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# DISSERTATION

Titel der Dissertation

## Modulation of cardiac sodium channel variants by Fyn tyrosine kinase

Verfasser

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# Introduction

## 1.1 Background

Ion channels are intrinsic membrane proteins that control the flow of specific ions across the cellular membrane. All ion channels are large transmembrane proteins. In living cells there are 300 types of ion channels (Gabashvili et al., 2007). Channels are ion selective and can differentiate between size and charge of the ions. Ion channels are classified based on the physical and chemical modulator of their gating properties. Some of the important groups of ion channels are listed below.

- Ligand-gated channels
- Voltage-gated channels
- Second messenger-gated channels
- Mechanosensitive channels
- Gap junctions

Voltage-gated ion channels in the cell membrane possess three different states: closed, open and inactivated. Ion channels in the cell membrane open or close depending on the cell membrane potential. Specific ions flow down their electrochemical gradient, when a channel is in an open state. The ion channel protein adopts different conformational states depending on the membrane potential. Ionic current is measured from the ion flow through an open channel. The best method to study this process is the patch clamp technique.

### 1.1.1 Cardiac action potential

The cardiac action potential is necessary for the electrical conduction system of the heart. Action potential of a single cardiac myocyte and the electrophysiological and mechanical function of the heart are interdependent. Various ion channels are responsible for the cardiac action potential.

The action potential is divided into five distinct phases corresponding to clearly recognizable landmarks in its contour.

Phase 0: Fast depolarization or upstroke

Phase 1: Fast initial repolarization

Phase 2: Plateau

Phase 3: Fast terminal repolarization

Phase 4: Electric diastole

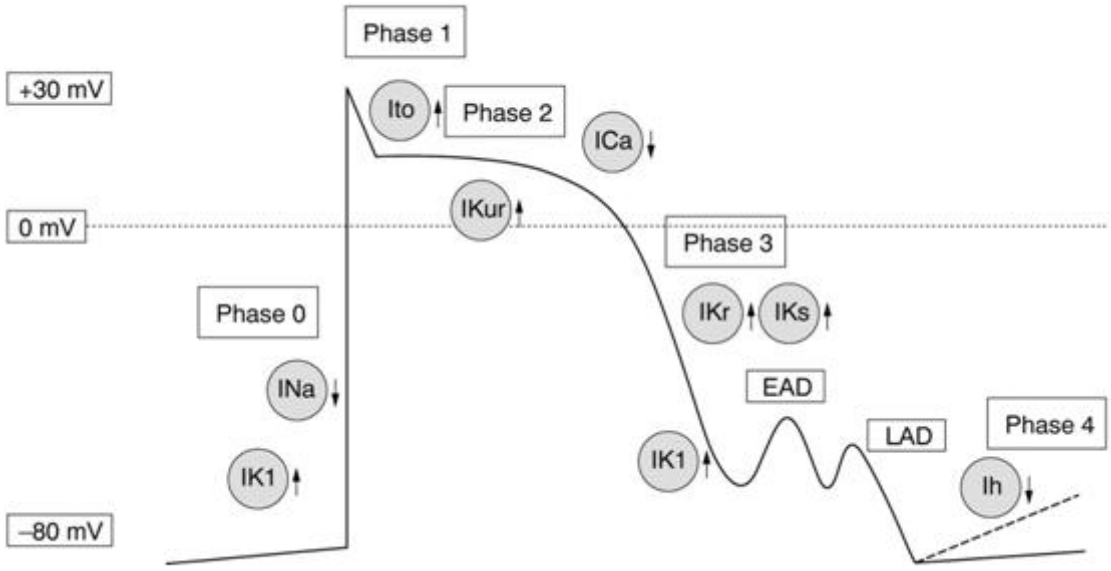


**Figure 1: Representation of an action potential from a ventricular myocardial cell showing all the possible currents responsible for different phases.**

An action potential is generated when the membrane potential is partially depolarized from the resting potential to the threshold potential.

**Table 1: Major currents during the cardiac ventricular action potential.**

| Ions | Current | Protein | Gene | Phase/Role |
|---|---|---|---|---|
| **$Na^+$** | $I_{Na}$ | $Na_V1.5$ | SCN5A | 0 |
| **$Ca^{2+}$** | $I_{Ca(L)}$ | $Ca_V1.2$ | CACNA1C | 0-2 |
| **$K^+$** | $I_{to1}$ | $K_V4.2/4.3$ | KCND2/KCND3 | 1, notch |
| **$K^+$** | $I_{Ks}$ | $K_V7.1$ | KCNQ1 | 2,3 |
| **$K^+$** | $I_{Kr}$ | hERG ($K_V11.1$) | KCNH2 | 3 |
| **K+** | $I_{K1}$ | $K_{IR}2.1/2.2/2.3$ | KCNJ2/ KCNJ12/KCNJ4 | 3,4 |

## *1.2 Voltage-gated ion channels*

Hodgkin and Huxley performed experiments on the ionic events responsible for the action potential, and with their classical equations they described the conductance and currents quantitatively (Hodgkin and Huxley, 1952b). The rising phase generation in the action potential was analyzed and explained by a conductance to sodium ions.

The eel electroplax, was the source where the first voltage-dependent ion channel was isolated and purified (Agnew et al., 1978). Several years later the sequence for the eel sodium channel was deduced from its mRNA (Noda et al., 1984). The voltage-dependent potassium channel (KvAP) from *Aeropyrum pernix* is the first X-ray crystallographic structure solved at a resolution of 3.2 Å (Jiang et al., 2003). Till now this forms the template for modelling other voltage-gated sodium, calcium and potassium channels. This research was awarded with the Nobel Prize in 2003. A basic pattern emerged from all these sequences: the functional channels are made up of four subunits ($K^+$ channels) or one protein with four homologous domains ($Na^+$ and $Ca^{2+}$ channels).

In this thesis I will focus on the voltage-gated sodium channel.

## 1.3 Voltage-gated sodium channels

Voltage-gated sodium channels ($Na_V$) belong to a family of membrane proteins that selectively conduct sodium due to changes in membrane potential. Sodium channels have various functional and pharmacological properties in different tissues and species (Mandel, 1992). Some sodium channels are not voltage-gated, such as the epithelial sodium channel (ENaC), which is responsible for sodium transport, and it is unrelated to other $Na_V$ channels (Catterall, 2000; Yu and Catterall, 2003).

### 1.3.1 Structure of voltage-gated sodium channels

$Na_V$ channels have a large, complex multimeric structure that is comprised of a core α-subunit and auxiliary function modifying β-subunits (Catterall, 2000) (Figure 2). These auxiliary subunits are involved in cellular signalling, channel trafficking, cell adhesion, stability of the membrane, gating modulation, and they may be targets for proteases involved in disease (Fahmi et al., 2001; Isom, 2001; Isom et al., 1994; Isom et al., 1992; Isom et al., 1995; Morgan et al., 2000; Zhou et al., 2000). Although $Na_V$ channels have auxiliary subunits, channel function, such as channel opening, ion selection and inactivation, is controlled by the core α-subunit. The central core is formed by a four domain-folding (DI-DIV) pattern, which determines the ion selectivity and conductance. Each domain is composed of six transmembrane helices (S1-S6). The S4 of each domain is the voltage sensor; it contains a distinct amino acid sequence, which has a positively charged residue followed by two hydrophobic residues. The re-entrant P-loop connecting S5-S6 forms a narrow and selective ion pore (Kass, 2006). Two negatively charged amino acids are present in an analogous position in all four domains, which form the receptor site and selectivity filter. The mammalian sodium channel resembles a subunit from the potassium channel of primitive bacteria (Ren et al., 2001). Substitution of a lysine at position 1,422 in repeat III and/or an alanine at position 1,714 in repeat IV of rat sodium channel II alters ion selectivity such that the channel takes on properties of a calcium channel. These data suggest that these residues constitute a portion of the selectivity filter (Heinemann et al., 1992).

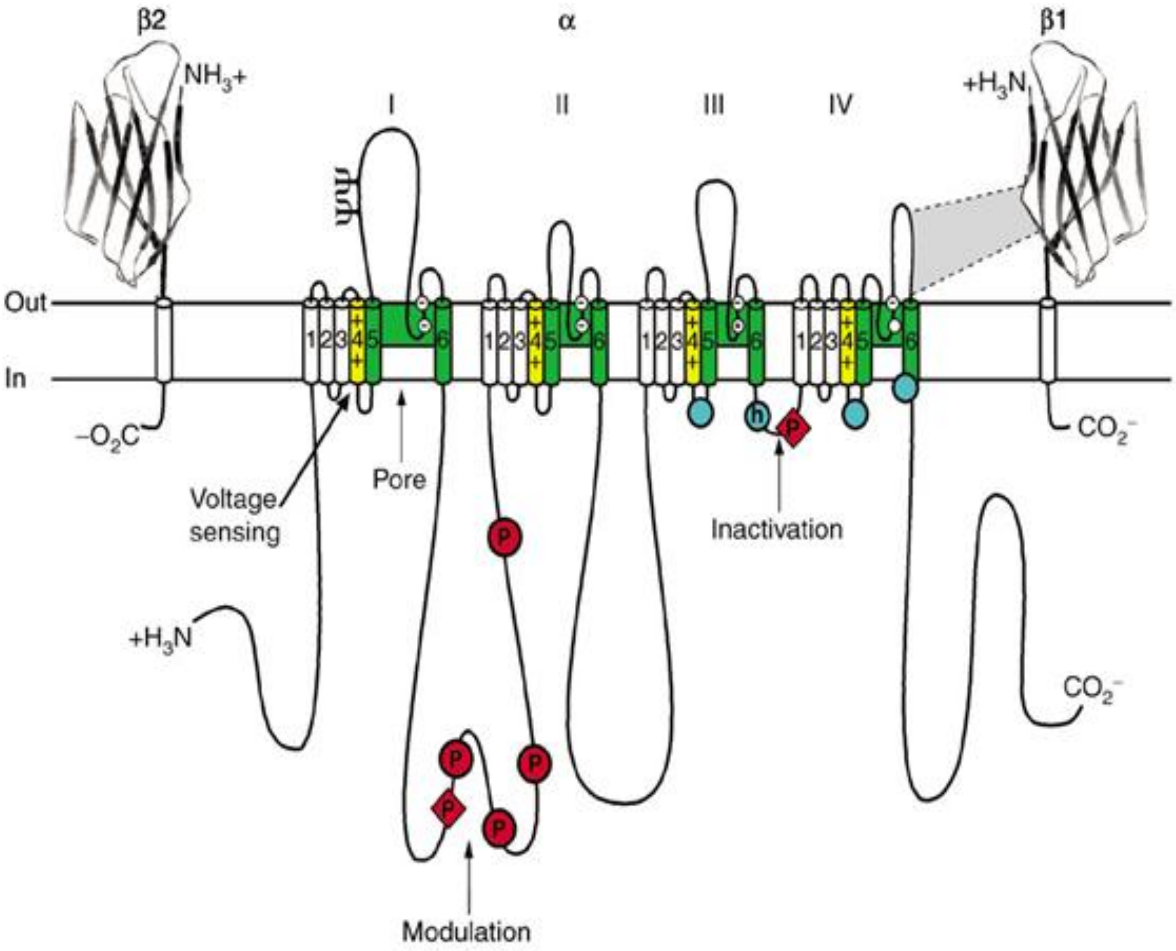


**Figure 2: A schematic representation of the voltage-gated sodium channel showing all four domains (DI-DIV) of the α-subunit and two β-subunits.**

Each domain has 6 transmembrane helices with a voltage senor S4 (in yellow) and two pore lining segments S5 and S6 (in green). Blue circles in the intracellular loops of domains III and IV indicate the inactivation gate IFM motif and its receptor (h, inactivation gate); P, phosphorylation sites (in red circles: sites for protein kinase A; in red diamonds: sites for protein kinase C); ψ, probable *N*-linked glycosylation sites. Adapted from Yu and Catterall (2003).

### 1.3.2 Sodium channel gating

The sodium channel gates between three functional states: resting, active and inactivated – in a manner controlled by the membrane potential (Hodgkin and Huxley, 1952a). Channel opening (activation) is caused by the outward movement of the voltage sensors (S4) in each of the four domains in response to depolarization of the membrane potential. The movement of the voltage sensors is somehow coupled to opening of the channel, allowing entry of $Na^+$ ions. The short duration of channel activation is due to rapid inactivation. Inactivation is controlled by amino acids within the cytoplasmic loop between domains III and IV (George, 2005). Sodium channels undergo a slow inactivation if the membrane is depolarized for a long duration (Vilin and Ruben, 2001). This slower inactivation affects the amount of channels in the activation state. The detailed molecular mechanism of voltage-gated sodium channel gating is documented (Catterall, 1991, 2000; Denac et al., 2000; French and Horn, 1983; Hodgkin and Huxley, 1952a; Kuhn and Greeff, 1999; Marban et al., 1998; Mitrovic et al., 1995; Morgan et al., 2000; Romine et al., 1974; Tomaselli et al., 1995; Yang et al., 1996).

### 1.3.3 Voltage-gated sodium channel subtypes

There are nine sodium channel subtypes that are categorised by amino acid sequence and channel function (Table 1). These nine subtypes are broadly classified into three groups. The first group of channels is located in the central nervous system (CNS) and is comprised of the $Na_V1.1$, $Na_V1.2$, $Na_V1.3$ and $Na_V1.6$ sodium channels. The $Na_V1.3$ channel is highly expressed in the dorsal root ganglion (DRG). All four of the sodium channel subtypes are located on chromosome 2 (Catterall et al., 2005; Malo et al., 1994), and they are sensitive to tetrodotoxin (TTX) with a nanomolar $IC_{50}$ value (Goldin, 2001). The next group of sodium channel subtypes is more diverse and is present on chromosome 3. This group is comprised of the cardiac $Na_V1.5$ channel subtype and the nociceptive neuron channel subtypes $Na_V1.8$ and $Na_V1.9$. These latter subtypes are TTX-resistive with micromolar $IC_{50}$ values (Goldin, 2001). Single amino acid change from aromatic tyrosine or phenylalanine in chromosome 2 located subtypes to hydrophilic cystine, as present in $Na_V1.5$, reduces the TTX sensitivity 200 fold (Satin et al., 1992). On the other hand, change from phenylalanine to serine,

which is present in $Na_V1.8$ and $Na_V1.9$, results in even higher resistance to TTX (Sivilotti et al., 1997). TTX-resistant subtypes differ from TTX-sensitive subtypes in that they have slow inactivation kinetics and a single amino acid substitution in the pore-lining region of domain I (Catterall et al., 2005; Rogers et al., 2006). The third group of sodium channel subtypes is the intermediate group, which is composed of the TTX-sensitive skeletal muscle channel $Na_V1.4$ and the DRG and sympathetic ganglion sodium channel $Na_V1.7$ (Catterall et al., 2005; Rogers et al., 2006).

Sodium channels remain important targets for the development of novel drugs to treat many neurological, muscular and cardiac disorders. To date, numerous isoforms have been identified. Yet, it is unclear whether there are additional sodium channel isoforms. Advances in molecular biology have made it possible to discover channel mutations that occur in clinical disorders. However, additional analysis of the signalling pathways and molecules that modulate sodium channels is necessary. Proteins that are associated with sodium channels and the site of interaction should be identified in order to have a better understanding of channel function. Phenotype-based research should be performed to elucidate the role of sodium channels in physiology and disease. Lastly, since there is no crystal structure of mammalian sodium channels, understanding the structure-function relationship of sodium channels is hindered. Recent studies have shown that the sodium channel is far more complex than anticipated in terms of its function and association with other signalling molecules.

## 1.4 Channelopathies

Research of the molecular properties of sodium channels has elucidated mutations that cause multiple inherited hyper-excitability diseases in humans – considerably unexpected since a priori mutations might be expected to produce primarily hypo-excitability. These disorders are termed channelopathies. The first channelopathy involving sodium channels was found in skeletal muscle. Other channelopathies have been reported in cardiac and neuronal subtypes. A brief overview of review articles and original papers dealing with the various channelopathies is given in Table 2, followed by reviewing very recent publications in the subsequent section.

**Table 2: Different sodium channel isoforms and their associated channelopathies.**

| Isoform | Gene | Location | Channelopathies | References |
|---|---|---|---|---|
| $Na_V1.1$ | SCN1A | CNS and DRG | • Generalized epilepsy with febrile seizure<br>• Dravet syndrome | (Heron et al., 2007; Lossin, 2009) |
| $Na_V1.2$ | SCN2A | CNS and DRG | • Generalized epilepsy with febrile seizure<br>• Dravet syndrome<br>• Benign familial neonatal-infantile seizure | (Herlenius et al., 2007; Heron et al., 2007; Misra et al., 2008; Sugawara et al., 2001) |
| $Na_V1.3$ | SCN3A | embryos, DRG and CNS | | (Chen et al., 2000; Cummins and Waxman, 1997) |
| $Na_V1.4$ | SCN4A | skeletal muscle | • Potassium-aggravated myotonia<br>• Paramyotonia congenita<br>• Hyperkalemic periodic paralysis<br>• Hypokalemic periodic paralysis 2 | (Heine et al., 1993; Orrell et al., 1998)<br>(Koch et al., 1995; Ptacek et al., 1992; Wu et al., 2001)<br>(Bendahhou et al., 1999; Ptacek et al., 1993)<br>(Bendahhou et al., 2000; Bulman et al., 1999; Davies et al., 2001) |
| $Na_V1.5$ | SCN5A | heart, embryos and DRG | • Long QT syndrome<br>• Brugada syndrome<br>• Conduction dysfunction<br>• Sinus node dysfunction<br>• SIDS<br>• Atrial fibrillation | (Bennett et al., 1995; Goldenberg and Moss, 2008; Heron et al., 2009)<br>(Benito et al., 2009; Brugada et al., 2009; Chen et al., 1998)<br>(Schott et al., 1999)<br>(Benson et al., 2003; Lei et al., 2008)<br>(Makielski, 2006; Skinner et al., 2005; Wedekind et al., 2006)<br>(Ellinor et al., 2008; McNair et al., 2004; Olson et al., 2005) |
| $Na_V1.6$ | SCN8A | DRG and CNS | In *jolting* mice- inherited cerebellar ataxias | (Kohrman et al., 1996) |
| $Na_V1.7$ | SCN9A | DRG and sympa-thetic ganglion | • Primary erythermalgia<br>• Paroxysmal extreme pain disorder<br>• Insensitivity to pain | (Drenth et al., 2005; Han et al., 2006; Harty et al., 2006; Yang et al., 2004)<br>(Fertleman et al., 2006)<br>(Cox et al., 2006) |
| $Na_V1.8$ | SCN10A | DRG | • Neuropathic injury of DRG<br>• Trigeminal ganglia model of neuropathic pain<br>• In human cases radicular pain | (Boucher et al., 2000; Coward et al., 2000; Dib-Hajj et al., 1998)<br>(Eriksson et al., 2005)<br>(Abe et al., 2002; Coward et al., 2000) |
| $Na_V1.9$ | SCN11A | DRG and CNS | • Neuropathic injury of DRG<br>• Trigeminal ganglia model of neuropathic pain<br>• In human cases radicular pain | (Boucher et al., 2000; Coward et al., 2000; Dib-Hajj et al., 1998)<br>(Eriksson et al., 2005)<br>(Abe et al., 2002; Coward et al., 2000) |

### 1.4.1 Cardiac sodium (SCN5A) channelopathies

Impulse conduction in the atria, His-Purkinje system and the ventricle is sustained by the transient increase in sodium permeability (Hoffman and Cranefield, 1960). Cardiac sodium channels are the target of antiarrhythymic drugs (Grant et al., 1984; Hondeghem and Katzung, 1977). Disturbances in conduction and life-threatening arrhythmias are caused by decreased sodium channel function (Cascio, 2001; Tomaselli and Zipes, 2004).

$Na_V1.5$ is a 2016 amino acid protein. Mutations in SCN5A, which encodes the primary sodium channel in cardiac tissues, cause sodium channel dysfunction and are associated with a number of unrelated arrhythmic syndromes, such as long QT syndrome (LQTS) (Bennett et al., 1995; Goldenberg and Moss, 2008), Brugada syndrome (BrS) (Benito et al., 2009; Brugada et al., 2009; Chen et al., 1998), conduction dysfunction (Schott et al., 1999), sinus node dysfunction (Benson et al., 2003; Lei et al., 2008), sudden infant death syndrome (SIDS) (Makielski, 2006; Skinner et al., 2005; Wedekind et al., 2006) and atrial fibrillation (Ellinor et al., 2008; McNair et al., 2004; Olson et al., 2005).

Sodium channel mutation causes cardiac arrhythmia by one of the two mechanisms:

a) *Loss-of-function (LoF) mutations:* The consequences of these mutations are non-functional channels or rapidly inactivating channels. This results in a decrease of available sodium current during the fast depolarization phase.
b) *Gain-of-function (GoF) mutations:* These mutations cause an increase in inactivation reversibility in the late component of the sodium current. In addition, these mutations prolong action potential duration and the QT interval.

## 1.5 Cardiac sodium channel splice variants

Exons coding for the cardiac sodium channel subtype undergo alternative splicing which eventually can produce biochemically, pharmacologically and functionally distinct sodium channels (Choi et al., 2010; Gazina et al., 2010; Kerr et al., 2008; Makielski et al., 2003; Schirmeyer et al., 2010; Schroeter et al., 2010; Tan et al., 2005). Figure 3 shows different splice variants of the cardiac sodium channel.

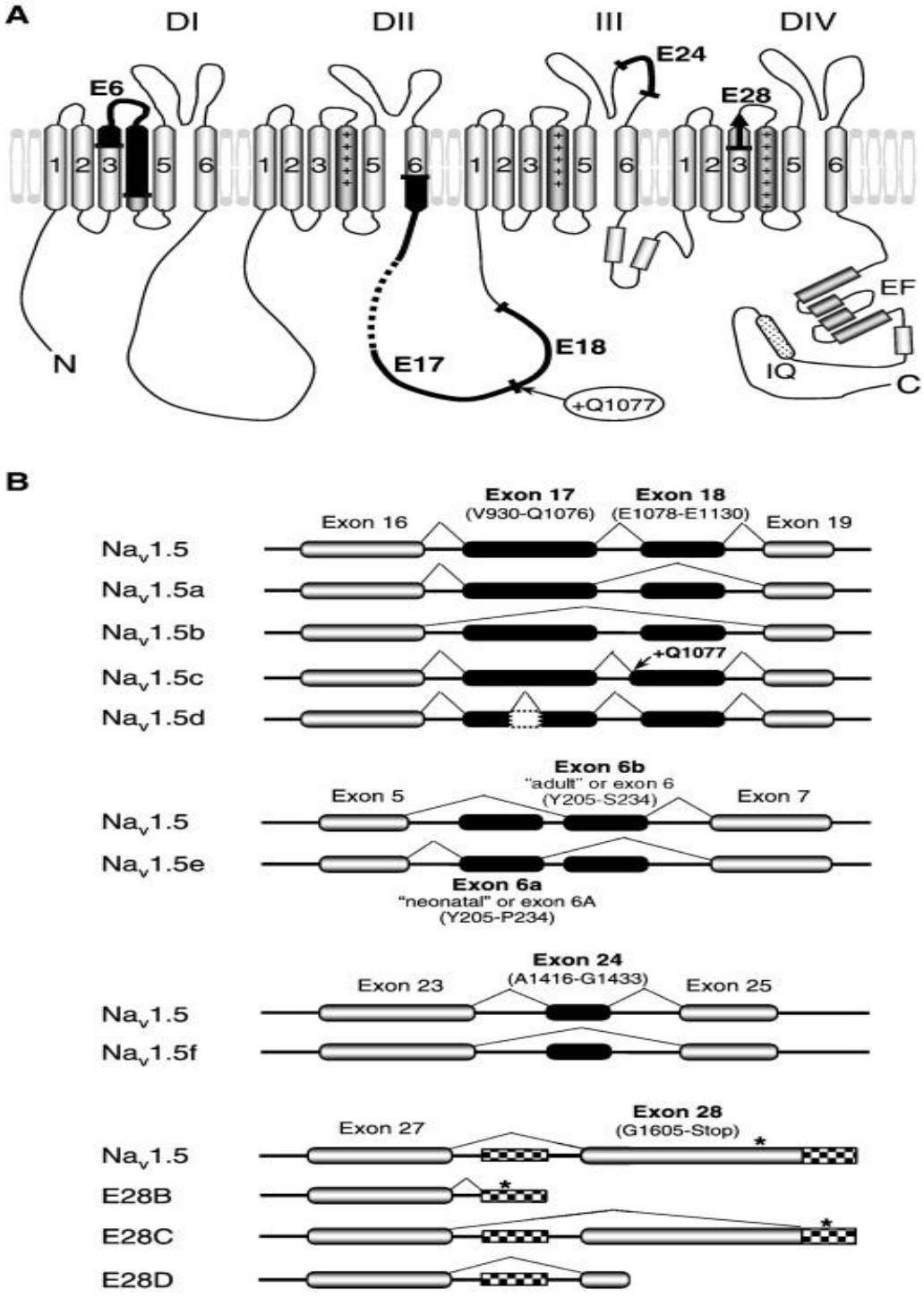


**Figure 3: Structure of $Na_V1.5$ splice variants.**

(A) Proposed membrane topology of $Na_V1.5$. (B) Alternative splicing of $Na_V1.5$ results in exon skipping ($Na_V1.5a$, $Na_V1.5b$, $Na_V1.5f$), in alternative usage of the exon 18 splice acceptor site and the extension of this exon by a CAG trinucleotide coding for Q1077 ($Na_V1.5c$), in partial deletion of exon 17 ($Na_V1.5d$), in the alternative usage of one of two exon 6 variants ($Na_V1.5e$), in abnormal exon 27/exon 28 splicing (variants E28B, E28C) or in premature transcript termination (E28D; last amino acid is G1642). The original numbering system of hH1 with 2016 amino acids (Gellens et al., 1992) is used in the sequence numbering. Reproduced from Schroeter et al. (2010).

### 1.5.1 Functional $Na_V1.5$ splice variants

*$Na_V1.5a$* was the first functional splice variant with the deletion of exon 18, which encodes for 53 amino acids of the $D_{II-III}$ linker (Zimmer et al., 2002). This variant is a species–specific expression in the mammalian heart. It is not expressed in human, whereas it is expressed in pig, rat and mouse (Blechschmidt et al., 2008). Electrophysiological studies show similar kinetics to mouse $Na_V1.5$ when expressed in HEK293 cells (Zimmer et al., 2002).

*$Na_V1.5c$* is the most abundant splice variant in the human heart. A glutamine at position 1077 by a 5′-extension of exon 18 (trinucleotide CAG), makes the splice variant $Na_V1.5c$ (Makielski et al., 2003). Electrophysiological properties of Q1077 and ΔQ1077 were alike (Makielski et al., 2003). Recent studies show that this splice variant plays an important role in SCN5A channelopathies like LQTS and BrS (Cocquet et al., 2006; Wang et al., 2007). The predicted phosphorylation site for CK2 ('Casein kinase 2', regulated through protein-protein interactions and changes in its concentration (Rodriguez et al., 2008)) is destroyed when Q1077 is included (Kerr et al., 2004). ΔQ1077 is the most abundant variant present in human heart. It is present in 45% of the human population (Makielski et al., 2003) (Table 3). Physiology of this splice variant is uncertain.

*$Na_V1.5d$* is a splice variant, with alternative exon 17 splicing as shown in Figure 3. Electrophysiological measurement shows that $Na_V1.5d$ significantly altered the kinetics, with depolarized steady-state activation and inactivation and 20% reduction in peak $I_{Na}$ (Camacho et al., 2006; Schroeter et al. 2010).

Functional splice variant *$Na_V1.5e$* is present is human brain (Ou et al., 2005) and in human breast cancer cells (Brackenbury et al., 2007; Brackenbury et al., 2008; Fraser et al., 2005). $Na_V1.5e$ variant is alternative usage of 'neonatal' exon 6a. Shift of the steady-state activation to more depolarized potentials, slower recovery from inactivation and reduced availability are the electrophysiological differences between $Na_V1.5e$ and $Na_V1.5$ (Onkal et al., 2008).

### 1.5.2 Non-functional $Na_V1.5$ splice variants

Non-functional splice variant *$Na_V1.5b$* is generated by deletion of exon 17 and 18 which codes for the C-terminal end of DIIS6 that forms the large portion of the intracellular loop $L_{DII-DIII}$ (Zimmer et al., 2002). It is expressed in mouse heart but it is not expressed in rat, pig and human heart (Blechschmidt et al., 2008).

*$Na_V1.5f$* is a non-functional splice variant present in various rat tissues and in human brain. $Na_V1.5f$ is characterized by deletion of exon 24 and thus removal of 18 amino acids in the DIII pore region (Schroeter et al., 2010; Wang et al., 2009; Wang et al., 2008).

C-terminal truncated splice variants (E28B, E28C and E28D) of $Na_V1.5$ were the splice variant which showed first evidence in the pathophysiology of the human heart (Shang et al., 2008; Shang et al., 2007). The mechanism of splicing is uncertain. All three novel C-terminal truncated splice variants were transcribed in human lymphoblasts, and one of them was expressed in human skeletal muscle (E28D). None of the truncated variants were observed in rats and mice (Schroeter et al., 2010; Shang et al., 2008; Shang et al., 2007).

## 1.6 Src tyrosine kinase

Tyrosine phosphorylation plays a vital role in the regulation of a variety of biological responses, which includes cell proliferation, migration, differentiation and survival (Thomas and Brugge, 1997). The protein tyrosine kinase (PTK) encompasses a diverse spectrum of proteins which mediate the above responses, as well as the receptors which activate them (Thomas and Brugge, 1997). There are many different distinct families of tyrosine kinases which execute the responses that lead to complex extensive cross talk between different receptor pathways. One such large family of cytoplasmic tyrosine kinases which is capable of communicating with a large number of different receptors is the Src protein tyrosine kinase (Src PTK) (Brugge and Erikson, 1977). The prototype of Src was first identified as the transforming protein (v-Src) of the oncogenic retrovirus, Rous sarcoma virus (Brugge and Erikson, 1977). V-Src is the mutant variant of a cellular protein ubiquitously expressed and highly

conserved over evolution. In 1978, two groups of researchers found that Src proteins possess protein tyrosine kinase activity, which led to deep investigation of Src on cell proliferation and its kinase activity (Collett and Erikson, 1978; Levinson et al., 1978).

### 1.6.1 Members of Src tyrosine kinase and its cellular location

Ten proteins were identified in this large Src PTK family which have structural features and significant amino acid sequence homology to Src. The ten members are Fyn, Yes, Yrk, Blk, Fgr, Hck, Lyn and the Frk subfamily proteins Frk/Rak and Iyk/Bsk (Brown and Cooper, 1996; Cance et al., 1994; Oberg-Welsh and Welsh, 1995; Thuveson et al., 1995). The Src PTK is further divided into three major groups based on their expression. Src, Fyn and Yes are expressed in almost all tissues (Brown and Cooper, 1996). The second group consists of hematopoietic cell expressed Src PTK Blk, Fgr, Hck, Lck and Lyn (Bolen and Brugge, 1997). The third subgroup, Frk/Rak and Iyk/Bsk Src PTK, is expressed in epithelial-derived cells (Cance et al., 1994; Lee et al., 1994; Oberg-Welsh and Welsh, 1995; Thuveson et al., 1995). Thus, Src-PTKs can function in many distinct cells and in distinct sub-cellular locations.

### 1.6.2 Functional regions of Src tyrosine kinase

Src PTKs are 52-62 kDa proteins composed of six distinct functional regions (Figure 4) (Brown and Cooper, 1996)

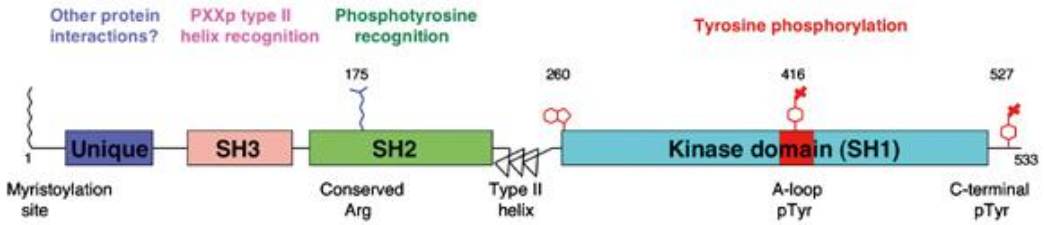


**Figure 4: Organization of Src tyrosine kinase domain structure**

The Figure shows all 6 different functional regions as explained in the text. Reproduced from Boggon and Eck (2004).

*I.* *Src Homology (SH) 4 domain*

This domain is a 15-amino acid sequence which contains signals for lipid modification of Src PTKs. Glycine at position 2 is responsible for the addition of myristic acid moiety, which is involved in targeting Src PTKs to cellular membranes (Resh, 1993). The cysteine residues in this domain are subjected to palmitylation (Resh, 1993; Thomas and Brugge, 1997).

*II.* *Unique region*

The name suggests its properties; this domain is unique and distinct for each member. It is proposed that this domain is involved in mediating interactions with receptors or proteins that are specific for each family member (Morgan et al., 1989; Winkler et al., 1993). It is also speculated that this region is involved in modulating proteins: protein interactions or regulation of catalytic activity (Thomas and Brugge, 1997).

*III.* *SH3 domain*

SH3 domains of Src PTK are composed of 50 amino acids. It is very important for intra- and intermolecular interactions that regulate Src catalytic activity, Src localization and recruitment of substrates. All SH3 domains target a proline-rich core consensus motif P-X-X-P (Feng et al., 1994; Rickles et al., 1995; Yu et al., 1994). Amino acids surrounding the prolines play a vital role in additional affinity and specificity for individual SH3 domains. Binding affinities of SH3 domains are in the micro-molar range. These affinities are strengthened *in vivo* by additional contacts with the target protein and other domains of Src (Cohen et al., 1995; Thomas and Brugge, 1997).

*IV.* *SH2 domain*

The SH2 domain is also an important domain involved in the regulation of the catalytic activity of Src PTKs, as well as the localization of Src and its binding proteins (Cohen et al., 1995; Pawson, 1995). All SH2 domains bind to short contiguous amino acid sequences containing phosphotyrosine. The specificity of individual SH2 domains lies in the 3-5 residues following the phosphotyrosine (Pawson, 1995). Structural analysis of SH2 revealed that the ligand-binding surface of SH2 domains is composed of two pockets. One pocket contacts the phosphotyrosine , the other pocket contacts the +3 amino acid residue following the phosphotyrosine (Songyang et al., 1993).

V. *Kinase domain (SH1)*

This domain possesses tyrosine Y-416, specific for protein kinase activity. The autophosphorylation site is very important for the regulation of kinase activity (Hubbard et al., 1994; Johnson et al., 1996; Knighton et al., 1991a; Knighton et al., 1991b). Phosphorylation of Tyr-416 stimulates complete activation of Src and provides a binding site for SH2 domains of other cellular proteins. The SH3 domain interacts with sequences in the kinase domain, as well as with sequences in the linker region that lies between the SH2 and kinase domain (Sicheri et al., 1997; Xu et al., 1997).

VI. *Short negative regulatory region*

This segment plays an important role in the activation of src kinase. It has the critical tyrosine residue Tyr 527. The SH2 domain interacts with pTyr 527 (Src) and adjacent residues in the negative regulatory tail (Brown and Cooper, 1996). Y527 in c-Src, and the corresponding tyrosine in other Src PTKs, are the primary sites of tyrosine phosphorylation *in vivo*. Kinase activity is reduced when pTyr 527 is phosphorylated and bound to the SH2 domain (Brown and Cooper, 1996).

Apart from all these domains the very important SH2 and SH3 domains have four principal functions in the regulation of Src kinase (Brown and Cooper, 1996):

1. They constrain the activity of the enzyme via intramolecular contacts.
2. Proteins that contain SH2 or SH3 ligands can bind to the SH2 or SH3 domains of Src and attract them to specific cellular locations.
3. Displacement of the intramolecular SH2 or SH3 domains results in activation of Src kinase activity.
4. Proteins containing SH2 or SH3 ligands can enhance their ability to function as substrates for Src protein–tyrosine kinase.

### 1.6.3 Structure of Src PTK and activation of tyrosine kinase

The Src kinase domain consists of the characteristic bilobed protein kinase architecture as illustrated in Figure 5 (Glass et al., 1997; Xu et al., 1997). The human

Src gene encodes a protein of 536 amino acids, and the chicken Src gene encodes a 533-residue protein. The small amino-terminal lobe of the kinase consists of residues 267–337 and is involved in anchoring and orienting ATP. This smaller lobe has mainly antiparallel β-sheet structure (Roskoski, 2004). Residues 341–520 form the large carboxyl-terminal lobe which is responsible for binding the protein substrate, and part of the ATP-binding site occurs in this lobe. It has predominantly α-helical structure (Roskoski, 2004). The cleft between the two lobes is the catalytic site for Src kinase. The two lobes move relative to each other and can open or close the cleft. Active site residues are from both the small and large lobes of the kinase; hence changes in the orientation of the two lobes can promote or restrain activity (Roskoski, 2004).

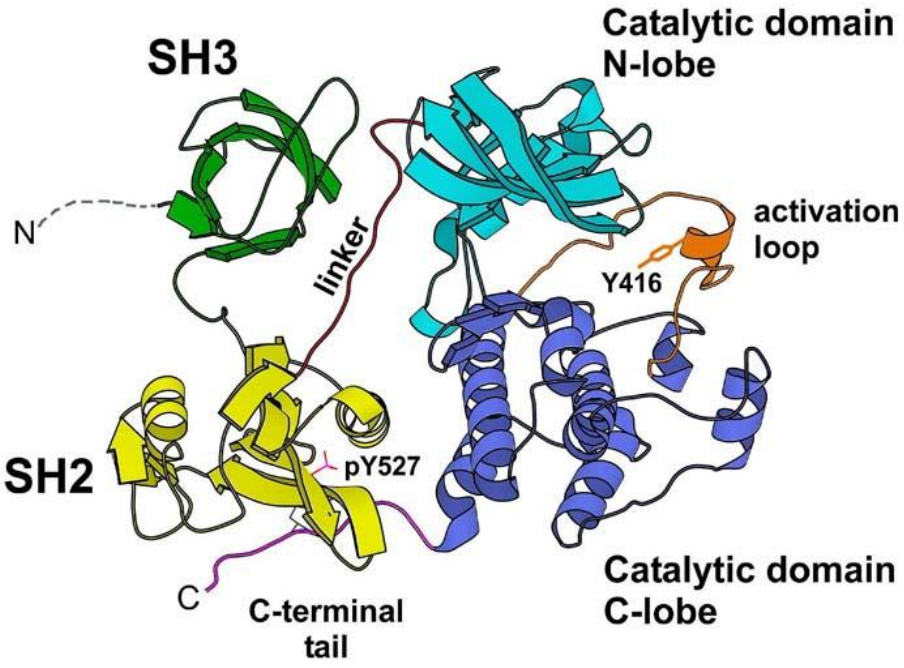


**Figure 5: Ribbon diagram illustrating the structure of human Src.**

This figure is reproduced from Roskoski (2004) and Xu et al. (1999).

The network controlling Src is a three step mechanism described by Harisson: the latch, the clamp, and the switch (Harrison, 2003).

A latch is formed when SH2 domain binds to phosphotyrosine 527 in the C-terminal tail. Tyr-527 is phoshrylated by *csk* kinase (Nada et al., 1991). This latch in turn stabilizes the attachment of the SH2 domain to the large lobe. Then the SH3 domain

contacts the small lobe. Prolines in the linker between the SH2 and kinase domains function as a motif that binds the SH3 domain and attaches the SH3 domain to the small kinase lobe. The assembly of the SH2 and SH3 domains behind the kinase domain is the clamp. This prevents the opening and closing of the cleft between the small and large lobes. The switch is the kinase-domain activation loop; the activation loop can switch between active and inactive conformations.

### 1.6.4 Mechanism of Src activation

Src tyrosine kinase is activated by unlatching, unclamping and switching.

In the inactive state, Tyr 416, which is present in the activation loop, is segregated and is not a substrate for phosphorylation by another kinase (Roskoski, 2004). The protein is unlatched when phosphotyrosine 527 dissociates or is displaced from the SH2-binding pocket. When the protein is unlatched the clamp no longer locks the catalytic domain in an inactive conformation (Harrison, 2003; Xu et al., 1999). This dissociation allows dephosphorylation by enzymes such as protein tyrosine phosphatase-α (PTPα) (Brown and Cooper, 1996). This in turn allows the activation loop to assume their active conformations. Tyr416 can then undergo autophosphorylation by another Src kinase molecule. Following autophosphorylation, the enzyme is stabilized in its active state (Roskoski, 2004).

Studies show that substitution of Tyr-527 by another amino acid residue leads to activation of c-Src (Kmiecik and Shalloway, 1987). The inhibition of the csk gene activity also stimulates activity of PTK of the Src family (Imamoto and Soriano, 1993). Phosphorylation at Tyr-416 in Src (or homologous amino acid residues in other tyrosine kinases) is necessary for complete activation of most kinases studied so far. In the absence of phosphorylation, the activating loop acquires different conformations, which often inhibit protein-protein interactions. In this active conformation the loop forms a part of the site recognized by the substrates (Xu et al., 1997).

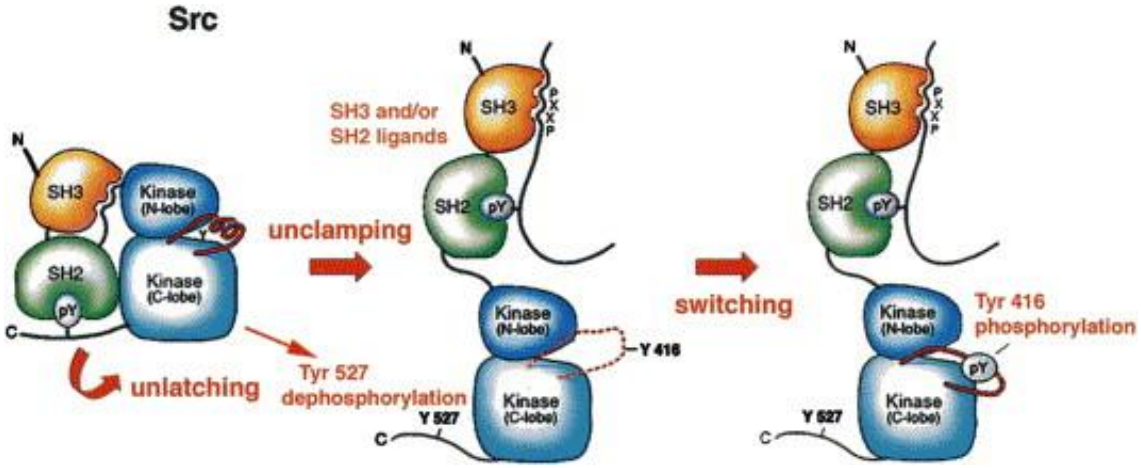


**Figure 6: The activation mechanism of Src tyrosine kinase.**

Reproduced from Roskoski (2004) and Xu et al. (1999).

## 1.7 Src kinase and ion channels

Both Src family and receptor tyrosine kinases are known to be potent modulators of ion channels (Levitan, 1994; Siegelbaum, 1994). Modulation by Fyn and other Src-family tyrosine kinases on potassium channels, calcium channels, sodium channels and glutamate receptors has been reported earlier. Studies show that potassium channel $K_V2.1$ is inhibited by Fyn tyrosine phosphorylation (Sobko et al., 1998; Tsai et al., 1999). Tyrosine phorsphorolated $K_V1.5$ interacts with the SH3 domain of Src kinase (Holmes et al., 1996a; Nitabach et al., 2001). $K_V1.2$ currents are inhibited by tyrosine phosphorylation (Peretz et al., 2000; Wischmeyer et al., 1998). In 2003, Hou et al. showed that Src/Fyn binds and modulates voltage-gated calcium channels (Hou et al., 2003). Cellular plasticity, a form of plasticity that modifies the input–output relationships of the entire neuron, is mediated by modulation of voltage-gated sodium, calcium, and potassium channels by tyrosine posphorylation/ dephosphorylation (Cantrell and Catterall, 2001).

### 1.7.1 Src tyrosine kinase and voltage-gated sodium channels

Physiological and biochemical studies show that sodium channels are regulated by tyrosine kinase. Sodium channel studies have shown that the kinase activity enhances the intrinsic slow inactivation gating process, and thereby reduces the availability of channels (Cantrell and Catterall, 2001; Carr et al., 2003; Chen et al., 2006). Tyrosine phosphorylation and dephosphorylation have also been implicated in rapid sodium channel modulation (Hilborn et al., 1998; Ratcliffe et al., 2000). Tyrosine phosphorylation of these sodium channels is associated with a hyperpolarizing shift in steady-state inactivation, resulting in fewer available channels for generating an action potential. Recent studies done with sodium channels show that Fyn kinase binds to the rat brain sodium channel $Na_V1.2$, enhances fast inactivation, and mediates inhibition of sodium currents by brain-derived neurotrophic factor (BDNF), acting through the neurotrophin receptor tyrosine receptor kinase B (TrkB) (Ahn et al., 2007). The molecular mechanism underlying the fast inactivation on $Na_V1.2$ with Fyn tyrosine kinase is also reported. Fyn kinase binds to a Src homology 3 (SH3)-binding motif in the second half of the intracellular loop connecting domains I and II ($L_{DI–DII}$) of $Na_V1.2$. Mutation of that SH3-binding motif prevents Fyn binding and Fyn enhancement of fast inactivation of sodium currents. Fast inactivation of the closely related $Na_V1.1$ channel is not modulated by Fyn. These channels do not contain a SH3-binding motif in $L_{DI–DII}$.

In cardiac sodium channels, very few studies show that they behave oppositely from their neuronal counterparts. Hyperpolarizing shift is observed in the inactivation-voltage relationship, when tyrosine kinase inhibitors are added to cardiac myocytes (Wang et al., 2003). This suggests that the phosphorylated form of the cardiac channel displays enhanced excitability. In 2005, Richard Horn's group (Ahern et al., 2005) expressed SCN5A clones (hH1) in HEK cells. They found that Fyn shifts the inactivation-voltage relationship towards more hyperpolarizing potentials and the kinase activity dead mutant of Fyn reversed the effect.

### 1.7.2 Physiological relevance of cardiac sodium channels and Src family tyrosine kinase modulation

Cardiac sodium channel $Na_V1.5$ (Maier et al., 2002) and Src tyrosine kinase (Holmes et al., 1996b; Toyofuku et al., 2001) are accumulated at the adherens junctions. Adherens junctions are made up of protein complexes linking the cell membranes and cytoskeletal elements within and between cells. Adherens junctions are responsible for the electrical coupling between cardiac myocytes (Ahern et al., 2005). Gap junctions in cardiac myocytes are reduced because of the c-Src-mediated tyrosine phosphorylation of Connexin-43. It also decreases the stability of Connexin-43 at the cell surface as well as in the whole cell (Toyofuku et al., 2001). Non-pathological stimulation of adrenergic ligands (Ma and Huang, 2002), angiotensin II (Sadoshima et al., 1995), epidermal growth factor (Wu et al., 2000) or insulin (Zhang and Hancox, 2003) are induced by tyrosine kinase activity in the heart.

### 1.7.3 Modulation of sodium channel variants by Fyn tyrosine kinase

In cardiac sodium channel variants, there is evidence which shows that sodium currents behave oppositely to their neuronal counterparts. In 2005, Ahern et al. showed that Fyn tyrosine kinase shifts the inactivation kinetics to more depolarizing potentials in HEK cells expressing $Na_V1.5$ (hH1 clone). Later in 2007, Beacham et al. argued that the cardiac sodium channel does not have equivalent SH3 domain binding residues compared to the neuronal counterpart, so that the fast inactivation is shifted to more depolarizing potentials. Y1495 in the loop $L_{DIII-DIV}$ is required for phosphorylation, in turn leading to the shift in inactivation. Equivalent Y1495 in the neuronal sodium channel is the site for phosphorylation.

## 1.8 Nomenclature

**Table 3: Different cardiac sodium channel variants**

International Human Genome Sequencing Consortium (IHGSC) sequence and reference sequence; hH1, hH1b and hH1c are previous cDNA clones of SCN5A; Accession No., GenBank nucleotide accession numbers, amino acid frequency (AAF), and variant name, name relative to SCN5A (defined herein as identical to IHGSC), AA No. indicates amino acid position number in the protein, using the full-length numbering consistent with the IHGSC databases. Population frequency indicates estimated percentage of channels in the study population.

| **Common Name** | **IHGSC** | **hH1** | **hH1b** | **hH1c** | **AAF** |
|---|---|---|---|---|---|
| **Accession No.** | **AC137587** | **M77235** | **AF482988** | **AY148488** | |
| **AA. No. 558** | H | H | R | H | 70% H |
| **559** | T | T | T | T | 100% T |
| **618** | L | L | I | L | 100% L |
| **1027** | R | Q | R | R | 100% R |
| **1077** | Q | Q | Δ | Δ | 65% Δ |
| **Variant name** | **SCN5A [Q1077]** | **R1027Q** | **[H558R; L618I;Q1077del]** | **Q1077del** | |
| **Population Frequency** | **25%** | **0%** | **0%** | **45%** | |

# Aims

The main aim of this project is to investigate the role of the Glutamate1077 residue in the intracellular loop ($L_{DII-III}$) connecting Domain II and III of the voltage-gated cardiac sodium channel in modulation by Fyn tyrosine kinase. In particular I address the following questions:

1. The role of Q1077 in $L_{DII-III}$ in the modulation of the sodium channel by Fyn tyrosine kinase?
2. Can Q1077 mutation affect the modulation by Fyn?
3. What is the potential SH3 binding motif in $Na_V1.5$ which differs from $Na_V1.2$ in modulation by Fyn kinase?
4. What are the potential residues involved in modulation of different sodium channel isoforms by Fyn kinase?
5. What are the unique amino acid sequences in the loop $L_{DII-III}$ of cardiac sodium channel, which makes it different from other sodium channel subtypes?

# Methods and Materials

## 3.1 Molecular Biology

### 3.1.1 DNA clones

Q1077Pre and Q1077Del clones in *pcDNA3-N were kindly provided by* Prof. Jonathan C Makielski, University of Wisconsin, Madison. Fyn tyrosine kinase active (ΔTyr 527) clone pCS2-c-Fyn$^{CA}$ and tyrosine kinase dead (K299M) clone pCS2-c-Fyn$^{KD}$ were gifts from Dr Richard Horn, Jefferson Medical College, USA. πH3-CD8 was a gift from Dr. B. Seed, Harvard Medical School, Boston, MA, USA

### 3.1.2 DNA amplification and isolation

Subcloning Efficiency™ DH5α™ (Invitrogen®) competent E. coli cells were used to transform the gifted cDNAs. Transformation was performed according to the manufacturer's protocol. The transformed cells were plated on LB Ampicilline plates. Then the transformed clones were picked from the LB plates and grown in SOC medium overnight. The overnight culture was used to isolate cDNA. Isolation of cDNA from the overnight culture was done using QIAGEN® mini and midiprep kits according to the manufacturer's protocol. Using Nanodrop spectrophotometer the concentration of the purified DNA from QIAGEN® mini and midiprep was obtained. cDNA were verified using restriction enzyme digestion and run on an agarose gel electrophoresis.

The components of the LB Ampicillin plates were:

- 10 g Tryptone Yeast Extract
- 5 g Yeast Extract
- 10 g NaCl
- 20 g Agar
- 1 l double distilled $H_2O$ and autoclaved

10 ml of Ampicillin (concentration 100 mg/ml) was added and 7-10 ml were poured in each plate.

The components of the SOC medium were:

- 5 g Yeast Extract
- 20 g Tryptone
- 0.5 g NaCl
- 1 l double distilled $H_2O$ and autoclaved

10 ml filter-sterilized (0.2 µm filter) solution of 1M $MgCl_2$, 1M $MgSO_4$ and 2M glucose was added.

### 3.1.3 Site-directed mutagenesis

Site-directed mutagenesis was performed using Stratagene Quick Change Site-Directed Mutagenesis Kit according to the manufacturer's protocol. Q1077Present (GenBank AC137587) $Na_V1.5$ cDNA was used as the template for the mutation process. Both forward and reverse primers were designed using Stratagene primer design program. The forward primer and reverse primers for all the mutants are given in Table 4. Primers were ordered from Sigma® Life Sciences.

**Table 4: Forward and reverse primers for Q1077 mutations.**

| Oligo Name | Sequence 5' to 3' |
|---|---|
| | |
| Q1077A Forward | CCAGCAAGCAGGCGGAATCCCAGC |
| Q1077A Reverse | GCTGGGATTCCGCCTGCTTGCTGGAC |
| | |
| Q1077K Forward | GAGTCCAGCAAGCAGAAGGAATCCCAGCC |
| Q1077K Reverse | CAGGCTGGGATTCCTTCTGCTTGCTGGACTC |
| | |
| Q1077P Forward | GTCCAGCAAGCAGCCGGAATCCCAGCCTG |
| Q1077P Reverse | CAGGCTGGGATTCCGGCTGCTTGCTGGAC |
| | |
| Q1077Y Forward | GGAGTCCAGCAAGCAGTATGAATCCCAGCCTGTGT |
| Q1077Y Reverse | ACACAGGCTGGGATTCATACTGCTTGCTGGACTCC |

The reagents were added according to the manufacturer's instruction manual in 200 µl Polymerase chain reaction (PCR) tubes. Then the tubes were placed in the PCR machine, with programmed cycling parameters given below in Table 5.

**Table 5: PCR cycling parameters Quick Change Site-Directed Mutagenesis.**

| Segment | Cycle | Temperature | Time |
|---|---|---|---|
| 1 | 1 | 95ºC | 2 minutes |
| 2 | 18 | 95ºC | 20 seconds |
| | | 95ºC | 10 seconds |
| | | 95ºC | 6.1 minute |
| 3 | 1 | 95ºC | 5 minutes |

The PCR product was then transformed into the XL10-Gold ultra-competent cells according to the transformation protocol provided by the manufacturer. The transformed cells were plated on LB Ampicillin plates for more than 16 hours. Then

the transformed clones were picked from the LB plates and grown in SOC medium overnight. The overnight culture was used to isolate cDNA. Isolation of cDNA from the overnight culture was done using QIAGEN® mini and midiprep kits according to the manufacturer's protocol. Using Nanodrop spectrophotometer from Thermo Scientific, the concentration of the purified DNA from QIAGEN® mini and midiprep was obtained. The sequence of the DNA was verified using restriction enzyme digestion and sequencing.

## 3.2 Cell Culture

### 3.2.1 Cells

Human embryonic kidney tsA-201 cells with a passage number less than 25 were used for the experiments.
HEK293 stable cells encoding for Q1077Present (GenBank AC137587) and Q1077 Deleted (GenBank AY148488) were obtained from Prof. Jonathan C. Makielski, University of Wisconsin, Madison.

### 3.2.2 Cell culture media

DMEM-F-12 (Dulbecco's modified Eagle's medium/nutrient mixture F12 Ham) was purchased from Sigma-Aldrich GmbH for tsA-201cells. MEM (Modified Eagle's medium), MEM Sodium Pyruvate, L-Glutamine, Non-essential Amino Acids, and Geneticin were purchased from Sigma-Aldrich GmbH for stably transfected cells. Fetal calf serum (FCS) and Fetal bovine serum (FBS) was purchased from Sigma-Aldrich GmbH. PBS without $Ca^{2+}/Mg^{2+}$ 10X: 2.00 g KCl, 2.0 g $KH_2PO_4$, 80.0 g NaCl, 27.07 g $Na_2PO_4.2H_2O$, 1000 ml distilled $H_2O$. Mixed to dissolve, this solution was diluted 1:10 with distilled $H_2O$, sterile filtrated before use and stored at 4 °C.
Trypsin/EDTA 10X: 0.25 g trypsin (1:250), 0.20 g EDTA, 10 ml PBS without $Ca^{2+}/Mg^{2+}$ 1X. Mix to dissolve. This solution was sterile filtrated and stored at –20 °C, and diluted 1:10 with sterile PBS without $Ca^{2+}/Mg^{2+}$ 1X before use.

### 3.2.3 Sub-culturing cells

#### *3.2.3.1 Stably transfected cells*

Stably transfected cells were grown on tissue culture flasks until 70% confluent cells. Medium was aspirated from plates, then cells were washed with 2 ml 1x PBS, twice. 2 ml trypsin (0.25% trypsin, 0.02% EDTA) was added and incubated for 2 min at 37°C. 3 ml of medium was added to quench and transferred to a 15 ml tube. The tube was centrifuged for 2 min at 1,000 RPM. After the medium was aspirated off the plates, 5 ml medium was added to resuspend the cells. 1/10 of the cell/media mixture was added to a new plate with fresh medium. The flask was placed in a 37°C incubator and checked every 2-3 days for growth. Cells for transfection were plated on Petri dishes (Falcon) at 30–50% confluence ~16 h before transfection. For cryostorage the resuspended cells were added to a medium (without antibiotics) containing 10% FBS and 5% DMSO.

**Table 6: Medium composition for culturing stably transfected cells.**

| Reagent | Volume |
|---|---|
| MEM | 500 ml bottle |
| MEM Sodium Pyruvate | 5 ml |
| L-Glutamine | 5 ml |
| MEM non-essential AA solution | 5 ml |
| FBS (Fetal Bovine Serum) | 50 ml |

#### *3.2.3.2 tsA Cells*

Human embryonic kidney tsA-201 cells were grown at 5% $CO_2$ and 37 °C to 70% confluence in Dulbecco's modified Eagle's/F-12 medium supplemented with 10% fetal bovine serum (FBS). Cells were split using trypsin/ EDTA and plated on 35-mm Petri dishes (Falcon) at 30–50% confluence ~16 h before transfection.

### 3.2.4 Transfection

#### *3.2.4.1 Transient transfection of stably transfected HEK cells*

Before transfection, the medium was replaced with fresh medium, and the cells were transiently transfected with cDNAs of either 1µg cDNA of $Fyn^{CA}$ or $Fyn^{KD}$ with πH3-CD8 cDNA using the QIAGEN polyfect transfection reagents according to the manufacturer's protocol. Cells were incubated overnight at 37°C in 5% $CO_2$. After 12–16 h, the medium was replaced, and the cells were allowed to recover for 9–24 h before experiments. Anti-CD8-coated beads (Dynal, Oslo, Norway) were used to identify transfected cells (Jurman et al., 1994).

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#### *3.2.4.2 Transient transfection of mutant clones*

Sodium channels were transiently expressed for electrophysiological analysis by transfecting tsA-201 cells in 35 mm dishes with 1.2 µg of cDNA encoding the $Na_V1.5$ (Genebank AC13758) α- subunit mutants Q1077A, Q1077P, Q1077Y, Q1077K and πH3-CD8 cDNAs with or without 0.1 µg of $Fyn^{CA}$ or $Fyn^{KD}$ cDNA, using the QIAGEN polyfect transfection reagents according to the manufacturer's protocol. Anti-CD8-coated beads (Dynal, Oslo, Norway) were used to identify transfected cells (Jurman et al., 1994).

## 3.3 Electrophysiology

### 3.3.1 Patch pipettes

Patch pipettes were made in three stages: pipette pulling, heat polishing and filling. The patch pipettes were pulled from borosilicate glass 1.5mm O.D. X 0.86mm I.D. (Harvard Apparatus, UK) using a programmed Flaming/Brown micropipette puller (P-87, Sutter Instrument Company, CA, USA). The pipette tips were heat polished on a Micro-Forge model MF-79 (Narishige Scientific Instrument Lab., Tokyo, Japan). The pipette tip polishing was observed at 16 x 35 magnification using

a compound microscope with a long distance objective (Leitz Biomed, Wetzlar, Germany). This procedure further reduced the tip opening to a final resistance of ≤ 5-8 MΩ. The pipette solution was back filled using hypodermic needles of size 0.60 X 60mm 23GX2-3/8" (Enosa, ROSE GmBh, Germany). The pipette solution must be filtered before using with a pore size equivalent to 0.2 mm in diameter (Sartorius, Göttingen, Germany). The pipette resistance was controlled before patch formation. Pipettes with a resistance lower than 1.5 MΩ were rejected and also pipettes with a resistance higher than 10 MΩ. Usually, the pipette resistance between 1.5-3.5 MΩ was used for whole cell patch clamp experiments.

### 3.3.2 Patch clamp solutions

Macroscopic $I_{Na}$ was recorded using the whole cell patch-clamp technique.

#### *3.3.2.1 Stably transfected HEK cells*

The bath (extracellular) solution contained (in mM) 20 NaCl, 120 $C_5H_{14}ClNO$, 4 KCl, 1.8 $CaCl_2$, 0.75 $MgCl_2$, and 5 HEPES (pH 7.4 with NaOH). The pipette solution contained (in mM) 120 CsF, 20 CsCl, 5 EGTA, and 5 HEPES (pH 7.4 set with CsOH).

#### *3.3.2.2 tsA-201 cells with Q1077 mutants*

The bath (extracellular) solution contained (in mM) 140 NaCl, 4 KCl, 1.8 $CaCl_2$, 0.75 $MgCl_2$, and 5 HEPES (pH 7.4 with NaOH). The pipette solution contained (in mM) 120 CsF, 20 CsCl, 5 EGTA, and 5 HEPES (pH 7.4 set with CsOH).

### 3.3.3 Experimental set-up

The patch clamp set-up consisted of the microscope (Axiovert 100, Carl Zeiss, Germany), amplifier (Axopatch 200B, Axon Instruments, CA, USA), headstage (CV 203BU Axon Instruments, CA, USA), pipette holder (HL-U, Axon Instruments, Inc.,

Foster City, Ca, USA), micromanipulator (Patchman, Eppendorf, Hamburg, Germany), computer, metal cage and vibration isolation table (Newport Corporation, Irvine, CA, USA). The silver wire was chlorinated every week before using as an electrode.

Membrane currents were recorded with an Axopatch 200B amplifier (Axon Instruments). Data were acquired using pCLAMP 10.2. Peak $I_{Na}$ was obtained after passive leak subtraction. Data were digitized at 100 kHz and were low pass filtered at 10 kHz. Parameter fits were obtained using Origin 7.0.

### 3.3.4 Pulse protocol

*Pulse protocol for activation curves*

Current was elicited by a clamp from a holding potential of -140 mV to various test pulses (-60, -55, -50, -45, -40, -35, -30, -25, -20, -15, -10, 0, +10, +20, +30, +40, +50, +60, +70 mV) for 24 ms. 10 s was given between each sweep.

*Pulse protocol for inactivation curves*

Current recordings were obtained when the cells were clamped from a holding potential of -140 mV to 100 ms prepulses at -130, -120, -110, -105, -100, -95, -90, -85, -80, -75, -70, -65, -60, -50, -40, -30, -20, -10, and 0 mV, followed by a 24 ms test pulse to 0 mV.

### 3.3.5 Data acquisition and analysis

For the study of peak current-voltage relationships, data were normalized to the peak $I_{Na}$ in each data set. The current-voltage (I-V) curves were fitted according to the following modified Boltzmann function: $G_{Na} = [1 + \exp (V_{1/2}\text{-}V)/ k_{act}]^{-1}$, where $V_{1/2}$ and $k_{act}$ are the mid-point and the slope factor, respectively, and $G_{Na} = I_{Na}/(V\text{-}V_{rev})$ where $V_{rev}$ is the reversal potential. For voltage dependence of “steady-state” inactivation, $I_{Na}$ was obtained in response to a test depolarization to 0 mV from a holding potential of –150 mV, followed by a 1 sec conditioning step to the various conditioning potentials ($V_c$). In order to normalize the capacity transients a 0.2 ms step back to -150 mV was applied before a test depolarization. The voltage dependent

availability from inactivation was determined by fitting the data to the Boltzmann function: $I_{Na}= I_{Na\text{-}max}\ [1+ \exp\ (V_c\text{-}V_{1/2})/k_{inact}]^{-1}$, where $V_{1/2}$ and $k_{inact}$ are the midpoint and the slope factor, respectively, and V is the membrane potential.

## 3.4 Bioinformatics Analysis

Sequences of all the sodium channel variants were obtained from NCBI protein database. Later the sequences were aligned using the CLUSTALX: Multiple sequence alignment program (Larkin et al., 2007; Thompson et al., 1994) with default parameter. Then the aligned sequence was corrected manually if there was a mismatch.

# Results

Previous results published on the modulation of the sodium channel by Fyn show that active Fyn shifts the steady-state inactivation in $Na_V1.2$ (Beacham et al., 2007) and $Na_V1.5$ (Ahern et al., 2005) without affecting the steady-state activation kinetics. Therefore, particular attention was payed on studies of the steady-state inactivation kinetics of cardiac sodium channel variants (Q1077 and ΔQ1077) in presence and absence of Fyn.

## 4.1 Voltage-dependent kinetic parameters for Q1077 and ΔQ1077 cardiac sodium channel variants

Modulation of sodium current kinetics by Fyn tyrosine kinases on stably expressed Q1077 and ΔQ1077 variants in HEK-293 cells was investigated. Figure 7 shows the original current traces obtained with Q1077 and ΔQ1077 variants. From the Figure it is evident that ΔQ1077 has an increased peak $I_{Na}$ compared to the Q1077 variant.

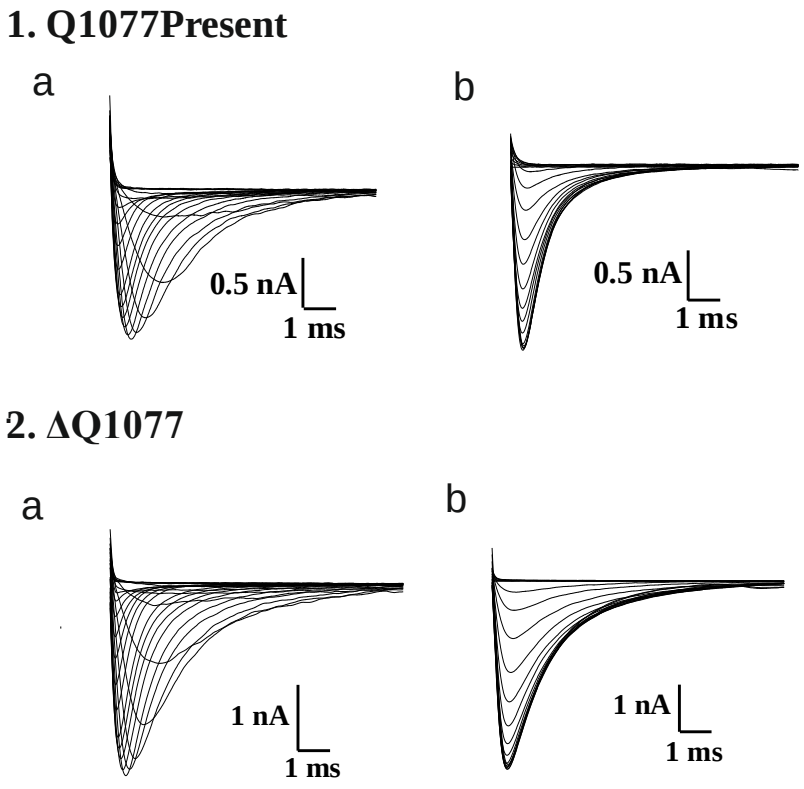


**Figure 7: Original traces illustrating steady-state activation and steady-state inactivation of sodium channel currents of both (1) Q1077Present and (2) ΔQ1077.**

In both variants, (1) and (2), (a) represents the activation current elicited from a holding potential of -140 mV to various test pulses (-60, -55, -50, -45, -40, -35, -30, -25, -20, -15, -10, 0, +10, +20, +30, +40, +50, +60, +70) for 24 ms. 10 s was given between each sweep. (b) In both (1) and (2) current recordings are shown, which were obtained when the cells where clamped from a holding potential of -140 mV to 100 ms prepulses at -130, -120, -110, -105, -100, -95, -90, -85, -80, -75, -70, -65, -60, -50, -40, -30, -20, -10, and 0 mV, followed by a 24 ms test pulse to 0 mV.

Table 7 shows the voltage-dependent kinetic parameters for both Q1077 and ΔQ1077 variants. There was no significant difference in the activation kinetics among both the variants. However, the $V_{1/2}$ steady-state inactivation of ΔQ1077 was observed at more depolarized potentials (-79.39 ± 0.38 mV) than that of the Q1077 variant (-88.21 ± 0.31 mV) ($p<0.001$). The slope ($K_{inact}$) of the inactivation curve of ΔQ1077 (5.20 ± 0.27 mV) also differs from that of Q1077 (6.55 ± 0.21 mV) ($p<0.001$). These results indicate that the glutamate residue (Q) at position 1077 plays a role in the steady-state inactivation of the cardiac sodium channel. As shown in Figure 7 and Table 7 the

peak $I_{Na}$ of ΔQ1077 (3.78 ± 0.11 nA) was larger than that of the Q1077Present variant (1.92 ± 0.10 nA) ($p<0.001$). The decay of $I_{Na}$ at 0 mV, for the portion of the $I_{Na}$ trace after 90% of peak, was fitted using double exponential decay function. The values obtained from the fit are also tabulated in Table 7. The results on the decay of $I_{Na}$ indicate that the fast component of the time constant ($\tau_f$) in ΔQ1077 (1.69 ± 0.14 ms) is significantly different ($p<0.001$) from that of the Q1077Present variant (0.79 ± 0.46 ms) (Table 7, Figure 7).

**Table 7: Voltage-dependent kinetic parameters for Q1077 and ΔQ1077 cardiac sodium channel variants.**

Data are given as the means ± SEM, obtained from curve fitting to n experiments. Activation, inactivation, and decay were obtained according to the protocol described in the Materials and Methods section. For the decay of $I_{Na}$ at 0 mV, the portion of the $I_{Na}$ trace after decay to 90% of the peak current was fit to a sum of exponentials: $I_{Na}(t) = A_f \text{ X } \exp -t/\tau_f + A_s \text{ X } \exp -t/\tau_s + \text{offset}$, where t is time, $\tau_f$ and $\tau_s$ represent the time constants of the fast and slow components, and $A_f$ and $A_s$ are amplitudes of fast and slow components, respectively.

* Significant difference (ANOVA) for Q1077 and ΔQ1077 stably transfected cells $p<0.001$

| | **ΔQ1077** | **Q1077Present** |
|---|---|---|
| **Inactivation** | | |
| $V_{1/2,}$ mV | -79.39 ± 0.38* | -88.21 ± 0.31 |
| Slope, mV | 5.20 ± 0.27* | 6.55 ± 0.21 |
| n | 9 | 8 |
| **Activation** | | |
| $V_{1/2,}$ mV | -48.16 ± 0.20 | -47.65 ± 0.29 |
| Slope, mV | 3.44 ± 0.10 | 3.60 ± 0.10 |
| n | 5 | 4 |
| **Peak Current** | | |
| $I_{Na,}$ nA | 3.78 ± 0.11* | 1.92 ± 0.10 |
| n | 8 | 4 |
| **Decay ( 0 mV)** | | |
| $A_f$ | 0.87 ± 0.11 | 0.85 ± 0.03 |
| $\tau_{f,}$ ms | 1.69 ± 0.14* | 0.79 ± 0.46 |
| $\tau_{s,}$ ms | 3.78 ± 0.15 | 4.09 ± 0.90 |
| n | 7 | 5 |

Figure 8 shows the Boltzmann fitted curves of both steady-state activation and inactivation of (A) Q1077 and (B) ΔQ1077 cells on the normalized $I_{Na}$ and $G_{Na}$ obtained from the original traces using the inactivation and activation pulse protocol as described in Material and Methods.

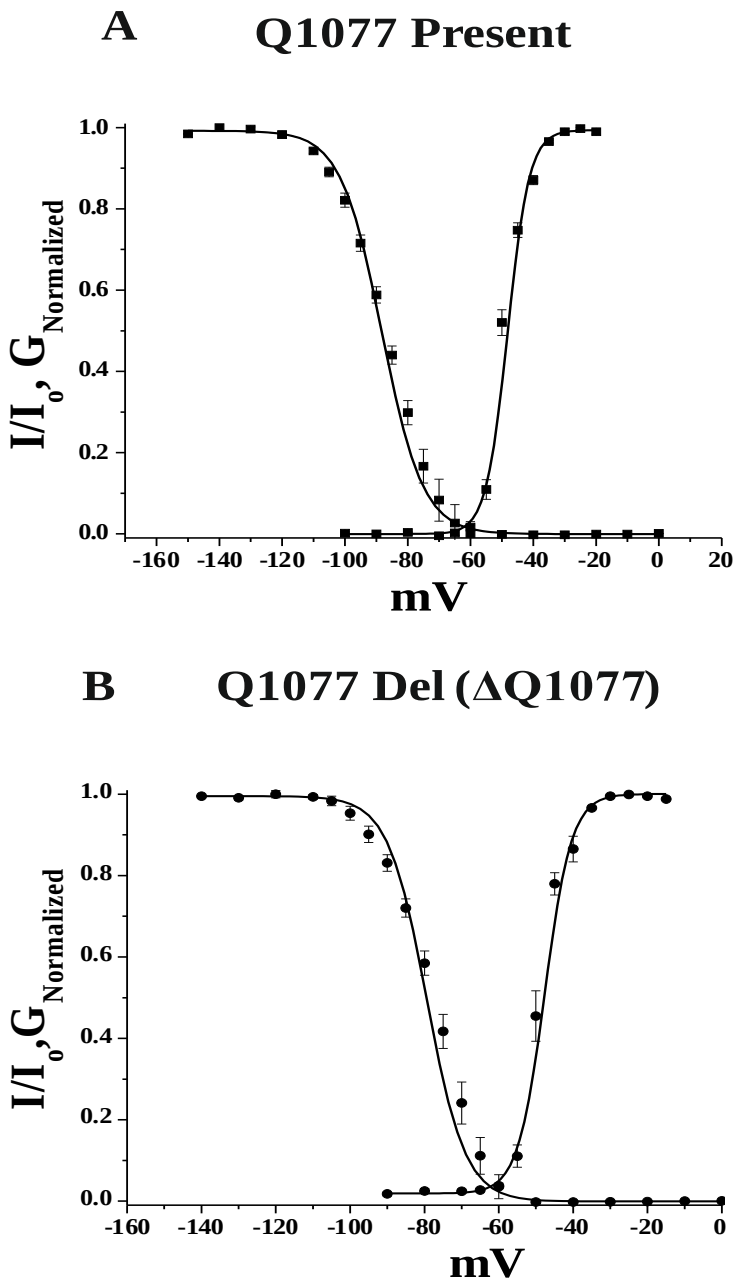


**Figure 8: Boltzmann fitted steady-state inactivation and activation curves of (A) Q1077Present and (B) ΔQ1077 stably transfected cells.**

Boltzmann fitting, using the functions as described above, was done on the normalized values from original traces.

## 4.2 Inactivation properties of ΔQ1077 and Q1077 with $Fyn^{CA}$, $Fyn^{KD}$ and tyrosine kinase inhibitor PP2

To test whether Fyn tyrosine kinase can differently modulate ΔQ1077 and Q1077, the stably transfected cells were transiently transfected with $Fyn^{CA}$ and $Fyn^{KD}$. The constitutively active mutant, $Fyn^{CA}$ lacks its inhibitory carboxyl terminus (amino acids upstream of 525 were deleted), whereas the kinase dead mutant $Fyn^{KD}$ has a single point mutation at position K299M (Ahern et al., 2005; Nitabach et al., 2001). Figure 9 shows the steady-state inactivation curves of the sodium current from stably expressed cells (ΔQ1077 and Q1077), either alone or transiently transfected with active $Fyn^{CA}$ or dead $Fyn^{KD}$, or $Fyn^{CA}$ incubated with the potent Src tyrosine kinase inhibitor PP2.

Experiments with $Fyn^{CA}$ on Q1077Present caused a 16-mV depolarizing shift of the steady-state inactivation curve (Figure 9 and Table 9). The $V_{½}$ inactivation of Q1077Present was -88.21 ± 0.31 mV (n=8) and the $V_{½}$ inactivation of Q1077Present with $Fyn^{CA}$ was shifted to -72.98 ± 0.06 mV ($p<0.001$, n=6). This result indicates that the shift in steady-state inactivation of Q1077Present in presence of $Fyn^{CA}$ is similar to the hH1 cardiac sodium channel variant modulation by $Fyn^{CA}$. In the hH1 cardiac sodium channel, $Fyn^{CA}$ caused a 5-mV shift of the steady-state inactivation curve from a $V_{½}$ of -94.9 ± 2.4 mV to -89.4 ± 0.9 mV (Ahern et al., 2005). To prove that this shift was due to Fyn, Q1077Present cells were transiently transfected with $Fyn^{KD}$. The data were confirmed as no shift was observed with $Fyn^{KD}$ (-87.13±0.29, n=4). Further evidence was obtained with the kinase inhibitor PP2. PP2 inhibited the Fyn action and reversed back the depolarizing shift from -72.98 ± 0.06 mV caused by $Fyn^{CA}$ to -88.41 ± 0.09 mV (n=3). These results obtained with Q1077Present in presence of $Fyn^{CA}$, $Fyn^{KD}$ and Src tyrosine inhibitor PP2 show that the depolarizing shift was caused by Fyn tyrosine kinase.

Our experiments with $Fyn^{CA}$ on ΔQ1077 caused a 9-mV hyperpolarizing potential shift of the steady-state inactivation curve (Figure 9 and Table 9) from an average $V_{½}$ of -79.39 ± 0.38 mV (n=9) for ΔQ1077 stably expressed cells to -88.51 ± 0.31 mV in the presence of $Fyn^{CA}$ (n=5) ($p < 0.001$). The hyperpolarizing shift caused by $Fyn^{CA}$ was similar to that observed in the $Na_V1.2$ channel, in which a 5-mV shift from -64.2 ± 0.42 mV to -70.1 ± 0.32 mV was caused by Fyn (Beacham et al., 2007). When the

ΔQ1077 cells were transiently transfected with $Fyn^{KD}$ no shift of the steady-state inactivation curve was observed (n=6). This study confirms that the hyperpolarizing shift was caused by $Fyn^{CA}$. To substantially confirm that the shift in the inactivation of ΔQ1077 was due to Fyn, the cells which were transfected with $Fyn^{CA}$ were treated with 1 µmol/L of the Src kinase inhibitor PP2. The kinase inhibitor PP2 inhibited the Fyn action and reversed back the $Fyn^{CA}$-caused hyperpolarizing shift from -88.51 ± 0.31 mV to -78.52 ± 0.14 mV. This clearly confirms that the hyperpolarizing shift was caused by Fyn tyrosine kinase.

No significant difference was seen in the peak $I_{Na}$ and the decay of current in both Q1077Present and ΔQ1077 with and without $Fyn^{CA}$ (Table 9). However, from analysis of steady-state inactivation it was evident that Q1077Present and ΔQ1077 cardiac sodium channels behave differently in the modulation by Fyn tyrosine kinase.

**Table 8: Kinetic parameters for Q1077 and ΔQ1077 cardiac sodium channel variants with and without $Fyn^{CA}$.**

| | **ΔQ1077** | **ΔQ1077 + $Fyn^{CA}$** | **Q1077Present** | **Q1077 + $Fyn^{CA}$** |
|---|---|---|---|---|
| **Peak Current** | | | | |
| $I_{Na,}$ nA | 3.78 ± 0.11 | 3.97 ± 0.28 | 1.92 ± 0.10 | 1.71 ± 0.26 |
| n | 8 | 3 | 4 | 4 |
| **Decay ( 0 mV)** | | | | |
| $A_f$ | 0.87 ± 0.11 | 0.84 ± 0.12 | 0.85 ± 0.03 | 0.89 ± 0.49 |
| $\tau_{f,}$ ms | 1.69 ± 0.14 | 1.45 ± 0.21 | 0.79 ± 0.46 | 0.96 ± 0.38 |
| $\tau_{s,}$ ms | 3.78 ± 0.15 | 4.13 ± 0.63 | 4.09 ± 0.90 | 4.62 ± 0.16 |
| n | 7 | 3 | 5 | 4 |

**Table 9: Half-maximal inactivation of both Q1077 and ΔQ1077 with $Fyn^{CA}$, $Fyn^{KD}$ and with $Fyn^{CA}$ + PP2.**

Data are derived from fits of Boltzmann function to normalized inactivation curves determined from individual cells as described in Materials and Methods. * $p<0.001$.

| Variant | Half-maximal inactivation ($V_{1/2}$) (mV) | $K_{Inact}$ (mV) | n |
|---|---|---|---|
| | | | |
| Q1077Present | -88.21 ± 0.31 | 5.55 ± 0.21 | 8 |
| Q1077Present + $Fyn^{CA}$ | -72.98 ± 0.06* | 6.73 ± 0.35* | 6 |
| Q1077Present + $Fyn^{KD}$ | -87.13 ± 0.29 | 5.18 ± 0.24 | 4 |
| Q1077Present + $Fyn^{CA}$ + PP2 | -88.41 ± 0.09 | 5.91 ± 0.63 | 3 |
| | | | |
| ΔQ1077 | -79.13 ± 0.38 | 5.76 ± 0.27 | 9 |
| ΔQ1077 + $Fyn^{CA}$ | -88.21 ± 0.31* | 7.63 ± 0.18* | 5 |
| ΔQ1077 + $Fyn^{KD}$ | -80.61 ± 0.36 | 5.97 ± 0.28 | 6 |
| ΔQ1077 + $Fyn^{CA}$ + PP2 | -78.88 ± 0.14 | 6.28 ± 0.37 | 2 |

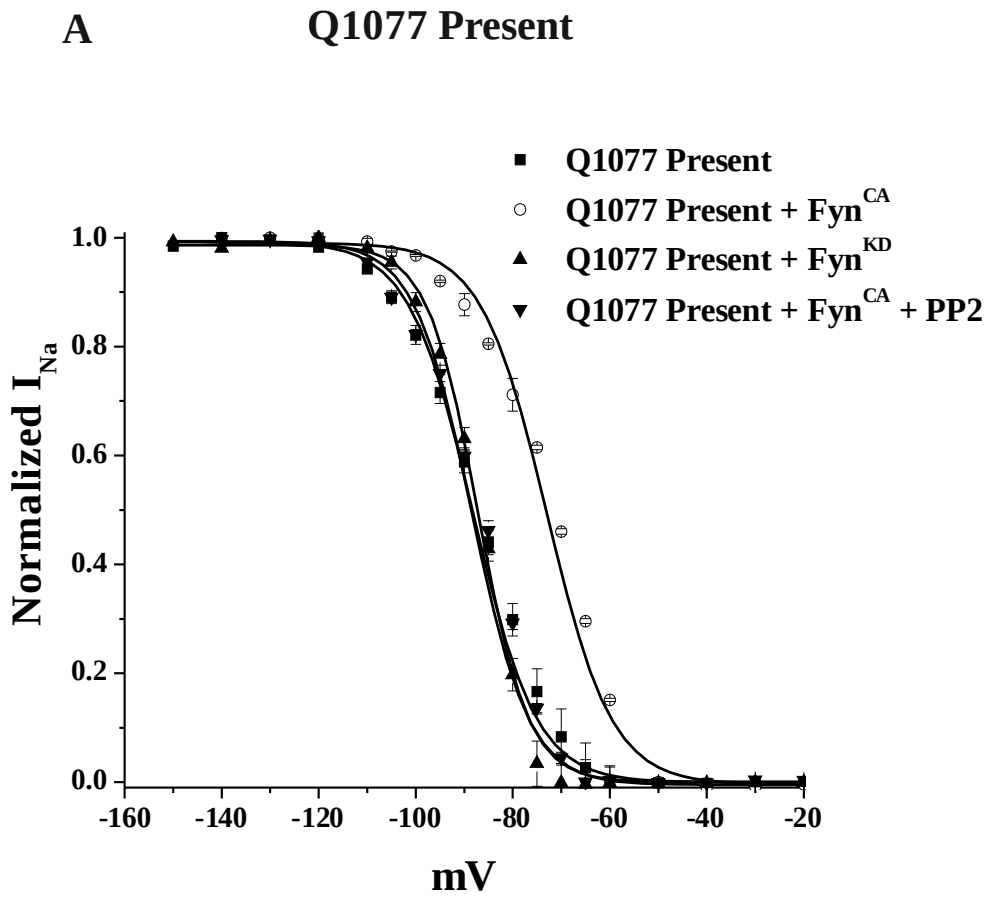


**Figure 9A: Expression of catalytic active Fyn$^{CA}$ alters steady state inactivation curves in stably expressed Q1077Present and ΔQ1077 cells.**

A. Steady-state inactivation properties of stably expressed Q1077Present cardiac sodium channel current with Fyn$^{CA}$. Solid line in (A) with filled squares corresponds to the Boltzmann fit of current recorded from stably expressed Q1077Present cells. The $V_{1/2}$ of Q1077Present is -88.21 ± 0.31 mV. The Boltzmann fit curve with open circles represents data obtained from stably expressed Q1077Present cells with Fyn$^{CA}$. $V_{1/2}$ of Q1077Present expressed with Fyn$^{CA}$ is -72.98 ± 0.06 mV. When Q1077Present cells were expressed with Fyn$^{KD}$, represented by filled up-pointing triangles, the $V_{1/2}$ is -87.13 ± 0.29 mV. To test the Src tyrosine kinase inhibitor action, the Q1077Present cells tranfected with Fyn$^{CA}$ were incubated with the specific tyrosine kinase inhibitor PP2 (1 µmol/L) for 30 minutes before current recording. The curve with filled down-pointing triangles denotes the Boltzmann fit of Q1077Present in presence of Fyn$^{CA}$ and the specific tyrosine kinase inhibitor PP2; the $V_{1/2}$ is -88.41 ± 0.09 mV.

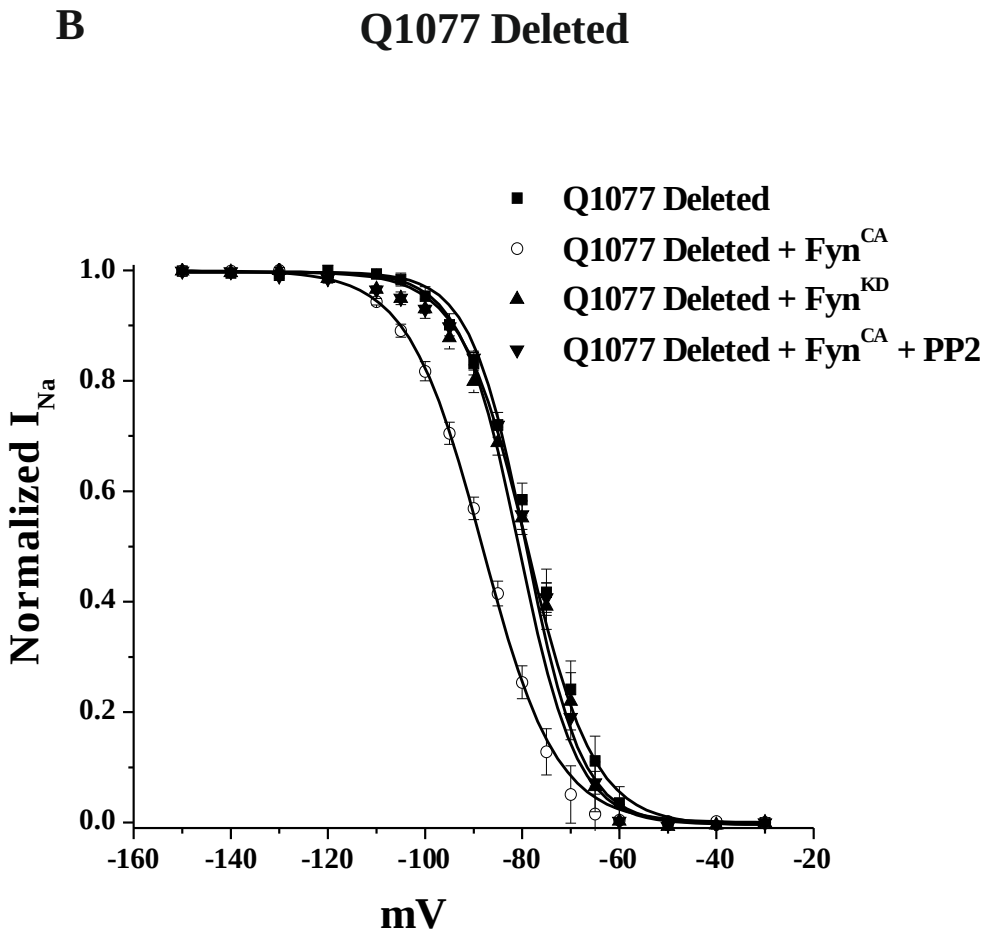


**Figure 9B:**
B. Similar inactivation curves for ΔQ1077 cells are shown as explained above for Q1077Present cells. Boltzmann fit of stably expressed ΔQ1077 cells is denoted with filled squares. The $V_{1/2}$ inactivation of ΔQ1077 is -79.39 ± 0.38 mV. The inactivation curve of stably expressed ΔQ1077 with $Fyn^{CA}$ is represented by open circles. $V_{1/2}$ for ΔQ1077 expressed with $Fyn^{CA}$ is -80.76 ± 0.36 mV. The inactivation curve for ΔQ1077 cells expressed with $Fyn^{KD}$ is represented by filled up-pointing triangles; the $V_{1/2}$ is -80.76 ± 0.36 mV. The Boltzmann fitted inactivation curve for the ΔQ1077 cells in presence of $Fyn^{CA}$ and PP2 is depicted with filled down-pointing triangles. $V_{1/2}$ inactivation, when treated with PP2, is -78.52 ± 0.14 mV.

## 4.3 Activation Properties of ΔQ1077 and Q1077 with $Fyn^{CA}$

To determine whether $Fyn^{CA}$ can modulate the steady-state activation of ΔQ1077 and Q1077Present, the cells were transfected with $Fyn^{CA}$, and the normalized conductance of ΔQ1077 and Q1077 with or without $Fyn^{CA}$ was analysed. The steady-state activation of ΔQ1077 and Q1077Present in presence or absence of $Fyn^{CA}$ is shown in Figure 10 and Table 10.

**Table 10: Half-maximal activation and slope of both Q1077 and ΔQ1077 with $Fyn^{CA}$.**

Data derived from fits of a Boltzmann function to normalized activation curves, determined from individual cells as described in Materials and Methods.

| Variant | Half-maximal activation ($V_{1/2}$) (mV) | $K_{Act}$ (mV) | n |
|---|---|---|---|
| | | | |
| Q1077 | -48.91 ± 0.22 | 3.87 ± 0.13 | 5 |
| Q1077 + $Fyn^{CA}$ | -49.17 ± 0.23 | 4.05 ± 0.23 | 3 |
| | | | |
| ΔQ1077 | -47.65 ± 0.29 | 3.69 ± 0.10 | 4 |
| ΔQ1077 + $Fyn^{CA}$ | -48.56 ± 0.63 | 4.07 ± 0.59 | 3 |

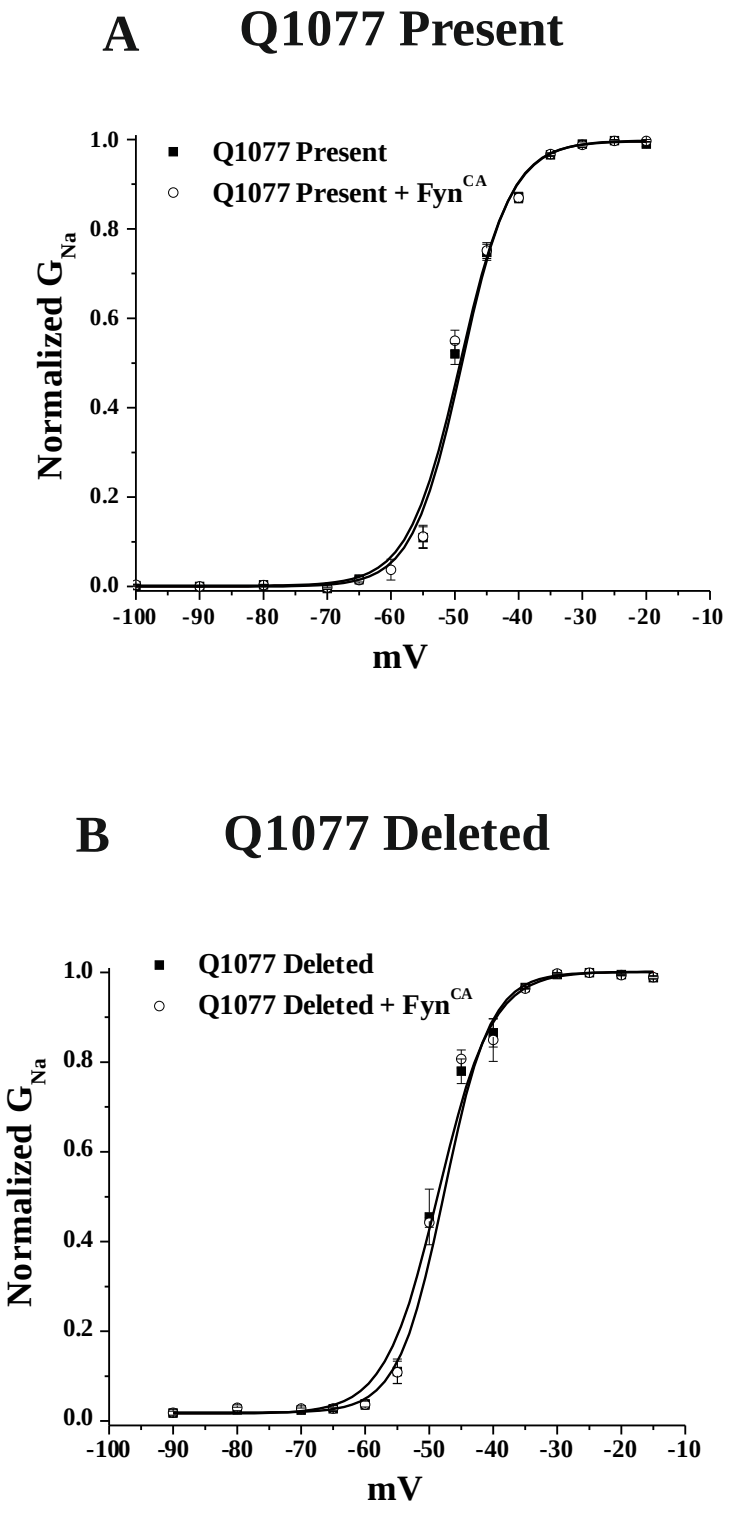


**Figure 10: Steady-state activation curves of stably expressed Q1077 and ΔQ1077 cardiac sodium channels.**

Boltzmann functions fit to normalized conductance were determined from individual cells as described in Materials and Methods.

Figure 10 shows the activation curves of Q1077Present in absence and presence of $Fyn^{CA}$. $Fyn^{CA}$ did not cause any shift of the activation curve. Similarly, in ΔQ1077 $Fyn^{CA}$ also did not alter the activation curve (Table 10).

These data clearly show that activation kinetics of the two variants, Q1077Present and ΔQ1077, is not modulated by Fyn. These results were consistent with earlier published results on Fyn modulation of steady state activation of $Na_V1.2$ and $Na_V1.5$ (hH1) sodium channels (Ahern et al., 2005; Beacham et al., 2007).

## 4.4 Sequence alignment of sodium channel subunits of the intracellular loop $L_{DII\text{-}DIII}$

To ascertain the uniqueness of cardiac sodium channel sequence, the amino acid sequences of all the nine sodium channel isoforms were aligned using the multiple sequence alignment CLUSTAL X program. The Pfam database is a large collection of protein families, each represented by multiple sequence alignments and hidden Markov models (Finn et al., 2010). According to pfam06512 database (Marchler-Bauer et al., 2011), amino acids of the cardiac sodium channel between positions 953-1214 form one of the sodium ion-transport associated regions of the channel. In the predicted cardiac sodium channel structure (George et al., 1992), amino acids from position 953 to 1214 also form the intracellular loop connecting the DII and DIII region ($L_{DII\text{-}DIII}$) of the cardiac sodium channel. Since Q1077 is present in the intracellular loop connecting domains II and III, amino acid sequences of this loop of all the sodium channel isoforms were aligned and analysed (Figure 11).

From Figure 11 it is evident that the Q1077 residue is located in the amino acid sequence 1075-KQQESQP-1081, which is unique in the cardiac sodium channel. In $L_{DII\text{-}DIII}$ the amino acid sequences 1047-GEQPGQGTPGDPEPVCVPIAVAE-1053 and 1116-QQWKAEPQAPGCGETP-1132 are also unique among different sodium channel isoforms (Figure 11).

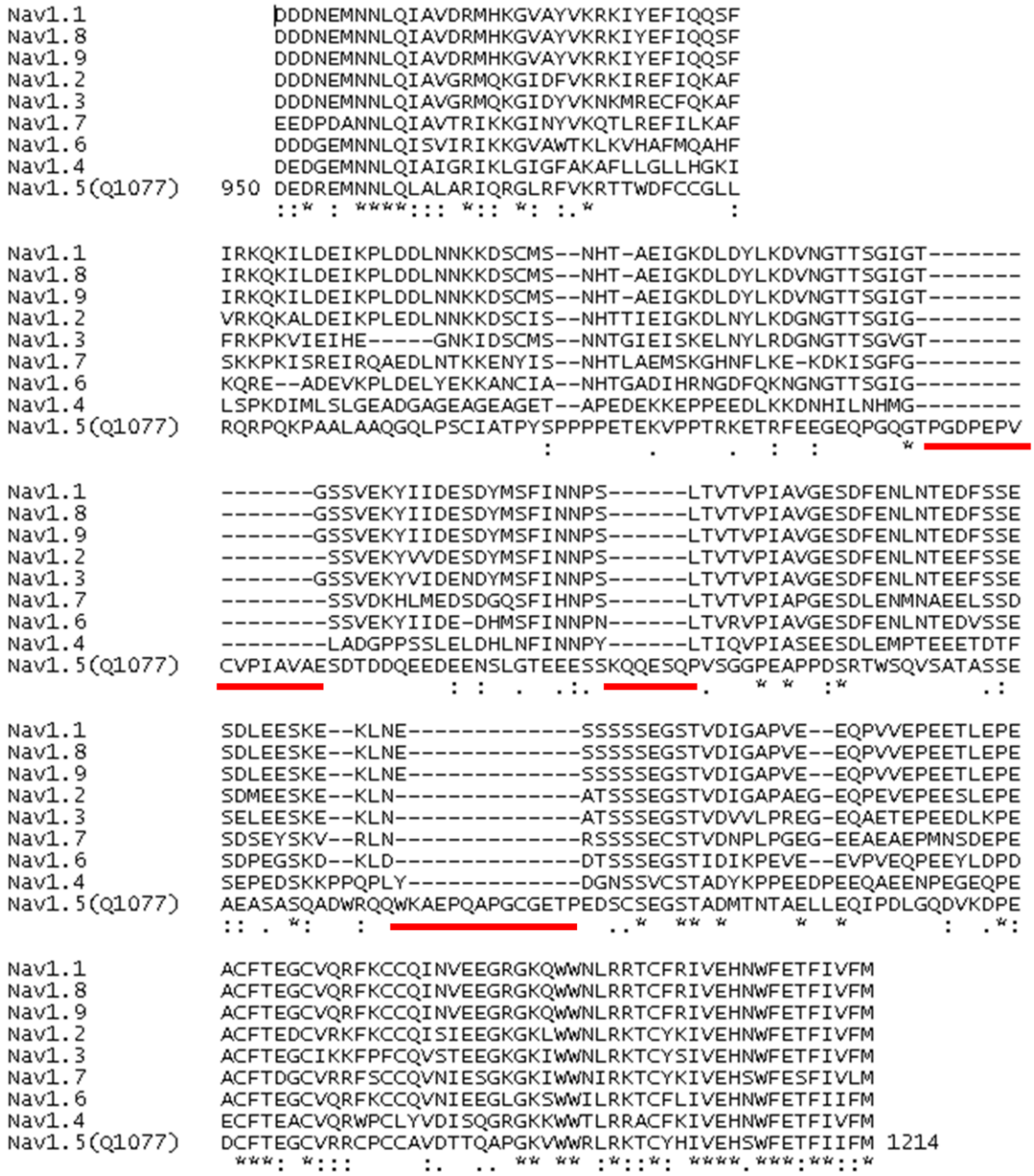


**Figure 11: Alignment of the intracellular loop of sodium channel subtypes connecting Domain II and III ($L_{DII-DIII}$).**

Amino acid numbering of cardiac sodium channel and the proposed structure (George et al., 1992) forms the base of the alignment. Corresponding $L_{DII-DIII}$ residues of the other sodium channel subtypes were used for the alignment.

Asterisks denote consensus amino acids, colons denote high similarity of amino acids, and dots denote less similarity among the aligned amino acids. No symbol denotes lack of similarity among amino acids.

## 4.5 Kinetics of Q1077 cardiac sodium channel mutants

To analyse the importance of Q1077 in the cardiac sodium channel, the amino acid glutamate was replaced with different amino acids using the Stratagene Quick Change Site-Directed Mutagenesis Kit with the Q1077Present cDNA as the template. The Q1077 was mutated to lysine (Q1077K), alanine (Q1077A), proline (Q1077P), and tyrosine (Q1077Y). The steady-state inactivation kinetics was obtained as described in the Materials and Methods section.

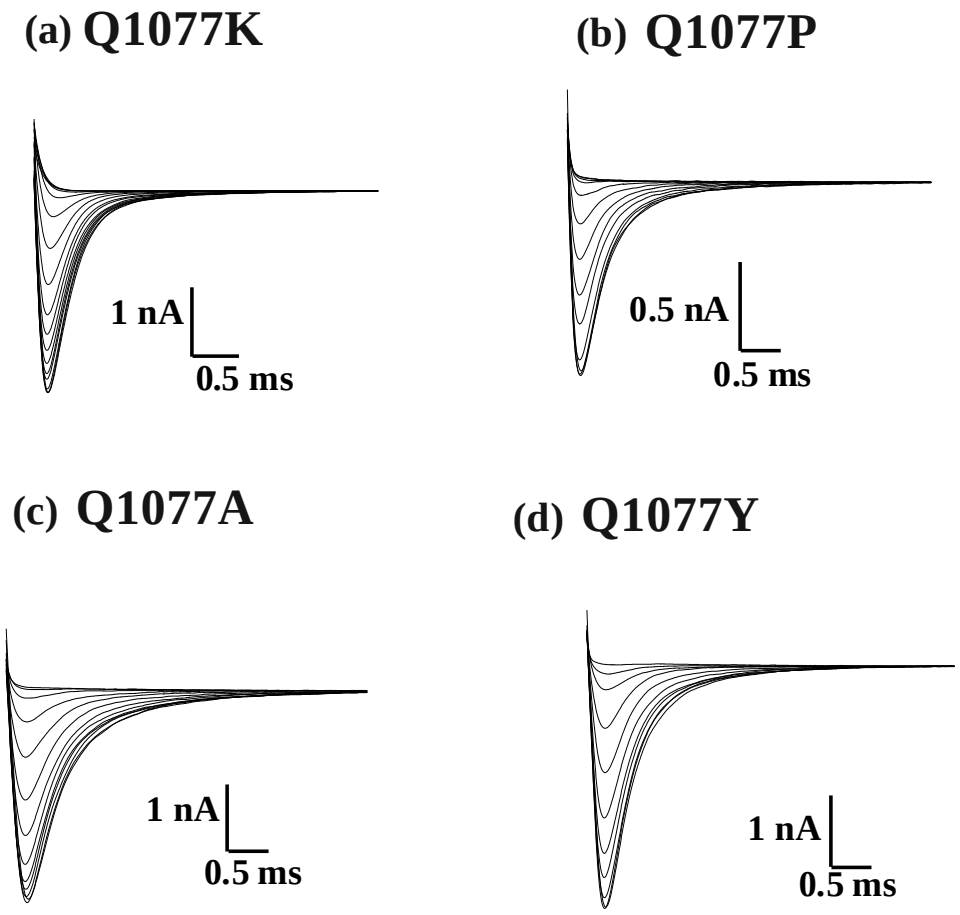


**Figure 12: Original traces illustrating steady-state inactivation of the transiently transfected mutants at position Q1077 in tsA-201 cells:**
(a) Q1077K, (b) Q1077P (c) Q1077A (d) Q1077Y. Current recordings are shown, which were obtained when the cells were clamped from a holding potential of -140 mV to 100 ms prepulses at -150, - 140, -130, -120, -110, -105, -100, -95, -90, -85, -80, -75,-70,-65, -60, -50, -40, -30, -20, -10, and 0 mV, followed by a 24 ms test pulse to 0 mV.

From Figure 12 it is evident that the peak $I_{Na}$ of Q1077P was smaller than that of the other counterparts. To obtain detailed inactivation kinetics, the original traces were fitted with Boltzmann functions as done for Q1077Present and ΔQ1077 (Table 11).

**Table 11: Kinetic parameters for all four mutants of the Q1077 cardiac sodium channel.**

§ ($p<0.001$) Significant difference (ANOVA) of the mutants relative to Q1077Present.
* ($p<0.001$) Significant difference (ANOVA) among all 4 different Q1077 mutants.

| | **Q1077K** | **Q1077P** | **Q1077A** | **Q1077Y** |
|---|---|---|---|---|
| **Inactivation** | | | | |
| $V_{1/2,}$ mV | -97.47 ± 0.23*§ | -88.21 ± 0.47 | -101.61 ± 0.30*§ | -90.43 ± 0.35 |
| Slope, mV | 6.13 ± 0.22 | 7.16 ± 0.32 | 6.39 ± 0.25 | 6.49 ± 0.32 |
| *n* | 3 | 4 | 6 | 4 |
| **Peak Current** | | | | |
| $I_{Na,}$ nA | -3.59 ± 0.92 | 0.81 ± 0.09* | -4.48 ± 0.87 | -3.24 ± 0.51 |
| *n* | 4 | 3 | 3 | 3 |
| **Decay ( 0 mV)** | | | | |
| $A_f$ | 0.94 ± 0.27 | 0.64 ± 0.26 | 0.69 ± 0.25 | 0.86 ± 0.43 |
| $\tau_{f,}$ ms | 0.53 ± 0.76 | 0.76 ± 0.11 | 0.65 ± 0.24 | 0.78 ± 0.63 |
| $\tau_{s,}$ ms | 2.84 ± 0.33 | 2.44 ± 0.44 | 5.45 ± 0.70* | 2.13 ± 0.14 |
| *n* | 3 | 4 | 3 | 3 |

As illustrated in Table 11 the steady-state inactivation of mutants Q1077A and Q1077K was shifted to more hyperpolarizing potentials. The $V_{1/2}$ inactivation of Q1077K was -97.47 ± 0.23 mV (n=3) and for Q1077A it was -101.61 ± 0.30 mV (n=6), which was statistically different ($p<0.001$) from the Q1077Present variant (-88.21 ± 0.31 mV, n=8). In contrast, there was no statistically significant difference in the steady-state inactivation kinetics of Q1077P (-88.21 ± 0.47 mV, n=4) and Q1077Y (-90.43 ± 0.35 mV, n=4) compared to the Q1077Present variant (-88.21 ± 0.3 mV, n=8). This indicates that the mutation at position 1077 with alanine and lysine (Q1077A and Q1077K) altered the $V_{1/2}$ inactivation. The peak $I_{Na}$ of Q1077P

(0.81 ± 0.09 nA, n=3) was statistically different ($p<0.001$) from that of the other mutants Q1077K (-3.59 ± 0.92 nA, n=4), Q1077A (-4.48 ± 0.87 nA, n=3) and Q1077Y (-3.24 ± 0.51 nA, n=3). Thus, the mutation from glutamine at position 1077 to proline significantly reduced the peak $I_{Na}$. For the decay of $I_{Na}$ at 0 mV, the portion of the $I_{Na}$ trace measured after 90% of peak was fit to a sum of exponentials and the parameters are listed in Table 11. The decay time constant of the slow component ($\tau_s$) for Q1077A was significantly ($p<0.001$) different from that of the other 1077 mutants (Q1077K, Q1077P and Q1077Y).

## 4.6 Inactivation kinetics of Q1077 mutants with $Fyn^{CA}$ and $Fyn^{KD}$

To test the modulation of Q1077 mutants by $Fyn^{CA}$ and $Fyn^{KD}$, the mutant clones were co-transfected with $Fyn^{CA}$ and $Fyn^{KD}$ in tsA-201 cells. Original traces were obtained using the inactivation protocol as described in the Materials and Method section. The obtained traces were then analysed and fitted using Boltzmann function. The results were plotted (Figure 13) and tabulated (Table 12). The difference in the modulation by $Fyn^{CA}$ among different Q1077 mutants is presented in the following sections.

### 4.6.1 Modulation of inactivation kinetics of Q1077K mutant with $Fyn^{CA}$ and $Fyn^{KD}$

When the neutral amino acid glutamine at position Q1077 was replaced with the basic residue lysine (Q1077K), the inactivation was shifted to more hyperpolarizing potentials (-97.47 ± 0.23 mV, n=6). Cells co-transfected with $Fyn^{CA}$ produced a statistically significant ($p<0.001$) hyperpolarizing shift from -97.47 ± 0.23 mV to -102.95 ± 0.43 mV (n=3). This result was similar to that of the $Na_V1.2$ sodium channel modulation by Fyn and ΔQ1077 stably tranfected cell modulation by Fyn. Q1077K with $Fyn^{KD}$ ($V_{1/2}$: -97.63 ± 0.98 mV, n=3) did not produced any shift of the inactivation curve. This strongly emphasises that the modulation in inactivation was due to Fyn.

### 4.6.2 Modulation of inactivation kinetics of Q1077A mutant with $Fyn^{CA}$ and $Fyn^{KD}$

The polar glutamine residue at position 1077 was replaced with the unpolar residue alanine (Q1077A). Q1077A also produced a hyperpolarizing shift, which was even larger (13.4 mV) compared to the other three mutants (Table 12). Cells co-transfected with $Fyn^{CA}$ produced a statistically significant hyperpolarizing shift from -101.61 ± 0.30 mV (n=6) to -118.02 ± 0.36 mV (n=3). When the cells were co-transfected with $Fyn^{KD}$ no statistically significant shift in the inactivation ($V_{1/2}$: -105.33 ± 0.51 mV) was observed. The results obtained for inactivation of $I_{Na}$ of Q1077A were in line with the Q1077K mutant, but the shift caused due to Q1077A mutant was larger (Table 12).

### 4.6.3 Modulation of inactivation kinetics of Q1077P mutant with $Fyn^{CA}$ and $Fyn^{KD}$

The aliphatic glutamine residue at position 1077 in the cardiac sodium channel was replaced with the heterocyclic amino acid residue proline (Q1077P). The steady-state inactivation of the Q1077P mutant (-88.21 ± 0.47 mV, n=6) was not shifted. When the Q1077P mutant was co-transfected with $Fyn^{CA}$, a statistically significant ($p<0.001$) hyperpolarizing shift from -88.21 ± 0.47 mV to -97.71 ± 0.38 mV (n=4) was produced. The hyperpolarizing shift was not observed in the Q1077P mutant transfected with $Fyn^{KD}$ (-88.85 ± 0.89 mV, n=4). This hyperpolarizing shift caused by the mutant Q1077P with $Fyn^{CA}$ was less compared to the Q1077A mutant, but more pronounced than with the Q1077K mutant.

### 4.6.4 Modulation of inactivation kinetics of Q1077Y mutant with $Fyn^{CA}$ and $Fyn^{KD}$

The aromatic amino acid residue tyrosine was substituted instead of the aliphatic glutamine residue at position 1077 (Q1077Y). Similar to mutant Q1077P, the steady-state inactivation of Q1077Y was also not shifted (-90.43 ± 0.35 mV, n=4). When the Q1077Y mutant was co-transfected with $Fyn^{CA}$, a statistically significant

($p<0.001$) hyperpolarizing shift from -90.43 ± 0.35 mV to -99.79 ± 0.18 mV (n=3) was observed. Such a hyperpolarizing shift was not seen when the cells were co-transfected with $Fyn^{KD}$ (-90.45 ± 0.32 mV, n=3).

**Table 12: Half-maximal inactivation of all four Q1077 mutants with $Fyn^{CA}$ and $Fyn^{KD}$.**

Data are derived from fits of Boltzmann function to normalized inactivation curves.

| Mutant | Half-maximal inactivation ($V_{1/2}$) (mV) | $K_{Inact}$ (mV) | n |
|---|---|---|---|
| | | | |
| Q1077Y | -90.43 ± 0.35 | 6.49 ± 0.32 | 4 |
| Q1077Y + $Fyn^{CA}$ | -99.79 ± 0.18* | 7.98 ± 0.13* | 3 |
| Q1077Y + $Fyn^{KD}$ | -90.45 ± 0.32 | 6.62 ± 0.59 | 3 |
| | | | |
| Q1077P | -88.21 ± 0.47 | 7.16 ± 0.32 | 6 |
| Q1077P + $Fyn^{CA}$ | -97.71 ± 0.38* | 7.41 ± 0.14 | 4 |
| Q1077P + $Fyn^{KD}$ | -88.85 ± 0.89 | 6.65 ± 0.53 | 4 |
| | | | |
| Q1077K | -97.47 ± 0.23 | 6.13 ± 0.22 | 6 |
| Q1077K + $Fyn^{CA}$ | -102.95 ± 0.43* | 8.96 ± 0.24* | 3 |
| Q1077K + $Fyn^{KD}$ | -97.63 ± 0.98 | 6.82 ± 0.63 | 3 |
| | | | |
| Q1077A | -101.61 ± 0.30 | 6.39 ± 0.25 | 6 |
| Q1077A + $Fyn^{CA}$ | -118.02 ± 0.36* | 6.84 ± 0.17 | 3 |
| Q1077A + $Fyn^{KD}$ | -105.33 ± 0.51 | 5.73 ± 0.28 | 5 |

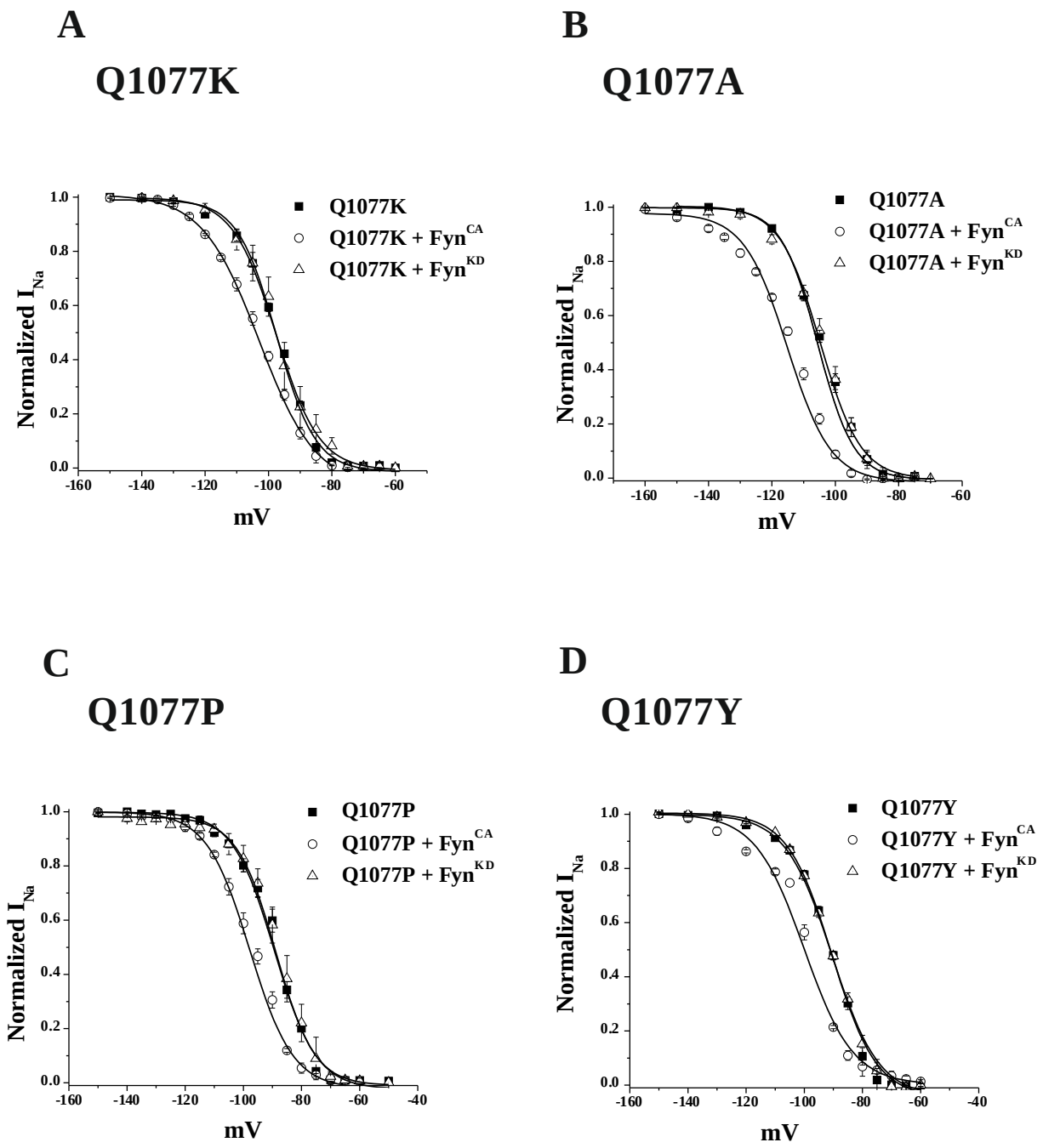


**Figure 13: Expression of catalytic active Fyn$^{CA}$ alters steady-state inactivation kinetics of Q1077 mutants.**

Boltzmann function fitted steady-state inactivation curves of four Q1077 mutants - (A) Q1077K, (B) Q1077A, (C) Q1077P, (D) Q1077Y with Fyn$^{CA}$ and Fyn$^{KD}$ are shown. Solid lines with filled squares correspond to Boltzmann fits of the respective transiently transfected mutant. Cells expressing both, Q1077 mutants and Fyn$^{CA}$, are represented by open circles. Q1077 mutants expressed with Fyn$^{KD}$ are represented by open triangles. Number of experiments is given in Table 12.

Thus, the steady-state inactivation curves of all four different Q1077 mutants were shifted to more hyperpolarizing potentials when they were co-expressed with $Fyn^{CA}$. This hyperpolarizing shift in inactivation is similar to the modulation of $Na_V1.2$ and ΔQ1077 cardiac sodium channels caused by Fyn tyrosine kinase.

### 4.6.5 Correlation between the mutant amino acid properties and the shift in inactivation curves.

There is a linear correlation ($R^2 = 0.99$) when the hydrophobicity of an amino acid side chain is plotted against the difference in shifts of the inactivation curves in presence and absence of $Fyn^{CA}$ (Figure 14).

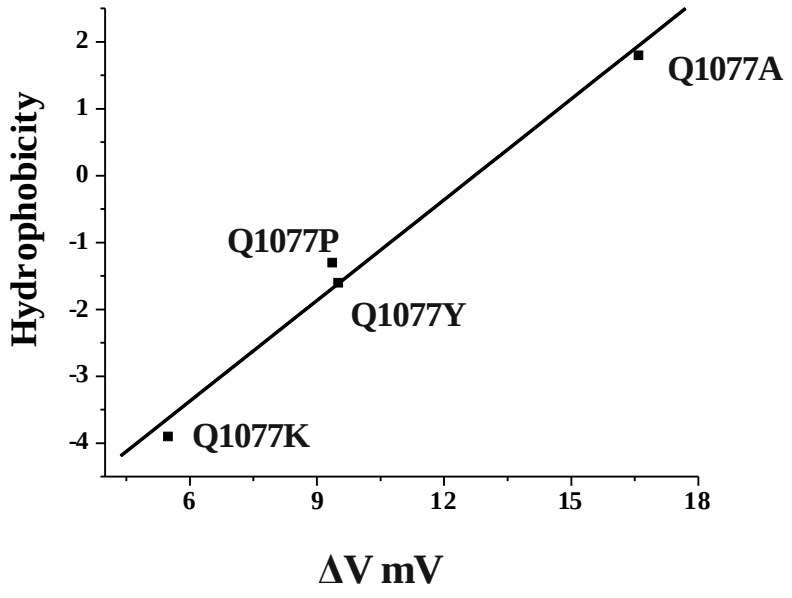


**Figure 14: Plot showing the linear relationship ($R^2 = 0.99$) between the hydrophobicity of an amino acid and the difference in shift of the inactivation curves ($\Delta V = V_{1/2, Mutant} - V_{1/2, Mutant + Fyn^{CA}}$).**

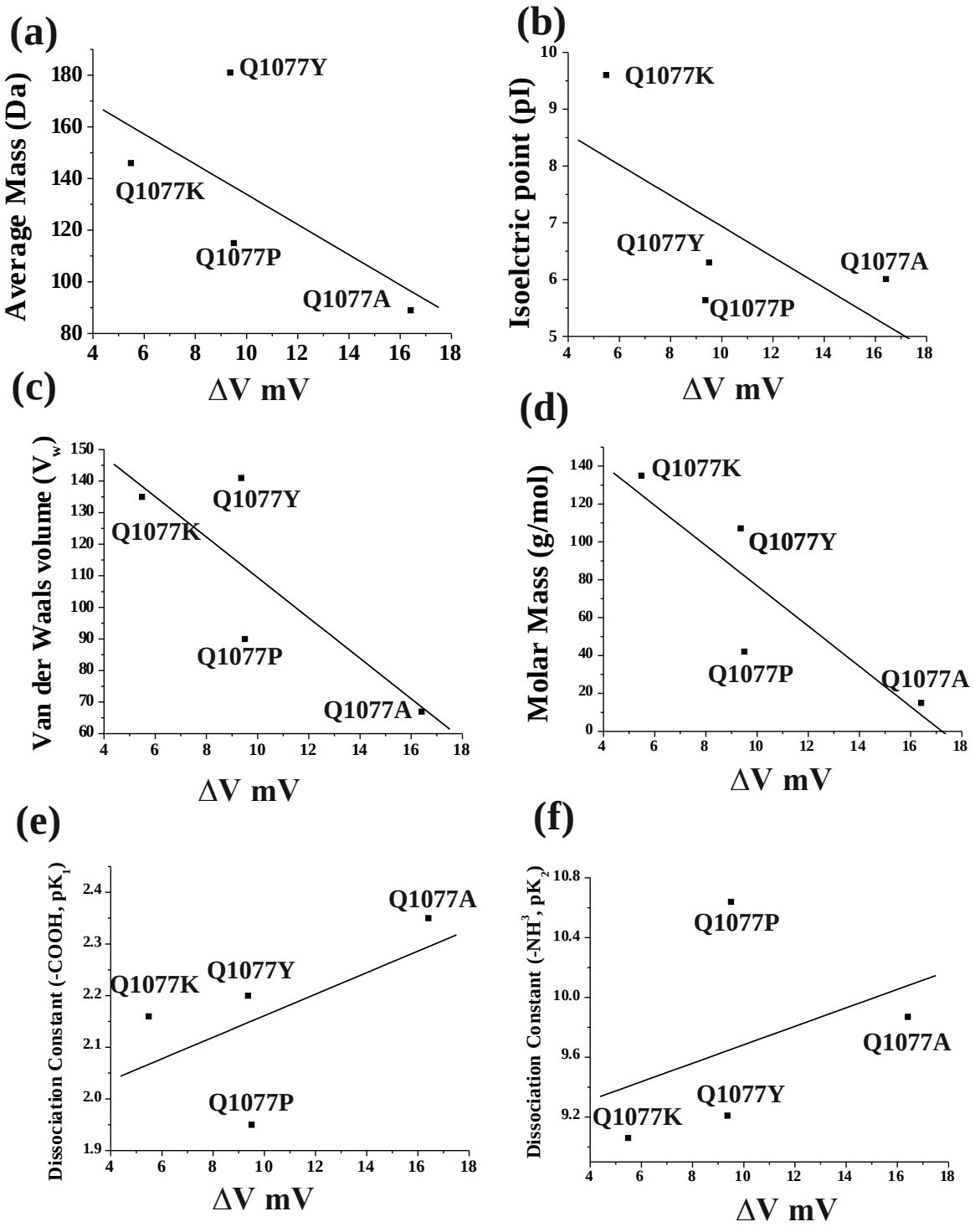


**Figure 15: Plot showing the linear relationship between different properties of amino acids and the difference in shift of the inactivation curves**

(a) average mass ($R^2$ = 0.45), (b) isoelectric point ($R^2$ = 0.45), (c) Van der Waals volume ($R^2$ = 0.67), (d) molar mass ($R^2$ = 0.76), (e) dissociation constant (α-COOH) $pK_1$ ($R^2$ = 0.33) and (f) dissociation constant (α-$NH_3$) $pK_2$ ($R^2$ = 0.16) of an amino acid and the difference in shift of the inactivation curves ($\Delta V = V_{1/2, Mutant} - V_{1/2, Mutant + Fyn^{CA}}$) is shown.

Linear correlation graphs of different properties of the amino acids plotted against the difference in shift of the inactivation curves (ΔV mV) show weak or no correlation (Figure 15). Only molar mass ($R^2$ = 0.76) and the Van der Waals volume (Vw) ($R^2$ = 0.67) were weakly correlated, whereas the other parameters like average mass ($R^2$ = 0.45), isoelectric point ($R^2$ = 0.45), dissociation constant $pK_1$ ($R^2$ = 0.33) and $pK_2$ ($R^2$ = 0.16) were poorly correlated.

# Discussion

In this study, it is shown that the voltage-gated sodium channel variants are modulated differently by Fyn, which is a member of the large Src tyrosine kinase family. The effect of Fyn tyrosine kinase has been evaluated by shifts observed in the steady-state inactivation curves of the sodium channel variants. The evidence of this difference in the modulation by Fyn tyrosine kinase is further supported by mutating the glutamine residue (Q1077) in the intracellular loop ($L_{DII-DIII}$) to four different residues (A, P, K and Y). The amino acid sequence of the intracellular loop ($L_{DII-DIII}$) of all the sodium channel isoforms was analyzed and the uniqueness of the cardiac sodium channel to other sodium channel isoforms is documented. Lastly, a search for putative Src tyrosine kinase SH3 and SH2 binding motifs in the amino acid sequence of the cardiac sodium channel was performed.

## 5.1 Splice variant specificity of Fyn modulation of cardiac sodium channels

Our results indicate that Fyn regulates inactivation of different cardiac sodium channel variants differently. The Q1077Present variant shifted the inactivation curve to more depolarizing potentials (15 mV), while the ΔQ1077 variant shifted the inactivation curve to more hyperpolarizing potentials (9 mV). Studies on the hH1 polymorphic cardiac sodium channel variant shifted the inactivation curve with Fyn to more depolarizing potentials (5 mV) (Ahern et al., 2005). The hH1 sodium channel variant is composed of 2016 amino acids, and it has a glutamine residue at position

1027 compared to Q1077Present, which has an arginine residue at this position. ΔQ1077 (Q1077del) is a splice variant of the Q1077Present variant where an additional glutamine is inserted at position 1077 by a 5′-extension of exon 18 by the trinucleotide CAG (Makielski et al., 2003). The peak $I_{Na}$ of ΔQ1077 is larger than in hH1, and $V_{1/2}$ inactivation appeared at more depolarized potentials (+5 mV) than in the hH1 variant. The activation curve is not shifted in both the ΔQ1077 and hH1 variant (Makielski et al., 2003). This suggests that Q1077 and surrounding residues may play a role in the steady-state inactivation kinetics of the cardiac sodium channel.

There is also evidence that the ΔQ1077 variant behaves differently in the background of cardiac mutations causing channelopathies. The loss of function mutation G1406R in the cardiac sodium channel, which causes Brugada syndrome, shows difference in the partial expression defect with ΔQ1077 and Q1077 in the background. These data suggest that there might be a partial trafficking defect, expression defect or gating abnormalities due to loss of function of the G1406R mutation (Tan et al., 2006). Therefore, intracellular trafficking might play an important role in the difference of modulation.

The putative site for CK2 (casein kinase 2) phosphorylation is reported to be (S/T)XX(E/D) (Meggio and Pinna, 2003). This sequence -SKQE- is present in loop $L_{DII-DIII}$ in the ΔQ1077 variant of the $Na_V1.5$ cardiac sodium channel at position 1074 to 1077. Inserting a glutamine residue (Q1077Present) abolishes the putative phosphoacceptor site for CK2 (Kerr et al., 2004).

There is also a study which reports that ΔQ1077 is present in 45% of the $Na_V1.5$ transcript in human hearts, and the Q1077Present variant transcript is present in 25% of the human hearts (Makielski et al., 2003). The hH1 cardiac sodium channel transcript (Accession No. M77235), on which modulation by Fyn was studied (Ahern et al., 2005), is not present (0%) in the human population (Makielski, 2006). Thus, based on these previously published data and our own results on the modulation of inactivation of ΔQ1077 and Q1077Present by Fyn, we speculate that the glutamine residue at position 1077 and the neighbouring residues in loop $L_{DII-DIII}$ could play a role in the difference of modulation of the cardiac sodium channel by Fyn.

## 5.2 Mutational effects at position 1077

Our results indicate that substitution of aliphatic glutamine at position 1077 to aromatic tyrosine or heterocyclic proline does not have any effect on inactivation kinetics, as it was similar to the Q1077Present variant. Whereas, when aliphatic and polar glutamine was mutated to aliphatic and hydrophobic alanine or to the polar and basic amino acid lysine there was a shift in $V_{1/2}$ inactivation observed compared to Q1077Present. In the case of Q1077A, alanine is a hydrophobic residue and it is ambivalent; that means it can be inside or outside the protein accessible area. This could lead to different hydrophobic interactions between other nearby residues, in turn hindering the inactivation process. In Q1077K, lysine substitution shifted the $V_{1/2}$ inactivation to more hyperpolarizing potentials compared to the Q1077Present variant. The lysine residue has the ability to interact with the solvent readily, which might have played a role in shifting the inactivation curve to more hyperpolarizing potentials. Q1077P and Q1077Y, which are bulky residues, could hinder the folding of loop $L_{DII-DIII}$ which is the ion transport associated region.

Our results on mutation at position 1077 revealed that all the amino acid mutations from glutamine to alanine, lysine, tyrosine and proline caused a shift of the respective inactivation curves to more hyperpolarizing potentials with Fyn as observed in ΔQ1077 (Figure 9) and $Na_V1.2$ (Beacham et al., 2007). However, there is different extent of the hyperpolarizing shift among different mutations. The mutation with alanine caused a very large hyperpolarizing shift by Fyn, and the least hyperpolarizing shift was produced by the positively charged residue lysine. There was also a strong linear correlation ($R^2$=0.99) between the hydrophobicity of the mutated amino acids and the extent of the Fyn-caused hyperpolarizing shift. Highly hydrophobic residues are present in the SH2 domain of the Src tyrosine kinase, and in addition also the peptide binding site of SH3 is composed of a hydrophobic groove. When Q is present at position 1077 there might be hindrance to the hydrophobic interaction between the SH domains and the cardiac sodium channel. There is weak linear correlation observed with molar mass ($R^2$=0.76) or the Van-der-Waals volume ($V_w$) (volume of the union of overlapping spheres) ($R^2$=0.67), respectively, and the difference in the hyperpolarizing shifts caused by Fyn. $V_w$ plays an important role in the packing and folding of a protein (Richards, 1977). Thus, from our mutational analysis we can

conclude that hydrophobicity, and to a lesser extent, the Van-der-Waals volume ($V_w$) of the amino acids is vital in the different modulation of the sodium channel by Fyn.

## 5.3 Uniqueness of cardiac sodium channel intracellular loop $L_{DII-DIII}$

From the performed sequence analysis it is obvious that the glutamine residue at position 1077 is unique among different sodium channel isoforms (Figure 11). There are three short unique amino acid sequences present in the cardiac sodium channel compared to other sodium channel isoforms. The glutamine at position 1077 is present in one of these unique sequences of the cardiac sodium channel as shown in the Figure 11. Glutamine at position 1076 and 1077 is surrounded by the charged amino acids lysine at position 1075 and glutamic acid at position 1078. In turn, both of these charged amino acids are surrounded by the polar residue serine. Thus, the two glutamine residues are hidden in between the two polar serine residues, which might hinder the flexibility of the sodium channel. This serine residue has the ability to form a hydrogen bond with other polar residues. It has the property of either accepting or donating a hydrogen bond. So, an additional glutamine at position 1077 might hinder the flexibility of the loop $L_{DII-DIII}$. This might play an anchoring role in facilitating the SH domains of the Src tyrosine kinase to bind to the cardiac sodium channel.

Recent studies on MOG1 (multicopy suppressor of *gsp1*), which is present in the nucleus and the cytosol of cardiac cells (Marfatia et al., 2001), demonstrate that it plays an important role in the regulation of nuclear protein trafficking (Wu et al., 2008). It showed that MOG1 is an important cofactor for optimal expression of $Na_V1.5$ at the cell membrane. When MOG1 and $Na_V1.5$ were co-expressed in HEK293 cells, there was only an increase in the peak $I_{Na}$ with no change in the biophysical characteristics. The interaction site for MOG1 in $Na_V1.5$ is the loop connecting domain II and domain III ($L_{DII-DIII}$) (Abriel and Kass, 2005; Wu et al., 2008). Ankyrins are intracellular proteins which target and stabilize the membrane proteins including ion channels, transporters, exchangers and cell adhesion molecules in various tissues (Cunha and Mohler, 2006). Ankyrin-G binds to the -VPIAXXSD-motif in loop $L_{DII-DIII}$ of the cardiac sodium channel (Abriel and Kass, 2005; Mohler et al., 2004). SCN5A mutation (pE1053K) in Brugada syndrome patients leads to

disruption of the interaction between $Na_V1.5$ and ankyrin-G (Mohler et al., 2004). Thus, these studies on different intracellular proteins, which modulate the sodium channel, reveal that $L_{DII-DIII}$ is an essential target. From our results on cardiac sodium channel variants, modulation by Fyn suggests that the protein-protein interaction site for the sodium channel and Fyn should be present in $L_{DII-DIII}$.

## 5.4 Possible binding sites for the SH2 domain in the cardiac sodium channel

Our results on the possible SH3 domain binding, poly-proline motif show that there are five such motifs in loop $L_{DII-DIII}$. As shown in Figure 15, the $Na_V1.2$ does not contain any proline-rich motif in $L_{DII-DIII}$. Thus, this loop $L_{DII-DIII}$ could play an important role in SH3 binding and modulation of the cardiac sodium channel by Fyn. In $Na_V1.2$ the consensus sequence for the potential binding site of the Fyn SH2 domain, after phosphorylation, is present in the N- and C-terminal (Beacham et al., 2007). The sequence for the potential SH2 binding site in $Na_V1.2$ is reported as 66-YGDI-69 in the N-terminal, and 1893-YEPI-1896 in the C-terminal. In the cardiac sodium channel corresponding sites were analysed using the sequence alignment program. There, the C-terminal -YGDI- is replaced by 68-YGNP-, whereas the N-terminal has a corresponding 1988-YEPI-1992 sequence (Beacham et al., 2007). Similarly, in the $Na_V1.2$ channel there is a Y730 which is located in $L_{DI-DII}$ near the SH3 binding motif (-RVLPILP-), which is required for optimal regulation by Fyn (Beacham et al., 2007). This Y730 residue is conserved in all other sodium channel isoforms except the cardiac sodium channel. In the cardiac sodium channel the corresponding Y730 is replaced by N688 (asparagine). In $Na_V1.2$, it was speculated that the SH2 motifs in the N- and C-terminal and the SH3 motif in $L_{DI-DII}$ play a role in maintaining a high local concentration of Fyn. But in the cardiac sodium channel there is only one SH2 binding motif in the C-terminal and several putative SH3 binding motifs in $L_{DII-DIII}$. Earlier studies show that the Fyn SH2 domain binds to the -pYQQI- motif of the target protein (Songyang et al., 1993). However, there are some other SH2 domains which bind to different patterns. The growth factor receptor-bound protein 2 (Grb2) SH2 domain binds to the -pYXNX- motif of the target protein

(Rahuel et al., 1996), whereas the SH2 domain of the growth factor phospholipase C-γ1 (PLC-γ1) binds to hydrophobic residues that follow pTyr (Ji et al., 1999). Thus, Fyn could also bind to different SH2 motifs in the cardiac sodium channel other than the putative SH2 binding motif in $Na_V1.2$. Putative SH2 binding motifs are situated in the C-terminal of the cardiac sodium channel, because there are motifs (1811-YDSV-1814 and 1977-YSVL-1980) apart from the conserved -YEPI-, which have a hydrophobic residue at the pY+3 residue. The pY+3 residue is tightly bound to the hydrophobic pocket, and this pocket is critical for the peptide specificity (Brändén and Tooze, 1999). Thus, valine in the 1811-YDSV-1814 motif and leucine in the 1977-YSVL-1980 motif might form a tight hydrophobic interaction with the hydrophobic pocket of the SH2 domain. Mutational studies on these motifs will give an insight view on the SH2 binding pockets of the cardiac sodium channel.

## 5.5 Putative SH3 domain binding sites in sodium channels

The SH3 domain consists of a five-stranded up-and-down antiparallel β sheet. The peptide binding site is made up of a hydrophobic groove (Brändén and Tooze, 1999). The SH3 domain of the Src tyrosine kinase binds to the target peptides which have proline-rich motifs (Lim, 1996; Yu et al., 1994). When the peptide binds to the SH3 domain, proline-rich segments adopt a left-handed poly-proline type II helix with three amino acids per turn. There are three ridges in the poly-proline helix of which two contact the SH3 domain of the protein tyrosine kinase. The SH3 domain of the Src tyrosine kinase contains a group of acidic amino acids and less hydrophobic amino acid residues (Brändén and Tooze, 1999). All known SH3 target proteins carry a consensus sequence -PXXP-. When v-src is expressed with the human voltage gated potassium channel subtype ($hK_V1.5$) (Holmes et al., 1996b), it is phosphorylated. It was found that $hK_V1.5$ contains two SH3 binding motifs (RPLPPLP) between amino acid residues 65 and 82 of the channel protein (Holmes et al., 1996b; Tamkun et al., 1991).

In $Na_V1.2$ the SH3 binding motif for Fyn is present in $L_{DI\text{-}DII}$ (Figure 17) (Beacham et al., 2007). Insertion of a glycine residue in this motif (-PTLP- to -PTGLP-) nullifies the modulation of $Na_V1.2$ by Fyn (Beacham et al., 2007). In Figure 17, the other

putative SH3 binding motifs are also shown; there is no proline-rich motif present in $L_{DII-DIII}$, but two putative SH3 binding proline-rich motifs are present in the N-terminal region. In the cardiac sodium channel there are five -PXXP- motifs in $L_{DII-DIII}$. The cardiac sodium channel has one -PXXP- motif in the loop $L_{DI-DII}$, which is different from that of $Na_V1.2$. Two of the -PXXP- motifs in $L_{DII-DIII}$ of the cardiac sodium channel are present in the unique sequence found when aligned with other sodium channel isoforms (Figure 16). This suggests that the putative SH3 domain binding motif of the cardiac sodium channel is present in loop $L_{DII-DIII}$. These studies reveal that the SH3 domain binding domain of the $Na_V1.2$ and $Na_V1.5$ is different. Thus, this could contribute to the difference observed in the modulation by Fyn tyrosine kinase.

Cardiac sodium channel sequence analysis reveals five putative SH3 binding domains in loop $L_{DII-DIII}$ (Figure 16), but there is no proline-rich motif in $L_{DII-DIII}$ of the $Na_V1.2$ sodium channel (Figure 17). Whereas, for the $Na_V1.2$ channel it is proven that there is one SH3 binding motif present in the $L_{DI-DII}$. However, the same motif is not present in $Na_V1.5$. Inserting a residue in the SH3 motif of $Na_V1.2$ alters the modulation of inactivation kinetics by Fyn. According to suggested proline-rich motifs binding the SH3 domain of Src tyrosine kinases (Yu et al., 1994), in the cardiac sodium channel out of five putative motifs, the sequences 986-RQRPQKP-992, 1120-KAEPQAP-1126 and 1008-PYSPPPP-1014 in $L_{DII-DIII}$ are most likely to form the SH3 binding site for Fyn kinase (Figure 16). The first two putative motifs have a charged amino acid residue (R and K) two residues (any amino acid) before the first proline of the proline-rich motif which is responsible for the SH3 domain binding groove (Brändén and Tooze, 1999; Yu et al., 1994). The third, above mentioned, proline rich motif contains four continuous proline residues making it an ideal candidate for SH3 domain binding. Apart from the previously mentioned proline-rich motifs there are two other SH3 binding -PXXP- motifs present in $L_{DII-DIII}$. The two proline-rich motifs in $L_{DII-DIII}$ are 1039-PGDPEP-1044 and 1086-PEAPP-1090; these can also form the SH3 domain binding motif, but it lacks the charged R/K residue, two residues before the first proline of the -PXXP- motif. But the 1086-PEAPP-1090 is in very close proximity to the glutamine residue at position1077. Hence, the consideration of this motif for SH3 binding should not be left out. Apart from $L_{DII-DIII,}$ there is one weak SH3 binding, proline-rich motif (PLAP) in loop $L_{DI-DII}$ (Figure 16). Insertion mutation

or removing the entire motifs will give an insight view on the SH3 binding site of the cardiac sodium channel.

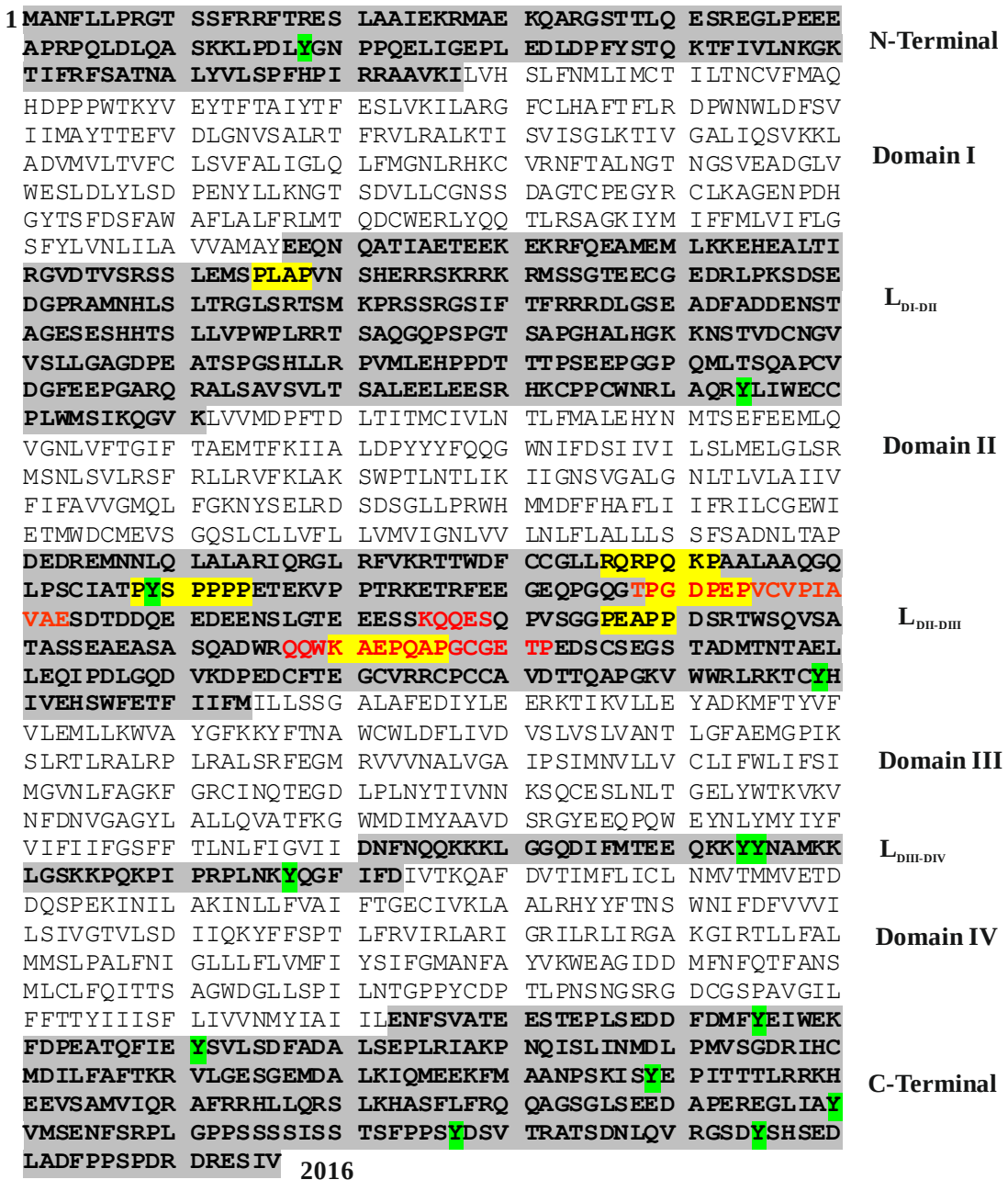


**Figure 16: Amino acid sequence of the Q1077 (Accession Number AC137587) cardiac sodium channel.**

The cardiac sodium channel numbering and the proposed structure (George et al., 1992) forms the base of different structural parts. The membrane embedded parts and the extracellular loops of domains I-IV are not highlighted. The intracellular parts of the sodium channel are highlighted in grey. The unique sequences in $L_{DII-DIII}$ are highlighted in red. The possible SH3 domain binding motifs (proline-rich) are highlighted in yellow. The intracellular tyrosine residues are marked in green.

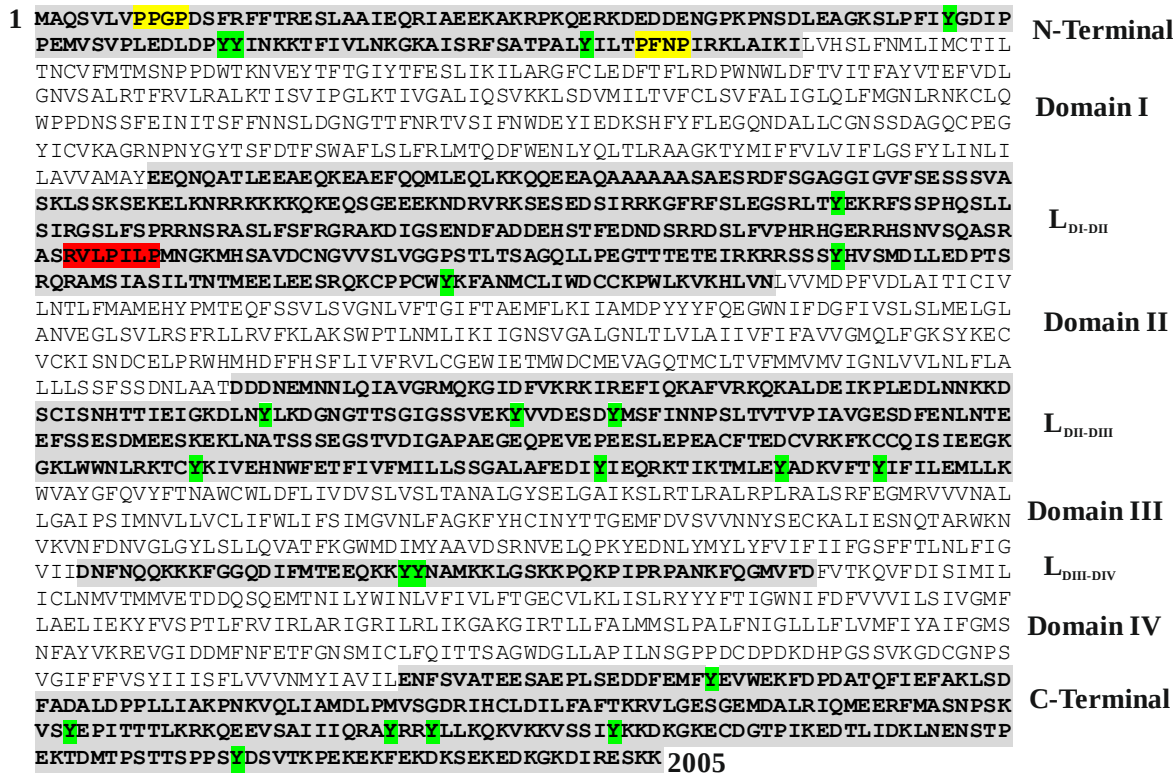


**Figure 17: Amino acid sequence of the $Na_V1.2$ (Accession Number NP_001035232) neuronal sodium channel.**

The membrane embedded parts and the extracellular loops of domains I-IV are not highlighted. The intracellular parts of the sodium channel are highlighted in grey. The SH3 binding site in $Na_V1.2$ loop $L_{DI-DII}$ is highlighted in red. The other possible SH3 domain binding motifs (proline-rich) are highlighted in yellow. The tyrosine residues are marked in green.

## 5.6 Importance of the tyrosine residues in sodium channel for Fyn tyrosine kinase

In both $Na_V1.5$ and $Na_V1.2$, the tyrosine residue in the inactivation loop is essential for the modulation of the sodium channel by Fyn (Ahern et al., 2005; Beacham et al., 2007). In the neuronal sodium channel isoform $Na_V1.2$, tyrosine residues Y1497 and Y1498 in the inactivation loop ($L_{DIII–DIV}$) play an important role in the regulation by Fyn, whereas in the cardiac sodium channel $Na_V1.5$, tyrosine at position Y1495 in $L_{DIII-DIV}$ is the residue responsible for the modulation. When Y1495 is substituted to phenylalanine (Y1495F), the shift caused by Fyn is abolished in cardiac sodium channel (hH1). Mutation in $Na_V1.2$ tyrosine residue Y1498F annuls

the effect of Fyn. Our analysis on $L_{DIII-DIV}$ of the sodium channel isoforms reveal that 95% of the amino acids were conserved in $L_{DIII-DIV}$. The tyrosine residue, which is present in $L_{DIII-DIV}$, is conserved in all sodium channel isoforms (Fig. 14 and 15, Appendix alignment). It plays an important role in the modulation of sodium channels by Fyn. Although the tyrosine residue is conserved in the inactivation loop, Fyn modulates the sodium channel isoforms differently.

$Na_V1.1$ is not modulated by Fyn (Beacham et al., 2007), whereas in $Na_V1.2$ Fyn shifts the inactivation curve to more hyperpolarizing potentials (Beacham et al., 2007). In hH1 (Ahern et al., 2005) and Q1077Present (Figure 9 and Table 9) cardiac sodium channel variants, Fyn shifts the inactivation curves to more depolarizing potentials. In the ΔQ1077 (Figure 9 and Table 9) cardiac sodium channel variant and the Q1077A, Q1077Y, Q1077P and Q1077K mutants, the inactivation curves are shifted to more hyperpolarizing potentials. Thus, although all sodium channels have a conserved tyrosine residue in $L_{DIII-DIV}$, which is responsible for the modulation, different sodium channels are modulated differently by Fyn.

In loop $L_{DII-DIII}$, there are two tyrosine residues, one at the distal end and one embedded in the proline-rich motif -PYSPPPP-. In $Na_V1.2$, there is Y730 in $L_{DI-DII}$, which is in close proximity to the SH3 binding domain that is involved in the regulation of Fyn (Beacham et al., 2007). In the cardiac sodium channel five putative SH3 binding sites are present in $L_{DII-DIII}$. The tyrosine residues at position 1008 and 1118 in $L_{DII-DIII}$ could contribute to the regulation of Fyn tyrosine kinase. Y1008 is unique in $Na_V1.5$ compared to other sodium channel isoforms. The distal end tyrosine residue Y1118 in $L_{DII-DIII}$ is conserved in $Na_V1.2$, $Na_V1.7$, $Na_V1.3$ and cardiac sodium channel $Na_V1.5$. Hence, these two tyrosine residues in $L_{DII-DIII}$ of the cardiac sodium channel might also be involved in the regulation of the sodium channel by Fyn.

# Conclusion

Results from the thesis proclaim that Q1077Present and ΔQ1077 encoding for cardiac sodium channel ($Na_V1.5$) variants are differently regulated by Fyn tyrosine kinase. In Q1077Present, Fyn shifted the inactivation curve to more depolarizing potentials, whereas in ΔQ1077 it was shifted to more hyperpolarizing potentials. Mutational analysis at position 1077 revealed the role of glutamine at this position, which is important for the modulation by Fyn. Fyn shifted the inactivation curves of all mutants (Q1077A, Q1077K, Q1077P and Q1077Y) to more hyperpolarizing potentials. Hydrophobicity of the substituted amino acid is one of the prominent factors in the extent of the shift. Sequence alignment showed that the region around $L_{DII\text{-}DIII}$ is unique and the glutamine residue at position 1077 is present in one of the unique segments of $L_{DII\text{-}DIII}$. Results from this thesis speculate that the putative SH3 binding site should be situated in $L_{DII\text{-}DIII}$, and the SH2 binding site in the C-terminal. These data will be pertinent in understanding the role of Q1077 and its surrounding amino acids, which are present in the transport associated region ($L_{DII\text{-}DIII}$). Results show that $L_{DII\text{-}DIII}$ of the cardiac sodium channel is pivotal in Fyn binding and regulation of function.

# Abbreviations

| | |
|---|---|
| AF | Atrial Fibrillation |
| BrS | Brugada syndrome |
| $Ca_v$ | Voltage-gated calcium channel |
| CK2 | Casein Kinase 2 |
| CNS | Central Nervous System |
| DI-DIV | Voltage-gated sodium channel domain (I-IV) |
| DRG | Dorsal Root Ganglion |
| GoF | Gain-of-function |
| $hNa_V$ | Human voltage gated sodium channel |
| $I_{Na}$ | Sodium Current |
| $K_V$ | Voltage gated potassium channel |
| LB | Lysogeny broth |
| LQT | Long QT syndrome |
| LoF | Loss-of-function |
| mRNA | Messenger ribonucleic acid |
| $Na^+$ | Sodium ion |
| $Na_V$ | Voltage gated sodium channel |
| $Na_V1.5_X$ | cardiac sodium channel splice variants |
| PTK | Protein tyrosine kinase |
| $rNa_V$ | Rat voltage gated sodium channel |
| S1-S6 | Trans-membrane helices (1-6) |
| SA | Sinoatrial |
| SCNXA | Voltage gated sodium channel (X- Subtype) |
| SH2 | Src Homology 2 |
| SH3 | Src Homology 3 |
| SIDS | Sudden infant death syndrome |
| SOC | Super optimal broth (modified) |
| Src tyrosine kinase | Sarcoma tyrosine kinase |
| SSS | Sick Sinus Syndrome |
| TTX | Tetrodotoxin |
| WT | Wild type |

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# Appendix

## 9.1 Abstract

Voltage-gated sodium channels are very large membrane proteins. They are encoded by ten genes in mammals. Sodium channels are a crucial component of excitable tissues; hence, they are a target for various drugs and toxins. $Na_V1.5$ is the cardiac voltage-gated sodium channel involved in the rapid depolarizing phase of the cardiac action potential. This isoform is also involved in the propagation of electrical impulses in the heart. Out of nine $Na_v1.5$ splice variants, four of them were functional. The splice variant ΔQ1077 and Q1077Present variant of $Na_V1.5$ are present in 45% and 25 % of human population, respectively. Cardiac sodium channels are modulated by different intracellular proteins. Tyrosine kinases are one of the potent intracellular modulators of ion channels. Fyn, a member of the Src tyrosine kinase family, modulates sodium channel isoforms distinctly.

Results on the Q1077Present and the ΔQ1077 variants show different steady-state inactivation in presence of catalytic active $Fyn^{CA}$. While the Q1077Present variant shifted the inactivation curve to more depolarizing potentials, the ΔQ1077 variant shifted the inactivation curve to more hyperpolarizing potentials, similar to neuronal sodium channel $Na_V1.2$. The Fyn-induced shift in inactivation of Q1077Present was similar to the modulation observed in the hH1 polymorphic cardiac sodium channel variant with Fyn. The Src tyrosine kinase inhibitor PP2 reversed back the shifts caused by $Fyn^{CA}$ in both Q1077Present and ΔQ1077 cardiac sodium channel variants. Moreover, the kinase dead mutant $Fyn^{KD}$ did not shift both the ΔQ1077 and Q1077Present inactivation curves. The activation curves of both Q1077Present and ΔQ1077 were not affected by active Fyn. Sequence analysis of all the sodium channel isoforms reveal that the Q1077glutamine is present in one the three unique sequences in the intracellular loop connecting the domain DII and DIII, which forms the 'sodium ion transport associated' region. Point mutation at position 1077 from glutamine to alanine, lysine, tyrosine and proline shifted the inactivation curves to more hyperpolarizing potentials with $Fyn^{CA}$. Linear correlation was observed between the

hyperpolarizing shift caused by $Fyn^{CA}$ and the hydrophobicity of the amino acids' side chains. Putative SH2 and SH3 binding sites for Fyn in the cardiac sodium channel were also determined. Five unique proline-rich motifs were identified, which are potential SH3 binding sites for Fyn in the cardiac sodium channel. Putative SH2 binding motifs for Fyn were found in the C-terminal of $Na_v1.5$.

These data will be pertinent in understanding the role of Q1077 and its surrounding amino acids. The intracellular loop $L_{DII\text{-}DIII}$, which is distinct in cardiac sodium channels from other sodium channel isoforms, forms one of the 'sodium ion transport associated' regions. Thus, $L_{DII\text{-}DIII}$ plays a pivotal role in regulating cardiac sodium channel binding to various intracellular signaling molecules.

## 9.2 Zusammenfassung

Spannungabhängige Natriumkanäle sind sehr große Membranproteine. In Säugetieren werden sie von zehn Genen kodiert. Natriumkanäle spielen eine wichtige Rolle in erregbaren Zellen und sie sind deshalb ein wichtiger Angriffspunkt für Arzneistoffe und Toxine. $Na_V1.5$ ist der spannungsabhängige Natriumkanal im Herzen, wo er für die rasche Depolarisationsphase im Aktionspotential verantwortlich ist und auch an der Reizweiterleitung beteiligt ist. Von den neun Spleißvarianten von $Na_V1.5$ sind vier funktionell. Die Spleißvariante ΔQ1077 und die Q1077Present Variante des kardialen Natriumkanals sind in 45% bzw. 25% der Bevölkerung anzutreffen. Kardiale Natriumkanäle werden durch unterschiedliche intrazelluläre Proteine moduliert. Tyrosinkinasen sind eine der potenten Modulatoren verschiedener Ionenkanäle. Fyn, das zur Src Tyrosinkinase-Familie gehört, moduliert verschiedene Natriumkanal-Isoformen auf unterschiedliche Weise. Die mit den Q1077Present und ΔQ1077 Varianten erhaltenen Ergebnisse zeigten unterschiedliche Inaktivierung in Gegenwart katalytisch aktiver $Fyn^{CA}$. Während die Q1077Present Variante die Inaktivierungskurve in die depolarisierende Richtung verschob, trat die halbmaximale Inaktivierung bei ΔQ1077 bei negativeren Potentialen auf, ähnlich wie beim neuronalen Natriumkanal $Na_V1.2$. Die durch $Fyn^{CA}$ bedingte Verschiebung der Inaktivierungskurve in der Q1077Present Variante war vergleichbar mit der durch $Fyn^{CA}$ hervorgerufenen Modulation in der hH1 polymorphen kardialen Natriumkanal-Variante. Mit der Kinase-toten Mutante $Fyn^{KD}$ kam es weder bei ΔQ1077 noch bei Q1077Present zu einer Verschiebung der Inaktivierungskurven. Weiters konnte in beiden Varianten die durch $Fyn^{CA}$ bedingte Verschiebung der Inaktivierungskurven durch den Src Tyrosinkinase-Hemmer PP2 aufgehoben werden. Die Aktivierungkurven beider Varianten, Q1077Present und ΔQ1077, wurden durch $Fyn^{CA}$ nicht beeinflusst. Eine Sequenzanalyse aller Natriumkanal-Isoformen zeigte, dass Glutamin in Position 1077 in einer der drei für $Na_V1.5$ einzigartigen Sequenzen in der intrazellulären Schleife liegt, die die Domänen DII und DIII verbindet und die "Natriumionentransport-assoziierte" Region bildet. Eine Punktmutation in Position 1077 von Glutamin zu Alanin, Lysin, Tyrosin oder Prolin verschob die Inaktivierungskurven in Gegenwart von $Fyn^{CA}$ zu negativeren Potentialen. Es konnte

eine lineare Korrelation zwischen dem Ausmaß der Verschiebung der Inaktivierungskurven durch $Fyn^{CA}$ und der hydrophoben Eigenschaft der Aminosäuren-Seitenketten beobachtet werden. Mögliche SH2 und SH3 Bindungsstellen für Fyn im kardialen Natriumkanal wurden ebenfalls identifiziert. Fünf spezifische, prolinreiche Motive, die als SH3 Bindungsstelle für Fyn im kardialen Natriumkanal fungieren könnten, wurden aufgefunden. Mögliche SH2 Bindungsmotive für Fyn wurden im C-Terminus von $Na_V1.5$ identifiziert.

Diese Daten tragen zum Verständnis der Rolle des Q1077 und den angrenzenden Aminosäuren bei. Die intrazelluläre Schleife $L_{DII-DIII}$ des kardialen Natriumkanals, die sich wesentlich von der anderer Natriumkanal-Isoformen unterscheidet, bildet eine der "Natriumionentransport-assoziierten" Regionen. Deshalb spielt $L_{DII-DIII}$ eine wichtige Rolle bei der Bindung intrazellulärer Signalmoleküle an den Natriumkanal.

## 9.3 Alignment

```
Nav1.1          ---MEQTVLVPPGPDSFNFFTRESLAAIERRIAEEKAKNPKPD----KKDDDENGPKPNS
Nav1.8          ---MEQTVLVPPGPDSFNFFTRESLAAIERRIAEEKAKNPKPD----KKDDDENGPKPNS
Nav1.9          ---MEQTVLVPPGPDSFNFFTRESLAAIERRIAEEKAKNPKPD----KKDDDENGPKPNS
Nav1.2          ---MAQSVLVPPGPDSFRFFTRESLAAIEQRIAEEKAKRPKQER---KDEDDENGPKPNS
Nav1.3          ---MAQALLVPPGPESFRLFTRESLAAIEKRAAEEKAKKPKKE----QDNDDENKPKPNS
Nav1.7          -----MAMLPPPGPQSFVHFTKQSLALIEQRIAERKSKEPKEE----KKDDDEEAPKPSS
Nav1.6          ---MAARLLAPPGPDSFKPFTPESLANIERRIAESKLKKPPKADGSHREDDEDSKPKPNS
Nav1.4          MARPSLCTLVPLGPECLRPFTRESLAAIEQRAVEEEARLQRNK----QMEIEEPERKPRS
Nav1.5          ----MANFLLPRGTSSFRRFTRESLAAIEKRMAEKQARGSTTLQESREGLPEEEAPRPQL
                       * * *...:  ** :*** **:* .* : :        .   ::   :*

Nav1.1          DLEAGKNLPFIYGDIPPEMVSEPLEDLDPYYINKKTFIVLNKGKAIFRFSATSALYILTP
Nav1.8          DLEAGKNLPFIYGDIPPEMVSEPLEDLDPYYINKKTFIVLNKGKAIFRFSATSALYILTP
Nav1.9          DLEAGKNLPFIYGDIPPEMVSEPLEDLDPYYINKKTFIVLNKGKAIFRFSATSALYILTP
Nav1.2          DLEAGKSLPFIYGDIPPEMVSVPLEDLDPYYINKKTFIVLNKGKAISRFSATPALYILTP
Nav1.3          DLEAGKNLPFIYGDIPPEMVSEPLEDLDPYYINKKTFIVMNKGKAIFRFSATSALYILTP
Nav1.7          DLEAGKQLPFIYGDIPPGMVSEPLEDLDPYYADKKTFIVLNKGKTIFRFNATPALYMLSP
Nav1.6          DLEAGKSLPFIYGDIPQGLVAVPLEDFDPYYLTQKTFVVLNRGKTLFRFSATPALYILSP
Nav1.4          DLEAGKNLPMIYGDPPPEVIGIPLEDLDPYYSNKKTFIVLNKGKAIFRFSATPALYLLSP
Nav1.5          DLQASKKLPDLYGNPPQELIGEPLEDLDPFYSTQKTFIVLNKGKTIFRFSATNALYVLSP
                **:*.*.** :**: *  ::. ****:**:*  :***:*:*:**:: **.** ***:*:*

Nav1.1          FNPLRKIAIKILVHSLFSMLIMCTILTNCVFMTMSNPPDWTKNVEYTFTGIYTFESLIKI
Nav1.8          FNPLRKIAIKILVHSLFSMLIMCTILTNCVFMTMSNPPDWTKNVEYTFTGIYTFESLIKI
Nav1.9          FNPLRKIAIKILVHSLFSMLIMCTILTNCVFMTMSNPPDWTKNVEYTFTGIYTFESLIKI
Nav1.2          FNPIRKLAIKILVHSLFNMLIMCTILTNCVFMTMSNPPDWTKNVEYTFTGIYTFESLIKI
Nav1.3          LNPVRKIAIKILVHSLFSMLIMCTILTNCVFMTLSNPPDWTKNVEYTFTGIYTFESLIKI
Nav1.7          FSPLRRISIKILVHSLFSMLIMCTILTNCIFMTMNNPPDWTKNVEYTFTGIYTFESLVKI
Nav1.6          FNLIRRIAIKILIHSVFSMIIMCTILTNCVFMTFSNPPDWSKNVEYTFTGIYTFESLVKI
Nav1.4          FSVVRRGAIKVLIHALFSMFIMITILTNCVFMTMSDPPPWSKNVEYTFTGIYTFESLIKI
Nav1.5          FHPIRRAAVKILVHSLFNMLIMCTILTNCVFMAQHDPPPWTKYVEYTFTAIYTFESLVKI
                :  :*: ::*:*:*::*.*:** ******:**:  :** *:* ******.*******:**

Nav1.1          IARGFCLEDFTFLRDPWNWLDFTVITFAYVTEFVDLGNVSALRTFRVLRALKTISVIPGL
Nav1.8          IARGFCLEDFTFLRDPWNWLDFTVITFAYVTEFVDLGNVSALRTFRVLRALKTISVIPGL
Nav1.9          IARGFCLEDFTFLRDPWNWLDFTVITFAYVTEFVDLGNVSALRTFRVLRALKTISVIPGL
Nav1.2          LARGFCLEDFTFLRDPWNWLDFTVITFAYVTEFVDLGNVSALRTFRVLRALKTISVIPGL
Nav1.3          LARGFCLEDFTFLRDPWNWLDFSVIVMAYVTEFVDLGNVSALRTFRVLRALKTISVIPGL
Nav1.7          LARGFCVGEFTFLRDPWNWLDFVVIVFAYLTEFVNLGNVSALRTFRVLRALKTISVIPGL
Nav1.6          IARGFCIDGFTFLRDPWNWLDFSVIMMAYITEFVNLGNVSALRTFRVLRALKTISVIPGL
Nav1.4          LARGFCVDDFTFLRDPWNWLDFSVIMMAYLTEFVDLGNISALRTFRVLRALKTITVIPGL
Nav1.5          LARGFCLHAFTFLRDPWNWLDFSVIIMAYTTEFVDLGNVSALRTFRVLRALKTISVISGL
                :*****:  ************ ** :** ****:***:***************:**.**

Nav1.1          KTIVGALIQSVKKLSDVMILTVFCLSVFALIGLQLFMGNLRNKCIQWPP----------T
Nav1.8          KTIVGALIQSVKKLSDVMILTVFCLSVFALIGLQLFMGNLRNKCIQWPP----------T
Nav1.9          KTIVGALIQSVKKLSDVMILTVFCLSVFALIGLQLFMGNLRNKCIQWPP----------T
Nav1.2          KTIVGALIQSVKKLSDVMILTVFCLSVFALIGLQLFMGNLRNKCLQWPP----------D
Nav1.3          KTIVGALIQSVKKLSDVMILTVFCLSVFALIGLQLFMGNLRNKCLQWPP----------S
Nav1.7          KTIVGALIQSVKKLSDVMILTVFCLSVFALIGLQLFMGNLKHKCFRN------------
Nav1.6          KTIVGALIQSVKKLSDVMILTVFCLSVFALIGLQLFMGNLRNKCVVWP------------
Nav1.4          KTIVGALIQSVKKLSDVMILTVFCLSVFALVGLQLFMGNLRQKCVRWPPPFNDTNTTWYS
Nav1.5          KTIVGALIQSVKKLADVMVLTVFCLSVFALIGLQLFMGNLRHKCVRNFT-----------
                **************:***:***********:*********::**.
```

```
Nav1.1          NASLEEHSIEKN-ITVNYNGTLINETVF----------EFDWKSYIQDSRYHYFLEGFLD
Nav1.8          NASLEEHSIEKN-ITVNYNGTLINETVF----------EFDWKSYIQDSRYHYFLEGFLD
Nav1.9          NASLEEHSIEKN-ITVNYNGTLINETVF----------EFDWKSYIQDSRYHYFLEGFLD
Nav1.2          NSSFEINITSFFNNSLDGNGTTFNRTVS----------IFNWDEYIEDKSHFYFLEGQND
Nav1.3          DSAFETNTTSYFNGTMDSNGTFVNVTMS----------TFNWKDYIGDDSHFYVLDGQKD
Nav1.7          --SLENNETLES---------IMNTLES----------EEDFR------KYFYYLEGSKD
Nav1.6          ---------------INFNESYLENGTK----------GFDWEEYINNKTNFYTVPGMLE
Nav1.4          NDTWYGNDTWYGNEMWYGNDSWYANDTWNSHASWATNDTFDWDAYISDEGNFYFLEGSND
Nav1.5          -------------ALNGTNGSVEADGLV----------WESLDLYLSDPENYLLKNGTSD
                                                     .         .   *  :

Nav1.1          ALLCGNSSDAGQCPEGYMCVKAGRNPNYGYTSFDTFSWAFLSLFRLMTQDFWENLYQLTL
Nav1.8          ALLCGNSSDAGQCPEGYMCVKAGRNPNYGYTSFDTFSWAFLSLFRLMTQDFWENLYQLTL
Nav1.9          ALLCGNSSDAGQCPEGYMCVKAGRNPNYGYTSFDTFSWAFLSLFRLMTQDFWENLYQLTL
Nav1.2          ALLCGNSSDAGQCPEGYICVKAGRNPNYGYTSFDTFSWAFLSLFRLMTQDFWENLYQLTL
Nav1.3          PLLCGNGSDAGQCPEGYICVKAGRNPNYGYTSFDTFSWAFLSLFRLMTQDYWENLYQLTL
Nav1.7          ALLCGFSTDSGQCPEGYTCVKIGRNPDYGYTSFDTFSWAFLALFRLMTQDYWENLYQQTL
Nav1.6          PLLCGNSSDAGQCPEGYQCMKAGRNPNYGYTSFDTFSWAFLALFRLMTQDYWENLYQLTL
Nav1.4          ALLCGNSSDAGHCPEGYECIKTGRNPNYGYTSYDTFSWAFLALFRLMTQDYWENLFQLTL
Nav1.5          VLLCGNSSDAGTCPEGYRCLKAGENPDHGYTSFDSFAWAFLALFRLMTQDCWERLYQQTL
                 **** .:*:* ***** *:* *.**::****:*:*:****:******** **.*:* **

Nav1.1          RAAGKTYMIFFVLVIFLGSFYLINLILAVVAMAYEEQNQATLEEAEQKEAEFQQMIEQLK
Nav1.8          RAAGKTYMIFFVLVIFLGSFYLINLILAVVAMAYEEQNQATLEEAEQKEAEFQQMIEQLK
Nav1.9          RAAGKTYMIFFVLVIFLGSFYLINLILAVVAMAYEEQNQATLEEAEQKEAEFQQMIEQLK
Nav1.2          RAAGKTYMIFFVLVIFLGSFYLINLILAVVAMAYEEQNQATLEEAEQKEAEFQQMLEQLK
Nav1.3          RAAGKTYMIFFVLVIFLGSFYLVNLILAVVAMAYEEQNQATLEEAEQKEAEFQQMLEQLK
Nav1.7          RAAGKTYMIFFVVVIFLGSFYLINLILAVVAMAYEEQNQANIEEAKQKELEFQQMLDRLK
Nav1.6          RAAGKTYMIFFVLVIFVGSFYLVNLILAVVAMAYEEQNQATLEEAEQKEAEFKAMLEQLK
Nav1.4          RAAGKTYMIFFVVIIFLGSFYLINLILAVVAMAYAEQNEATLAEDKEKEEEFQQMLEKFK
Nav1.5          RSAGKIYMIFFMLVIFLGSFYLVNLILAVVAMAYEEQNQATIAETEEKEKRFQEAMEMLK
                *:*** *****:::**:*****:********** ***:*.: * ::** .*:  :: :*

Nav1.1          KQQEAAQQAATAT----ASEHSREPSA--AGRLSDSSSEASKLSSKSAKERRNRRKKRKQ
Nav1.8          KQQEAAQQAATAT----ASEHSREPSA--AGRLSDSSSEASKLSSKSAKERRNRRKKRKQ
Nav1.9          KQQEAAQQAATAT----ASEHSREPSA--AGRLSDSSSEASKLSSKSAKERRNRRKKRKQ
Nav1.2          KQQEEAQAAAAAASA--ESRDFSGAGG--IGVFSESSSVASKLSSKSEKELKNRRKKKKQ
Nav1.3          KQQEEAQAVAAAS-A--ASRDFSGIGG--LGELLESSSEASKLSSKSAKEWRNRRKKRRQ
Nav1.7          KEQEEAEAIAAAA----AEYTSIRRSR--IMGLSESSSETSKLSSKSAKERRNRRKKKNQ
Nav1.6          KQQEEAQAAAMATSAGTVSEDAIEEEGEEGGGSPRSSSEISKLSSKSAKERRNRRKKRKQ
Nav1.4          KHQEELEKAKAAQ-----------------------------------------------
Nav1.5          KEHEALTIRGVDT------------------VSRSSLEMSPLAPVNSHERRSKRRKR--
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Nav1.1          KEQSGGEEKDEDE-FQKSESEDSIRRKGFRFSIEGNRLTYEKRYSSPHQSLLSIRGSLFS
Nav1.8          KEQSGGEEKDEDE-FQKSESEDSIRRKGFRFSIEGNRLTYEKRYSSPHQSLLSIRGSLFS
Nav1.9          KEQSGGEEKDEDE-FQKSESEDSIRRKGFRFSIEGNRLTYEKRYSSPHQSLLSIRGSLFS
Nav1.2          KEQSGEEEKNDR--VRKSESEDSIRRKGFRFSLEGSRLTYEKRFSSPHQSLLSIRGSLFS
Nav1.3          REHLEGNNKGERDSFPKSESEDSVKRSSFLFSMDGNRLTSDKKFCSPHQSLLSIRGSLFS
Nav1.7          KKLSSGEEKGDAEKLSKSESEDSIRRKSFHLGVEGHRRAHEKRLSTPNQSPLSIRGSLFS
Nav1.6          KELSEGEEKGDPEKVFKSESEDGMRRKAFRLPD--NRIG--RKFSIMNQSLLSIPGSPFL
Nav1.4          -ALEGGEADGDP-------------------------------------------------A
Nav1.5          --MSSGTEECGEDRLPKSDSEDGPR------------------AMNHLSLTRGLSRTSM
                       .

Nav1.1          PRRNSRTSLFSFRG--RAKDVGSENDFADDEHSTFEDNESRRDSLFVPRRHGERRNSNL-
Nav1.8          PRRNSRTSLFSFRG--RAKDVGSENDFADDEHSTFEDNESRRDSLFVPRRHGERRNSNL-
Nav1.9          PRRNSRTSLFSFRG--RAKDVGSENDFADDEHSTFEDNESRRDSLFVPRRHGERRNSNL-
Nav1.2          PRRNSRASLFSFRG--RAKDIGSENDFADDEHSTFEDNDSRRDSLFVPHRHGERRHSNV-
Nav1.3          PRRNSKTSIFSFRG--RAKDVGSENDFADDEHSTFEDSESRRDSLFVPHRHGERRNS---
Nav1.7          ARRSSRTSLFSFKG--RGRDIGSETEFADDEHSIFGDNESRRGSLFVPHRPQERRSSNI-
Nav1.6          SRHNSKSSIFSFRGPGRFRDPGSENEFADDEHSTVEESEGRRDSLFIPIRARERRSSYSG
Nav1.4          HGKDCNGSLDTSQG-----------------------------------------------
Nav1.5          KPRSSRGSIFTFRR----RDLGSEADFADDENSTAGESESHHTSLLVPWPLRRTSAQGQ-
                  :... *: : :
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Nav1.1          ----SQTSRSSRMLAVFPANGKMHSTVDCNGVVSLVGGPSVPTSPVGQLLP---------
Nav1.8          ----SQTSRSSRMLAVFPANGKMHSTVDCNGVVSLVGGPSVPTSPVGQLLP---------
Nav1.9          ----SQTSRSSRMLAVFPANGKMHSTVDCNGVVSLVGGPSVPTSPVGQLLP---------
Nav1.2          ----SQASRASRVLPILPMNGKMHSAVDCNGVVSLVGGPSTLTS-AGQLLP---------
Nav1.3          ------------------------------------------------------------
Nav1.7          ----SQASRSP---PMLPVNGKMHSAVDCNGVVSLVDGRSALMLPNGQLLP---------
Nav1.6          YSGYSQGSRSSRIFPSLRRSVKRNSTVDCNGVVSLIGGPGSHIG--GRLLP---------
Nav1.4          ------------------------------------------------------------
Nav1.5          -------PSPGTSAPGHALHGKKNSTVDCNGVVSLLGAGDPEATSPGSHLLRPVMLEHPP


Nav1.1          -EGTTTETEMRKRRSSSFHVSMDFLEDPSQRQRAMSIASILTNT-VEELEESRQKCPPCW
Nav1.8          -EGTTTETEMRKRRSSSFHVSMDFLEDPSQRQRAMSIASILTNT-VEELEESRQKCPPCW
Nav1.9          -EGTTTETEMRKRRSSSFHVSMDFLEDPSQRQRAMSIASILTNT-VEELEESRQKCPPCW
Nav1.2          -EGTTTETEIRKRRSSSYHVSMDLLEDPTSRQRAMSIASILTNT-MEELEESRQKCPPCW
Nav1.3          -NGTTTETEVRKRRLSSYQISMEMLEDSSGRQRAVSIASILTNT-MEELEESRQKCPPCW
Nav1.7          -EGTTNQIHK-KRRCSSYLLSEDMLNDPNLRQRAMSRASILTNT-VEELEESRQKCPPWW
Nav1.6          -EATT-EVEIKKKGPGSLLVSMDQLASYGRKDRINSIMSVVTNTLVEELEESQRKCPPCW
Nav1.4          -------------------------EKGAPRQSSSGDSGISDA-MEELEEAHQKCPPWW
Nav1.5          DTTTPSEEPGGPQMLTSQAPCVDGFEEPGARQRALSAVSVLTSA-LEELEESRHKCPPCW
                                         .     :  *  * ::.: :*****:::**** *

Nav1.1          YKFSNIFLIWDCSPYWLKVKHVVNLVVMDPFVDLAITICIVLNTLFMAMEHYPMTDHFNN
Nav1.8          YKFSNIFLIWDCSPYWLKVKHVVNLVVMDPFVDLAITICIVLNTLFMAMEHYPMTDHFNN
Nav1.9          YKFSNIFLIWDCSPYWLKVKHVVNLVVMDPFVDLAITICIVLNTLFMAMEHYPMTDHFNN
Nav1.2          YKFANMCLIWDCCKPWLKVKHLVNLVVMDPFVDLAITICIVLNTLFMAMEHYPMTEQFSS
Nav1.3          YRFANVFLIWDCCDAWLKVKHLVNLIVMDPFVDLAITICIVLNTLFMAMEHYPMTEQFSS
Nav1.7          YRFAHKFLIWNCSPYWIKFKKCIYFIVMDPFVDLAITICIVLNTLFMAMEHHPMTEEFKN
Nav1.6          YKFANTFLIWECHPYWIKLKEIVNLIVMDPFVDLAITICIVLNTLFMAMEHHPMTPQFEH
Nav1.4          YKCAHKVLIWNCCAPWLKFKNIIHLIVMDPFVDLGITICIVLNTLFMAMEHYPMTEHFDN
Nav1.5          NRLAQRYLIWECCPLWMSIKQGVKLVVMDPFTDLTITMCIVLNTLFMALEHYNMTSEFEE
                 : ::  ***:*   *:..*. : ::*****.** **:**********:**: ** .*.

Nav1.1          VLTVGNLVFTGIFTAEMFLKIIAMDPYYYFQEGWNIFDGFIVTLSLVELGLANVEGLSVL
Nav1.8          VLTVGNLVFTGIFTAEMFLKIIAMDPYYYFQEGWNIFDGFIVTLSLVELGLANVEGLSVL
Nav1.9          VLTVGNLVFTGIFTAEMFLKIIAMDPYYYFQEGWNIFDGFIVTLSLVELGLANVEGLSVL
Nav1.2          VLSVGNLVFTGIFTAEMFLKIIAMDPYYYFQEGWNIFDGFIVSLSLMELGLANVEGLSVL
Nav1.3          VLTVGNLVFTGIFTAEMVLKIIAMDPYYYFQEGWNIFDGIIVSLSLMELGLSNVEGLSVL
Nav1.7          VLAIGNLVFTGIFAAEMVLKLIAMDPYEYFQVGWNIFDSLIVTLSLVELFLADVEGLSVL
Nav1.6          VLAVGNLVFTGIFTAEMFLKLIAMDPYYYFQEGWNIFDGFIVSLSLMELSLADVEGLSVL
Nav1.4          VLTVGNLVFTGIFTAEMVLKLIAMDPYEYFQQGWNIFDSIIVTLSLVELGLANVQGLSVL
Nav1.5          MLQVGNLVFTGIFTAEMTFKIIALDPYYYFQQGWNIFDSIIVILSLMELGLSRMSNLSVL
                :* :*********:*** :*:**:*** *** ******.:** ***:** *: :..****

Nav1.1          RSFRLLRVFKLAKSWPTLNMLIKIIGNSVGALGNLTLVLAIIVFIFAVVGMQLFGKSYKD
Nav1.8          RSFRLLRVFKLAKSWPTLNMLIKIIGNSVGALGNLTLVLAIIVFIFAVVGMQLFGKSYKD
Nav1.9          RSFRLLRVFKLAKSWPTLNMLIKIIGNSVGALGNLTLVLAIIVFIFAVVGMQLFGKSYKD
Nav1.2          RSFRLLRVFKLAKSWPTLNMLIKIIGNSVGALGNLTLVLAIIVFIFAVVGMQLFGKSYKE
Nav1.3          RSFRLLRVFKLAKSWPTLNMLIKIIGNSVGALGNLTLVLAIIVFIFAVVGMQLFGKSYKE
Nav1.7          RSFRLLRVFKLAKSWPTLNMLIKIIGNSVGALGNLTLVLAIIVFIFAVVGMQLFGKSYKE
Nav1.6          RSFRLLRVFKLAKSWPTLNMLIKIIGNSVGALGNLTLVLAIIVFIFAVVGMQLFGKSYKE
Nav1.4          RSFRLLRVFKLAKSWPTLNMLIKIIGNSVGALGNLTLVLAIIVFIFAVVGMQLFGKSYKE
Nav1.5          RSFRLLRVFKLAKSWPTLNTLIKIIGNSVGALGNLTLVLAIIVFIFAVVGMQLFGKNYSE
                ****************** ************************************.*.:

Nav1.1          CVCKIASDCQLPRWHMNDFFHSFLIVFRVLCGEWIETMWDCMEVAGQAMCLTVFMMVMVI
Nav1.8          CVCKIASDCQLPRWHMNDFFHSFLIVFRVLCGEWIETMWDCMEVAGQAMCLTVFMMVMVI
Nav1.9          CVCKIASDCQLPRWHMNDFFHSFLIVFRVLCGEWIETMWDCMEVAGQAMCLTVFMMVMVI
Nav1.2          CVCKISNDCELPRWHMHDFFHSFLIVFRVLCGEWIETMWDCMEVAGQTMCLTVFMMVMVI
Nav1.3          CVCKINDDCTLPRWHMNDFFHSFLIVFRVLCGEWIETMWDCMEVAGQTMCLIVFMLVMVI
Nav1.7          CVCKINDDCTLPRWHMNDFFHSFLIVFRVLCGEWIETMWDCMEVAGQAMCLIVYMMVMVI
Nav1.6          CVCKINQDCELPRWHMHDFFHSFLIVFRVLCGEWIETMWDCMEVAGQAMCLIVFMMVMVI
Nav1.4          CVCKIALDCNLPRWHMHDFFHSFLIVFRILCGEWIETMWDCMEVAGQAMCLTVFLMVMVI
Nav1.5          LRD--SDSGLLPRWHMMDFFHAFLIIFRILCGEWIETMWDCMEVSGQSLCLLVFLLVMVI
                       .  ****** ****:***:**:***************:**::** *:::****
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Nav1.1          GNLVVLNLFLALLLSSFSADNLAATDDDNEMNNLQIAVDRMHKGVAYVKRKIYEFIQQSF
Nav1.8          GNLVVLNLFLALLLSSFSADNLAATDDDNEMNNLQIAVDRMHKGVAYVKRKIYEFIQQSF
Nav1.9          GNLVVLNLFLALLLSSFSADNLAATDDDNEMNNLQIAVDRMHKGVAYVKRKIYEFIQQSF
Nav1.2          GNLVVLNLFLALLLSSFSSDNLAATDDDNEMNNLQIAVGRMQKGIDFVKRKIREFIQKAF
Nav1.3          GNLVVLNLFLALLLSSFSSDNLAATDDDNEMNNLQIAVGRMQKGIDYVKNKMRECFQKAF
Nav1.7          GNLVVLNLFLALLLSSFSSDNLTAIEEDPDANNLQIAVTRIKKGINYVKQTLREFILKAF
Nav1.6          GNLVVLNLFLALLLSSFSADNLAATDDDGEMNNLQISVIRIKKGVAWTKLKVHAFMQAHF
Nav1.4          GNLVVLNLFLALLLSSFSADSLAASDEDGEMNNLQIAIGRIKLGIGFAKAFLLGLLHGKI
Nav1.5          GNLVVLNLFLALLLSSFSADNLTAPDEDREMNNLQLALARIQRGLRFVKRTTWDFCCGLL
                **********************:*.*:* ::* : ****::: *:: *: :.*          :

Nav1.1          IRKQKILDEIKPLDDLNNKKDSCMS--NHT-AEIGKDLDYLKDVNGTTSGIGT-------
Nav1.8          IRKQKILDEIKPLDDLNNKKDSCMS--NHT-AEIGKDLDYLKDVNGTTSGIGT-------
Nav1.9          IRKQKILDEIKPLDDLNNKKDSCMS--NHT-AEIGKDLDYLKDVNGTTSGIGT-------
Nav1.2          VRKQKALDEIKPLEDLNNKKDSCIS--NHTTIEIGKDLNYLKDGNGTTSGIG--------
Nav1.3          FRKPKVIEIHE-----GNKIDSCMS--NNTGIEISKELNYLRDGNGTTSGVGT-------
Nav1.7          SKKPKISREIRQAEDLNTKKENYIS--NHTLAEMSKGHNFLKE-KDKISGFG--------
Nav1.6          KQRE--ADEVKPLDELYEKKANCIA--NHTGADIHRNGDFQKNGNGTTSGIG--------
Nav1.4          LSPKDIMLSLGEADGAGEAGEAGET--APEDEKKEPPEEDLKKDNHILNHMG--------
Nav1.5          RQRPQKPAALAAQGQLPSCIATPYSPPPPETEKVPPTRKETRFEEGEQPGQGTPGDPEPV
                                        :       .     .  :  :      *

Nav1.1          -------GSSVEKYIIDESDYMSFINNPS------LTVTVPIAVGESDFENLNTEDFSSE
Nav1.8          -------GSSVEKYIIDESDYMSFINNPS------LTVTVPIAVGESDFENLNTEDFSSE
Nav1.9          -------GSSVEKYIIDESDYMSFINNPS------LTVTVPIAVGESDFENLNTEDFSSE
Nav1.2          --------SSVEKYVVDESDYMSFINNPS------LTVTVPIAVGESDFENLNTEEFSSE
Nav1.3          -------GSSVEKYVIDENDYMSFINNPS------LTVTVPIAVGESDFENLNTEEFSSE
Nav1.7          --------SSVDKHLMEDSDGQSFIHNPS------LTVTVPIAPGESDLENMNAEELSSD
Nav1.6          --------SSVEKYIIDE-DHMSFINNPN------LTVRVPIAVGESDFENLNTEDVSSE
Nav1.4          --------LADGPPSSLELDHLNFINNPY------LTIQVPIASEESDLEMPTEEETDTF
Nav1.5          CVPIAVAESDTDDQEEDEENSLGTEEESSKQQESQPVSGGPEAPPDSRTWSQVSATASSE
                               : :  .  .:.        .   * *  :*          .:

Nav1.1          SDLEESKE--KLNE-------------SSSSSEGSTVDIGAPVE--EQPVVEPEETLEPE
Nav1.8          SDLEESKE--KLNE-------------SSSSSEGSTVDIGAPVE--EQPVVEPEETLEPE
Nav1.9          SDLEESKE--KLNE-------------SSSSSEGSTVDIGAPVE--EQPVVEPEETLEPE
Nav1.2          SDMEESKE--KLN--------------ATSSSEGSTVDIGAPAEG-EQPEVEPEESLEPE
Nav1.3          SELEESKE--KLN--------------ATSSSEGSTVDVVLPREG-EQAETEPEEDLKPE
Nav1.7          SDSEYSKV--RLN--------------RSSSSECSTVDNPLPGEG-EEAEAEPMNSDEPE
Nav1.6          SDPEGSKD--KLD--------------DTSSSEGSTIDIKPEVE--EVPVEQPEEYLDPD
Nav1.4          SEPEDSKKPPQPLY-------------DGNSSVCSTADYKPPEEDPEEQAEENPEGEQPE
Nav1.5          AEASASQADWRQQWKAEPQAPGCGETPEDSCSEGSTADMTNTAELLEQIPDLGQDVKDPE
                :: . *:   :                 ..*  ** *     *  *       :  .*:

Nav1.1          ACFTEGCVQRFKCCQINVEEGRGKQWWNLRRTCFRIVEHNWFETFIVFMILLSSGALAFE
Nav1.8          ACFTEGCVQRFKCCQINVEEGRGKQWWNLRRTCFRIVEHNWFETFIVFMILLSSGALAFE
Nav1.9          ACFTEGCVQRFKCCQINVEEGRGKQWWNLRRTCFRIVEHNWFETFIVFMILLSSGALAFE
Nav1.2          ACFTEDCVRKFKCCQISIEEGKGKLWWNLRKTCYKIVEHNWFETFIVFMILLSSGALAFE
Nav1.3          ACFTEGCIKKFPFCQVSTEEGKGKIWWNLRKTCYSIVEHNWFETFIVFMILLSSGALAFE
Nav1.7          ACFTDGCVRRFSCCQVNIESGKGKIWWNIRKTCYKIVEHSWFESFIVLMILLSSGALAFE
Nav1.6          ACFTEGCVQRFKCCQVNIEEGLGKSWWILRKTCFLIVEHNWFETFIIFMILLSSGALAFE
Nav1.4          ECFTEACVQRWPCLYVDISQGRGKKWWTLRRACFKIVEHNWFETFIVFMILLSSGALAFE
Nav1.5          DCFTEGCVRRCPCCAVDTTQAPGKVWWRLRKTCYHIVEHSWFETFIIFMILLSSGALAFE
                 ***: *:::     :.  .. ** ** :*::*: ****.***:**::***********

Nav1.1          DIYIDQRKTIKTMLEYADKVFTYIFILEMLLKWVAYGYQTYFTNAWCWLDFLIVDVSLVS
Nav1.8          DIYIDQRKTIKTMLEYADKVFTYIFILEMLLKWVAYGYQTYFTNAWCWLDFLIVDVSLVS
Nav1.9          DIYIDQRKTIKTMLEYADKVFTYIFILEMLLKWVAYGYQTYFTNAWCWLDFLIVDVSLVS
Nav1.2          DIYIEQRKTIKTMLEYADKVFTYIFILEMLLKWVAYGFQVYFTNAWCWLDFLIVDVSLVS
Nav1.3          DIYIEQRKTIKTMLEYADKVFTYIFILEMLLKWVAYGFQTYFTNAWCWLDFLIVDVSLVS
Nav1.7          DIYIERKKTIKIILEYADKIFTYIFILEMLLKWIAYGYKTYFTNAWCWLDFLIVDVSLVT
Nav1.6          DIYIEQRKTIRTILEYADKVFTYIFILEMLLKWTAYGFVKFFTNAWCWLDFLIVAVSLVS
Nav1.4          DIYIEQRRVIRTILEYADKVFTYIFIMEMLLKWVAYGFKVYFTNAWCWLDFLIVDVSIIS
Nav1.5          DIYLEERKTIKVLLEYADKMFTYVFVLEMLLKWVAYGFKKYFTNAWCWLDFLIVDVSLVS
                ***::.::.*: :******:***:*::****** ***:  :************* **:::
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Nav1.1          LTANALGYSELGAIKSLRTLRALRPLRALSRFEGMRVVVNALLGAIPSIMNVLLVCLIFW
Nav1.8          LTANALGYSELGAIKSLRTLRALRPLRALSRFEGMRVVVNALLGAIPSIMNVLLVCLIFW
Nav1.9          LTANALGYSELGAIKSLRTLRALRPLRALSRFEGMRVVVNALLGAIPSIMNVLLVCLIFW
Nav1.2          LTANALGYSELGAIKSLRTLRALRPLRALSRFEGMRVVVNALLGAIPSIMNVLLVCLIFW
Nav1.3          LVANALGYSELGAIKSLRTLRALRPLRALSRFEGMRVVVNALVGAIPSIMNVLLVCLIFW
Nav1.7          LVANTLGYSDLGPIKSLRTLRALRPLRALSRFEGMRVVVNALIGAIPSIMNVLLVCLIFW
Nav1.6          LIANALGYSELGAIKSLRTLRALRPLRALSRFEGMRVVVNALVGAIPSIMNVLLVCLIFW
Nav1.4          LVANWLGYSELGPIKSLRTLRALRPLRALSRFEGMRVVVNALLGAIPSIMNVLLVCLIFW
Nav1.5          LVANTLGFAEMGPIKSLRTLRALRPLRALSRFEGMRVVVNALVGAIPSIMNVLLVCLIFW
                * ** **::::*.*****************************:*****************

Nav1.1          LIFSIMGVNLFAGKFYHCINTTTGDR-FDIEDVNNHTDCLKLIE-RNETARWKNVKVNFD
Nav1.8          LIFSIMGVNLFAGKFYHCINTTTGDR-FDIEDVNNHTDCLKLIE-RNETARWKNVKVNFD
Nav1.9          LIFSIMGVNLFAGKFYHCINTTTGDR-FDIEDVNNHTDCLKLIE-RNETARWKNVKVNFD
Nav1.2          LIFSIMGVNLFAGKFYHCINYTTGEM-FDVSVVNNYSECKALIE-SNQTARWKNVKVNFD
Nav1.3          LIFSIMGVNLFAGKFYHCVNMTTGNM-FDISDVNNLSDCQALG----KQARWKNVKVNFD
Nav1.7          LIFSIMGVNLFAGKFYECINTTDGSR-FPASQVPNRSECFALMN-VSQNVRWKNLKVNFD
Nav1.6          LIFSIMGVNLFAGKYHYCFNETSEIR-FEIEDVNNKTECEKLMEGNNTEIRWKNVKINFD
Nav1.4          LIFSIMGVNLFAGKFYYCINTTTSER-FDISEVNNKSECESLMH--TGQVRWLNVKVNYD
Nav1.5          LIFSIMGVNLFAGKFGRCINQTEGDLPLNYTIVNNKSQCESLNL--TGELYWTKVKVNFD
                **************:  *.* *     :    * * ::*  *         * ::*:*:*

Nav1.1          NVGFGYLSLLQVATFKGWMDIMYAAVDSRNVELQPKYEESLYMYLYFVIFIIFGSFFTLN
Nav1.8          NVGFGYLSLLQVATFKGWMDIMYAAVDSRNVELQPKYEESLYMYLYFVIFIIFGSFFTLN
Nav1.9          NVGFGYLSLLQVATFKGWMDIMYAAVDSRNVELQPKYEESLYMYLYFVIFIIFGSFFTLN
Nav1.2          NVGLGYLSLLQVATFKGWMDIMYAAVDSRNVELQPKYEDNLYMYLYFVIFIIFGSFFTLN
Nav1.3          NVGAGYLALLQVATFKGWMDIMYAAVDSRDVKLQPVYEENLYMYLYFVIFIIFGSFFTLN
Nav1.7          NVGLGYLSLLQVATFKGWTIIMYAAVDSVNVDKQPKYEYSLYMYIYFVVFIIFGSFFTLN
Nav1.6          NVGAGYLALLQVATFKGWMDIMYAAVDSRKPDEQPKYEDNIYMYIYFVIFIIFGSFFTLN
Nav1.4          NVGLGYLSLLQVATFKGWMDIMYAAVDSREKEEQPQYEVNLYMYLYFVIFIIFGSFFTLN
Nav1.5          NVGAGYLALLQVATFKGWMDIMYAAVDSRGYEEQPQWEYNLYMYIYFVIFIIFGSFFTLN
                *** ***:**********  ********   . ** :* .:***:***:***********

Nav1.1          LFIGVIIDNFNQQKKKFGGQDIFMTEEQKKYYNAMKKLGSKKPQKPIPRPGNKFQGMVFD
Nav1.8          LFIGVIIDNFNQQKKKFGGQDIFMTEEQKKYYNAMKKLGSKKPQKPIPRPGNKFQGMVFD
Nav1.9          LFIGVIIDNFNQQKKKFGGQDIFMTEEQKKYYNAMKKLGSKKPQKPIPRPGNKFQGMVFD
Nav1.2          LFIGVIIDNFNQQKKKFGGQDIFMTEEQKKYYNAMKKLGSKKPQKPIPRPANKFQGMVFD
Nav1.3          LFIGVIIDNFNQQKKKFGGQDIFMTEEQKKYYNAMKKLGSKKPQKPIPRPANKFQGMVFD
Nav1.7          LFIGVIIDNFNQQKKKLGGQDIFMTEEQKKYYNAMKKLGSKKPQKPIPRPGNKIQGCIFD
Nav1.6          LFIGVIIDNFNQQKKKFGGQDIFMTEEQKKYYNAMKKLGSKKPQKPIPRPLNKIQGIVFD
Nav1.4          LFIGVIIDNFNQQKKKLGGKDIFMTEEQKKYYNAMKKLGSKKPQKPIPRPQNKIQGMVYD
Nav1.5          LFIGVIIDNFNQQKKKLGGQDIFMTEEQKKYYNAMKKLGSKKPQKPIPRPLNKYQGFIFD
                ****************:**:****************************** ** ** ::*

Nav1.1          FVTRQVFDISIMILICLNMVTMMVETDDQSEYVTTILSRINLVFIVLFTGECVLKLISLR
Nav1.8          FVTRQVFDISIMILICLNMVTMMVETDDQSEYVTTILSRINLVFIVLFTGECVLKLISLR
Nav1.9          FVTRQVFDISIMILICLNMVTMMVETDDQSEYVTTILSRINLVFIVLFTGECVLKLISLR
Nav1.2          FVTKQVFDISIMILICLNMVTMMVETDDQSQEMTNILYWINLVFIVLFTGECVLKLISLR
Nav1.3          FVTRQVFDISIMILICLNMVTMMVETDDQGKYMTLVLSRINLVFIVLFTGEFVLKLVSLR
Nav1.7          LVTNQAFDISIMVLICLNMVTMMVEKEGQSQHMTEVLYWINVVFIILFTGECVLKLISLR
Nav1.6          FVTQQAFDIVIMMLICLNMVTMMVETDTQSKQMENILYWINLVFVIFFTCECVLKMFALR
Nav1.4          LVTKQAFDITIMILICLNMVTMMVETDNQSQLKVDILYNINMIFIIIFTGECVLKMLALR
Nav1.5          IVTKQAFDVTIMFLICLNMVTMMVETDDQSPEKINILAKINLLFVAIFTGECIVKLAALR
                :**.*.**: **.************.: *.     :*  **::*: :** * ::*: :**

Nav1.1          HYYFTIGWNIFDFVVVILSIVGMFLAELIEKYFVSPTLFRVIRLARIGRILRLIKGAKGI
Nav1.8          HYYFTIGWNIFDFVVVILSIVGMFLAELIEKYFVSPTLFRVIRLARIGRILRLIKGAKGI
Nav1.9          HYYFTIGWNIFDFVVVILSIVGMFLAELIEKYFVSPTLFRVIRLARIGRILRLIKGAKGI
Nav1.2          YYYFTIGWNIFDFVVVILSIVGMFLAELIEKYFVSPTLFRVIRLARIGRILRLIKGAKGI
Nav1.3          HYYFTIGWNIFDFVVVILSIVGMFLAEMIEKYFVSPTLFRVIRLARIGRILRLIKGAKGI
Nav1.7          HYYFTVGWNIFDFVVVIISIVGMFLADLIETYFVSPTLFRVIRLARIGRILRLVKGAKGI
Nav1.6          HYYFTIGWNIFDFVVVILSIVGMFLADIIEKYFVSPTLFRVIRLARIGRILRLIKGAKGI
Nav1.4          QYYFTVGWNIFDFVVVILSIVGLALSDLIQKYFVSPTLFRVIRLARIGRVLRLIRGAKGI
Nav1.5          HYYFTNSWNIFDFVVVILSIVGTVLSDIIQKYFFSPTLFRVIRLARIGRILRLIRGAKGI
                 **** .**********:****  *:::*:.**.***************:***::*****
```

```
Nav1.1          RTLLFALMMSLPALFNIGLLLFLVMFIYAIFGMSNFAYVKREVGIDDMFNFETFGNSMIC
Nav1.8          RTLLFALMMSLPALFNIGLLLFLVMFIYAIFGMSNFAYVKREVGIDDMFNFETFGNSMIC
Nav1.9          RTLLFALMMSLPALFNIGLLLFLVMFIYAIFGMSNFAYVKREVGIDDMFNFETFGNSMIC
Nav1.2          RTLLFALMMSLPALFNIGLLLFLVMFIYAIFGMSNFAYVKREVGIDDMFNFETFGNSMIC
Nav1.3          RTLLFALMMSLPALFNIGLLLFLVMFIYAIFGMSNFAYVKKEAGIDDMFNFETFGNSMIC
Nav1.7          RTLLFALMMSLPALFNIGLLLFLVMFIYAIFGMSNFAYVKKEDGINDMFNFETFGNSMIC
Nav1.6          RTLLFALMMSLPALFNIGLLLFLVMFIFSIFGMSNFAYVKHEAGIDDMFNFETFGNSMIC
Nav1.4          RTLLFALMMSLPALFNIGLLLFLVMFIYSIFGMSNFAYVKKESGIDDMFNFETFGNSIIC
Nav1.5          RTLLFALMMSLPALFNIGLLLFLVMFIYSIFGMANFAYVKWEAGIDDMFNFQTFANSMLC
                ****************************::****:****** * **:*****:**.**::*

Nav1.1          LFQITTSAGWDGLLAPILNSKPPDCDPNKVNPGSSVKGDCGNPSVGIFFFVSYIIISFLV
Nav1.8          LFQITTSAGWDGLLAPILNSKPPDCDPNKVNPGSSVKGDCGNPSVGIFFFVSYIIISFLV
Nav1.9          LFQITTSAGWDGLLAPILNSKPPDCDPNKVNPGSSVKGDCGNPSVGIFFFVSYIIISFLV
Nav1.2          LFQITTSAGWDGLLAPILNSGPPDCDPDKDHPGSSVKGDCGNPSVGIFFFVSYIIISFLV
Nav1.3          LFQITTSAGWDGLLAPILNSAPPDCDPDTIHPGSSVKGDCGNPSVGIFFFVSYIIISFLV
Nav1.7          LFQITTSAGWDGLLAPILNSKPPDCDPKKVHPGSSVEGDCGNPSVGIFYFVSYIIISFLV
Nav1.6          LFQITTSAGWDGLLLPILN-RPPDCSLDKEHPGSGFKGDCGNPSVGIFFFVSYIIISFLI
Nav1.4          LFEITTSAGWDGLLNPILNSGPPDCDPNLENPGTSVKGDCGNPSIGICFFCSYIIISFLI
Nav1.5          LFQITTSAGWDGLLSPILNTGPPYCDPTLPNS-NGSRGDCGSPAVGILFFTTYIIISFLI
                **:*********** ****  ** *.    :. .. .****.*::** :* :*******:

Nav1.1          VVNMYIAVILENFSVATEESAEPLSEDDFEMFYEVWEKFDPDATQFMEFEKLSQFAAALE
Nav1.8          VVNMYIAVILENFSVATEESAEPLSEDDFEMFYEVWEKFDPDATQFMEFEKLSQFAAALE
Nav1.9          VVNMYIAVILENFSVATEESAEPLSEDDFEMFYEVWEKFDPDATQFMEFEKLSQFAAALE
Nav1.2          VVNMYIAVILENFSVATEESAEPLSEDDFEMFYEVWEKFDPDATQFIEFAKLSDFADALD
Nav1.3          VVNMYIAVILENFSVATEESAEPLSEDDFEMFYEVWEKFDPDATQFIEFSKLSDFAAALD
Nav1.7          VVNMYIAVILENFSVATEESTEPLSEDDFEMFYEVWEKFDPDATQFIEFSKLSDFAAALD
Nav1.6          VVNMYIAIILENFSVATEESADPLSEDDFETFYEIWEKFDPDATQFIEYCKLADFADALE
Nav1.4          VVNMYIAIILENFNVATEESSEPLGEDDFEMFYETWEKFDPDATQFIAYSRLSDFVDTLQ
Nav1.5          VVNMYIAIILENFSVATEESTEPLSEDDFDMFYEIWEKFDPEATQFIEYSVLSDFADALS
                *******:*****.******::**.****: *** ******:****: :  *::*. :*.

Nav1.1          PPLNLPQPNKLQLIAMDLPMVSGDRIHCLDILFAFTKRVLGESGEMDALRIQMEERFMAS
Nav1.8          PPLNLPQPNKLQLIAMDLPMVSGDRIHCLDILFAFTKRVLGESGEMDALRIQMEERFMAS
Nav1.9          PPLNLPQPNKLQLIAMDLPMVSGDRIHCLDILFAFTKRVLGESGEMDALRIQMEERFMAS
Nav1.2          PPLLIAKPNKVQLIAMDLPMVSGDRIHCLDILFAFTKRVLGESGEMDALRIQMEERFMAS
Nav1.3          PPLLIAKPNKVQLIAMDLPMVSGDRIHCLDILFAFTKRVLGESGEMDALRIQMEDRFMAS
Nav1.7          PPLLIAKPNKVQLIAMDLPMVSGDRIHCLDILFAFTKRVLGESGEMDSLRSQMEERFMSA
Nav1.6          HPLRVPKPNTIELIAMDLPMVSGDRIHCLDILFAFTKRVLGDSGELDILRQQMEERFVAS
Nav1.4          EPLRIAKPNKIKLITLDLPMVPGDKIHCLDILFALTKEVLGDSGEMDALKQTMEEKFMAA
Nav1.5          EPLRIAKPNQISLINMDLPMVSGDRIHCMDILFAFTKRVLGESGEMDALKIQMEEKFMAA
                 ** :.:** :.** :*****.**:***:*****:**.***:***:* *:  **::*:::

Nav1.1          NPSKVSYQPITTTLKRKQEEVSAVIIQRAYRRHLLKRTVKQASFTYNKNKIK--GGANLL
Nav1.8          NPSKVSYQPITTTLKRKQEEVSAVIIQRAYRRHLLKRTVKQASFTYNKNKIK--GGANLL
Nav1.9          NPSKVSYQPITTTLKRKQEEVSAVIIQRAYRRHLLKRTVKQASFTYNKNKIK--GGANLL
Nav1.2          NPSKVSYEPITTTLKRKQEEVSAIIIQRAYRRYLLKQKVKKVSSIYKKDKGK--ECDGTP
Nav1.3          NPSKVSYEPITTTLKRKQEEVSAAIIQRNFRCYLLKQRLKNISSNYNKEAIK--GRIDLP
Nav1.7          NPSKVSYEPITTTLKRKQEDVSATVIQRAYRRYRLRQNVKNISSIYIKDGDR--D-DDLL
Nav1.6          NPSKVSYEPITTTLRRKQEEVSAVVLQRAYRGHLARR--------------------GFI
Nav1.4          NPSKVSYEPITTTLKRKHEEVCAIKIQRAYRRHLLQRSMKQASYMYRHSHDG--SGDDAP
Nav1.5          NPSKISYEPITTTLRRKHEEVSAMVIQRAFRRHLLQRSLKHASFLFRQQAGSGLSEEDAP
                ****:**:******:**:*:*.*  :** :* :  ::                    .

Nav1.1          IKEDMIIDRI-------NENSITEKTDLTMSTAACPPSYDRVTKPIVEK---------HE
Nav1.8          IKEDMIIDRI-------NENSITEKTDLTMSTAACPPSYDRVTKPIVEK---------HE
Nav1.9          IKEDMIIDRI-------NENSITEKTDLTMSTAACPPSYDRVTKPIVEK---------HE
Nav1.2          IKEDTLIDKL-------NENSTPEKTDMTPSTTS-PPSYDSVTKPEKEK---------FE
Nav1.3          IKQDMIIDKL-------NGNSTPEKTDGSSSTTS-PPSYDSVTKPDKEK---------FE
Nav1.7          NKKDMAFDNV-------NENSSPEKTDATSSTTS-PPSYDSVTKPDKEK---------YE
Nav1.6          CKKTTSNKLE-------NGGTHREKKESTPSTAS-LPSYDSVTKPEKEKQQR------AE
Nav1.4          EKEGLLANTMSKMYGHENGNSSSPSPEEKGEAGDAGPTMGLMPISPSDT--A------WP
Nav1.5          EREGLIAYVMSEN----FSRPLGPPSSSSISSTSFPPSYDSVTRATSDNLQVRGSDYSHS
                 ::                .     . . .:    *: . :. .  :.
```

```
Nav1.1          QEGKDEKAKGK-------
Nav1.8          QEGKDEKAKGK-------
Nav1.9          QEGKDEKAKGK-------
Nav1.2          KDKSEKEDKGKDIRESKK
Nav1.3          KDKPEKESKGKEVRENQK
Nav1.7          QDRTEKEDKGKDSKESKK
Nav1.6          EGRRERAKRQKEVRESKC
Nav1.4          PAPPPGQTVRPGVKESLV
Nav1.5          EDLADFPPSPDRDRESIV
```

## 9.4 Curriculum Vitae

**PERSONAL DETAILS**


| | |
|---|---|
| First Name | Gowri Shankar |
| Last Name | Bagavananthem Andavan |
| Date of Birth | 25 November 1983 |
| Place of Birth | Chennai, India |
| Nationality | Indian |
| Email | gowri.bagavananthem.andavan@univie.ac.at |


**ACADEMIC PROFILE**

| | INSTITUTE | YEAR |
|---|---|---|
| Primary and Secondary Schooling | Sindhi Model Senior Secondary School, Chennai India. | June 1988 - April 1999 |
| Higher school Certificate | Jaigopal Garodia Higher Secondary School Chennai, India | June 1999 - April 2001 |
| Bachelor of Technology | Centre for Biotechnology (CBT), Anna University, Chennai, India. | June 2001 - May 2005 |
| Master of Science (Research) | University of Sydney, Sydney, Australia. | March 2006 - March 2007 |

**GRANT**

1. **First Contact Initiative Grant from European Society of Cardiology (ESC)**.Feb 2011-March 2011.

**SCIENTIFIC PAPERS**

1. **Voltage Gated Sodium Channels: mutations, channelopathies and subtype specific targets**
   **Gowri Shankar Bagavananthem Andavan** and Rosa Lemmens-Gruber
   *Current Medicinal Chemistry*, **2011**, 18(3):377-97.

2. **Essential structural features of TNF-α lectin-like domain derived peptides for activation of amiloride-sensitive sodium current in A549 cells.**
   Parastoo Hazemi, Susan J. Tzotzos, Bernhard Fischer, **Gowri Shankar Bagavananthem Andavan**, Hendrick Fischer, Helmut Pietschmann, Rudolf Lucas, Rosa Lemmens-Gruber.
   *Journal of Medicinal Chemistry*, **2010**, 53(22):8021-9.

3. **Cyclodepsipeptides from marine sponges: natural agents for drug research.**
   **Gowri Shankar Bagavananthem Andavan** and Rosa Lemmens-Gruber
   *Marine Drugs*, **2010**, *8*(3), 810-834.

4. **A Structural and Phylogenetic Analysis of Cytochrome P450**
   **B.A.Gowri Shankar**, Michael Charleston, Michael Murray and David Hibbs *International Proceedings of the 15th International Conference on Cytochrome P450: Biochemistry, Biophysics, Functional Genomics*, **2007**, 75-80.

5. **Ion-pairs in non-redundant protein structures**
   **B.A.Gowri Shankar**, R.Sarani, Daliah Michael, P.Mridula, C.Vasuki Ranjani, G.Sowmiya, B.Vasundhar, P.Sudha, J.Jeyakanthan, D.Velmurugan and K.Sekar. *J. Biosci.*, **2007**, *32(4)*, 693-704.

## POSTER PRESENTATIONS

1. **ESC Congress 2010,** 28 August - 1 September, Stockholm, Sweden. ***Title*****:** *Modulation of cardiac voltage gated sodium channel splice variants with its auxiliary subunit by Fyn tyrosine kinase.*

2. **Frontiers in Cardiovascular Biology**, Berlin, Germany, 16-19 July 2010, ***Title:*** *Cardiac sodium channel variants and fyn tyrosine kinase modulation.*

3. **XX$^{th}$ World Congress of the International Society of Heart Research 2010,** May 13-16 2010, Kyoto, Japan. ***Title:*** *Fyn tyrosine kinase modulation on cardiac voltage gated sodium channel splice variants.*

4. **54$^{th}$ Biophysical Society's Annual Meeting** at The Moscone Convention Center 20-24 February 2010, San Francisco, California. ***Title:*** *Modulation of $Na_v1.5$ varients by Src tyrosine kinase.*

5. **15th International Conference on Intelligent System for Molecular Biology (ISMB)** *Jointly with* **European Conference on Computational Biology (ECCB)** on 21-25 July, 2007, Vienna, Austria.

6. **11th Annual International Conference on Research in Computational Molecular Biology (RECOMB 2007),** 21-25 April, 2007, San Francisco Bay, California.

7. **Fifth Asia-Pacific Bioinformatics Conference**, 15-17 January, 2007, Hong Kong.

8. **4th Annual Rocky Mountain Bioinformatics Conference**, 1-6 December, 2006, Aspen/Snowmass, Colorado.

9. **Bioinformatics Australia 2006,** Sydney, 21-22 November, 2006.

## SCHOLARSHIPS

1. Travel grant from the **ESC Council on Basic Cardiovascular Science** to attend ESC Congress 2010, Stockholm, Sweden.
2. International **ISHR Travel Grant** to attend XXth World Congress of the International Society of heart Research 2010, Kyoto, Japan.
3. International Travel Award from **Biophysical Society** to attend and present my data in 54th Annual Meeting at San Francisco, California.
4. Travel scholarship from **RECOMB 2007** committee, to attend RECOMB 2007 in San Francisco Bay, California, USA.
5. BioinfoSummer 2006, **ICE-EM Scholarship** to attend summer symposium in Bioinformatics, ANU, Canberra; Australia.
6. Travel scholarship to attend **PhD Winter School** in Data Mining & Bioinformatics, Lorne, Victoria, Australia
7. Travel scholarship to attend **APAC Summer School in Computational Science** 2006, Brisbane, Australia.
8. Chief Minister Meritorious Award to students to continue their undergraduate studies, Chennai, India.

## TEACHING

- Demonstrating **Medicinal Chemistry** PHAR2632 in Faculty of Pharmacy, University of Sydney. (March 2007 – June 2007)
- Demonstrating **Medicinal Chemistry** PHAR2618 in Faculty of Pharmacy, University of Sydney. (July 2006 – November 2006)