Genetics of neuropathic pain

Summary

Neuropathic pain (NeP) is a complex condition usually resulting from damage or disease of the central and/or peripheral nervous system. It is a common manifestation in several disorders altering the somatosensory nervous system, including Small Fiber Neuropathy (SFN) and peripheral diabetic neuropathy (DN), affecting up to 10% of general population. NeP is associated with multiple comorbidities and especially its psychiatric and emotional component negatively influences patients' quality of life and well-being, often leading to anxiety and depression. Current pain management is based on symptomatic treatment and many of the patients cannot achieve pain relief with available analgesics. This creates a huge need for novel, targeted therapies for better clinical outcome, however lack of knowledge about underlying mechanism and pathophysiology impedes effective drug development. It is also clear that genetic predisposition is playing a role and multiple genes have been shown to be associated or causally involved with pain. Voltage gated sodium ion channels (VGSC) are the best studied genes with a clearly documented role in pain pathophysiology, however only part of the patients have dysfunctional VGSC due to a pathogenic genetic variant. For the majority of NeP patients the underlying genetic cause is not known. Therefore, the work presented in this thesis aimed to identify novel genetic variants and potential NeP related genes via NGS of patient cohorts and to establish the functional role of possible pathogenic variants in novel genes.

Chapter 1 provides a general introduction of neuropathic pain. This chapter reviews different aspects of NeP, including clinical manifestations, pathophysiology, causes, diagnostics and treatment strategies. Moreover, two phenotypes associated with neuropathic pain; SFN and DN are being discussed in more detail. The second part of the chapter focusses on the genetic background of NeP and elaborates on the involvement of VGSCs in pain etiology. It also covers the detection of novel disease genes using NGS technology and the challenges of functionally testing Variants of Unknown Significance (VUS) role. The chapter ends with the aims and an outline of the thesis.

In the past years, a role for ion channel genes in painful neuropathy has become evident, making them important novel therapeutic targets. **Chapter 2** elaborates about current state of knowledge on the involvement of ICG. Ion channels expressed in dorsal root ganglion neurons are potentially analgesic pharmacological targets in sensory signalling (e.g., *TRPV1*), regulation of neuronal excitation (potassium

channels), action potential transmission (sodium channels), generation of involuntary action potentials (HCN channels), mediating thermal pain (Anoctamin), pH modulation (ASIC channels), and neurotransmitter release (calcium channels). Therefore, understanding the role of variants in ion channel genes in neuropathic pain is important to personalize this broad range of potential molecular pain-control targets. The most investigated channels in relation to pain are voltage-gated sodium ion channels, however, they are involved in less than 20% of the patients with neuropathic pain. In this chapter, we focus on other ion channel genes and their involvement in painful neuropathies. The calcium auxiliary subunits and TRP channel genes, particularly *TRPV1*, have been thoroughly investigated as cause of and therapeutic target for pain. However, pain alleviation has not been achieved, probably due the heterogeneous genetic background in these patients. Studies on the genetic factors involving these ion channels may improve our knowledge of the common molecular mediators of pain and neuropathy, leading to personalized therapeutic approaches for painful neuropathies and paving the way toward new targets to be investigated.

The possible implication of 15 ion channel genes (ICG) and related genetic variants potentially affecting channel function have been investigated in **Chapter 3.** We performed molecular inversion probes-next generation (MIP-NGS) sequencing of 5 transient receptor potential cation channels, 8 potassium channels and 2 calcium-activated chloride channel genes in 222 painful- and 304 painless-DN patients, showing 12 painful-DN (5.4%) patients showed potentially pathogenic variants (five nonsense/frameshift, seven missense, one out-of-frame deletion) in *ANO3* (n = 3), *HCN1* (n = 1), *KCNK18* (n = 2), *TRPA1* (n = 3), *TRPM8* (n = 3) and *TRPV4* (n = 1) and 14 painless-DN patients (4.6%-three nonsense/frameshift, nine missense, one out-of-frame deletion) in *ANO1* (n = 1), *KCNK18* (n = 3), *KCNQ3* (n = 1), *TRPA1* (n = 2), *TRPM8* (n = 1), *TRPV1* (n = 3) and *TRPV4* (n = 3). We observed that painful-DN patients with ion channel gene variants reported higher maximal pain and painful-DN patients with TRP variants had severe pain combined with abnormal thermal thresholds. In **Chapter 4**, we used the same gene panel to perform MIP-NGS of 414 SFN patients that did not have VSCG variant. The sequencing resulted in identification of potentially pathogenic heterozygous variants in twenty patients (4.8%) in *ANO3* (n = 2), *KCNK18* (n=2), *KCNQ3* (n=2), *TRPA1* (n=7), *TRPM8* (n=3), *TRPV1* (n=3) and *TRPV3* (n=2). Again, patients with ion-channel gene variants, including individuals with TRP variants, reported more severe pain compared to patients without such variants. Our results described in **Chapter 3** and

Chapter 4 strongly suggest extensive ICG variants implication in neuropathic pain, however these findings require functional validation.

To identify novel pain genes and specific genetic variants (**Chapter 5**), we created panel of 592 gene candidates, selected from the literature because of their association with pain, genes from genetic pain databases and Human Phenotype Ontology database and Online Mendelian Inheritance in Man (OMIM) based on association with (painful) peripheral neuropathy symptoms. We identified 4 different heterozygous pathogenic variants (c.684C>G, c.2159T>A, c.2567G>A and a combination of c.2794A>C, c.2971G>T and c.5756A>G) in *SCN9A*, and 3 different heterozygous likely pathogenic variants in 3 genes; *AR* (c.1792A>G), *MFN2* (c.1384T>C) and *SCN11A* (c.1744G>A). In total, 40 different variants of uncertain clinical significance (VUS) in 37 genes; n=17, 42.5% variants in genes involved in neurotransmission, n=16, 40% in ion channel genes, n=6, 15% in metabolism related genes and n=1, 2.5% variant in gene involved in immune response. As variant classification is largely based on pathogenicity prediction tools and literature data, the experimental studies of these variants is of great interest.

Using next-generation sequencing, we identified possible pathogenic variants [VUS; c.638C>T, p.(S213F) and c.1357A>G, p.(I453V)] in the Anoctamin 3 (ANO3) gene in NeP patients. To get further insight in the possible role of these VUS in pain pathophysiology, we worked on an experimental model and functional read outs for these ANO3 VUS (**Chapter 6**). First, using Site Directed Mutagenesis we generated mutated ANO3 in an expression vector. In order to functionally test these variants, we selected HEK293 and HT-29 cell lines, in which according to literature and databases ANO3 was not expressed. Surprisingly, Western blot did not show significant protein overexpression, but unexpectedly also the untransfected cells displayed ANO3 protein. Therefore, we tested a number of cell lines for ANO3 expression and muscle stem cells, called mesoangioblasts (MABs) were the only cell line not expressing ANO3 protein at all. The MABs were transfected, but we did not obtain sufficient material for WB to confirm protein expression, probably due to a poor transfection efficiency. As ANO3 belongs to Ca^{2+}-activated Cl- channels, we have been developing a functional assay based on calcium measurement. In conclusion, awaiting the results of the functional studies, we cannot draw a definite conclusion on the role of these ANO3 variants in NeP, but everything is now in place to be able to perform the required experiments.

Chapter 7 discusses the key findings of this thesis and elaborates on the challenges of functional validation of VUS detected during NGS. Several *in vitro* and *in vivo* models are being described including their advantages and limitations in terms of VUS testing. **Chapter 8** provides overview of the research chapters together with scientific and social impact of this work.