Modelling the schizophrenia-related aggregation of NPAS3 and CRMP1 in neuroblastoma cells

Samardžija, Bobana

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SVEUČILIŠTE U RIJECI

ODJEL ZA BIOTEHNOLOGIJU

Diplomski sveučilišni studij

„Istraživanje i razvoj lijekova"

Bobana Samardžija

Modeliranje agregacije proteina NPAS3 i CRMP1 povezanih sa šizofrenijom
u stanicama neuroblastoma

Diplomski rad

Rijeka, 2020.

Mentor: dr. sc. Nicholas Bradshaw

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In front of the Committee:

1. Assistant Prof. Jelena Ban

2. Assistant Prof. Željko Svedružić

3. Assistant Prof. Nicholas Bradshaw

This thesis has 48 pages, 12 figures, 7 tables and 56 citations.

Abstract

Chronic mental illness affects millions of people every year, while its origins and mechanisms remain unclear. Since symptoms of neurodegenerative and mental disorders are similar, our research focuses on protein aggregation. Protein aggregation changes the structure of protein and can cause loss of function or gain of a novel toxic function. There are several proteins characterized as aggregating in mental illnesses, of which we focused on Collapsin Response Mediator Protein 1 (CRMP1) and Neuronal PAS Domain Protein 3 (NPAS3). We expressed variants of mentioned proteins in neuroblastoma cell line and assessed them by immunofluorescence.

CRMP1 has been detected as insoluble in brains of mental disorder patients and seen to aggregate in cell culture. In our research we wanted to confirm aggregation of the long and short CRMP1 variants. The long variant is seen to aggregate alone, while short variant is only known to co-aggregate with other proteins. Since the long variant od CRMP1 has a longer N-terminal section unlike the short variant, we investigated its truncation too. However, we did not see aggregation of long and short CRMP1 variants when expressed alone, nor the effect of N-terminal truncations. Thus, we believe CRMP1 has greater tendency to co-aggregate with other proteins and the following research should explore it in more detail.

Meanwhile, genetic studies have implicated a mutated version of NPAS3 in mental disorders. Also, mutant NPAS3 has been seen as insoluble in cell culture. We examined aggregation of full-length wild type and mutated NPAS3 variants, along with the major NPAS3 structural regions. We observed that mutated NPAS3 does not enter the nucleus and therefore cannot perform its normal function as transcription factor. Interestingly, we have seen that the bHLH1 region of NPAS3 can aggregate by itself and is stabilised by presence of the PAS region. Our results confirmed that NPAS3 aggregation is a consequence of mutation, but there could be other mechanisms which include disruption of bHLH1 region.

Next steps should include further investigation of bHLH1 and PAS regions of NPAS3, with regards to mutation and translocation. Finally, this and similar research could result in model of aggregation for both proteins, which could be translated to other proteins implicated in mental illness.

Key words: protein aggregation, NPAS3, CRMP1, mental illness, mental disorders

Sažetak

Milijuni ljudi obolijevaju od kronične mentalne bolesti godišnje, dok su uzorci i mehanizmi još uvijek nepoznati. Simptomi neurodegenerativnih i mentalnih poremećaja su slični, zbog čega se naše istraživanje temelji na agregaciji proteina. Agregacija proteina mjenja strukturu proteina te može dovesti do gubitka funkcije ili stjecanja nove toksične funkcije proteina. Postoji nekoliko proteina čija je agregacija opisana u mentalnim poremećajima, a u ovom radu istražili smo protein posrednik odgovora kolapsina 1 (eng. Collapsin Response Mediator Protein 1 ili CRMP1) i neuronski protein s PAS domenom 3 (eng. Neuronal PAS Domain Protein 3 ili NPAS3). Eksprimirali smo verzije spomenutih proteina u staničnoj liniji neuroblastoma i analizirali ih putem immunofluorescencije.

U mozgovima pacijenata s mentalnim poremećajima, CRMP1 je detektiran u netopljivoj frakciji te je utvrđeno da agregira u staničnoj kulturi. U našem istraživanju željeli smo potvrditi agregaciju duge i kratke verzije CRMP1. Do sada je viđeno kako duga verzija može sama agregirati, dok kratka verzija agregira u prisutnosti drugih proteina. Duga verzija se razlikuje od kratke verzije po svom N-terminalnom dijelu, zbog čega smo dodatno istražili i skraćene verzije. Međutim, nismo vidjeli agregaciju duge ni kratke verzije kao ni utjecaj N-terminalog dijela. Shodno tome, vjerujemo kako CRMP1 ima veću sklonost prema ko-agregaciji s drugim proteinima, što je potrebno detaljnije istražiti u budućnosti.

Međutim, genetska istraživanja su potvrdila prisutnost mutirane verzije proteina NPAS3 u mentalnim poremećajima te je dokazana njegova netopljivost u staničnoj kulturi. Istražili smo agregaciju divlje i mutirane verzije NPAS3 te agregaciju glavnih regija proteina NPAS3. Primijetili smo kako mutirana verzija proteina NPAS3 ne ulazi u jezgru, zbog čega ne može djelovati kao transkripcijski faktor. Zanimljivo je kako smo vidjeli da bHLH1 regija može samostalno agregirati te je stabilizirana prisustvom PAS regije. Naši rezultati su potvrdili kako je agregacija NPAS3 posljedica mutacije, no

moguće je da postoje i drugi mehanizmi agregacije koji uključuju ometanje bHLH1 regije. Slijedeći koraci bi trebali uključivati daljnje istraživanje bHLH1 i PAS regija, uzimajući u obzir mutaciju i translokaciju povezanu s proteinom NPAS3. U konačnici, slična istraživanja mogu dovesti do modela agregacije, koji bi se mogao primijeniti i na druge proteine implicirane u mentalnim poremećajima.

Ključne riječi: agregacija proteina, NPAS3, CRMP1, mentalne bolesti, mentalni poremećaji

Acknowledgements

In this part of thesis, I would like to take time to appreciate my mentor, dr.sc. Nicholas Bradshaw, for giving me a warm welcome to the lab. His enormous patience allowed him to answer all my questions without hesitation, no matter how repetitive or simple they have been. In past year, he continued to be supportive and to believe in my work, even when I did not. Working in his lab and writing this thesis allowed me to acquire new skills, but more important – it filled me up with optimism for future research. Many thanks to Fred Berry, at University of Alberta for providing me with NPAS3 constructs used in this research. Secondly, I would like to thank my fellow lab mates: Giovanna, Ines, and Maja, for all of their support. Enormous thanks go to my lab partner Beti, for lending me a hand (and a voice) in long lab hours.

Alongside with her, there were my friends Ana, Ivan, Iris, Jerko, Marta, Matea R., Mateja and Patrik, who helped me stay sane during most stressful periods. My research and whole education at university would never be as wonderful as it was without my dear colleagues and friends Danijel, Vladimir, Filip, Katarina, Karla, Ivona and Sanja. You were like a family during past six years and I am proud of how much we all grown together as well as excited to see where our paths lead us. In addition, I would like to thank my dear friends Josip, Ilijana, Matea, Marta, Sara, Lucija, Marina, Lana, Josipa, Katarina, Mirjana, Maja and Lucija.

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And I devote this paper to my sister Vedrana – she still is and forever will be the best thing that happened to me.

Table of Contents

LIST OF ABBREVIATIONS

aa	Amino acids
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
BD	Bipolar disorder
bHLH1	Basic helix loop helix
CMI	Chronic mental illness
CRMP1	Collapsin response mediator protein 1
CRMP1 Lv	Long variant of CRMP1
CRMP1 Sv	Short variant of CRMP1
FTD	Frontotemporal dementia
HD	Huntington's disease
MDD	Major depressive disorder
NPAS3	Neuronal PAS domain protein 3
NDs	Neurodegenerative diseases
PAS	Per-Arnt-Sim
PD	Parkinson's disease
SZ	Schizophrenia
TAD	Topologically associating domain

1 Introduction

A chronic mental illness (CMI) is any disorder that affects an individual's emotions and thoughts, decreasing their life quality and physical well-being. Examples of the most severe CMIs are major depressive disorder, schizophrenia, and bipolar disorder.

1.1 Major depressive disorder

Major depressive disorder (MDD), commonly known as depression, is a mood disorder with severe consequences on the everyday life of an individual. Episodes of depression include feelings of hopelessness and worthlessness, lack of motivation and energy, as well as sleep and appetite. These episodes ultimately lead to thoughts of self-harm and, in some cases, suicide. Suicide was one of 20 leading causes of death in 2015 with 788,000 victims (1.5% of deaths worldwide)[1].

Depression can affect patients in all age groups and now represents one of the leading causes of disability[2]. It is estimated that 4.4% of the global population (322 million people) suffers from depression, which is more prevalent in the female (5.1%) than the male (3.6%) population[1]. According to data from European Health Interview Survey (EHIS), carried out between 2014-2015, around 10% of population in Croatia has symptoms of depression[3]. Due to the complex genetic background, it is hard to identify a single cause of this illness. The heritability is estimated at 38%, based on twin studies[4]. Depression often begins in adulthood and can co-occur with other serious illnesses (such as cancer, diabetes, Parkinson diseases, eating disorders, HIV/AIDS). All this leads to worsening patients' life conditions, as well as complicating diagnostics and treatment[4]. Depression can be treated with medications (antidepressants) and psychosocial therapy, both separately and in combination. It is important to highlight that therapy is not always successful – 10-30 % of patients do not or poorly respond to therapy[5].

1.2 Schizophrenia

Schizophrenia (SZ) is a complex neuropsychiatric disorder, which includes a variety of symptoms, roughly divided in three groups: positive, negative and cognitive symptoms. Positive symptoms (novel changes which lead to the development of more severe symptoms) include hallucinations and delusions, which occur in the early stage of disease. Negative symptoms (previously observed behaviours that are now absent) include antisocial behaviour, disorganized speech and lack of motivation. The third group consists of cognitive symptoms such as poor memory, disorganized thinking and concentration difficulties. The symptoms of SZ typically emerge between the late teenage and early adult phases of life. For a disorder to be classified as SZ, symptoms must last for at least six months, with one month of active positive and negative symptoms.

The lifetime prevalence of schizophrenia appears to be approximately 0.3%-0.7%, with variations depending on population and ethnicity, while maintaining equivalent risks for both males and females[7]. All these symptoms complicate patients' quality of living, as employment and social contacts are endangered. Around 19% of patients diagnosed with SZ were in paid employment compared to average employment rate in the general population of 80%[8,9]. The rate of reported loneliness by patients with SZ and other psychotic disorders is two times higher than in the general population, highlighting loneliness as a major everyday challenge[10].

The low quality of life in SZ patients has been shown to increase suicide risk: approximately 5% of individuals with SZ die by committing suicide, while approximately 20% admit to attempting suicide on one or more occasions[11]. In addition, over half of patients with SZ have substance-related disorders (mostly daily smoking of cigarettes)[12]. Medical conditions such as diabetes and metabolic syndrome, as well as cardiovascular and pulmonary disease are more common in SZ patients than in the general population[13]. A cohort study done in Denmark showed how both SZ and BD reduce life expectancy (16-18 years shorter life expectancy in SZ patients

and 12-13 years for BD patients, compared to general population)[14]. The aetiology of SZ is still unclear, but there are some known genetic and environmental factors that increase the risk of the SZ. Genetic studies, such as twin studies carried out in Sweden and Denmark, identified the chance of inheriting SZ as around 79%[15]. As for the environmental factors, extreme stress (such as post-traumatic stress disorder - PTSD or famine), birth complications, life in urban residence and migrant status have been singled out as the most influential[16].

1.3 Bipolar disorder

Bipolar disorder (BD) is described as alternating episodes of depression and maniacal behaviour with highly elevated mood. In manic episodes, a patient is impulsive and energetic, while, in depressive episodes, the patient loses all motivation and interest for normal life activities, followed by thoughts about self-harm or suicide[17]. BD is usually diagnosed in late adolescence. As in SZ, there is no single cause of disease; both genetic and environmental factors contribute to the development of this disorder. Genetic studies based on twins and families have shown around 80% heritability, while childhood trauma surfaced as the most common environmental factor[18,19].

1.4 Challenges in the diagnosis and treatment of chronic mental illness

In 2019, the World Health Organisation (WHO) estimated that one in four people in the world will experience a mental disorder at some point of their lives[19]. In Croatia, mental disorders are the second most common cause of hospitalization in adults. Each year, Croatia spends around 160 million kuna on depression treatment and anti-depressant drugs[2]. Not only are mental illnesses affecting more and more people each day, they stay with the patient for the rest of their life. Busy lifestyles also carry a lot of daily stress,

which contributes to the development of mental illness. Meanwhile, current technologies in diagnostics and treatment of chronic mental illness are lacking. Not only is the diagnosis of mental illness solely based on standardized psychiatric evaluations, with no biological test yet available[6], but the current treatment of mental illnesses is also insufficient. Since symptoms of mental illness largely depend on patient's description of feelings, thoughts, and mood, it is hard to identify how efficient treatment is. When antidepressants and placebo treatment were compared, difference between antidepressant and placebo was present, however the overall efficiency was poor[20]. The efficacy of antidepressants depends on the severity of depression. It was shown that anti-depressant drugs improved symptoms in about 20 out of 100 people[14]. Previous studies indicate that the use of antipsychotics in treatment of acute and chronic SZ results with a lower risk of death (compared to no use)[23]. However, each patient responds differently to antipsychotics, while the risk for adverse reactions and treatment resistance grows with length of antipsychotic therapy[24].

So far, research has not offered a specific single cause or mechanism for disease development. Genetic studies offer some insight into the background of each disease, but most of the identified genes and variants overlap within the chronic mental illnesses group or with other neurological and psychiatric illnesses[24]. Biological studies are still in minority compared to genetic studies, although some are showing promising results. One of the emerging fields of research is protein aggregation in mental illnesses.

1.5 Protein aggregation

In order to have a fully functional protein, its structure must be preserved. Protein homeostasis (proteostasis) includes the production, folding, trafficking and degradation of proteins, all of which are tightly regulated under normal conditions. Formation of protein aggregates permanently changes the structure of a protein, which causes either a loss of function or a gain of a new, toxic function.

Loss of protein function can be a consequence of mutations, which causes protein instability and increase its propensity to aggregation[23].

Aggregates that acquire a new and toxic function can lead to the development of neurodegenerative diseases (NDs). Prominent examples of these are Alzheimer's, Parkinson's, and Huntington's disease, as well as amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). These diseases are caused by accumulation of different insoluble proteins, including Aβ peptide and tau protein in Alzheimer's disease, α-synuclein in Parkinson's disease, huntingtin in Huntington's disease and TAR DNA-binding protein 43 (TDP-43) in ALS and FTD[26]. Possible causes of this accumulation of protein aggregates include disturbance in regulation of cellular mechanisms involved in protein degradation, such as autophagy or degradation in proteasomes[25].

Incorrect proteasomal degradation of proteins has been linked with the aging processes, which is consistent with the larger prevalence of NDs in the older population compared to the younger population[25]. Proteasome dysfunction has also been implicated in SZ[28].

Aggregation of proteins can be observed in both *in vivo* and *in vitro* models. In *in vivo* animal models, proteins can form aggregates as a response to stress from the environment, such as traumatic brain injury. *In vitro* studies show the formation of aggregates under influence of stress factors, such as increased oxidative stress and dopamine[29].

In terms of symptoms, the early stages of NDs are similar to mental disorders (psychosis or SZ). A good example of this is FTD which mostly includes apathy, impulsive and repetitive behaviour – all symptoms of SZ. Therefore, it is possible to conclude that there are some shared elements between CMIs and NDs.

1.6 Proteins connected to aggregation in mental illness

Work done in *post-mortem* human cases, *in vivo* animal work, and *in vitro* analysis provided evidence of aberrant proteostasis. Out of all the candidate proteins misassembled in CMI, five proteins have been characterised so far that seem to be misassembled and/or aggregated in CMI: Disrupted in Schizophrenia 1 (DISC1), dysbindin-1, Trio-Binding Protein 1 (TRIOBP-1), Collapsin Response Mediator Protein 1 (CRMP1) and Neuronal PAS Domain Protein 3 (NPAS3)[29].

1.6.1 Disrupted in Schizophrenia 1 (DISC1)

The *Disrupted in Schizophrenia 1 (DISC1)* gene was first identified as a risk factor for the development of chronic mental illnesses after its discovery in a large Scottish family[30]. Some members of this family suffered from depression and SZ and were shown to be carriers of a balanced translocation between chromosomes 1 and 11, t(1:11), which was later found to disrupt the *DISC1* gene[31,32]. DISC1 is a scaffolding protein involved in numerous cell processes such as the maturation, migration and proliferation of neuronal cells, nuclear localization and transcriptional activity, as well as formation of protein complexes[33]. DISC1 was found in *post-mortem* brain samples of patients with affective disorder or SZ in the form of insoluble protein aggregates, but not in the control group[33]. A full-length human DISC1 protein has been overexpressed in a transgenic rat model and it showed signs of aggregation and displayed changes in dopamine neurotransmission (higher sensitivity to amphetamine and greater exploratory behaviour). Molecular investigations showed an increase in affinity and number of dopamine receptors on plasma membrane, all leading to an increase of dopamine in organism. Further work, this time involving cell lines, showed increased formation of DISC1 aggregates after treatment with dopamine[35].

1.6.2   Dysbindin-1

The *DTNBP1* gene encodes the dysbindin-1 protein, which is expressed in the central nervous system. In genetic studies, *DTNBP1* has been correlated with the cognitive deficits and negative symptoms of SZ[29]. Due to its active interaction with other proteins, dysbindin-1 modulates expression of two classes of neurotransmitter receptors: N-methyl-D-aspartate receptors (NMDARs) and dopaminergic D2 receptors. Dysbindin-1 also has a role in intracellular vesicular trafficking, where it is involved in the formation of lysosome-related organelle complex 1 (BLOC-1)[35]. In humans, dysbindin exists in three isoforms: dysbindin-1a, 1b, and 1c. Dysbindin-1a is the full-length version and is mainly expressed in postsynaptic densities (along with dysbindin-1c). Dysbindin-1b is exclusively found within synaptic vesicles. It is shorter due to the lack of a PEST sequence, which acts as a signal for protein degradation. Dysbindin-1a can co-aggregate with DISC1[34] while dysbindin-1b can aggregate on its own, forming aggregates which are transmissible and toxic [33]. Aggregation of dysbindin-1b can negatively affect BLOC-1 and causes impaired cognition[36]. Increased expression of dysbindin-1b mRNA was demonstrated in patients with SZ as compared to healthy control. In cell systems, increased expression of dysbindin-1b lead to increased formation of aggregomes, which can recruit dysbindin-1a, leading to the disrupted function of dysbindin-1[37].

1.6.3   Trio-binding protein 1 (TRIOBP-1)

Trio-binding protein 1 (TRIOBP-1, also known as Tara) is a TRIO and F-actin-binding protein (TRIOBP) isoform which regulates actin cytoskeletal organization. The proposed structure of TRIOBP-1 includes three separate domains: the N-terminal PH domain, central domain and C-terminal domain[38]. TRIOBP-1 was directly associated with aggregation in SZ. Firstly, the *TRIOBP* gene was implicated as risk factor for SZ when increased expression of transcripts encoding for TRIOBP-1 was found in the

brains of patients[40]. Next, insoluble fractions of brain tissue samples from patients with diagnosed SZ were used to immunize a mouse, thus producing monoclonal antibodies specific for aggregated proteins in the brains of SZ patients, one of which was specific for TRIOBP-1. Aggregation of TRIOBP-1 was confirmed in neuroblastoma cells[39]. Further research of TRIOBP-1 structure showed that its aggregation depends on a specific region of 25 amino acids, found in the central domain[36].

1.6.4   CRMP1

Collapsin response mediator proteins (CRMPs) are a group of the cytosolic phosphoproteins. The CRMPs are highly expressed during brain development, mostly in the embryonic state and the first week after birth. The CRMPs are mostly localised in neurites and axonal growth cones. On a cellular level, CRMPs are involved in apoptosis, cytoskeletal rearrangement and axonal branching[42]. Thus, CRMPs can be related to neuronal differentiation and axonal outgrowth[43]. Of the five CRMPs, CRMP1 and CRMP2 are the most known. CRMP2 was the first identified and it is more expressed in adult brain than other CRMPs[43]. CRMP2 has an important role in signal transduction as a mediator of the signalling molecule semaphorine 3A (Sema3A)[44].

Its role in CMI was indicated when decreased expression of CRMP2 were reported in the frontal cortex of *post-mortem* brains of patients diagnosed with SZ, BD or depression[45]. CRMP1 is one of the proteins connected to CMI in a hypothesis free assay due to its insolubility. Aggregomes of brain samples from patients diagnosed with SZ were used to produce monoclonal antibodies, that showed specificity to the aggregomes of SZ patients, compared to controls. One of these antibodies was specific for CRMP1[46]. Subsequent research verified the existence of CRMP1 in aggregomes of brain samples from patients with diagnosed SZ and BD, but not in brain samples of patients diagnosed with major depression or a control group[46].

CRMP1 exists in two variants: short (CRMP1 Sv) and long (CRMP1 Lv), with the short variant being more prevalent in the brain[46]. CRMP1 Sv by itself was not seen to aggregate in cell culture, unlike the longer variant[47]. However, aggregation of CRMP1 Sv has been detected when co-expressed with DISC1[46]. A similar result was seen when CRMP1 was co-expressed with huntingtin, leading to conclusion that CRMP1 may help with the aggregation of other proteins[48].
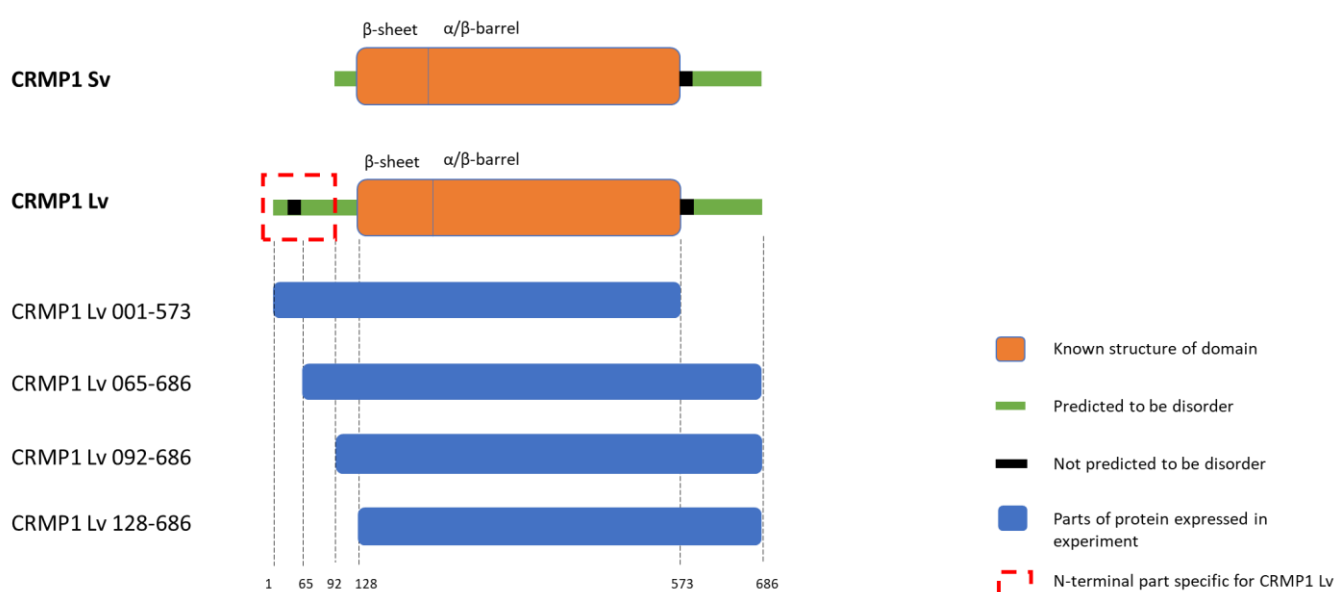

Figure 1 Diagram of CRMP1 structure for short (CRMP1 Sv) and long (CRMP1 Lv) variant, adapted from Bradshaw and Korth[29]. The regions of CRMP1 expressed in this thesis are shown (in blue).
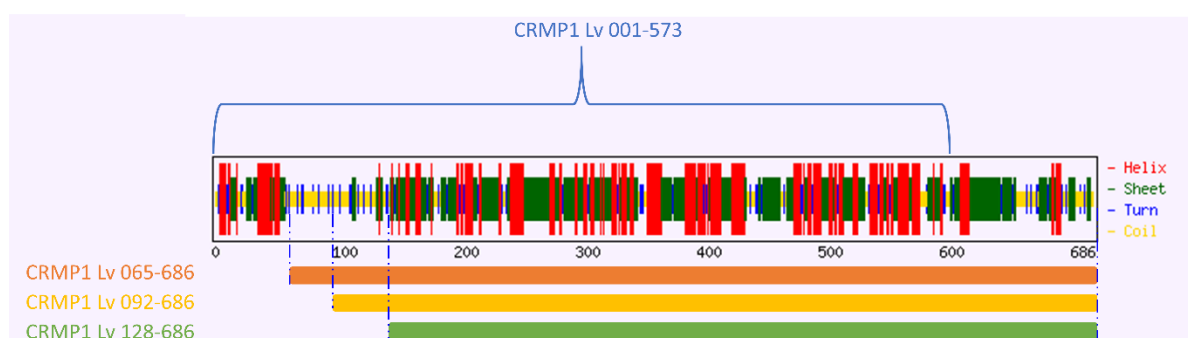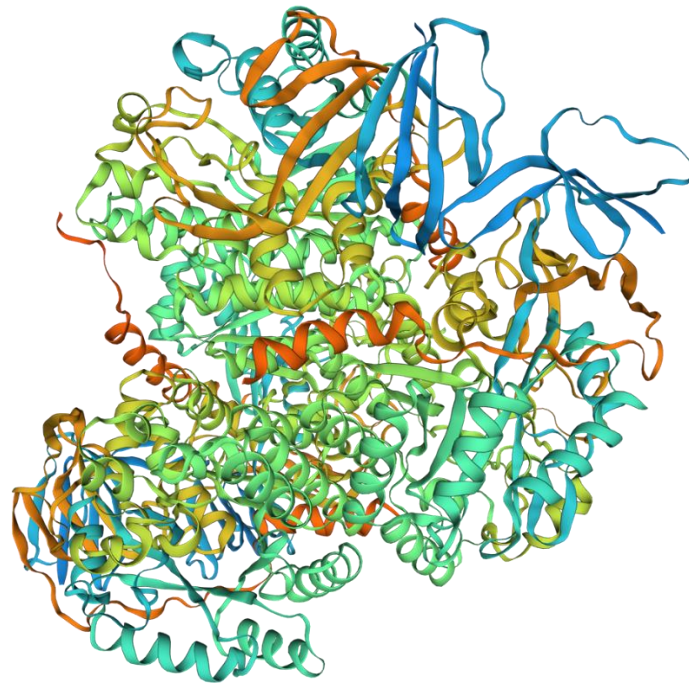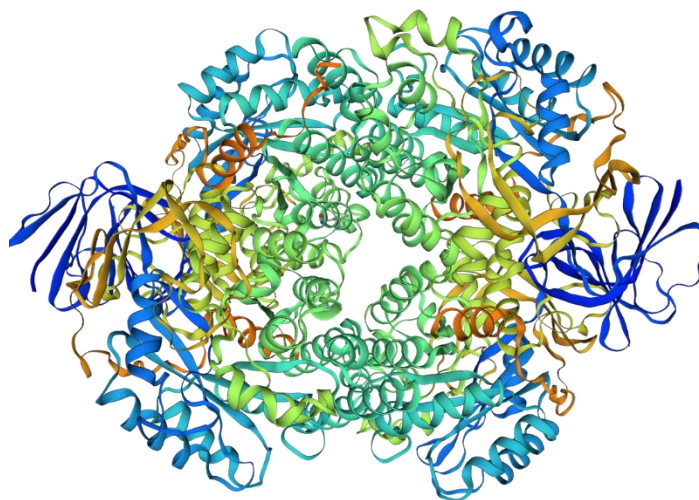

Figure 2 Diagram of CRMP1 secondary structure for CRMP1 Lv with the indicated regions of CRMP1 Lv expressed in this thesis. Diagram obtained by program CFSSP: Chou & Fasman Secondary Structure Prediction Server

Predictions for CRMP1 Lv sequence from – to :

1- 70 aa	211-260 aa	421-490 aa	631-686 aa
71-140 aa	261-350 aa	491-560 aa	
141-210 aa	351-420 aa	561-630 aa	

Figure 3 Secondary structure of CRMP1 Sv, predicted based on amino sequence in program Swiss Model ExPasy

Predictions for CRMP1 Sv sequence from – to :

1- 70 aa	211-260 aa	421-490 aa
71-140 aa	261-350 aa	491-560 aa
141-210 aa	351-420 aa	561-572 aa

Figure 4 Secondary structure of CRMP1 Sv, predicted based on amino sequence in program Swiss Model ExPasy

1.6.5　NPAS3

Neuronal PAS domain protein 3 (NPAS3) is a transcription factor mostly expressed in the brain. NPAS3 is involved in regulation of neurogenesis, metabolism, and circadian rhythms[29]. NPAS3 consists of three main subunits: bHLH (basic helix-loop-helix), two PAS (Period, Aryl hydrocarbon receptor, Single minded) domains (PAS-A and PAS-B) and TAD (transcriptional activation domain)[48]. bHLH is protein structural motif which is quite common among transcription factor and it is involved in binding to particular sequences in promoters of target genes. The PAS region (containing two PAS domains, PAS A and PAS B) regulates NPAS3 interaction ability[48]. One of the heterodimeric partners of NPAS3 is Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT), and the interaction between ARNT and NPAS3 is highly influenced by PAS A domain, while PAS B domain regulates normal gene regulatory function, ligand binding and protein-protein interaction with chaperone proteins[48, 49].
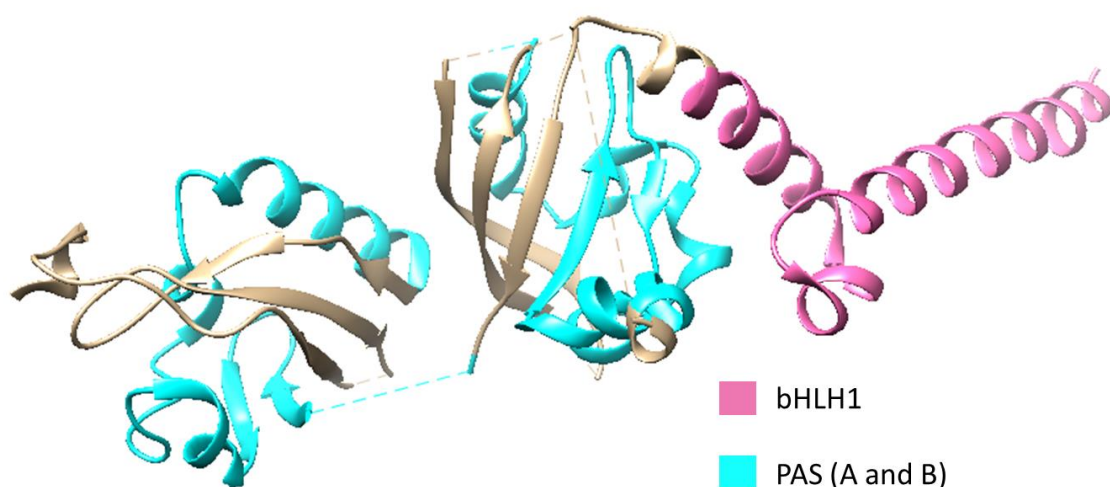

Figure 5 3D model of crystal structure of the bHLH and PAS domains of mouse NPAS3 with marked regions (see legend). Image is derived from PDB structure 5SY7, solved by Wu et al (2016) [49] and analysed in Chimera

The involvement of NPAS3 in SZ was first recognized after the discovery of a translocation in mother and daughter, both diagnosed with SZ[51]. The translocation may result in lack of NPAS3, or production of a truncated version of NPAS3 protein without its TAD and PAS domains, crucial for normal function.

Further research on lack of NPAS3 protein in knockout mice showed abnormal behaviour: increased locomotor activity and reduced recognition memory[51]. Other studies showed specific haplotypes of NPAS3 that increase the risk of SZ and BD[52], while efficacy of antipsychotic drugs was associated with single nucleotide polymorphism in NPAS3 through whole genome association study done on patients with SZ[53].

Genome-wide association studies have associated the gene *NPAS3* with both BD and SZ[54]. The V304I mutation in NPAS3 was found in a small family with diagnosed SZ[55]. Mutation V304I is in the PAS region of NPAS3 protein, between PAS-A and PAS-B, and is likely related to DNA binding capacity and protein stability. Mutated NPAS3 V304I was shown to be insoluble using *in vitro* systems as a purified recombinant protein, or in mammalian cell lines when over-expressed[56]. This implies it to be aggregating, although this has not yet been confirmed by microscopy.
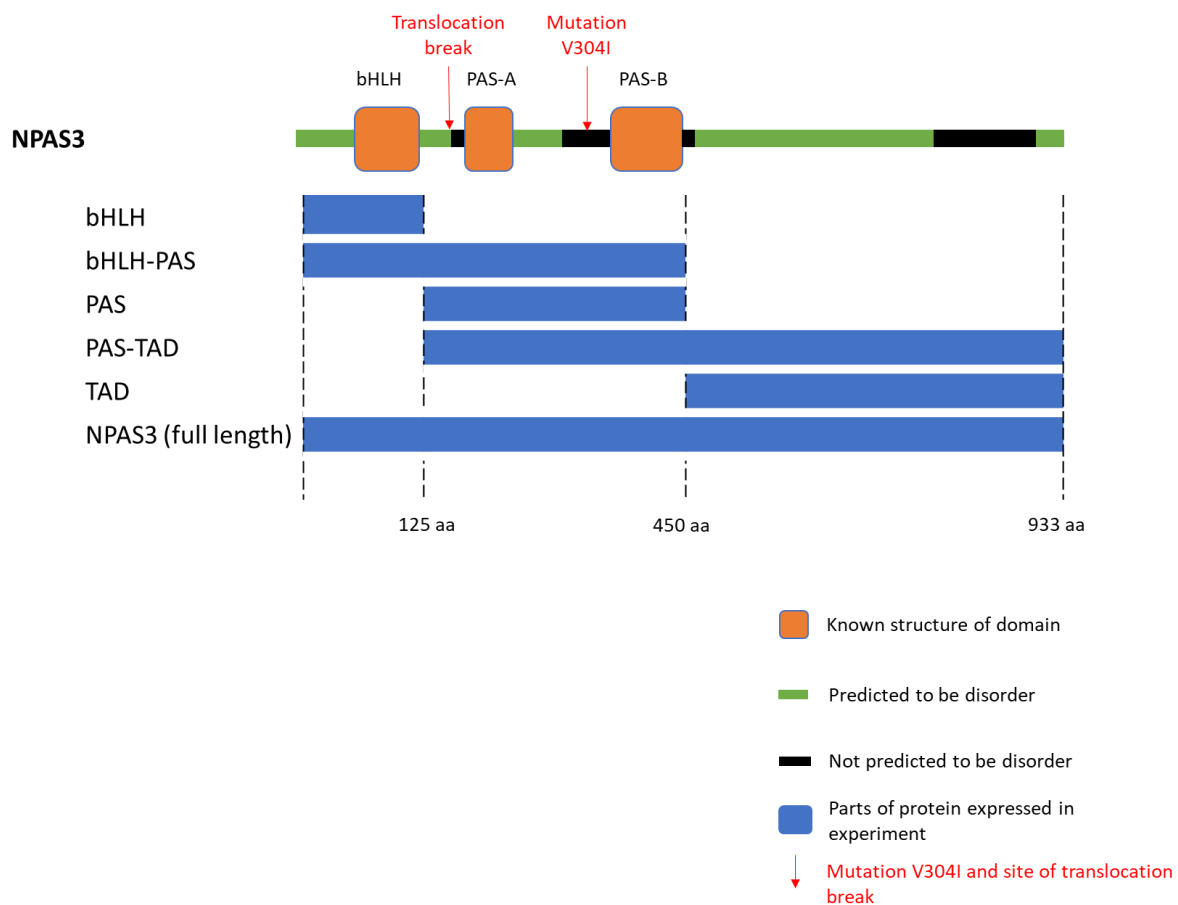

Figure 6 Diagram of structure for NPAS3 constructs, adapted from Bradshaw and Korth[29]. The regions of NPAS3 expressed in this thesis are shown (in blue).

2 Aim of thesis

Five proteins have been characterised so far that seem to be misassembled and/or aggregated in CMI: DISC1, dysbindin-1, TRIOBP-1, CRMP1 and NPAS3. In this thesis, the main focus is on CRMP1 and NPAS3. Both proteins are highly expressed in the brain and are involved in neurogenesis and neuronal differentiation. CRMP1 was found in the insoluble fraction of proteins in *post-mortem* brain samples of patients diagnosed with SZ and BD[47]. CRMP1 exists in two splice variants: short (CRMP1 Sv) and long (CRMP1 Lv) and they both have been seen to aggregate in cell culture by immunofluorescence. The CRMP1 Lv has been seen to aggregate when expressed alone, while the CRMP1 Sv showed potential to aggregate in cell culture when co-expressed with DISC1 and huntingtin[47]. On the other hand, NPAS3 was implicated in chronic mental illnesses primarily by genetic studies. The mutated NPAS3 V304I showed potential to aggregate *in vitro* as a purified recombinant protein, or in mammalian cell lines when over-expressed, but aggregation was not confirmed by immunofluorescence[56].

On these grounds, the aims of this thesis are:

1. To confirm whether CRMP1 Lv can aggregate by itself in neuroblastoma cell line, as seen in previous studies,

3. To investigate if CRMP1 Sv can aggregate by itself in neuroblastoma cell line,

4. To determine whether the N-terminal section of CRMP1 Lv, which is absent in CRMP1 Sv, is required for its aggregation,

5. To investigate whether NPAS3 V304I mutation is required for aggregation in neuroblastoma cell line, as predicted by previous studies.

6. To further research major NPAS3 regions and their possible aggregation.

3 Materials and methods

3.1 Materials

3.1.1   DNA plasmids

Table 1 List of DNA plasmids used

Vector	Protein encoded	Publication	Source
pdcDNA-Flag	CRMP1 Lv	Unpublished	Nicholas Bradshaw, Rijeka, Croatia
pdcDNA-Flag	CRMP1 Sv	Unpublished	Nicholas Bradshaw, Rijeka, Croatia
pdcDNA-Flag	CRMP1 Lv (1-573)	Unpublished	Nicholas Bradshaw, Rijeka, Croatia
pdcDNA-Flag	CRMP1 Lv (65-686)	Unpublished	Nicholas Bradshaw, Rijeka, Croatia
pdcDNA-Flag	CRMP1 Lv (92-686)	Unpublished	Nicholas Bradshaw, Rijeka, Croatia
pdcDNA-Flag	CRMP1 Lv (128-686)	Unpublished	Nicholas Bradshaw, Rijeka, Croatia
pI-HA	NPAS3 full length	Luoma and Berry (2018), BMC Molecular Biol	Fred Berry, Edmonton, Alberta, Canada
pI-HA	NPAS3 V304I	Luoma and Berry (2018), BMC Molecular Biol	Fred Berry, Edmonton, Alberta, Canada
pI-HA	NPAS3 (bHLH1 1-116)	Luoma and Berry (2018), BMC Molecular Biol	Fred Berry, Edmonton, Alberta, Canada
pI-HA	NPAS3 (PAS 116-450)	Luoma and Berry (2018), BMC Molecular Biol	Fred Berry, Edmonton, Alberta, Canada
pI-HA	NPAS3 (bHLH1-PAS 1-450)	Luoma and Berry (2018), BMC Molecular Biol	Fred Berry, Edmonton, Alberta, Canada
pI-HA	NPAS3 (TAD 451-933)	Luoma and Berry (2018), BMC Molecular Biol	Fred Berry, Edmonton, Alberta, Canada
pI-HA	NPAS3 (PAS-TAD 116-933)	Luoma and Berry (2018), BMC Molecular Biol	Fred Berry, Edmonton, Alberta, Canada

3.1.2   Antibodies

Table 2 List of primary antibodies used in Western blot and immunocytochemistry with supplier name, host, concentration, and dilution

Name	Type	Supplier	Host	Concentration	Dilution
Anti-FLAG M2	Monoclonal	Sigma	Mouse	1 mg/mL	1:1000
Anti-HA	Monoclonal	Sigma	Mouse	1 mg/mL	1:1000
Anti-NPAS3	Polyclonal	PromoKine	Rabbit	1 mg/mL	1:1000
Anti-CRMP1	Polyclonal	ProSci	Rabbit	1 mg/mL	1:1000
Anti-β-actin	Monoclonal	OriGene	Mouse	1 mg/mL	1:500

Table 3 List of secondary antibodies used in Western blot with supplier name, concentration, and dilution

Name	Supplier	Concentration	Dilution
Peroxidase Conjugated Affinity Purified Goat anti-Mouse IgG	Thermo Fischer Scientific	1 mg/mL	1:2000 for samples from HEK cells,

Table 4 List of secondary antibodies, cytoskeletal and nuclear stains used in immunocytochemistry with supplier name, concentration, and dilution

Name	Supplier	Concentration	Dilution
Alexa Fluor 594 Goat anti-Mouse IgG	Thermo Fischer Scientific	2 mg/mL	1:500
Phalloidin-iFluor 488 Reagent (cytoskeletal stain)	Abcam	1000x stock solutions	1:2000
DAPI (nuclear stain)	Sigma	1 mg/mL	1:500

3.1.3   Buffers and solutions

Buffers and solutions used in this thesis are listed below in in alphabetical order. Each of them was made in deionized distilled water (dH2O).

30% acrylamide solution

14.6 g acrylamide

0.5 g *N,N'*-methylbisacrylamide

Added dH$_2$O up to 100 mL

Cell lysis buffer

5 ml 10x PBS

5 ml 10% Triton X-100

1 mL 1 M Magnesium chloride
 (MgCl$_2$)

50 µL DNase I

50 µL 100 mM Phenylmethylsulfonyl fluoride
 (PMSF)

Added dH$_2$O up to 50 mL

D-MEM or D-MEM/F-12

500 mL D-MEM or D-MEM/F-12

50 mL Fetal calf serum

5 mL 100x penicillin /streptomycin solution

5 mL 100x non-essential amino acid solution

Elution buffer

1 mL Tris 1 M [pH 8.0]

0.2 mL EDTA

Added dH$_2$O up to 100 mL and adjusted pH to 8.0 if needed

**Fixation buffer
(PBS/4% Paraformaldehyde)**

8g Paraformaldehyde (PFA)

20mL 10x PBS

Added dH$_2$O up to 200 mL and adjusted to pH 7.4

For dissolving the PFA, the solution was heated to 50˚C and 1M NaOH was added periodically

LB media

10 g tryptone

5 g yeast extract

5 g NaCl

Mounting medium

0.25g Propyl gallate

5 mL 1M Tris [pH 8.8]

45 mL Glycerol

Added dH$_2$O up to 1 L, adjusted pH to 7.0 and sterilized by autoclaving

The solution was heated for dissolving and 50 µl DAPI was added afterwards (1 µL/mL).

Phosphate buffered saline (PBS) 10x stock solution

80 g NaCl

2 g KCl

14.4 g Na$_2$HPO$_4$

2.4 g KH$_2$PO$_4$

Added dH$_2$O up to 1 L and adjusted pH to 7.4

PBS-Tween

50 mL 10x PBS

250 mL Tween 20

Added dH$_2$O up to 750 mL

Permeabolisaiton buffer (PBS/Trition X-100)

10mL 10% Triton X-100

10mL 10x PBS

Added dH$_2$O up to 100 mL

Ponceau S stain

1 g Ponceau S

4 mL acetic acid

Added dH2O up to 200 mL

Protein loading buffer

6.25 1 M Tris [pH 6.8]

10 mL glycerol

20 mL 10% SDS

3.75 mL dH2O

Added approximately 5 mg of bromophenol blue

SDS-PAGE running buffer 10x stock solution

30 g Tris

144 g glycine

10 g SDS

Added dH$_2$O up to 1 L

SOB media

10 g tryptone

2.5 g yeast extract

0.25 g NaCl

5 mL 250 mM KCl

2.5 mL 2 M MgCl$_2$

Added dH$_2$O up to 250 mL, adjusted pH to 7.0 and sterilized by autoclaving

TAE buffer 50x stock solution

242 g Tris

18.61 g EDTA

57.1 mL acetic acid

Added dH$_2$O up to 1L

TE buffer

0.5 mL 1 M Tris [pH 7.4]

200 µL 0.25M EDTA

Added dH$_2$O up to 50 mL

Transfer buffer 10x stock solution

5.8 g Tris

2.9 g glycine

4 mL 10% SDS

200 mL methanol

Added dH$_2$O up to 1 L

3.1.4 Gels

Acrylamide running gel (handmade)

Table 5 Measurements for handmade acrylamide running gels

	dH$_2$O	30% acrylamide	1.5 M Tris [pH 8.8]	10% SDS	10% APS	TEMED
8%	5.5 mL	3.2 mL	3.0 mL	120 µL	120 µL	120 µL
10%	4.8 mL	3.9 mL	3.0 mL	120 µL	120 µL	120 µL
12%	3.9 mL	4.8 mL	3.0 mL	120 µL	120 µL	12 µL

Acrylamide stacking gel (handmade)

Table 6 Measurements for a handmade acrylamide stacking gel

dH$_2$O	30% acrylamide	1 M Tris [pH 8.8]	10% SDS	10% APS	TEMED
2.6 mL	1.0 mL	625 µL	50µL	50µL	50µL

Acrylamide stacking and running gels were hand casted using the Mini-PROTEAN Tetra Handcast Systems (Bio-Rad). The stacking gel was placed on top of the running gel. Stacking gel is used to create an ionic gradient that concentrates all the protein in one band. Once the protein reaches the more concentrated running gel with smaller pores, it will separate according to its molecular weight.

3.2 Methods

3.2.1   Growth of plasmids in bacterial culture

NEB5α (New England Biolabs) competent bacterial cells were transformed with plasmids encoding various fragments of NPAS3 and CRMP1. For each transformation 1 µL of plasmid was mixed with 50 µL of freshly thawed NEB5α bacteria in a 1.5 mL Eppendorf tube and incubated on ice for 30 minutes. Transformation occurred after heat shock at 42°C for 30 seconds, after which transformed bacteria recovered on ice for 5 minutes. Transformed bacteria were grown on LB agar plates with ampicillin (100 µg/mL) overnight in an incubator at 37°C. The next day, a colony was picked and grown in 3 mL LB media with ampicillin (100 µg/mL) in a shaking incubator at 37°C/150 rpm overnight. After the culture had grown, it was centrifuged (3700 rpm/15 min). The pellet was kept, and plasmid DNA was eluted from it according to the manufacturer's protocol with a Qiagen QIAprep Spin Miniprep Kit. The concentration of plasmid DNA in the samples was measured with a BioDrop µLITE spectrophotometer (absorbance wavelength 260 nm). Elution buffer was used as a blank probe and the concentrations were expressed in µg/mL.

3.2.2   Cell culture and transfection

The HEK293T human kidney cell line and the SH-SY5Y human neuroblastoma cell line were both grown in T25 flasks or 12-well plates. For HEK293T cells, D-MEM media (Pan Biotech) with 10% fetal calf serum, penicillin and streptomycin was used. For SH-SY5Y cells, D-MEM/F12 (Pan Biotech) was used with addition of 1x MEM non-essential amino acids, 10% fetal calf serum, penicillin, and streptomycin. Flasks and plates were kept in the incubator at 37°C/5% CO_2. The cells were split when the confluency reached 90% or more with Trypsin/EDTA (Pan Biotech).

The SH-SY5Y cell line was transfected with Lipofectamine 2000 (Invitrogen) and the HEK293T cell line with Metafectene (Biontex). For transfection of

HEK293T cells with NPAS3 constructs, two sets of solutions were prepared: one of DNA constructs in D-MEM 100 µL solutions (without serum nor antibiotics) with 0.5 µg of plasmid and other set of solutions in 100 µL DMEM with 2 µL of Metafectene per well. The prepared solutions were incubated for 5 minutes separated and then for 30 minutes combined. The solutions were then transferred and incubated in 12-well plates for a period of 6 hours in antibiotic and serum free D-MEM. After 6 hours, media were removed and replaced with fresh D-MEM containing the serum and antibiotics. After another 16 hours of incubation, transfected HEK293T cells were lysed, and cell lysates were used for Western blotting. The protocol for transfection of SH-SY5Y cells with NPAS3 constructs was the same except the D-MEM/F-12 media and Lipofectamine 2000 were used and the transfected cells were used for immunocytochemistry and microscopy.

Transfection of HEK293T or SH-SY5Y cell lines with CRMP1 constructs additionally included treatment with proteasome inhibitor MG-132 (TargetMol, 1 µM) for 4 hours after 6 hours incubation with Lipofectamine, DNA constructs and DMEM or DMEM/F-12. After 4 hours, cells were used either for Western blotting or immunocytochemistry and microscopy.

3.2.3   Cell lysis

The day after transfection, HEK293T cells were washed twice with 1x Phosphate Buffered Saline. Afterwards, 100 µL of Cell Lysis Buffer 1 was added per well and incubated for 5-6 minutes. Lysed cell suspensions were scraped of plate, transferred to 1.5 mL Eppendorf tubes and incubated on ice for 1 hour with occasional mixing. Samples were prepared for SDS-PAGE by adding Protein Loading Buffer, in the same amount as Cell Lysis Buffer, and 1M DTT (10% the volume of Cell Lysis Buffer). These were then heated at 95°C for 5 minutes to denature the proteins. Prepared samples were stored at -20°C.

3.2.4 SDS-PAGE and Western blotting

Acrylamide gels were used for SDS-PAGE and the protein samples acquired from cell lysis were loaded onto it, alongside a my-Budget Prestained Protein Ladder 10 kDa - 180 kDa (Bio-Budget Technologies GmbH, 0.2-0.4 µg/µL). The electrophoresis was run at 180 V for 45 minutes in SDS-PAGE Running Buffer. With this method, the protein samples were separated according to their molecular weight and the gel was then transferred to a Parablot PVDF membrane (Macherey-Nagel, 0.2 µm pore) with a Transblot Turbo system (Bio-Rad) which ran for 30 minutes. The membrane was then stained with Ponceau S solution to see the total protein. Following the transfer, the membrane was incubated for an hour on a shaker at room temperature, or overnight at 4°C, in 5% milk powder/PBS-Tween to block it. Proteins were detected at room temperature using a primary antibody (listed in table 2) diluted 1000-fold and incubation was done in PBS-T alone overnight. Primary antibodies were stored with 0.02% sodium azide at -20°C and re-used up to 3 times. Secondary antibodies (listed in table 3) were diluted 10,000-fold in PBS-T and incubated with the membrane for 1 hour at room temperature. Following the incubation of the membrane with each antibody at room temperature, the wash with PBS-T for 10 minutes three times. Protein bands on the membrane were visualized using Pierce ECL Prime Western Blotting Substrate (ThermoScientific) on a ChemiDoc MP Imaging System (Bio-Rad).

3.2.5   Immunocytochemistry and microscopy

Transfected SH-SY5Y cells growing on glass coverslips were gently washed with 0.5 mL PBS, fixed with 4% paraformaldehyde/PBS for 15 minutes and permeabilized with 1% Triton X-100/PBS for 10 minutes at room temperature. Coverslips were then washed three times with 1x PBS and blocked with 10% goat serum/PBS for 20 minutes. Blocking medium was removed and fixed cells were stained with primary antibody (listed in table 2) diluted 500-fold in 10% goat serum/PBS for 3 hours. Cells were washed three times with PBS over a period of 15 minutes and incubated with the secondary antibody, cytoskeletal and nuclear stain (listed in table 4) in 10% goat serum/PBS for 1 hour in the dark. The cells were washed three times with PBS and once dH_2O and attached to slides with Mounting Medium containing DAPI (Life Technologies) or with commercial Mounting Medium Fluoroshield (Sigma), in which case DAPI was added with secondary antibody. The coverslips were viewed on an Olympus IX83 fluorescent microscope under 20x and 60x magnifications. Images were taken using Hamamatsu Orca R2 CCD camera and CellSens software.

4   Results

Protein aggregation was previously implicated and proven in the development of neurodegenerative diseases and recently, a correlation with chronic mental illness has been shown. CRMP1 and NPAS3, on which we mainly focused in this thesis, are characterised as misassembled and/or aggregated in chronic mental illness. Plasmids encoding human proteins NPAS3 and CRMP1 and fragments thereof were expressed in human HEK293T cultured cells and human SH-SY5Y neuroblastoma cultured cells.

4.1 Verification of plasmid vectors to be used in cell culture research

In order to further investigate CRMP1 and NPAS3 *in vitro*, all plasmids to be used in this thesis were verified by Western blot after expression in mammalian HEK293 cell culture and lysis. Construct CRMP1 Sv seems to be express more strongly in HEK293T cells than CRMP1 Lv. Construct CRMP1 Lv (128-686) showed a slightly higher molecular weight band (~63 kDa) than expected (~61 kDa) (Figure 4) and it seems it is the most highly expressed construct of all. Other constructs showed bands at expected size and have a similar expression level.
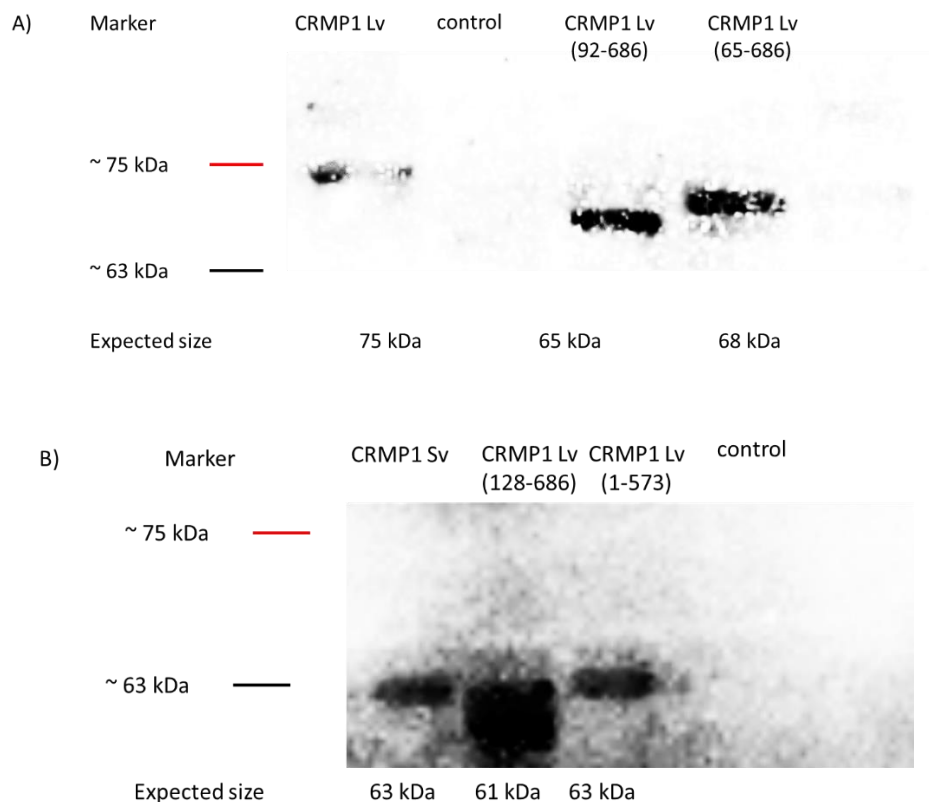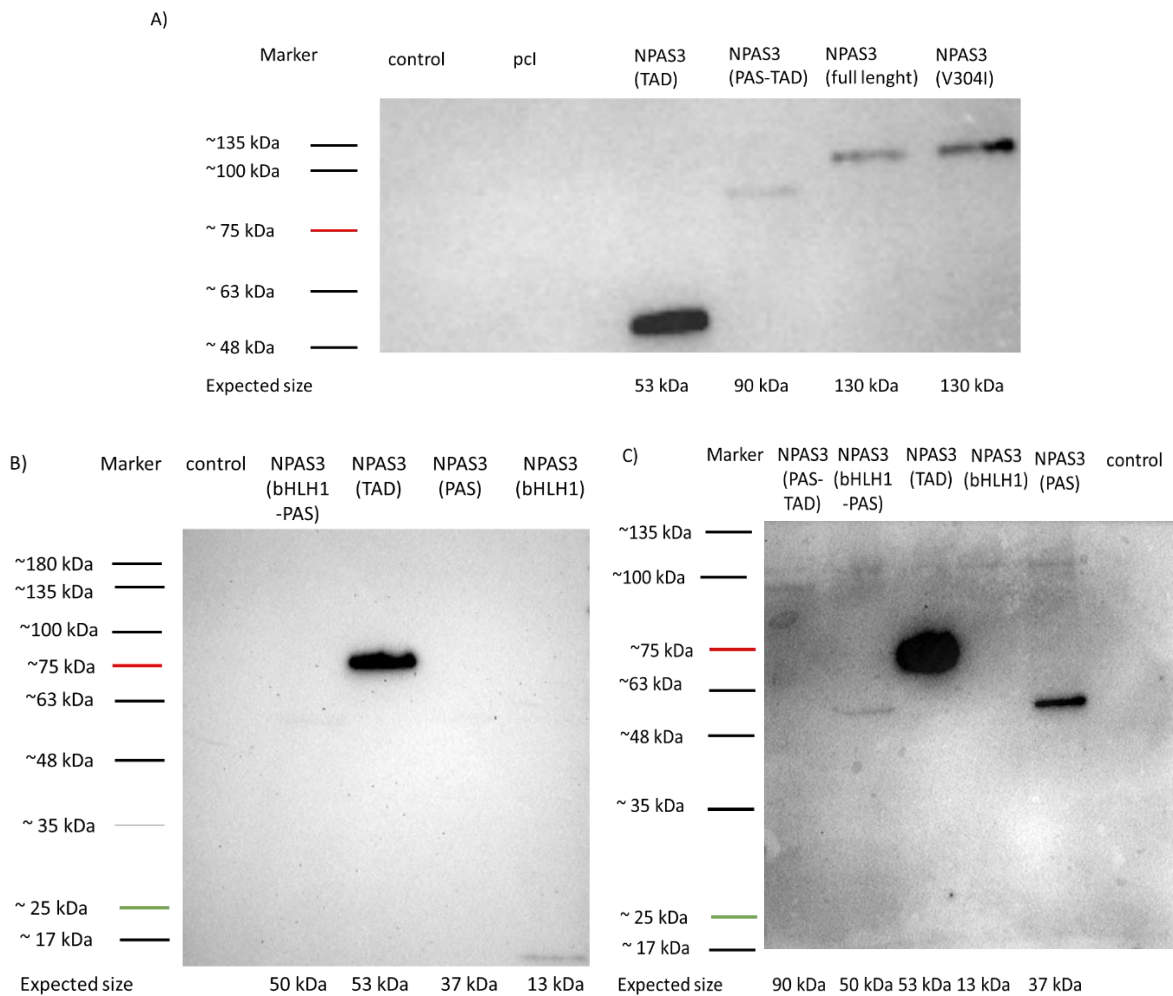
Figure 7 **Western Blot Analysis for anti-FLAG stained membranes with CRMP1 proteins expressed in HEK293T cells**. The membranes with proteins were stained with anti-FLAG primary antibody and then with a goat anti-mouse secondary antibody. The pictures were visualized with ECL Prime kit using ChemiDoc, and exposure time for both membranes was 60 seconds. The sizes were compared to Prestained Protein Ladder 10-180 kDa. Controls were lysates from mock transfected cells. The cells were transfected with 0.5 µg of plasmid DNA.

NPAS3 (bHLH1), NPAS3 (PAS-TAD) and NPAS3 (bHLH1-PAS) showed bands with lower intensity than the other NPAS3 constructs (Figure 5A-C). Mutant version of NPAS3 (V304I) is present at a higher level after expression in HEK293T cells than wild type full length NPAS3 (see Figure 5A). Construct NPAS3 (PAS) showed a higher molecular weight band (between 63 and 48 kDa) than expected (37 kDa), while other constructs showed bands that match expected size.

Figure 8 **Western Blot Analysis for anti-HA stained membranes with NPAS3 proteins expressed in HEK293T cells.** The membranes with proteins were stained with anti-HA primary antibody and then with a goat anti-mouse secondary antibody. The pictures were visualized with ECL Prime kit under ChemiDoc. Exposure time for pictures are: A) 120 seconds, B) 360 seconds, C) 143 seconds. The sizes were compared to Prestained Protein Ladder 10-180 kDa. Negative controls included lysates from mock transfected cells and cells containing only the empty pcI vector. The cells were transfected with 0.5 µg of plasmid DNA.

Western blot analysis showed all constructs were expressed properly, thus indicating those proteins are successfully expressed in SH-SY5Y neuroblastoma cell culture as well.

4.2 Neither CRMP1 Lv nor CRMP1 Sv were seen to form aggregates alone in SH-SY5Y cells

Previous studies showed how CRMP1 Lv is able to aggregate by itself[47], while CRMP1 Sv aggregate only when co-expressed with DISC1 and huntingtin[47]. In this thesis, we attempted to replicate aggregation of CRMP1 Lv and, in a similar manner, investigate aggregation of CRMP1 Sv. Successfully expressed proteins with FLAG tags were seen as bright red fluorescence, while cellular actin and nucleus were visualized with phalloidin (green) and DAPI (blue). The control were mock transfected cells that did not contain a FLAG tagged protein and, as such, showed no red staining. The transfection of full length and truncated versions of CRMP1 Lv proteins and CRMP1 Sv protein showed no clear signs of aggregation in transfected SH-SY5Y cells.
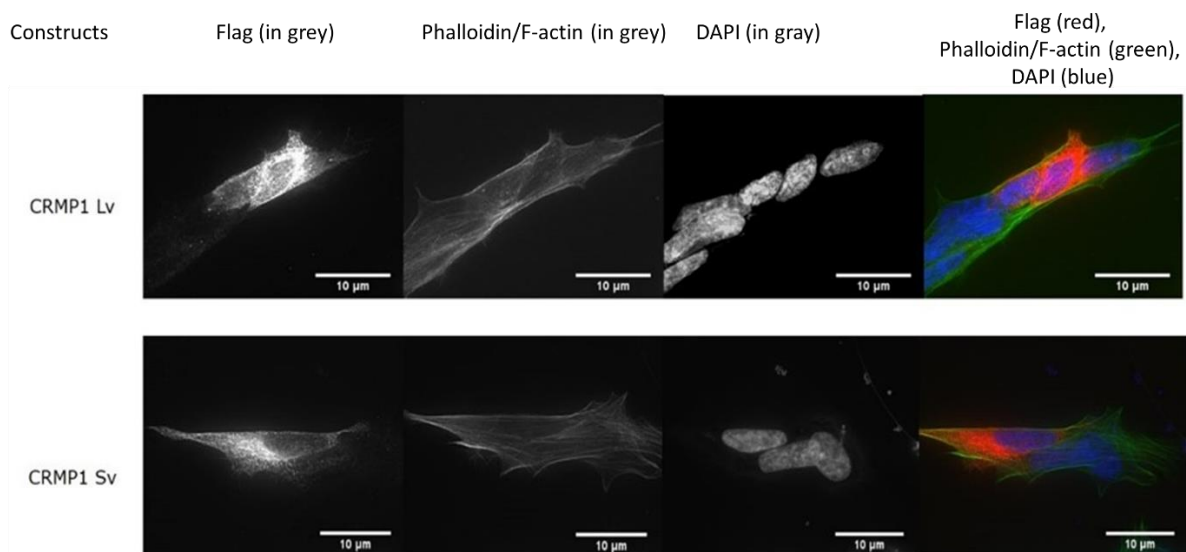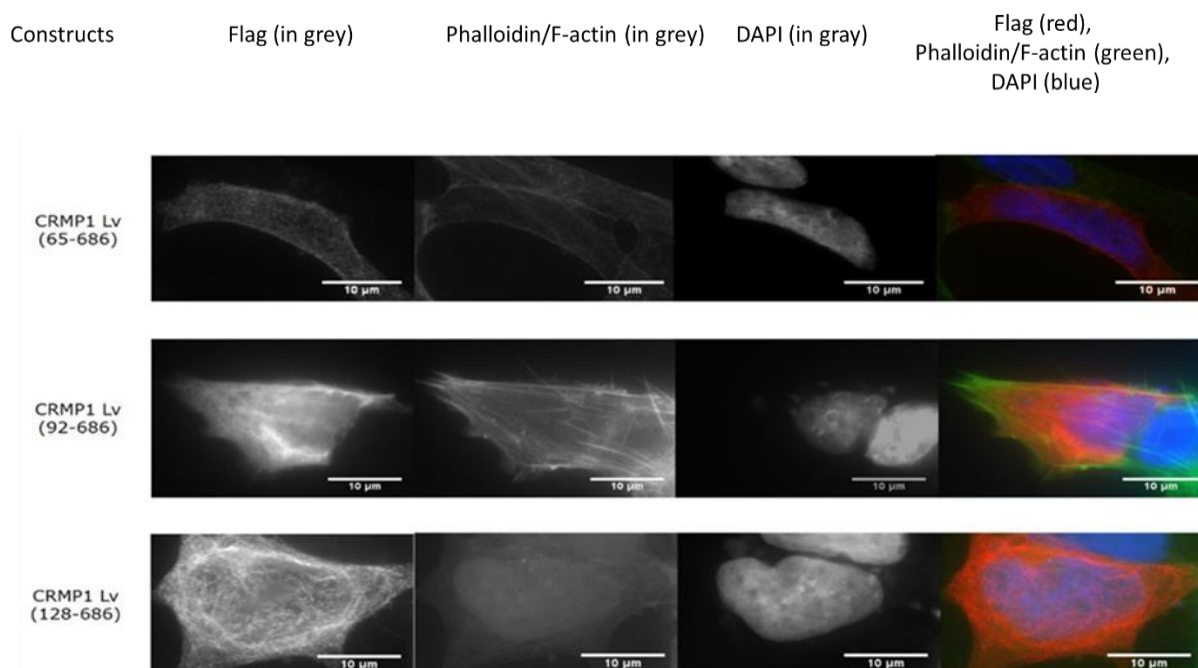

Figure 9 **Fluorescent microscopy of SH-SY5Y cells transfected with CRMP1 Lv and CRMP1 Sv proteins, of which neither one shows clear signs of aggregation.** The proteins were labelled with anti-FLAG primary antibody and goat anti-mouse 594 nm secondary antibody, shown as bright red signal on images. The DAPI was used to stain the nucleus (blue signal) and the phalloidin 488 nm was used to stain cellular actin (green signal). The obtained images were captured under 60x magnification on a fluorescent microscope with CellSens software. The scale bar represents 10 µm. All expressed proteins are shown as even distribution of red signal from full length either in the cytoplasm or the nucleus of transfected cells. All images are representative of two independent experiments.

4.3 N-terminal section of CRMP1 Lv showed no effect on CRMP1 Lv aggregation

Based on previous studies, which showed signs of aggregation for CRMP1 Lv, but not for CRMP1 Sv[47], when expressed alone, we hypothesized that the N-terminal section of CRMP1 Lv (1-128), which is absent in CRMP1 Sv, could affect aggregation of CRMP1. Plasmids encoding full length and truncated versions of CRMP1 were therefore expressed in SH-SY5Y cells, following the same protocols as in the previous subsection. None of the cells showed clear signs of aggregation upon transfection. The cells expressing CRMP1 Lv (1-573), a CRMP1 construct without C-terminal unstructured region, showed punctate patterns in the nucleus.

| Constructs | Flag (in grey) | Phalloidin/F-actin (in grey) | DAPI (in gray) | Flag (red), Phalloidin/F-actin (green), DAPI (blue) |
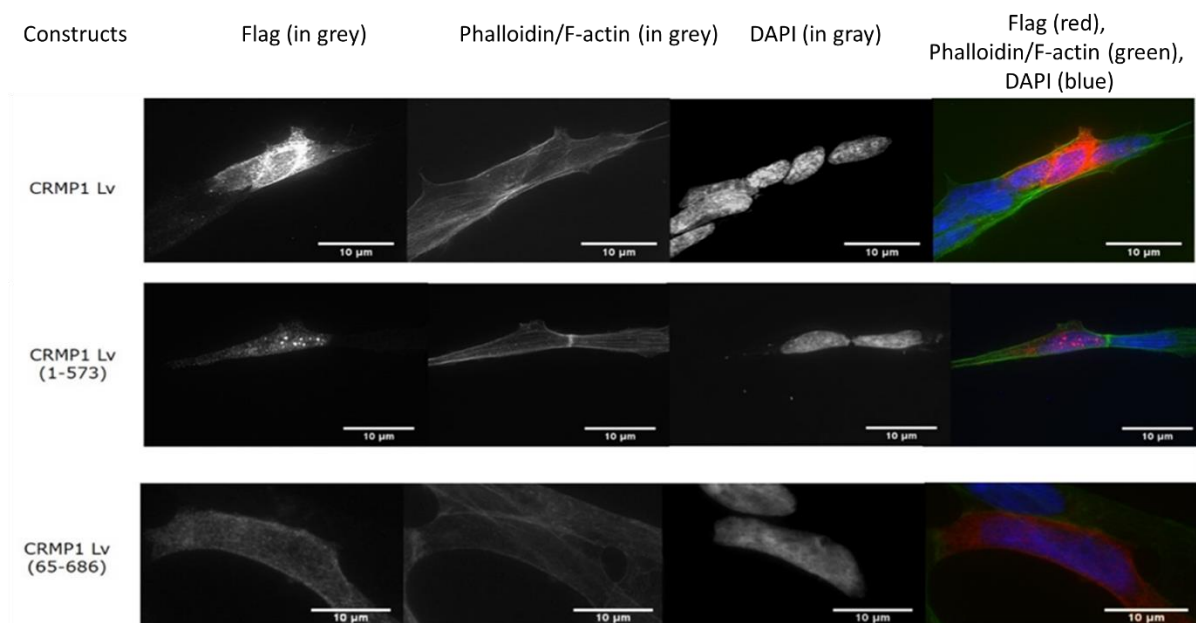
Figure 10 Fluorescent microscopy of SH-SY5Y cells transfected with full length and truncated CRMP1 Lv proteins, of which neither one shows clear signs of aggregation. The proteins were labelled with primary anti-FLAG antibody and secondary antibody Goat Anti-Mouse 594 nm, shown as bright red signal on images. The DAPI was used to stain the nucleus (blue signal) and the phalloidin 488 nm was used to stain cellular actin (green signal). The obtained images were captured under 60x magnification on a fluorescent microscope with CellSense software. The scale bar represents 10 µm. All expressed proteins are shown as even distribution of red signal from full length either in the cytoplasm or the nucleus of transfected cells. All images are representative of two independent experiments.

4.4 The V304I mutation affects NPAS3 aggregation in SH-SY5Y cells

Previous studies have shown that the V304I mutation in NPAS3 leads to insolubility of NPAS3 protein and thus, possibly, aggregation. In this thesis, we investigated the effect of V304I NPAS3 mutation on formation of visible aggregates in human SH-SY5Y neuroblastoma cell line. In a similar manner as to CRMP1, successfully expressed NPAS3 constructs were seen as bright red fluorescence. Cell integrity was confirmed with the fluorescent phalloidin, to visualise actin, and the nuclear stain DAPI. The controls were

mock transfected cells that did not express any HA tagged proteins and as such, showed no red staining. Aggregates of NPAS3 (V304I) can be seen as red dots the in cytoplasm of transfected cells. Also, we observed that NPAS3 (V304l) does not enter the nucleus and is retained in cytoplasm. In comparison, the cells transfected with wild type NPAS3 protein show even distribution of red signal of expressed protein in the nucleus of transfected cells. Taking into account the fact that NPAS3 is a transcription factor, signal from nucleus is compatible with expected results.
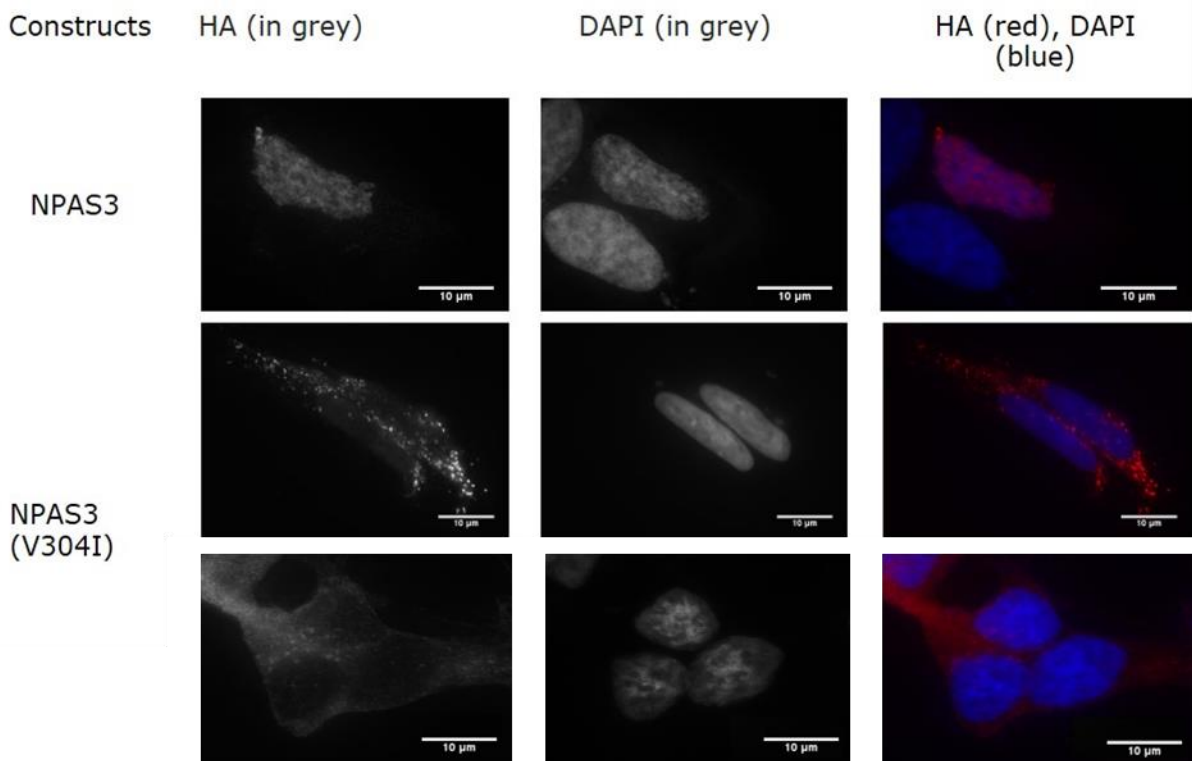
Figure 11 **Fluorescent microscopy of SH-SY5Y cells transfected with mutated and wild type NPAS3 protein, of which only cells with mutated NPAS3 show signs of aggregation**. The proteins were labelled with primary anti-HA antibody and secondary antibody Goat Anti-Mouse 594 nm, shown as bright red signal on images. The DAPI was used to stain the nucleus (blue signal). The obtained images were captured under 60x magnification on a fluorescent microscope with CellSense software. The scale bar represents 10 μm. Images for NPAS3 transfection are representative of three independent experiments, and for NPAS3 (V304I) images represent two successful transfection out of three.

4.5 NPAS3 aggregation in cells occurs through the bHLH domain, indicating mutation V304I NPAS3 is not essential for aggregation

From previous studies it is still unclear if NPAS3 aggregation requires the presence of the V304I mutation or is possible without it.

To address that question, plasmids encoding full length (wild type and mutant, 933 aa) and regions of NPAS3 (bHLH1 1-125 aa, bHLH1-PAS 1-450 aa, PAS 116-450, TAD 451-933, PAS-TAD 116-933) were expressed in SH-SY5Y cells (shown in figure 3). The expression of plasmids followed the same protocols as in the previous subsection.

Clear signs of aggregation (red dots in cytoplasm) were seen in SH-SY5Y cells transfected with the bHLH1 region of NPAS3 protein. Non-aggregating full length NPAS3 protein was primary seen in nucleus.

 Other regions of the NPAS3 protein expressed alone (the PAS or TAD regions) showed no signs of aggregation, with the PAS region expressed in whole cell and TAD in the nucleus only.

The combined bHLH1 and PAS region showed no signs of aggregation and was expressed throughout the whole cell.
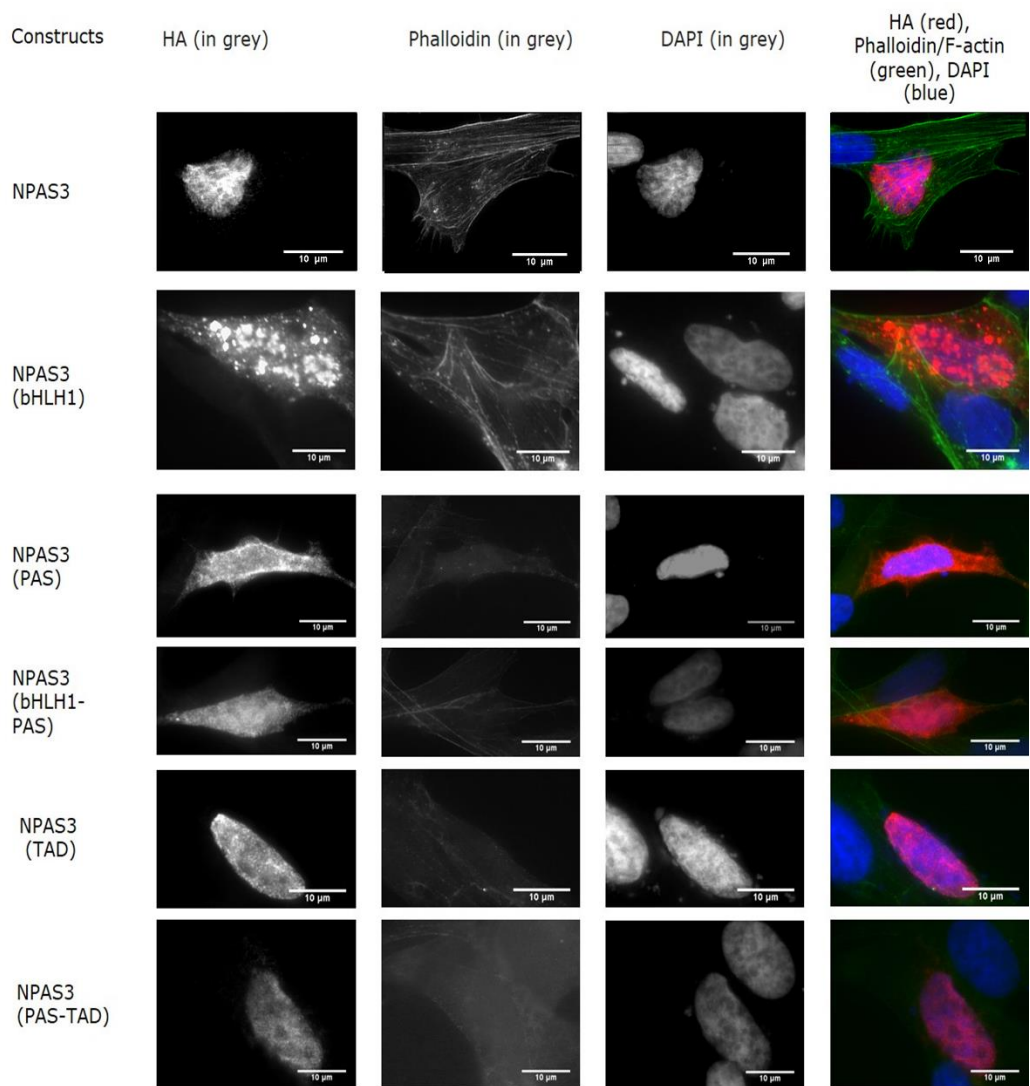
Figure 12 **Fluorescent microscopy of SH-SY5Y cells transfected with truncated NPAS3 proteins and full length NPAS3 protein, of which only bHLH1 region shows clear signs of aggregation.** The proteins were labelled with anti-HA primary antibody and goat anti-mouse 594 nm secondary antibody, shown as bright red signal on images. The DAPI was used to stain the nucleus (blue signal) and phalloidin 488 nm was used to stain cellular actin (green signal). The obtained images were captured under 60x magnification on a fluorescent microscope with CellSense software. The scale bar represents 10 μm. All images are representative of three independent experiments.

5 Discussion

5.1 The short and long version CRMP1 showed no clear signs of aggregation, nor did the N-terminal section of CRMP1 Lv show an effect on aggregation

CRMP1 has a major role in brain development, as one of the proteins which is highly expressed in the brain and involved in neuronal differentiation and axonal outgrowth[42,43]. CRMP1 was implicated in chronic mental illnesses as a result of hypothesis-free proteomic studies, where it was singled out as one of the insoluble proteins in brain samples from patients diagnosed SZ and BD[45,46]. Thus, CRMP1 was added to the list of proteins considered to aggregate in chronic mental illnesses, along with DISC1 and dysbindin. DISC1 has been investigated previously for its connection to SZ by genetic research[30,31,32], but it was also found as insoluble in brain samples from patients with SZ and affective disorders[33]. There are two variants of CRMP1: the long (CRMP1 Lv) and short (CRMP1 Sv) variants. Experiments by Bader et al. showed that CRMP1 Lv was able to aggregate by itself, unlike CRMP1 Sv, which aggregated when co-expressed with DISC1[46].

We therefore hypothesised that aggregation of CRMP1 Lv was dependant on the N-terminal section of CRMP1 Lv, amino acids 1-128, which is the only region present in CRMP1 Lv, but absent in CRMP1 Sv. Thus, we tested both versions of CRMP1: CRMP1 Lv and CRMP1 Sv by expressing them in the human neuroblastoma cell line SH-SY5Y, to try to replicate previous experiments. We also included truncated versions of CRMP1 Lv in order to investigate the effect of the N-terminal section of CRMP1 Lv.

In our research, we did not see any clear signs of aggregation for CRMP1 Lv, nor for CRMP1 Sv or for any of the N-terminally truncated versions of CRMP1 Lv. The cells expressing CRMP1 Lv (1-573), a long variant of CRMP1 without the unstructured C-terminus, showed punctate patterns in the nucleus. This punctuate pattern cannot be considered as aggregation, since

protein aggregates would not be able to fit through the nuclear pores and therefore enter the nucleus. Instead, they likely represent localisation to specific components of the nucleus, such as the nucleoli.

For their experiments, Bader et al. over-expressed CRMP1 Lv and CRMP1 Sv fused with GFP. GFP is a large protein tag (approx. 27 kDa) that, due to its size, could affect the aggregation propensity of proteins they are fused to[46]. In our case, CRMP1 proteins were over-expressed in plasmids with a much smaller FLAG tag, which is less likely to interfere with CRMP1 expression and function. Another difference between our research and the one conducted by Bader et al. was the cell line. Bader et al. used CAD cells which originate from mouse catecholaminergic neuronal tumour[46], while we choose a more relevant cell line, the human neuroblastoma cell line SH-SY5Y. Therefore, our approach provides more a more accurate point of view.

It is worth mentioning it still is not an ideal system, which would include expression of proteins in neuronal cultures or proteins without any tags. Since previous studies have showed CRMP1 can aggregate when co-expressed with other proteins (e. g. DISC1[46] and huntingtin[47]), the next experiments could include co-expression of our plasmids with DISC1 in the human neuroblastoma cell line. Our constructs of both isoforms of CRMP1, as well as the truncated versions of CRMP1, would separately be expressed with full length DISC1 or NPAS3. Immunofluorescence microscopy would then be used to assess colocalization and coaggregation of CRMP1 and DISC1 or NPAS3 in cells after expression.

5.2 The bHLH1 domain could play a key role in NPAS3 aggregation

NPAS3 is a transcription factor that contains three important regions: bHLH1, TAD and PAS (the PAS region contains two functional domains: PAS A and PAS B). In healthy cells, NPAS3 is thought to be a heterodimer, in which the PAS domains determine the interaction partner and the bHLH1 domain enables homodimerization with other NPAS3 proteins or heterodimerization with other interaction partners that also contain bHLH1 domains[48]. NPAS3 acts as a regulator in neurogenesis, metabolism, and circadian rhythms[29].

In our research, we have seen that the bHLH1 region is capable of aggregation when expressed alone. However, the expression of bHLH1 region together with PAS region showed no clear evidence of aggregation. These results imply that PAS region provides a stabilising effect, which aligns with the previously indicated importance of a functional PAS region in healthy circumstances.

The other region or combination of regions tested (TAD, PAS, PAS-TAD) showed no signs of aggregation by themselves. Expression of the PAS region in the cytoplasm and nucleus matches predictions, since it is the NPAS3 region involved in protein interaction and ligand binding[48]. The TAD region is responsible for modulating gene expression of the target gene and contains an NLS (nuclear localization signal)[48], which directs it to nucleus, as supported by our research. Expression of combined PAS and TAD regions in the nucleus can also be explained by presence of the NLS in the TAD region[48].

5.3 Implications of the work for NPAS3 aggregation in mental illness

NPAS3 was first implicated in SZ after the discovery of a translocation between chromosomes 9 and 14 in a family case, involving a mother and daughter diagnosed with SZ. Both had the translocation, leaving them with one copy of NPAS3 broken in two between the bHLH1 and PAS regions[50,51]. The translocation would lead to production of a truncated protein, which would contain bHLH1, but without the PAS and TAD regions. This version of the protein could either bind to promoters but lack function, or else bind to other NPAS3 proteins to form non-functional proteins.

However, cells contain surveillance mechanisms which identify and degrade incorrect mRNA and protein species. There are therefore two possible scenarios: in one case truncated mRNAs and/or truncated proteins would be successfully degraded and the general level of NPAS3 proteins would be halved compared to normal individuals with intact, functional chromosome 14[51], similar to the DISC1 translocation[31,32].

In the other scenario, the truncated protein is not successfully degraded and continues to exist, and possibly interfere with, the cell.

Subsequent genetic research linked the gene *NPAS3* with BD and SZ[54], while a single nucleotide polymorphism in NPAS3 was associated with efficacy of antipsychotic drugs[53].

More recent studies described a mutation, V304I, in NPAS3 in a family with SZ[55]. Mutated NPAS3 protein showed signs of insolubility, both when tested as a purified recombinant protein using systems, and when overexpressed in mammalian cells[56], leading to the assumption that it probably aggregates. This assumption had not yet been confirmed with microscopy.

It is interesting to note that mutation V304I disrupts the protein between the two PAS domains. Compared to patients with the translocation, these patients would express the full-length NPAS3 protein, but their PAS region would be disrupted, which could affect the function of protein. In both cases, the importance of the PAS region for the normal function of NPAS3 is proven.

Previous studies showed that mutated NPAS3 was insoluble in mammalian cells upon overexpression, suggesting it to be aggregating[56]. Our research confirmed that mutated NPAS3 V304I forms visible aggregates in neuroblastoma cell culture and is present in cytoplasm, unlike full-length wild type NPAS3 which enter the nucleus. However, we also see aggregation of the bHLH1 region alone, and this construct is especially interesting since it resembles the protein that would be produced from translocation chromosome[50,51].

Therefore, it is possible to hypothesise that the translocation may cause mental illness because the short variant of NPAS3 with bHLH1 region aggregates faster than it can be degraded, thus accumulating in the cell, and gaining a new, toxic function. To summarise, NPAS3 aggregation was previously only implied as a consequence of the V304I NPAS3 mutation, but we have now also seen aggregation after overexpression of a non-mutated region (bHLH1) which is stabilized by presence of the PAS region. Therefore, our results strongly imply that aggregation could also occur through other mechanisms (if other mutations, translocation, or cellular stresses interfered with the function of the PAS region).

Furthermore, research in this thesis provides more insight into the NPAS3 translocation and it suggests that the V304I mutation may cause aggregation by stopping PAS region from stabilizing the protein.

5.4 Open questions and future research

Since NPAS3 seems to be a more promising direction for the future into protein aggregation in mental illness than CRMP1, based on results of this thesis, there are two major questions that need answers: A) Does the truncated NPAS3 produced by the translocation aggregate, as the bHLH1 region was seen to in this research? B) How does the NPAS3 V304I mutation affect the stabilising function of PAS region?

To answer the first question, future experiments should start with testing plasmids including NPAS3 region encoded with exons 1-4, to generate a protein that matches the one produced by the translocation chromosome. In a similar manner to the research done in this thesis, the protein would be expressed in the SH-SY5Y cells and investigated for aggregation with fluorescence microscopy.

To answer the second question, experiments would include cloning the bHLH1-PAS region (1-450) with the V304I mutation introduced. These would be expressed in SH-SY5Y and screening for aggregation with fluorescent microscopy.

There is also a need to confirm if NPAS3 V304l mutation is unique in the brain, as seen in previously described family case[55], or is it more common.

Another possible future approach in investigation of NPAS3 as an aggregating protein could include bioinformatic analysis. Using bioinformatic tools, the structure of NPAS3 regions could be modelled and the interactions between them observed, given that the structure of bHLH1 and PAS regions are known[48].

Based on cell culture and bioinformatics results, in the long-term experiments should include live animal models. One possible experiment would involve overexpression of wild type and mutated NPAS3 in the fruit fly *Drosophila melanogaster* and monitoring locomotor activity and social recognition.

Previous study with NPAS3 knock out mice model showed correlation between lack of NPAS3 and increased locomotor activity with reduced recognition memory[50]. Nevertheless, results of mentioned future research could give some insights into the mechanism of aggregation for other proteins implicated in mental illness.

6 Conclusion

In this Master's thesis, the potential for aggregation of NPAS3 and CRMP1 was investigated. Previous research showed that the long and short variant of CRMP1 could aggregate, either alone or with other proteins.

Still, we did not see signs of aggregation when those variants were expressed from a vector containing a small protein tag in neuroblastoma cell line. Also, truncation of long version of CRMP1 at N-terminal section showed no effect on protein aggregation.

In comparison to previous studies, the work presented here used a cell line more similar to neurons and the tag less likely to affect results, providing a better model for investigation of CRMP1 aggregation.

Our next experiments include co-expression of vectors used in this research and other proteins implicated in mental disorders, such as DISC1.

Concerning NPAS3, we have two new findings which were not presented before. First of all, we have confirmed that mutated full-length NPAS3 behaves differently in cells than the wild type. Based on this, it is possible that the V304I mutation is preventing entrance in nucleus, which prevents NPAS3 to act as a transcription factor and at the same time it accumulates in cytoplasm and eventually aggregates.

We can even hypothesise that the mutant version of NPAS3 could be misassembling and could behave like a "seed" for aggregation of wild type NPAS3, as seen with Aβ peptide and tau protein in Alzheimer's disease and TDP-43 in ALS and other conditions[26].

On the other hand, we have seen that non-mutated region (bHLH1) is capable of aggregation by itself and stabilised when the PAS region is present.

Based on this, we can hypothesize that NPAS3 aggregation may be able to occur through different mechanism, not only the previously described mutation.

If that is the case, it implies NPAS3 aggregation is not limited to genetic predispositions and it could be a general risk factor for whole population. To answer this question, we are looking into aggregation of NPAS3 and CRMP1 in *post-mortem* brains samples.

Future research in this field could give some insights into the mechanism of aggregation for other proteins implicated in mental illness.

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8 List of figures

9 List of tables

Financial support

Bobana
Samardžija

DATE OF BIRTH:
25/01/1996

CONTACT

Nationality: Serbian

Gender: Female

📍 Javornik 21
44440 Dvor, Croatia

✉ bobana.samardzija@gmail.com

📞 (+385) 921502649

🖱 www.linkedin.com/in/bobana-samardzija

https://orcid.org/0000-0001-7745-2337

https://www.researchgate.net/profile/Bobana_Samardzija

WORK EXPERIENCE

05/2016 - 06/2018

Student Teaching Assistant in courses Analytical Chemistry and Bioassays

Department of Biotechnology, University of Rijeka

Preparation of material for lab practicum (solutions, laboratory equipment)

Helping students to apply theoretical knowledge and develop laboratory skills

Rijeka, Croatia

EDUCATION AND TRAINING

01/10/2017 – CURRENT – Rijeka, Croatia

Masters degree in "Drug research and development"

University of Rijeka, Department of Biotechnology

01/03/2020 – 30/06/2020 – Wilhelm-Johnen-Straße, Jülich, Germany

Internship in Institute of Biological Information Processing-Structural Biochemistry (IBI-7)

Forschungszentrum Jülich GmbH

1. Basic theoretical knowledge in the fields of:

- biophysical methods used in the characterisation of proteins (DLS) and protein interactions (ITC)

-protein expression (bacterial hosts, possibly cell-free system) and purification

- protein crystallisation for structure determination
- data acquisition, processing and visualisation in X-ray crystallography

2. Knowledge and practical experience in the fields of:
- primer design for the specific regions of the DISC-1 gene and their cloning in the expression vector for the MBP fusion protein

- DNA expression and purification

3. Competence to judge suitability of methods to a given problem based on their strengths and weaknesses

EQF level 2 | https://www.fz-juelich.de/portal/EN/Home/home_node.html

10/2015 – 07/2017 – Rijeka, Croatia

● **Bachelor's Degree Biotechnology and drug research**
Department of Biotechnology, University of Rijeka

Expert courses (General, Analytical and Organic Chemistry, Biochemistry, Pharmacology, Microbiology, Immunology)

In silico methods for modeling molecules

Final paper thesis:

Title: Influence of Alternative and Conventional Therapy on Changing Fatty Acid Composition in Diabetic Liver

Mentor: dr. sc. Jasminka Giacometti

Laboratory for natural compounds and metabolomics

Learned analytical methods: gas chromatography (GC)

Published paper:

Giacometti, Jasminka; Milin, Čedomila; Ćuk, Mira; Samardžija, Bobana; Radosević-Stašić, Biserka: Partial Hepatectomy and Diets Enriched with Olive and Corn Oil Altered the Phospholipid Fatty Acid Profile in the Spleen, Journal of Nutrition and Food Science Forecast, 1 (2018), 1005, 9

Field(s) of study
  ○ Life sciences

● **Pharmacy technician**
Viktorovac Highschool

General

- Croatian, English, Latin language; Mathematics; Biology; Physics,

Occupational

- Anatomy and Physiology; General,Organic and Analytical Chemistry; Biochemistry; Industrial manufacture of drugs; Pharmaceutical chemistry with pharmacology; Medical microbiology; Botanics with pharmacognosy; Pharmaceutical technology with cosmetology; Natural healing means

Field(s) of study
  ○ Health

2016 – 2020

- **Presentations**

  ○ Attending of Digital International Student Congress Of (bio)Medical Sciences 2020 in a poster session:

Samardžija B, Bradshaw NJ: Mechanism of aggregation of NPAS3, which is implicated in mental illnesses

  ○ Active participation at the 9th Student Congress of Neuroscience NeuRi 2019

Samardžija, B, Dashi G., Odorcic M, Bradshaw NJ: Review of protein-specific aggregation connected to schizophrenia

  ○ Active participation at the Students' Congress "Nutrition and Clinical Dietotherapy" with international participation:

in 2016: P.Pavletić, V. Štimac, B. Samardžija, M. Matešić: Comparison of polyphenols of cocoa mass of different origin

in 2017: Samardžija, B: Influence of Alternative and Conventional Therapy on Changing Fatty Acid Composition in Diabetic Liver

LANGUAGE SKILLS

MOTHER TONGUE(S): Croatian

English

Listening	Reading	Spoken production	Spoken interaction	Writing
C1	C1	C1	C1	C1

NETWORKS AND MEMBERSHIPS

- **Memberships**

  Member of Association of students of biotechnology in Rijeka

  (USBRI-Udruga Studenata Biotehnologije Sveučilišta u Rijeci)

  - Project manager for Summer School of Chemistry in University Department of Biotechnology in Rijeka.

COMMUNICATION AND INTERPERSONAL SKILLS

Communication and interpersonal skills

Highly proficient in spoken and written English and capable of
fast adaption to a multicultural environment,

both developed during volunteering at international sport events (European
University Games 2016, World University Championship Handball 2018,
European University Championship Beach Handball 2019)

and while working in German institute (Internship at the Institute of
Biological Information Processing- Structural Biochemistry (IBI-7) in
Forschungszentrum Jülich GmbH 2020)

JOB-RELATED SKILLS

Job-related skills

Autumn School of Scientific Writing (November 2017)

Society for clinical application of neuroscience

Education about literature search, writing and publishing scientific articles,
papers, case reports and scientific posters.

VOLUNTEERING

Volunteering

Projects at University Department's level:

Volunteer at Association USBRi (Association of Biotechnology Students Rijeka)

Note-able activities: Science Festival at University of Rijeka and Science Picnic in Zagreb

Projects at university level:

Member of the Student Union in University of Rijeka:

Mention-able projects: Student Day Festival (SDF)

Volunteer at Unisport Rijeka (University sport association)

Projects at city level in Rijeka:

Street race "Homo si teć" (2016-2019)

Projects on national level in Croatia

Croatia's Davis Cup by BNP Paribas semifinal clash between USA and Croatia in Zadar 2018.

International volunteering projects:

European University Games (EUG) 2016, World University Championship Handball 2018, European University Championship Beach Handball 2019

All volunteering activities are presented in my Volunteering book.