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The Use of Monogenic Disease to Study Basal and Disease Associated Mechanisms

with Focus on NGF Dependent Pain Insensitivity and ISCU Myopathy

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To my family

Abstract

Monogenic diseases make excellent models for the study of gene functions and basal cellular mechanisms in humans. The aim of this thesis was to elucidate how genetic mutations affect the basal cellular mechanisms in the monogenic diseases Nerve growth factor (NGF) dependent pain insensitivity and Iron-Sulphur cluster assembly protein U (ISCU) myopathy.

NGF dependent pain insensitivity is a rare genetic disorder with clinical manifestations that include insensitivity to deep pain, development of Charcot joints, and impaired temperature sensation but with no effect on mental abilities. The disease is caused by a missense mutation in the *NGFβ* gene causing a drastic amino acid substitution (R221W) in a well-conserved region of the protein. NGF is secreted in limited amounts by its target tissues and is important for the development and maintenance of the cholinergic forebrain neurons as well as the sensory and sympathetic neurons. To reveal the underlying mechanisms of disease we performed functional studies of the mutant NGF protein. We could show that mutant NGF was unable to induce differentiation of PC12 cells as a consequence of impaired secretion. Furthermore, mutant NGF had different intracellular localisation compared to normal NGF and resided mostly in its unprocessed form proNGF. Mature NGF and proNGF have different binding properties to the receptors TrkA and p75. Individuals with mutations in *TRKA* are, aside from pain insensitive mentally affected; therefore it has been proposed that the R221W mutation mainly affects the interaction with p75. In agreement with this, we could show that R221W NGF was able to bind and activate TrkA whereas the interaction with p75 was impaired as compared to normal NGF.

ISCU myopathy is a monogenic disease where the affected patients suffer from severe exercise intolerance resulting in muscle cramps and sometimes severe lactic acidosis. The disease is caused by a point mutation in the last intron of the Iron sulphur cluster assembly gene, *ISCU,* resulting in the inclusion of a part of the intron in the mRNA. ISCU functions as a scaffold protein in the assembly of iron-sulphur (Fe-S) clusters important for electron transport in Kreb's cycle and the respiratory chain. We have shown that ISCU is vital in mammals since complete knock-down of *Iscu* in mice results in early embryonic death. The deletion of ISCU homologous in lower organisms has also been shown fatal. In spite this central role in energy metabolism the disease is restricted to the patient's skeletal muscles while other energy demanding organs seem unaffected. To address this contradiction we examined if tissue-specific differences in the splicing of mutant *ISCU* could explain the muscle-specific phenotype. We could show that the splicing pattern did, indeed, differ with more incorrectly spliced ISCU in muscle compared to other tissues. This was accompanied by a decrease in Fe-S containing proteins in muscle, while no decrease was observed in other tissues. Alternative splicing is more common then previously thought and may depend upon interacting factors and/or differences in the surrounding milieu. To reveal plausible mechanisms involved in the tissue-specific splicing we identified nuclear factors that interacted with the region where the mutation was located. Five interacting factors were identified, out of which three affected the splicing of ISCU. PTBP1 was shown to repress the incorrect splicing while IGF2BP1 and RBM39 repressed the formation of normal transcript and could also counteract the effect of PTBP1. IGF2BP1 was the only factor that showed higher affinity to the mutant sequence making it a possible key factor in the incorrect splicing of the mutant *ISCU* gene.

Together, these results offer important insights into the cellular mechanisms causing these diseases. We found impaired secretion and inaccurate sorting of NGF to be cellular mechanisms contributing to NGF dependent pain insensitivity while tissue-specific splicing of *ISCU* was found to be the event contributing to the phenotype of ISCU myopathy.

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List of Papers

This thesis is based on the papers listed below, which will be referred to in the text by the corresponding Roman numerals (I-IV).

I **Larsson E**, Kuma R, Norberg A, Minde J, Holmberg M, "Nerve growth factor R221W responsible for insensitivity to pain is defectively processed and accumulates as proNGF", Neurobiol Dis., 33(2):221-228, 2009

II **Larsson E**, Fahey MS, Watson JJ, Holmberg M, Dawbarn D, Allen SJ, "Purification and characterization of the Nerve Growth Factor R221W mutant causing insensitivity to pain", Manuscript

III Nordin A, **Larsson E**, Thornell LE, Holmberg M, "Tissue-specific splicing of ISCU results in a skeletal muscle phenotype in myopathy with lactic acidosis, while complete loss of ISCU results in early embryonic death in mice", Hum Genet.,129(4):371-8, 2011

IV Nordin A, **Larsson E**, Holmberg M," The defective splicing caused by the ISCU intron mutation in patients with myopathy with lactic acidosis is repressed by PTBP1 but can be de-repressed by IGF2BP1. Hum Mutat. In press

Abbreviations

APP	Amyloid precursor protein, human gene symbol
BDNF	Brain-derived neurotrophic factor
BDNF	Brain-derived neurotrophic factor, human gene symbol
CIPA	Congenital insensitivity to pain with anhidrosis
CNS	Central nervous system
DES	Desmin, human gene symbol
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmatic reticulum
ERK1/2	Extracellular signal regulated kinase 1 and 2
Fe-S	Iron-sulphur
FPLC	Fast protein liquid chromatography
FXN	Frataxin, human gene symbol
GRP78	Glucose regulated protein 78
HEK 293	Human embryonic kidney 293
HSAN	Hereditary and autonomic neuropathy
IGF2	Insuline-like growth factor 2
IGF2BP1	Insuline-like growth factor 2 mRNA binding protein 1
ISCU	Iron sulphur cluster protein U
ISCU	Iron sulphur cluster protein U, human gene symbol
Iscu	Iron sulphur cluster protein U, mouse gene symbol
kDa	kilo Dalton
KOMP	Knock-out mouse project
MMPs	Matrix metalloproteinases
mRNA	messenger RNA
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor
NGFβ	Nerve growth factor beta, human gene symbol
Ngf	Nerve growth factor, mouse gene symbol
NT-3	Neurotrophin-3
NT-4/5	Neurotrophin-4/5
NTRK1	Neurotrophic tyrosine receptor kinase type 1, human gene symbol
PC12	Pheochromocytoma 12
PE	Primary erythromelalgia
PEPD	Paroxysmal extreme pain disorder
PNS	Peripheral nervous system
PSEN1	Presenilin 1, human gene symbol
PSEN2	Presenilin 2, human gene symbol

PTB	Polypyrimidine tract binding
PTBP1	Polypyrimidine tract binding protein 1
RBM39	RNA-binding protein 39
R221W	Arginine 221 tryptophane
SCN9A	Sodium channel voltage-gated type IX alpha subunit, human gene symbol
SDH	Succinate dehydrogenase
SDHB	Succinate dehydrogenase subunit B
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophorecis
SFRS14	Splicing factor arginine/serine-rich 14
SgII	Secretorygranin II
SLC25A3	Solute carrier family 25 member 3, human gene symbol
SREs	Splicing regulatory elements
TGN	*Trans*-Golgi Network
TrkA	Tropomyosin-related kinase A
TrkA	Tropomyosin-related kinase A, mouse gene symbol
tPA	tissue plasminogen activator
VPS10	Vacuolar protein sorting 10
V66M	Valine 66 methionine

Introduction

Much of our current knowledge on specific proteins and their normal and pathological functions is based on information gained from their role in genetic disease. With the discovery of disease-causing genes, important knowledge regarding the gene product and the associated pathways can be obtained by functional studies involving different cellular and animal models; as well as by biochemical analysis. The focus of this thesis has been to study the basic and disease associated mechanism of two disease genes i) the Nerve growth factor beta gene, *NGFβ,* that causes insensitivity to pain, and ii) the Iron sulphur cluster assembly protein U gene, *ISCU*, that causes exercise intolerance. Both diseases originate from the northern part of Sweden.

Background

Genetics in Disease

Genetic diseases can conform to the Mendelian laws of inheritance, or they can be more complex, involving the interaction of genetic and environmental factors (age, blood pressure, infections) as well as lifestyle choices (smoking, sedentary lifestyle). Most of the focus nowadays is upon common complex diseases because they affect large proportions of the population. In addition to environmental factors, they involve multiple genes, which, in most cases, contribute only slightly to the disease development which therefore make them difficult to dissect. The identification of genes contributing to the phenotype of a complex disease can be achieved but requires a large patient cohort, extensive genotyping and bioinformatic analysis. Once an association or linkage has been found the challenge is to find the causative gene, but because the variation in each disease gene is normally much less drastic than seen in monogenic disease, and not necessarily located in the obvious gene related sequences, this may often prove difficult. Mendelian inheritance of many common diseases exists but is rare, representing only few percent of cases. The identification of genetic factors that cause familial forms of complex disease is, however, more straightforward and may increase our understanding of the pathogenic mechanisms involved in the common forms of complex disease. One example is Alzheimer's disease where the identification of mutations in *APP*, *PSEN1* and *PSEN2*, linked to familial forms of the disease, has contributed to a deeper understanding of the underlying pathogenic mechanism [1].

Mendelian diseases, also denoted monogenic, are characterised by a strong correlation between the disease phenotype and a specific mutation in a single gene. Many early onset diseases are caused by a drastic mutation in the coding region (exon) of the disease gene, resulting in amino acid substitutions or truncations with severe effects on the function of the resulting protein. However, mutation located in a non-coding (intron) or regulatory region might also lead to drastic phenotypes due to differential splicing or regulation of the gene. Monogenic diseases are most often inherited in a dominant or recessive manner. Autosomal recessive inheritance is often due to a loss-of-function mutation where one functional copy of the gene is enough to sustain a normal phenotype. Therefore two copies of the disease gene are needed in order to cause the disease. However, if the recessive trait is located on a sex chromosome (X or Y), male carriers will develop the disease since they only carry a single copy of each sex

chromosome. Dominant traits are often due to a gain-of-function mutation presenting the protein with new toxic properties making a single copy of the mutant gene enough to cause disease.

Monogenic diseases may be found more frequently in some geographical regions due to the population structure caused by founder effects, limited immigration or low mobility during history. Founder effect is an extreme variant of genetic drift, created when a small group of individuals founds a new population thereby causing a reduction of the gene pool. Such populations are often found in remote or isolated areas such as islands and river valleys. The population of northern Sweden has expanded rapidly from small founder populations and subpopulations have also arisen due to the relative isolation in each of the larger river valleys. This has created a population where genetic drift, founder effects and inbreeding has resulted in a number of recessive and dominant diseases, some with unusually high frequencies and some unique to this area [2]. This, in combination with the Swedish church records and the socialised Swedish health-care system, has provided excellent conditions to identify disease causing genes. Genetic studies in this population has led to the successful identification of disease genes for several monogenic diseases, as well as loci for some complex diseases including type 1 and type 2 diabetes [3-6]. Among the monogenic disease genes identified in this population are the genes for the two diseases that will be discussed in this thesis, NGF dependent pain insensitivity [7] and ISCU myopathy [8, 9].
The identification of disease-causing genes has proven to be a useful tool for gaining insight into the development and pathology of disease and it can also increase knowledge of the normal function of the protein in question. The aim of this thesis was to identify the cellular mechanisms contributing to the disease-associated phenotype in patients with NGF dependent pain insensitivity and ISCU myopathy.

Nerve Growth Factor (NGF) Dependent Pain Insensitivity

A large family suffering from pain insensitivity has been identified in the county of Norrbotten in northern Sweden. Three individuals, all with consanguineous parents, were found to suffer from a severe form of pain insensitivity. In the severe form of the disease, patients presented with multiple painless fractures in the lower extremities already during childhood. They were also seen to develop severe neuropathic joint

destructions, Charcot joints, at an early age (Figure 1). Clinical evaluations have shown that the patients also suffer from impaired temperature sensation, making it difficult to distinguish between warm and hot stimuli. They are able to perceive touch, and they do not suffer from any serious autonomic deficiency, including anhidrosis (inability to sweat), or any intellectual disabilities. One of the patients has, however, been reported to suffer from gastrointestinal disturbances and a slight urinary incontinence, that may be associated with the disease. Sural nerve biopsies revealed a moderate loss of thin myelinated nerve fibres (Aδ fibers) that are associated with temperature, pressure and fast pain, and a severe reduction of the unmyelinated C fibres, involved in temperature and slow conducting pain. The separate nerve fascicles fiber densities was found to be uniform which indicates an early developmental reduction of fibres rather than acquired nerve damage [7, 10].

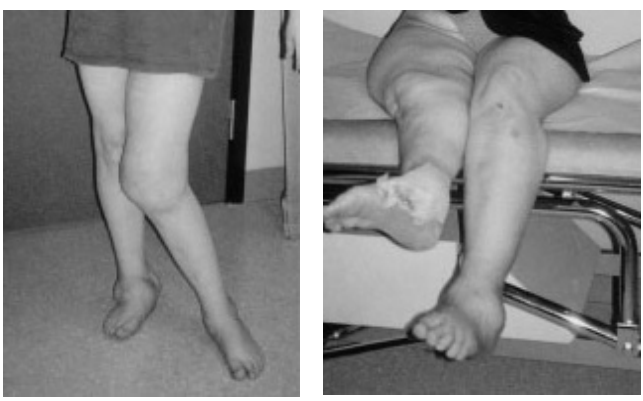

Figure 1. Neuropathic joint destruction in Swedish patients suffering from NGF dependent pain insensitivity. *Reprinted with permission from John Wiley and Sons.*

The disease has been shown to be caused by a missense mutation (661C>T) in the nerve growth factor beta (*NGFβ*) gene. The mutation results in a drastic amino acid substitution from a basic arginine to a non-polar tryptophan at position 221 in the protein, a region that is highly conserved in NGF of different species as well as in the related neurotrophins [7]. The three severe cases are homozygous for the *NGF* mutation, and are also the only individuals that have been shown to carry two copies of the disease gene. Patients heterozygous for the mutation display a more heterogeneous phenotype. The clinical picture varies from asymptomatic cases to those with minor reduction of nerve fibres and an adult neuropathic joint destruction

[11]. This suggests an underlying gene dosage effect for the mutation. Based on the phenotype the disease has been classified as being hereditary sensory and autonomic neuropathy V (HSAN V) (OMIM 608654)[7, 10].

Recently, a second *NGFβ* mutation that causes pain insensitivity in humans was discovered [12]. In this case, five children with related parents of Bedouin decent were shown to be homozygous for a frameshift mutation (680C>A) + (681_682delGG). The mutation resulted in the replacement of the 15 terminal amino acids with 43 novel amino acids in the resultant protein [12]. The Bedouin cases, apart from being pain insensitive, also suffer from self-mutilation, mild mental retardation as well as other neurological deficiencies that have not been observed in the Swedish patients [12]. The frameshift mutation thus seems to have a more drastic effect on the properties of the NGF protein than that caused by the Swedish missense mutation. In this thesis NGF dependent pain insensitivity will further be referred to the Swedish R221W NGF mutation.

NGF

NGF belongs to a family of structurally related proteins, denoted neurotrophins for their trophic properties in the development and maintenance of the nervous system. Other family members include brain-derived neurotrophic factor (BDNF), neurotrophic factor 3 (NT-3) and neurotrophic factor 4/5 (NT-4/5). Additional family members have been identified in bony fishes but no equivalent gene sequence has been identified in invertebrates [13].

NGF and the Nervous System

NGF is important for the development of neurons in the peripheral nervous system (PNS) and the central nervous system (CNS). The developing neurons compete for limited amounts of NGF which are synthesised and secreted from the target tissues and organs. Bound NGF is endocytosed and translocated from the axon terminal to the cell body in a retrograde fashion. Only those neurons that obtain sufficient trophic support survive while those who do not will undergo programmed cell death (apoptosis). In this way the number of neurons that innervate a region is matched to the area and requirements of the target field (Figure 2) [14].

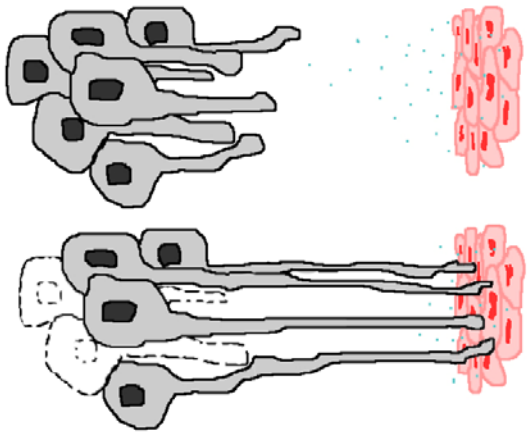

Figure 2. The limited amounts of secreted NGF by a target tissue match the requirements of innervating neurons.

Sympathetic and sensory neurons are recruited by a large number of NGF-producing cells in the PNS and peripheral tissues. These include different cells in the skin, smooth muscle cells, and exocrine cells like the testis, ovary, pituitary and thyroid, as well as exocrine glands. In the CNS cholinergic neurons in the basal forebrain, as well as neurons in the hippocampus and the striatum, produce and secrete NGF [13, 15, 16].

NGF is also produced by immune cells such as the T and B lymphocytes, mast cells and macrophages which furthermore have been shown to express the p75 and TrkA receptors [17-19]. The expression of NGF is increased in response to inflammation and TrkA and p75 receptor binding results in activation of NF-κB leading to the expression of several proinflammatory proteins [20].

NGF Processing

NGF is initially synthesised as a precursor protein, pre-proNGF. The pre-peptide directs the protein to the endoplasmatic reticulum (ER) where it folds and is subjected to post-translational modifications. After translocation to the *trans*-Golgi network (TGN), proNGF is either secreted or processed to

mature NGF by furin or pro-hormone convertases prior to secretion [21-23]. Cleavage of proNGF in the extracellular space is accomplished by plasmin or the matrix metalloproteinases (MMPs) [24]. Fully processed NGF has a molecular weight of ~13,5 kDa while proNGF have a molecular weight ranging from 25 to 40 kDa depending on different processing and post-translational modifications. By X-Ray chrystallography NGF was found to form a cystein-knot by three disulfide bonds, a structure that stabilises the folding of the protein [25]. Both proNGF and mature NGF have biological activities as homodimers [26, 27]. NGF has a basic isoelectric point (9,2-9,3) which is thought to limit its rate of action [13].

NGF Receptors

Mature NGF bind two different types of transmembrane receptors, tropomyosin-related kinase A (TrkA) and the neurotrophin receptor p75. The receptors can act independently or together to mediate biological events. TrkA belongs to a family of receptor tyrosine kinases, which also include the other neurotrophic factor receptors, TrkB and TrkC. p75 is a member of the tumor necrosis receptor superfamily and binds all neurotrophins with similar affinity. NGF/TrkA interaction mediates a homodimerisation of the receptors resulting in transphosphorylation of the tyrosine residues in the intracellular kinase domain. This in turn causes an intracellular response regulating cell growth and survival [28]. When p75 and TrkA are co-expressed, the affinity for NGF is enhanced making cells more NGF sensitive [29]. ProNGF has also been shown to bind to TrkA but with a highly reduced affinity compared to mature NGF [30]. ProNGF has instead been shown to be the preferred p75 ligand [24]. Studies have shown that proNGF induce p75 mediated apoptosis upon tissue injuries [31, 32]. Sortilin, a receptor from the VPS10 family, was later discovered to bind the prodomain of proNGF. Sortilin and p75 have been found to act together by forming a proNGF binding complex through which apoptosis is transduced [33] (Figure 3).

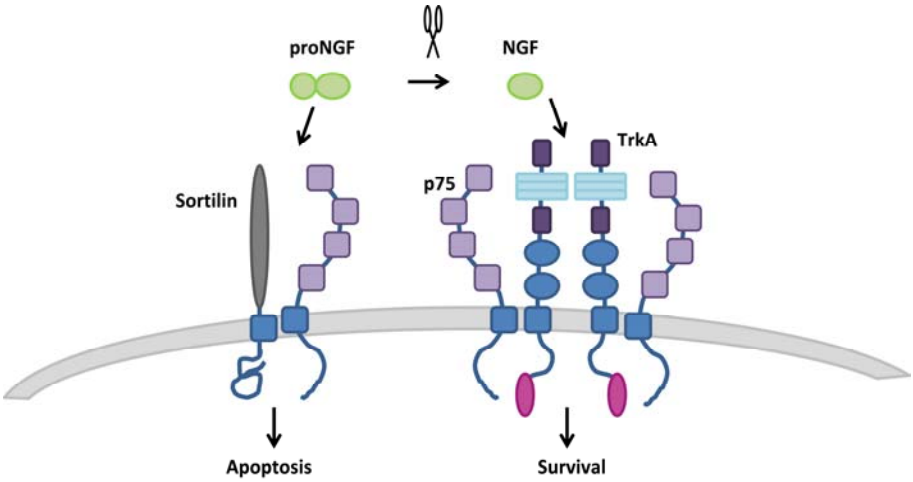

Figure 3. The NGF receptors TrkA, p75 and Sortilin activate different down-stream signalling pathways either promoting cell-survival or apoptosis.

NGF in Mice Models

Knockout of *Ngf* in mice results in a severe reduction of sensory and sympathetic neurons, and not surprisingly, these mice show almost no reaction to noxious stimuli. The complete loss of NGF, furthermore, results in death at only a couple of weeks following birth. The heterozygous mice are however both viable and fertile but show a minor reduction of neurons in the PNS and their reaction to heat-induced pain is also slightly impaired [34].

In transgenic mice where the *Ngf* gene has been coupled to an epidermis-activated promoter, the overexpression of NGF in the skin results in an increased innervation of both sensory and sympathetic neurons in this tissue [35]. As a consequence of the NGF overexpression, these mice suffer from hyperalgesia (elevated pain sensitivity) and respond to lower thresholds of mechanical and thermal stimuli [36].

Monogenic Pain Diseases

Disorders where pain sensitivity is impaired or absent altogheter are very rare. The sensation of pain is unpleasant and it teaches the individual from the early stages in life to avoid potentially harmful actions. Hence pain is an important mechanism since it protects the individual from noxious stimuli and tissue damage. The identification of genes that cause different pain disorders has provided significant knowledge about the normal physiological mechanisms of pain in humans and offered potential new therapeutics in the treatment of pain.

As previously mentioned, NGF dependent pain insensitivity in the Swedish family was classified as HSAN V based on clinical findings. HSAN is a classification of disorders, comprising five subtypes, characterised by the loss of pain sensation. The different subtypes are genetically and clinically heterogeneous but sural biopsies have shown that the development and survival of the autonomic and sensory neurons, mainly the Aδ and C-fibers, are affected in all these disorders. These neurons constitute the two major classes of nociceptors where the myelinated Aδ fibers conduct the first, sharp pain and the unmyelinated, small diameter C fibers conduct the second, duller pain (Figure 4). The nociceptors fire only when the stimuli have reached a noxious level. These neurons innervate both the target organs and the spinal cord and the cell bodies are found in the dorsal root ganglion (DRG) or trigeminal ganglion [37].

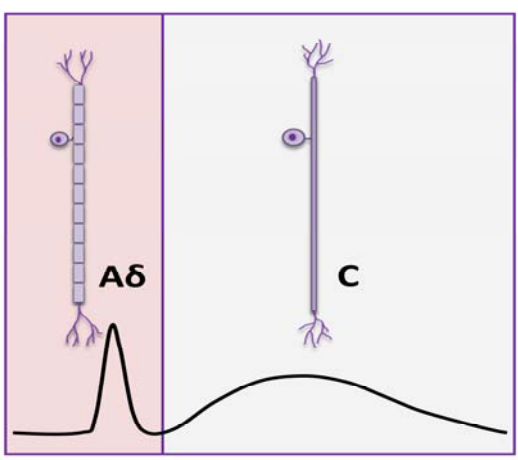

Figure 4. Myelinated Aδ fibers conduct the first, sharp pain and the unmyelinated, small diameter C fibers conduct the second, duller pain.

HSAN IV (OMIM 256800), also denoted congenital insensitivity to pain with anhidrosis (CIPA) is characterised by loss of pain sensation, impaired temperature sensation, anhidrosis and mental retardation. The patients are insensitive to both deep and superficial pain which leads to repeated painless fractures as well as self-mutilation. Motor functions and the ability to respond to touch are normal. Their inability to sweat can be detrimental in hot environments or in cases of fever since the body lacks the ability to cool. Children with CIPA exhibit severe learning disabilities and emotional problems which indicate CNS involvement in these patients [38, 39]. The disease is inherited in an autosomal recessive fashion and is caused by mutations in the NGF receptor TrkA gene *NTRK1*. Mutations that either affects the extracellular binding domain or the intracellular signal-transduction domain of TrkA have been reported [40]. The TrkA signalling pathway has been shown to be important in the development of the sensory and the peripheral nervous system. Knockout of the *TrkA* gene in mice results in a similar phenotype as that seen in CIPA patients with a decreased response to noxious stimuli and severe cell loss in the sensory and sympathetic ganglia, and the cholinergic basal forebrain. However, neurophysiological and behavioural studies cannot be performed on these mice since complete loss of TrkA results in the death of these animals within a few weeks of birth [41]. Interestingly, patients that suffer from CIPA display a more severe phenotype than the NGF dependent pain insensitivity patients.

In 2006, Cox and co-workers identified the gene for a “pure” form of insensitivity to pain without neuropathy in three families of Pakistani decent. The autosomal recessive disease in these families was found to be caused by mutations in the *SCN9A* gene. The mutations resulted in the loss of function of the $Na_v1.7$ protein, the α-subunit in the tetrodotoxin-sensitive voltage-gated sodium channel and the disease was thus referred to as channelopathy associated insensitivity to pain (OMIM 243000) [42]. The $Na_v1.7$ sodium channels are expressed in sympathetic and peripheral sensory neurons but also in the epithelia of the olfactory [43, 44]. Since the discovery, additional mutations in *SCN9A* have been reported in cases suffering from the same disease [45, 46]. These patients experience no pain in any part of their body, which results in painless fractures and the development of neuropathic joints and bone deformities. Furthermore, self-mutilating of the lip, tongue and fingers commonly occur during childhood. In addition to this cuts, bruises and burning injuries are frequently seen in such patients. Their ability to perceive touch and distinguish hot from cold is normal, as well as their mental abilities [42, 45], however these patients are

unable to perceive odours [44]. Complete knockout of $Na_v1.7$ in mice leads to early death, while tissue-specific knockout in the nociceptive neurons results in loss of inflammatory pain [43]. Individuals with gain-of-function mutations in the *SCN9A* gene instead suffer from elevated pain syndromes [47, 48]. In primary erythromelalgia (PE) (OMIM 133020), warm stimuli, moderate exercise or even standing results in a burning pain sensation and inflammation mainly in the feet, lower legs and hands of the affected patients [47]. Patients suffering from paroxysmal extreme pain disorder (PEPD) (OMIM 167400) show a different clinical phenotype with sudden episodes of burning pain in the rectal, ocular and in the area of the lower jaw often followed by skin flushing [48]. The missense mutations underlying these disorders results in altered activation of the sodium channel. Mutations causing PE lowers the activation thresholds and PEPD mutations cause a defective inactivation of the channel [48, 49]. Furthermore, a common polymorphism in the *SCN9A* gene has been linked to an enhancement of C-fiber pain sensitivity in carriers. The polymorphism results in an amino acid substitution in a part of $Na_v1.7$ with unknown function [50].

Pain Therapeutics

Attention for new possible targets in pain has been raised with the discovery of these disorders. By blocking $Na_v1.7$ which is expressed preferentially in the sensory and sympathetic neurons, many side effects could be circumvented. Toxins from the venom of different species of tarantulas have been shown to block sodium channels but so far none of them have been shown to specifically block the $Na_v1.7$ channel. These toxins could however provide great models for structure and function during studies in the pursuit for specific $Na_v1.7$ molecules [51]. A new pharmacological molecule that block the $Na_v1.7$ in animal models with persistent neuropathic pain have shown longer lasting and better pain-killing effects than morphine-like substances [52]. Painful stimuli can also be circumvented by targeting the NGF/TrkA signalling pathway. This can be accomplished by molecules that either remove free NGF or prevent TrkA binding or activation. The administration of anti-NGF antibodies or a TrkA-IgG fusion protein in animal models of different pain states has proved to markedly reduce hyperalgesia and pain [53]. Furthermore, the early administration of a Trk inhibitor has proven to attenuate the cancer-induced pain in a mouse model of bone cancer pain [54]. In humans, a phase 3 trial, where a humanised monoclonal NGF antibody (tanezumab) was administrated to patients suffering from

osteoarthritis, showed promising results. A significant reduction in pain was noted in patients that received the antibody compared to cases administrated with a placebo [55]. However, it has been reported that 16 of the participants in this clinical trial needed joint replacement and therefore this trial has been put on hold until the reason for the progressively worsening of the osteoarthritis in these patients has been established [56]. Meanwhile there are still ongoing clinical trials where antibodies against NGF are tested in the treatment of pain. This proves that further research concerning NGF is important and perhaps that analysis of the R221W NGF protein found in the Swedish family could contribute to this.

ISCU Myopathy

ISCU myopathy (OMIM #255125) is a rare genetic disease which originates from the counties of Ångermanland and Västerbotten in northern Sweden. It is transmitted in an autosomal recessive fashion and to date a total of 30 homozygous patients have been identified. The disease phenotype mainly involves the skeletal muscles. Affected patients suffer from exercise intolerance where even a minor workload results in painful muscle cramps, tachycardia and dyspnoea (shortness of breath). Biochemical analysis of blood samples has shown increased levels of lactate and pyruvate even after low physical activity. The poor physical capacity becomes evident during childhood and remains throughout life. Extreme physical exercise or extreme diets like fasting can trigger severe episodes of the disease. A severe episode is marked by severe lactic acidocis, widespread fatigue coupled with nausea and vomiting, resting dyspnoea and tachycardia as well as severe muscle weakness and occasionally even pareses. Dark urine can also be observed as a result of breakdown of damaged muscles, which in turn can lead to kidney failure. A severe episode, in which the patient often becomes bedridden for several weeks, is often followed by a period of full recovery. In a few cases, the severe state of the disease has proven fatal [57, 58]. Biochemical and histological findings from the patients showed defects of proteins involved in the energy metabolism of the cell. Low levels of both Succinate dehydrogenase (SDH), a protein complex involved in the respiratory chain and Krebs cycle, and the mitochondrial enzyme aconitase, was found [59, 60]. Because these enzymes contain Fe-S clusters it was hypothesised that defects in the Fe-S cluster machinery was the underlying cause of the disorder. In agreement with this, the disease was shown to be caused by a mutation in the last intron of the *ISCU* gene (IVS5+382G>C) [8, 9]. *ISCU* encodes the Iron-Sulphur Cluster assembly protein U, a scaffold protein that

functions in Fe-S cluster assembly and transfer [61]. The protein has a central role in the formation of Fe-S clusters which are then delivered to target proteins important for various processes in the cell, including Krebs cycle and the respiratory chain (Figure 5) [62, 63]. The intronic mutation in the *ISCU* gene enhances a weak splice acceptor site which results in the inclusion of intron sequence in the messenger RNA (mRNA). This in turn will cause the introduction of a 15 novel amino acid and a premature stop in the ISCU protein [8, 9]. Two differently sized intron inserts, 86 and100 base pair respectively, with diverging donor splice sites has been found in mRNA from ISCU myopathy patient tissue [64].

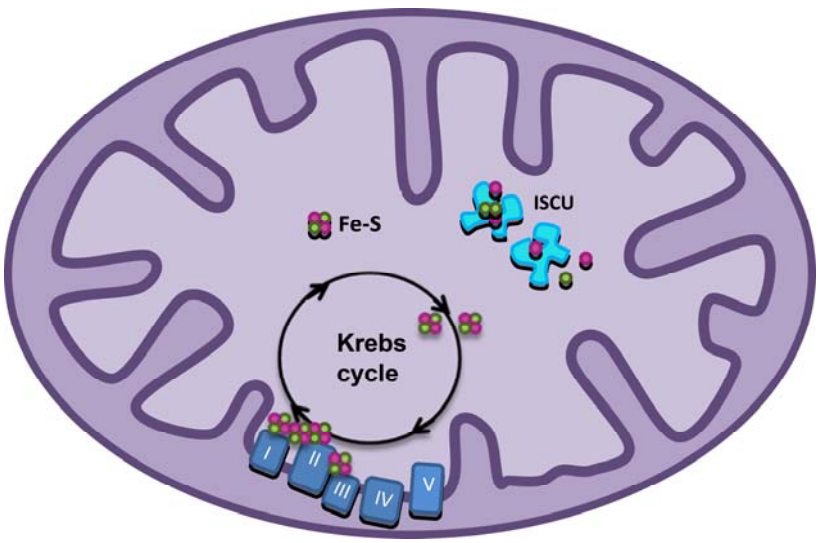

Figure 5. ISCU assembles Fe-S clusters which are delivered to proteins involved in the energy metabolism in the mitochondrion.

Gene Diversity through Alternative Splicing

The human genome was initially expected to hold at least 100 000 genes, but the number turned out to be drastically lower and the current estimate is 20 000-25 000 protein-coding genes [65]. This should be compared with the genome of some lower organisms like the nematode *Caenorhabditis elegans* and the fruitfly *Drosophila melanogaster* that hold roughly 19 000 [66] and 14 000 genes respectively [67]. However, the complexity of an organism is not only dependent on the sheer number of genes but also on the regulation of the genes and their products on a transcriptional/post-transcriptional and a translational/post-translational level. Most human genes are made up by protein coding exons that are intervened by stretches of non-coding introns and flanked by regions that contain elements of importance for the regulation of the gene. After transcription of a gene to pre-mRNA, the molecule is subjected to post-transcriptional modifications such as splicing. Splicing is the event where the exons are joined after the removal of the introns from the pre-mRNA resulting in a continuous protein-coding sequence. It has been shown that a single gene can encode several different proteins due to alternative splicing of the pre-mRNA. Today it is believed that over 90 percent of the human genes are alternatively spliced [68] which thereby greatly expands the human proteome without increasing the number of genes.

The Mechanism of Splicing

The mechanism of splicing is complex and involves numerous RNA and protein interacting factors. The splicing is performed by the spliceosome which discriminates between exons and introns due to short nucleotide consensus sequences, the donor (5') and acceptor (3') splice sites, situated in the exon/intron boundaries. Another important regulatory element is the branch point sequence which can be found 18 to 40 nucleotides upstream from the acceptor splice site. These three splice signalling motifs are present in every intron and are recognised numerous times during the process of splicing [69, 70]. Additional splicing regulatory elements (SREs) are present in the pre-mRNA. *Cis*-acting SREs serving either as splicing enhancers or inhibitors depending on whether the associating *trans*-acting regulatory proteins suppress or activate splice sites or the assembly of the splicosome (Figure 6) [70]. Developmental and tissue-specific expression of the regulatory proteins contributes to the alternative splicing of the pre-mRNA [71, 72].

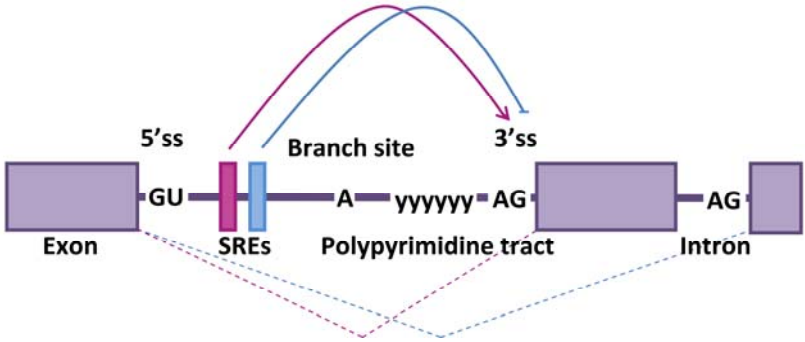

Figure 6. Consensus sequences and regulatory elements in the pre-mRNA affect which parts are included in the mRNA.

Monogenic Diseases Coupled to Aberrant Splicing

The outcome of mutations that alter consensus sequences or regulatory elements can be the retention of intronic sequences in the mRNA as in the case of ISCU myopathy where a polypyrimidine tract is strengthened due to a G (purine) to C (pyrimidine) substitution [8, 9]. Mayr and co-workers recently showed that a homozygous mutation in the intron of *SLC25A3* gene resulted in a myopathy and cardiomyopathy due to deficiencies in the mitochondrial phosphate carrier in a family from Turkey [73]. The mutation created a novel acceptor splice site which resulted in the introduction of eight base pairs in the mRNA leading to a frame-shift and a premature stop codon. A small proportion of the wild-type mRNA of the mitochondrial phosphate carrier was found in the skeletal muscles which might explain the milder phenotype seen in these patients compared to patients with a mutation in the muscle specific exon 3A in the *SLC25A3* gene [73, 74]. Donor and acceptor splice site mutations in the *DES* gene encoding the desmin protein involved in contractile action of heart, skeletal and smooth muscles has been shown to result in cardiac and skeletal myopathy due to exon skipping in this gene [75]. The inactivation of the splice sites results in the exclusion of the third exon which holds 96 base pairs and therefore does not interrupt the reading-frame. The resulting mutant protein was however shown to be non-functional [75]. Other intronic mutations that do not result in an alteration of the splicing product can also result in drastic disease phenotypes. Trinucleotide repeats in the first intron of the Frataxin gene *FXN* is the cause of the autosomal recessive mitochondrial disorder Friedreich's ataxia (OMIM 229300) [76, 77]. The trinucleotide expansion results in the formation of heterochromatin-structure which leads to decreased promoter accessibility and thus the silencing of the gene [78].

Aim of this Thesis

The overall aim of this thesis was to study the genes causing two monogenic diseases; NGF dependent pain insensitivity and ISCU myopathy in order to increase our understanding of the basal and disease associated mechanisms.

The specific aims were as follows:

To determine how the R221W NGF causes pain insensitivity and how this may explain the restricted phenotype, and more specifically:

- Analyse the cellular fate of the R221W NGF;
- Investigate how the R221W mutation affects the ability of NGF to interact and activate its receptors TrkA and p75;
- Purify R221W proNGF in order to determine its biological properties.

To determine the cellular mechanism contributing to the skeletal muscle phenotype of ISCU myopathy by, more specifically:

- Analyse the functional consequences of complete loss of *Iscu* in mice;
- Examine the possible role of tissue-specific splicing in the development of the muscle-specific phenotype;
- Identify factors that are important for the development of the tissue-specific phenotype.

Results and Discussion

Functional Analysis of Mutant NGF Causing Pain Insensitivity

Patients with a missense mutation (661C>T) in the *NGFβ* gene have been shown to be insensitive to deep pain. The mutation results in a drastic amino acid substitution in a region of the protein that is highly conserved among the related neurotrophins. Sural nerve biopsies from the homozygous patients showed a moderate loss of thin myelinated Aδ nerve fibres and a severe reduction of unmyelinated C-fibres [7]. The mutation responsible for NGF dependent pain insensitivity results in a less severe phenotype than that seen in CIPA patients, which carries mutations in the NGF receptor *TRK1* gene. These patients also suffer from pain insensitivity and, in addition display cognitive malfunctions and other neurological impairments [39]. The fact that the amino acid substitution is located in a region of NGF that has been shown to interact with the p75 receptor [79] suggests that the mutation primarily affects the binding to this receptor. Because the Swedish cases that suffer from NGF dependent pain insensitivity show no mental disabilities, functional studies on the R221W NGF protein could provide important insights into the development of the peripheral and central nervous system.

Biological Activity of R221W NGF

Because the patients display a loss of peripheral sensory neurons we suspected that the biological activity of the protein was impaired by the R221W substitution. In order to test the biological function of the mutant protein, the ability to differentiate PC12 cells were analysed. PC12 cells express both TrkA and p75 on the cell surface and this cell line has been extensively used to study neurite outgrowth upon NGF stimulation (Figure 7).

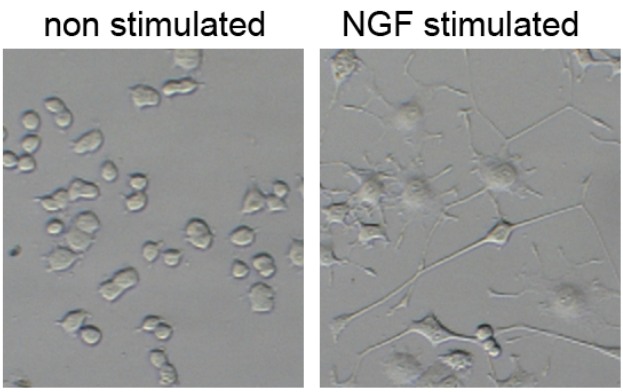

Figure 7. PC12 cells before and after NGF stimulation.

PC12 cells were transfected with expression vectors containing either wild-type or the mutant NGF sequence followed by scoring and documentation of the neurite outgrowth over a period of four days. By day four we found that approximately 90 percent of the wild-type NGF expressing cells were differentiated while only around four percent of the mutant NGF expressing cells were differentiated (Figure 1, **paper I**). In MOCK transfected cells only two percent were differentiated at the same time point. The difference observed between mutant and MOCK transfected cells was significant, indicating that some biological activity was retained in the mutant protein.

The inability of mutant NGF to induce PC12 cell differentiation could be a consequence of one or more effects on the protein including;

i) increased degradation
ii) defective secretion
iii) impaired receptor interaction

To determine whether the mutation affected the levels of secretion of NGF, we performed Western blot on conditioned media and cell lysate from two different cell lines; PC12 and COS-7 cells with neuronal origin and non-neuronal origin, respectively. Both cell lines secrete proteins via the constitutive pathway where secretory granules by default are transported to the periphery of the cell where they fuse. PC12 cells also secrete proteins via an activity dependent secretory pathway where vesicles fuse with the plasma membrane upon transmitter release [80]. We found, as was expected, high

levels of wild-type mature NGF and trace amounts of proNGF in the conditioned media from both cell lines. However, the secretion of mutant NGF was found to be impaired, with only trace amounts of mutant mature NGF secreted from both cell lines and limited amounts of secreted mutant proNGF from PC12 cells (Figure 2 A and B, **paper I**). The secretion of mutant NGF showed a 20 and 30-fold decrease from COS-7 and PC12 cells, respectively, compared to wild-type NGF (Figure 2 C, **paper I**). Furthermore, we found that the majority of NGF remained in the cell as proNGF (Figure 2 A and B, **paper I**). The fate of the mutant protein was confirmed by pulse-chase studies (Figure 3, **paper I**). The fact that mutant NGF stimulated PC12 cell differentiation to some degree, suggested that the small amount of secreted mutant NGF was biologically active. To investigate if mutant protein could bind and activate the TrkA receptor, phosphorylation of the downstream target ERK1/2 was analysed by Western blot on lysate from PC12 cells either stimulated with wild-type or mutant NGF. We found that stimulation with mutant NGF resulted in an activation of ERK1/2; however at a reduced level compared to that obtained by wild-type NGF (Figure 8, **paper I)**. This result indicates that the R221W substitution does not completely abolish the interaction with TrkA and that some biological activity therefore is sustained. Together these findings can explain the low, but significant, differentiation of PC12 cells by mutant NGF. The impaired secretion of mature NGF is most likely the major cause of loss of Aδ and C-fibres seen in the patients [7, 10]. This is also in agreement with the neurotrophic hypothesis that states that NGF depending neurons which do not receive enough trophic support undergo apoptosis [13, 81]. Interestingly, the Swedish patients show no mental retardation indicating no impairment in the development and survival of the CNS neurons. This implies that other mechanisms are of importance for the NGF dependent development of the CNS compared to the PNS.

Processing and Sorting of R221W proNGF

Because the major part of mutant NGF was found to accumulate as proNGF we hypothesised that R221W NGF may be cleavage resistant. To test this mutant proNGF was obtained from COS-7 cell lysate and were incubated with different concentrations of the proNGF convertases, furin and plasmin. Furin has been shown to process proNGF to mature NGF in the cell [21, 23] and plasmin to convert the proprotein in the extracellular space [24]. As expected, we found that the amount of wild-type proNGF was decreased with increasing concentrations of the convertases. However, the same result was

obtained for mutant proNGF (Figure 4, **paper I**) suggesting that the accumulation of mutant proNGF was not a consequence of impaired convertase accessibility due to the R221W substitution. This further implies that secreted mutant proNGF could actually be processed to mature NGF by plasmin in the extracellular space and therefore possibly adding to the development of specific neurons. It has in fact been shown that proNGF is co-localised with tPA, a regulator of the plasmin activation, in numerous pyramidal neurons of the cerebral cortex of rats and that it is released from CNS cortical neurons in an activity-dependent manner together with the protease cascade complex necessary for the cleavage in the extracellular space [82]. It has further been suggested that incomplete cleavage of proNGF might redirect this form to the activity dependent secretory pathway in hippocampal neurons [80].

The intracellular processing of proNGF occurs in the secretory vesicles by furin or other pro-hormone convertases after translocation from the *trans*-Golgi network. Because mutant proNGF is not cleavage resistant we hypothesised that it instead is incorrectly sorted, thereby escaping processing and secretion. To test this, the cellular localisation of the NGF protein was investigated by immunostaining. Cells expressing either the wild-type or the mutant NGF protein were analysed using antibodies discriminating between all NGF forms and proNGF. We found no specific differences in localisation between wild-type and mutant NGF in COS-7 cells. In PC12 cells the wild-type protein was mainly detected around the nucleus and in the processes as mature NGF. As expected, the major part of mutant NGF was found as proNGF, however it was not localised to specific compartments, but rather spread throughout the cytoplasm. Some mature mutant NGF was detected at the processes in differentiated PC12 cells (Figure 5, **paper I**). Furthermore, we confirmed a co-localisation between wild-type NGF and the dense-core secretory granule marker secretorygranin II (SgII) at the processes and around the nucleus. A minor amount of mutant NGF was also co-localised with SgII at the processes, although the majority of the protein was not (Figure 6 A, **paper I**). In order to analyse the concentration of NGF secreted from the activity dependent secretory vesicles, differentiated PC12 cells were transfected and stimulated with KCl and the conditioned media was assessed by ELISA. We observed an increased concentration of wild-type NGF after the treatment whereas only a minor elevation of mutant NGF was detected (Figure 6 B, **paper I**). These results show that a minor amount of mutant proNGF is co-localised with SgII and targeted for an activity dependent secretion in PC12 cells whereas the major part is not, supporting our hypothesis that the R221W NGF evades

processing and secretion due to an impaired sorting. Interestingly, studies have shown that a polymorphism in *BDNF* (V66M) results in a decreased regulated secretion of BDNF in cultured hippocampal neurons whereas the constitutive secretion is unaffected [83]. Furthermore, impaired intracellular trafficking and regulated secretion was observed from primary cortical neurons and neuro-secretory cells but not from vascular smooth muscle cells [84]. These findings propose that the intracellular sorting of proteins to different compartments are sensitive to alterations in the protein. Interestingly, the V66M polymorphism has only been reported in disorders of the CNS, including schizophrenia [85], Alzheimer's disease [86] and bipolar disorders[87] indicating that the activity dependent secretory pathway is of great importance in the CNS neurons.

Intracellular Responses to R221W proNGF

Because mutant proNGF can be detected throughout the cytoplasm and not co-localised with specific secretory vesicles we wanted to investigate whether mutant proNGF was recognised as a misfolded protein targeted for degradation by the proteasome. Folding of polypeptides in the ER is aided by numerous proteins, including the heat shock protein GRP78. This protein is up-regulated as a consequence of an overload of misfolded protein. Proteins that are targeted for degradation are ubiquitinated and sent to the proteasome for destruction. We analysed the secretion of the protein after inhibition of the proteasome and found that the inhibition did not increase the secretion of mutant NGF whereas a slight increase of wild-type NGF was observed (Figure 7 C, **paper I**). Furthermore, no obvious up-regulation of the heat shock protein GRP78 was observed in cells expressing wild-type or mutant NGF (Figure 7 A, **paper I**). These results indicate that mutant proNGF is not recognised as a misfolded protein, targeted for degradation by the proteasome, but that it is incorrectly sorted and therefore not present in the secretory vesicles. The fate of mutant NGF might, however, depend on the tissue or cell type expressing it. We observed more secreted mutant proNGF from the PC12 cells and less protein in the cell when compared with COS-7 cells. These two cell types have different secretory properties. PC12 cells secrete protein through both a constitutive and an activity dependent pathway implicated in the secretion of neurotrophins from CNS neurons [82, 84]. This, in combination with the fact that NGF/proNGF signalling is complex with opposing effects depending upon the interacting receptors and targets, might explain why the NGF dependent pain insensitive patients show a severe decrease of peripheral neurons but no impairment of the CNS.

Interestingly, a novel mutation in the *NGFβ* gene has recently been discovered that have been shown to cause a more drastic phenotype in the affected patients than that observed in the Swedish patients. Apart from pain insensitivity, this phenotype also includes anhidrosis and mental retardation. This novel mutation causes a frameshift and the replacement of the 15 terminal amino acids with 43 novel amino acids [12]. This mutation has also been shown to affect the secretion of the protein [12].

R221W NGF and Receptor Interaction

We showed previously that mutant NGF secreted from PC12 cells can bind and activate TrkA and thereby also the downstream target ERK1/2 in PC12 cells. However, the level of phosphorylation was lower in the cells stimulated with mutant NGF compared to wild-type NGF. The difference in activation of the downstream target obtained with mutant NGF could nonetheless be a result of decreased binding to the p75 receptor. The mutation causing NGF dependent insensitivity to pain is positioned in a region highly conserved among the neurotrophins [7] which all bind p75 with similar affinity. It has also been shown that this region is in fact important for the interaction with the p75 receptor [79]. To determine if the lower level of ERK1/2 phosphorylation produced by mutant NGF in PC12 cells was due to a decreased affinity for p75, cells only expressing TrkA or both TrkA and p75 were stimulated with wild-type or mutant NGF. We found, as expected, wild-type NGF to cause a substantial phosphorylation of ERK1/2 in cells expressing both p75 and TrkA while mutant NGF produced a weaker phosphorylation (Figure 1, **paper II**). In contrast to this, no significant difference in the ability to activate ERK1/2 was observed between wild-type and R221W NGF in cells only expressing TrkA (Figure 1, **paper II**) suggesting that the R221W substitution in NGF primarily alters the affinity for the p75 receptor.

Purification of R221W proNGF from *E.coli*

Because only limited amounts of mutant NGF could be obtained from PC12 and COS-7 cells we set out to purify larger quantities of this protein using an *Escherichia coli* (*E.coli*) expression system. The fact that the majority of mutant NGF in the cell exists in the pro-form suggests that proNGF may be the biologically more relevant form in patients; we therefore prioritised the purification of proNGF over mature NGF. *E.coli* cells were transformed with

constructs encoding either wild-type or R221W proNGF and to minimize the processing from proNGF to mature NGF, the furin cleavage site was destroyed by point mutations in both constructs. The protein was purified and solubilised from inclusion bodies and the inclusion body proteins were refolded whereby proNGF were purified by fast protein liquid chromatography (FPLC). The purity of the collected fractions of R221W proNGF were analysed by SDS-PAGE and tested on an acid native gel and the biophysical characteristics were similar to wild-type proNGF (Figure 2, **paper II**). Interestingly, during the process of refolding we found that the R221W proNGF were prone to aggregate and mutant protein was furthermore eluted from the CM Sepharose S column with a different retention time than the wild-type protein. The final yield of mutant proNGF was much lower than the amount of wild-type proNGF. These observations indicate that the R221W substitution results in a protein with fundamentally different folding properties.

Biological Activity of *E.coli* Expressed R221W proNGF

In order to analyse if the purified mutant proNGF was biologically active we again used the PC12 differentiation assay. Wild-type proNGF has previously been shown able to induce differentiation in PC12 cells although with a reduced efficiency compared to mature NGF [30]. PC12 cells were stimulated with mutant proNGF and assessed for neurite outgrowth over four days. By day four we found that approximately six percent of the cells stimulated with mutant protein were differentiated (Figure 3, **paper II**). However, barely three percent of the non-stimulated cells were found differentiated by the same time point. This result was in accordance with that obtained from mutant NGF expressing PC12 cells. However, this result was found to be due to a drastic impairment of the secretion of the protein (Figure 1 and 2, **paper I**) while 100 ng/ml of purified mutant proNGF was administrated to the cells in this case, suggesting that only a small fraction of the R221W proNGF was biologically active. The mutant protein was later found to be more or less inactive when different concentrations of R221W proNGF caused similar levels of ERK1/2 phosphorylation as non-stimulated cells in cells only expressing TrkA (Figure 4, **paper II**). Previous experiments have shown that wild-type proNGF could be expressed and purified from *E.coli*, HEK 293 cells or by using a baculovirus/insect cell system without affecting the outcome of receptor interactions due to different posttranslational modifications [88]. The replacement of the R221 amino acid with any hydrophobic amino acid has previously been shown to result in low yields of

purified protein [89]. This, together with our findings that PC12 cells expressed R221W NGF is able to bind TrkA, implies that the eukaryotic folding machinery is necessary in the folding of R221W proNGF.

In vitro studies by Capsoni and co-workers performed on a NGF mutant, where the arginine was replaced by glutamic acid, indicate that this position of the protein is critical in the activation of TrkA and its downstream targets. The phosphorylation of residue Tyr490 was decreased by 70 percent compared to wild-type activation [90]. However, the same group showed earlier by Surfance Plasmon Resonance that the dissociation constant of this protein to TrkA was identical to that of the wild-type protein [89].

To date we have shown that mutant NGF can activate ERK1/2, to a similar level to wild-type NGF, in cells only expressing TrkA. In cells that express both TrkA and p75, the level of activation by R221W NGF was at a lower level compared to the wild-type which suggests that the mutation may alter the p75 binding properties but leave the binding to TrkA intact. However, there is still a lot we do not know about how the R221W NGF interact with different combinations of receptors and what role the origin of cell type expressing the protein play.

Functional Analysis of Mechanisms Contributing to the Tissue-Specific Phenotype Seen in Patients with ISCU Myopathy

ISCU myopathy has been shown to be caused by an intron mutation in the *ISCU* gene leading to the incorporation of intron sequence into the mRNA [8, 9]. The result of the defective splicing of ISCU is a Fe-S cluster protein deficiency, affecting enzymes involved in Krebs cycle and the respiratory chain. These defects lead to severe exercise intolerance in the affected patients. Interestingly, the phenotype seems to be restricted to the skeletal muscles while other energy-demanding organs are unaffected.

Knock down of *Iscu* in mice

ISCU has been shown to be important for the formation of Fe-S clusters involved in Krebs cycle and the respiratory chain [62, 63] and the knock-down of *ISCU* homologs in lower organisms have proven to be detrimental [61, 91, 92]. The observation that the phenotype seen in patients suffering from ISCU myopathy is restricted to the skeletal muscles, while other energy demanding organs seem unaffected, might indicate that a back-up system has evolved in higher organisms. To determine the importance of ISCU in mammals, *Iscu* null mice were generated from embryonic stem cell lines obtained from the knock-out mouse project (KOMP) repository. The heterozygous mice showed no difference in physical and behavioural phenotype compared to their wild-type littermates. Furthermore, the protein levels of Fe-S containing aconitase and the SDH subunit SDHB were at normal levels in the heterozygous mice. However, a reduction of 20–30 percent in the levels of ISCU was seen in all tissues examined compared to wild-type mice (Figure 5 B, **paper III**) which indicates an up-regulation from the wild-type *Iscu* allele. In order to obtain *Iscu* null offspring the heterozygous mice were interbred, but no *Iscu* null mice were obtained (n=100). The stage of embryonic death was examined by dissecting and genotyping E7.5-10.5 embryos (n=45) but no homozygous null mice was detected at any stage. We did, however observe two embryos out of nine with the *Iscu*$^{-/-}$ genotype, at the pre-implantation stage E 3.5 (Figure 5 A, **paper III**) proving ISCU to be of vital importance already at an early stage in the development in mammals. This shows that the evolutionary role of ISCU has been preserved in organisms ranging from yeast to mammals. Furthermore,

these findings suggest that the mutation causing ISCU myopathy in human does not completely abolish the function of the protein.

Protein Expression

As the disease does not result in a systemic phenotype we wanted to examine if the levels of ISCU protein differed between muscle and non-affected tissues. As expected ISCU was essentially absent in patient muscle but, in contrast, ISCU was detected in all other tissues examined such as heart, liver and kidney samples (Figure 2 A, **Paper III**). The levels of ISCU protein in heart and muscle tissue from patient were then compared with the levels in controls. These results again demonstrated that the levels of ISCU in patient muscle tissue were almost absent compared to the levels seen in the controls. Surprisingly, the levels of ISCU in patient heart tissue were also drastically reduced as compared to the levels in controls (Figure 2 B, **Paper III**). To investigate if the decrease of ISCU seen in patient heart tissue affected the levels of aconitase and SDHB, heart tissue from patient and controls were analysed by Western blot. We found that the levels of these Fe-S cluster containing proteins were similar in the heart tissue from both patient and controls (Figure 4, **paper III**) in contrast to muscle where both SDHB and aconitase levels were decreased. These results are in agreement with the muscle specific phenotype seen in the ISCU myopathy patients. However, it has been shown that patients that are compound heterozygous for the Swedish mutation and a missense mutation in exon 3 of the *ISCU* gene display a more severe phenotype which also includes cardiomyopathy [64]. This finding indicates that ISCU is important for normal heart function and suggests that tissue-specific regulatory mechanisms contribute to the phenotype seen in the Swedish patients.

Tissue-Specific Splicing

As the mutation results in the enhancement of a cryptic splice site in the last intron of the *ISCU* gene, leading to the inclusion of a pseudo exon in the mRNA, we hypothesised that the disease phenotype was due to tissue-specific splicing. This hypothesis was tested by analysing the splicing pattern of ISCU in muscle, heart and liver tissue from ISCU myopathy patient and controls. We found that almost 80 percent of the total ISCU mRNA was in the form of mutant mRNA in patient muscle, compared to seven percent in

the controls (Figure 1, **Paper III**). This result explains the extremely low level of functional ISCU protein found in patient muscle. In contrast to this, in patient heart tissue the majority of the ISCU transcript was correctly spliced, with the wild-type transcript representing 70 percent of total ISCU. In liver tissue 54 percent of the transcript was correctly spliced (Figure 1, **Paper III**). These results support our hypothesis that the muscle-specific phenotype is due to tissue-specific splicing of the mutant ISCU transcript. The observation that 70 percent of ISCU is correctly spliced in combination with lower levels of total ISCU mRNA could explain the low levels of ISCU protein seen in patient heart tissue, but since no sign of a defect Fe-S cluster assembly was evident based on the normal levels of SDH and aconitase the level of wild-type ISCU found in patient heart tissue seems sufficient to uphold a normal heart phenotype. Furthermore, this is in line with our findings from heterozygous *Iscu* null mice which are indistinguishable from their wild-type littermates even though they show a 20 – 30 percent reduction in ISCU protein levels. Our results are also supported by previous findings of differences in RNA processing of ISCU mRNA between muscle, myoblasts, fibroblasts and blood [93]. Together these results argue that alternative splicing is the mechanism responsible for the relatively mild phenotype in ISCU myopathy patients.

Identification of Factors Interacting with the Region Harbouring the Mutation

Following this, we wanted to identify the factors involved in the defective and tissue-specific splicing of mutant ISCU. First we set out to identify proteins that interact with the region containing the mutation and determine if there were any differences in the interactions between wild-type and mutant *ISCU* sequence. We could show that nuclear factors interacted with both the wild-type and mutant sequence, using a RNA-gel-shift assay (Figure 1 A, **Paper IV**). The interactions with the mutant sequence were, however, more pronounced which suggests that the mutation increases the affinity for one or more nuclear factors. In order to identify the interacting proteins, the RNA/protein complexes were purified and separated by SDS-PAGE and visualised by silver staining. The protein binding pattern between mutant and wild-type sequence did not differ, with the exception of a band around 75 kDa that showed a higher intensity with the mutant sequence (Figure 1 B, **Paper IV**). The proteins were excised from the gel and five RNA-binding proteins were identified by mass spectrometry: matrin 3, SFRS14, IGF2BP1,

RBM39 and PTBP1. SFRS14, RBM39 and PTBP1 were known splicing factors whereas matrin 3 and IGF2BP1 had not previously been implicated in the splicing process (Table S1, **Paper IV**). IGF2BP1 was identified as the protein interacting more strongly with the mutant intron sequence. By Western blotting IGF2BP1 was verified to have a higher affinity to the mutant sequence than the wild-type sequence, compared to PTBP1 which bound both sequences with equal affinity (Figure 1 C, **Paper IV**).

Identification of factors regulating the tissue-specific splicing of ISCU

In order to examine whether the identified factors could actually affect the splicing pattern of the *ISCU* gene, a minigene containing either the wild-type or mutant intron sequence was constructed (Figure S1, **Paper IV**). RD4 cells were transfected with the minigene, alone or with the different factors, and the splicing pattern was analysed by RT-PCR. The splicing pattern was not altered when the minigene was co-expressed with either matrin 3 or SFRS14 (Figure 2 A-C, **Paper IV**). However, PTBP1 drastically decreased the incorrect splicing of both the mutant and wild-type minigene. PTB has previously been shown to act as a repressor of alternatively spliced exons by associating to polypyrimidine tracts [94, 95]. As the mutation causing ISCU myopathy is positioned in a region of the intron which resembles a polypyrimidine tract, this result was not surprising. The co-expression of the minigene with either IGF2BP1 or RBM39, on the other hand, resulted in a decrease of the total amount of both wild-type and mutant ISCU transcript (Figure 2 A-C, **Paper IV**). The decreased levels of total ISCU transcript was in agreement with previous findings showing decreased levels of ISCU mRNA in ISCU myopathy patient muscle [9, 93]. Furthermore, we found that the decrease was more pronounced for the normal transcript shifting the ratio towards the mutant transcript in the presence of IGF2BP1 and RBM39 (Figure 2 A-C, **Paper IV**). Because PTBP1 and IGF2BP1 or RBM39 had opposing effects, we further analysed the outcome of the splicing pattern with the co-expression of these factors. IGF2BP1 and RBM39 were shown to counteract the PTBP1 repression of incorrect splicing (Figure 2 D and E, **Paper IV**). This result suggests that IGF2BP1 and RBM39 might associate with the intron region and thereby block the binding of PTBP1 resulting in the formation of the mutant transcript. IGF2BP1 has previously been shown to have affinity for polypyrimidine tracts and the ability, under certain conditions, to compete with PTB for the binding of this tract in the mRNA of IGF2 [96]. The fact that IGF2BP1 bound the sequence harbouring the intron

mutation with higher affinity than the wild-type sequence, accompanied by the fact that it favours the formation of the transcript holding the pseudo-exon suggest that this factor may be a key player in the tissue-specific splicing causing the non-systemic phenotype seen in the ISCU myopathy patients. In line with this IGF2BP1 show a tissue-specific expression of IGF2BP1 during development, with high levels expressed in muscle and epithelia but an absence in the brain [96]. The fact that RBM39 showed equal affinity to both the mutant and wild-type sequence indicates that this factor does not contribute to the tissue specific phenotype seen in the ISCU myopathy patients.

Conclusions

- The R221W mutation results in an impaired secretion of mature NGF and is, most likely, the reason for the loss of nociceptive neurons in patients with NGF dependent pain insensitivity.

- The low amount of secreted mature NGF is due to incorrect intracellular sorting and the accumulation of unprocessed R221W proNGF inside the cell.

- R221W mutation results in impaired p75 signalling, while the ability to activate TrkA signalling is not significantly affected.

- The R221W substitution alters the folding properties of the protein, when purified from *E.coli* inclusion bodies.

- Complete loss of ISCU in mice results in early embryonic death while heterozygous *Iscu*$^{+/-}$ mice are indistinguishable from their wild-type littermates.

- The muscle-specific phenotype in patients with ISCU myopathy is most likely due to tissue-specific splicing.

- One RNA binding factor, IGF2BP1, bind the mutant ISCU intron region with higher affinity than the wild-type sequence.

- The aberrant splicing of ISCU is repressed by PTBP1 but can be de-repressed by IGF2BP1 and RBM39.

Populärvetenskaplig Sammanfattning

Mycket av dagens kunskap gällande specifika proteiner och dess normala respektive patologiska funktion kommer från studier av deras roll i genetiska sjukdomar. När en sjukdomsorsakande gen blivit identifierad kan viktig information om dess genprodukt, samt associerade proteiner och biologiska processer erhållas genom biokemiska analyser, cellulära studier och studier i djurmodeller. Syftet med denna avhandling var att studera basala och sjukdomrelaterade mekanismerna associerade med de gener/proteiner som orsakar två ovanliga monogena sjukdomar, smärtokänslighet respektive myopati med laktacidos,

Smärtokänslighet har identifierats i en familj som härstammar från en liten by i Norrbotten. Sjukdomen är mycket ovanlig med endast ett fåtal drabbade individer. De svårast drabbade patienterna är inkapabla att känna djup smärta och uppvisar nedsatt temperaturkänslighet, symptom som startar redan i tidig barndom. Smärtokänsligheten har för patienterna resulterat i flertalet smärtfria frakturer samt svåra ledskador som en följd av felbelastning. Sjukdomen orsakas av en mutation i genen som kodar för proteinet Nerve growth factor (NGF). Mutationen ger upphov till ett drastiskt aminosyrautbyte (R221W) i en del av proteinet som är väl konserverad mellan NGF i olika arter samt mellan besläktade proteiner. NGF produceras och utsöndras av målorganen och dess roll är betydande för utveckling och överlevnad av specifika neuroner i det perifera och centrala nervsystemet. Nervbiopsier har visat att patienterna har ett minskat antal av de smärtförmedlande NGF-beroende nerverna. I arbete **I** studerade vi hur R221W mutationen påverkade funktionen av NGF och fann att proteinet felsorterades inne i cellen vilket sedermera resulterade i en ackumulering av proNGF, en större variant av proteinet med andra egenskaper. Detta medförde en kraftigt minskad utsöndring av NGF vilket kan ses som en möjlig förklaring till det reducerade antalet sensoriska nervceller funna hos patienterna. En annan *NGF* mutation har visats orsaka en allvarligare form av sjukdom där de drabbade patienter förutom smärtokänslighet även lider av anhidros (oförmåga att svettas) samt nedsatt mental förmåga. Dessa symptom återfinns även hos patienter med mutationer i genen som kodar för NGF receptorn TrkA. Det faktum att de svenska patienterna inte lider av någon mental störning antyder att R221W mutationen inte påverkar utvecklingen av neuroner kopplade till det centrala nervsystemet. NGF förmedlar sin effekt genom att binda till TrkA receptorn varpå signaler om utveckling och överlevnad förmedlas till nervcellen. Celler som även

uttrycker receptorn p75 på dess yta binder NGF hårdare vilket därmed gör dem extra mottagliga för NGF. I motsats till NGF har den större varianten proNGF visats förmedla apoptos (programmerad celldöd) vid inbindning till p75 receptorn. Vi fann i arbete **II** att celler som ensamt uttryckte TrkA receptorn kunde binda R221W NGF i jämförbar utsträckning som normalt NGF medan de celler som både uttryckte TrkA och p75 band mutant NGF med sämre förmåga än normalt NGF. Detta resultat antyder att R221W mutationen främst påverkar inbindningen till p75 receptorn vilket även styrks av det faktum att mutationen är belägen i en del av proteinet som visats vara delaktig i interaktionen med receptorn. Vi fann även i försök med att rena mutant proNGF från bakterier att R221W mutationen fundamentalt ändrade sättet proteinet veckades på vilket tyder på att proteinveckningsprocesser associerade med eukaryota celler är avgörande för proteinets funktionalitet.

Den andra sjukdomen som studerats i denna avhandling är Myopati med svår lactacidos som identifierats i familjer med rötter i Ångermanland och Västerbotten. De drabbade patienterna har visats vara ”motionsintoleranta” vilket yttrar sig genom svår laktacidos och värkande muskelkramper efter måttfull fysisk aktivitet eller extrema dieter så som fasta. Sjukdomen uppenbaras under barndomen då de drabbade patienterna har svårt att hålla jämna steg med jämnåriga kamrater. Sjukdomen orsakas av en mutation i genen som kodar för proteinet Iron-Sulphur cluster assembly protein U. *ISCU* genen är uppdelad i protein-kodande (exon) och icke-kodande (intron) segment och normalt tas endast exonerna med i mallen för proteintillverkning (mRNA) i en process som kallas splicing. Den sjukdomsorsakande mutationen är belägen i en intron och gör att en del av intronen känns igen som en exon och därför tas med i mRNA:t vilket i sin tur resulterar i ett icke funktionsdugligt protein. Normalt sätter ISCU proteinet samman järn- och svavel- grupper, komponenter som visats vara viktiga för proteiner delaktiga i cellens energimetabolism. Sjukdomen har visats vara vävnadsspecifik och drabbar främst patienternas skelettmuskler medan andra energikrävande organ så som hjärta och lever verkar opåverkade. I arbete **III** undersökte vi orsaken till den vävnadsspecifika sjukdomsbilden och fann att majoriteten av mRNA:t i skelettmusklerna från patient innehöll intronregionen medan det normala mRNA:t dominerade i de andra undersökta vävnaderna vilket resulterade i normala nivåerna av järn-svavel innehållande proteiner i dessa vävnader. Vi kunde även visa att total avsaknad av ISCU inte är förenligt med liv då inte ett embryo kunde identifieras efter implantationsstadiet efter så kallad ”knock down” av *Iscu* genen i mus. I arbete **IV** var syftet att identifiera faktorer som bidrog till den

vävnadsspecifika splicingen av ISCU. Vi identifierade fem olika proteiner som interagerade med intronregionen och kunde visa att tre av dessa; PTBP1, IGF2BP1 och RBM39 påverkade splicingmönstret. PTBP1 motverkade inkluderandet av intronregionen medan IGF2BP1 och RBM39 motverkade formationen av normalt mRNA och därigenom ändrade fördelningen mellan mutant och normalt mRNA. Den enda av dessa faktorer som visade sig associera mer med den mutanta gensekvensen var IGF2BP1 vilket tyder på att denna RNA bindande faktor har en nyckelroll i den vävnadsspecifika splicingen av ISCU.

Sammanfattningsvis har dessa studier bidragit till viktiga insikter vilket gör gällande cellulära mekanismer som orsakar de två olika sjukdomarna. Felaktig sortering och kraftigt minskad utsöndring av NGF bidrar till smärtokänslighet medan vävnadsspecifik splicing av ISCU orsakar sjukdomsbilden som uppvisas av patienter med myopati med svår laktacidos.

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