Maastricht University

A zebrafish model of small-fiber neuropathy

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Valorisation

Clinical relevance

In the last few years, variants in *SCN9A*, *SCN10A* and *SCN11A* have been identified to be causative of small-fiber neuropathy (SFN) (Faber et al., 2012a, Faber et al., 2012b, Huang et al., 2014). The variant frequencies reported for these genes are based on relatively small SFN patient cohorts making it difficult to study correlations between phenotypes and genotypes. Insights in the variant frequency and eventually characterization of variants of Na_v channels increases the utility of genetic screening for clinical care and emphasizes the need for the development of tailored treatments with specific sodium channel blockers. Furthermore, certainty about the origin of symptoms, as well as, genetic counseling by which the patient and relatives are informed about the possibility of developing and transmitting the disease, is of great importance for patients with pure SFN.

In this thesis, we report that 11.6% of pure SFN patients harbor (potentially) pathogenic VGSC variants and we propose that genetic screening of *SCN9A, SCN10A* and *SCN11A* should be considered in all pure SFN patients, independently of clinical features or underlying condition. We also showed that erythromelalgia-like symptoms and warmth-induced pain were significantly more common in patients harboring VGSC variants. Further extending this cohort in the near future will lead to the identification of more potentially pathogenic variants which will allow studying detailed correlations between VGSC and the underlying conditions. This will help us to better understand the disease and identify patients with such condition that are at high risk for developing a neuropathy. However, a drawback is that current methods to demonstrate pathogenicity unambiguously, like electrophysiology, are not always (financially) possible or able to keep up with the high number of variants of uncertain clinical significance (VUS) in these patients. The identification of hotspots in these genes, the presence of the same potentially pathogenic variants in unrelated SFN patients or segregation analysis can solve this issue for some of these variants, however, many will still remain VUS. To solve this problem, we set out to develop a zebrafish model that can be of great importance in characterizing these VUS. To assess the effects of variants we set up a panel of two read-outs reflecting SFN in zebrafish, being nerve density and behavioral responses. Nerve density was studied using a transgenic line in which the sensory neurons are GFP-labelled. For the behavioral experiments, a temperature-controlled water compartment was developed, which allowed quantification of the behavioral response to temperature changes. By overexpressing human pathogenic

SCN9A variants and comparing the outcome with the effect of the overexpression of WT-*SCN9A* we demonstrated that the zebrafish model has this potential of characterizing VUS in SFN in a medium throughput manner. However, as also described in **chapter 6** an extensive validation of our model is necessary before this model can be implemented in diagnostics. Such validation involves testing more known pathogenic variants (Table 1 **Chapter 6**), demonstrating the accuracy to predict pathogenicity of our model, based on one of the read-outs or both. Also, non-pathogenic variants must be included which provides information about the false positive rate of our model. As injecting WT-*SCN9A* cDNA does not change nerve density nor temperature sensitivity, this seems promising. Eventually, if properly validated, VUS can be tested to define their pathogenicity, which at first must be followed-up by additional tests to confirm or rule out pathogenicity. The successful establishment of our SFN model by overexpression of *SCN9A* variants raises the question whether a similar model for variants in the two other voltage-gated sodium channels (*SCN10A* and *SCN11A*) causative of SFN can be established as well. If so, a similar approach for optimizing and validating the assays as described for *SCN9A* should be followed (Table 1 **Chapter 6**). As described in **chapter 6** the use of transgenic zebrafish models is another possibility but has several limitations. Since the zebrafish has proven to be an excellent model for drug discovery, our SFN zebrafish model has the potential to test fast the efficacy and toxicity of novel compounds with potential analgesic properties (Yoganantharjah and Gibert, 2017, Vaz et al., 2018). Therefore, our *in vivo* model has the potential for identifying novel therapeutic interventions but can also support in the development of sodium channel blockers.

Application of our read-out model to study other mechanisms involved in SFN.

We have demonstrated that our read-out panel is not only specific for SFN caused by *SCN9A* variants **(Chapter 5)**. Therefore, our panel can be used to further unravel other mechanisms causative of SFN. There are many causes of SFN which can be divided into hereditary causes, metabolic causes, vitamin deficiency, neurotoxic exposure, infections, immunological causes and idiopathic SFN (Terkelsen et al., 2017). The many (genetic) tools that are nowadays available make it possible to study these mechanisms in further detail. A loss-of-function of the gene of interest can be studied in a transient manner (morpholinos and crispants) or creating a stable genetic line with CRISPR/Cas9 (Stainier et al., 2017, Liu et al., 2017).

Alternatively, a loss-of-function mutant, if available, can be ordered from the Zebrafish Mutation Project (ZMP), which aims to create a knockout allele in every protein-coding gene in the zebrafish genome. Although our read-out panel has many advantages, there are also limitations to this set-up. Since our panel can only be used for larvae up to 5dpf, our model is not applicable for studying complex chronic disorders that develop at juvenile or adult stage. Therefore, it is necessary to translate our set-up to a juvenile or adult setting. This is possible and requires similar modifications to the ZebraCube (Viewpoint, Lyon, France), designed to quantify adult zebrafish behavior, as we applied to our ZebraBox (Viewpoint, Lyon, France). These modifications will enable us to study the effects of temperature-related activity in adult zebrafish. Besides, an adult model will allow us to study nerve densities of Aδ- and C-fibers instead of RB neurons (Sneddon et al., 2003). Nerve density can be studied in a skin biopsy after staining of the intra-epidermal nerve fibers with the PGP9.5 antibody in zebrafish as performed in the diagnosis of SFN (McCarthy et al., 1995).

Application of our temperature assay to study other pain disorders.

In **chapter 3**, **4** and **5**, we describe a temperature assay which was used to test the temperature response of zebrafish larvae. This temperature sensitivity assay was developed in collaboration with Maastricht Instruments and is used as an add-on to the ZebraBox system and is currently commercially available (Fig.1) (Viewpoint, Lyon, France and Maastricht Instruments BV., Maastricht, The Netherlands). This newly developed add-on enables us to rapidly increase the water temperature in the water compartment and allows us to study zebrafish behavior towards temperature response. Since SFN patients have an aberrant temperature threshold and symptoms like thermal allodynia, we used the setup mainly in the context of the pain-related disorder small-fiber neuropathy (Hoeijmakers et al., 2012). However, the use of this assay is not limited to SFN and has far more potential. This assay can be of interest to study mechanisms involved in other pain-related disorders. For instance, a genome-wide association study has revealed that genetic variations in genes associated with dopamine (*COMT*, *GCH1* and *DRD2*) probably play a role in the development of chronic postsurgical pain (Montes et al., 2015). To further unravel this similar (genetic) tools, as we used to study SFN in zebrafish, can be applied to study the effect of a loss-of-function or a gain-of-function of one of these genes in the involvement of this pain disorder.

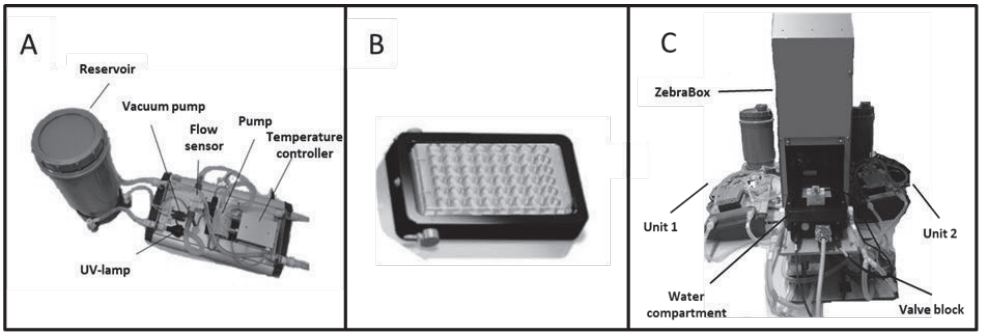

Figure. 1. Newly developed temperature assay which can be used as an add-on to the ZebraBox. Panel A. The water reservoir contains a tank which feeds the water compartment. The controller controls flow rate, temperature and water quality (UV sterilization). Panel B. Because two different water reservoirs like depicted in panel A can feed the water compartment it is possible to rapidly increase the water temperature of the compartment containing a 48-well plate (Top view water compartment). Panel C. Overview of the entire setup including the ZebraBox (Viewpoint).

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