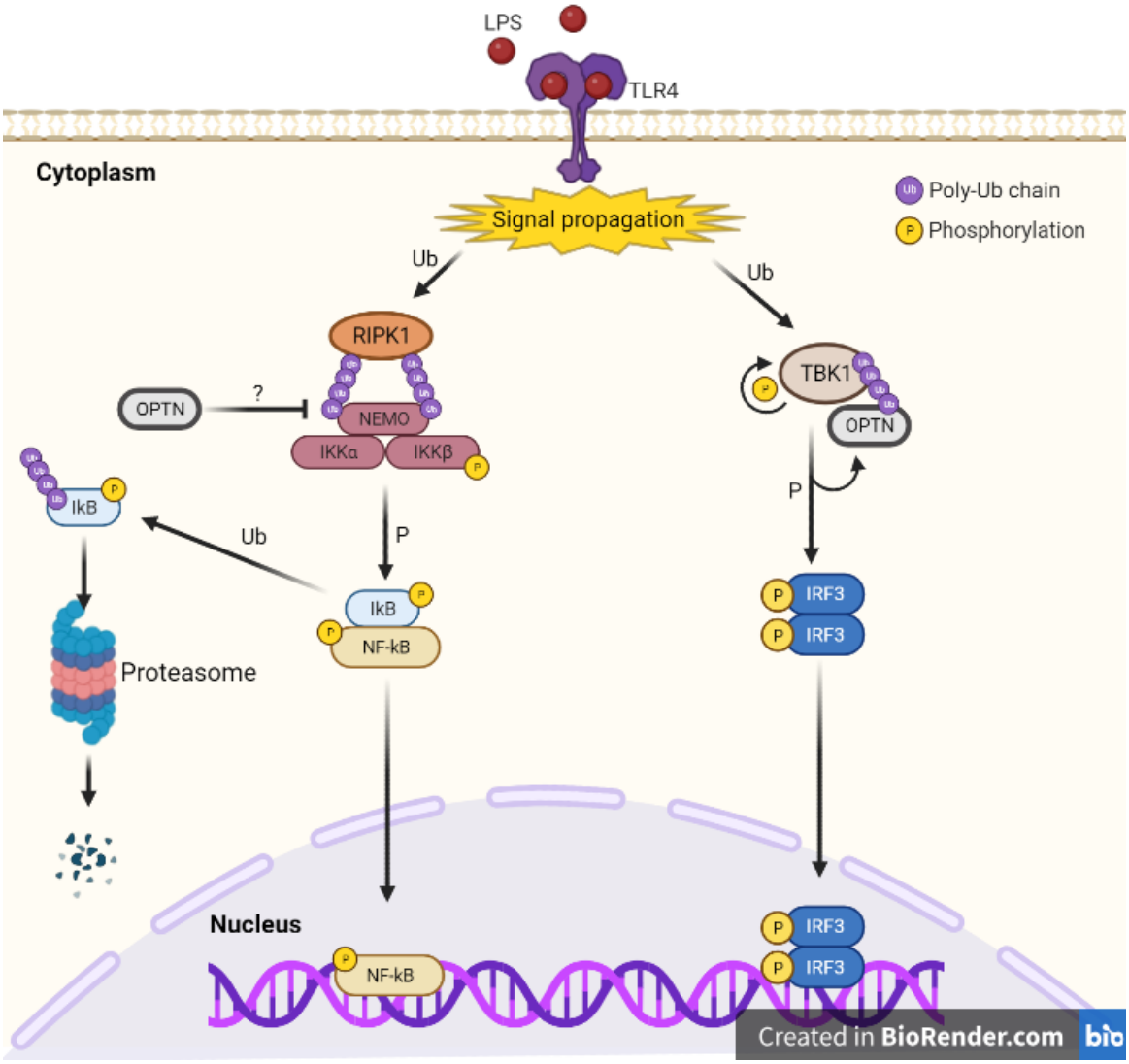

Figure 3. Inflammatory pathways leading to activation of NF-κB and IRF3 transcription factors. The picture represents the activation of two inflammatory pathways NF-κB and TBK1 upon LPS stimulation of TLR4. DAMPs also activate these pathways. In the NF-κB pathway, ubiquitination of RIPK1 permits IKK complex recruitment. IKK complex is then activated by IKKβ phosphorylation, which then phosphorylates IκB and NF-kB. Phosphorylated IκB is ubiquitinated causing release of NF-κB. NF-κB translocates to the nucleus and induces proinflammatory gene transcription. In the TBK1 pathway, TBK1 gets ubiquitinated and transautophosphorylated. TBK1 interacts with optineurin. TBK1 phosphorylates both itself and optineurin creating a positive feedback loop. Activated TBK1 phosphorylates IRF3. Phosphorylated IRF3 dimerises and is

translocated to the nucleus. IRF3 then promotes expression of IFN related genes. The figure was drawn in BioRender.

2. Thesis Goals

Upon finding pathogenic optineurin mutations in ALS patients, several mouse models were generated to explain its role in disease pathogenesis. In this thesis we tested if inflammation would trigger neurodegeneration in Optn^{470T} mouse model. In our laboratory, Markovinovic et al. previously aimed to determine the role of optineurin in NF-κB and TBK1 signalling pathways of young Optn^{470T} mice in response to acute LPS-mediated activation of TLR4 in the motor cortex (32). Of importance to this study is that they did not detect microgliosis or neurodegeneration in WT and Optn^{470T} mice upon acute LPS stimulation. Therefore, we aimed to continue the *in vivo* investigation of the relationship between Optn^{470T} mice and inflammatory signalling in the LSC upon the same peripheral (intraperitoneal) LPS stimulation. Such LPS application stimulates the peripheral innate immune response, which in turn affects the CNS. We focused on the potential changes in the LSC as it is the only region affected in classical ALS mouse model mSOD1 (6).

The specific goals were:

- To assess the activation status of glial cells (microglia, and astrocytes) in the presence and absence of acute peripheral inflammation (induced by intraperitoneal LPS application after 24 h); this was measured by immunofluorescence of microglial activation marker Iba1, and astrocyte activation marker GFAP.
- To test if Optn^{470T} mutation by itself or acute peripheral inflammation triggers neurodegeneration (i.e. total neuronal loss, motor neuron loss, cytoplasmic TDP-43 aggregates and nuclear depletion of TDP-43) in the LSC; this was measured by immunofluorescence of the neuronal marker neuronal nuclear protein (NeuN), motor neuron-LSC specific marker choline acetyltransferase (ChAT) and phosphorilated-TDP-43 (p-TDP-43), respectively.

3. Materials and methods

3.1. Mice

Mice carrying optineurin C-terminal truncation ($Optn^{470T}$), were generated as previously described (28). C57BL/6 wild-type (WT) mice (from Jackson; bred in the Animal facility at the Faculty of Medicine, University of Rijeka) were used as controls. Young adult (7-9-week-old) male mice were used for all experiments. Animals were taken care of correspondingly to European Communities Council Directive of 24 November 1986 (86/609/EEC) and institutional and national instructions. All conducted experiments were authorised by the Ethics Committees of the Department of Biotechnology, Medical School of the University of Rijeka, and the Ministry of Agriculture of the Republic of Croatia.

3.2. Reagents

EmeraldAmp GT PCR Master mix (#RR310A) was from Takara and Gel star nucleic acid gel stain (#50535) was from Lonza. Primary antibodies used for immunofluorescence analysis were anti-GFAP (#ab53554) from Abcam, anti-Iba1 (#019-19741) from Wako, anti-NeuN (#12943) from Cell Signalling, anti-ChAT (#AB144P) and anti-p-TDP-43 (#SAB4200223) from Sigma Aldrich. Alexa Fluor 488 and Alexa Fluor 555- conjugated secondary antibodies used were from Invitrogen. Lipopolysaccharide (LPS; *E. coli* O111:B4, #4391) was from Sigma Aldrich.

3.3. Mice genotyping

3.3.1. DNA isolation from murine tails

Mice tails were digested by a lysing buffer (10 μM Tris, 10 μM ethylenediaminetetraacetic acid (EDTA), 0.15 M NaCl, 0.2% sodium dodecyl sulphate) and proteinase K (0.4mg/mL) and incubated on Thermomixer C for 4 h at 55°C and 900 rpm. Residual tail hair was removed by centrifugation for 1 minute at 4°C and 1200 rpm. To induce DNA precipitation, isopropanol was added to collected supernatants, and the samples were centrifuged for 10 minutes. Supernatants were discarded. Ethanol (70%) was added to the remaining pellet, followed by another

round of 10-minute centrifugation. The supernatants were discarded once again. Isolated DNA pellets were then dissolved in water during 1 h incubation at 55°C and 900 rpm on Thermomixer C. The DNA concentrations were established by using a UV/VIS spectrophotometer BioDrop-DUO (BioDrop) at the wavelength of 260 nm.

3.3.2. Polymerase chain reaction (PCR)

The PCR was conducted by using the "EmeraldAmp GT PCR Master mix" kit on the Mastercycler nexus GSX1 (Eppendorf) in 35 cycles. Each PCR tube contained 1 µl of DNA sample and 19 µl of appropriate master mix (Emerald M. Mix Buffer, with the appropriate forward (FW) and reverse (RW) primers, resuspended in miliQ H_2O). The primers used for detection of WT Optn and Optn^{470T} mice and the PCR program are shown in Tables 1 and 2.

Table 2. PCR primer sequences used for WT and Optn^{470T} mice genotyping

	WT Optn	Optn^{470T}
Forward primer	5'-GCTACCATGCTCAGCCAGAGTTTC-3'	5'-GCTACCATGCTCAGCCAGAGTTTC-3'
Reverse primer	5'-GGCTTCAGGGATGCATGAATC-3'	5'-GGCTTCAGGGATGCATGAATC-3'

Table 3. WT and Optn^{470T} mice genotyping PCR program

	Temperature	Time	
Initial denaturation	95°C	30s	
Denaturation	95°C	30s	35X
Primer annealing	55°C	30s	
Annealing	72°C	60s	
Final extension	72°C	10min	
Cooling	+4°C	∞	

3.3.3. DNA electrophoresis on agarose gel

To distinguish between WT and Optn^{470T} mice, we ran electrophoresis on 2% agarose gel, which was prepared in 1X TAE buffer (diluted from 50X stock; 5.4 mM Tris, 0.13% CH_3COOH, 1.1 mM EDTA). The 2% agarose gel was heated in a microwave oven and briefly cooled; 1 µl of Gel star nucleic acid gel stain was used for DNA visualisation on gel. The DNA ladder and the samples were loaded on the gel and ran for 30 minutes on 110 V. Results were acquired on ChemiDocTM MP Imaging System (Bio-Rad).

3.4. Immunofluorescence

Young adult male mice received intraperitoneally either control solution, phosphate buffer saline (PBS), or 5 mg/kg of LPS diluted in PBS. After 24 hours, prior to perfusion of mice, they were intraperitoneally anaesthetised with 0.1 mg/g ketamine (Richter Pharma AG, Austria) and 0.02 mg/g xylazine (Alfasan International, Netherlands) mixture. The mice were perfused with PBS and fixed with 4% paraformaldehyde (PFA). Spinal cords were removed, postfixed in 4% PFA overnight (O/N) and preserved in 30% sucrose to induce tissue dehydration. The lumbar part of the spinal cord was embedded in O.C.T compound and cut to 25 µm thick slices with Leica CM1850 cryostat. To prevent cryo-damage, spinal cord sections were stored in tissue preservation solution (30% ethylene glycol, 30% glycerol, 24.4 mM phosphate buffer, pH = 7.4). On the first day, tissue slices were both permeabilized and blocked (10% Horse serum and 0.25% Triton X-100 in PBS) for 1 h at room temperature (RT). Tissue slices were then incubated with primary antibodies in an antibody probing solution (1% horse serum and 0.25% Triton X-100 in PBS) O/N at RT. The primary antibody dilutions used in the experiments were: anti-Iba1 1:500, anti-GFAP 1:200, anti-NeuN 1:200, anti-ChAT 1:100 and anti-p-TDP-43. The next day, tissue slices were washed with PBS and incubated with the appropriate secondary antibody for 1 h at RT. All secondary antibodies were used in a 1:200 dilution. After incubation with secondary antibodies, slices were washed again and stained with DAPI (500 ng/mL) for 15 minutes, and then incubated with True Black (20X stock diluted to 1X in 70% ethanol) for 30 seconds to quench

autofluorescence. Slices were mounted in Vectashield. All images were obtained on Olympus IX83 fluorescent microscope (10x and 20x objectives, Z stacking (30 stacks, 1 µm step size)). Image analysis was performed in ImageJ software (National Institute of Health, USA). In brief, after the scale bar was set, the cells were marked with the threshold option Otsu in ImageJ after which the values of integrated density (mean fluorescence intensity; MFI) and cell body area (CBA) were measured. The longest process length of each microglial cell was measured by "freehand line" option.

3.5. Statistics

The statistical analysis was conducted by using two-way ANOVA with Sidak's multiple comparisons test in GraphPad Prism Software 8.0.1 (San Diego, CA, USA). The results were considered statistically significant if the p-value was < 0.05.

4. Results

4.1. Genotyping of Optn^{470T} mice

We genotyped Optn^{470T} mice by the PCR technique. We detected a band around 288 bp in size which represents the Optn^{470T} genotype, corresponding to that of the Optn^{470T} positive control mice. Additionally, we detected a band at around 363 bp in size which represents the genotype of WT mice, as the band size correlates with the band size of positive control WT mice (Fig. 4). Altogether, the Optn^{470T} mice were successfully confirmed, and the results obtained from mice of Optn^{470T} genotype can be compared to mice of Optn WT genotype.

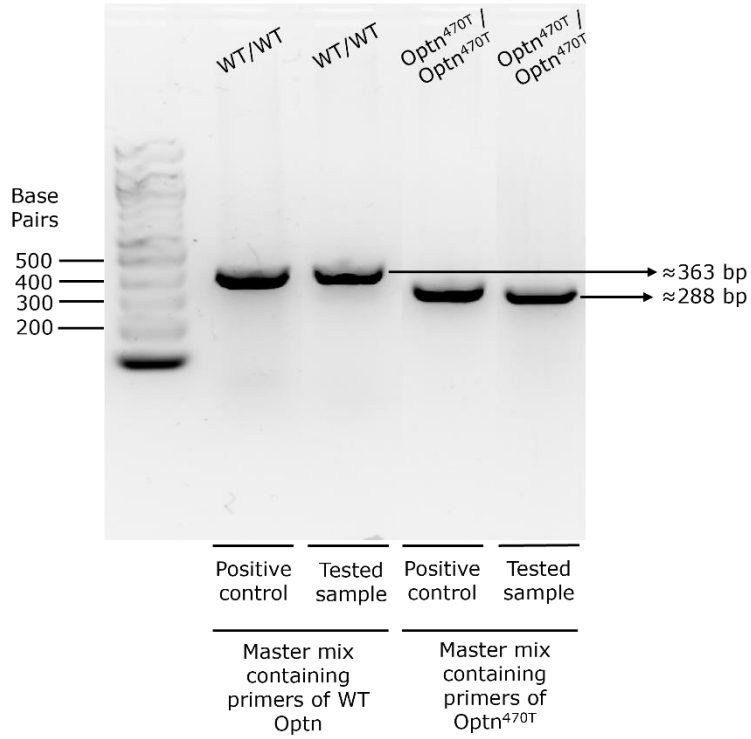

Figure 4. Genotyping of Optn^{470T} mice. DNA from tails of WT and Optn^{470T} mice was isolated and evaluated for a presence or absence of Optn^{470T} truncation by PCR. WT and Optn^{470T} positive controls and tested samples are indicated, along with their corresponding genotype and the size of the amplified DNA fragments marked in base pairs.

4.2. LPS-induced microgliosis and astrocytosis were not increased in Optn^{470T} compared to WT mice

To determine if the peripheral immune stimulation will differently affect microglial activation in the LSC of WT compared to Optn^{470T} mice, we stimulated young adult male mice with LPS (Fig. 5 A-G). Microglia of unstimulated (PBS control) WT mice were not activated. The same was observed in Optn^{470T} mice, indicating no differences between genotypes in the baseline conditions. Upon LPS stimulation of WT mice we observed microglial activation as an increase in microglial cell body area (Fig. 5 E) and shortening of the processes (Fig. 5 F). We did not observe a significant change in MFI (Fig. 5 G). Notably though, microglial activation in Optn^{470T} mice upon LPS challenge was similar to WT counterparts. No differences in astrocyte activation were present in unstimulated conditions between the genotypes (Fig. 6 A-E). Furthermore, we did not find evidence for astrocyte activation in either WT and Optn^{470T} mice upon LPS stimulation. Additionally, no differences were present between genotypes. However, a significant decrease in astrocyte activation (p value = 0,02) was observed in WT LPS mice (Fig. 6 E) compared to WT PBS mice, for which we lack an explanation at present. Therefore, Optn^{470T} mice did not differ to WT mice in microglial and astrocyte activation, suggesting that single acute peripheral LPS administration is insufficient to elicit a potential hyperinflammatory response of microglia and astrocytes in the LSC of Optn^{470T} mice, which was similar to the previous findings in the motor cortex in the brain (32).

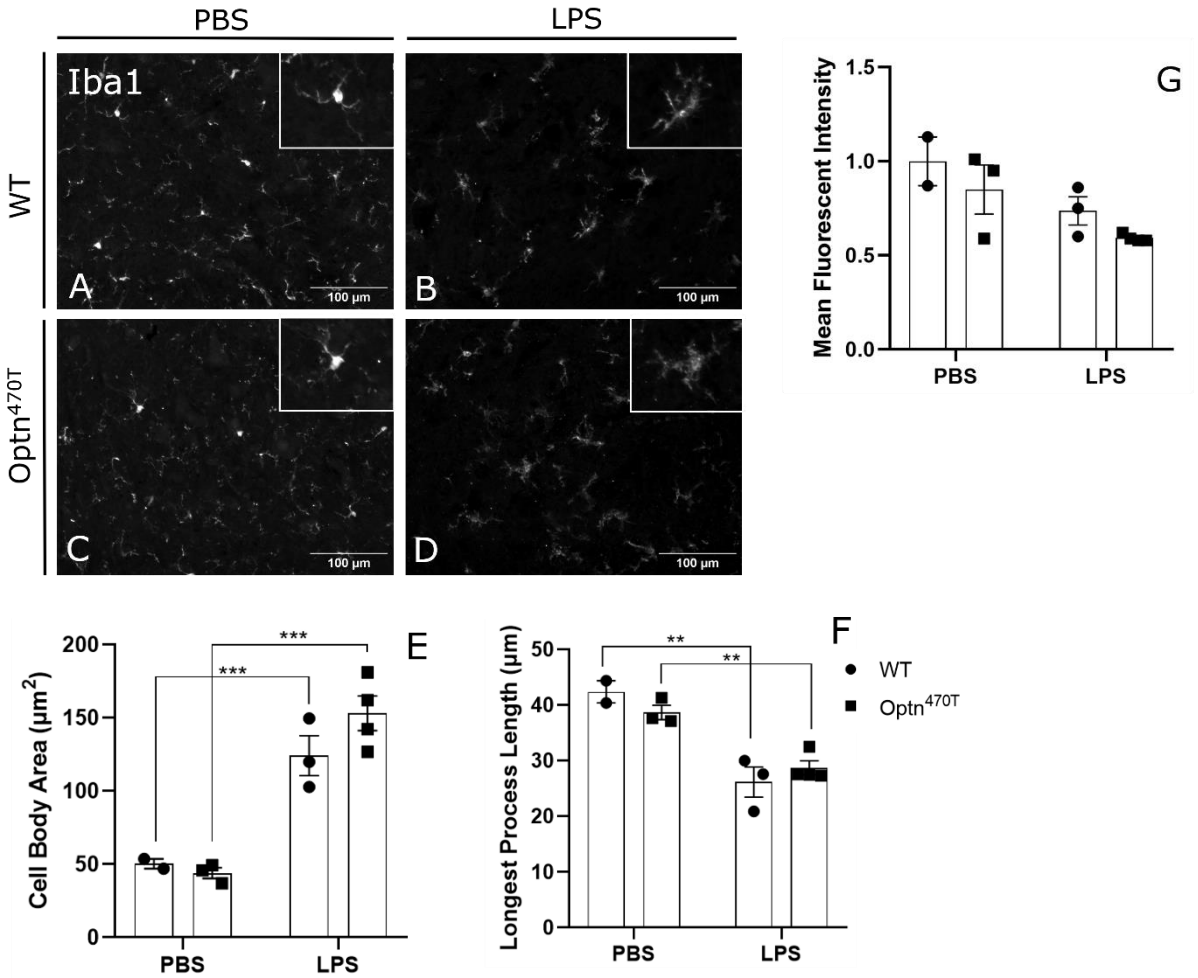

Figure 5. LPS-induced microgliosis was not exaggerated in Optn^{470T} mice. Male 7-9-week-old WT and Optn^{470T} mice were stimulated with LPS (5 mg/kg) or PBS (control) for 24 h. Lumbar spinal cord sections were stained for microglial marker Iba1 (Fig. 5 A-D). Cell body area (Fig. 5 E), longest process length (Fig. 5 F) and mean florescence intensity (Fig. 5 G) were calculated with the ImageJ software. The data are presented as mean ± SEM from 2-4 WT and Optn^{470T} mice (Two-way ANOVA; ** $p < 0.01$, *** $p < 0.001$).

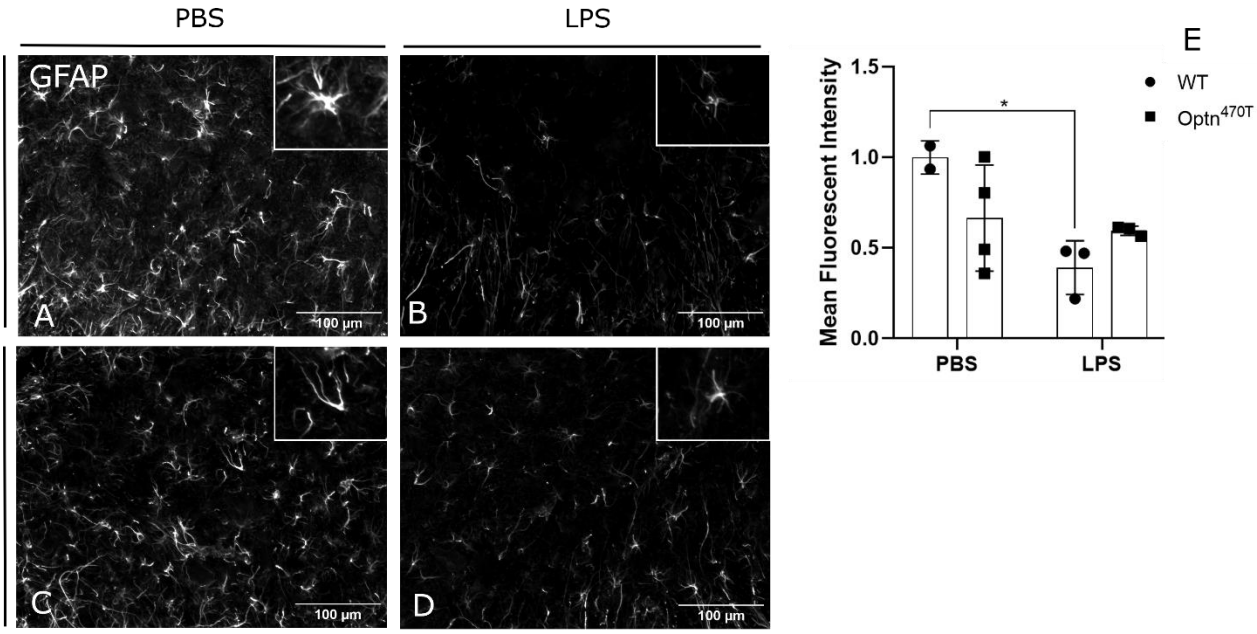

Figure 6. Acute LPS stimulation decreased GFAP expression in WT mice. Male 7-9-week-old WT and Optn^{470T} mice were stimulated with LPS (5 mg/kg) or PBS (control) for 24 h. Spinal cord sections were stained for astrocyte marker GFAP (Fig. 6 A-D). MFI was calculated with ImageJ software (Fig. 6 E). The data are presented as mean ± SEM from 2-4 WT and Optn^{470T} mice (Two-way ANOVA; * $p < 0.05$).

4.3. Acute LPS stimulation does not induce neurodegeneration in WT and Optn^{470T} mice

To further study if the acute LPS stimulation of the peripheral immune system can trigger neurodegeneration, we counted the number of neurons in the ventral horn of the lumbar part of the spinal cord. Tissue sections were stained for a neuronal marker neuronal nuclear protein, NeuN and a spinal cord motor neuron-specific marker choline acetyltransferase, ChAT (Fig. 7 A-D, Fig. 8 A-D). We observed no significant decrease in neither total neuron numbers nor motor neuron numbers between PBS and LPS treatment (Fig. 7 E, Fig. 8 E) in WT mice. The same was observed between PBS and LPS treatment for the Optn^{470T} genotype. Ultimately, acute LPS stimulation of the peripheral immune system could not induce neurodegeneration in neither WT nor Optn^{470T} male mice.

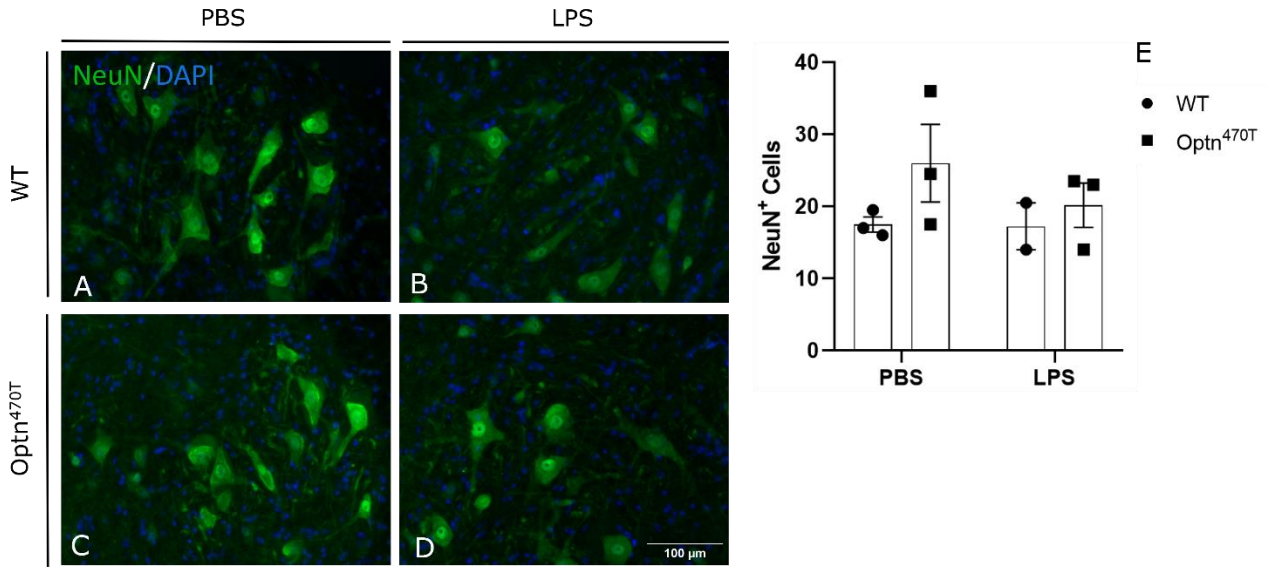

Figure 7. Acute LPS stimulation does not induce neurodegeneration in WT and Optn^{470T} mice. Male 7-9-week-old WT and Optn^{470T} mice were stimulated with LPS (5 mg/kg) or PBS (control) for 24 h. Spinal cord sections were stained for a neuronal marker, neuronal nuclear protein (NeuN) (Fig. 7 A-D). Neuron numbers were assessed with ImageJ software (Fig. 7 E). Cell nuclei were distinguished by DAPI positivity. The data are presented as mean ± SEM from 2-3 WT and Optn^{470T} mice (Two-way ANOVA).

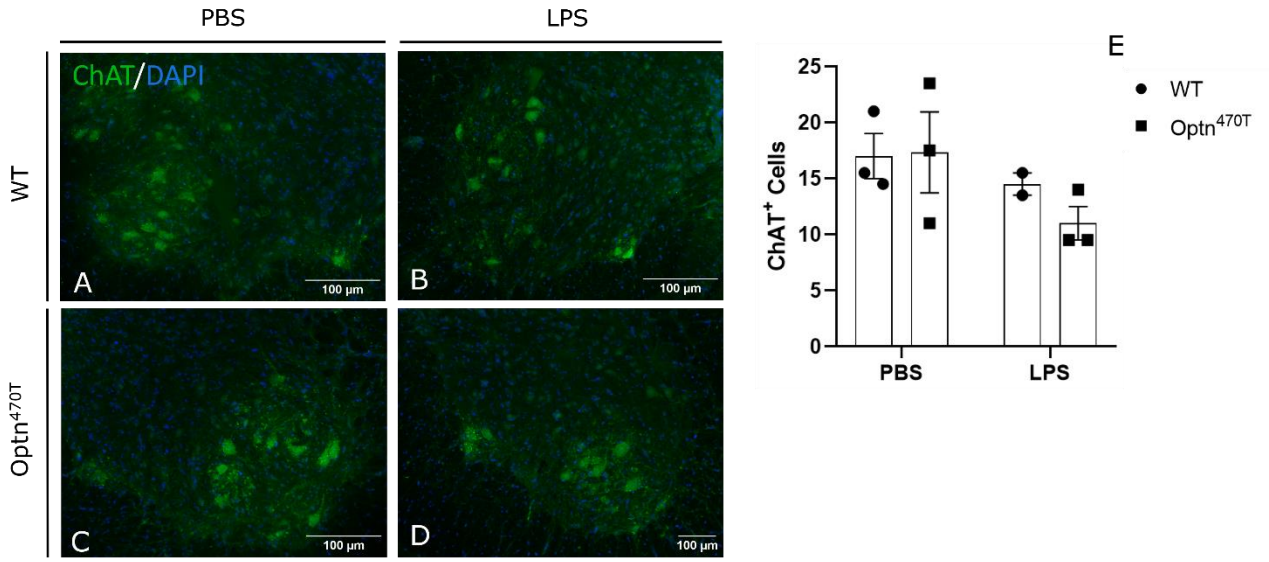

Figure 8. Acute LPS stimulation does not induce motor neuron degeneration in WT and Optn^{470T} mice. Male 7-9-week-old WT and Optn^{470T} mice were stimulated with LPS (5 mg/kg) or PBS (control) for 24

h. Lumbar spinal cord sections were stained for spinal cord motor neuron marker, choline acetyltransferase (ChAT) (Fig. 8 A-D). Motor neuron numbers were assessed with ImageJ software (Fig. 8 E). Cell nuclei were distinguished by DAPI positivity. The data are presented as mean ± SEM from 2-3 WT and Optn^{470T} mice (Two-way ANOVA).

4.4. Optn^{470T} mice do not show p-TDP-43 cytoplasmic mislocalisation and aggregation upon acute LPS stimulation

Given that we were unable to detect neuronal loss, we next sought for another hallmark of ALS, the phosphorylated TDP-43 cytoplasmatic aggregates (7). We observed nuclear p-TDP-43 staining in WT mice for both PBS and LPS treatments, but no cytoplasmic p-TDP-43 inclusions (Fig. 9 A-D). The same was observed in Optn^{470T} mice. Additionally, we measured nuclear p-TDP-43 depletion by MFI assessment. No significant p-TDP-43 nuclear depletion was observed (Fig. 9 E) in the LSC of WT mice for both non-stimulated and stimulated conditions. Similar results were observed in Optn^{470T} mice for both treatments. Altogether, no TDP-43 cytoplasmatic aggregates and significant nuclear TDP-43 losses were found in neither PBS- nor LPS-stimulated animals, suggesting that acute peripheral immune stimulation did not lead to TDP-43 pathology, which is characteristic for ALS patients.

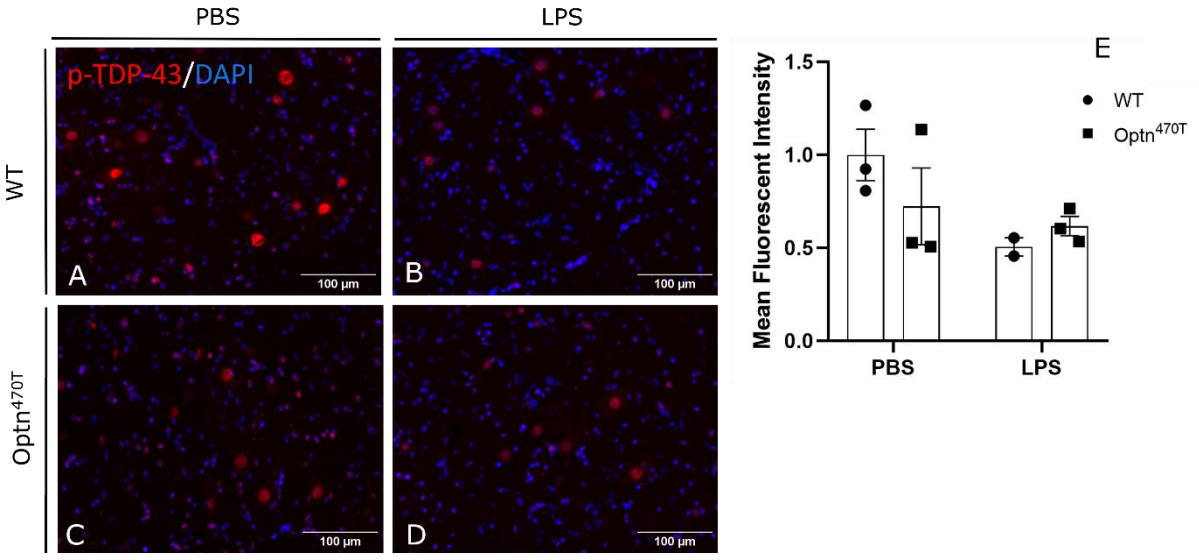

Figure 6. Optn^{470T} mice do not show p-TDP-43 mislocalisation and aggregation upon acute LPS stimulation. Male 7-9-week-old WT and Optn^{470T} mice were stimulated with LPS (5 mg/kg) or PBS (control) for 24 h. Spinal cord sections were stained for p-TDP-43 (Fig. 9 A-D). MFI was assessed with ImageJ software (Fig. 9 E). Cell nuclei were distinguished by DAPI positivity. The data are presented as mean ± SEM from 2-3 WT and Optn^{470T} mice (Two-way ANOVA).

5. Discussion

Prior to this study, Markovinovic et al. did not detect increased neuroinflammatory pathology in the brains of young adult male Optn^{470T} mice compared to WT mice at steady state or upon acute LPS challenge *in vivo* (32). These findings are in line with the reports which also documented an absence of ALS-like pathology in the cortices of young mSOD1 transgenic mice (37). These mice represent the classical models of ALS since they replicate an early spinal onset of disease, characterised by motor neuron loss (6). Moreover, the mSOD1 mouse is a relevant model for observing inflammation in ALS given that microglia in the brain and SC exhibit hyperinflammation following acute LPS challenge (38,39). To inspect if young adult Optn^{470T} male mice would present a similar phenotype in the ventral horns of the LSC, we resumed the study of neuroinflammatory pathology initiated by Markovinovic et al. However, our findings in the LSC were consistent with those of Markovinovic et al. in the motor cortices. We observed that optineurin deficiency by itself does not alter microglial activation in the LSC. Furthermore, although we detected an expected increase in microglial cell body area and process shortening upon LPS challenge, we were unable to identify differences in Optn^{470T} microglial activation compared to their WT counterparts. The discrepancies between Optn^{470T} microglia and mSOD1 microglia may be attributed to the earlier onset of neurodegeneration in the mSOD1 mice.

As an additional microglial activation parameter, we measured the MFI of the microglial activation marker Iba1. The staining intensity of Iba1 was formerly reported to increase with microglial activation and ALS disease progression (40). However, this was not the case in our study as no significant MFI changes were present when comparing LPS and PBS treatments, including comparisons between both genotypes. Although this was not statistically significant, the trends showed a small decrease for the LPS conditions. These observations can be attributed to many factors. First, Iba1 staining appears to be somewhat stronger in WT mice suggesting that

there might have been an artefact present in the test system during immunofluorescence. This is of note since all the result were normalised to WT PBS mice. Second possibility is that the storage time of tissues was too long since we used tissues stored by Markovinovic et. al in 2018. Long storage time was reported to decrease immunoreactivity (41). To confirm these results the experiment would have to be repeated. Unfortunately, we were unable to do so for this thesis due to the limited tissue availability and time constraints. In the future experiments the tissues should be stained for multiple microglial markers to improve the assessment of their activation. It is imprecise to label the microglia as activated based on the usage of only one marker. Microglial induction is a graded process, which consists of different morphologies and varied molecular expression (42). In addition to Iba1, some of the other notable markers that can be used to characterise microglial activation include transmembrane protein 119 (TMEM119), purinergic receptor P2Y G protein-coupled 12 (P2YG12), sialic acid binding Ig-like lectin (Siglec-H) and major histocompatibility complex class II (MHC II) (43,44). TMEM119 and P2YG12 show decreased expression upon microglial induction, whereas MHC II and Siglec-H are upregulated. Comparison of two markers of opposite expression (e.g. TMEM119 and Iba1), enables a more reliable distinction between resting (i.e. unaffected) and activated microglia. Moreover, cytokine profiling of microglia might be more relevant for characterising their activation phenotype as cytokine production precedes morphological changes (45). Markovinovic et al assessed the levels of proinflammatory cytokine TNF-α *in vitro* to determine the activity of NF-kB pathway (32). Even though TNF-α levels were elevated following LPS stimulation, no differences were observed between WT and Optn^{470T} genotype indicating an absence of hyperinflammation. Overall, the observations regarding microglial activation in the LSC of Optn^{470T} mice show consistency with the findings obtained from the cortices of the Optn^{470T} mice.

Currently, very few studies directly addressed astrocyte pathology in relation to *OPTN* mutations in ALS. Astrocyte activity is traditionally linked to increased expression of GFAP (46). Upregulation of this marker can also be a result of astrocyte involvement in neuroinflammatory processes. In the SC of transgenic GFAP-luciferase mice, astrocytes were reported have an increased GFAP production after being subjected to systemic LPS after 24 hours (47). As such, we assessed if astrocytes of Optn^{470T} mice would present any variations in activation for stimulated and unstimulated conditions compared to their WT counterparts. Interestingly, by measuring the MFI of GFAP, we detected an absence of astrocytosis upon LPS stimulation for both WT and Optn^{470T} mice. In addition, we observed a significant decrease in astrocytosis for WT mice when compared to PBS control. Moreover, a decreasing trend in stimulated Optn^{470T} mice compared to unstimulated was present, however unsignificant. Although GFAP has traditionally been used to mark astrocyte activation, newer findings have found that it is not an accurate marker (48). Similar to our results, in one mSOD1 astrocyte-neuron co-culture study established from the SC of mSOD1 transgenic mice, the expression of GFAP in astrocytes was reported to be decreased (49). They proposed that the decrease in GFAP levels might have been a consequence of S100 calcium-binding protein B (S100B) upregulation which affects the assembly of GFAP monomers. These astrocytes were marked as aberrant, and their presence was further confirmed on the SC tissues of mSOD1 transgenic mice. Additionally, a study conducted on human stem cell-derived retinal ganglion cells carrying a mutation implicated in glaucoma, E50K *OPTN*, also demonstrated decreased GFAP levels (50). Curiously, there was also a report of no significant changes in GFAP upon systemic LPS administration in mice (45). Based on these findings GFAP is potentially unable to label all reactive astrocytes. As in microglia, the usage of other astrocyte activation markers such as S100B, as well as cytokine profiling would be more preferential in determining their activity. Nevertheless, this does not exclude the possibility of artefacts in the test system and other potential problems related to tissue

age. Astrocyte activity is yet to be assessed on brain cortices of young Optn^{470T} mice *in vivo* as this was outside the scope of research conducted by Markovinovic et al.

TDP-43 pathology is a hallmark of ALS, as most patients are positive for p-TDP-43 cytoplasmic aggregates, which is coupled to nuclear depletion of TDP-43 (7,8). Moreover, TDP-43 has been reported to aggregate in the CNS tissue of deceased patients carrying E478G and Q398X *OPTN* mutations (25,36). In our study, the LSC tissues of young Optn^{470T} mice were negative for p-TDP-43 cytoplasmic inclusions in both LPS-stimulated and unstimulated conditions. We also report a decreasing trend for TDP-43 nuclear depletion in stimulated Optn^{470T} mice. However, the decrease was unsignificant and the results might not be reliable given the high mouse-to-mouse variability. These observations were not in line with the results of Kurashige et al. They utilised older 8-to-24-month-old Optn$^{-/-}$ mice and detected mislocalisation and cytoplasmic aggregation of p-TDP-43 (35). Although this is an interesting finding and could argue that this model phenocopies some of the ALS features, it is of note that the study of Kurashige et al. also had high mouse-mouse variability and low mouse numbers. Notably, our discrepancies with this study could have been attributed to the difference in mice age and the usage of a different p-TDP-43 antibody. However, the recent results from our laboratory, in which we used the same antibodies as Kurashige et al. on a larger cohort of mice, strongly argue that there is no TDP-43 pathology in unmanipulated Optn^{470T} mice at one and two years of age (unpublished results from our laboratory (Mohovic and Peradinovic et al.), allowing for the possibility that Optn$^{-/-}$ and Optn^{470T} mice are not equal or that study by Kurashige et al. was not statistically powered to provide reliable conclusions.

Since we did not observe hyperinflammation or cytoplasmic TDP-43 aggregates in the LCS of Optn^{470T} at baseline conditions and upon LPS stimulation, it was no surprise that the total neuron numbers and motor neuron numbers of Optn^{470T} mice remained preserved. This resembles the

results obtained from analysing the brain cortices of Optn^{470T} mice, which also demonstrated no changes in neuronal counts (Markovinovic et al., unpublished). However, the research conducted on brain cortices could not have reported specific motor neuron losses given that the available markers are unable to target motor neurons in the brain. Other optineurin deficiency models, such as Optn$^{-/-}$, also showed retained neuron numbers in the brain and SC of young mice, which aligned with our findings in young Optn^{470T} mice (34). In contrast, Kurashige et al., who demonstrated TDP-43 pathology in Optn$^{-/-}$ mice, additionally reported a significant reduction of motor neurons in the LSC, although with no motoric symptoms (35). A follow up study should be performed to address this issue since other optineurin mouse models exhibited minimal to no neurological impairments during ageing (31,34). This may initially indicate that Optn^{470T} mice might have a slower onset ALS pathogenesis due to the absence of microgliosis and astrocytosis in the CNS tissue of young Optn^{470T} mice. However, recently, unstimulated one-to-two-year-old Optn^{470T} mice underwent an in-depth neuropathological investigation of the brain cortices and the LSC (unpublished results from our laboratory (Mohovic and Peradinovic et al.)). Similar to our unstimulated young Optn^{470T} mice, the old unstimulated Optn^{470T} mice did not demonstrate abnormal glial activation, motor neuron losses or motoric symptoms. The authors suggested that optineurin deficiency and ageing are insufficient to elicit ALS-like pathology.

Overall, optineurin deficiency itself does not provoke a hyperinflammatory response in the LSC of young Optn^{470T} mice, nor does it exaggerate it after being subjected to LPS. Our results align with the hypothesis that optineurin contributes to disease by a loss of function mechanism given that young Optn^{470T} mice did not suffer neurodegeneration and are negative for toxic cytoplasmic aggregates. Nevertheless, optineurin deficiency model studies are yet to address whether the neurotoxic properties of mutated optineurin can somehow be induced. The deleterious effects can potentially be triggered by subjecting the optineurin mouse models to a long-term

stimulus, such as chronic infection. Another possibility is that the optineurin deficient mice should carry mutations in other ALS genes to manifest the disease phenotype.

6. Conclusion

In conclusion, our study aimed to investigate the potential neuroinflammatory pathology in the LSC of young adult male Optn^{470T} mice. Our findings were consistent with the previous research conducted on Optn^{470T} mice of all ages. We report an absence of hyperinflammation, i.e. no increase in microgliosis and astrocytosis, in Optn^{470T} compared to WT mice in both LPS-stimulated and unstimulated conditions. We further propose that the usage of multiple activation markers and cytokine profiling would provide more comprehensive information regarding the glial cell activation phenotypes. Additionaly, we did not observe TDP-43 pathology in the LSC of Optn^{470T} mice as the tissues were not positive for cytoplasmic p-TDP-43 inclusions. This demonstrated the discrepancy with the previous study conducted in Optn$^{-/-}$ mice. However, the findings from the latter study do not align with the unpublished data from our laboratory, in which an absence of TDP-43 pathology in optineurin deficient mice was demonstrated. We also report no total neuron or motor neuron loss in the LSC of Optn^{470T} and WT mice for both LPS and PBS conditions.

The limitations of our study include tissue storage time, tissue availability, the potential artifacts in the test system that occurred during immunofluorescence, and the usage of only one marker to assess glial cell activation. Due to these difficulties, a follow up study on fresh tissues will have to be performed, which will further evaluate the results reported here.

Overall, our study provides information regarding the neuroinflammatory response and degenerative processes following LPS stimulation, associated with optineurin deficiency in ALS. We propose the need for research which would inspect the consequences of exposure of optineurin deficient mice to a long-term chronic stimulus and/or generation of optineurin deficient models which carry an additional ALS-linked mutation. These types of studies would possibly provoke the optineurin-mediated mechanisms of disease.

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EDUCATION AND TRAINING

10/2020 – CURRENT Rijeka, Croatia
BACHELOR OF BIOTECHNOLOGY AND DRUG RESEARCH Department of biotechnology

09/2016 – 07/2020 Bjelovar, Croatia
STUDENT Language Gymnasium, Secondary School of Languages

LANGUAGE SKILLS

Mother tongue(s): **CROATIAN**

Other language(s):

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken production	Spoken interaction	
ENGLISH	C1	C1	C1	C1	C1
GERMAN	A2	A2	A2	A2	A2
JAPANESE	A2	A2	A2	A2	A2

Levels: A1 and A2: Basic user; B1 and B2: Independent user; C1 and C2: Proficient user

ADDITIONAL INFORMATION

ORGANISATIONAL SKILLS

Symposiums & Scientific events Organizing committee, support crew, Neuroart exhibition welcome speech

Project „Reconnect Science with the Blue Society" - activity "European Reasearcher's Night" Organization & presentation

INTERNSHIP

08/2021
Understanding the (eco) toxicological role of selected SLC uptake and MATE efflux transporters in zebrafish using functional genomics tools - DANIOTRANS (IP-2019-04-1147; PI: dr.sc. Tvrtko Smital)

Institute for Medical Research and Occupational Health, Molecular Toxicology Unite the description...

08/2018
Re-programmed; Transgenic Cells

AbbVie Deutschland GmbH & Co. KG

CONFERENCES AND SEMINARS

30/06/2022 – 03/07/2022 – Department of Biotechnology, University of Rijeka
Symposium on Inflammation and Proteinopathy in ALS/FTD Spectrum Disorder

VOLUNTEERING

2021 – CURRENT University of Rijeka, Department of biotechnology, UsbRi, Croatia
Activity "Student-Mentor" Support and guidance for freshmen

17/04/2021 University of Rijeka, Department of biotechnology, UsbRi, Croatia
Activity "Traveling Scientists Demonstration, explanation of various experiments to kindergarteners

HONOURS AND AWARDS

2022
State scholarships for students in STEM academic disciplines – Croatian Government, Ministry od education

2023
Scholarship for Excellence – University of Rijeka

HOBBIES AND INTERESTS

Drawing

Table tennis

Theatre

DRIVING LICENCE

Driving Licence: B