Stress as a factor affecting aggregation of NPAS3 and other proteins related to mental illness

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SVEUČILIŠTE U RIJECI

ODJEL ZA BIOTEHNOLOGIJU

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"Biotehnologija u medicini"

Mihaela Bergman

Stres kao faktor utjecaja na agregaciju NPAS3 i ostalih proteina prisutnih u mentalnim bolestima

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1. Izv. prof. dr. sc. Rozi Andretić Waldowski
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3. Doc.dr.sc. Nicholas J. Bradshaw

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Abstract

Chronic mental illness are some of the leading cause of disability worldwide. They include major depressive disorder, bipolar disorder and schizophrenia. Schizophrenia has the most severe symptoms, with an estimated prevalence of 24 million people in the general population. Chronic mental illness are characterized by both genetic and non-genetic risk factors, however, their underlying biological cause remains relatively unknown. Several candidates have been implicated in protein aggregation pathology of chronic mental illness, including TRIOBP-1, DISC1, CRMP1 and NPAS3. In this thesis, we investigate the effect of environmental stress factors on aggregation of these proteins, when into cultured cells. Initially we were focusing on treatment protocol optimization and settled on stress treatment for 3 hours. The results suggest that TRIOBP-1, DISC1 and NPAS3 show signs of aggregation when following application of stress. However, NPAS3 showed the most interesting results, where it clearly mislocalized from nuclei to the cytoplasm forming insoluble aggregates. The tests were repeated, showing aggregation of NPAS3 after it was treated with sodium arsenide, iron (II) chloride or zinc acetate, as well as potential aggregation when treated with calcium chloride and an MG132 proteasome inhibitor. Environmental stress factors potentially do affect protein aggregation, which can be used as a new approach to investigate the effect on chronic mental illness. Further cell culture research could provide us with initial results and could be used as a model for observing stress in chronic mental illness based on these findings. Further research should be focused on stress treatment protocol modification, as well as the implication of other stress factors, such as heat shock, as the high temperature resulted in a quantitative test failure, where most of the transfected cells showed signs of NPAS3 aggregation.

Key words: chronic mental illness, NPAS3, protein aggregation, schizophrenia

Sažetak

Kronične mentalne bolesti jedan su od vodećih uzroka smetnji širom svijeta. One uključuju depresivni poremećaj, bipolarni poremećaj i shizofreniju. Shizofreniju karakteriziraju nateži simptomi, te je procijenjena prevalencija kod 24 miljuna ljudi u populaciji. Kronične mentalne bolesti karakteriziraju genski faktori i faktori koji nemaju genski uzrok, međutim, njihov temeljni biološki uzrok ostaje relativno nepoznat. Nekoliko kandidata povezanih s patologijom agregacije proteina kod mentalnih bolesti uključuju TRIOBP-1, DISC1, CRMP1 i NPAS3. U ovom diplomskom radu istražujemo učinak stresnih faktora okoliša na agregaciju ovih proteina u staničnim kulturama. U početku smo se usredotočili na optimizaciju protokola za tretiranje stanica stres faktorima i odlučili smo se na tretiranje u trajanju od 3 sata. Rezultati ukazuju da TRIOBP-1, DISC1 i NPAS3 agregiraju nakon tretiranja stres faktorima. Međutim, NPAS3 je pokazao najzanimljivije rezultate, gdje se jasno vidi premještaj iz jezgre stanice u citoplazmu, stvarajući netopljive agregate. Ponovljena testiranja potvrđuju NPAS3 aggregaciju nakon tretiranja s natrijevim arsenidom, željezovim (II) kloridom i cink acetatom, kao i potencijalnu agregaciju nakon tretiranja kalcijevim kloridom i inhibitorom proteasoma MG132. Okolišni stres faktori potencijalno utječu na agregaciju proteina, te se mogu koristiti kao novi pristup istraživanja učinka stres faktora na kronične mentalne bolesti. Daljnja istraživanja na staničnim kulturama mogla bi nam dati početne rezultate i koristiti se kao model za promatranje učinka stresa kod kroničnih mentalnih bolesti na temelju ovih otkrića. Daljenja istraživanja bi trebala biti usmjerena na modifikaciju protokola za tretiranje stanica, kao i korištenje drugih faktora stresa, primjerice toplinski šok, obzirom da je visoka temperatura rezultirala neuspješnim kvantitativnim eksperimentom, gdje je većina transficiranih stanica pokazivala znakove NPAS3 agregacije.

Ključne riječi: kronične mentalne bolesti, NPAS3, agregacija proteina, shizofrenija

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1. Introduction

1.1. Chronic mental illness

Bipolar disorder, major depressive disorder, and schizophrenia, collectively referred to as chronic mental illness (CMI), are some of the leading causes of disability globally[1]. Neuropsychiatric conditions impose an increasing burden on society, and are complex, heterogeneous conditions characterized by both genetic and non-genetic elements[2].

1.1.1. Major depressive disorder

Major depressive disorder (MDD), sometimes referred to simply as depression, is a condition characterized by clear-cut changes in the mood, diminished interest or pleasure in daily activities, feeling of worthlessness and sadness, and general loss of energy which severely affect the everyday life of an individual[3]. There are multiple symptoms of this disorder which may differ between individuals, although they should last for at least 2 weeks to be classified as MDD. It affects about 6% of the adult population worldwide each year, and, according to the Global Burden of Disease Consortium, it is the second leading contributor to global disease burden, expressed in both developed and non-developed countries[4]. MDD in adolescence is a risk factor for substance abuse, psychosocial impairment in adulthood, and suicide, as it is estimated that 8% of adolescents diagnosed with MDD had attempted suicide in their young adulthood[5]. According to GWAS (genome-wide association studies), 120 genes have been identified as likely risk factors[6]. Furthermore, the contribution of genetic factors' to MDD is estimated to be around 35%, with higher heritability shown in twin and family-based studies compared to single-nucleotide polymorphism-based studies. A large number of rare mutations

are involved in disease, but twin studies estimate in total genetic contribution is much higher than the common varients can explain by GWAS studies. Additionally, environmental factors such as emotional, sexual, or physical abuse highly correlate with a risk of MDD development[7].

1.1.2. Bipolar disorder

Bipolar disorder (BD) is a chronic affective disorder characterized by persistent and striking mood swings with alternating episodes of mania and depression[3]. The illness affects more than 1% of the general population and is one of the main causes of functional and cognitive impairment in affected individuals[8]. It usually appears in late adolescence but occasionally, mothers can be diagnosed during pregnancy or after childbirth. The risk factors that contribute to the development of BD are both genetic and environmental[9]. Heritability correlating with BD development is estimated to be around 80% based on adoption, twin and family studies. Despite the high association with genetic factors, according to GWAS and large meta-analyses, the results to date explain only 1-2% of the heritability[10]. Another meta-analysis study was conducted based on environmental stress factors such as emotional, physical, and sexual abuse. The evidence suggests that the patients who have been the victims of emotional abuse during their childhood are 4 times more likely to develop BD compared to the control groups[9].

1.1.3. Schizophrenia

Schizophrenia is a severe psychiatric disorder characterized by significant impairment in emotional responses, cognitive processing, and social function. It is associated with a variety of symptoms typically divided into

three categories. The psychotic symptoms are characterized by changes in a perception of reality which includes hallucinations, delusions, as well as movement, and thought disorder[3]. The majority of patients usually tend to lose motivation or interest in social life activities, as well as experiencing emotional expression, defined as negative symptoms. Cognitive symptoms refer to a deficit in memory processing, attention, and concentration, disabling individuals to perform daily activities such as attending school classes or working. The illness is commonly diagnosed in early adulthood and its long-term impairments are associated with a high health care burden[11]. According to WHO, 0.32 % of the general population (24 million people) are affected by this condition, but only 31.3% of these are receiving mental care treatment[12]. In one GWAS study conducted on 76,755 schizophrenia patients and 243,649 control participants, 4 genes (*SP4*, *FAM120A*, *GRIN2A* and *STAG1*) were identified to have a convergence of common variant associations, suggesting a strong relationship between their altered function and development of the disease. These results suggest that neurons form the basis of the condition's pathophysiology because common variant associations are referred to genes expressed in synapses of both inhibitory and excitatory neurons of the central nervous system. This is associated with functions related to synaptic differentiation, organization, and transmission, which may be disrupted by gene alteration. However, these variants refer only to a small subgroup of schizophrenia patients[6]. Hence, it is likely that the genetic approach is not providing enough relevant evidence for the target research, emphasizing that schizophrenia is a polygenic disorder in most cases[6]. The complexity of these conditions suggests that both genetic and environmental factors play a key role in, the still largely unknown, pathology of the disease. A particular large-scale twin study was conducted through the access of two nationwide registers of twins in Denmark where they tested the heritability of schizophrenia. The evidence showed a heritability rate of 79% in identical twins, demonstrating a substantial genetic predisposition risk having a significant influence. Furthermore, a concordance rate of 33% in non-

identical twins is much higher than the rate in the general population[13,14]. However, the rate is not completely dependent on genetic components, meaning that environmental factors might play a role in the penetrance of schizophrenia as well[14].

1.2. Role of environmental factors in CMI

Mental illness are characterized by significant overlap in their symptomology and genetic predisposition. Environmental factors in combination with high-risk genes, can have a significant contribution to the occurrence of psychiatric conditions. One example of an environmental factor is infection, for instance by *T. gondii*, a protozoan parasite that infects humans and other warm-blooded species. Recent studies report significant relevance between infection and bipolar disorder phenotype, as there were increased levels of antibodies found in blood samples of bipolar disease and other CMI patients. This infection is associated with behavioral alteration in rodents. Furthermore, research evidence indicates a presence of metabolic alteration, as dopamine production is increased due to infection. This biochemical process is similar to potential manic episodes characteristic of bipolar disorder. Symptoms of cognitive decline as a result of local inflammation were present in both CMI-suffering individuals and people experiencing T.gondii invasion. The substantial evidence of this parasitic infection suggests that environmental factors can have a powerful impact on mental disorder development[15].

Recent studies have suggested neuroinflammation which occurs due to viral or bacterial infection can play a significant role in triggering CMI pathology. As a response to the infection, immune system cell recruitment begins locally in the brain, where certain pro-inflammatory cytokines are released. These events have a tissue-destructive ability, which may cause particular symptoms present in psychotic phenotypes. This evidence is supported by

higher levels of pro-inflammatory markers identified in schizophrenia patients[16].

Another noteworthy factor is stress caused by urbanization and migration. Meta-analysis has shown that individuals living in cities with large population have an increased rate of psychotic symptoms compared to people living in rural areas[17]. A higher risk of developing psychotic disorders can also be noticed in the migrant population compared to a control group. Considering this, particular stressful social events could be one of the trigger external factors for the development of such deteriorating conditions[18]. Childhood trauma is one of the major factors participating in schizophrenia and bipolar disorder development. Emotional neglect is the most common type of trauma, resulting in severe impairment in social and cognitive functions, and complications with learning and memory processing[19]. It is associated with aberrant size and function of brain compartments, such as a decrease in hippocampal size and increased activity of the amygdala[20].

1.3. Protein aggregation

As the genetic approach has so far only had limited success in identifying new diagnostic and therapeutic targets, another approach is required, from a different scientific perspective that might help in a better understanding of the biological mechanisms occurring in these conditions. Recent studies implicate/propose that aberrant proteostasis could be the starting point for mental illness background explanation. In post-mortem samples of patients that suffered from neurodegenerative diseases, particular protein deposits have been detected[21]. For instance, Alzheimer's disease is characterized by tau neurofibrillary tangles and beta-amyloid peptide aggregates, making them the hallmarks of this disorder. These deposits are formed by the misassembly of insoluble protein fractions in the brain, promoting neuronal cell death if not treated properly[22]. Early-stage neurodegenerative disorders

have common features with CMI. For instance, initial behavioral symptoms present in both cases, making clinical diagnosis more complicated[23,24]. Insoluble protein fractions were detected in brain tissue samples from a subset of CMI patients, similar to protein deposits forming in neurodegenerative conditions. This evidence proposes abnormal protein homeostasis as a potential approach to understanding the pathology of the disease[25]. Protein aggregates are formed after misassembling or misfolding, which leads to defective protein[26].

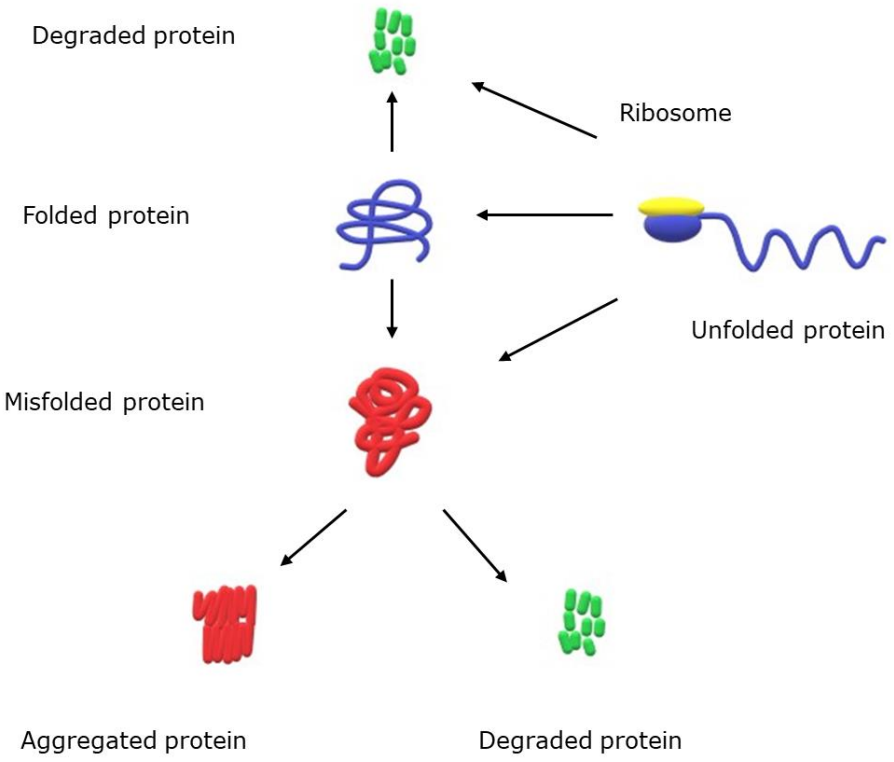

Figure 1. Schematic representation of protein misfolding and aggregation resulting in cell accumulation as insoluble protein fractions. Adapted from Chaturvedi *et al.* 2016[27].

Protein aggregation is used as an approach due to its implication in other clinical abnormalities, such as cardiovascular and pulmonary diseases, cancer, and neurodegenerative disorders. Disfunction in the ubiquitin-

protease mechanism has been detected in schizophrenia patients, suggesting it could be a hallmark of aberrant proteostasis in their brains. This process enables the elimination of misfolded and toxic proteins from cells. The regulation of protein homeostasis is essential for normal cell function. This biological background could be a potential reason for unexplained impairment in psychiatry phenotypes[25].

1.4. Proteins associated with chronic mental illness

Research conducted on *in vitro* samples, *in vivo* animal testing, and post-mortem human brain samples offer strong evidence of aberrant proteostasis in mental illness. Potentially aggregating proteins characterized in this manner include Trio-Binding Protein 1 (TRIOBP-1), Disrupted in Schizophrenia 1 (DISC1), Collapsin Response Mediator Protein 1 (CRMP1), and Neuronal PAS Protein 3 (NPAS3)[28–31]. Additionally, the EH domain-containing protein 3 (EHD3) protein was identified in schizophrenia-affected brain tissue samples. These protein candidates seem to misfold and/or form insoluble aggregates the brains of patients with schizophrenia orother chronic mental illnesses[26].

1.4.1. Trio-Binding Protein 1 (TRIOBP-1)

TRIOBP-1, also referred to as Tara, is one of several TRIOBP isoforms important in the regulation of the actin filaments in the cytoskeleton. Its initial function is to bind to F-actin, a base part of the cytoskeleton, formed by globular G-actin unit polymerization. Following F-action binding, TRIOBP-1 can inhibit/suppress its depolymerization, preventing it from returning to the G-actin form. This regulation pathway plays a role in the mobility and development of neuronal cells. Hence, an effective regulatory pathway is

essential for balance in neuronal environment[32]. TRIOBP-1 forms insoluble aggregates in brain samples of patients suffering from schizophrenia. It is assumed that this occurred as a result of coiled-coil domain interactions between a few TRIOBP-1 proteins, accumulating and forming larger protein clusters[33].

1.4.2. Disrupted in Schizophrenia 1 (DISC1)

DISC1 was identified as a candidate protein in a particular Scottish family with a clinical history of mental illness. Mutation of the DISC1 gene is located as a translocation between balanced chromosomes 1 and 11 t(1; 11), causing the disruption[28]. It is a scaffolding protein involved in the development and signaling pathways, as well as cytoskeletal regulation in neuronal cells. DISC1 has shown aggregation properties in brain tissue samples from patients suffering from various CMI, and the aggregates show cell-to-cell transmission[34]. *In vivo* transgenic rat study with expressed DISC1 confirmed the presence of insoluble aggregates. Furthermore, tested animals had a significant increase in dopamine, as well as an extreme increase or decrease in D2 receptors, suggesting that dopamine metabolic pathways might be disrupted in their schizophrenia[35].

1.4.3. Collapsin Response Mediator Protein 1 (CRMP1)

CRMP1 is a neuronal regulator of synaptic development, signaling and function. It is known to form insoluble proteins in schizophrenia and bipolar disorder-affected patients, but not in major depressive disorder patients or controls. Evidence shows that the short version of CRMP1 (CRMP1sv) co-aggregates with DISC1, forming visible aggregates both in cell culture and in post-mortem samples of schizophrenia-derived brains. This suggests that

CRMP1 might have a higher affinity for aggregation when co-localized with aggregating DISC1 protein. However, the long version of CRMP1 (CRMP1lv) does not have significant aggregation properties in brain tissue samples, although it forms aggregates in cell culture studies[30]. Recent studies propose CRMP1 as a factor in insoluble misfolded protein formation in Huntington's disease (HD), emphasizing its ability to suppress aggregation in this condition. This potential discovery could be implicated in the development of therapeutic strategies for HD and other neurodegenerative diseases[36].

1.4.4. EH domain-containing protein 3 (EHD3)

Recently, a new protein found to have the effect of misfolding/aggregation in chronic mental illness is EHD3. The gene for this protein is situated/localized on chromosome 2p22-23. EHD3 is expressed in endocytic vesicles, suggesting an implication in endocytic signaling transport. However, it is predominantly expressed on tubules due to the N-terminal domain of this protein, essential for endocytic tubular localization[37]. Another function dependency derived from the research establishing the endocytosis effect of D1 receptor recycling[38]. This process is potentially associated with cognitive impairment of the dopamine system in schizophrenia. Initial clinical studies proposed increased activity of dopamine transmission linked to hallucinations and delusions, characterized as positive symptoms in schizophrenia[39]. Purified EHD3 plasmids from post-mortem brain samples transfected in SH-Y5Y neuroblastoma cells form visible insoluble aggregates in the cytoplasm. Hence, this indicated the potential of this multifunctional protein to form insoluble aggregates and initiates biological dysfunctions related to schizophrenia development (Dashi & Bradshaw, unpublished).

1.4.5. Neuronal PAS Protein 3 (NPAS3)

NPAS3 is a brain-expressed protein and has a function as a transcription factor in the nucleus. Its expression is disrupted by a chromosomal translocation identified in a small family where mother and daughter were affected with schizophrenia[40,41]. Evidence from GWAS conducted on psychiatric patients emphasizes a potential risk of this gene identified as 1 of 226 genes associated with BD, as well as 1 of 9 genes additionally expressed in the dorsolateral prefrontal cortex from patients suffering from BD[42]. A point mutation, where a valine amino acid was substituted with isoleucine (V304I), was detected in a small family, in which 3 family members were affected with schizophrenia, and one with major depressive disorder (Figure 1)[43]. This mutation leads to a mutant/abnormal version of NPAS3 protein, which was repeatedly seem to aggregate both as purified recombinant protein and when over-expressed in cell culture[31]. A potentially critical region of the protein, close to genes associated with protein stability, DNA binding affinity, dimerization and other neurological functions regarding its transcriptional activity, was found to cause its aggregation[44]. Hence, this aggregation is potentially responsible for NPAS3 dysfunction[31].

A recent study conducted in our laboratory suggests that the point mutation might not be the only potential cause of protein misfold and aggregation. Post-mortem brain samples of 40 individuals were purified, revealing insoluble protein fractions containing full-length NPAS3 protein in approximately 70% of the fractions. Although the enrichment was low in original whole-brain samples, the reason might be that this protein is highly expressed in the early neurodevelopment stage, following a gradual decrease in protein level through adulthood. Knowing that aberrant proteostasis is common in other neurodegenerative diseases, a relevance between proteins with similar physiological functions might be the key to underlying pathological events in these conditions[45]. For instance, TDP-43, associated with ALS, is a nuclear protein as well as NPAS3, characterized

by aggregate formation in the cytoplasm instead of the nucleus in the early stages of the disease. Once the insoluble aggregates form, TDP-43 remains in the cytoplasm, losing its ability to translocate back into the nucleus[46,47]. In a similar manner, neuroblastoma cells were transfected and analyzed by fluorescence microscope. Both wild-type and mutant version NPAS3 were mainly expressed in the nucleus. However, in some cases proteins were identified in the cytoplasm, supporting the idea of nuclear protein aggregation both in schizophrenia and ALS[45].

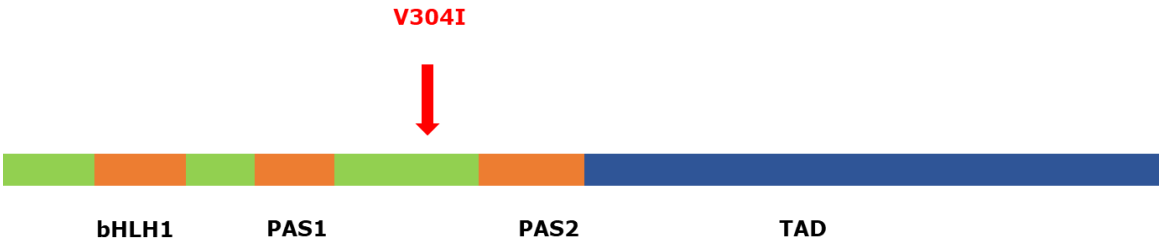

Figure 2. Domain structure of NPAS3 and the location of V304I mutation. Adapted from Samardžija et al (2021)[45]

Environmental factors affection the aggregation

Since proteins regulate every biochemical process in our body, a proper way of folding into compact structures/conformation is crucial for proteostasis. However, some external circumstances such as environmental stress or mutation can induce protein misfolding/aggregation[48,49]. For instance, oxidative stress appears to be an important factor leading to an imbalance of protein homeostasis. The process is based on reactive oxygen species (ROS) chemical interactions with protein residues. ROS are commonly present in organisms, and are formed through a wide range of chemical reactions. Once formed, they are able to attack and damage all cellular molecules, including lipids, DNA, and proteins. Our cellular system has developed a strategy for ROS removal through the stock of antioxidants that are able to convert ROS into neutralized derivates. Hence, it is

understandable that the production and scavenging of ROS, more precisely, pro-oxidative and antioxidative reactions should be in balance in order to maintain a proper cell regulation system. However, this highly efficient mechanism is able to repair only a specific group of proteins containing sulfur derivates on their oxidized amino acid residues. Other oxidized proteins are subjected to proteolytic degradation by specific intracellular mechanisms, such as the ubiquitin-proteasome pathway. If this system is not sufficient enough, or if ROS are over-produced, the oxidized protein content will increase, eventually leading to protein misfolding and aggregation[50]. These aberrant-structured proteins are implicated in the progression of various disorders, emphasizing the importance of further investigation of the mechanisms related to this phenomenon[31].

One of the inducers of ROS generation in the brain is iron. It is a redox-active metal associated with the regulation of metabolic homeostasis. Iron is an important cofactor of catalytic reactions in the human brain. Imbalance in the homeostasis of cellular iron can lead to mitochondria dysfunction and activation of microglia in the brain. However, the potential to perform one-electron reactions, following a generation of oxidative stress is associated with ALS development[51]. The feature of this disorder is characterized by the accumulation of aggregated protein deposits. The most abundant protein identified in aggregated inclusions of majority ALS cases is TDP-43[52]. It is a regulator of gene expression, located inside the nucleus. However, inclusions of aggregated TDP-43 were detected in the cytoplasm in all sporadic cases of ALS (85-95% of ALS cases), emphasizing the propensity of TDP-43 to mislocalize in this disorder[53]. Wild type TDP-43 also mislocalizes as a result of oxidative stress treatment with sodium arsenide, implicating that aggregation can be induced by stress *in vitro*[54]. It is demonstrated that ROS are associated with the dislocation of the protein in the cytoplasm, followed by insoluble aggregate formation. This can potentially induce neuronal toxicity pathways, leading to the degradation and progressive death of neurons[53].

2. Aims of the thesis

Recent studies have proposed several proteins that might misassemble/aggregate in CMI, including TRIOBP-1, DISC1, CRMP1, EHD3 and NPAS3. The focus of this thesis is to test if stress factors can affect the potential of these proteins to aggregate. Our hypothesis is that one or more of them will have an aggregation caused by stress factors. Given that TDP-43 aggregates when relocated in the cytoplasm in ALS or as a response to stress induction, it would be interesting to test NPAS3 (nuclear protein as well as TDP-43) using this particular assay[53,54]. The stress factors used in experiments are sodium arsenide, iron(II) chloride, calcium chloride, zinc acetate and proteasome inhibitor MG132, applied in different concentrations depending on cell susceptibility. The proteins were analyzed to identify which were most affected by stress factors *in vitro*. The proteins that showed an expression response to the treatment were followed by additional testing with the most effective factors. These studies could provide a potential discovery regarding environmental stress affecting high-risk aggregating proteins associated with CMI and therefore explain the complex pathological mechanism behind these neurological disorders.

Hence, the aims of this thesis are:

1. To investigate which protein (TRIOBP-1, DISC1, CRMP1, EHD3, NPAS3) has the most striking effect after stress factor treatment,
2. To determine which stress factors affect protein aggregation in cell culture,
3. To determine the concentations of this stress factor that are effective at inducing aggregation.

3. Materials and Methods

3.1. Enzymes, stains and commercially prepared kits

QIAprep Spin Miniprep Kit (QIAGEN), Gateway LR Clonase II Enzyme Mix (Thermo Fisher Scientific), InstantBlue Protein Stain (Expedeon), my-Budget DNA/RNA Stain Green (Bio-Budget Technologies GmbH), Proteinase K Solution (Thermo Fisher Scientific), Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific), my-Budget Gel Extraction Kit (Bio-Budget), T4 DNA Ligase (New England Biolabs)

3.2. Size Markers

my-Budget 1 kb DNA Ladder (200 µg/mL) (Bio-Budget Technologies GmbH): 13 blunt-ended fragments in the range from 250 bp (base pairs) to 10 kbp (kilobase pairs).

my-Budget 100 bp + 1.5 kb DNA Ladder (200 µg/mL) (Bio-Budget Technologies GmbH): 10 blunt-ended fragments in the range from 100 bp to 1000 bp, with an additional fragment at 1500 bp.

my-Budget Prestained Protein Ladder 10-180 kDa (0.2-0.4 µg/µL) (Bio-Budget Technologies GmbH): proteins in size range between 10 kDa and 180 kDa, with an additional fragment at 1500 bp and is used for Western blot transfers performed in Tris-Glycine buffer.

3.3. Antibodies

Table 1. List of primary antibodies used in Western blot and cell staining (immunocytochemistry) complete with supplier's name, host species, concentration, and dilution

Name	Type	Supplier	Host	Concentration	Dilution
Anti-HA	Monoclonal	Sigma	Mouse	1 mg/mL	1:1000
Anti-Flag M2	Monoclonal	Sigma	Mouse	1 mg/mL	1:1000
Anti-GFP	Monoclonal	Sigma	Mouse	1 mg/mL	1:1000

Table 2. List of secondary antibodies, cytoskeletal and nuclear stains used in cell staining (immunocytochemistry) and Western blot complete with supplier's name, concentration, and dilution

Name	Supplier	Concentration	Dilution
Alexa Fluor 594 Goat anti-Mouse IgG	Thermo Fischer Scientific	2 mg/mL	1:500
Alexa Fluor 555 Goat anti-Mouse IgG	Thermo Fischer Scientific	2 mg/mL	1:500
Alexa Fluor 488 Goat anti-Mouse IgG	Thermo Fischer Scientific	2 mg/mL	1:500
Phalloidin-iFluor 488 Reagent	Abcam	1000x stock solutions	1:500

(cytoskeletal stain)			
DAPI (nuclear stain)	Sigma	1 mg/mL	1:500
Peroxidase Conjugated Affinity Purified Goat anti-Mouse IgG (GAM)	Thermo Fischer Scientific	1 mg/mL	1:10 000 for samples from HEK cells

3.4. DNA plasmids

Table 3. List of the DNA plasmids used

Vector	Encoded protein	Source
pdcDNA-FlagMyc	Flag-TRIOBP-1	Nicholas Bradshaw & Carsten Korth, Düsseldorf
pdcDNA-FlagMyc	Flag-DISC1	Beti Zaharija & Nicholas Bradshaw, Rijeka
pdcDNA-FlagMyc	Flag-CRMP1 Sv	Nicholas Bradshaw, Rijeka
pdcDNA-FlagMyc	Flag-EHD3	Giovanna Dashi & Nicholas Bradshaw, Rijeka
pdcDNA-FlagMyc	Flag-NPAS3	Mihaela Bergman & Nicholas Bradshaw, Rijeka

pDEST-CMV-N-EGFP	EGFP-TRIOBP-1	Maja Juković & Nicholas Bradshaw, Rijeka
pDEST-CMV-N-EGFP	EGFP-DISC1	Beti Zaharija & Nicholas Bradshaw, Rijeka
pDEST-CMV-N-EGFP	EGFP-CRMP1 Sv	Beti Zaharija & Nicholas Bradshaw, Rijeka
pDEST-CMV-N-EGFP	EGFP-EHD3	Beti Zaharija & Nicholas Bradshaw, Rijeka
pDEST-CMV-N-EGFP	EGFP-NPAS3	Beti Zaharija & Nicholas Bradshaw, Rijeka
pCI-HA	HA-NPAS3 full length	Fred Berry, Edmonton, Alberta, Canada
pdcDNA-FlagMyc	(Empty destination vector)	BCCM/LMBP (4705)
pDEST-CMV-N-EGFP	(Empty destination vector)	Robin Ketteler, University College London, UK, Addgene clone 122842

3.5. Stress factors

Table 4. List of the stress factors used in experiments with the solvent used for each stress factor

Name	**Concentration (final)**	**Solvent**
Sodium arsenide	5 µM	dH_2O
Iron (II) chloride	30 µM	dH_2O
Calcium chloride	1000 µM	dH_2O
Zinc acetate	1000 µM	dH_2O
MG132 (proteasome inhibitor)	10 µM	DMSO

3.6. LR clonase reaction

LR clonase recombination reaction was used to generate expression vectors. Briefly, in the LR reaction, an entry vector containing a gene of interest was recombined with the destination vector (pdcDNA-FlagMyc or pDEST-CMV-N-EGFP). The LR clonase reaction mixture (150 ng destination vector, 100 ng entry vector, TE buffer to a total of 9 µL, 1 µL LR clonase) was prepared and incubated at 25°C for 1 hour. The reaction was terminated by adding 1 µL of Proteinase K solution. After incubation at 37°C for 10 minutes, the samples were either stored at -20°C or directly used for bacterial transformation into NEB5α (New England Biolabs) competent *E.coli* cells.

3.7. Growth of plasmids in bacterial culture

Plasmid constructs containing a gene of interest (*TRIOBP-1, DISC1, CRMP1 Sv, EHD3* or *NPAS3*) were transformed into competent bacteria. For each transformation 1 µL of plasmid was pipetted in a sterile 1.5 mL Eppendorf tube and mixed with 50 µL of freshly thawed NEB5α cells. The tube was incubated on ice for 30 minutes, followed by heat shock transformation (30 sec/42°C) to open membrane pores and incorporate foreign DNA. At that point, the transformed bacteria were allowed to recover on ice for 5 minutes. Optionally, plasmid constructs that carry a gene for kanamycin antibiotic resistance were mixed with 250 µL of LB media (10 g tryptone, 5 g yeast extract, 5 g NaCl, dH_2O added up to 1 L, pH adjusted to 7.0) and incubated for 1 hour (37°C/250 rpm). Transformed bacteria were grown on LB agar plates (1 g tryptone, 0.5 g yeast extract, 0.5 g NaCl, 1.5 g Agar, dH_2O added up to 100 mL) containing the antibiotic of interest. The constructs with Flag, EGFP and HA destination vectors were grown on LB agar plates containing ampicillin (100 µg/mL) and placed in an incubator at 37°C overnight. The next day, an individual bacterial colony was picked from the LB agar plate and transferred into a 15 mL Falcon tube containing 3 mL of LB media with ampicillin (100 µg/mL). The samples were incubated overnight (37°C/250 rpm).

Bacteria that had grown overnight were harvested by centrifugation at 3700 rpm (15 min/4°C). The supernatant was discarded and the pellet was resuspended in buffer P1 according to the manufacturer's instructions (Qiagen QIAprep Spin Miniprep Kit). The sample was transferred to a 1.5 mL Eppendorf tube and mixed with buffer P2 that lysed the bacterial cells. Buffer N3 was added to neutralise the suspension within 5 minutes after which it was centrifuged (10 min/13 000 rpm). The supernatant was transferred to the QIAprep 2.0 spin column and centrifuged (1 min/11 000 rpm). After every subsequent centrifugation, the flow-through in the spin column was discarded. The column was washed with buffer PE and

centrifuged (1min/11 000 rpm) two times in a row to remove the residual wash buffer. The column was then placed in a sterile 1.5 mL Eppendorf tube followed by the addition of 50 µL TE buffer (0.5 mL 1 M Tris pH 7.4, 200 µL 0.25 M EDTA, dH_2O added up to 50 mL) preheated at 50°C to elute the plasmid DNA. The tube with a column inside was incubated at 25°C for 1 minute and underwent final centrifugation (1min/13 000 rpm). The column was discarded from the tube that contained a purified plasmid DNA. The concentration of plasmid was measured on a BioDrop µLITE spectrophotometer, with absorbance wavelength set at 260 nm. Elution TE buffer was used as a blank probe. For each concentration measurement, 1 µL of the sample was used and concentrations were expressed in µg/mL.

3.8. Cell culture and transfection

The HEK293T human kidney cell line and SH-SY5Y human neuroblastoma cell line were grown in T25 flasks or 12-well plates. HEK293T cells were grown in D-MEM media (ThermoFisher) containing 10% CCS (Cosmic Calf Serum), penicillin and streptomycin solution. SH-SY5Y cells were grown in D-MEM/F-12 (Gibco DMEM; ThermoFisher) containing 10% FCS (foetal calf serum), penicillin and streptomycin solution and 1x MEM non-essential amino acids. Cell cultures were kept in sterile conditions in an incubator (37°C/5% CO_2). Cells were split using Trypsin/EDTA enzyme (Pan Biotech) when the confluency in T25 flasks reached over 90%.

The HEK293T cell line was transfected with Metafectene (Biotex) and SH-SY5Y cell line with Metafectene Pro (Biotex).

For the transfection protocol, two sets of solutions were prepared; the first contained 0.5 µg of plasmid DNA and 100 µL D-MEM -/- (without serum or antibiotics), and the second 2 µL of the transfecting reagent (Metafectene /Metafectene Pro) per well. Solutions were incubated at room temperature for 5 minutes. After the incubation, 100 µL of the solution containing

transfecting reagent was added to each tube containing DNA plasmid and was placed in an incubator for 30 minutes(37°C/5% CO_2). The media with serum and antibiotics was removed from the wells and replaced with D-MEM -/- media. Solutions were pipetted in wells and incubated for a total of 6 hours. After the incubation, the media was replaced with fresh media containing serum and antibiotics. 12-well plates were incubated overnight.

Subsequent experiments for SH-SY5Y cells were cell stressing treatment, cell staining (immunocytochemistry) and fluorescent microscopy. Transfection of the HEK293T cells was followed by cell lysis and Western blotting.

3.9. Cell stressing treatment and immunocytochemistry

Solutions containing stress factors were thawed 15 minutes before the stress treatment. The day after the transfection, the media was replaced with fresh media (containing serum and antibiotics) and treated with a stress factor solution. The samples were placed in an incubator for 3 hours (37°C/5% CO_2). After the incubation, SH-SY5Y cells growing on a glass coverslip were gently washed with 1x PBS (Phosphate buffered saline; 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4, dH_2O added up to 1L, pH adjusted to 7.4) per well, fixed with Fixation Buffer (8 g paraformaldehyde, 20 mL 10x PBS, dH_2O added up200 mL, pH adjusted to 7.4) for 15 minutes and permeabilized with Permeabilizing Buffer (10 mL 10x PBS, 10 mL 10% Triton X-100, dH_2O added up to 1 L) for 10 minutes. Cells were gently washed three times with 1x PBS and blocked in 10% goat serum/PBS solution for 45 minutes. After the incubation, a blocking solution was removed and cells were treated with a primary antibody solution (Table 1). The primary antibody was diluted in 10% goat serum/PBS solution and incubated on the coverslips for 4 hours. Following the incubation, cells were washed 3 times with 1x PBS and treated with secondary antibody. The

solution contained a DAPI antibody to stain the nucleus and fluorescent phalloidin to stain the actin cytoskeleton (Table 3), each diluted 500-fold in 10% goat serum/PBS solution. Cells were incubated for 1 hour in the dark. After the incubation, coverslips were washed 3 times with 1x PBS and additionally with dH_2O to remove excess salt. Coverslips were removed for the wells and attached to slides with commercial Mounding Medium Flourshield (Sigma). The cells on the coverslips were visualized on an Olympus IX83 fluorescent microscope under 60x magnification.

3.10. Cell lysis

The day after the transfection, HEK293T cells were washed twice with 1x PBS. After the washing, PBS was removed and 100 µL of Cell Lysis Buffer (5 mL 10x PBS, 5 mL 10% Tripton x-100, 1 mL 1M $MgCl_2$, 50 µL DnaseI, 50 µL 100 mM Phenylmethyl-sulphonyl fluoride, dH_2O added up to 50 mL) was applied to each well and incubated on ice for 5 minutes. The Cell Lysis Buffer solution was completed with a protease inhibitor cocktail (1x concentration) and Dnase I (0.5 µL per 1 mL of the buffer). Lysed cell suspensions were collected from the surface of the plate into 1.5 mL Eppendorf tubes and incubated on a rotor for 30 minutes. Samples were prepared for SDS-PAGE by adding Protein Loading Buffer (6.25 mL 1M Tris pH 6.8, 10 mL glycerol, 20 mL 10% SDS, 3.75 mL dH_2O, 5 mg bromophenol blue) in the same volume as Cell Lysis Buffer and 1M DTT (10% of the volume of Cell Lysis Buffer). The tubes were heated on the thermo-block at 95°C for 5 minutes to denature the proteins. Prepared samples were ready to be used for SDS-PAGE or stored at -20°C.

3.11. SDS-PAGE and Western blotting

Acrylamide running gel

Table 5. Measurement for handmade 8% and 10% acrylamide running gels

	dH_2O	30% acrylamide	1.5M Tris [pH 8.8]	10% SDS	10% APS	TEMED
8%	5.5 mL	3.2 mL	3.0 mL	120 µL	120 µL	12 µL
10%	4.8 mL	3.9 mL	3.0 mL	120 µL	120 µL	12 µL

Acrylamide stacking gel

Table 6. Measurements for a handmade acrylamide stacking gel

dH_2O	30% acrylamide	1M Tris [pH 6.8]	10% SDS	10% APS	TEMED
2.6 mL	1 mL	625 µL	50 µL	50 µL	5 µL

Acrylamide running and stacking gels were handmade by using the Mini-PROTEAN Tetra Handcast system (BioRad). The stacking gel has a lower polyacrylamide concentration; therefore it is placed on the top of the running gel to create an ionic gradient that concentrates the protein in a single band. Once the protein reaches a more concentrated running gel with smaller pores, it will separate according to its molecular weight.

Firstly, handmade acrylamide running gel (Table 4) and stacking gel (Table 5) were prepared using 30% acrylamide solution (14.6 g acrylamide, 0.5 g *N,N'*-methylbisacrylamide, dH_2O added up to 100 mL). SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was performed to separate proteins by their molecular weight. Protein samples and my-

Budget Prestained Protein Ladder 10kDa-180 kDa marker (Bio-Budget Technologies GmbH, 0.2-0.4 µg/µL) were thawed and loaded onto 8% (for proteins encoded by pdcDNA-Flag, pCI-HA vectors) and 10% (for proteins encoded by pDEST-CMV-N-EGFP vectors) gels. The gels were run at 180 V for 45 min in 1x SDS-PAGE 1x running buffer (10x stock solution; 30 g Tris, 144 g glycerine, 10 g SDS, dH_2O added up to 1 L). After the electrophoresis was finished, the proteins were transferred from gel to a Parablot PVDF membrane (Macherey-Nagel, 0.2 µm pore) by using the Trans-Blot Turbo Transfer System (BioRad) which ran for 30 minutes at 25 V and 0.5 A when one gel was used and 1.0 A when two gels were used. The membranes were then stained with Ponceau S solution (1 g Ponceau S, 4 mL acetic acid, dH_2O added up to 200 mL) to visualize the total protein. Following the transfer, the membranes were incubated overnight at 4°C or placed on a shaker for one hour at room temperature in 5% milk powder/PBS-Tween solution to block it. After the blocking, the proteins were detected using a primary antibody (Table 1) diluted 2000-fold (anti-Flag M2 and anti-HA) or 1000-fold (anti-GFP) in PBS-Tween overnight at 4°C or incubated for 4 hours at room temperature. Membranes were then washed 3 times over 30 minutes using PBS-Tween (100 mL 10x PBS, 900 mL Mili-Q H_2O, 500 µL Tween 20). Secondary antibody was prepared 10 000-fold (Table 3) in PBS-Tween solution and membranes were incubated for 1 hour at room temperature. After the incubation, the membranes were washed 3 times over 30 minutes using PBS-Tween. Pierce ECL Prime Western Blotting Substrate (ThermoScientific) was used to visualize protein bands on a ChemiDoc MP Imaging System and Image Lab (BioRad).

3.12. DNA gel electrophoresis

Agarose gel

- 0.5 g agarose, 50 mL 1x TAE buffer, 0.5 µL DNA stain

DNA gel electrophoresis was used to separate plasmid DNA based on their size. Agarose gels were mixed and heated in short intervals to fully melt. After the mixture was cool enough, DNA Stain Green was added to the gel which was then placed inside the electrophoresis tank filled with 1x TAE buffer (50x stock solution; 242 g Tris, 18.61 g EDTA, 57.1 mL acetic acid, dH_2O added up to 1L). The marker was prepared by pipetting 0.5µL 10x FastDigest buffer, 2.5 µL my-Budget 1 kb DNA Ladder (200 mg/mL) (Bio-Budget Technologies GmbH) and 7 µL of dH2O, and samples were prepared by pipetting 1 µL 10x FastDigest buffer, 2µL of DNA and 7 µL of dH2O. 10 µL of each sample was loaded in the gel and run at 180 V for 20 minutes and visualized using the ChemiDoc MP Imaging System and Image Lab (BioRad).

4. Results

It has been proven that environmental stress can influence protein aggregation in neurodegenerative disorders[15–20]. Since the insoluble protein fractions have been found in a subset of post mortem brain samples from psychiatric patients, we are investigating the stress factors' effect on protein aggregation using an *in vitro* assay. Plasmids encoding proteins that are known to aggregate in chronic mental illness were generated and transfected in the human kidney cell line (HEK293T) and human neuroblastoma cell line (SH-SY5Y), and then subjected to stress factors.

4.1 Verification of protein size by Western blot

Firstly, the plasmids generatedwere tested by Western blot, after expression in HEK293T cells and cell lysis. Flag-tagged plasmids showed the expected size on membranes (Figure 3). TRIOBP-1 showed a molecular weight of 75 kDa, DISC1 of 105 kDa, CRMP1 Sv of 67 kDa and EHD3 of 65 kDa (Figure 3A). On the other membrane, Flag-NPAS3 wild type was expressed showing a molecular weight of 125 kDa, slightly higher than expected (Figure 3B). A potential reasons for this result could be post-translational modifications, which can increase a molecular weight of the protein.

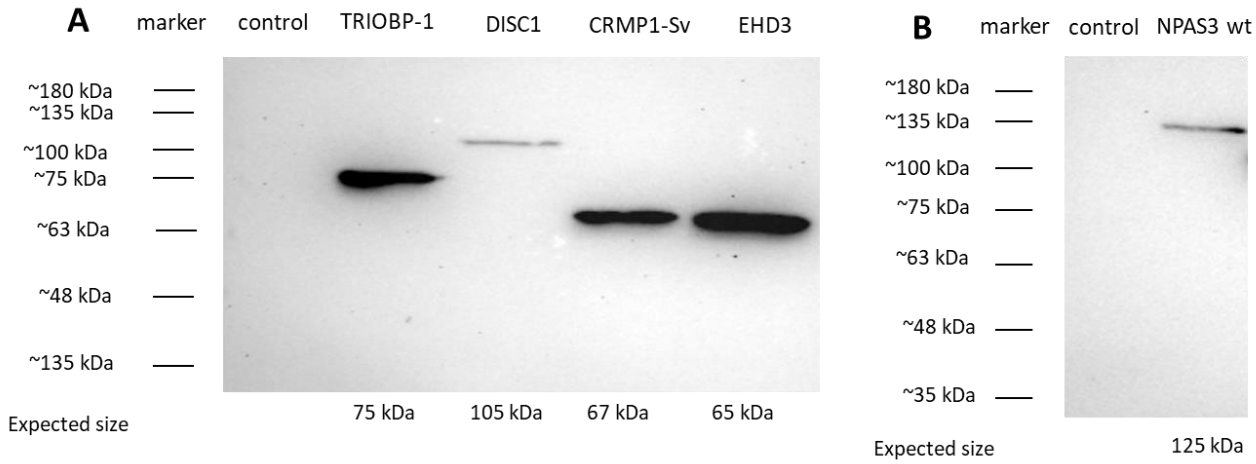

Figure 3. Western Blot analysis for anti-Flag M2 stained membranes with TRIOBP-1, DISC1, CRMP1 Sv, EHD3 and NPAS3 wild type, expressed in HEK293T cells. The membranes were stained with anti-Flag M2 primary antibody. The membranes were stained using goat anti-mouse (GAM) secondary antibody and visualized using ECL Prime Kit and ChemiDoc software. The exposure time for each of the membranes was 120 seconds. The protein marker used for molecular weight size comparison was Prestained Protein Ladder (10-180 kDa). Control represented lysates from mock treated transfected cells. Cells were transfected with 0.5 µg of the plasmid.

EGFP-fused proteins were tested using Western Blot and visualized on membranes (Figure 4A, 4B). The pDEST-CMV-N-EGFP expression vector expresses proteins with a bigger molecular weight than pdcDNA-FlagMyc expression vector, due to large size of EGFP. Hence, all the expressed plasmids show a bigger size on membrane bands. The molecular weight of TRIOBP-1, DISC1 and CRMP1 Sv showed the expected size on the membrane; 100 kDa, 130 kDa and 110 kDa. On the other hand, EGFP-EHD3 showed a lower molecular weight than expected of 95 kDa instead of the 105 kDa, expected. EGFP-DISC1 showed a lower expression level of plasmid than expected (Figure 4A). On the other membrane, EGFP-NPAS3 wild type showed an expected molecular weight size of 150 kDa, respectively (Figure 4B).

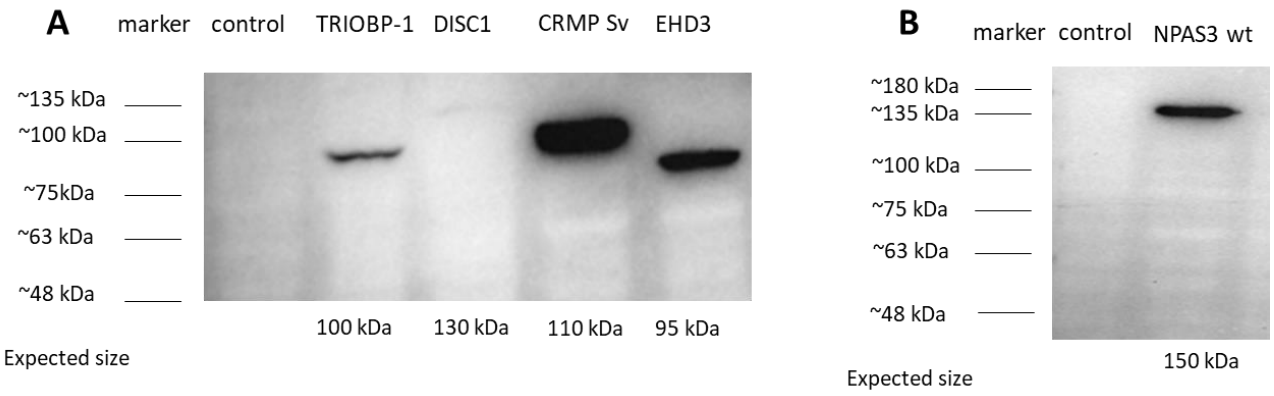

Figure 4. Western Blot analysis for anti-GFP stained membranes with TRIOBP-1, DISC1, CRMP1 Sv, EHD3 and NPAS3 wild type, expressed in HEK293T cells. The membranes were stained with anti-GFP primary antibody. The membranes were stained using goat anti-mouse (GAM) secondary antibody and visualized using ECL Prime Kit and ChemiDoc software. The exposure time for the membrane visualization was 300 seconds for membrane A) and 120 seconds for membrane B). The protein marker used for molecular weight size comparison was Prestained Protein Ladder (10-180 kDa). Control represented lysates from mock treated transfected cells. Cells were transfected with 0.5 µg of plasmid.

NPAS3 wild type was also expressed using pCI-HA expression vector and visualized on Western blot membrane. The construct showed the expected molecular weight size of 120 kDa (Figure 5).

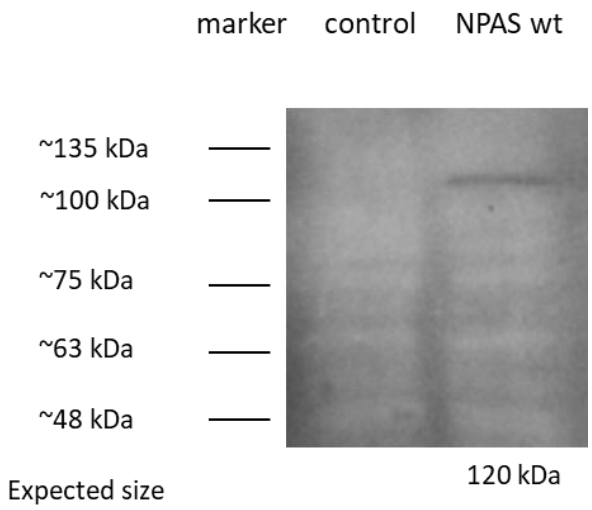

Figure 5. Western Blot analysis for anti-HA stained membrane with NPAS3 wild type, expressed in HEK293T cells. The membrane was stained with an anti-HA primary antibody. The membrane was stained using goat anti-mouse (GAM) secondary antibody and visualized using ECL Prime Kit and ChemiDoc software. The exposure time for the membrane visualization was 300 seconds. The protein marker used for molecular weight size comparison was Prestained Protein Ladder (10-180 kDa). Control represented lysates from mock treated transfected cells. Cells were transfected with 0.5 µg of plasmid.

4.2. Stress factor protocol optimization

The experimental protocol was generated by comparison of similar studies conducted on *in vitro* stress treatment and protein aggregation. Those studies were mostly focused on protein aggregation in neurodegenerative disorders[55–57]. We prepared stock solutions for each stress factor. The constructs that were tested for stress treatment protocol were TRIOBP-1, DISC1, CRMP1 Sv, EHD3 and NPAS3 wild type cloned in pdcDNA-FlagMyc expression vector and NPAS3 wild type cloned in pCI-HA expression vector. Initially, the cells were transfected and then immediately treated with stress factors during an 18-hour incubation. The samples were stress-treated a day after plasmid transfection. Next, the samples were fixed, permeabilized, and stained with primary and secondary antibodies, following the coverslip

preparation for the fluorescent microscopy. Initial concentrations of stress factors used in the experiments were 1000 μM for iron (II) chloride, calcium chloride and zinc acetate, 50 μM for sodium arsenide and 10 μM for MG132 proteasome inhibitor mixed with media solution, based on previous publications. The negative controls were treated only with cell culture media, without using stress factors. Most of the cells treated with these concentrations seem to be overexposed to the oxidative stress shock, resulting in cell death and deformation. For instance, when treated with sodium arsenide, Flag-CRMP1 Sv showed signs of aggregation, but the cell cytoskeleton lost its form, suggesting cell death (Figure 6A). This concentration and stress factor incubation period was fatal for Flag-NPAS3 expressed cells. The results show nucleus and cytoskeleton decline and cell death (Figure 6B). Furthermore, most of the samples showed a high number of dead cells, even though they were transfected. However, a low number of cells were successfully treated without visible oxidative stress damage, although without signs of protein aggregation (Figure 6C).

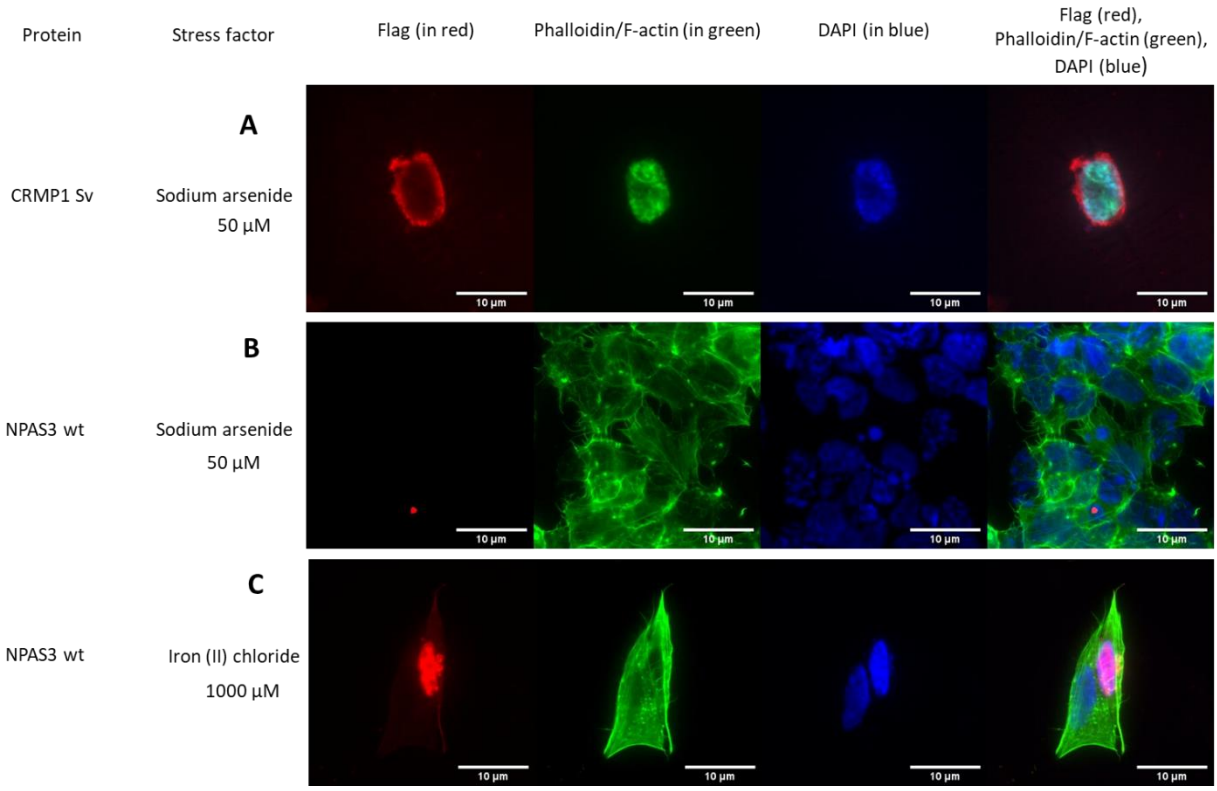

Figure 6. Fluorescent microscopy of SH-SY5Y cells transfected with CRMP1 Sv and NPAS3, stress treated for 18 hours with sodium arsenide and iron (II) chloride, showing the effect of oxidative stress damage. The proteins were labelled with anti-FlagM2 primary antibody and goat anti-mouse 555 nm secondary antibody, indicating the red signal. DAPI was used to stain the nuclei and phalloidin 488 nm was used to stain cellular actin. The obtained images were captured under 60x magnification on a florescent microscope using CellSens software. The scale bar represents 10 µm. CRMP1 Sv and NPAS3 did not show signs of aggregation as the cells were damaged due to stress factor treatment. All the images were obtained as a result of 2 independent experiments.

In order to optimize the protocol, we refined our approach. We treated cells with stress factors for 6 hours, instead of 18 hours. The cell death rate was lower compared to previous experiments (Figure 7). However, the cells still looked damaged due to stress treatment exposure. For instance, when treated with zinc acetate, cells would lose their normal structure, as the membrane would shrink around the nucleus (Figure 7A). Furthermore, the nucleus looked like it was no longer intact in some cases when treated with calcium chloride (Figure 7B).

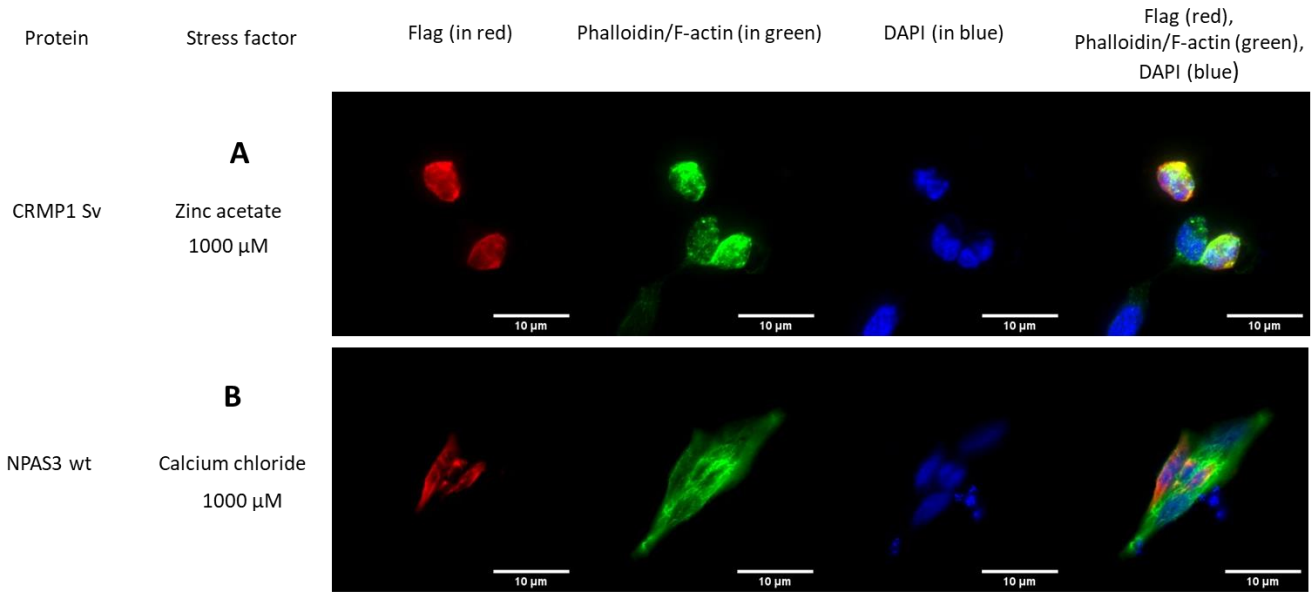

Figure 7. Examples of SH-SY5Y cells transfected with CRMP1 Sv and NPAS3, stress treated for 6 hours with zinc acetate and calcium chloride, showing the effect of oxidative stress damage. The proteins were labelled with anti-FlagM2 primary antibody and goat anti-mouse 555 nm secondary antibody, indicating the red signal. DAPI was used to stain the nuclei and phalloidin 488 nm was used to stain cellular actin. The obtained images were captured under 60x magnification on a florescent microscope using CellSens software. The scale bar represents 10 µm. CRMP1 Sv and NPAS3 did not show signs of aggregation as the cells were damaged due to stress factor treatment. All the images were obtained as a result of 3 independent experiments.

Since the cells had clear signs of oxidative stress damage, it was essential to modify the protocol further. We decided to stress treat transfected neuroblastoma cells for a period of 3 hours to see if that exposure results in cell damage or if the cells maintain health. The cells were not damaged in most cases, which is essential for protein aggregations to occur, as the transfection itself could only be carried out if the cells are healthy (Figure 8). The results indicate that the stress treatment for 3 hours was the most suitable, due to cells looking healthy. Furthermore, neuroblastoma cells show a clear sign of aggregation in some cases. For instance, when the cells were stress treated with sodium arsenide, NPAS3 showed signs of translocation to the cytoplasm, indicating the aggregation process has begun[45] (Figure 8B).

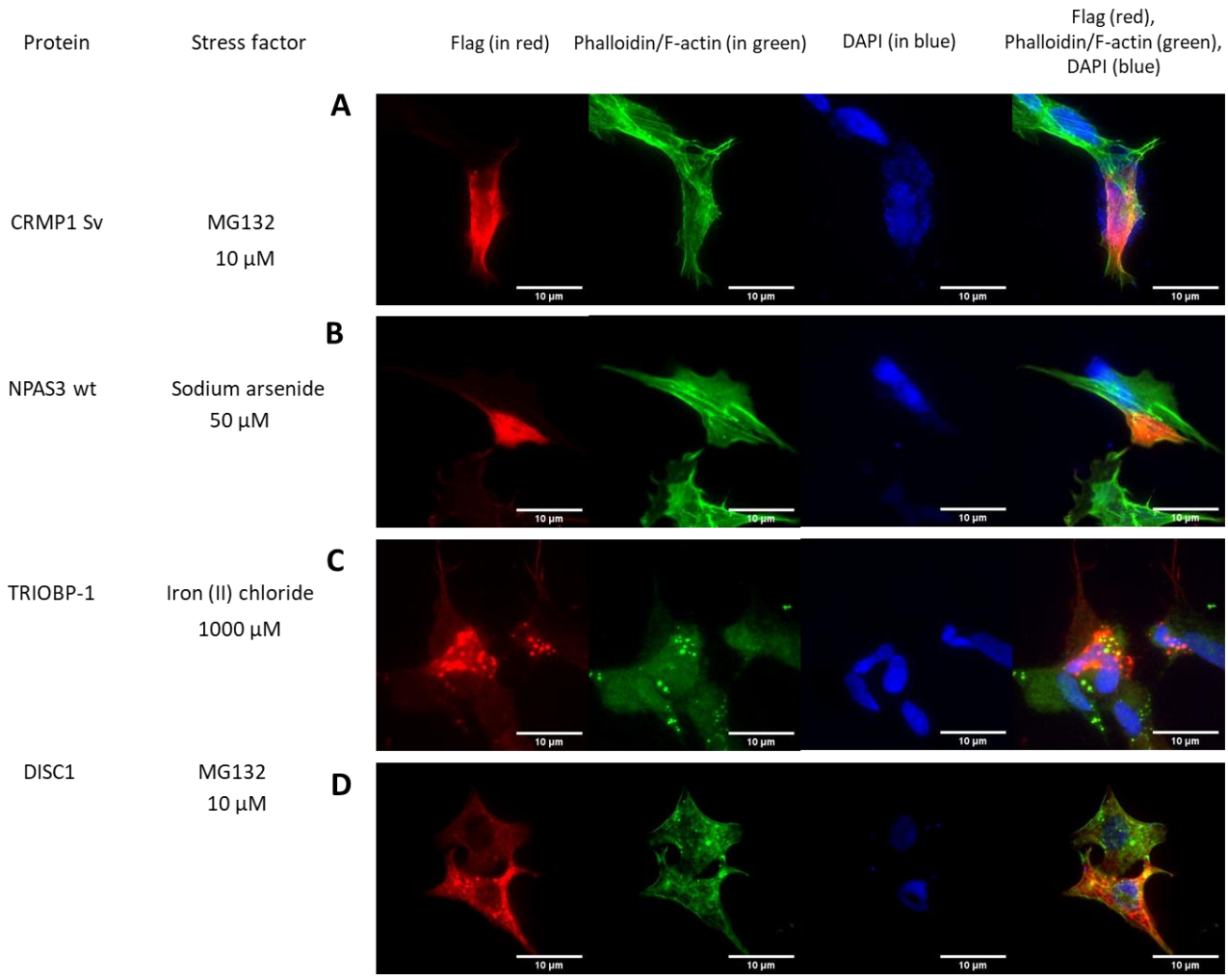

Figure 8. Examples of SH-SY5Y cells transfected with CRMP1 Sv, NPAS3, TRIOBP-1 and DISC1, stress treated for 3 hours with MG132, sodium arsenide and iron (II) chloride, showing the stress treatment effect. The proteins were labelled with anti-FlagM2 primary antibody and goat anti-mouse 555 nm secondary antibody, indicating the red signal. DAPI was used to stain the nuclei and phalloidin 488 nm was used to stain cellular actin. The obtained images were captured under 60x magnification on a florescent microscope using CellSens software. The scale bar represents 10 µm. Out of 4 proteins of interest shown on A-D, only CRMP1 Sv does not show any signs of stress treatment inducing the aggregation. All the images were obtained as a result of 3 independent experiments.

4.3. Aggregation of stress-treated proteins expressed with a Flag tag

In order to investigate the effect of oxidative stress effect on protein aggregation, it was essential to find out which expression vector is suitable

for protein visualization, offering a strong signal. We started by using the plasmids encoded in pdcDNA-FlagMyc expression vector, as the protein produced is smaller than the equivalent encoded by the pDEST-CMV-N-EGFP vector, making it easier to obtain the signal. EGFP tag vector is more likely to make smaller proteins aggregate, as it interacts with their shape, potentially obtaining false results regarding induced aggregation properties. All the Flag-generated plasmids that were used in the stress treatment experiments are listed in Table 7. All of the samples were stress treated for 3 hours. Most of the results indicated ”unclear” aggregation, normally because the cells were dead due to the intensive effect of oxidative stress or the aggregates could not be visualized. Additional treatment experiments are required to provide clear results on stress factors' effect on insoluble protein formation.

Table 7. Results on protein aggregation from plasmids encoded in pdcDNA-FlagMyc expression vector, transfected in SH-SY5Y cells and stress treated for 3 hours.

	TRIOBP-1	**DISC1**	**CRMP1 Sv**	**NPAS3**
Untreated cells	Aggregation	Aggregation	Unclear	Unclear
Sodium arsenide	Increased aggregation	Unclear	Unclear	Aggregation
Iron (II) chloride	Increased aggregation	Unclear	Unclear	Aggregation
Calcium chloride	Unclear	Unclear	Unclear	Unclear
MG132	Unclear	Increased aggregation	No aggregation	Unclear
Zinc acetate	Unclear	Unclear	Unclear	Aggregation

4.4. NPAS3 as a protein affected by stress treatment

Following the stress treatment protocol, we analyzed the results regarding the oxidative stress effect on the aggregation. Out of the 5 proteins of interest, NPAS3 had the most interesting results on stress treatment, with clear aggregation properties shown in initial experiments with sodium arsenide, iron (II) chloride and zinc acetate, while the protein did not form aggregates when expressed without an added stress factor (Table 7). It has previously been suggested that NPAS3 aggregation might be affected by sodium arsenide[45]. In this thesis, we took other stress factors into consideration, with the aim to screen them and optimize their application methods in experiments.

Follow-up experiments required testing if other destination vectors could assist with better visualization of insoluble proteins via fluorescent microscope. We switched to a new NPAS3 construct, using pCI-HA expression vector, which showed a much stronger signal compared to Flag-tagged plasmids. Hence, HA-NPAS3 wild type was used in follow-up experiments. Furthermore, the concentrations of stress factors were particularly high, which resulted in cell death in many cases. Hence, the concentrations of sodium arsenide and iron (II) chloride were diluted. Concentrations of sodium arsenide ranged from 50 nM to 50 μM, as for iron (II) chloride ranged from 10 μM to 1 mM. Samples treated with iron (II) chloride showed aggregation properties in most cells when treated with a concentration of 30 μM, where the NPAS3 translocated in the cytoplasm (Figure 9B). Following the same principle, samples treated with 5 μM of sodium arsenide had the most cells with aggregating NPAS3 in the cytoplasm (Figure 9C). The findings suggest that these concentrations have the most oxidative stress effect on NPAS3, resulting in protein function loss and diffusion in the cytoplasm of the cell. Due to interesting results regarding iron (II) chloride and sodium arsenide treatment, a quantitative

assay for both stress factors was attempted, but failed due to technical reasons, as will be discussed later.

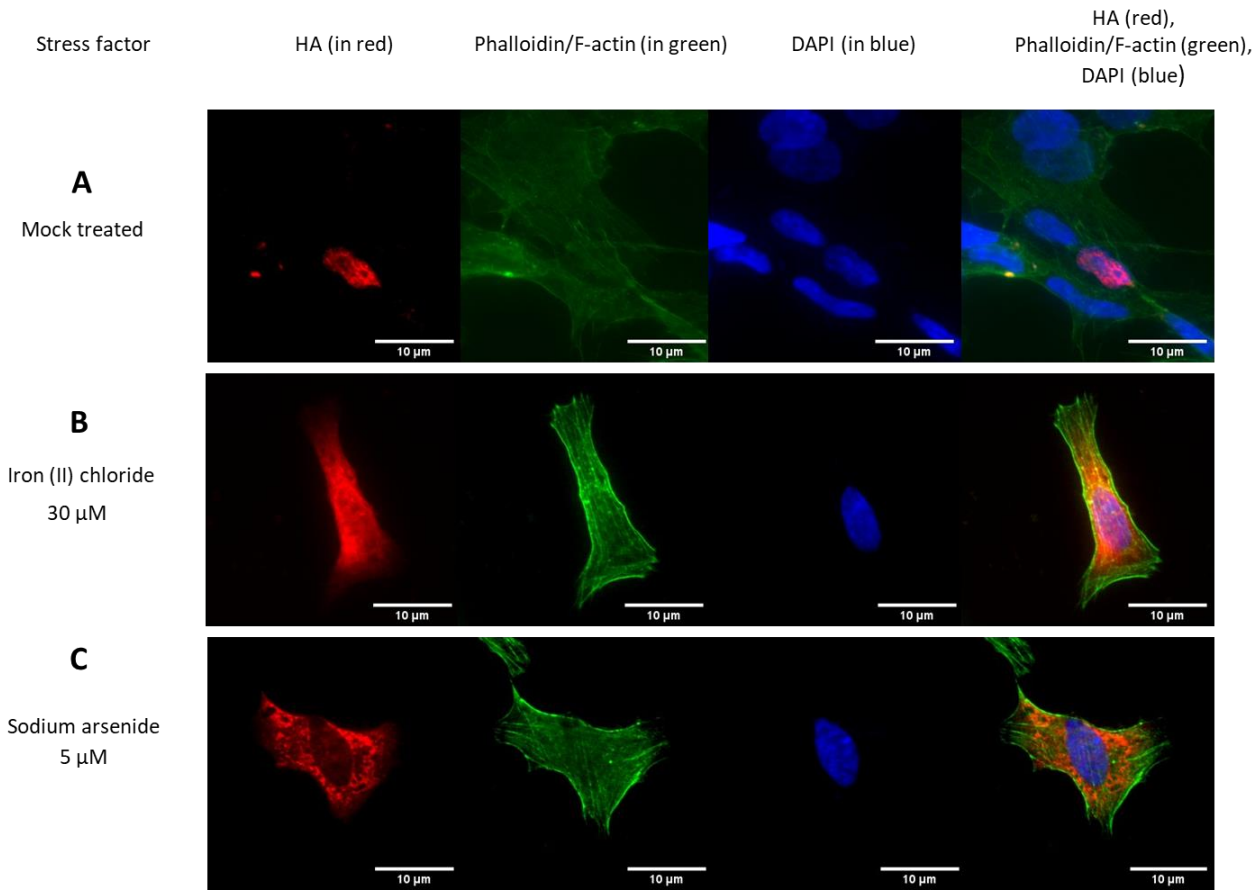

Figure 9. Fluorescent microscopy of SH-SY5Y cells transfected with NPAS3, stress treated for 3 hours with sodium arsenide and iron (II) chloride, showing the stress treatment effect. The proteins were labelled with anti-HA primary antibody and goat anti-mouse 555 nm secondary antibody, indicating the red signal. DAPI was used to stain the nuclei and phalloidin 488 nm was used to stain cellular actin. The obtained images were captured under 60x magnification on a florescent microscope using CellSens software. The scale bar represents 10 µm. Cells treated with 30 µM of iron (II) chloride and 5 µM of sodium arsenide show the most oxidative stress effect on NPAS3. The NPAS3 is translocated to the cytoplasm. Mock-treated cells do not show signs of aggregation, as the NPAS3 is detected in nuclei. All the images were obtained as a result of 3 independent experiments.

Calcium chloride and MG132 have previously shown no clear effect in inducing NPAS3 aggregation when the Flag vector was used (Table 7). To

confirm the results, we decided to stress-treat HA-NPAS3 transfected cells with the same concentration. On the first attempt, cells show no effect on NPAS3 aggregation after the treatment (Figure 10). However, the images show the results from 2 independent experiments. Hence, the experiments were repeated to confirm the results.

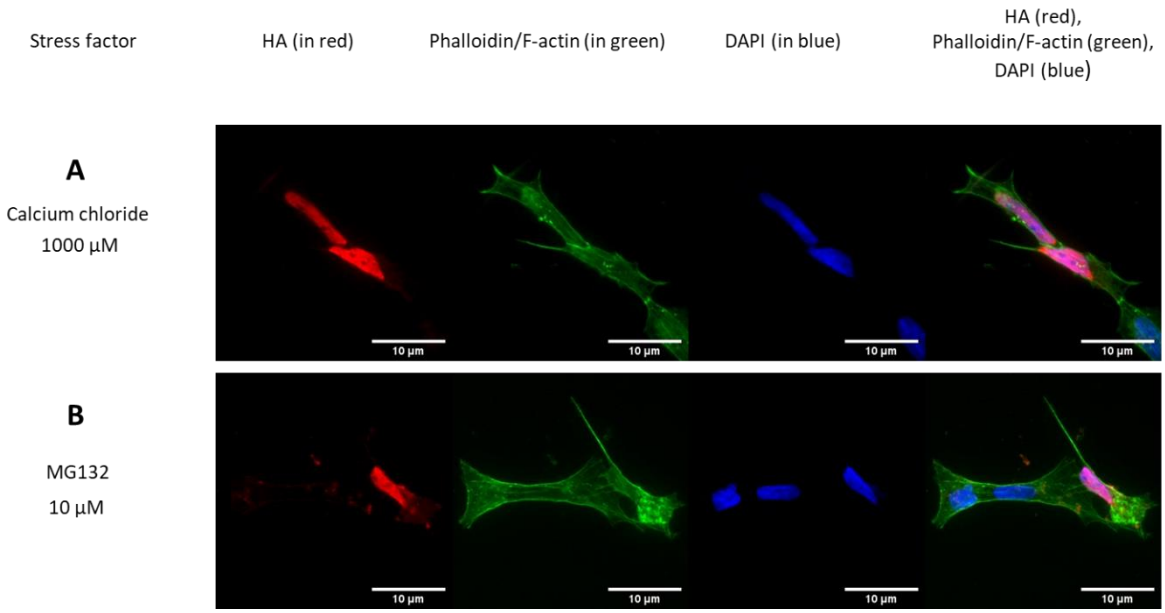

Figure 10. Fluorescent microscopy of SH-SY5Y cells transfected with NPAS3, stress treated for 3 hours with calcium chloride and MG132, showing the stress treatment effect. The proteins were labelled with anti-HA primary antibody and goat anti-mouse 555 nm secondary antibody, indicating the red signal. DAPI was used to stain the nuclei and phalloidin 488 nm was used to stain cellular actin. The obtained images were captured under 60x magnification on a florescent microscope using CellSens software. The scale bar represents 10 µm. Cells were treated with 1000 µM of calcium chloride and 10 µM of MG132. Cells do not show signs of aggregation, as the NPAS3 is detected in nuclei. All the images were obtained as a result of 2 independent experiments.

However, the repeated experiments showed different results (Figure 11). NPAS3 was aggregating in most of the transfected cells, with 1000 µM of calcium chloride and 10 µM of MG132 used as stress treatment concentrations. The results suggest that these stress factors potentially affect the aggregation of NPAS3. Furthermore, we decided to confirm the

zinc acetate effect on NPAS3 proteostasis. The results suggest that zinc acetate does induce protein aggregation, as the proteins were localized in the cytoplasm instead of the nuclei. However, oxidative stress resulted in the damaging of cellular actin, indicating that the zinc acetate concentration of 1000 µM might be too high for stress treatment (Figure 11C).

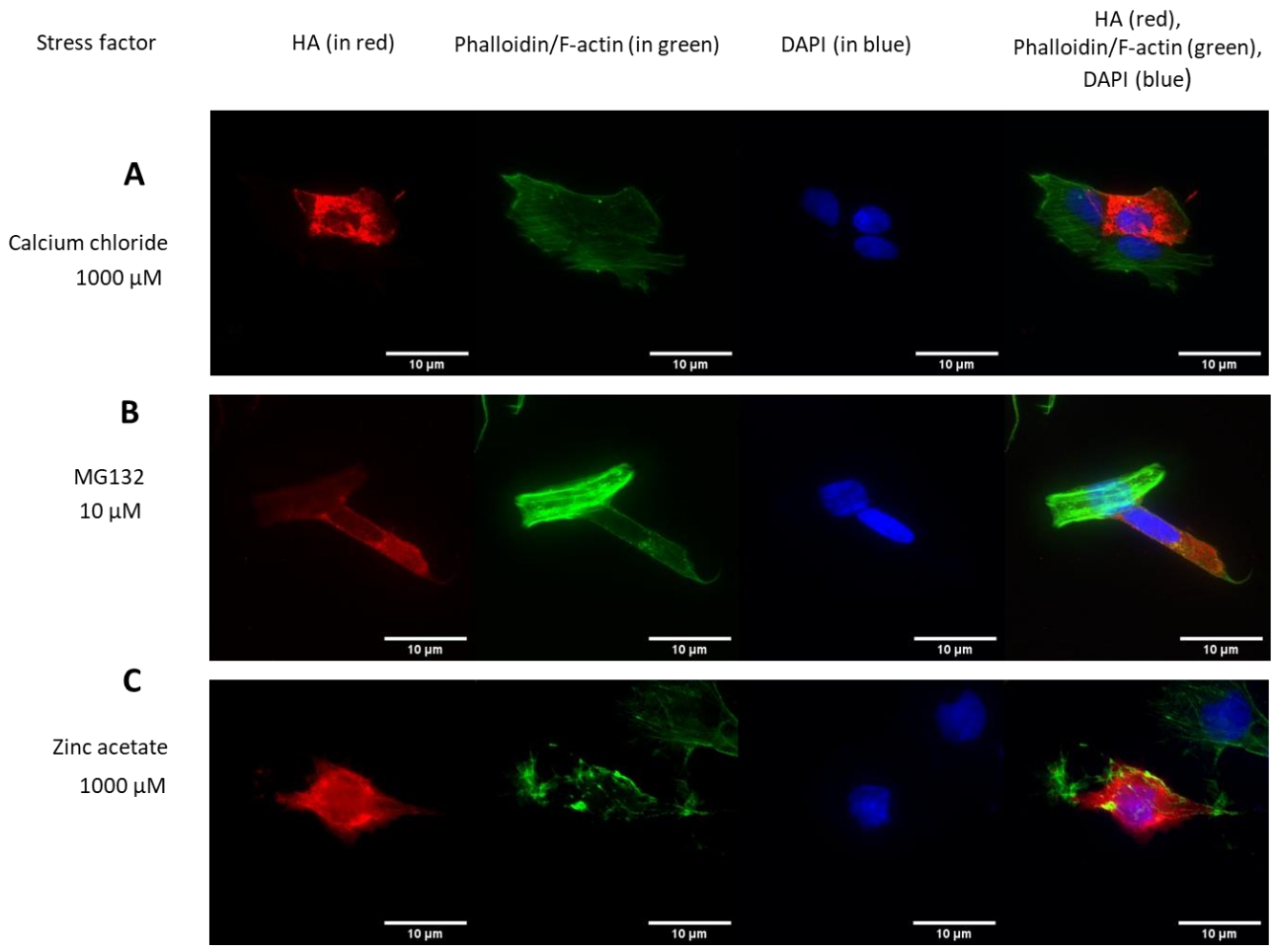

Figure 11. Fluorescent microscopy of SH-SY5Y cells transfected with NPAS3, stress treated for 3 hours with calcium chloride, MG132 and zinc acetate, showing the stress treatment effect. The proteins were labelled with anti-HA primary antibody and goat anti-mouse 555 nm secondary antibody, indicating the red signal. DAPI was used to stain the nuclei and phalloidin 488 nm was used to stain cellular actin. The obtained images were captured under 60x magnification on a florescent microscope using CellSens software. The scale bar represents 10 µm. Cells were treated with 1000 µM of calcium chloride, 10 µM of MG132 and 1000 µM of zinc acetate. The NPAS3 is translocated to the cytoplasm in all the proteins shown on A-C. Zinc acetate had a damaging effect on the cellular actin, as shown on A. All the images were obtained as a result of 3 independent experiments.

To summarize our results, the repeated aggregation properties have been seen when the NPAS3 expressed cells were treated with sodium arsenide, iron (II) chloride and zinc acetate. However, zinc acetate was toxic to the cell structure, suggesting the protocol modification for follow-up investigation. Furthermore, zinc acetate, calcium chloride and MG132 have not yet been tested with different concentrations, as is the case for sodium arsenide and iron (II) chloride (Table 8).

Table 8. Stress factors used for stress treatments on HA-NPAS3 expressed SH-SY5Y cells, results in their effect on protein and minimum concentration resulting the effect

Stress factor	**Sodium arsenide**	**Iron (II) chloride**	**Calcium chloride**	**MG132**	**Zinc acetate**
Effect	Aggregation	Aggregation	Potential aggregation	Potential aggregation	Aggregation
Minimum concentration Tested that caused aggregation	5 μM	30 μM	1000 μM	10 μM	1000 μM

5. Discussion

Chronic mental illnesses are known to have a wide range of risk factors involved in the progression and severity of psychiatric conditions. Stress factors have been implicated in *in vitro* experiments modelled in other studies. Furthermore, there is clear evidence implicating oxidative modulation of protein as a pathological inducer of protein aggregation in various diseases[50]. Reactive oxygen species can be generated by several factors; for instance, irradiation (ultraviolet light, x-rays), air pollutants (ozone, cigarette smoke, NO_2), and metabolic products in our organism (lipid peroxidation products, glycation products, oxidized electron transport carriers). A recent meta-analysis conducted on 731 schizophrenia patients suggests a glucose homeostasis disruption when compared to healthy controls. This can increase the risk for type 2 diabetes due to higher levels of glucose in blood plasma and neuronal insulin resistance. As a primary source of energy, glucose is transported into the brain via ETC (electron transport chain) where it is metabolized to ATP. This essential metabolic process might also be altered through antipsychotic administration which affects glucose regulation as a drug side effect. Further research is required to understand the link between the impairment of glucose-metabolic pathways and the pathophysiological background of CMI[50]. A particular study demonstrated α-synuclein samples treated with 100 μM of calcium chloride for 24 hours[55]. The treated samples show predominately larger α-synuclein particles (10-20 nm) when compared to control group (5 nm). Another study conducted on α-synuclein demonstrated iron stress treatment[56]. The samples were treated with 2 mM of iron, and incubated for 1 hour at 37°C, showing that iron promotes the formation of globular protein aggregates both in mutant and wild type α-synuclein. A previous study suggested that the mutant version of NPAS3, a protein associated with mental illness, is more prone to mislocalize in the cytoplasm, where it forms insoluble aggregates[31]. However, it is an extremely rare mutation,

where the amino acid valine is substituted with isoleucine (V304I). NPAS3 is a nuclear protein, meaning its functions in the nuclei of the cell. When it mislocalizes, it forms insoluble aggregates in the cytoplasm, where it loses its transcriptional function and becomes unable to return to nuclei. A recent study suggests that the aggregation is not present only in a mutant type of NPAS3[45]. The results from purified protein fractions of the brains of suicide victims and depression-affected patients have demonstrated NPAS3 wild type forms and insoluble protein fractions. Here, we decided to focus on this version of NPAS3.

Modelled on previous studies, one of the stress factors tested in this thesis is iron (II) chloride[56]. It showed an effect of induced NPAS3 aggregation when applied at a minimum concentration of 30 μM (Figure 9B). The experiment was repeated 3 times, suggesting the potential of protein translocation in cytoplasm and function loss when exposed to this stress factor. Iron (II) chloride seemingly showed an effect of inducing aggregation of TRIOBP-1 as well (Figure 8C). Another stress factor used in our research is sodium arsenide. It has previously been shown that this stress factor could potentially alter the NPAS3 function and form insoluble proteins outside of the nuclei[45]. We decided to conduct the treatment testing for a period of 3 hours. The results suggest that sodium arsenide does induce insoluble NPAS3 formation in the cytoplasm, showing that this stress factor can potentially stimulate the disruption of NPAS3 homeostasis in the brains of chronic mental illness suffering patients (Figure 9C). In a study where the human α-synuclein was treated with iron cations (Fe^{2+}), it has been demonstrated that it promotes *in vitro* aggregation, resulting in the formation of early-stage oligomers, characteristic for Parkinson's disease[56]. They have suggested that the accumulation of iron in the brains of patients can interfere with protein domains of plasma membranes, which can affect protein signaling regulation, leading to disruption of neurotransmission homeostasis. However, this has not yet been tested in CMI studies.

Zinc acetate has also shown clear signs of inducing protein aggregation of NPAS3 in the cytoplasm of neuroblastoma cells (Figure 11C). A previous study suggests zinc as one of the environmental toxic inducers of protein misassembly and aggregation, following the formation of Lewy bodies in Parkinson's disease[57]. However, its underlying effect resulted in cytoskeletal damage, suggesting that the concentration or the exposure time of the stress treatment is too high for the cells (Figure 11C). The current protocol should be modified to further investigate the possible effect of insoluble protein formation.

Another notable stress factor used in this thesis is calcium chloride. As already mentioned, it has been implicated in α-synuclein studies, suggesting an increase in protein aggregates formation[55]. Calcium chloride did not show any effect on cell aggregation at first (Figure 10A). However, follow-up experiment results clearly indicate that calcium chloride has properties of induced NPAS3 aggregation (Figure 11A). The NPAS3 experiments were conducted by using the HA tag vector. It has a similar size to the Flag tag vector, making it easier to obtain the identification signal of NPAS3 protein on a fluorescent microscope. EGFP tag vector has not been used in stress treatment experiments, as it is more likely to interfere with expressed proteins, stabilizing them and making them more prone to aggregation.

To summarize, metal ions that have been tested in this thesis are iron, zinc and calcium, having the effect of induced aggregation that should be tested further in the future.

MG132 is a proteasome inhibitor, affecting the proteasomal degradation pathway, resulting in intracellular accumulation of aggregated proteins. It is known that patients suffering from schizophrenia have lower proteasomal activity compared to control groups[58]. Hence, we decided to test this effect in our experiments. In this case, the transfected neuroblastoma cells were not stress treated, but rather tested on how well they can handle the stress.

We induced the inhibition of proteasomal activity, normally responsible for protein degradation. A recent study suggests that treatment with MG132 at 10 μM, incubated for 18 hours, induces the aggregation of the DISC1 protein[59]. We decided to apply the same concentration using neuroblastoma cells with expressed NPAS3, but treated for only 3 hours. Initial results suggest that MG132 did not induce NPAS3 aggregation, as the protein is detected in the nuclei (Figure 10B). However, repeated experiments suggest that NPAS3 does aggregate when treated with MG132, suggesting that blocking the protein degradation pathway in neuroblastoma cells and contribute to the accumulation of misfolded proteins (Figure 11B). Hence, this suggests that NPAS3 can aggregate without stress treatment, as the proteasome that would normally contain it is not functioning. MG132 has shown the properties of aggregation in DISC1 and CRMP1 Sv expressed neuroblastoma cells (Figure 8A, 8D). However, this effect should be investigated further in the future. Furthermore, it would be interesting to test the combination of MG132 (proteasome inactivation) and other stress factors (iron for instance), to see if they could co-induce protein aggregation.

It is noteworthy that all the tested stress factors have shown some kind of alternation in NPAS3 proteostasis or the cell structure. However, systematic testing should be obtained to generate a suitable protocol for each stress factor in the future. Zinc acetate has shown a strong toxic effect on the cytoskeleton, resulting in cell death (Figure 11C). As this factor is seen to form NPAS3 aggregated repeatedly (Table 8), the future perspective should be focused on the stress treatment with lower concentrations and incubation period. Furthermore, calcium chloride and MG132 have shown a potential effect on NPAS aggregation (Table 7). However, the stress testing should be repeated to fully investigate the effect of these stress inducers, as the results were not repeatable. On the other hand, MG132 and calcium chloride have not been tested on neuroblastoma cells using variable concentrations, nor has zinc acetate (Table 8). Hence, experimenting with

different concentrations and incubation periods is essential in order to confirm the aggregation properties of these stress factors. Furthermore, quantitative/blinded tests are required to verify these results.

As mentioned in the results, an attempt was made to quantify the effect of iron (II) chloride and sodium arsenide using a blinded assay. However, this failed due to technical problems (a lack of air conditioning over the weekend in the laboratory), leading to the cells experiencing a high temperature. Due to the temperature modification, the NPAS3 aggregated at a much higher level regardless of whether a stress factor was applied, the cells were in effect stressed already. The heat shock could be a potential reason for this phenomenon, which could be tested in follow-up research as a new stress factor. Another test that should be taken into a consideration is a treatment with stress factors on endogenous NPAS3. This could provide evidence that NPAS3 does aggregate when stress tested, without the impression that these results are artificial due to induced overexpression of the protein. The cell-based studies regarding stress factors usage should be emphasized as a starting point of environment and trauma effect on the development of psychiatric disorders in patients. It has been demonstrated that those factors can increase the disease risk[15–19]. However, a clear link between a stress factors treatment and those factors is required to fully understand the disease progression on a cellular level.

It is safe to say that environmental stress factors do affect NPAS3 protein aggregation on a cellular level. However, the protocols for testing this must be further optimized, with the follow-up experiments regarding the stress treatment period, different concentrations and constant laboratory conditions (temperature) in order to obtain sustainable results. This investigation could lead us to potential discoveries of environmental stress effect on protein aggregation in chronic mental illness, and help us understand the underlying pathological pathway of these disorders regarding external conditions which can induce these conditions in a subset of suffering individuals.

6. Conclusions

Environmental stress has been implicated in neurodevelopmental disorders investigations regarding aberrant proteostasis of particular markers of these conditions[55,56]. Furthermore, it has also been implicated in studies affecting protein aggregation in chronic mental illness[45,59]. However, there is not a lot of data supporting this evidence.

In this thesis, we decided to test the effect of stress on neuroblastoma cells expressing proteins that have a tendency to aggregate in a subset of chronic mental illness suffering patients.

Out of the tested proteins, CRMP1 Sv and DISC1 showed clear signs of aggregation when stress treated with MG132, as well as TRIOBP-1 when stress treated with iron (II) chloride. The initial aims of this thesis were focused on screening the proteins with stress factors, and determining which protein and stress factor show the most interesting effect. Further investigation is required to test all the proteins with an optimized protocol in order to confirm these initial results. NPAS3 has shown the most interesting results based on aggregation induced by stress factors treatment. Sodium arsenide, iron (II) chloride and zinc acetate affected the aggregation properties of NPAS3 in repeated experiments, inducing the translocation of the nuclear protein to the cytoplasm of the cell. The results on calcium chloride and MG312 stress treatment showed a potential effect on NPAS3 aggregation. However, these findings should be fully investigated, as the results were not repeatable to confirm the effect of these stress factors on NPAS3 aggregation.

These experiments were the first broad attempt to test the protein aggregation in mental illness by stress factor treatment, previously only tested to a limited extent one protein[45,59]. Based on our results, we suggest that NPAS3 is affected by stress factor treatment in cell culture studies. There are clear signs of induced aggregation after the stress treatment.

Other proteins have the potential to aggregate when stress treated as well, yet further investigation is required to validate the obtained results. These findings are potential initiators of a new investigation perspective, focused on environmental stress treatment of specific proteins that are known to aggregate in a subset of brain samples from psychiatric patients. Future discoveries could lead us to understand the pathological mechanisms of external factors affecting the pathways of protein aggregation and give us an insight with the aim to develop new drugs and therapies suitable for individuals worldwide.

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Protein Aggregation of NPAS3, Implicated in Mental Illness, Is Not Limited to the V304I Mutation

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Listening	Reading	Spoken production	Spoken interaction	Writing
A2	A2	A1	A1	A1

DIGITAL SKILLS

My Digital Skills

Comunication Platforms (zoom, skype, google hangouts, etc.) / Skilled internet user / Adaptability / Accessibility / Communicativeness / Responsibility / Motivated / Organizational and planning skills / Gmail / Team-work oriented / Microsoft Office / Reliability

VOLUNTEERING

2016 – CURRENT

Biotechnology Student Association at University of Rijeka

University of Rijeka, Department of Biotechnology

09/2019 – 02/2020

Laboratory volunteer

University of Rijeka, Department of Biotechnology, Bradshaw lab

Researching protein aggregation in mental illness.
Methods: Human samples and mammalian cell culture.

STUDENT JOBS

10/2021 – 07/2022

Sales associate

https://textilehouse.hr/

06/2017 – 10/2018

Airport check in agent

https://www.airport-dubrovnik.hr/

06/2015 – 10/2015

Assistant chef

https://www.hotel-cavtat.hr/

DRIVING LICENCE

Driving Licence: B