Novel approaches to target sodium channel trafficking in cardiomyocytes

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Chapter 1

Introduction and scope of this thesis

As a pump, the heart is responsible for circulating blood throughout the body. It beats regularly at a rate of approximately 60 times per minute in humans. During every heartbeat, an electrical impulse is propagated throughout the heart resulting in the cardiac excitation–contraction coupling process. Cardiac voltage-gated sodium (Na^+) channels ($Na_v1.5$) play a crucial role in the regular electrical activity of the heart. They allow the influx of Na^+ ions through the plasma membrane of cardiomyocytes (CMs) leading to the rapid upstroke of the cardiac action potential (AP), which triggers the initiation and propagation of APs throughout the whole heart. The cardiac AP is composed of 5 phases: Phase 0 is the depolarization phase caused by activation of $Na_v1.5$ leading to the inward Na^+ current (I_{Na}), followed by an early transient repolarization phase (Phase 1) where I_{Na} inactivates and fast transient voltage-gated outward K^+ channels (generating $I_{to,f}$) activate. Phase 2 represents the plateau phase, characterized by activation of L-type voltage-gated calcium (Ca^{2+}) channels (Ca_v), leading to Ca^{2+} influx which consequently induces Ca^{2+}-release from the sarcoplasmic reticulum, crucial for excitation-contraction coupling. Once Ca_v channels inactivate, the outward rapid and slow rectifier potassium (K^+) currents I_{Kr} and I_{Ks} cause repolarization, resulting in phase 3, and this, with the help of the inwardly rectifying I_{K1}, brings the membrane voltage back to the resting potential (Phase 4)[1].

$Na_v1.5$: structure, function and regulation

$Na_v1.5$ structure and function

$Na_v1.5$, encoded by the *SCN5A* gene, is a transmembrane protein consisting of 2016 amino acids forming the α-subunit of the cardiac voltage-gated Na^+ channel. $Na_v1.5$ is composed of 4

homologous domains (DI-DIV) each of which consists of 6 transmembrane segments (S1-S6), linked by intracellular and extracellular loops (**Figure 1A**).

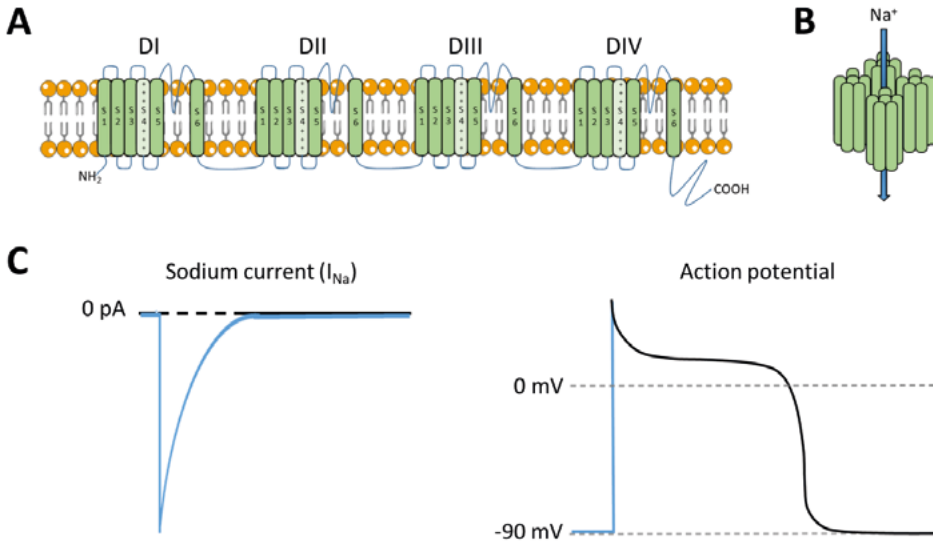

Figure 1. $Na_v1.5$ protein structure and function. A, Schematic representation of $Na_v1.5$ protein structure, the α-subunit of the cardiac sodium channel encoded by *SCN5A*. **B**, Visualization of $Na_v1.5$ 3D structure. **C**, Sodium current generated by $Na_v1.5$ (left panel) and its effect on the action potential (right panel) in ventricular cardiomyocytes.

The domains fold into a specific 3D structure, creating a pore, connecting the extracellular space to the cytoplasm (**Figure 1B**). The S1–S4 helices include the voltage sensing domains (VSD), with the positively charged S4 segments acting as voltage sensor. The S5 and S6 helices form the central pore domain and are connected by extracellular P-loops, which act as an ion filter allowing only Na^+ ions to pass through[2]. Na^+ channels are dynamic proteins that undergo rapid structural rearrangements in response to changes in the electrical field across the sarcolemma, a process

known as “gating”. Upon membrane depolarization, the S4 segments move toward the extracellular space. This causes the pore of the channel to open (activation) allowing a brief but large influx of Na^+ into the cell (peak I_{Na}), which results in the AP upstroke (**Figure 1C**). Activation of the channel lasts only few milliseconds and is followed by fast inactivation, a non-conducting state from which the channel cannot re-open. Membrane repolarization is necessary to allow Na^+ channels to recover from inactivation to the resting state (closed state), from which they can re-open during the next cardiac cycle[3]. Similar to activation, inactivation is usually fast, occurring within few milliseconds, in which a portion of the cytoplasmic linker connecting DIII and DIV occludes the pore. However, in case of prolonged membrane depolarization, $Na_v1.5$ can go into a slow inactivation state which is believed to involve conformational changes of the pore, although the exact mechanism still need to be fully elucidated[2,4,5].

Regulation of $Na_v1.5$ function

Regulation of sodium channels occurs via transcriptional regulation of *SCN5A* mRNA levels and through post-translational modifications of $Na_v1.5$, including phosphorylation, glycosylation, and ubiquitination[6]. In addition, $Na_v1.5/I_{Na}$ function is modulated by temperature and intracellular ion concentrations[3]. $Na_v1.5$ furthermore associates with regulatory subunits (β1-β4), which modulate the gating properties of the channel, regulate its oligomerization and expression at the membrane. Moreover, these accessory β subunits function as cell adhesion molecules in terms of interaction with extracellular matrix, regulation of cell migration, cellular aggregation, and interaction with the cytoskeleton[2,7]. In addition to these β-subunits, $Na_v1.5$ associates with many other interacting proteins within a macromolecular complex, as further discussed below.

Subcellular localization of $Na_v1.5$ in cardiomyocytes

Subcellular distribution of $Na_v1.5$ in cardiomyocytes

$Na_v1.5$ expression varies within the cardiac conduction system, with low expression in the nodal tissues and high expression in the intraventricular conduction system. Furthermore, a gradient of expression and channel availability across the ventricular wall (transmural gradient) is present, with low functional $Na_v1.5$ availability in the subepicardium compared to subendocardium[8]. At a cellular level, CMs consist of two main regions: i) the intercalated disc (ID), the region where cell-cell communications take place allowing the mechanical coupling within CMs through desmosomes and adherens junctions, and ii) the lateral membrane (LM) that consists of crests, with a dome shape, interspersed by grooves, which are invaginations of the membrane. $Na_v1.5$ is differently distributed within these subcellular domains: $Na_v1.5$ is more abundant at the ID, generating a larger I_{Na} with different gating properties as compared to the LM[9]. Within the LM, at the crest region, functional $Na_v1.5$ do not distribute homogenously. In fact, in this region are found $Na_v1.5$ clusters with the highest density within the LM surrounded by areas lacking functional $Na_v1.5$[10,11] (**Figure 2**). Usually crest-crest distance is ≈2 µm but it can change in pathological conditions affecting the structure of the CM membrane[12,13]. At the grooves, $Na_v1.5$ associates with costameres, the Z-line associated structures that connect sarcomeres (the basic contractile unit of CMs) to the sarcolemma and the extracellular matrix.

Subcellular diversity of $Na_v1.5$-interacting proteins

$Na_v1.5$ associates with, and is modulated by, a number of interacting proteins. Over the last decade, it has become increasingly clear that the components of $Na_v1.5$ macromolecular

complexes differ between subcellular domains within CMs. At the ID, $Na_v1.5$ associates with proteins such as N-cadherin (a cell adhesion protein), connexin-43 (involved in intracellular coupling) and the desmosomal proteins plakophilin-2 and desmoglein-2[9,10,14,15]. In the grooves of the LM, $Na_v1.5$ is associated with proteins such as syntrophin, dystrophin and the calcium/calmodulin-dependent serine protein kinase (CASK)[9,13,15,16] (**Figure 2**). Dystrophin connects costameres to cytoskeleton network through α-actinin[13]. As discussed below, the functional relevance of these interacting proteins is underscored by the fact that their dysfunction has been shown to impact on I_{Na}, conduction, cardiac structure and/or arrhythmogenesis.

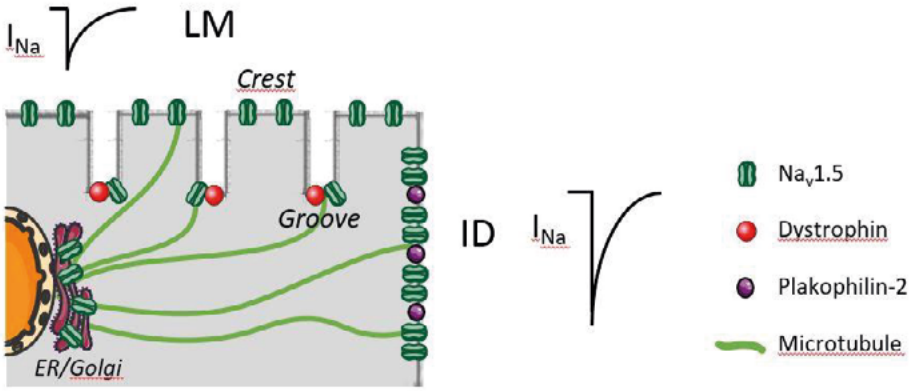

Figure 2. $Na_v1.5$ trafficking and subcellular localization. Schematic representation of $Na_v1.5$ trafficking from the ER/Golgi to the membrane via microtubules and association with microdomain-specific $Na_v1.5$-associated proteins on the membrane of cardiomyocytes (CMs). LM, lateral membrane; ID, intercalated disc; I_{Na}, sodium current.

$Na_v1.5$ trafficking in cardiomyocytes

Following translation, $Na_v1.5$ is translocated to the endoplasmic reticulum (ER) where molecular chaperones help the correct folding of the protein. Once quality control misfolding checkpoints are passed, $Na_v1.5$ can exit the ER and traffic to the Golgi for further modifications, and ultimately to the CM membrane via vesicular transport on microtubules (MTs) (**Figure 2**). MTs consist of dynamic polymers of α- and β-tubulin, are part of the cytoskeletal network and are involved in many cell functions such as maintenance of cell shape, polarization, mitosis, axonemal-based motility and morphogenesis[17]. Furthermore, MTs act like railways inside the cell, along which motor proteins such as kinesin and dynein carry cargo (i.e. vesicles containing proteins) towards the CM membrane, and are therefore crucial for the trafficking of proteins, including $Na_v1.5$. MT function is regulated by MT-associating proteins, including MT plus-end tracking proteins which bind to the MT plus-ends extending towards the cell membrane[15]. MTs undergo several post-translational modifications such as detyrosination/re-tyrosination, acetylation, glutamylation and glycosylation, which regulate MT dynamics. In the detyrosination-re-tyrosination cycle, a tyrosine at the carboxyterminal end of the α-tubulinof MTs is removed by a tubulin tyrosine carboxypeptidase (TCP), shown to be a complex of vasohibin and the small vasohibin binding protein (VASH-SVBP)[18,19] and added back by tubulin-tyrosine ligase (TTL)[20]. A number of cardiac pathological conditions, including Duchenne muscular dystrophy (DMD), hypertrophic cardiomyopathy (HCM) and heart failure (HF)[12,21,22], are characterized by increased MT detyrosination associated with alterations in calcium handling and CM contraction-relaxation coupling[12,21,22]. In addition, increased MT detyrosination may also affect I_{Na} by impacting on $Na_v1.5$ trafficking. Indeed, the speed by which motor proteins carry their cargo to the membrane

is lower on detyrosinated as compared to tyrosinated MTs[23-25]. As a consequence, $Na_v1.5$ trafficking towards the membrane may be slower, explaining (at least in part) the decrease in I_{Na} observed in conditions associated with increased MT detyrosination (i.e. DMD, HF)[23-25]. Additionally, it has been shown that the interaction between cytoplasmic linker CLIP170 and MT end-binding protein 1 (EB1), two MT plus (+)-end tracking proteins known to be important for the delivery of $Na_v1.5$ to the membrane[26], is facilitated on tyrosinated MTs[27,28]. Furthermore, CAP-Gly, another MT plus (+)-end tracking protein involved in trafficking of $Na_v1.5$, was found to be more present on tyrosinated MTs[29]. Hence, in the setting of increased MT detyrosination, the release and delivery of $Na_v1.5$ onto the membrane may be compromised, additionally contributing to I_{Na} reduction[21,22,30-33].

Cardiac disorders associated with $Na_v1.5$ dysfunction

Inherited disorders associated with SCN5A mutations

Mutations in *SCN5A* are associated with inherited primary electrical disorders caused by either a gain of Na^+ channel function (i.e. long QT syndrome type 3, LQT3), or a loss of Na^+ channel function (Brugada syndrome, cardiac conduction disease). Some mutations, such as *SCN5A*-1795insD, lead to a so-called "overlap syndrome", with affected patients displaying features of LQT3, Brugada syndrome and/or conduction disease[34]. LQT3 is characterized clinically by a prolonged QT interval on the electrocardiogram (ECG) and a high risk for sudden cardiac death due to ventricular arrhythmias occurring mostly during rest or sleep (at slow heart rates)[35]. At the cellular level, LQT3 is characterized by a prolonged AP due to increased late I_{Na} (I_{NaL}), a small inward current that persists throughout the AP plateau, caused by a defect in channel

inactivation. Brugada syndrome is clinically characterized by an elevation of the ST-segment in the precordial leads of the ECG and electrophysiologically by a decreased peak I_{Na} that increases the risk for ventricular arrhythmias and sudden cardiac death, in young and mainly male subjects (<40 years old)[35]. The *SCN5A*-1795insD mutation, investigated in this thesis, comprises an insertion of 3 nucleotides (TGA) at position 5537 of *SCN5A* leading to an insertion of aspartic acid (1795insD) in the C-terminal domain of $Na_v1.5$. This mutation was identified in a large Dutch family, with affected patients displaying an overlap syndrome of LQT3, Brugada syndrome and/or conduction disease[35-37]. Studies in *SCN5A*-1795insD human induced pluripotent stem-cell derived cardiomyocytes (hiPSC-CMs)[36] and mice with the mouse homolog mutation *Scn5a*-1798insD[36] have demonstrated that the mutation leads to both a decreased I_{Na} and an increased I_{NaL}, explaining the observed overlap phenotype[37].

Acquired disorders associated with $Na_v1.5$ dysfunction

In addition to inherited disorders, $Na_v1.5$ dysfunction also occurs in a number of acquired disorders, such as myocardial ischaemia and heart failure. Decreased peak I_{Na} secondary to membrane depolarization in ischemic tissue or alterations in channel expression or function may lead to conduction slowing and re-entrant ventricular arrhythmias. On the other hand, increased I_{NaL},caused by defective $Na_v1.5$ inactivation, can prolong AP duration and alter intracellular calcium homeostasis, inducing early and/or late afterdepolarisations and consequent pro-arrhythmia[15]. The complex nature of $Na_v1.5$ subcellular re-distribution within CMs plays a significant role in the pathophysiology of acquired cardiac diseases. In fact, remodeling of the ID, LM, and microtubule network (in particular, increased MT detyrosination) has been observed in

failing CMs associated with alterations in $Na_V1.5$ cluster size and I_{Na} within the distinct subcellular microdomains[12].

Disorders affecting $Na_v1.5$-interacting proteins

The components of the $Na_V1.5$ macromolecular complex (described above) are important for $Na_V1.5$ function and integrity, both at the LM and the ID. Indeed, dysfunction of $Na_V1.5$-interacting proteins has been shown to cause contractile, structural and/or electrical alterations in the heart. DMD is caused by mutations leading to loss of dystrophin from the LM, resulting in calcium dysregulation and I_{Na} reduction in CMs in addition to an increased risk for arrhythmias and cardiomyopathy [10]. Mutations in *SNTA1* gene encoding syntrophin (located at the LM) have been associated with long QT syndrome secondary to a gain of Na^+ channel function[38]. Mutations in genes encoding for desmosomal proteins (for instance, *PKP2* encoding plakophilin-2) that associate with $Na_V1.5$ at the ID lead to arrhythmogenic right ventricular cardiomyopathy, also known as arrhythmogenic cardiomyopathy (ACM)[39]. In addition to structural alterations in cell-to-cell contact and intracellular calcium homeostasis, ACM may also be associated with decreased I_{Na} which may potentially contribute to arrhythmogenesis.

Towards novel therapeutic strategies targeting $Na_v1.5$ trafficking

(Limited) Therapeutic strategies to prevent sodium channel (dys)function

Clinical management of patients with *SCN5A* mutations is primarily directed at preventing pro-arrhythmic triggers, including avoiding certain drugs and treating fever in Brugada syndrome patients, and pacemaker therapy (to avoid bradycardia) and beta blockade in individuals with

LQT3[40,41]. Patients who remain at risk for ventricular arrhythmias and sudden cardiac death may be implanted with an ICD (internal cardioverter-defibrillator); however, ICD implantation is associated with side-effects such as infection and inappropriate shocks[40]. The sodium channel blocker and class 1b anti-arrhythmic drug mexiletine may decrease mutation-induced I_{NaL} and as a result shorten the QT-interval and prevent arrhythmias in some LQT3 patients[41,42]. In contrast, there are as yet no pharmacological options to increase I_{Na}. Hence, there is an unmet need to develop novel strategies to enhance and restore $Na_v1.5$ function and peak I_{Na}. In particular, promoting membrane localization of $Na_v1.5$ by targeting its normal, endogenous trafficking pathway may provide an efficient and safe therapeutic approach. This thesis explores two potential targets to enhance $Na_v1.5$ membrane trafficking: protein folding in the ER and modulation of MT dynamics.

Pharmacological chaperones

Pharmacological chaperones are molecules that can serve as scaffolds to stabilize misfolded proteins, preventing their degradation and promoting their exit from the ER and subsequent trafficking to the cell membrane[43]. Certain sodium channel blockers, including mexiletine, may also act as chaperones: incubating cells for 24-48 hours with mexiletine enhances trafficking of $Na_v1.5$ to the membrane and consequently increases peak I_{Na}[44-47]. However, most previous studies were performed in heterologous expression systems such as HEK293 cells and used very high, non-therapeutic concentrations of mexiletine (>100 μM), a dose at which acute inhibition of peak I_{Na} may occur in addition to non-cardiac side-effects. Hence, the potential clinical use of mexiletine as a pharmacological approach to increase peak I_{Na} requires investigation of its efficacy at (sub)therapeutic concentrations (10 μM or lower) in a cardiomyocyte environment.

Targeting the MT network

As described above, MT detyrosination occurring in the setting of certain pathological conditions is associated with a number of deleterious consequences, including intracellular calcium dysregulation, altered CM contractility and I_{Na} reduction. Previous work from our group has shown that taxol, an anti-cancer drug, decreased I_{Na} while increasing the amount of detyrosinated MTs[48]. Hence, pharmacological interventions aimed at achieving the opposite, i.e. reducing MT detyrosination may restore the disrupted balance within the CM, increase I_{Na} and ultimately prevent arrhythmias. Parthenolide (PTL), a compound obtained from the feverfew plant, has been used for centuries to treat pain, fever, headache[30] and is now mainly known for its anti-inflammatory and anti-cancer properties[31]. PTL is also known to be able to decrease the amount of detyrosinated MTs in cells and has demonstrated beneficial effects on CM contraction and calcium handling[21,22,32,33]. However, its effect on I_{Na} have as yet not been investigated.

Scope of this thesis

The scope of this thesis is the investigation of new potential strategies aimed at restoring $Na_v1.5/I_{Na}$ dysfunction. In particular, the thesis investigates trafficking pathways as novel therapeutic targets, while at the same time exploring their remodeling in the setting of pathological conditions as well as their modulatory effect on $Na_v1.5$ subcellular (re)distribution. **Part I** focuses on the therapeutic potential of mexiletine in inherited primary electrical disorders, both as a pharmacological chaperone and I_{NaL} inhibitor. In **Chapter 2,** we investigated whether mexiletine can rescue the double phenotype associated with the overlap mutation *SCN5A*-1795insD, exploring the chronic and acute effects of a therapeutic concentration of mexiletine

(10 μM) on I_{Na} and I_{NaL} in HEK293 cells as well as hiPSC-CMs from a *SCN5A*-1795insD patient. Following our observation that chronic mexiletine incubation also increased I_{Na} in HEK293 cells expressing wild-type *SCN5A*, we next assessed the acute and chronic effects of mexiletine in non-diseased hiPSC-CMs in **Chapter 3**. In **Chapter 4**, we investigated whether mexiletine at concentrations lower than 10 μM may still increase I_{Na}, and explored the potential mechanisms by which mexiletine exerts its effects.

Part II of the thesis focuses on inherited disorders affecting $Na_v1.5$-interacting proteins associated with reduced I_{Na}, assessing the therapeutic potential to increase I_{Na} by reducing MT detyrosination. Using sophisticated methods such as super-resolution imaging and macropatch, we furthermore explored the modulatory impact of MT remodeling on subcellular $Na_v1.5$ distribution and function. In **Chapter 5**, we used PTL to investigate the link between MT detyrosination, I_{Na}, and $Na_v1.5$ cluster size and density in both LM and ID in dystrophin-deficient *mdx* mice (a model of DMD). In **Chapter 6**, we showed for the first time the presence of increased MT detyrosination levels in a well-established mouse model of ACM (PKP2cKO), and investigated the effects of PTL on membrane stiffness and subcellular $Na_v1.5$ distribution and function.

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