Physical Mechanisms and Biological Consequences of Voltage-gated Sodium Channel Modulation by FHF Proteins

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Physical Mechanisms and Biological Consequences of Voltage-gated Sodium Channel Modulation by FHF Proteins

By Yue Liu

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2017
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Voltage-gated Sodium Channel Modulation by FHF Proteins

By Yue Liu

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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THE CITY UNIVERSITY OF NEW YORK
Abstract

Physical Mechanisms and Biological Consequences of Voltage-gated Sodium Channel Modulation by FHF Proteins

By Yue Liu  Thesis Advisor: Dr. Mitchell Goldfarb

Purpose: FHF1A and FHF1B were compared to derivative proteins bearing the epilepsy-associated mutation for their ability to raise the voltage dependence of Nav inactivation. We found that the epilepsy missense FHF1 mutation is gain-of-function, enabling aberrant FHF1 isoforms to further elevate the voltage at which sodium channels inactivate. These findings offer a clear rationale for how the mutation promotes epilepsy. To investigate the physical mechanism and biological consequences of A-FHF-mediated Nav LTI, we did functional testing of A-FHF proteins bearing amino acid substitutions along with microinjection of an antibody that specifically blocks A-FHF-mediated Nav LTI. We found that A-FHF proteins bear an N-terminal motif that employs cationic and aliphatic residues to induce and maintain the Nav long-term inactivated state. Furthermore, antibody blockade of Nav LTI
mediated by endogenous A-FHFs in hippocampal pyramidal neurons enhances neural excitability by suppressing spike frequency accommodation. **Conclusions:** FHFs can naturally promote or limit neuronal excitability. When the pro-excitatory function of FHF1 proteins is enhanced by mutation, epilepsy could be triggered. Neuronal excitability can also be enhanced if A-FHF mediated Nav LTI is suppressed.
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Abbreviations

Ab Antibody
A-FHF A-type fibroblast growth factor homologous factor
AIS Axon initial segment
BSA Bovine albumin serum
BSS Hank’s Balanced Saline Solution
CT C-terminus
CTD C-terminal domain
CV Conditioning voltage
DIV days in vitro
DMEM Dulbecco’s modified Eagle’s medium
DMSO Dimethyl sulfoxide
DRG dorsal root ganglion
ECL Enhanced chemiluminescence
EDTA Ethylenediaminetetraacetic acid
EOEE early-onset epileptic encephalopathy
ESC Embryonic stem cell
FBS Fetal Bovine Serum
FGF Fibroblast growth factor
FHF Fibroblast growth factor homologous factor
GEFS+ generalized epilepsy with febrile seizures plus
GFP Green fluorescent protein
GSK3 Glycogen synthase kinase 3
HBSS Hanks’ Balanced Saline Solution
HEK293T Human embryonic kidney cell line with SV40 Large T-antigen
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP Horseradish Peroxidase
Ig Immunoglobulin
IP Immunoprecipitation
IRES Internal ribosome entry site
LQT long-QT syndrome
MEK MAPK/ERK Kinase, Mitogen-activated protein kinase kinase
MEM Minimum Essential Media
Nav Voltage gated sodium channels
NeoR Neomycin resistance (selectable marker)
Neuro2A Neuroblastoma cell line
PBS Phosphate buffered saline
PCR Polymerase chain reaction
PGK Phosphoglycerate kinase
PVDF Polyvinylidene Fluoride
RV Reporting voltage
SCA human spinocerebellar ataxia
scFv Single-chain variable fragment
SFA Spike frequency accommodation
SK Calcium-activated small conductance potassium channel
TBS Tris buffered saline
TBST Tris buffered saline with 0.1% Tween 20
TEA Tetraethylammonium chloride
tpA Triple poly-adenylation sequence
TTX Tetrodotoxin
TTXr Tetrodotoxin resistant
VH Variable region of the heavy chain
VL Variable region of the light chain
WT Wild-type
Chapter 1 Introduction

1.1 Voltage-gated Ion Channels

Voltage-gated ion channels are transmembrane proteins that conduct ions through cell membrane in response to changes in membrane voltage (potential). These transmembrane proteins are crucial for excitable cells, including neurons and myocytes, to generate electrical events that allow neuron-to-neuron communication and muscular contraction. One of the characteristics of voltage-gated ion-channels is their selectivity to a specific type of ions, such as sodium (Na\(^+\)), potassium (K\(^+\)), calcium (Ca\(^{2+}\)), and chloride (Cl\(^-\)) (Catterall, 2000; Purves D, 2001).

1.1.1 Structure

Voltage-gated ion channels are organized into a structure forming a central pore through which ions can pass down electrochemical gradients. In addition to the central pore, two other important functional units of voltage-gated ion channels are the voltage sensor and the gate (Bezanilla, 2005). Voltage-gated potassium channels comprise four transmembrane α subunits (tetramers) forming a central pore, whereas voltage-gated sodium channels and calcium channels comprise a single pore-forming α subunit containing four homologous domains—a pseudotetramer. The transmembrane segments (S1-S6) are folded into α-helical structures. The fifth and sixth transmembrane segments (S5 and S6) form the selectivity filter and pore of the channel, and S1-S4 form the voltage sensor of the channel that underlies gating mechanisms (Bezanilla, 2005). Besides pore-forming α subunits of voltage-gated ion channels, there are also regulatory
proteins such as β subunits and fibroblast growth factor homologous factors (FHF s), which regulate the expression and functions of α subunits.

1.1.2 Functions

Voltage-gated ion channels dictate cell excitability. Voltage-gated sodium channel activation contributes to the rising phase of action potentials, while voltage-gated potassium channel activation, together with inactivation of sodium channels, are the major mediators for the falling phase of action potentials. In some neurons, generation of action potentials also rely on voltage-gated calcium channels. Besides generating electrical signals, calcium that flows into the cell can also affect a large range of intracellular biochemical processes, such as release of neurotransmitters at synapses and contraction of cardiomyocytes (Purves D, 2001).

1.2 Voltage-gated sodium channels

Voltage-gated sodium channels (Nav s) initiate the rising phase of action potentials in excitable cells, including neurons in the nervous system and myocytes in cardiac and skeletal muscle. Sodium current and its involvement in action potential were first identified by Nobel Prize laureates Hodgkin and Huxley in 1952 (Hodgkin and Huxley, 1952a; Hodgkin and Huxley, 1952b; Hodgkin and Huxley, 1952c; Hodgkin and Huxley, 1952d; Hodgkin and Huxley, 1952e) and the Nav protein was molecularly characterized in 1980’s by Catterall and his collaborators (Catterall, 2012).
1.2.1 Molecular Structure of NaVs and Functional Units

Figure 1 Schematic diagrams of molecular structures of voltage-gated sodium channels (A) Voltage-gated sodium channel (Na\textsubscript{v}) comprises a pore-forming \(\alpha\) subunit, which is composed of four homologous domains (DI~DIV). Each domain consists of six transmembrane segments (S1~S6). S5, S6, and the reentrant P loop (S5-P-S6, green) from each domain form a central conducting pore, and S4 segments of all domains (pink) form a voltage sensor. The hydrophobic residues isoleucine, phenylalanine, and methionine (IFM) at the intracellular linker between domain III and IV form a fast inactivation gate (yellow). (B) The \(\alpha\) subunit can be associated with accessory proteins, including \(\beta\) subunits (purple) and FHF\(\text{s}\) (red).

Na\textsubscript{v} comprises a large pore-forming \(\alpha\) subunit (Na\(\alpha\)) (~2000 amino acids, 260 kD) and modulatory proteins, including \(\beta\) subunits (Na\(\beta\)) and fibroblast growth factor homologous factors (FHF\(\text{s}\)).

**Organization of the \(\alpha\) Subunit**— The \(\alpha\) subunit consists of four homologous domains (DI~IV) (Catterall, 2000). Each domain is organized into six \(\alpha\)-helical transmembrane segments (S1~6) (Noda et al., 1984). The functional units of
Navα include a central pore, a selectivity filter, and a voltage sensor. S5, S6, and the reentrant P loop (S5-P-S6) from each domain form a central conducting pore (Figure 1, green) (Payandeh et al., 2011).

**Ion Selectivity Filter**—The selectivity filter comprises a ring of four key residues, aspartate, glutamate, lysine, and alanine, from the pore-forming domains (Sun et al., 1997). Unlike potassium channels that select K⁺ directly with a rather small pore (Zhou et al., 2001), Nav with a much larger pore selects hydrated Na⁺ ions that could fit in the high-field-strength site at the extracellular end of the pore, based on the crystal structure of sodium channels from bacteria (Payandeh et al., 2011).

**Voltage Sensor**—S4 segments of all domains form the voltage sensor (Figure 1, pink), which surrounds the central pore and contains cationic residues. Depolarization can trigger outward movement of the positively charged residues in the voltage sensor, which causes rolling movement of S1-S3 and bending and twisting of S5 and S6. This S4 movement-driven conformational change of the channel opens the ion-conducting central pore in a camera aperture-like manner (Catterall, 2010; Catterall, 2014b; Cummins et al., 1993; Pathak et al., 2007).

**Fast Inactivation Gate**—Within milliseconds following the conformational change of Nav toward activation, the fast inactivation gate obstructs the conducting pore and prevents sodium influx (Catterall, 2000; Goldfarb, 2012). The fast inactivation gate is formed by the hydrophobic residues isoleucine, phenylalanine, and methionine (IFM) on the intracellular linker between domain III and IV (Figure 1, yellow) (West et al., 1992). Nav can recover from fast inactivation upon repolarization, which propels inward
movement of the voltage sensor. This dislocation of the voltage sensor deactivates \( \text{Nav} \) and disrupts the \( \text{Nav} \)'s structure that is locked in the fast inactivation state. Therefore, \( \text{Nav} \) must deactivate first before recovery from fast inactivation (Kuo and Bean, 1994).

**Various Isoforms of the \( \alpha \) subunit**— In mammalian cells, nine isoforms of \( \text{Nav}\alpha \) have been identified. The encoded channels show some differences in their structure, function, and distribution. Of all nine isoforms, \( \text{Nav}1.4 \) is selectively expressed in the skeletal muscle; \( \text{Nav}1.5 \), in cardiomyocytes; \( \text{Nav}1.7 \), \( \text{Nav}1.8 \), and \( \text{Nav}1.9 \) are preferably expressed in primary sensory neurons in the dorsal root ganglion (DRG); and the rest are expressed both in the central nervous system and peripheral nervous system (Kwong and Carr, 2015). The conductance of \( \text{Nav}1.1–1.4 \), \( \text{Nav}1.6 \), and \( \text{Nav}1.7 \) are sensitive to tetrodotoxin (TTX), whereas \( \text{Nav}1.5 \), 1.8, and 1.9 are TTX-resistant (Catterall et al., 2005).

**\( \beta \) subunits**— The mammalian genome encodes four types of \( \beta \) subunits (\( \beta1–\beta4 \)), which can modulate the gating of \( \text{Nav} \) and stabilize their localizations (Catterall, 2012). \( \beta \) subunit is a single \( \alpha \)-helical transmembrane protein, containing an extracellular N-terminal region with an immunoglobulin (Ig) domain (Brackenbury and Isom, 2011). As accessory proteins of the pore-forming \( \alpha \) subunits, \( \beta \) subunits function to modulate the trafficking, gating (Calhoun and Isom, 2014), and pharmacology of the channel (Lenkowski et al., 2003). The extracellular Ig domain suggests that \( \beta \) subunits may also function as cell adhesion molecules and contribute to neurites growth (O'Malley and Isom, 2015).
1.2.2 Four Types of Nav Inactivation

Navα can be in closed, activated (open) and inactivated states. Inactivation is a state that occurs during depolarization and prevents channel conduction until recovery transition follows membrane repolarization. The precise and dynamic transitions among channels’ closing, activation and inactivation control membrane excitability and enable proper functions of neurons and myocytes.

Four types of inactivation have been characterized (Table 1): fast inactivation mediated by an intrinsic α subunit intramolecular mechanism, classical slow inactivation also mediated by α subunit-intrinsic mechanism, open-channel block and resurgent unblock mediated by β4 subunit, and fast-onset long-term inactivation mediated by A-type variants of FHF proteins. Except for slow inactivation, the other three types of inactivation occur within milliseconds but differ in recovery time. Fast inactivation is caused by the intrinsic particle located between DIII and DIV (West et al., 1992). On the contrary, slow inactivation is mediated by the voltage sensor in S4 of four domains (Silva and Goldstein, 2013a; Silva and Goldstein, 2013b; Ulbricht, 2005). Open-channel block is induced by β4 subunit, which only docks into the pore of open channels. However, upon repolarization, the docked β4 subunit will be expelled immediately thus the channels become available at that moment and generate a transient resurgent current prior to deactivation (return to the closed state). Open-channel block is pro-excitatory and facilitates high-frequency firing (Grieco et al., 2005; Raman and Bean, 1997), which may be due to the resurgent sodium current generated upon repolarization or simply by the near-instantaneous return of channels to an available closed state. Lastly, the N-termini of
A-type isoforms of FHFs (A-FHFs) can capture sodium channels into a long-term inactivated state (Dover et al., 2010; Goldfarb, 2012).

**Table 1** Comparison of four types of $\text{Na}_V$ inactivation

<table>
<thead>
<tr>
<th>$\text{Na}_V$ Inactivation</th>
<th>Types</th>
<th>Effector</th>
<th>Onset speed</th>
<th>Recovery speed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intrinsic to $\text{Na}_V\alpha$</td>
<td>Fast inactivation</td>
<td>DIII~DIV loop</td>
<td>Fast</td>
<td>fast</td>
</tr>
<tr>
<td></td>
<td>Slow inactivation</td>
<td>Voltage sensor in S4</td>
<td>Slow</td>
<td>very slow</td>
</tr>
<tr>
<td>Induced by accessory proteins</td>
<td>Open-channel block</td>
<td>$\text{Na}_V\beta 4$</td>
<td>Fast</td>
<td>very fast</td>
</tr>
<tr>
<td></td>
<td>Long-term inactivation</td>
<td>A-FHFs</td>
<td>Fast</td>
<td>slow</td>
</tr>
</tbody>
</table>

**1.2.2.1 Fast Inactivation**

Within milliseconds following activation, $\text{Na}_V$ fast inactivation occurs when the inner mouth of the channel’s pore is blocked by the intracellular loop connecting DIII and DIV (Stuhmer et al., 1989; West et al., 1992). This loop between $\text{Na}_V$ DIII and DIV directly participates in fast inactivation, since antibodies against this loop slow the $\text{Na}_V$ fast inactivation process (Vassilev et al., 1988). Mutations of the three clustered hydrophobic residues in the DIII/DIV loop, IFM (Figure 1, yellow), completely abolish fast inactivation (West et al., 1992). Mutations in the DIII/DIV loop in the cardiac $\text{Na}_V$ affect fast inactivation and cause congenital Long QT syndrome (LQT) (Bennett et al., 1995; Wang et al., 1995), an inherited disease that predisposes patients to sudden death from cardiac arrhythmias.
The voltage dependence of Nav fast inactivation can be shifted by accessory proteins. Navβ1 can cause a negative shift of the fast inactivation’s voltage dependence (An et al., 1998; Spampanato et al., 2004; Wallner et al., 1993), whereas most FHFs cause a positive shift (Dover et al., 2010; Lou et al., 2005; Wittmack et al., 2004). Accordingly, Navβ1 promotes faster inactivation and reduces sodium current, whereas FHFs impede fast inactivation and enhance sodium current. However, the physical mechanisms by which these accessory proteins modulate Nav fast inactivation are unknown.

1.2.2.2 Slow Inactivation

In contrast to fast inactivation with a rapid onset, slow inactivation develops in hundreds of milliseconds and the recovery process is very slow (Rudy, 1978). Slow inactivation is believed to result from conformational changes in the ion selectivity filter (Balser et al., 1996; Todt et al., 1999), S6 segment (Chen et al., 2006), and the voltage sensor (Silva and Goldstein, 2013a; Silva and Goldstein, 2013b). According to crystal structures of sodium channels in a slow-inactivated state from bacteria, two of the S6 segments move toward the center of the pore, and two of the S6 segments, on the contrary, move away. The asymmetric movements of S6 segments cause the conducting pore to collapse, which may contribute to the stability of the slow-inactivated state of Nav (Payandeh et al., 2012; Zhang et al., 2012).

1.2.2.3 Long-term Inactivation

FHF2A was shown to cause a rapid-onset inactivation of Nav with a slow recovery rate (Rush et al., 2006). Later, our lab identified that all A-FHFs can induce a
rapid-onset inactivation with slow recovery via the highly conserved N-termini, in competition with fast inactivation. Due to this type of inactivation’s rapid onset and slow recovery, our lab termed it as long-term inactivation (LTI) to distinguish it from slow inactivation that relies on a different mechanism (Dover et al., 2010).

1.2.2.4 Open-channel Block

Open-channel block is induced by the β4 subunit, (Lewis and Raman, 2014), which only blocks open channels (Wang et al., 2006). However, upon repolarization, the associated β4 subunit will be expelled immediately before deactivation can occur. Therefore, upon the dissociation of β4 subunit, the channel briefly reopens and generates a resurgent current. Sodium channels that can generate resurgent current can enhance high-frequency firing of neurons (Grieco et al., 2005; Raman and Bean, 1997). While resurgent current itself has been offered as the basis for enhanced excitability (Grieco et al., 2005; Raman and Bean, 1997), the mechanism for enhanced excitability may more simply be due to the instantaneous recovery of channels to a non-inactive, available state. β4 subunit-mediated open-channel block competes with the channel’s intrinsic fast inactivation, since compromised fast inactivation enhances resurgent current by favoring β4 block of the channel (Lewis and Raman, 2013; Wang et al., 2006).

1.2.3 Nav Blockers and Toxins

Navs are targets of many local anesthetics, anticonvulsants, and antiarrhythmics. Many Nav blockers, including lidocaine, amitriptyline, and lamotrigine, are marketed as medications to treat pain and cough (Fertleman et al., 2006; Fischer et al., 2009; Slaton et
al., 2013). However, these drugs block various isoforms of Na universally, and the lack of selectivity of these blockers raises safety issues since they risk blocking cardiac and muscular sodium channels. Topical administration of these blockers could be a solution. Moreover, pharmaceutical companies are also developing sodium channel blockers or antibodies that specifically target a specific isoform.

Resurgent current has been shown to enhance neuronal excitability and facilitate high-frequency firing (Grieco et al., 2005; Raman and Bean, 1997). This pro-excitatory resurgent current may be involved in paroxysmal extreme pain disorder (Theile et al., 2011). Interestingly, anandamide could selectively block resurgent current (Theile and Cummins, 2011), which could be a therapeutic reagent candidate against this disease.

Navs are also targets of neurotoxins secreted by a variety of animals, including cone snail, puffer fish, scorpions, spiders, and octopi, as a defense strategy in nature. These toxins can either decrease or increase channel activity by blocking the pore, decreasing fast inactivation, or causing a negative shift in the voltage dependence of activation (Catterall et al., 2007; Stevens et al., 2011).

1.2.4 Nav Functions and Diseases

Navs are selectively permeable to sodium ions in response to depolarization of membrane potential. Sodium influx further depolarizes membrane potential. Therefore, Navs mediate the rising phase of action potentials in excitable cells and thus are important in generating and conducting electrical signals. Mutations of Nav can lead to various inherited disorders termed as sodium channelopathies, such as epilepsy (Catterall, 2014a), chronic pain (Dib-Hajj et al., 2007), and arrhythmia (Terrenoire et al., 2007).
In the central nervous system, Navs are critical in excitation-inhibition balance of the brain. Since Navs are expressed in both excitatory and inhibitory neurons, enhanced Nav activity in these two populations of cells will have entirely opposite effect on the overall excitation-inhibition balance. Indeed, both loss-of-function and gain-of-function mutations in genes encoding pore-forming α subunits of Nav have been reported in epilepsy (Catterall et al., 2010; Estacion et al., 2014; Kamiya et al., 2004; Veeramah et al., 2012). In the peripheral nervous system, Navs are crucial in sensory transmission, and dysfunctions of Navs can lead to diseases such as pain disorders. For example, Nav1.7, Nav1.8, and Nav1.9, expressed in DRG sensory neurons, are important in human pain disorders (Dib-Hajj et al., 2015; Waxman, 2013) and are vital drug targets for pain treatment. Considering the trafficking of these channels from the endoplasmic reticulum to plasma membrane, excessive trafficking in pathological conditions may be a therapeutic target to treat pain disorders (Bao, 2015). In the heart, loss-of-function Nav1.5 mutations have been reported to cause Brugada syndrome (Calleo et al., 2013; Nielsen et al., 2013), whereas gain-of-function mutations in Nav1.5 causes Long QT syndrome (Bennett et al., 1995; Wang et al., 1995).

Moreover, Navs are also expressed in non-excitable cells, such as astrocytes, microglia, macrophages, and cancer cells, which suggests “noncanonical roles” of Navs (Black and Waxman, 2013). Blocking Navs can significantly affect many functions and processes in these non-excitable cells—phagocytosis of macrophages (Carrithers et al., 2009); migration of dendritic cells (Kis-Toth et al., 2011), microglia (Black et al., 2009), oligodendrocyte precursor cells (Tong et al., 2009), and T lymphocytes (Fraser et al., 2004); and invasiveness and metastasis of cancer cells (Fraser et al., 2005; Yildirim et al., 2005).
2012). However, how Navs contribute to and regulate these processes remains largely unknown.

1.3 Fibroblast Growth Factor Homologous Factors (FHF)

Fibroblast growth factor homologous factors (FHF) are cytosolic proteins that share strong sequence and structure similarity to fibroblast growth factors (FGF). However, the biochemical and functional characteristics of these two groups of proteins are quite different from each other. FGFs are secreted extracellularly, whereas FHF remains in the cytoplasm and bind to various protein targets, including Navs. My research focuses on studying FHF/Nav interaction and its functional consequences.

1.3.1 FHF Are Structurally but Not Functionally Homologous to FGF

FHF bears sequence and structural homology to FGF. However, the biochemical and functional characteristics of these protein families appear to be completely different (Olsen et al., 2003). The homologous region is folded into a \( \beta \)-trefoil structure, and this region of FHF is tethered to the Nav tail, whereas that of FGF cannot bind with Nav (Goldfarb, 2005). FGFs are secreted and bind to receptors on the cell membrane and act as mitogens, chemo-attractants, and mediators of cellular differentiation (Goldfarb, 1990). In contrast, cytosolic proteins FHF artificially applied extracellularly cannot activate any of the FGF receptors (Olsen 2003). Instead, FHF bind to various protein targets in the cytoplasm, including Junctophilin-2 (Hennessey et al., 2013b), IB2 Kinase Scaffold (Schoorlemmer and Goldfarb, 2001), and most, if not all, Nav isoforms (Dover et al., 2010; Liu et al., 2001; Liu et al., 2003; Lou et al., 2005; Rush et al., 2006) to modulate their functions.
1.3.2 FHF Genes, Isoforms, and Expression

Four genes encoding FHFs in vertebrates have been characterized: \textit{fhf1 (fgf12)}, \textit{fhf2 (fgf13)}, \textit{fhf3 (fgf11)}, and \textit{fhf4 (fgf14)}. For all genes, alternative transcription initialization sites and alternative splicing of mRNA give rise to at least two protein isoforms, A and B, with different N-termini for each gene. The \textit{fhf2} gene encodes five distinct isoforms (Munoz-Sanjuan et al. 2000). Different neurons in the nervous system express different repertoire of FHFs. For example, cerebral cortical neurons and hippocampal pyramidal neurons express all four FHFs, cerebellar granule neurons and spinal motor neurons do not express FHF2, and FHF4 is not present in periphery sensory neurons (Atlas; Goldfarb, 2005)(Allen Brain Atlas).

1.3.3 FHF Functions and Human Diseases

1.3.3.1 FHFs—a Double-edged Sword: Interaction with Nav

FHFs can affect membrane excitability of excitable cells through modulating the gating of sodium channels. FHFs are tethered to Nav in the cell through the binding between the channel’s C-terminal domain (CTD) and the \(\beta\)-trefoil core conserved in all FHFs (Goetz et al., 2009; Wang et al., 2012). Interestingly, most of the residues in the \(\beta\)-trefoil core of FHFs involved in the binding with Nav\(\alpha\) are diverged in FGFs, which do not exhibit Nav\(\alpha\) binding capacity (Goetz et al., 2009). Tethered to the tail of Navs, FHFs can modulate the availability of sodium channels either through impeding fast inactivation or by inducing long-term inactivation (LTI) (Dover et al., 2010; Goldfarb et al., 2007; Rush et al., 2006).
**FHFs enhance the membrane excitability by impeding Nav fast inactivation** — In the presence of most FHFs, the intrinsic fast inactivation of Navs occurs at a more positive membrane potential (FHFs cause a positive shift of \( V_{1/2} \) for fast inactivation) and the recovery from fast inactivation is faster, thus there are more sodium channels available at a given voltage and time (Lou et al., 2005; Rush et al., 2006; Wittmack et al., 2004). A consequence of FHFs’ impediment to fast inactivation is enhanced neuronal excitability. This has been clearly demonstrated in \( Fhf4^{-/-} \) and \( Fhf1^{-/-}Fhf4^{-/-} \) cerebellar granule and Purkinje cells as loss of repetitive firing and the requirement of greater current input to drive spiking (Bosch et al., 2015; Goldfarb et al., 2007; Shakkottai et al., 2009). It has been shown that mutations in the channel-binding interface of the \( \beta \)-trefoil core deprive FHFs of their modulatory function on fast inactivation (Goetz et al., 2009). If FHF modulation of Nav fast inactivation is compromised, sodium current will be decreased and membrane excitability will be reduced—which may lead to neurological disorders. A missense mutation in \( FHF4 \) has been identified in patients with spinocerebellar ataxia (van Swieten et al., 2003) the disease phenotypes have been recapitulated in mice with this mutation (Goldfarb et al., 2007). Recently, a collaborative study between our and Glenn Fishman’s labs show that FHF2 protects mice against fever-induced conduction failure and cardiac arrhythmia by inhibiting temperature-dependent increase of Nav1.5’s fast inactivation rate (Park et al., 2016). FHF2 loss of function has also been shown to cause temporal lobe epilepsy in mice (Puranam et al., 2015).

**A-FHFs reduce membrane excitability by causing Nav LTI** — In contrast, A-type FHFs (A-FHFs) can decrease Nav\( \alpha \) availability by capturing sodium channels into a long-term inactivated (LTI) state (Table 1) (Dover et al., 2010; Rush et al., 2006). The
rapid-onset Navα inactivation with long-term recovery was first discovered in hippocampal neural dendrites and was suggested to promote dendritic spike attenuation in these cells (Colbert et al., 1997; Jung et al., 1997). Thereafter, FHF2A was shown to cause rapid-onset LTI of Navα (Rush et al., 2006). Our lab has demonstrated that all A-FHFs can induce LTI of sodium channels via a highly conserved N-terminal domain in competition with intrinsic fast inactivation (Dover et al., 2010).

Accordingly, FHFs are a double-edged sword, since they can either increase Navα availability/neuronal excitability by impeding fast inactivation via β-trefoil core or decrease it by capturing sodium channels into LTI via the N-termini conserved in all A-FHFs.

1.3.3.2 Other Functions of FHFs

Interaction with IB2 Protein — In addition to Nav, FHFs can also bind to Islet brain-2 (IB2) (Schoorlemmer and Goldfarb, 2002), a putative MAP kinase scaffold protein in brain and pancreatic islet cells (Negri et al., 2000). FHFs may serve as cofactors to recruit the MAP kinase to scaffold proteins (Goldfarb, 2005). Another hypothetical function for FHF/IB2 interaction is that IB2 may deliver FHFs to presynaptic terminals. The presynaptic localization is critical for FHF4 to control parallel fiber–Purkinje neuron synaptic transmission in the cerebellum (Tempia et al., 2015) and Schaffer collateral–CA1 synaptic plasticity in the hippocampus (Xiao et al., 2007).

Interaction with Junctophilin-2 Protein — In addition to Nav and IB2, a third type of protein that FHFs can bind to is junctophilin-2, which can organize the localization of L-type Ca^{2+} channels in cardiomyocytes. Knockdown of FHF2 results in
aberrant localization of calcium channels and reduces calcium current, which leads to shortened cardiac action potentials (Hennessey et al., 2013b).

1.3.3.3 FHFs and Human Diseases

Reports for human diseases involving FHF gene mutations are limited. *FHF4* gene mutation has been reported in the neurodegenerative disorder spinocerebellar ataxia (SCA) (van Swieten et al., 2003) through a dominant-negative reduction of Na⁺ (Laezza et al., 2007) and Ca²⁺ currents (Yan et al., 2013), which affects neuronal intrinsic excitability (Goldfarb et al., 2007) and synaptic transmission, respectively. In addition, X-chromosome deletion in *FHF2* gene was found in Wildervanck syndrome (Abu-Amero et al., 2014); Translocation between chromosomes X and 14 with a breakpoint on the X chromosome affecting *FHF2* gene was reported in genetic epilepsy and febrile seizures plus (GEFS+) (Puranam et al., 2015).

Interestingly, a mutation in cardiac sodium channel Nav1.5 that affects FHF interaction leads to atrial and ventricular arrhythmia, cardiac arrest, and sudden cardiac death. The mutant channel shows gain-of-function in primary myocytes: weaker interaction with FHFs leads to a larger positive shift of voltage-dependence of fast activation and larger sodium current (Musa et al., 2015).

1.4 Mechanisms of FHF Functions in Normal and Epileptic Brain

FHFss control neuronal excitability by modulating Nav function. FHF4s raise the voltage dependence of Nav fast inactivation to enhance excitability, whereas A-FHF4s also
capture Navs into LTI, potentially to reduce excitability. My doctoral research focuses on addressing FHF balance of neuronal excitability in normal and epileptic brains.

Our collaborator Dr. Gunnar Buyse had found a single missense mutation affecting the β-trefoil core of FHF1 in epileptic patients, and Chapter 3 will illustrate the roles of the mutant FHF1 proteins in the epileptic brain by using genetic, molecular, and electrophysiological approaches. Chapter 4 will describe physical mechanisms and biological functions of A-FHF-induced Nav LTI. The following questions will be addressed: 1) What are the essential individual residues in the A-FHF LTI particle? 2) Does the A-FHF LTI particle function as an open-channel blocker? 3) What are the cellular and subcellular expression patterns of A-FHFs? 4) What are biological functions of Nav LTI? Chapter 5 is the continuation of the two projects described in Chapter 3 and 4, and it will explore genetic strategies to further study physiological functions of FHFs and their connections to disease phenotypes.

My research aims to provide better insights into understanding Nav fast inactivation and LTI mechanisms and functions. This knowledge may provide significant diagnostic and therapeutic potential for various diseases, such as epilepsy, arrhythmias, and pain disorders.
Chapter 2 Methods

2.1 Plasmids and Mutagenesis

The cDNA of TTX-resistant murine sodium channel \( \text{Nav}1.6^{\text{TTXr}} \) (\( \text{Nav}1.6^{Y371S} \)) was cloned into the bicistronic vector pIRESneo3 (Clontech) previously (Dover et al., 2010; Rush et al., 2006). Human \( \text{Nav}1.5 \) cDNA in the pcDNA3.1 vector was provided by R. Kass. Fast-inactivation-defective sodium channels were generated by substituting an essential residue in the DIII/DIV inactivation loop, F1478 in \( \text{Nav}1.6^{\text{TTXr}} \) and F1486 in \( \text{Nav}1.5 \) to glutamine (Q) (Catterall, 2000; Dover et al., 2010; Eaholtz et al., 1999; West et al., 1992). Point mutations were introduced with complementary mutagenic primers and the PfuTurbo DNA polymerase (Stratagene). Construction of FHF and ZsGreen1 Fluorescent Proteins into the bicistronic vector pIRES2-ZsGreen1 (Clontech) was described previously (Catterall, 2000; Dover et al., 2010). FHF2A mutations (I5A, L9A, I10A, R11Q, K13Q, R14Q, R17Q, R11Q/R14Q, R11Q/R17Q, and R14Q/R17Q), FHF1A mutation (R114H), and FHF1B mutations (R52H, R52A, and R52G) were generated with complementary mutagenic primers. Plasmids with desired mutations were confirmed by DNA sequencing. Cre expressing plasmid, pBS-CMV-NLS-Cre, was a gift from K. Kelly. pBigT and pROSA26-PA plasmids were from the lab of F. Costantini (Srinivas et al., 2001).

2.2 Peptides

N-terminally acetylated peptide corresponding to FHF2A residues 2–18 (AAAIASSLIRQKRQARE; F2A2-18) and unmodified peptide corresponding to Navβ4
residues 154-167 (KKLITFILKKTREK; β4_{154-167}) were custom synthesized, purified by high-performance liquid chromatography, and confirmed by mass spectroscopy (ChinaPeptides).

2.3 Antibodies

2.3.1 Monoclonal and Polyclonal Antibodies

Mouse anti-Pan-FHF-A monoclonal antibody (NeuroMab clone N235/22) (IgG_{2b}) was generated against F2A_{2-18} peptide in collaboration with the University of California (UC) Davis/NIH NeuroMab Facility. Rabbit anti-FHF2 C terminus polyclonal antibody was produced previously (Schoorlemmer and Goldfarb, 2002). Mouse anti-ankyrin G monoclonal antibody (IgG1) was purchased from Santa Cruz Biotechnology. Rabbit anti-GFP polyclonal antibody (IgG) was from Abcam. Mouse Anti-Pan-Sodium Channel monoclonal antibody (IgG1) was from Sigma. Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Jackson ImmunoResearch. Fluorescent secondary antibodies along with TOPRO iodide were purchased from Invitrogen.

2.3.2 Single-chain Variable Fragment (scFv)

Single-chain variable fragment (scFv), also termed intrabody, can be expressed intracellularly and thus can be used for immunizing cytosolic proteins. We created an scFv against the N-termini of A-FHFs. It was generated by connecting the variable regions of heavy (VH) and light chains (VL) from the monoclonal antibody connected with a short flexible linker. For more details, please refer to Section 5.2 in Chapter 5.
2.4 Transfection

2.4.1 Nav and FHF Expression in Neuro2A cells

Neuro2A cells were cultured in DMEM medium supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 4 mM L-glutamine, and 5% fetal bovine serum (FBS). Nav and FHFs were expressed in Neuro2A cells by Lipofectamine (LFN2000) (Invitrogen)-mediated plasmid transfection (Dover et al., 2010; Lou et al., 2005) at a 2:1 ratio of Nav- and FHF-expressing plasmids. For immunoblot of FHFs, transfected cells were lysed after 24 h culture. For electrophysiology, transfected cells were trypsinized, plated onto coverslips, and maintained for 24–48 h before transfer to a recording chamber.

2.4.2 Nav, FHF, and scFv Expression in HEK293T cells

HEK293T cells were cultured in DMEM medium supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 4 mM L-glutamine, and 5% FBS. Nav, FHFs, and EGFP-scFv were expressed in cells by Lipofectamine-mediated transfection at a 4:2:4 ratio of Nav-, FHF-, and scFv-expressing plasmids. For coimmunoprecipitation of EGFP-scFv and FHFs, transfected cells were lysed after 48 h culture.

2.5 Western Blot

Transfected cells were rinsed with ice-cold PBS and lysed in ice-cold Triton lysis buffer (20 mM Tris pH7.4, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 2 mM Na pyrophosphate, 1 mM Na orthovanadate, 10% glycerol, 1% Triton X-100, and freshly added 1 mM phenylmethanesulfonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml...
leupeptin) (Whitmarsh et al., 1998). Protein concentration was measured with Bradford assay. Cell lysates were then electrophoresed through precast 4% to 20% gradient polyacrylamide gels (Pierce Protein Biology Product, Thermo Scientific), transferred to PVDF membranes, and blocked in Tris buffered saline with 0.1% Tween 20 (TBST) containing 10% FBS. The PVDF membranes were incubated with TBST containing 5% bovine albumin serum (BSA) and primary antibody (1 μg/ml), rinsed three times with TBST, incubated with TBST containing 5% BSA and HRP-conjugated secondary antibody (1:5000), and rinsed three times in TBST and twice in TBS. The immunosignals were detected with enhanced chemiluminescent (ECL) substrate and autoradiography.

2.6 Coimmunoprecipitation

Rabbit anti-GFP polyclone antibody and mouse anti-Pan-FHF-A monoclonal antibody were used to immunoprecipitate EGFP-scFv and FHF2A from cell lysates, respectively. The antigen-antibody complex was pulled out of the solution by protein G sepharose beads (GE Healthcare Life Sciences), rinsed three times with ice-cold Triton lysis buffer, and then eluted by heating in Laemli sample buffer with β-mercaptoethanol at 50°C. The protein samples were always kept on ice before elution. Eluted samples (IP) and total protein lysates (input) were analyzed by Western blot. Co-immunoprecipitated A-FHFs with EGFP-scFv were detected by mouse anti-Pan-FHF-A monoclonal antibody (1 μg/ml, NeuroMab); co-immunoprecipitated sodium channels with A-FHFs or A-FHF−EGFP-scFv were detected by mouse anti-pan-NaV monoclonal antibody (1 μg/ml, Sigma).
2.7 Immunohistochemistry

Cryosections of 20 μm, from 4% paraformaldehyde-fixed mouse brain, and 200 μm vibratome brain slices, fixed after electrophysiological recordings, were permeabilized in 0.5% Triton X-100, blocked with 10% horse serum in PBS, and incubated with primary and then fluorescent secondary antibodies. TOPRO iodide (1:1000, Invitrogen) was used to stain nuclear DNA before brain samples were mounted and scanned with confocal microscopy (Leica Instruments).

2.8 Electrophysiological Recordings of Nav-derived Sodium Current in Neuro2A Cells

All recordings were performed on transiently transfected Neuro2A cells, identified by the green fluorescence of ZsGreen1. Coverslips with cells were placed in a recording chamber filled with O2:CO2 (95:5)-bubbled extracellular solution under a Nikon EF600 microscope. Glass pipettes filled with intracellular solutions were used to record sodium current from cells. The extracellular solution for isolating Nav1.6TTXr or Nav1.6TTXr-F1478Q-generated currents contained the following (in mM): 109 NaCl, 26 NaHCO3, 4.7 KCl, 11 glucose, 1.2 MgCl2, 2 sodium pyruvate, 3 myo-inositol, 2 CaCl2, 0.2 CdCl2, 10 HEPES, and 0.001 TTX (buffered to pH 7.2 with NaOH). The intracellular pipette solution contained (in mM) 104 CsF, 50 tetraethylamine chloride (TEA), 10 HEPES, 5 glucose, 2 MgCl2, 10 EGTA, and 2 Na2ATP, 0.2 Mg-GTP (buffered to pH 7.2 with CsOH). Sodium ion reversal potential was decreased to record Nav1.5-F1486Q-derived current: the modified extracellular solution with NaCl reduced to 29mM was supplemented with 80mM choline chloride to balance the molarity, and the pipette
solution with high Na\(^+\) contained (in mM) 100 NaF, 20 NaCl, 30 TEA, 10 HEPES, 5 glucose, 2 MgCl\(_2\), 10 EGTA, 2 Na2ATP, and 0.2 NaGTP (buffered to pH 7.2 with NaOH). In some experiments, pipette solutions were supplemented with 1mM peptide(s) or antibody (0.5mg/ml).

Voltage clamp and current recordings were performed with an Axopatch 200B amplifier, Digidata 1322 digital/analog interface, and pCLAMP9 software (Molecular Devices) at room temperature. After a tight seal (>5 G\(\Omega\)) between the pipette and cell, whole-cell configuration was obtained by brief gentle suction. For experiments with peptides or antibodies in pipette solutions and their negative controls, series resistance was 7–14 M\(\Omega\) after pipettes broke into cells. For experiments analyzing FHF mutagenesis, series resistance was 2.5–5 M\(\Omega\). Recording signals were filtered at 5 kHz and digitized at 10 kHz or 20 kHz. To measure evoked sodium currents, capacitive and leak currents were subtracted during data acquisition using the pre-sweep hyperpolarizing P/N method in pCLAMP9 software.

2.9 Protocols of Electrical Stimulation

Protocols for measuring the voltage dependence of sodium channel activation and steady-state inactivation, long-term inactivation accumulation, and long-term inactivation recovery rate were described previously (Dover et al., 2010) and modified in some experiments. Details of these protocols are briefly described below.
2.9.1 The Voltage Dependence of Activation

A 17-sweep protocol with the holding command at -90 mV and a 50ms variable test voltage (-60+5(n−1) mV) was used to measure the voltage dependence of sodium channel activation. The peak sodium current (I_{Na-peak}) was measured at and plotted against each test voltage, and the maximal sodium conductance was calculated from the Ohmic/linear portion of the plot (-5 to 10 mV). The percentage of activated channels at different test voltages was fitted to Boltzmann equation, \( f(\text{Vtest}) = \frac{1}{1 + e(\text{V}_{1/2} - \text{Vtest})/k} \) -1 + C, to obtain \( V_{1/2} \) of activation (the voltage at which 50% of the channels were activated) and the slope \( k \).

2.9.2 The Voltage Dependence of Steady-state Inactivation

The 21-sweep protocol to measure the voltage dependence of sodium channel steady-state inactivation (fast inactivation) with holding voltage at -110 mV contained a 60 ms variable conditioning voltage (CV) (-110+5(n−1) mV) and a 20 ms 0 mV reporting voltage (RV). In each sweep, peak sodium current at the RV was measured, and the fraction of channels available (not inactivated) at the RV equaled \( \frac{I_{Na-peak}(\text{V}_{test})}{I_{Na-peak}(-110 \text{ mV})} \). To obtain \( V_{1/2} \) and \( k \) values for inactivation, data points were fitted to the Boltzmann equation.

2.9.3 Long-term Inactivation Accumulation (LTI)

A protocol with -90 mV holding command and four cycles of 16 ms 0 mV separated by 40 ms -90 mV recovery phases was used to measure long-term inactivation (LTI) accumulation of NaV1.6^{TTXr} generated currents. For NaV1.5-F1486Q generated
sodium current, the recovery phases and holding command were at -100 mV, and each of
the four depolarization cycles was 48 ms 70 mV. Percentages of channels recovered at
the second, third, and fourth cycles were calculated by peak sodium currents over the
maximal current elicited by the first cycle of depolarization. Percentages of recovered
channels were plotted against depolarization cycle numbers.

2.9.4 The Voltage Dependence of Na\textsubscript{v1.6}\textsuperscript{TTXr-F1478Q} Activation and LTI

A 29-sweep protocol with a holding command at -90 mV, a 5 ms variable CV (-
90+5(n−1) mV) step, a 40 ms -90 mV recovery phase, and a 10 ms a 0 mV RV was used
to measure the voltage dependence of Na\textsubscript{v1.6} \textsuperscript{TTXr} (F1478Q) activation (peak currents at
CVs plotted against CVs) and the level of LTI (percentages of available channels at the
RV plotted against CVs). Percentages of maximal activation and LTI against various CVs
were plotted on the same graph.

2.9.5 Na\textsubscript{v} Recovery Rate from LTI

A 20-sweep protocol with a holding command at -90 mV, three cycles of
depolarization (two 20 ms 0mV and one 10 ms 0 mV) with 10 ms recovery phases, a
recovery phase of variable durations, 20 + 100(n−1) ms, and a 10 ms 0 mV pulse was
used to measure the Na\textsubscript{v} recovery rate from LTI. In each sweep, the first three
depolarizations induced LTI accumulation, and fractions of recovered channels were
calculated as \frac{I_{Na-peak}(4)}{I_{Na-peak}(1)}. The recovery time in sweep 1 was set as t =0.
Fractions of recovered channels in all sweeps were then fitted to the exponential recovery
equation: Recovered (t) = 1 −A\text{fast}(e^{−t/\tau_{fast}}) −A\text{slow}(e^{−t/\tau_{slow}}) − C. The protocol used
to measure the recovery rate in the presence of various FHF2A mutants was modified to
make the variable durations as 20n ms. The latter protocol was further modified with the holding command at -100 mV to measure the recovery rate of Nav1.5-F1486Q.

2.10 Statistical Analysis of Electrophysiological Data

All calculated values were expressed as mean ± SEM. Statistical significance between two comparable conditions was calculated by two-tailed unpaired Student’s t test.

2.11 Culture and Infection of Hippocampal Neurons

Hippocampal neurons were cultured from rat embryos. A pregnant rat at E18 was sacrificed in CO₂ and decapitated. The brains from the embryos were dissected and placed in ice-cold sterile HBSS. The meninges were removed and the hippocampi were cut out with a scalpel. The brain tissue was digested in papain medium containing 100 units/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES, 1 mg L-cysteine (papain activator), and 40 µl papain suspension in 5ml HBSS. The complete papain medium was incubated for 15 min at 37°C and sterilized with 0.22 µm filter. The digestion was terminated by BSA. Digested hippocampi were resuspended in MEM containing 0.6% glucose and 10% FBS and triturated with three polished Pasteur pipettes from large to small opening to dissociate neurons. The cells were counted with a hemocytometer and plated at a density of 5×10⁴ cells/well on poly-L-lysine-coated coverslips in a 24-well plate. After neurons attached to the coverslips, the medium was replaced with Neurobasal medium containing 50% glial-conditioned medium, 1% penicillin, streptomycin, 0.25% glutamine, 2% B27 supplement, and 0.5% FBS. 10µM cytosine arabinoside and 10µM
deoxycytidine were added to the medium two days later. Viruses, expressing a single-chain variable fragment (scFv) against A-FHFs, were added to the neurons at DIV10. The images of the infected neurons were taken at DIV13 when axon initial segments matured.

2.12 Transgene Targeting to the ROSA26 Locus of Embryonic Stem Cells (ESCs)

2.12.1 Culture of JM8A3 ESCs

Ultra-low passage JM8A3 embryonic stem cells (ESCs) (Pettitt et al., 2009) were generated by the trans-NIH Knock-Out Mouse Project (KOMP) and obtained from the UC DAVIS KOMP Repository (www.komp.org). These ESCs are derived from the C57BL/6N mouse JM8 parental line and can be cultured without feeder cells. Mice generated from these cells are heterozygous for agouti allele (A/a) and their coat color will be agouti.

The cells were thawed, plated on 0.1% gelatin coated dishes, and cultured with feeder-free ESC culture medium containing 1% penicillin, streptomycin, 2 mM glutamine, nonessential amino acids (HyClone), 10 mM HEPES (pH 7.2), 0.01 mM β-mercaptoethanol, 13% heat-inactivated FBS, 0.5 ml ESGRO leukemia inhibitory factor supplement, and 0.5 ml MEK/GSK3 inhibitor set (2i) (EMD Millipore) in high-glucose (4.5 g/L) DMEM (Life Technology).

2.12.2 Electroporation of ESCs

When cells grew rapidly, they were trypsinized and the density was adjusted to 2×10^7 cells/ml. 0.5 ml of cell suspension and 15 µg of linearized plasmid DNA were
added into a 0.4 cm cuvette (Bio-Rad). Electroporation was performed at 0.4 kV and 125 μF (Bio-Rad Gene Pulser). Transfected cells were incubated at room temperature for 10 min and plated to 10 cm 0.1% gelatin coated culture dishes. Selection medium with 350 μg/ml G418 was added 24 h later. The selected clones were picked, trypsinized, and passed to 96-well plates for cryopreservation and 24-well plates for DNA extraction. The freezing medium for ESC cryopreservation contained 10% FBS, 20mM HEPES, and 10% DMSO in DMEM. The ESC lysis buffer for DNA extraction contained 100 μg/ml proteinase K, 100 mM Tris (pH 8.5), 5 mM EDTA (pH 8.0), 0.2% SDS, and 200 mM NaCl. To precipitate DNA from lysed ESCs, 0.5 ml isopropanol was added to each well. Precipitated DNA was then retrieved with sealed Pasteur glass pipettes, rinsed in 70% and 100% ethanol sequentially, and dissolved in TE at 37°C overnight.

2.12.3 Genotyping and Cre Induction

The dissolved genomic DNA was adjusted to 100 ng/μl and digested (10 μl) with KpnI, NheI, and SacI in NEB Buffer 1.1 at 37°C overnight in a 20 μl reaction. The digested genomic DNA (1 μl) was used as the template in a 25 μl PCR reaction including 5.0 μl 5X OneTaq GC Buffer (NEB), 2.5 μl OneTaq High GC Enhancer, 20 μM dNTPs, 0.2 μM primers, and 0.04 U/μl OneTaq Polymerase. The primers used in PCR reactions were the sense primer, ROSA-KI-For, which annealed to the 5’ homology arm (sequence: CCTAAAGAAGAGGCTGTGCTTTGG) and the antisense primer, CAG-US-Rev, which annealed to the upstream of the CAG promoter within the ROSA-CAG plasmids (sequence: GCCATTTCGGTAAGTTATGTAACG). The cycling process of PCR contained an initialization step at 94°C for 3 min; 40 cycles of denaturation at 94°C for
15 sec, annealing at 50°C for 1 min, and elongation at 68°C for 2 min; and a final elongation step at 68°C for 3 min. PCR products were electrophoresed in 1.0% agarose gel.

Positive clones were thawed and spread to multiple plates for the following purposes: 1) cryopreservation; 2) DNA extraction, to validate the result of genotyping; and 3) Cre transfection, to confirm whether the transgene in ESCs is Cre inducible. pBS-CMV-NLS-Cre plasmid was introduced into ESCs via Lipofectamine-mediated transfection strategy. After 2 h transfection, cells were recovered in ESC medium for 0.5 h and then trypsinized and plated to gelatin coated coverslips. After 72 h culture, cells were placed under a microscope to validate Cre induction, indicated by ZsGreen fluorescence signal.
Chapter 3 Molecular Mechanisms of the Gain-of-function *FHF1*

Mutation Mediated Early-onset Epileptic Encephalopathy

3.1 Aberrant FHF4s Are Potential Culprits in Nav-Related Neurological Disorders

The gating of voltage-gated sodium channels (Nav) can be modulated by accessory proteins including β subunits and fibroblast growth factor homologous factors (FHF4s). These modulators in aberrant forms can be potential culprits in causing neurological disorders involving Nav dysfunctions. Indeed, a mutation in the Navβ1 subunit encoding gene (SCN1B) that affects the gating of Nav has been reported in generalized epilepsy with febrile seizures plus (GEFS+) (Wallace et al., 1998).

As Nav accessory proteins, FHF4s bind to the cytoplasmic Nav tail and modulate the expression and gating of Nav. FHF4s exert a pro-excitatory effect on neurons and cardiomyocytes by increasing sodium current through impeding Nav fast inactivation and enhancing sodium channel expression (Goldfarb et al., 2007; Lou et al., 2005; Rush et al., 2006). Excitation deficits resulting from a *FHF4* loss-of-function mutation may be the physiological mechanism for human spinocerebellar ataxia (SCA) (Goldfarb et al., 2007; Shakkottai et al., 2009). *Fhf1* and *Fhf2* loss-of-function mutations are causative or associative with cardiac arrhythmia in humans and mice (Hennessey et al., 2013a; Park et al., 2016), and *FHF2* loss-of-function causes temporal lobe epilepsy (Puranam et al., 2015).
It is not hard then to speculate that mutations in FHF1s causing a gain-of-function in this pro-excitatory effect may lead to over-excitation of neurons. If affected cells are excitatory neurons, this over-excitation may result in neurological disorders, such as epilepsy. This speculation remained a mental exercise until a pediatric neurologist, Gunnar M. Buyse, from the Center for Child Neurology at University Hospitals Leuven, Belgium, reached out to our lab a couple of years ago with a new finding in a type of epilepsy that afflicts children during neonatal and early infantile periods. It is termed early-onset epileptic encephalopathy (EOEE). Affected children experience severe recurrent seizures, which are usually unmanageable by antiepileptic medications. Additionally, patients also suffer from developmental retardation or regression, exhibiting as serious cognitive, neurologic, and behavioral deficiencies (Berg et al., 2010).

The etiology of EOEEs is largely undetermined (Tavyev Asher and Scaglia, 2012), and a few identified genetic mutations involve SCN8A, a gene that encodes the Na\textsubscript{v}1.6 subtype of the pore-forming \(\alpha\) subunit. These mutations increase sodium current and thus enhance neuronal membrane excitability (Estacion et al., 2014; Veeramah et al., 2012).

Dr. Buyse’s team carried out whole-exome sequencing and found no mutation in sodium channels, or other molecules affecting neuronal intrinsic excitability and synaptic transmission, such as STXBP1, KCNQ2, and SLC2A1. Instead, a missense mutation in the \(FHF1\) gene was found. Dr. Buyse contacted my mentor, Dr. Goldfarb, a founder and expert in the FHF field. This began a collaboration whereby I introduced missense mutations into FHF1 expression plasmids to identify the molecular mechanisms of EOEEs.
3.2 Clinical Phenotypes of EOEE and the Identified FHF1 Gene Mutation

Dr. Buyse’s patients affected by EOEEs were two children in a Caucasian family quintet. The proband was the first child (female, born at term, birthweight 2.92 kg) in a spontaneous twin pregnancy, with the other fetus lost at the end of the first trimester. The child appeared normal until the onset of tonic seizures, which developed at day 14, largely during sleep. The seizures were incontrollable by various antiepileptic medications. The patient developed extreme intellectual impairment, acquired microcephaly, axial hypotonia, ataxia (limbs), severe feeding difficulties, cerebral visual damage, and absent speech development. She could sit without support at 24 months old, but could never stand or walk. Cerebellar atrophy was detected by brain MRI at 6 years old. The proband died of status epilepticus at 7 years old. The other patient (male) had similar clinical phenotypes, with the onset of epilepsy at 4 weeks old and cerebellar atrophy detected at 3 years old. The second patient died at 3.5 years old, but the cause of death was not clear.

Whole-exome sequencing identified a heterozygous missense mutation in the FHF1 gene (C to T) on chromosome 3 (1167G to A in mRNA). It was the only variant shared by the affected children but not present in their unaffected parents, younger sibling, and common population databases (1000 Genomes, the NHLBI Exome Variant Server, and the EXaC browser). The absence of this mutation in other family members may be explained by the mosaicism in one of the parent’s germinal cells, potentially caused by sporadic mutation. This mutation is in exon 3 of the FHF1 gene, which encodes part of the protein β-trefoil core structure, and the resultant mutant proteins
include FHF1A-R114H and FHF1B-R52H. The affected residue, arginine, is highly conserved (e.g. R114 in FHF1A, R52 in FHF1B, R110 in FHF2A, and R57 in FHF2B) in all FHFs (Figure 2A). This arginine is located in the \( \beta \)-trefoil core domain of FHFs, and its side chain protrudes into a concave structure formed by a histidine and an aspartate in the C-terminal domain of Nav (Wang et al., 2012). Therefore, this arginine plays an important role in the binding between FHFs and Nav (Figure 2B).

**Figure 2** The mutated residue is highly conserved in FHF family and a key component in binding with the Nav tail

**(A)** The affected residue (R114 in A-isoform, R52 in B-isoform) in EOEE is highly conserved in all FHFs as shown by the sequence alignment of FHFs’ core domains. **(B)** Ribbon diagram of the binding interface between FHF and Nav. The arginine (blue) interacts with an aspartate acid and a histidine (red) in the Nav C-terminal domain in the cytoplasm (Wang et al., 2012).
3.3 Molecular Mechanisms of the EOEE Involving the *FHF1* Gene Mutation

The voltage dependence of Nav intrinsic fast inactivation is shifted to a more depolarized direction by most FHFs. The rate of fast inactivation is also slowed in the presence of FHFs. Therefore, at a given voltage and time, there are more available sodium channels and thus sodium current is larger (Lou et al., 2005; Rush et al., 2006). FHFs’ impediment to fast inactivation increases neuronal excitability and enables repetitive firing of granule neurons in the cerebellum (Goldfarb et al., 2007).

FHFs’ modulation of Nav fast inactivation relies on the Nav binding interface in their β-trefoil core structure. Mutations of eight amino acids in β-trefoil core (octa-mutant) prevented FHF2A from binding with the channel. Electrophysiology study revealed that both FHF2A and FHF2B octa-mutants failed to raise the voltage-dependence of Nav fast inactivation (Goetz et al., 2009). Therefore, the binding between FHFs and Nav C-terminal domain is essential in FHFs’ pro-excitatory effect.

In our study, the missense mutation in *FHF1* gene led to the mutation of arginine, a residue within the β-trefoil core domain, to histidine in FHF1 proteins. As shown in Figure 2B, this arginine residue is a part of FHF/Nav binding interface. Previous study demonstrated that the mutation of this arginine to glycine reduced the affinity of FHF/Nav binding (Goetz et al., 2009), but its effect on FHFs’ modulation of Nav fast inactivation was never tested. Dr. Buyse’s new finding of the FHF1 R to H mutation identified in the EOEE led my research back to resolve the functional effect of the mutation with mutagenesis and electrophysiological approaches. The R to H mutation was introduced into constructs expressing FHF1A or FHF1B. The plasmid expressing
WT or mutant A- or B-isoforms of FHF1 (FHF1A-R114H and FHF1B-R52H) and that expressing Na\(_{\text{v}}\)1.6 were introduced into Neuro2A cells by transient DNA transfection. Whole-cell voltage clamp protocols were used to measure the voltage dependence of Na\(_{\text{v}}\)1.6 activation and inactivation.

### 3.3.1 FHF1A-R114H Raises the Voltage Dependence of Na\(_{\text{v}}\)1.6 Fast Inactivation in a Gain-of-function Manner, but Does Not Affect Activation Gating

To test the effect of EOEE missense mutation on FHF1A modulation of Na\(_{\text{v}}\)1.6, the voltage dependence of Na\(_{\text{v}}\)1.6 fast inactivation was measured by patching the cells expressing Na\(_{\text{v}}\)1.6 and WT or mutant FHF1A in a whole-cell configuration. The patched cells were stimulated with a series of voltage commands. Each voltage command had a various conditioning voltage (CV) followed by a constant reporting voltage (RV). At the RV, the reduction of sodium current reflects the fractions of the channels that have entered fast inactivation during CV.

At any given CV, there was a higher percentage of maximal sodium current elicited by the RV in the presence of FHF1A-WT compared to no FHF. This fraction of sodium current was even larger in the presence of FHF1A-R114H (Figure 3A). \(V_{1/2}\) for fast inactivation refers to the voltage at which 50% of the channels are fast inactivated. In the absence of FHF, \(V_{1/2}\) for Na\(_{\text{v}}\)1.6 fast inactivation FHF was -81.4±1.6 mV (Figure 3B), and FHF1A-WT induced a depolarizing shift in the voltage dependence of Na\(_{\text{v}}\)1.6 fast inactivation (Figure 3C) with \(V_{1/2}\) at -63.4±1.6 mV. FHF1A-R114H produced much stronger channel modulation, with \(V_{1/2}\) at -56.4±0.9 mV (\(p=0.004\), FHF1A-R114H vs FHF1A-WT).
Figure 3 The voltage dependence of Na\textsubscript{v}1.6 fast inactivation and activation in the absence of FHF or in the presence of FHF1A-WT or FHF1A-R114H

(A) Superimposed sodium currents (upper) at the reporting voltage (RV) following several selected conditioning voltages (CVs) (red: -85 mV, green: -75 mV, and blue: -65 mV; lower). The percentage of maximal sodium current following a given CV is higher with FHF1A compared to that with no FHF, and this percentage is even higher with FHF1A-R114H. (B) $V_{1/2}$ for Na\textsubscript{v}1.6 fast inactivation (the voltage at which 50% of the channels are fast inactivated) without FHF is -81.4±1.6 mV (n=7); with FHF1A-WT, -63.4±1.6 mV (n=7); and with FHF1A-R114H, -56.4±0.9 mV (n=8). (C) The voltage dependence of Na\textsubscript{v}1.6 steady-state inactivation (fast inactivation). Dotted lines indicate color-coded CVs in (A).
The voltage dependence of Na$_V$1.6 activation (generated by plotting currents at CVs against CV) shows no difference among the three groups. **, p<0.01; ***, p<0.001.

Shifts in the voltage dependence of Nav activation have been shown to be associated with many sodium channelopathies (Cummins et al., 1993; Green et al., 1998; Mitrovic et al., 1995), and some mutations affecting Nav activation are also involved in GEFS+ (Lossin et al., 2003; Spamanato et al., 2003). Therefore, I also analyzed the voltage dependence of Na$_V$1.6 activation by plotting the peak conductance generated by CV against the CV. This assay showed that the R to H mutation did not cause a notable change in the activation gating of Na$_V$1.6 (Figure 3D).

3.3.2 FHF1B-R52H also Showed a Gain-of-function in Raising the Voltage Dependence of Na$_V$1.6 Fast Inactivation

Similarly, the EOEE missense mutation was introduced into FHF1B to compare Nav modulation by WT and mutant FHF1B isoforms. The FHF1B mutation had a dramatic gain-of-function effect on Nav1.6 inactivation gating (Figure 4). For example, at -75 mV of the CV, there was 25% maximal sodium current at the RV in the absence of FHF, this fraction increased to 50% in the presence of FHF1B-WT, and it further rose to 80% with FHF1B-R52H (Figure 4A). FHF1B-WT caused a depolarizing shift in the voltage dependence of Nav1.6 fast inactivation, from $V_{1/2}$ at -81.4±1.6 mV to -74.9±1.3 mV. FHF1B-R52H showed a stronger effect in the modulation, raising the $V_{1/2}$ to -65.8±1.3 mV ($p=0.0002$, FHF1B-R52H vs FHF1B-WT) (Figure 4B and C). Like the mutation in FHF1A, the R to H substitution in FHF1B did not cause a notable change in the gating Nav1.6 activation (Figure 4D).
Figure 4 The voltage dependence of Na\textsubscript{v}1.6 fast inactivation and activation in the absence of FHF or in the presence of FHF1B-WT or FHF1B-R52H

(A) Superimposed sodium currents (upper) at the RV following several selected CVs (red: -85 mV, green: -75 mV, and blue: -65 mV; lower). The percentage of maximal sodium current following a given CV is higher in the presence of FHF1B compared to no FHF, and this percentage of maximal current is even higher in the presence of FHF1B-R52H. (B) $V_{1/2}$ for Na\textsubscript{v}1.6 fast inactivation without FHF is -81.4±1.6 mV (n=7); with FHF1B-WT, -74.9±1.3 mV (n=8); and with FHF1B-R52H, -65.8±1.3 mV (n=9). (C) The voltage dependence of Na\textsubscript{v}1.6 fast inactivation (steady-state inactivation). Dotted lines indicate color-coded CVs in (A). (D) The voltage dependence of Na\textsubscript{v}1.6 activation shows no difference among the three groups. **, p<0.01; ***, p<0.001.
3.3.3 The Gain-of-function of FHF1 mutants in EOEE Arose from Loss of the Arginine

To test whether the gain-of-function of the EOEE associated mutation is due to the presence of the substituted histidine or the loss of the arginine, I compared the effects of FHF1B-R52H, FHF1B-R52A, and FHF1B-R52G isoforms on sodium channel inactivation gating. FHF1B-R52A was chosen because the analogous R→A substitution in FHF4 has been reported as loss-of-function (Yan et al., 2014), and FHF1B-R52G was chosen because this substitution reduces the affinity of FHF/Nav binding (Goetz et al., 2009) and would also be predicted to display a loss-of-function phenotype.

As shown in Figure 5, FHF1B-R52G also caused an enhanced depolarizing shift in the voltage dependence of Nav1.6 fast inactivation, with \( V_{1/2} \) at -70.7±0.7 mV. FHF1B-R52A (\( V_{1/2} =67.1±1.5 \) mV) was as potent as FHF1B-R52H in raising the voltage dependence. Like R to H substitution, the R to G or A substitution did not lead to a notable change in the gating of Nav1.6 activation (Figure 5D).
Figure 5 The voltage dependence of Na\textsubscript{V}1.6 fast inactivation and activation in the presence of FHF1B-WT, FHF1B-R52G, and FHF1B-R52A

(A) Superimposed sodium currents (upper) during the RV following several selected CVs (red: -85 mV, green: -75 mV, and blue: -65 mV; lower). The percentage of maximal sodium current following a given CV is higher with FHF1B-R52G or FHF1B-R52A compared to that with FHF-WT. (B) $V_{1/2}$ for Na\textsubscript{V}1.6 fast inactivation with FHF1B-WT is -74.9±1.3 mV (n=8); with FHF1B-R52G, -70.7±0.7 mV (n=9); and with FHF1B-R52A, 67.1±1.5 mV (n=8).

(C) The voltage dependence of Na\textsubscript{V}1.6 steady-state inactivation (fast inactivation). Dotted lines indicate color-coded CVs in (A). The voltage dependence of fast inactivation in the presence of FHF1B-R52H is added for comparison. (D) The voltage dependence of Na\textsubscript{V}1.6 activation shows no difference among the three groups. **, p<0.01; ***, p<0.001.
These functional results were unexpected. The affected arginine residue in FHF1 is highly conserved in all FHF proteins and is part of the essential residues that form the binding interface between FHFs and the tail of Nav (Goetz et al., 2009; Wang et al., 2012). Other mutations in the β trefoil core domain of FHF that affect the binding also negatively affect FHF’s modulation of Nav fast inactivation (Goetz et al., 2009). However, my result here showed the mutation of the arginine in the core domain of FHFs had a positive effect on modulating the gating of Nav fast inactivation, regardless of what the substitutions were.

This surprising result suggests that whereas the FHF binding to the tail of Nav is essential for the modulation of Nav inactivation gating, the specific FHF arginine side-chain interactions with Nav may operate in a way to restrict FHF’s modulation of the fast inactivation. Once this restriction conferred by the arginine is released in neurons under pathological conditions, as is the case of the EOEE in my research, there is stronger modulation of Nav fast inactivation by FHFs and thus more sodium current, which will in turn increase neuronal excitability. It is possible that a slightly weaker binding between FHF and Nav somehow allows stronger modulation of Nav fast inactivation, as schematized in Figure 6. Alternatively, FHF modulation of Nav inactivation may be tuned by both the strength of Nav binding and by a secondary effect on the channel, such as promoting a conformation change; despite somewhat weaker channel-binding affinity of arginine-substituted FHF, an enhanced effect on the channel conformation change is responsible for greater functional modulation.
3.4 In Vivo Recapitulation of the EOEE

My results revealed, compared to FHF1A-R114H, FHF1B-R52H showed a stronger gain-of-function modulation of $N_{av}$ fast inactivation. Therefore, another collaborator of our lab, Dr. Peter de Witte, from the Laboratory for Molecular Biodiscovery, Department of Pharmaceutical and Pharmacological Sciences, University of Leuven, Belgium, established an in vivo system in zebrafish larvae transiently overexpressing the mutant FHF1B in the brain. Local field potential recordings indicated that recurrent epileptiform discharges occurred in 50% of the animals overexpressing the mutant FHF1B (fhf1b1-R56H in zebrafish), and this percentage was significantly higher than that of the animals overexpressing fhf1b1-WT (27.8%) or the control animals only injected with vectors without FHFs (17.5%).

The zebrafish larvae overexpressing the mutant FHF had a higher probability of displaying epileptiform electrical activities than the group with the WT FHF. Therefore, these in vivo studies substantiate the epileptic potential of the FHFs with the EOEE-associated missense mutation. This result recapitulates the FHF1 gain-of-function as the mechanism of the EOEE from molecular and cellular level to the organismal level.
3.5 Summary

Dr. Buyse has identified a missense mutation in the \textit{FHF1} gene in two cases of familial EOEE with progressive cerebellar atrophy. The mutation resulted in mutant proteins FHF1A-R114H and FHF1B-R52H. Affected patients showed tonic seizures, extreme intellectual impairment, microcephaly, axial hypotonia, ataxia, severe feeding difficulties, cerebral visual damage, and no speech development.

My mutagenesis and electrophysiological results showed that the EOEE-linked FHF1 R to H mutation caused a gain-of-function on modulating Nav fast inactivation (Figure 7). This may increase neuronal excitability and lead to over-excitation, which provides a potential explanation for the occurrence of epilepsy. Prolonged over-excitation may result in neuronal toxicity and contribute to the progressive encephalopathy. These in vitro results also demonstrate a surprising relationship between FHF/Nav binding strength and the gating of Nav fast inactivation.

Dr. de Witte recapitulated the epileptic potential of the mutant FHF1 at the organismal level. The zebrafish larvae overexpressing the mutant FHFs were more likely to exhibit epileptiform electrical activities than the WT-FHF-overexpressing group. These in vivo results further support the FHF1 gain-of-function mechanism in the EOEE.

This collaboration study reported the first neurological disease linked with FHF1 proteins and with an FHF gain-of-function. These findings were published in \textit{Neurology} with me as a co-first author (Siekierska et al., 2016). More recently, a clinical genetics consortium has reported that the FHF1 R->H mutation has also occurred as three other
independent de novo mutations in children with EOEE, thereby defining an FHF1 Epileptic Syndrome (Al-Mehmadi et al., 2016).

**Figure 7** Summary diagram of the gain-of-function mutation in FHF1 proteins

(A) FHF1s can inhibit Na\textsubscript{v} fast inactivation. (B) The R to H mutation in FHF1 empowers the mutant proteins (FHF1A-R114H and FHF1B-R52H) to inhibit fast inactivation to a much stronger degree. Therefore, more Na\textsubscript{v}s will be available at a given membrane voltage and within a specific time window. This will then result in more sodium influx, which can increase membrane excitability.
Chapter 4 Physical Mechanisms and Biological Functions of A-type

FHF-induced Long-term Inactivation

A type of rapid-onset Nav inactivation with long-term recovery was discovered in hippocampal neural dendrites and was believed to promote spike attenuation in dendrites (Colbert et al., 1997; Jung et al., 1997). Subsequently, FHF2A was shown to cause rapid-onset long-term inactivation (LTI) of Nav (Rush et al., 2006). Thereafter, our lab has identified that all A-type FHFs (A-FHFs) can induce LTI via the highly conserved N-termini, and that the LTI particle competes with the intrinsic fast inactivation particle for inactivation (Dover et al., 2010).

4.1 Aliphatic and Cationic Residues in the A-FHF N-terminus Are Essential in Inducing and Maintaining Nav Long-term Inactivation

A-FHFs can induce Nav LTI via clusters of aliphatic (hydrophobic) and cationic (basic) residues in the highly conserved N-termini (Dover et al., 2010). All tested A-FHFs, including FHF1A, FHF2A, and FHF4A, can cause LTI of Nav1.6. Additional experiments also show that FHF2A can induce LTI of Nav1.5. Moreover, both FHF1A and FHF2A have been shown to induce LTI of endogenous sodium channels, Nav1.2, 1.3, 1.4, and 1.7 (Lou et al., 2005), in Neuro2A cells (Dover et al., 2010). Cluster mutations of aliphatic or cationic residues in FHF2A N-terminus prevent FHF2A from inducing LTI (Dover et al., 2010).

To provide a more refined and clearer molecular basis for LTI, I introduced mutations of single or double aliphatic and cationic amino acids in the FHF2A LTI
particle and expressed various FHF2A mutants and TTX-resistant Nav1.6 in Neuro2A cells. To study the effect of these mutations on LTI, I used electrophysiological approaches (voltage clamp) in a whole-cell configuration with TTX to block endogenous sodium current, Cd²⁺ to block calcium current, and TEA to block potassium current to isolate Nav1.6TTXr-derived sodium currents (Dover et al., 2010) with a LTI accumulation protocol.

4.1.1 Crucial Aliphatic Residues in the A-FHF LTI Particle

To determine the contribution of individual aliphatic residues in the FHF2A N-terminus to Nav1.6 LTI, single mutations of aliphatic residues were engineered in FHF2A-expressing construct (Figure 8A). Neuro2A cells were transfected with various FHF2A mutants and TTX-resistant Nav1.6, and electrophysiology voltage clamp was performed to record sodium current with a four-cycle depolarization protocol (Figure 8B). Representative current traces were shown in Figure 8C, and LTI of all FHF2A mutants are plotted in Figure 8D.

As shown in Figure 8 and Table 2, in the absence of FHF2A four cycles of depolarization triggered an equal amount of sodium currents (Figure 8C, left). Nav1.6 LTI induced by FHF2A was shown by the accumulative loss of channel availability through cycles of depolarization (Figure 8C, middle). The substitution of L9 or I10 (Figure 8C, right) to alanine completely abolished Nav1.6 LTI accumulation, whereas the I5A substitution partially impaired accumulative LTI (Figure 8D). None of these mutations caused a notable change in FHF2A’s modulation of Nav1.6 fast inactivation (Table 2).
Figure 8 Crucial aliphatic residues in the A-FHF LTI particle

(A) FHF2A N-terminal sequence. Dark shaded are conserved in all A-FHFs. Light shaded are conserved in three of the four A-FHFs. Single-residue mutations are shown above the sequence. (B) Voltage-clamp protocol to record sodium channel LTI accumulation. (C) Representative Na\textsubscript{v}1.6 current traces from Neuro2A cells in the absence of FHF, with FHF2A-WT, and with FHF2A-I10A. (D) LTI accumulation induced by WT or mutant FHF2A. Mutations in the aliphatic residues reduce or abolish the LTI. The number of cells tested in each group is indicated in parentheses.
### Table 2 The effect of aliphatic residue mutations in FHF2A N terminus on Na\textsubscript{v} LTI

<table>
<thead>
<tr>
<th>FHF2A</th>
<th>No FHF</th>
<th>WT</th>
<th>I5A</th>
<th>L9A</th>
<th>I10A</th>
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<tr>
<td><strong>LTI</strong></td>
<td>Percentage /1\textsuperscript{st} cycle</td>
<td></td>
<td></td>
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<tr>
<td>Cycle 2</td>
<td>93.4 ± 2.9</td>
<td>68.7 ± 2.5</td>
<td>76 ± 1.2</td>
<td>93.2 ± 1.0</td>
<td>95.1 ± 1.4</td>
</tr>
<tr>
<td>Cycle 3</td>
<td>89.9 ± 0.9</td>
<td>52.0 ± 3.1</td>
<td>69.3 ± 1.0</td>
<td>93.4 ± 1.6</td>
<td>96.0 ± 1.5</td>
</tr>
<tr>
<td>Cycle 4</td>
<td>89.8 ± 1.5</td>
<td>46.8 ± 2.9</td>
<td>64.1 ± 2.0</td>
<td>91.2 ± 1.4</td>
<td>92.5 ± 2.2</td>
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<tr>
<td><strong>t test</strong></td>
<td>***</td>
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<tr>
<td>(vs WT)</td>
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<tr>
<td><strong>V\textsubscript{1/2}-fast</strong> (mV)</td>
<td>-76.0 ± 1.5</td>
<td>-59.3 ± 1.5</td>
<td>-56.8 ± 1.7</td>
<td>-58.3 ± 1.6</td>
<td>-57.6 ± 0.7</td>
</tr>
<tr>
<td><strong>n</strong></td>
<td>6</td>
<td>5</td>
<td>8</td>
<td>5</td>
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</table>

LTI, long-term inactivation; V\textsubscript{1/2}-fast: V\textsubscript{half} of the voltage dependence of fast inactivation; **, p < 0.01; ***, p < 0.001.

#### 4.1.2 Crucial Cationic Residues in the A-FHF LTI Particle

To investigate the role of individual cationic residues in the FHF2A N-terminus in the Na\textsubscript{v}1.6 LTI, glutamine substitutions of these residues were engineered in the FHF2A-expressing construct (Figure 9A). Voltage clamp was performed to record sodium current with the four-cycle depolarization protocol (Figure 8B). The results were shown in Figure 9 and Table 3. Representative current traces were shown in Figure 9B–E, and LTI induced by the FHF2A mutants were plotted in Figure 9F.
Figure 9 Crucial cationic residues in the A-FHF LTI particle

(A) FHF2A N-terminal sequence. Dark shaded residues are conserved in all A-FHFs. Light shaded ones are conserved in three of the four A-FHFs. Single-residue mutations are shown above the sequence. (B) Voltage-clamp protocol to record Na\textsubscript{v}1.6 LTI accumulation. (C) Representative Na\textsubscript{v}1.6 current traces from Neuro2A cells with FHF2A-WT, FHF2A-R14Q, FHF2A-R17Q, or FHF2A-R14Q/R17Q. (D) Na\textsubscript{v}1.6 LTI accumulation
induced by WT or mutant FHF2A. Single mutations reduce or abolish LTI. Double mutations have a stronger effect. The number of cells tested in each group is indicated in parentheses.

WT FHF2A induced Nav1.6 LTI shown by accumulative loss of channel availability through cycles of depolarization (Figure 9B). Single mutations of R11, K13, R14, and R17 to glutamine in the FHF2A N-terminus caused mild impairment of LTI accumulation (Figure 9C, D, and F; Table 3).

| Table 3 | The effect of single mutations of cationic residues in FHF2A N terminus on Nav LTI |
|---------|---------------------------------|----------------|----------------|----------------|----------------|
|         | FHF2A                          | R11Q           | K13Q           | R14Q           | R17Q           |
| LTI     | Percentage /1st cycle           |                |                |                |                |
| Cycle 2 | 79.1 ± 4.0                      | 72.0 ± 1.9     | 75.4 ± 3.6     | 78.8 ± 3.1     |
| Cycle 3 | 73.4 ± 3.4                      | 62.0 ± 2.8     | 68.6 ± 1.9     | 73.6 ± 2.9     |
| Cycle 4 | 67.1 ± 2.7                      | 55.7 ± 2.6     | 63.9 ± 2.8     | 68.6 ± 3.4     |
| t test  | ***                            | *              | **             | ***            |
| (vs WT) |                                |                |                |                |
| $V_{1/2\text{-fast}}$ (mV) | -60.9 ± 1.4                 | -58.1 ± 1.1    | -62.0 ± 1.9    | -56.1 ± 0.7    |
| n       | 5                               | 7              | 5              | 5              |

LTI, long-term inactivation; $V_{1/2\text{-fast}}$: $V_{\text{half}}$ of the voltage dependence of fast inactivation; *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Double substitutions of the above tested arginine residues at position 11, 14, and 17 to glutamines were also introduced to the FHF2A-expressing construct. The double mutants FHF2A-R11Q/R14Q and R11/R17Q had a stronger inhibitory effect on LTI accumulation than the single mutants (Figure 9F). FHF2-R14Q/R17Q completely suppressed the accumulative LTI (Figure 9E and F; Table 4).

None of these mutations cause a notable change in FHF2A’s modulation of Nav1.6 fast inactivation (Table 3 and 4).

<table>
<thead>
<tr>
<th>Table 4</th>
<th>The effect of double mutations of cationic residues in FHF2A N terminus on Nav&lt;sub&gt;V&lt;/sub&gt; LTI</th>
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<tr>
<td></td>
<td>FHF2A</td>
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<td>LTI</td>
<td>Percentage /1&lt;sup&gt;st&lt;/sup&gt; cycle</td>
</tr>
<tr>
<td>Cycle 2</td>
<td>90.4 ± 3.5</td>
</tr>
<tr>
<td>Cycle 3</td>
<td>89.5 ± 2.6</td>
</tr>
<tr>
<td>Cycle 4</td>
<td>88.5 ± 3.6</td>
</tr>
<tr>
<td>t test (vs WT)</td>
<td>***</td>
</tr>
<tr>
<td>&lt;sup&gt;V&lt;/sup&gt;1/2-fast (mV)</td>
<td>-59.8 ± 1.6</td>
</tr>
<tr>
<td>n</td>
<td>5</td>
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</tbody>
</table>

LTI, long-term inactivation; <sup>V</sup>1/2-fast: <i>V</i><sub>half</sub> of the voltage dependence of fast inactivation; ***: p < 0.001.
4.1.3 The Aliphatic and Cationic Residues in A-FHF s Are Also Crucial in
Maintaining Nav LTI

In the previous section, I have shown that the aliphatic and cationic residues in
FHF2A N-terminus are essential in induction of Nav LTI. To investigate whether these
mutations are also crucial in maintenance of LTI, the recovery rate of Nav from LTI in
the presence of various FHF2A mutants was also assayed.

Nav1.6 long-term recovery rates in the presence of FHF2A WT and mutants were
measured by the protocols shown in Figure 10A and B (lower). Due to quicker recovery
of sodium channels from LTI in the presence of the mutants, the step increase of the
variable duration was shortened to 20 ms from 100 ms in the recording protocol (Figure
10B, lower).

The representative current traces in Figure 10A and B showed that Nav1.6
recovered much faster in the presence of FHF2A-R11Q/R17Q than FHF2A-WT. The
graphic display of Nav1.6 long-term recovery rates in the presence of various FHF2A in
Figure 10C and D showed that all tested FHF2A mutants, which impaired the induction
of Nav1.6 LTI, accelerated channels’ recovery from impaired LTI.

In Figure 11, recovery rates of Nav from LTI were plotted against the percentage
of channels at the 4th cycle over the 1st cycle depolarization from Table 2, 3, and 4. It
showed that a faster recovery rate of Nav1.6 from LTI correlates with a larger fraction of
available channels, or, less LTI accumulation.
Therefore, these aliphatic and cationic residues in the FHF2A N-terminus are essential in both induction and maintenance of Nav LTI.

**Figure 10** Na\textsubscript{v}1.6 recovers faster from LTI in the presence of FHF2A mutants. 

(A) Superimposed current traces to indicate the Na\textsubscript{v} recovery rate LTI in the presence of FHF2A-WT (upper). Voltage-clamp protocol (lower) consists of three rapid pulses of depolarization to induce LTI and a fourth reporting voltage after a variable recovery period. (B) Superimposed current traces to show the speed of Na\textsubscript{v}1.6 recovery from LTI in the presence of FHF2A-R11Q/R17Q (upper). Voltage-clamp protocol (lower) consists of three rapid pulses of depolarization to induce LTI and a fourth reporting voltage after a variable recovery period, and the step increase of the recovery duration is shortened to 20 ms from 100 ms. Na\textsubscript{v}1.6 recovers faster from LTI in the presence of FHF2AR11Q/R17Q (B) compared to FHF2A-WT (A). (C) The percentage of Na\textsubscript{v}1.6 recovered after various
durations with WT or mutant FHF2A. (D) The recovery rate of Na\textsubscript{v}1.6 in different groups. The number of cells tested in each group is indicated in parentheses.

![Graph showing recovery rates of Na\textsubscript{v}1.6](image)

**Figure 11** Faster recovery rate of Nav1.6 from LTI correlates with a higher percentage of available sodium current.

Recovery rates of Nav1.6 in the presence of FHF2A WT or variants are plotted against percentages of maximal currents during the 4\textsuperscript{th} cycle recorded with the LTI accumulation protocol. A higher percentage of sodium current reflects reduced LTI accumulation. All FHF2A mutations affecting Nav1.6 LTI accumulation accelerate the Na\textsubscript{v}1.6 recovery rate from LTI.

![Image of A-FHF Ab and FHF2-CT Ab](image)

**Figure 12** Validation of FHF2A mutants’ expression in transfected Neuro2A cells

Lysates from Neuro2A cells expressing FHF2A WT and variants are immunoblotted with antibodies (Ab) recognizing either the N-terminus of A-FHFs or FHF2 C-terminus (FHF2-CT). A-FHF Ab only recognizes FHF2A variants when R11 and R14 are intact, suggesting
these two residues are critical in the binding epitope. FHF2-CT Ab recognizes all FHF2A variants.

Moreover, to exclude the possibility of absent expression of FHF2A mutants in the recorded cells, the cells that were transfected with WT or various FHF2A mutants were lysed and Western blot was performed. The lysates were electrophoresed in duplicate gels: one was immunoblotted with A-FHF Ab; the other, with FHF2-CT Ab. A-FHF Ab recognizes the N-terminus that is highly conserved in all A-FHFs; whereas FHF2-CT only recognizes the C-terminus of FHF2. As shown in Figure 12, A-FHF Ab failed to recognize the FHF2A mutants with glutamine substitution at R11 or R14, suggesting these two residues are crucial constituents of the antigen epitope (Figure 12, upper). However, FHF2-CT Ab recognized all FHF2A (Figure 12, lower). The immunoreactivity of FHF2-CT Ab with all FHF2A mutants, together with the retained ability of the mutants to cause positive shifts in the voltage dependence of sodium channel fast inactivation (Table 2, 3, and 4), validates that the suppressed LTI was resultant from mutations rather than from absent expression of the mutants in transfected Neuro2A cells.

4.2 The A-FHF LTI Particle Functions as an Open-channel Blocker

Both Nav fast inactivation and LTI have a rapid onset, but the recovery time for LTI is much longer than that for fast inactivation (Table 1). In addition, as for Nav1.6 in the presence of FHF2A, fast inactivation occurs even before channels are open ($V_{1/2}$: -66.3 mV), whereas LTI ($V_{1/2}$: -46.7 mV) needs a much greater membrane depolarization. While the $V_{1/2}$ for A-FHF-induced Nav LTI was reported to be 7 mV more negative than
that for Nav activation (V_{1/2}: -39.1 mV) (Dover et al., 2010), we still speculated LTI may only occur when Navs are open.

### 4.2.1 FHF2A Only Blocks Nav in Its Open State

The protocol used to measure the voltage dependence of LTI has a variable conditioning voltage (CV) and a constant reporting voltage (RV) separated by a 40 ms recovery phase. At the RV, reduction of sodium current reflects the fractions of Navs that have entered LTI during the CV (Dover et al., 2010).

To test the hypothesis that LTI only occurs in the open state of Nav, two modifications to a previous protocol (Dover et al., 2010) were made, to more precisely compare the voltage dependence of Nav activation and LTI (Figure 13A, lower): 1) TTX-resistant and fast inactivation defective sodium channel Nav1.6 (F1478Q) was used to minimize the involvement of fast inactivation, which will otherwise cause a negative shift in the apparent voltage dependence of channel activation; 2) The duration of the CV in the recording protocol was shortened to 5 ms. Nav activation is thus reported by sodium currents induced by CVs, and the level of LTI at a CV is measured by reduction of the sodium current at the RV, after a 40 ms recovery period, which allows Nav1.6 (F1478Q) to recover from any residual fast inactivation.

The rationale for the first modification is that fast inactivation affects the measurement of the real voltage dependence of other channel states (Gonoi and Hille, 1987). The rationale for the second modification is that a longer CV causes a negative shift in the apparent voltage dependence of LTI, since FHF2A continually captures more channels going through reversible open/close transitions into a stable LTI state.
In each of the representative sodium current traces (Figure 13A, upper) with the modified recording protocol (Figure 13A, lower), the sodium current elicited by a CV reflected channel activation, and reduction of sodium current during the RV reflected sodium channels that entered LTI during the CV. For CVs at negative potentials that did not activate channels (no current by a CV), no LTI occurred (no reduction of the current during the RV), as was the case of the CV at -50 mV (Figure 13A, upper; the black trace). Reduction of the current during the RV was only observed when a CV elicited a sodium current, as was the case of the CV at -35 mV (Figure 13A, upper; the magenta trace). This supports the argument that LTI only occurs when the channels are open. When a CV was further depolarized, more channels were activated, as shown by the gradually increasing sodium current at the CV, and more channels entered LTI, manifested by more reduction of the current during the RV (Figure 13A, upper; the orange, cyan, and green traces).

Figure 13 FHF2A induces sodium channel inactivation in the channel’s open state
(A) Representative superimposed current traces (upper) from fast inactivation-defective \(\text{Nav}_{1.6} (\text{F1478Q})\) in the presence of FHF2A elicited by several selected sweeps of stimulation from the protocol consisting of a 5 ms variable conditioning voltage (CV), a 40 ms recovery phase at -90 mV, and a reporting voltage (RV) at 0 mV (lower). (B) Coincident voltage dependence of channel activation and the degree of LTI (n=6). Voltages that activate sodium channels during the CV also trigger LTI, as shown by reduction of sodium current during the RV. Colored arrows indicate CVs shown in A (black, -50 mV; magenta, -35 mV; orange, -30 mV; cyan: -20 mV; and green: -5 mV).

The graphic display of the averaged voltage dependence of sodium channel activation and the level of LTI is shown in Figure 13B. The voltage dependence of channel activation \((V_{1/2}: 30.6 \pm 1.0 \text{ mV})\) and FHF2A-induced LTI \((V_{1/2}: 30.3 \pm 1.6 \text{ mV})\) matched with each other. This indistinguishable voltage dependence further suggests that the A-FHF LTI particle functions as an open-channel blocker.

4.2.2 Longer Open State of \(\text{Nav}\) Correlates with Greater LTI

The rationale for the second modification to the protocol shown in Figure 13A (lower) is that a longer CV causes a negative shift in the apparent voltage dependence of LTI, since FHF2A continually captures more channels in the reversible open/close state into a stable LTI state. Shortening the duration of CVs truly reflected the voltage dependence of LTI. The next question was whether a longer CV correlates with stronger LTI. To answer this question, several protocols containing CVs with various durations, 5 ms, 15 ms, and 60 ms (Figure 14A, B and C, lower), were used to revisit the relationship of sodium channel activation and LTI.
The CV at -35 mV triggered a similar amount of sodium currents regardless of the stimulation’s duration. However, the sodium currents during the RV gradually declined as the duration of the CV lengthened (Figure 14A, B and C, upper; the magenta trace). Therefore, the longer the sodium channels stay open, the greater the fraction of channels that become captured by FHF2A into a LTI state.

As shown by the graphic display of the averaged voltage dependence of sodium channel activation and the level of LTI in Figure 14D, the voltage dependence of channel activation ($V_{1/2}$: 30.6 ± 1.0 mV) and the FHF2A-induced LTI level ($V_{1/2}$: 30.3 ± 1.6 mV) matched with each other. However, the voltage dependence of the LTI level was shifted to a slightly hyperpolarized direction when the duration of the CVs was lengthened to 15 ms from 5 ms (Figure 14E). This negative shift became larger when CVs were lengthened to 60 ms (Figure 14F).
Figure 14 FHF2A captures more NaVs into LTI when channels open longer

(A, B, C) Representative superimposed current traces (upper) from fast inactivation-defective Na\(_{\text{v}}\)1.6 (F1478Q) in the presence of FHF2A elicited by several selected sweeps of stimulation from a protocol consisting of a 5 ms (A), 15 ms (B), or 60 ms (C) CV (the prepulse), a 40 ms recovery phase at -90 mV, and a RV at 0 mV (lower). (D, E, F) The voltage dependence of channel activation and the level of LTI (n=6). A longer CV
correlates with greater LTI, as shown by larger reduction of the sodium current during the RV. Colored arrows indicate CVs shown in A (black, -50 mV; magenta, -35; orange, -30 mV; cyan: -20 mV; and green: -5 mV).

The results from Figure 13 and 14 suggest that A-FHF LTI particles only block sodium channels in their open state. An open state of a longer duration enables more channels to be captured and blocked by A-FHF LTI particles.

4.2.3 The A-FHF LTI Particle Competes with β4 Blocking Particle for Binding with Nav

Very similar voltage dependent sodium channel transitions are needed to open the channel and to make it susceptible to A-FHF-induced LTI. These comparable voltage dependent transitional states suggest that A-FHF particle function as open-channel blockers to induce Nav to LTI. To further validate this statement, I tested whether this A-FHF LTI particle competes with a known open-channel blocker, Navβ4 subunit.

The known open-channel blocker, Navβ4, only docks into the pore of sodium channels in their open state. The docked β4 subunit will be expelled immediately upon repolarization; therefore, the channels briefly reopen and generate a resurgent current. This instantaneous resurgent current is pro-excitatory and facilitates high-frequency firing of neurons (Grieco et al., 2005; Khaliq et al., 2003; Raman and Bean, 1997; Wallace et al., 1998).

To test the mutual competition to block sodium channels between A-FHF particle and Navβ4 subunit, the involvement of fast inactivation was minimized by the introduction of a point mutation (F1486Q) in Nav1.5 that was expressed in Neuro2A cells. Nav1.5 was
used because ectopically expressed Nav1.6 does not readily generate Navβ4-induced resurgent current (Chen et al., 2008). Sodium reversal potential was reduced to -10 mV by changing the sodium concentration in the bath and pipette solutions, to increase the signal-to-noise ratio. A protocol of four depolarization cycles to 70 mV separated by 40 ms -100 mV intervals was used to investigate LTI accumulation (Figure 15A, lower) of Nav1.5 (F1486Q) expressed in Neuro2A cells with Navβ4 154–167 and/or F2A2-18 peptide(s).

To equalize the conditions of the competition between A-FHFs and Navβ4, instead of expressing the proteins in recorded cells, synthesized peptides corresponding to their blocking particles at the same concentration were used. A short peptide corresponding to a cytoplasmic segment of Navβ4 154–167 has been used to block Nav1.5 (F1486Q) expressed ectopically. This docked peptide promptly dissociates upon repolarization and generates a resurgent sodium current (Bant and Raman, 2010; Grieco et al., 2005; Wang et al., 2006). Previous work in our lab used F2A2-21 peptide to induce LTI of Nav1.5 and 1.6 (Dover et al., 2010). In this experiment, F2A2-18 peptide was used to induce LTI.

In the absence of peptide in the recording pipette, a series of depolarization to 70 mV elicited an equal amount of outward persistent currents, with weakly residual fast inactivating currents. (Figure 15A, upper; red arrows). Each repolarization triggered an instantaneous inward tail current before channel deactivation (Figure 15A, upper; green arrows).
In the presence of the 1mM β4 154–167 peptide in pipette, open channels were blocked upon each depolarization by the β4 peptide, leaving no persistent current (Figure 15B, red arrows). Upon repolarization, blocked channels briefly reopened and generated an inward resurgent current (Figure 15B, green arrows). Due to rapid dissociation of β4 154–167 peptide from the channels, each depolarization generated an equal amount of outward transient sodium currents (Figure 15B, red arrows).

In the presence of the F2A2-18 peptide in the pipette, the outward sodium current was terminated due to the entry of sodium channels into LTI. Previous work in our lab used F2A2-21 peptide to induce LTI of Nav 1.5 and 1.6 (Dover et al., 2010). Here a shorter version of the LTI particle, F2A2-18 peptide, is sufficient to induce LTI. With a much faster recovery rate of Nav 1.5 (F1486Q) from F2A2-18 peptide-induced LTI compared to that of Nav 1.6 with FHF2A protein (Figure 16), approximately 35% channels recovered within 40 ms at 100 mV (Figure 15C, red arrows). Due to the stable binding between F2A2-18 peptide and the channel, no resurgent current was generated upon repolarization (Figure 15C, green arrows).

In the presence of both peptides, there was a mutual competition for channel binding (Figure 15D). During the first cycle of depolarization, sodium channels were activated and inactivated by either F2A2-18 or β4 154–167 peptide, leaving no persistent current (Figure 15D, red). On one hand, some resurgent current was seen upon the first repolarization, reflecting the reopened channels that were blocked by β4 154–167 peptide during the first depolarization. However, resurgent current gradually decreased in subsequent repolarization cycles (Figure 15D and E), suggesting a progressive inhibition
of $\beta_{4_{154-167}}$ peptide and channel binding by F2A$_{2-18}$ peptide. On the other hand, the binding of $\beta_{4_{154-167}}$ peptide and channel significantly affected F2A$_{2-18}$ peptide-induced LTI (Figure 15D and F).

The reciprocal competition for sodium channel binding between A-FHF and Nav$\beta_{4}$ derived peptides confirms that the A-FHF LTI particle is an open-channel blocker, and it can compete with Nav$\beta_{4}$ for binding with the channel. These proteins may share overlapping docking sites within the open sodium channels. As open-channel blockers, Nav$\beta_{4}$ is pro-excitatory, whereas A-FHFs are pro-inhibitory. When both types of proteins are present in cells, it will be interesting to study which type of protein outcompetes the other and what the net effect will be on membrane excitability.
Figure 15 Functional competition between A-FHF and \( \beta 4 \) blocking particles

(A) Sodium currents are recorded in Neuro2A cells expressing fast inactivation-defective Nav1.5 (F1486Q). Sodium reversal potential was adjusted to -10 mV. Four cycles of depolarization to -70 mV were separated by 40 ms, -100 mV recovery phases (lower). Equal persistent outward sodium currents are elicited by all cycles of depolarization, followed by brief inward tail currents upon repolarization, in the absence of peptide in the recording pipette. (B) Equal outward sodium currents (red arrows) with 1 mM \( \beta 4_{154-167} \) peptide in the pipette are followed by equal inward resurgent currents (green arrows). (C) Outward transient currents (red arrows) are totally blocked in the presence of 1 mM
F2A_2–18 peptide in the pipette. Some channels recover from LTI after a 40 ms -100 mV interval, causing smaller outward sodium currents upon subsequent cycles of depolarization (red arrows). Stable F2A_2–18 binding prevents resurgent currents upon repolarization. (D) In the presence of β4_154–167 and F2A_2–18 (1 mM each), outward transient currents (red arrows) and inward resurgent currents (green arrows) both gradually decline. All channels are blocked by either β4_154–167 or F2A_2–18 during the first depolarization. β4_154–167 prevents maximal LTI induced by F2A_2–18. Stronger F2A_2–18 binding gradually inhibit β4_154–167-mediated resurgent currents. (E) Plot of resurgent currents from cells containing β4_154–167 with (n=6) or without (n = 5) F2A_2–18. Resurgent currents are significantly reduced upon repolarization 2, 3, and 4. (F) Plot of outward transient sodium currents from cells with no peptide (n = 4), F2A_2–18 (n = 6), or β4_154–167 + F2A_2–18 (n = 6). LTI induced by F2A_2–18 is significantly suppressed by β4_154–167, which allows a larger outward transient sodium current during the second depolarization.

*p<0.02, ***p < 0.0001.

**Figure 16** The recovery rate of Na_v1.5 (F1486Q) from LTI induced by F2A peptide

A voltage-clamp protocol consisting of three cycles of depolarization to induce LTI and a fourth reporting voltage (RV) separated by a variable recovery interval is used to measure
the Na\textsubscript{v}1.5 recovery rate from LTI induced by F2A peptide. Percentages of available channels at the RV are plotted against various recovery intervals.

### 4.3 Widespread Expression of A-FHFs in Juvenile Mouse Brain Neurons Implies Its Global Modulation of Neuronal Excitability

Spike frequency accommodation (SFA) is a common neuronal firing property found in many types of neurons (Chen et al., 2014; Faber and Sah, 2002; Miles et al., 2005), characterized by a gradually decreasing firing rate during a sustained depolarizing current injection. SFA provides a possible cellular mechanism for efficient neuronal coding which limits the redundancy in the sensory input in a natural environment (Brenner et al., 2000). In addition, SFA is more prevalent in aging hippocampal CA1 pyramidal neurons, which may explain the learning deficits in aging animals (Moyer et al., 1992). Interestingly, SFA decreases in both aging and young animals after trace eyeblink conditioning (Moyer et al., 2000).

Several possible mechanisms underlying SFA include the slow afterhyperpolarization (AHP) current (an unknown type of calcium-activated potassium current) (Andrade et al., 2012; Pedarzani and Storm, 1993), sodium-activated potassium current (Sanchez-Vives et al., 2000), M-type potassium current (Gu et al., 2005; Nigro et al., 2014; Otto et al., 2006; Peters et al., 2005), and slow inactivation of Nav (Fleidervish et al., 1996; Martina and Jonas, 1997). Calcium-activated small conductance potassium channels (SK) were suggested to mediate medium afterhyperpolarization and play an important role in SFA. Controversially, this argument has been challenged by some studies (Gu et al., 2008). For example, a recent finding has shown that SK’s defense
against hyperexcitability only becomes functional when Kv7/M channels are compromised (Chen et al., 2014).

A rapid onset “slow inactivation” (in contrast to the conventional slow inactivation with a slow onset) of Nav has been suggested to mediate early SFA in spinal motor neurons (Miles et al., 2005). Since LTI has a rapid onset and slow recovery, it is possible that SFA may be mediated by A-FHF-induced Nav LTI.

Previous studies have shown that different neurons express different repertoire of FHFs. For example, all four FHFs are expressed in cerebral cortical neurons and hippocampal pyramidal neurons, FHF2 is absent in cerebellar granule neurons and spinal motor neurons, and FHF4 is not present in periphery sensory neurons (Goldfarb, 2005). However, the expression of A-FHFs in the nervous system has not been systematically investigated. Therefore, immunohistochemistry was performed to study the expression of A-FHFs in the mouse brain with joint effort of my former colleague Dr. Kumar Venkatesan and me, to test the hypothesis that A-FHFs may mediate SFA.

Our results demonstrated a widespread expression of A-FHFs on neuronal axon initial segments (AIS), where Navs are highly concentrated and action potentials are initiated (Figure 17). The distribution of A-FHFs on the AIS was detected in many brain regions, including the hippocampal pyramidal layer (Figure 17A–C), subiculum (Figure 17D–F), cerebral cortex (Figure 17G–I), cerebellar cortex (Figure 17J–L), and motor neurons in the facial nucleus (Figure 17M–O).
A-FHFs were uniformly expressed in motor neurons and pyramidal neurons in the hippocampus and subiculum. However, the expression levels of A-FHFs among cerebral and cerebellar cortical neurons were highly variable (Figure 17G–L).

In consideration of the widespread expression of A-FHFs on the AIS of neurons in the brain, A-FHFs could be potential modulators of SFA.
Figure 17 A-FHFs are expressed on axon initial segments of neurons

Immunostaining of A-FHFs (green; A, D, G, J, M), AIS by ankyrin G (red) (B, E, H, K, N), and nuclei by TOPRO iodide in the mouse hippocampus CA1 pyramidal region (A–C), subiculum (D–F), cerebral cortex (G–I), cerebellar cortical granule layer (J–L), and facial motor nucleus (M–O). Merged images are shown in C, F, I, L, and O. A-FHFs are expressed on the AIS (co-staining is shown in yellow) in all stained CA1 and subicular
pyramidal neurons (A–F) and motor neurons (M–O), and in some (arrows) but not other (arrowheads) cerebral cortical neurons and cerebellar granule layer neurons (G–L). Scale bar, 5 μm. Credit: Dr. Kumar Venkatesan and Yue Liu.

4.4 A-FHF Monoclonal Antibody Blocks Nav LTI

To test whether A-FHFs contribute to SFA in neurons, an approach to block A-FHF-induced LTI was first developed. Previously, our lab has used an antibody against the N-terminal region of FHF2A to block LTI without affecting FHF modulation of Nav fast inactivation. That antibody was generated in rabbit with an N-terminally acetylated synthetic peptide corresponding to FHF2A residues 2–21 with additional GlyGlyCys at C-terminus and thus a polyclonal antibody, and it was tested with FHF2A (Dover et al., 2010).

The A-FHFs encoded by all four FHF genes share 78–100% homology through residues 1–18, and the conserved N termini function as blocking particles to capture Nav into a long-term inactivated state (Dover et al., 2010). In collaboration with the University of California (UC) Davis/NIH NeuroMab Facility, a monoclonal antibody (Ab) against the A-FHF N-terminal LTI particle (NeuroMab N235/22) was generated against a synthetic peptide corresponding to FHF2A residues 2-18.

To validate the function of this monoclonal A-FHF Ab, I used biochemical assays to test its immunoreactivity against various A-FHFs and electrophysiological approaches to investigate whether it can block Nav LTI induced by different types of A-FHFs.

Neuro2A cells were transfected with FHF1A, FHF2A, and FHF4A. Cells were then lysed and Western blot was performed with the extracted proteins to test the
immunoreactivity of A-FHF Ab against these A-FHFs. As shown in Figure 18, A-FHF Ab recognized all tested A-FHFs, but not ZsGreen control. An earlier result also demonstrated that R11 and R14 common to all four types of A-FHFs were among the reactive epitope residues (Figure 12).

![A-FHF Ab](image)

**Figure 18** Immunoreactivity of A-FHF antibody with FHF1A, FHF2A, and FHF4A. Lysates from Neuro2A cells expressing ZsGreen alone or with various A-FHFs are immunoblotted with N235/22 pan-A-FHF antibody (Ab). A-FHF Ab recognizes all tested A-FHFs (30kD).

The immunoreactivity of A-FHF Ab against all tested A-FHFs (Figure 18) makes A-FHF Ab a potential tool to block NaV LTI induced by various types of A-FHFs.

Electrophysiology was then performed for the functional validation of A-FHF Ab. To test whether A-FHF Ab can counteract the LTI effector region in N-termini of A-FHFs, Neuro2A cells were transfected with vectors expressing various A-FHFs and TTX-resistant sodium channel NaV1.6, the current generated by the transfected NaV1.6 was isolated with TTX, Cd^{2+}, and TEA, and a four-cycle depolarization protocol was used to trigger LTI accumulation (Dover et al., 2010) (Figure 19A).

NaV1.6 channels open and undergo fast inactivation upon depolarization, and during the 40 ms interval, almost all the fast-inactivated channels recover and become available to open upon the next cycle of depolarization. Therefore, in the absence of FHF, all cycles of depolarization triggered an equal amount of transient sodium currents.
(Figure 19B). In the presence of FHF2A, however, Nav1.6 was progressively captured into a long-term inactivated state through multiple cycles of depolarization, and this LTI accumulation remained unchanged after 40 min of recording (Figure 19C). When the A-FHF Ab (500 µg/ml) was added to the pipette, the patched cell was recorded first at 2 min after break-in, and again at 30–55 min post break-in. In the case of FHF2A, perfused A-FHF Ab significantly suppressed Nav1.6 LTI accumulation (Figure 19D and F).

FHF4A also induced a strong Nav1.6 LTI accumulation, whereas FHF1A caused a weaker accumulative LTI. Nav1.6 LTI accumulation caused by FHF1A or 4A was significantly inhibited by the perfused A-FHF Ab to recorded cells (Figure 19E and G). Although A-FHF Ab showed weaker immunological detection of FHF4A compared to FHF1A and 2A (Figure 18), the immunoreactivity was sufficient to cause a significant inhibition of FHF4A induced LTI (Figure 19G).
Figure 19 A-FHF antibody blocks Na\textsubscript{v}1.6 LTI induced by FHF1A, FHF2A, and FHF4A

(A) Voltage-clamp protocol to record LTI accumulation. (B) Equal sodium currents are generated by Na\textsubscript{v}1.6 upon all depolarization in the absence of FHFs. (C) Representative current trace shows classical LTI accumulation of Na\textsubscript{v}1.6 transfected to Neuro2A cells with FHF2A. (D) With A-FHF antibody (Ab) added to the pipette (500 \(\mu\text{g/ml}\)), LTI by FHF2A is blocked after Ab is perfused into the cell. (E–G) Plots of Na\textsubscript{v}1.6 currents in the presence FHF1A (E), FHF2A (F), and FHF4A (G) recorded at 2 min or >30 min post cell break-in with
or without A-FHF antibody (Ab) in the pipette. LTI is stable during the recording period without the Ab, but is almost abolished after the Ab perfuses into the cell. **p<0.002; ***p<0.001; n=5 or 6 cells for each group.

Most FHFs can cause a positive shift in the voltage-dependence of sodium channel fast inactivation to enhance membrane excitability (Dover et al., 2010; Goldfarb et al., 2007; Lou et al., 2005; Rush et al., 2006). Therefore, the role of FHFs in modulating sodium channels is complicated—FHFs can not only inhibit fast inactivation, but also cause Nav LTI in some cases depending on the endogenously expressed types of FHFs and membrane potentials. Before using A-FHF Ab as a tool to neutralize LTI, I validated the antibody’s specificity, to eliminate the possibility of any off-target effect, such as affecting FHFs’ modulation of fast inactivation.

Accordingly, the voltage-dependence of Nav fast inactivation was analyzed in cells expressing various A-FHFs by using recording pipettes with and without added A-FHF Ab. As shown in the inset of Figure 20A, the recording protocol contained a variable conditioning voltage (CV) and a constant reporting voltage (RV). Sodium channels that undergo fast inactivation during a CV will be reflected as reduced current during the RV. All A-FHFs induced a positive shift (~15 mV) in the voltage dependence of Nav1.6 steady-state (fast) inactivation (Dover et al., 2010) (Figure 20). A-FHF Ab did not cause any notable change in the modulation of fast inactivation by FHF1A (Figure 20A), FHF2A (Figure 20B), or FHF4A (Figure 20C).

A-FHF Ab can significantly suppress Nav LTI induced by FHF1A, FHF2A, and FHF4A (Figure 19) without affecting their modulatory effect on the gating of Nav fast inactivation (Figure 20). Therefore, A-FHF Ab can be used as a tool to neutralize the LTI
particle in the N-termini of A-FHFs expressed endogenously in neurons (Figure 17) that undergo spike frequency accommodation.

**Figure 20** A-FHF antibody has no effect on fast inactivation modulation by all tested A-FHFs.

N235/22 A-FHF monoclonal Ab has no effect on steady-state (fast) inactivation modulation by FHF1A (A), FHF2A (B), or FHF4A (C). The inset of I shows the protocol to measure the voltage dependence of fast inactivation. Non-inactivated channels are
reflected by the current measured at the 0 mV reporting voltage after the antibody had diffused into recorded cells (30-55 min after break-in). n=5 to 8 cells for each group.

4.5 A-FHFs Mediate Spike Frequency Accommodation in Hippocampal CA1 Pyramidal Neurons

Juvenile mouse hippocampal CA1 pyramidal neurons exhibit spike frequency accommodation (SFA) upon a constant current injection (Chen et al., 2014; Moyer et al., 1992; Thompson et al., 1985). Because a rapid onset “slow inactivation” of Nav has been suggested to mediate early SFA in spinal motor neurons (Miles et al., 2005) and A-FHFs are widely expressed on axon initial segments of mouse hippocampal CA1 pyramidal neurons (Figure 17), we hypothesized that A-FHFs may mediate SFA in hippocampal CA1 pyramidal neurons.

To test this hypothesis, I validated A-FHF Ab as a powerful and specific tool to block A-FHF-induced Nav LTI (Figure 19). My former colleague Dr. Kumar Venkatesan carried out current clamp on acutely dissociated mouse brain slice to record action potentials generated by hippocampal CA1 pyramidal neurons, and these firings (action potentials) exhibited SFA driven by a constant current stimulation. When he injected this A-FHF Ab to hippocampal CA1 pyramidal neurons, the perfused A-FHF Ab significantly suppressed SFA. He also used another monoclonal antibody against neuregulin as a control, and this Ab did not affect SFA of pyramidal neurons. In the voltage clamp recording mode, a series of depolarization induced accumulative LTI in ~25% of Nav. In the presence of the A-FHF Ab, LTI accumulation was significantly inhibited (Venkatesan et al., 2014).
Dr. Venkatesan’s findings suggested that cumulative long-term inactivation of sodium channels by A-FHFs could be an underlying mechanism for SFA. By gradually capturing more sodium channels into LTI, A-FHFs progressively raise action potential threshold and delay firings in hippocampal CA1 pyramidal neurons.

Therefore, progressively decreasing sodium conductance caused by A-FHFs (Venkatesan et al., 2014) and gradually increasing potassium conductance by Kv7.2 (Otto et al., 2006) collectively contribute to the phenomenon of SFA, reflecting gradually decreasing membrane excitability of hippocampal CA1 pyramidal neurons.

4.6 Summary

Figure 21 Physical mechanisms of NaV LTI induced by A-FHFs
(A) Essential aliphatic and cationic residues in the A-FHF N-terminus in inducing LTI. The A-FHF LTI particle functions as an open-channel blocker, which competes with β4 subunit for binding to NaV. (B) A-FHF antibody (Ab) blocks NaV LTI by binding to the N-terminus of
A-FHFs in Neuro2A cells. This functional validation of A-FHF Ab provides basis for the application of the Ab in neurons to block LTI induced by endogenous A-FHFs.

The individual aliphatic and cationic residues in the A-FHF LTI particle are essential in causing Nav LTI (Figure 8, 9, and 21A). The size of the letters in Figure 21A reflects the degree of impairment of LTI if the residue is mutated. These aliphatic and cationic residues are not only crucial in inducing LTI, but also in maintaining the long-term inactivated state—all these mutations accelerate sodium channel recovery from LTI. The importance of these residues in the maintenance of LTI supports that LTI and conventional slow inactivation are two distinct processes of sodium channels.

In addition, my research also demonstrates that the A-FHF LTI particle functions as an open-channel blocker, which only captures sodium channel from its open state into a long-term inactivated state (Figure 13). When sodium channels stay open longer, they are more likely to be captured by A-FHF LTI particles (Figure 14). As an open-channel blocker, the pro-inhibitory A-FHF LTI particle can compete with the known pro-excitatory open-channel blocker, Navβ4, for binding with sodium channels (Figure 15 and Figure 21A, black arrows).

Globally expressed on axon initial segments of mouse brain neurons (Figure 17), A-FHFs are speculated to play an important role in modulating firing patterns of these neurons. My research validates the monoclonal A-FHF antibody as a powerful and specific tool to block Nav LTI induced by FHF1A, FHF2A, and FHF4A without affecting their modulation of fast inactivation (Figure 19, 20, and 21B). This functional validation of A-FHF Ab facilitated my former colleague Dr. Kumar Venkatesan’s research to investigate the role of A-FHFs in spike frequency accommodation (SFA) in hippocampal
CA1 pyramidal neurons. He found that A-FHF Ab can block Nav LTI and significantly suppress the SFA.

The findings described above provide a better insight into understanding physical mechanisms of A-FHF-induced Nav LTI and its biological functions. The A-FHF LTI particle functions as an open-channel blocker, relying on the aliphatic and cationic residues in the N-terminus, to progressively arrest more channels and decrease sodium current. The accumulative loss of sodium channel availability caused by A-FHFs gradually raises the action potential threshold and delays the occurrence of firings, and this provides a molecular basis for SFA of many types of neurons. These results were published in *The Journal of Neuroscience* with me as a co-first author (Venkatesan et al., 2014).
Chapter 5 Genetic Strategies to Recapitulate Epileptic Encephalopathy with the *Fhf1* Missense Mutation and to Investigate Na\textsubscript{V} LTI Functions

5.1 Creating transgenic mice to Recapitulate Epileptic Encephalopathy with the *Fhf1* Missense Mutation

In Chapter 3, I have described that a gain-of-function missense mutation in the *FHF1* gene could lead to early onset epileptic encephalopathy (EOEE) (Siekierska et al., 2016). The EOEE-linked *FHF1* R to H mutation causes a greater shift of the voltage-dependence of Na\textsubscript{V} fast inactivation. This stronger inhibition of Na\textsubscript{V} fast inactivation will increase sodium current and enhance neuronal excitability. The higher neuronal excitability may lead to over-excitation, the occurrence of epilepsy, neuronal toxicity, and progressive encephalopathy.

Although the epileptic potential of the mutant FHF1 at the organismal level has been recapitulated in zebrafish larvae, recapitulating epilepsy and encephalopathy and investigating underlying mechanisms beyond the molecular level in transgenic mice will provide better insight into understanding the etiology of epilepsy and may provide significant diagnostic and therapeutic value for epilepsy. Therefore, we planned to create transgenic mice expressing the mutant FHF1 proteins.

5.1.1 Construction Strategy

To investigate which mutant isoform of FHF1 proteins leads to epilepsy, transgenic mice conditionally expressing either FHF1A-R114H or FHF1B-R52H will be
created. To understand which type of neurons are involved in the pathological process, spatial control of transgene expression can provide a better insight.

The Cre-loxP recombination system has been extensively used to generate conditional transgenic mice in specific tissues (Hoess et al., 1982; Rajewsky et al., 1996). Characterized in the virus bacteriophage P1, this site-specific recombination system comprises a DNA site termed lox (the locus of crossing-over) and a protein encoded by the phage, the Cre recombinase (Bezanilla, 2005; Sternberg et al., 1981). If a gene is flanked by two loxP sites in the same orientation, the Cre enzyme will excise that gene from the DNA. To create conditional transgenic mice, a triple polyadenylation sequence (tpA) flanked by loxP sites can be engineered upstream of a transgene, which remains silent (unexpressed) until Cre recombinase excises the stop signal. Therefore, we can generate transgenic mice with silent transgene following loxP flanked stop signal. By mating the animals with various mouse lines expressing the Cre recombinase in specific tissues or even specific subtypes of neurons, we can drive tissue- or neuron- specific expression of the transgene.

In addition to spatial control of a transgene, researchers may also need to manipulate the genetic modification in a temporal manner, since expression of a transgene at an early stage of development could be lethal or too toxic. For example, the afflicted patients in our study died at early ages, temporal control of transgene expression will bypass potential lethal phenotypes in transgenic animals and allow more functional studies of the mutation carried by the patients.
To bypass potential lethal phenotypes conferred by a transgene, a tamoxifen-inducible Cre-driver mouse strain can be used (Betz et al., 1996; Hoess et al., 1982; Ichise et al., 2016). By injecting tamoxifen to transgenic animals at a specific time point, we can control Cre recombination at a specific developmental stage of the animals. In this way, the transgene will remain silent until tamoxifen injection.

ROSA26 is a widely used locus for expressing transgenes in a ubiquitous manner (Soriano, 1999). A pBigT/ROSA26-PA targeting system (Srinivas et al., 2001) would allow introducing the FHF1 coding sequence with EOEE-associated mutation into a plasmid with the ROSA26 genomic flanking arms (Figure 22). Then we can target this ROSA26 plasmid into embryonic stem cells (ESCs).

**Figure 22** ROSA targeted conditional alleles with FHF1 R to H missense mutation

The coding sequence of mutant FHF1A (R114H) or FHF1B 1B (R52H) with IRESZsGreen (green) is cloned into XhoI and SacI sites of the BigT-CAG plasmid. A 6 kb fragment including a CAG enhancer promoter, beta-actin exon I, splice acceptor, loxP-flanked NeoR/tpA from pBigT-CAG is shuttled from this construct into PacI and Ascl sites of the ROSA26-PA vector, which is then linearized with PvuI and electroporated into ESCs. Scale bar: 1kb.
5.1.2 Molecular Cloning of FHF1A-R114H and FHF1B-R52H to pBigT-CAG and pROSA Vectors

We planned to use the pBigT (Addgene plasmid # 21270) /ROSA26-PA (Addgene plasmid # 21271) targeting system from Frank Costantini (Srinivas et al., 2001) to knock the gene expressing FHF1A-R114H or FHF1B-R52H coding sequence and IRES-ZsGreen into the ROSA26 locus of ESCs. pBigT vector contains a loxP-flanked DNA segment including a PGK-neo selectable marker (NeoR) and a triple polyadenylation sequence (tpA). A transgene can be shuttled to the downstream of the LoxP flanked tpA site. Then the fragment containing the silent transgene and a selectable marker can be cloned into the ROSA26-PA vector.

**Constructing pBigT-CAG** — pBigT/ROSA26-PA targeting vectors will allow conditional expression of a transgene under the endogenous ROSA26 promoter. Liqun Luo’s lab has inserted a strong CAG promoter and beta-actin exonI/IVS into ROSA targeting vectors to generate a much stronger conditional expression of transgene (Muzumdar et al., 2007). To enhance transgene expression, pBigT plasmid with a stronger promoter CAG was created by shuttling a fragment containing a CAG promoter, actin noncoding exon I, and the 5’ half of IVS1 derived from ROSA26-mT/mG, a gift from Liqun Luo (Addgene plasmid # 17787) to the PacI site of BigT vector (Addgene plasmid # 21270).

**Constructing pBigT-CAG-FHF1A-R114H-IRESZsGreen and pBigT-CAG-FHF1B-R52H-IRESZsGreen** — The bicistronic coding sequence of FHF1A-R114H or
FHF1B-R52H and IRESZsGreen was cloned into XhoI and SacI sites of the BigT-CAG plasmid (Figure 23). The constructs were confirmed by digestion and sequencing.

**Figure 23** Cloning FHF1A-R114H (or FHF1B-R52H)-IRESZsGreen to pBigT-CAG
The coding sequence of FHF1A-R114H (or FHF1B-R52H)-IRESZsGreen from the IRESZsGreen vector was cloned to XhoI and SacI sites of the pBigT-CAG vector.
Figure 24 Constructed BigT-CAG-FHF1A-R114H-ZsGreen and BigT-CAG-FHF1B-R52H-ZsGreen plasmids

The coding sequence of FHF1A-R114H or FHF1B-R52H with IRESZsGreen from the IRESZsGreen vector is cloned into XhoI and SacI sites of the BigT-CAG vector. Digested with PacI and Ascl restriction enzymes, a 6 kb fragment containing a CAG enhancer promoter, beta-actin exon I, splice acceptor, loxP-flanked NeoR/tpA, and the coding sequence of EGFP-scFv(N235/22) can be shuttled into PacI and Ascl sites of the ROSA26-PA vector.

Constructing pROSA-CAG-FHF1A-R114H-IRESZsGreen and pROSA-CAG-FHF1B-R52H-IRESZsGreen — The plasmid BigT-CAG-FHF1A-R114H (or FHF1B-R52H)-ZsGreen was digested with Ascl and PacI, and a resultant 6 kb fragment contained a CAG enhancer promoter, beta-actin exon I, splice acceptor, loxP-flanked DNA fragment including NeoR and a tpA sequence, and the coding sequence of EGFP-scFv(N235/22) (Figure 24). This fragment was cloned into Ascl and PacI sites of the ROSA26-PA plasmid (Addgene plasmid # 21271) (Figure 25).
Figure 25 Constructed ROSA-CAG-FHF1A-R114H-ZsGreen and ROSA-CAG-FHF1B-R52H-ZsGreen plasmids

The coding sequence of FHF1A or FHF1B mutant with IRESZsGreen within the 6 kb fragment containing a CAG enhancer promoter, beta-actin exon I, splice acceptor, and loxP-flanked NeoR/tpA upstream of it is shuttled into PacI and AscI sites of the ROSA26-PA vector. This construct is linearized with PvuI and electroporated into ESCs.

5.1.3 Transfection of pROSA-CAG-FHF1A-R114H and pROSA-CAG-FHF1B-R52H to ESCs

Ultra-low passage JM8A3 ESCs from the C57BL/6N mouse JM8 parental line were cultured without feeder cells. ESCs were electroporated with the PvuI-linearized ROSA-CAG-FHF1A-R114H-ZsGreen or ROSA-CAG-FHF1B-R52H-ZsGreen plasmid when cells grew rapidly. G418 was added the next day to select ESC clones that have integrated the transgene. DNA was extracted from selected clones for genotyping.
5.1.4 Screening of Positive clones

PCR was performed to screen positive clones with homologous recombination, which would allow the transgene to land to the ROSA26 locus of ESCs. In all 60 clones from either FHF1A or 1B transfected ESCs, at least four of each group showed a clear band after PCR reaction with one primer in the 5' homology arm and the other primer within the CAG promoter. Shown in Figure 26 were representative positive clones (A116, A147, A158, and A160; B103, B112, B130, and B160) with homologous recombination.

Figure 26 Genotyping of ESC clones electroporated with ROSA-CAG plasmids. Positive clones with homologous recombination of the transgene EGFP-scFv (E), FHF1A-R114H-ZsGreen (A), or FHF1B-R52H-ZsGreen (B). B114 is a negative clone. Molecular weight is indicated on the right.

5.1.5 Cre Induction

To confirm whether the recombined transgene in ESCs is Cre inducible, BS-CMV-NLS-Cre plasmid was transfected into ESCs. After 72 h culture, the cells were placed under a microscope to search for fluorescence.
All positive clones incorporating homologously recombined transgenes shown in Figure 27 generated a noticeable ZsGreen fluorescence signals upon Cre induction. For example, strong fluorescent signals were detected from cells derived from positive clone A116 (Figure 27B) and B160 (Figure 28B) after Cre transfection. Positive clones treated with mock transfection without the Cre plasmid did not show any positive ZsGreen signal (Figure 27A and 28A).

**Figure 27** The transgene expressing FHF1A-R114H-ZsGreen homologously recombined to the ROSA26 locus of ESCs is Cre-inducible

(A) ESCs cultures from the positive clone A116 post mock transfection without the Cre plasmid show no fluorescent signal. (B) ESCs cultures from the same clone post transfection with the Cre plasmid show strong fluorescent signals of ZsGreen, indicating expression of the transgene.
5.1.5 Depositing Positive Clones to Generate Transgenic Mice

Positive clones with homologous recombination of the transgene, FHF1A-R114H or FHF1B-R52H with IRESZsGreen, to the ROSA26 locus of ESCs were deposited to the Mouse Genetics and Gene Targeting Shared Resource Facility at Mount Sinai to generate transgenic mice.
5.1.6 An Alternative Construction Strategy: Allele Unmasking

However, the construction strategy described above is not an ideal recapitulation of EOEE patients’ genotype. The afflicted patients are heterozygous, whereas we are using knock-in strategy to overexpress the mutant FHF1 proteins. The true genotype may be reconstructed by generating CRISPR derived Fhf1\( ^+/R\rightarrow H \) mice. This strategy will only be feasible if the phenotypes are milder in mice to ensure lineage survival.

In case that CRISPR derived Fhf1\( ^+/R\rightarrow H \) mice are lethal, an alternative strategy is “conditional allele unmasking,” a technique that has been used to study the effects of conditional expression of heterozygous RAS oncogenic missense alleles on tumorigenesis (Tuveson et al., 2004). Briefly, mice with one allele that integrates the Cre-inducible exon 3 of Fhf1 with the missense mutation will be created. The mice can be mated with various neural Cre driver lines. In the absence of Cre, the mice will be Fhf1\( ^+/\) ; when mated with Cre driver lines, the mice will be Fhf1\( ^+/R\rightarrow H \). Using this strategy, we can reconstruct the true genotype of the EOEE patients, and by mating with different Cre driver lines, we can investigate which types of neurons contribute to epilepsy.

A DNA fragment containing exon 3 of mouse Fhf1 gene generated from a bacterial artificial chromosome vector with mouse Fhf1 gene (Figure 29A) will be cloned into the BluescriptSK plasmid, and the EOEE-associated R/H mutation will be introduced (Figure 29B). A loxP-flanked DNA fragment including PGK1 promoter, NeoR, and a tPA sequence (Figure 29D) will be cut at site PacI and SacII from the BigT-PaClloxP plasmid (Figure 29C). This fragment will then be blunted and cloned into the SwaI site of the BluescriptSK-Fhf1E3-R/H plasmid. This resultant LSL-FHF1(R/H) plasmid (Figure
30A) will be linearized at the KpnI site and electroporated into ESCs. The targeted locus is shown in Figure 30B.

Figure 29 Plasmid construction strategy for engineering FHF1 R/H conditional alleles in mice. **(A)** DNA fragment containing exon 3 of mouse Fhf1 gene. **(B)** The BluescriptSK-FHF1E3 plasmid generated by ligating mFHF1-Exon 3-Ncol fragment and Ncol-digested BluescriptSK plasmid with engineered Ncol site. The R/H mutation will be introduced. **(C)**
The BigT-PaCLoxP plasmid. (D) Blunted LoxP flanked fragment containing PKG-Neo-tpA digested from the BigT-PaCLoxP plasmid with PacI and SacII, and this fragment will be engineered into the Swal site of the BluescriptSK-FHF1E3(R/H) plasmid to generate the BluescriptSK-LSLPKG-Neo-tpA-FHF1E3 plasmid with R/H mutation shown in figure 30.

Figure 30 Targeting pLSL-FHF1(R/H) to Fhf1 genomic locus to generate a conditional endogenous Fhf1 (R/H) allele in mice
The plasmid BluescriptSK-LSLPKG-Neo-tpA-FHF1E3 (R/H), abbreviated as pLSL-FHF1(R/H) in (B), will be linearized with KpnI and electroporated to ESCs. Targeted locus will include an LSL (loxP-StopX3-loxP) cassette upstream of the missense-mutated exon 3. Cre will convert cells from $Fhf1^{+/+}$ to $Fhf1^{+/R\rightarrow H}$.

**5.2 Genetic Approaches for Intracellular Immunization of A-FHF LTI Particles**

In Chapter 4, I have described physical mechanisms and biological functions of voltage-gated sodium channel (Nav) long-term inactivation (LTI) induced by A-type fibroblast growth factor homologous factors (A-FHFs). The A-FHF LTI particle relies on the aliphatic and cationic residues in the N-terminus to progressively capture more channels from their open state into LTI and thus decrease sodium current. This progressive loss of sodium channels may be the potential molecular basis of spike frequency accommodation (SFA) of many types of neurons (Venkatesan et al., 2014).

In the research described above, a monoclonal antibody (Ab) against the N-terminus of A-FHFs (NeuroMab N235/22) was used to block the A-FHF LTI particle in vitro. To investigate broader physiological functions of Nav LTI, an in vivo model with Nav LTI blocked systematically or in a specific population of neurons needs to be established.
5.2.1 Constructing a Single-Chain Variable Fragment (scFv) to Block A-FHF LTI Particles

5.2.1.1 The Rationale

To develop an in vivo model to block A-FHF-induced Nav LTI, a traditional strategy would be disrupting genes expressing A-LTI particles. There are four genes encoding FHFs, *Fhf1, Fhf2, Fhf3*, and *Fhf4*, in vertebrates (Olsen et al., 2003). Therefore, disrupting all genes encoding FHFs is strenuous and exhausting. Fortunately, A-FHFs share 78–100% homology at the N-terminal LTI particle (Dover et al., 2010), and the A-FHF Ab potently and specifically blocks LTI particles of various A-FHFs without affecting their modulation of Nav fast inactivation (Figure 19 and 20). Therefore, if we can successfully express functional intracellular A-FHF antibody, it will provide a valuable tool to specifically perturb A-FHF LTI particles in vivo.

However, naturally occurring antibody contains four separately synthesized polypeptides (two heavy and two light chains) linked together by disulphide bonds, which are unlikely to form in the cytoplasmic reducing environment. The development of single-chain variable fragment (scFv) (also termed intrabodies) containing variable regions of heavy (VH) and light chains (VL) connected with a short flexible linker makes intracellular immunization conceivable (Bird et al., 1988). Despite removal of constant regions, lack of disulfide bonds, and introduction of a flexible linker, many scFv constructs retain specificity and affinity of their parent antibodies (Tanaka and Rabbitts, 2008). Therefore, designing a scFv construct expressing variable regions (VH and VL) of the A-FHF Ab may be an ideal alternative approach to disrupting all fours genes of FHFs.
5.2.1.2 Molecular Cloning of a scFv construct

With this non-canonical and exciting strategy in mind, we contacted UC Davis/NIH NeuroMab Facility that produced the monoclonal A-FHF Ab, and requested for hybridomas (N235/22) that express this antibody. After the hybridomas were received, they were sent to ProMab Biotechnologies, where the RNA from hybridomas (N235/22) was extracted and cDNA of A-FHF Ab’s VH and VL was synthesized, isolated, and sequenced. The cDNAs were sent back to our lab, and coding sequences of VH and VL were shown in Table 5.

PCR fragments of VH and VL were produced to include restriction enzyme sites on both ends of each fragment separated by a flexible linker Gly4Ser (G4S) (Schaefer, 2010) or a transcription terminator. With this PCR strategy, two PCR fragments were generated: EcoRI–G4S–VH–G4S–BspEI and BspEI–G4S–VL–TAG–BamHI. pEGFP-C1 vector was digested with single cutters EcoRI and BamHI within multiple cloning sites. The two PCR fragments and the digested vector was ligated to form a pEGFP-scFv construct as shown in Figure 29. The sequence of each scFv component was shown in Table 5. The construct was verified by DNA sequencing and would express a fusion protein EGFP-scFv if transfected to cells, in which the fluorescence of EGFP would indicate the expression of the scFv.
Figure 31 Diagram of constructing EGFP-scFv expressing plasmid

(A) Composition of a naturally occurring antibody. Red, light chain (VL); blue, heavy chain (VH); light color, variable regions. Inset: A single-chain variable fragment (scFv) can be generated by joining VH and VL with a flexible linker, such as Gly4Ser (G4S). (B) The map of the pEGFP-scFv construct. VH and HL of the monoclonal A-FHF antibody are cloned into multiple cloning sites of pEGFP-C1 vector (left). This construct will express a fusion protein of EGFP-scFv. This protein is expected to fold properly and retain the parent monoclonal antibody’s immunoreactivity to recognize the antigen epitope in the A-FHF LTI particle.
**Table 5** Sequences of scFv fragments

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<tr>
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<th>Sequence</th>
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<tbody>
<tr>
<td><strong>N235/22 VH</strong></td>
<td>CAGGTCCAGCTGGAGCAGTCAGGAGCTGAGCTGGTAAGGCTGGCATTCAG</td>
</tr>
<tr>
<td></td>
<td>GTGAAGGTGTCTCTGAAGGCTTCTGGAATACGCCTCTCATTACTTCTCATAGA</td>
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<td></td>
<td>GTGGGTAAAGCAGAGGCAGGCTGGACAGGGCCTTGAATGGATTGGAATGATTAA</td>
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5.2.1.3 Immunoreactivity of the Constructed scFv

To test whether the engineered fusion protein EGFP-scFv retained its parent monoclonal antibody’s immunoreactivity with the A-FHF LTI particle, different combinations of plasmids expressing EGFP-scFv or the EGFP control, FHF2A, and NaV1.5 were transfected to HEK293T cells. Cell lysates were immunoprecipitated with either a GFP or A-FHF antibody. Immunoprecipitated lysates were immunoblotted with an A-FHF or pan-NaV antibody. Total cell lysates (input) were loaded as control.

Binding between EGFP-scFv and FHF2A was detected by GFP-IP and A-FHF Ab blot; that between FHF2A and NaV1.5, AFHF-IP and pan-NaV blot; and formation of a ternary complex of all three proteins, GFP-IP and pan-NaV blot. The ternary complex was only readily detected in the first lane, in which EGFP-scFv, FHF2A, and NaV1.5 were all transfected (Figure 30A). As shown in Figure 30A, there was no obvious non-specific immunoreactivity between EGFP-scFv and NaV1.5 (lane 2, GFP-IP and pan-NaV blot), EGFP and FHF2A (lane 3, GFP-IP and A-FHF Ab blot), or EGFP and NaV1.5 (lane 3, GFP-IP and pan-NaV blot). Figure 30B illustrated formation of the ternary complex: the scFv immunoreacted with the N-terminus of FHF2A, the core structure of which is tethered to the tail of NaV1.5.
Figure 32 Single-chain variable fragment (scFv) of the monoclonal A-FHF antibody forms a ternary complex with FHF2A and Na\textsubscript{v}1.5 in transfected HEK293T cells

(A) HEK293T cells were transfected (TXF) with various combinations of plasmids expressing EGFP-scFv or EGFP control, FHF2A, and Na\textsubscript{v}1.5, as indicated below immunoblots. Cell lysates were immunoprecipitated with GFP (GFP-IP) or A-FHF antibody (AFHF-IP), as indicated on the top. Immunoprecipitated lysates or total lysates (input) were immunoblotted with an A-FHF or pan-Na\textsubscript{v} antibody. The EGFP-scFv–FHF2A complex was detected by GFP-IP and A-FHF Ab blot; the FHF2A–Na\textsubscript{v}1.5 complex, AFHF-IP and pan-Na\textsubscript{v} blot; and the ternary complex EGFP-scFv–FHF2A– Na\textsubscript{v}1.5, GFP-IP and pan-Na\textsubscript{v} blot.

(B) Diagram of the ternary complex EGFP-scFv–FHF2A– Na\textsubscript{v}1.5. GFP antibody (Ab) immunoprecipitates EGFP-scFv from cell lysates. The scFv immunoreacts with the N-terminus of FHF2A, the core structure of which is tethered to the tail of Na\textsubscript{v}1.5.

These results demonstrate that the cloned EGFP-scFv can stably bind to the A-FHF LTI particle and form a ternary complex with Nav through FHF2A.
5.2.2 Constructing a Viral Vector Expressing EGFP-scFv

After validating the immunoreactivity and stable binding of EGFP-scFv with FHF2A directly and Nav1.5 indirectly, expressing EGFP-scFv in a large amount in neurons was the next goal. An ideal scenario was to express abundant scFv in transgenic mice, while creating a lentiviral vector expressing scFv to infect neurons is also worth trying.

Due to their high infection rate, wide tropism (infecting both dividing and non-dividing cells), capability of integrating into genome, and low immune response in target cells, vesicular stomatitis virus G glycoprotein (VSV-G) pseudotyped lentiviral vectors are used to deliver transgene into cells and animals (Cronin et al., 2005). To minimize the risk of lentivirus as a transfer vector, the essential components to produce virus are split to multiple plasmids, including a transfer plasmid, an envelope plasmid, and packaging plasmid(s). The transfer plasmid is replication incompetent and contains transgene (e.g. EGFP-scFv) and genes for packaging, transduction, and integration of the plasmid into genome (Zufferey et al., 1998). Packaging plasmid(s) produce proteins to transcribe and package an RNA copy of the transfer plasmid into a virus. The envelope plasmid commonly encodes for VSV-G, an envelope protein that confers broad tropism to the packaged virus (Cronin et al., 2005). All these plasmids will be transfected to host cells to produce viruses.

5.2.2.1 Molecular Cloning of EGFP-scFv to a Lentiviral Vector

As described in the previous section, lentiviral vectors can infect both dividing and non-dividing cells, such as neurons; hence, a lentiviral vector may be a powerful tool
to deliver the transgene EGFP-scFv to neurons (Ding and Kilpatrick, 2013).

Calcium/calmodulin-dependent protein kinase II (CaMKII) promotor-based vectors have been shown to drive strong expression of transgene in glutamatergic pyramidal neurons (Dittgen et al., 2004). Therefore, we planned to use a lentiviral vector with a CaMKII promoter.

The lentivirus vector DRH229: FCK-QuasAr1-mO2 with a CamKII promoter was a gift from Adam Cohen (Addgene plasmid # 51629) and used as a vector to which EGFP-scFv was shuttled from the EGFP-scFv plasmid. Cohen’s lab cloned a voltage sensor protein, QuasAr1, into BamHI and EcoRI sites of the FCK vector (Hochbaum et al., 2014). However, BamHI and EcoRI sites are internal to the EGFP-scFv coding sequence. An alternative strategy is to use compatible overhangs of restriction enzymes. For example, BclI shares the same 5’ GATC overhand with BamHI, and MfeI has the same 5’ AATT overhang as EcoRI. Therefore, BclI and MfeI sites were engineered to 5’ of the EGFP and 3’ of the scFv coding sequence through PCR. This PCR product was digested with BclI and MfeI and ligated to BamHI and EcoRI digested pFCK vector. By using this cloning strategy (Figure 31), FCK-EGFP-scFv(N235/22) plasmid was generated and sent to the Neuroscience Gene Vector and Virus Core facility at the Stanford School of Medicine to produce lentivirus.
Figure 33 Construction of lentiviral plasmid FCK-EGFP-scFv(N235/22)

Primers including BclI and MfeI were used to amplify the EGFP-scFv coding sequence through PCR from the pEGFP-C1 vector. The digested PCR fragment was ligated to compatible overhangs of BamHI and EcoRI sites in the lentiviral vector FCK.

5.2.2.2 Expression of EGFP-scFv in Lentivirus-infected Primary Hippocampal Neurons

In Chapter 4, A-FHF-induced NaV LTI has been shown to be involved in spike frequency accommodation (SFA) of hippocampal pyramidal neurons (Venkatesan et al., 2014). Therefore, hippocampal pyramidal neurons became the first target to inject a lentiviral construct expressing EGFP-scFv. Upon receiving the lentivirus containing the FCK-EGFP-scFv construct, rat E17 hippocampal neurons were cultured and infected with the virus at DIV10. Infected neurons were given time for the viral genome to randomly integrate and detected under a fluorescence microscope at DIV13 when axon initial segments matured.
As shown in Figure 32, green fluorescence signal of the fusion protein EGFP-scFv could be detected from some infected neurons. EGFP-scFv was universally expressed in fluorescent neurons, including somas and arborized processes.

**Figure 34** Lentivirus-infected hippocampal neurons express EGFP-scFv

The fluorescence signal of the fusion protein EGFP-scFv expressed in cultured hippocampal neurons at DIV13 following an infection of lentivirus containing FCK-EGFP-scFv at DIV10. A single cell (A) and a pair of neurons (B) expressing EGFP-scFv in somas and processes.

### 5.2.3 Generating Transgenic Mice Expressing EGFP-scFv

After validating the immunoreactivity and stable binding of EGFP-scFv with FHF2A directly and Nav1.5 indirectly (Figure 30), we intended to create Cre-inducible transgenic mice that can express abundant scFv to block A-FHF LTI particles.

#### 5.2.3.1 Construction Strategy

As described in the previous section 5.1.1, a tamoxifen-inducible Cre-loxP recombination system can be used to create conditional transgenic mice in specific tissues at specific time points (Betz et al., 1996; Hoess et al., 1982; Ichise et al., 2016).
Accordingly, we can control Cre recombination to activate transgene expression at a specific developmental stage in a population of neurons. A pBigT-CAG/ROSA26-PA targeting system (Srinivas et al., 2001) was used to transfer the EGFP-scFv coding sequence into the ROSA26 locus of ESCs (Figure 33).

**Figure 35** ROSA targeted conditional expression of EGFP-scFv(N235/22)

The coding sequence of EGFP-tagged single-chain antibody against A-FHFs (blue) is cloned into NheI and NotI sites of the BigT-CAG plasmid. A 6 kb fragment including a CAG enhancer promoter, beta-actin exon I, splice acceptor, loxP-flanked NeoR/tpA from this construct is shuttled into PacI and AscI sites of the ROSA26-PA vector, which is then linearized with Sall and electroporated into ESCs. Scale bar: 1kb.

### 5.2.3.2 Molecular Cloning of EGFP-scFv to pBigT-CAG and pROSA Constructs

The pBigT (Addgene plasmid # 21270) /ROSA26-PA (Addgene plasmid # 21271) targeting system from Frank Costantini (Srinivas et al., 2001) was used to knock the gene expressing EGFP-scFv into the ROSA26 locus of ESCs. pBigT vector contains a loxP-flanked DNA segment including a PGK-neo selectable marker (NeoR) and a triple polyadenylation sequence (tpA). The transgene was shuttled to the downstream of the
LoxP flanked tpA site. Then a fragment containing the silent transgene and a selectable marker can be cloned into the ROSA26-PA vector.

**Figure 36** Construction of BigT-CAG-EGFP-scFv(N235/22) plasmid

The coding sequence of EGFP-scFv from the pEGFP-C1 vector was amplified through PCR adding NotI site downstream of the scFv coding sequence. The digested fragment was then shuttled to Nhel and NotI sites of the pBigT-CAG vector.
Constructing pBigT-CAG-EGFP-scFv(N235/22) — The pBigT/ROSA26-PA targeting vectors will allow conditional expression of the transgene from the endogenous ROSA26 promoter. Our goal was to express abundant scFv so this intrabody can saturate any endogenous A-FHF s present in neurons. Therefore, the pBigT-CAG vector described previously was used. The coding sequence of EGFP-scFv from the pEGFP-C1 vector was shuttled to NheI and NotI sites of the pBigT-CAG vector (Figure 34 and 35). The complete sequence of the insert was confirmed by DNA sequencing.

![Figure 37](image_url)

**Figure 37** Constructed BigT-CAG-EGFP-scFv(N235/22) plasmid and cloning strategy of the EGFP-scFv coding sequence into ROSA26-PA vector

The coding sequence of EGFP-scFv from the pEGFP-C1 vector is cloned into the BigT-CAG vector as illustrated in Figure 34. Digested with PacI and Ascl restriction enzymes, a 6 kb fragment containing a CAG enhancer promoter, beta-actin exon I, splice acceptor, loxP-
flanked NeoR/tpA, and the coding sequence of EGFP-scFv(N235/22) can be shuttled into the ROSA26-PA vector.

**Constructing pROSA-CAG-EGFP-scFv** — The plasmid BigT-CAG-EGFP-scFv(N235/22) was digested with AscI and PacI, and the resultant 6 kb fragment contained a CAG enhancer promoter, beta-actin exon I, splice acceptor, loxP-flanked DNA fragment including NeoR and a tpA transcriptional stop sequence, and the coding sequence of EGFP-scFv(N235/22) (Figure 35). This fragment was cloned into AscI and PacI sites of the ROSA26-PA plasmid (Addgene plasmid # 21271) (Figure 36).

![Diagram](image)

**Figure 38** Constructed ROSA-CAG-EGFP-scFv plasmid

The coding sequence of EGFP-scFv within a 6 kb fragment containing a CAG enhancer promoter from the pBigT-CAG vector, beta-actin exon I, splice acceptor, and loxP-flanked
NeoR/tpA upstream of it is shuttled into Pacl and Ascl sites of the ROSA26-PA vector. This construct is linearized with Sall, a single cutter in this construct.

5.2.3.3 The scFv Transgene Targeting to the ROSA26 locus of ESCs

Ultra-low passage JM8A3 ESCs from the C57BL/6N mouse JM8 parental line were cultured without feeder cells. While ESCs grew rapidly, they were electroporated with Sall-linearized pROSA-CAG-EGFP-scFv. G418 was added the next day to select ESC clones integrated with the linearized plasmid. DNA was extracted from selected clones and PCR was performed to screen positive clones with homologous recombination, which would allow the transgene to land to the ROSA26 locus of ESCs. In all 50 picked clones, at least three of them showed a clear band after PCR reaction with one primer in the 5' homology arm and the other primer within the CAG promoter. Shown in Figure 26 were representative positive clones (E8 and E22) with homologous recombination. However, none of the positive clones generated a noticeable EGFP fluorescence signal upon Cre induction. One possible reason is the EGFP-scFv may be too toxic for ESCs. However, we may bypass this lethal phenotype by inducing Cre recombination at a later stage of mouse development. This approach remains to be tested.

5.2.3.4 Reflection

We have successfully targeted the EGFP-scFv transgene to the ROSA26 locus of ESCs, although no signal of EGFP-scFv was observed upon Cre induction in screened positive clones. The cause of this remains to be investigated. However, the fluorescent signal of EGFP-scFv in lentivirus hippocampal neurons is encouraging and the viral
vector may turn out to be a powerful tool to immunize A-FHF LTI particles. I hope that our lab can recruit more people to validate and use this tool to continue my project.

It is interesting to look back on the last a few years of my life. Turning my head back and looking at those foot prints I left remind me that I still have a long way to go despite struggles, setbacks, and failures. Although mice that express the scFv may only be made on someone else’s watch, I have learned to maintain my emotional stability, remain optimistic, and embrace setbacks with hope. With this philosophy in mind, my doctoral training has taught me much more than doing scientific research—it has also prepared me as a person who values challenges as much as success and can stand up from obstacles and collect composure following achievements.

5.3 Summary

Construction strategies for transgenic mice described in this chapter is the continuation of chapter 3 and 4.

In Chapter 3, the epileptic potential of the mutant FHF1 at the organismal level has been recapitulated in zebrafish larvae (Siekierska et al., 2016). Since rodent models share great similarities with humans in terms of genetic, biological and behavioral traits, we plan to recapitulate the epileptic potential and the resultant encephalopathy and investigate the underlying mechanisms in mice. The strategy to create transgenic mice expressing the mutant FHF1 proteins was illustrated in Chapter 5.1. Positive ES clones with homologous recombination of the transgenes at the ROSA26 locus were screened, and the transgenes were Cre-inducible (Figure 27 and 28). Accordingly, positive clones
were sent to the Mouse Genetics and Gene Targeting Shared Resource Facility at Mount Sinai to undergo further screening and generate transgenic mice.

In Chapter 4, a monoclonal antibody (Ab) against the N-terminus of A-FHFs (NeuroMab N235/22) was used to block the A-FHF LTI particle in vitro to study biological functions of NaV LTI induced by A-FHFs (Venkatesan et al., 2014). To investigate broader physiological functions of NaV LTI, a strategy to create an intrabody (scFv) that can block A-FHF LTI particles was elaborated in Chapter 5.2. The scFv tagged with EGFP-scFv at the N-terminus can successfully and stably bind to FHF2A (Figure 30), and its lentiviral vector can infect cultured hippocampal neurons, in which fluorescent signal of EGFP can be detected (Figure 32). However, although homologously recombined to the ROSA26 locus of the positive ES clones (E8 and E22 in Figure 26), the transgene, EGFP-scFv(N235/22), could not be expressed upon Cre induction. Therefore, the project is discontinued.
Chapter 6 Discussion

6.1 Summary of Main Findings

The FHF protein family acts as a double-edged sword to modulate membrane excitability via leveraging Nav gating. On one hand, FHF1 proteins impede Nav fast inactivation and increase membrane excitability. Chapter 3 describes gain-of-function roles of mutant FHF1 proteins in modulating Nav fast inactivation in epileptic brains. The EOEE-associated FHF1 R to H mutation causes stronger impediment of FHF1 to Nav fast inactivation and increases sodium current. On the other hand, A-FHFs capture Nav to a long-term inactivated state to decrease membrane excitability. Chapter 4 demonstrates that the A-FHF LTI particle functions to progressively inactivate more channels and decrease sodium current. This LTI particle acts as an open-channel blocker, relying on the aliphatic and cationic residues in the N-terminus. Accumulative reduction of sodium current caused by Nav LTI gradually raises the threshold of action potentials, delays the occurrence of firings, and leads to spike frequency accommodation of hippocampal pyramidal neurons in the brain.

To study underlying mechanisms of the EOEE involving FHF1 mutation, Cre-inducible transgenes expressing the mutant FHF1 proteins are introduced to the ROSA26 locus of ESCs, and transgenic mice will be generated accordingly. To expand the scope of understanding biological functions of Nav LTI, single-chain variable fragment (scFv) containing VH and VL of the monoclonal antibody against the A-FHF LTI particle has been constructed with an EGFP tag at the N-terminus. EGFP-scFv’s binding with FHF2A is validated through co-immunoprecipitation experiments. A viral construct expressing
EGFP-scFv is produced and its infection of cultured hippocampal neurons is proved. The transgene encoding EGFP-scFv is targeted to the ROSA26 locus of ESCs; however, no expression of the transgene can be detected upon Cre induction.

6.2 The Gain-of-function Missense Mutation in FHF1 Associated with EOEE Expands Gene Repertoire for Neurological Disorders

6.2.1 FHF1s and Neurological Disorders

The findings of the gain-of-function FHF1 mutation in EOEE have expanded the rather limited human disease phenotypes related to FHF1s. The first report on the association between FHF1s and human diseases is a loss-of-function FHF4 gene mutation in spinocerebellar ataxia (SCA) (van Swieten et al., 2003). A decade later, deletion of the X-linked FHF2 gene was found in Wildervanck syndrome (Abu-Amero et al., 2014).

The first published record of the FHF gene in epilepsy is a translocation between chromosomes X and 14 with a breakpoint on the X chromosome affecting the FHF2 gene in genetic epilepsy and febrile seizures plus (GEFS+). This is speculated due to a loss-of-function of FHF2 in inhibitory interneurons in the hippocampus. The decreased excitability of inhibitory interneurons led to imbalance of excitation-inhibition, and this imbalance may contribute to enhanced excitability within local circuits of the hippocampus and the occurrence of epilepsy (Puranam et al., 2015). In contrast, the results described in Chapter 3 demonstrate that a gain-of-function FHF1 mutation can lead to epilepsy through raising the voltage dependence of Nav fast inactivation to a greater level.
6.2.2 The Gain-of-function Missense Mutation in FHF1 Associated with EOEE

Through whole-exome sequencing, our collaborator, Dr. Buyse, has identified a de novo heterozygous missense FHF1 mutation in two siblings with a fatal neurological disorder characterized by familial EOEE and progressive cerebellar atrophy. The mutation in the FHF1 gene in the EOEE results in mutant proteins FHF1A-R114H and FHF1B-R52H. My research demonstrates that the EOEE-linked FHF1 R to H mutation causes a gain-of-function on inactivation gating of Nav at the molecular level (Figure 3 and 4). This effect on channel gating can enhance neuronal excitability and result in over-excitation, which can probably explain the occurrence of epilepsy. Repetitive, extended over-excitation may cause neuronal toxicity and contribute to progressive encephalopathy in affected patients. In addition to recurrent seizures, affected patients also have other phenotypes, such as severe intellectual impairment, ataxia, and cerebral visual damage—possibly resultant from neurotoxicity-induced encephalopathy. The epileptic potential of the mutant FHF1 can be recapitulated at the organismal level. The in vivo results further support the FHF1 gain-of-function mechanism in the EOEE. This study reports the first neurological disease linked with FHF1 proteins and with an FHF gain-of-function. As the first neurological disease linked with FHF1 and with an FHF gain-of-function, our findings expand gene repertoire for neurological disorders.

6.2.3 Additional Cases of EOEE with the Missense Mutation in FHF1

Additionally, Berge Minassian, a neurologist in The Hospital for Sick Children, University of Toronto, has identified three additional unrelated patients with EOEE that carry the identical FHF1 R to H mutation (Al-Mehmadi et al., 2016). All affected patients
suffered from untreatable seizures and mild-to-severe intellectual impairment. These five cases collectively strengthen a causal relationship of the mutation and EOEE. This neurological disorder caused by the gain-of-function mutation in FHF1 may be treatable by personalized therapy. We hope more neurologists and pediatricians will screen for this locus when searching for abnormal genes in epilepsy for an early diagnosis. These cases lay a foundation for a genetic cause and molecular mechanisms of epilepsy, and more FHF mutations in epilepsy may be characterized in the future. If screened, afflicted patients may be treated by personalized gene therapy to downregulate expression of the mutant gene.

6.3 Modulation of Nav Fast Inactivation Relies on the Binding Interface between the Tail of Nav and FHF Core Domain

FHFs raise the voltage dependence of Nav fast inactivation of Nav and accelerate the channel recovery. Therefore, at a given membrane potential and time, more available Navs will generate a larger current (Lou et al., 2005; Rush et al., 2006; Wittmack et al., 2004). The enhanced sodium current by FHF1 and FHF4 proteins has been shown to favor neuronal excitability and facilitate repetitive firing of cerebellar granule and Purkinje neurons (Goldfarb et al., 2007; Shakkottai et al., 2009).

6.3.1 The Gain-of-function of the FHF1 Mutants in the EOEE Results from the Loss of the Arginine

The binding between FHFs and the tail of Nav is crucial for FHFs to modulate fast inactivation, since mutations of a cluster of residues in FHFs’ channel binding interface prevent FHFs from exerting this pro-excitatory function (Goetz et al., 2009).
Surprisingly, as described in Chapter 4, the EOEE-associated FHF1 missense mutation affects an arginine (R) residue critical in binding with Nav, but has a gain-of-function on modulating Nav fast inactivation. The EOEE-associated mutation substitutes an arginine residue in the β4-β5 loop (Figure 2A), a highly conserved residue located in FHF/Nav C-terminal domain binding interface (Figure 2B), to a histidine. Further mutagenesis and electrophysiological experiments show that this gain-of-function results from the loss of this arginine, regardless of the substitutions (histidine, glycine, and alanine) (Figure 5).

Hence, the specific arginine side-chain in FHF core domain may function to restrict FHF’s modulation of Nav fast inactivation. If this restriction gets released in neurons under pathological conditions, as is the case of the EOEE, FHFs will exhibit a gain-of-function in modulating Nav fast inactivation.

6.3.2 Gain-of-function Mutations of the Arginine’s Counterparts in Nav Lead to Human Diseases

The EOEE-associated mutation affects an arginine in the FHF core domain that is crucial in FHFs’ binding with Nav. This arginine protrudes its side chain into a depression formed by an aspartic acid and a histidine from the Nav C-terminal domain (Figure 2B). In the previous section, we have discussed the restrictive role of this arginine in FHFs’ modulation of Nav fast inactivation. Interestingly, the arginine’s counterparts—the aspartic acid and histidine—in the Nav tail also play modulatory roles in fast inactivation.
6.3.2.1 A Gain-of-function Mutation of the Aspartic Acid (D) in Nav in Epilepsy

A mutation of the aspartic acid (D1866Y) in the C-terminal domain of Nav1.1 is characterized in genetic epilepsy and febrile seizures plus (GEFS+). This missense mutation raises the voltage dependence of Nav1.1 fast inactivation in the absence of accessory proteins (Spampanato et al., 2004). β1 subunit causes a negative shift of the voltage dependence of Nav1.1 fast inactivation, whereas the D1866Y mutation significantly decreases this negative shift and leads to a gain-of-function of the mutant channel (Spampanato et al., 2004). However, it is unknown whether this gain-of-function relies on β1 subunit or FHFs or is independent of the accessory proteins to result in pathological changes of neurons in epileptic brains.

6.3.2.1 A Gain-of-function Mutation of the Histidine (H) in Nav in Cardiovascular Disease

A mutation of either the arginine in FHF core domain or the aspartic acid in the C-terminal domain of Nav can lead to a gain-of-function of Nav and cause epilepsy (Siekerska et al., 2016; Spampanato et al., 2003). Remarkably, a gain-of-function mutation of the histidine in the binding interface, H1849R, in the C-terminal domain (CTD) of cardiac sodium channel, Nav1.5, has also been reported in a five-generation family with a history of atrial and ventricular arrhythmias, cardiac arrest, and sudden cardiac death. The H1849R mutant form of Nav1.5 has reduced binding affinity. Electrophysiological studies demonstrate that H1849R causes a positive shift in the voltage dependence of fast inactivation. The resultant enhanced sodium current may explain the cardio phenotypes in affected patients (Musa et al., 2015).
6.3.3 Restrictive Roles of R-D/H Interactions within FHF/Nav Binding Interface in Membrane Excitability

Although complete destruction of the binding surface between FHF and Nav abolishes FHF’s modulation of Nav fast inactivation (Goetz et al., 2009), the mutation of the arginine from FHFs (Siekierska et al., 2016), the aspartic acid (Spampanato et al., 2004), or the histidine (Musa et al., 2015) from Nav leads to a gain-of-function of the channel, enhances membrane excitability, and induces epilepsy or cardio phenotypes. These results suggest that this region of the binding interface restricts membrane excitability by favoring Nav fast inactivation. Once this restriction is released under pathological conditions as described above, Nav fast inactivation will be inhibited and membrane excitability will be promoted. However, it remains mysterious how this interface exerts its restrictive roles, especially how D1866Y in Nav1.1 can cause a positive shift of the voltage dependence of Nav fast inactivation in the absence of accessory proteins. Another region within the FHF/Nav binding interface may also modulate Nav fast inactivation in a similar manner.

6.4 Epilepsy Can Result from Mutations in α subunit of Nav or Its Accessory Proteins

Nav comprises a large pore-forming α subunit and accessory proteins, such as β subunits and FHFs, and both can modulate the gating of Navα. β subunits modulate channels’ expression, gating (Calhoun and Isom, 2014), and pharmacology (Lenkowski et al., 2003). Through interacting with the CTD of Nav, FHFs can enhance membrane excitability via causing a positive shift of fast inactivation (Lou et al., 2005; Rush et al.,
2006; Wittmack et al., 2004), whereas β1 subunit can decrease excitability by causing a negative shift (Spampanato et al., 2004).

Previous studies have characterized mutations in genes encoding pore-forming α subunits of Nav in epilepsy (Catterall et al., 2010; Estacion et al., 2014; Kamiya et al., 2004; Veeramah et al., 2012). A mutation in SCN1B encoding the channel’s β1 subunit has also been shown to affect the gating of Nav in GEFS+ (Wallace et al., 1998). Recently, a mutation in the FHF2 gene has been identified in GEFS+ (Puranam et al., 2015). My collaboration work described in Chapter 3 links a missense mutation in the FHF1 gene to EOEE. All these findings suggest that any perturbation of Nav and its accessory proteins (β subunits and FHFs) could lead to abnormal neuronal excitability, which may trigger the occurrence of epilepsy.

Other genetic causes of epilepsy include loss-of-function mutations in genes encoding voltage-gated M-type potassium channels (Singh et al., 1998; Singh et al., 2003), and a gain-of-function mutation in the gene encoding large conductance calcium-sensitive potassium channel (Du et al., 2005).

6.5 Multiple Functions of the Nav C-terminal Domain

6.5.1 Modulatory Roles of the Nav C-terminal Domain in Fast Inactivation

Although FHFs and β1 subunit modulate Nav fast inactivation in completely opposite manners, both types of the modulation involve the Nav C-terminal domain. Several studies have demonstrated the modulatory roles of the Nav CTD in fast inactivation.
In addition to D1866Y in Nav1.1 (Spampanato et al., 2004), D1790G mutation in cardiac sodium channel Nav1.5 (corresponding to D1803 in Nav1.1) has been characterized in congenital long-QT syndrome (LQT) and shows strong effect on the channel’s gating in the presence of β1 subunit (An et al., 1998). However, in contrast to the gain-of-function mutation of D1866Y in Nav1.1, which causes a positive shift (10 mV) of the voltage dependence of Nav fast inactivation in the presence of β1 subunit (Spampanato et al., 2004), the LQT3 mutation D1790G in Nav1.5 C-terminal domain causes a strong negative shift (-16.3 mV) with β1 subunit co-expressed (An et al., 1998). These two aspartic acids are not very distant from each other; however, they obviously play opposite roles in modulating Nav fast inactivation. Additionally, the gain-of-function of D1866Y in Nav1.1 can be independent of β1 subunit, whereas D1790G’s loss-of-function in Nav1.5 relies on the presence of β1 subunit.

More studies provided a link between mutations in Nav1.5 CTD and LQT, including a substitution of D1840G (Benhorin et al., 1998) and an insertion of aspartic acid (1795D) (the affected patients also had Brugada syndromes) (Bezzina et al., 1999; Veldkamp et al., 2000). Moreover, a charged structured region of Nav1.5 C-terminal domain has been shown to stabilize the fast inactivation state and restrict membrane excitability (Cormier et al., 2002).

Todd Scheuer’s lab has constructed chimeric sodium channels using CTDs from different subtypes of sodium channels (Nav1.2 with faster fast inactivation from the brain, and Nav1.5 with slower fast inactivation from the heart). The results demonstrate that the CTD strongly influences the kinetics and voltage dependence of fast inactivation.
For example, replacing the tail of Na\textsubscript{V}1.5 with that from Na\textsubscript{V}1.2 speeds up the fast inactivation rate and causes a negative shift in its voltage dependence. Channel truncation experiments also reveals an inhibitory effect of the distal part of the C-terminal domain on fast inactivation: the truncated channels (Na\textsubscript{V}1.2-\delta K1890 and Na\textsubscript{V}1.5-\delta K 1888) show a much faster inactivation rate and a negative shift of the voltage dependence (Mantegazza et al., 2001).

6.5.2 Interactions between the Nav C-terminal Domain and Auxiliary Proteins

In addition to FHFs (Dover et al., 2010; Liu et al., 2001; Liu et al., 2003; Lou et al., 2005; Rush et al., 2006), other auxiliary proteins of Nav that interact with the CTD include β subunits (Spampanato et al., 2004) and Ca\textsuperscript{2+} sensor calmodulin (Mori et al., 2003). Na\textsubscript{V}α subunit interacts with β1 and β3 subunits non-covalently via their N- and C-termini (McCormick et al., 1998; Meadows et al., 2001; Spampanato et al., 2004); whereas it interacts with β2 and β4 covalently via a disulfide bond at the extracellular N-termini (Messner and Catterall, 1985; Yu et al., 2003). In the mammalian brain, Na\textsubscript{V}α subunit forms a heterotrimeric complex with two different β subunits (Messner and Catterall, 1985). Mutations in the Na\textsubscript{V} CTD that affect the binding with β subunits and calmodulin are reported in several human diseases. Yeast two-hybrid analysis has revealed a direct interaction between Nav CTD and β1 and β3 subunits. The D1866Y mutation in Nav1.1 CTD that reduces the Nav-β1 interaction and limits Nav fast inactivation is associated with epilepsy (Spampanato et al., 2004). A mutation (R1902C) in Nav1.2 characterized in autistic patients with seizures localizes to the calmodulin binding region in the channel’s C-terminal domain (Weiss et al., 2003).
Among all these auxiliary proteins of Nav α subunit, β1 subunit restricts membrane excitability by favoring fast inactivation, β4 subunit enhances membrane excitability via resurgent current, and FHFss affect membrane excitability in both ways. Since the D1866Y mutation in Nav1.1 CTD that weakens the interaction with β1 subunit is also the channel’s binding site with FHFss, there may be functional competition between these two types of auxiliary proteins. In contrast, since β4 is linked to Nav α via a disulfide bound at the N-terminus in the extracellular Ig domain and β4’s C-terminus interacts with the pore of the channel instead of the channel’s CTD, there should be no direct competition between β4 and FHFss or β1 for an interaction with Nav α CTD. The competition between the N-termini of A-FHFss and the C-terminus of β4 will be further discussed in Section 6.7 of current chapter.

6.6 Slow Inactivation and LTI Are Two Distinctive Processes

As described in Table 1, Nav requires a long time to recover from slow inactivation and LTI, compared to fast inactivation and β4 subunit-induced open-channel block. However, Nav slow inactivation and LTI are two distinctive processes. Slow inactivation is caused by intrinsic α subunit intramolecular mechanisms, whereas LTI is caused by A-FHFs. Additionally, the onset of LTI is much faster compared to that of slow inactivation.

More specifically, slow inactivation is induced by conformational changes in the ion selectivity filter (Balser et al., 1996; Todt et al., 1999), S6 segment (Chen et al., 2006), and voltage sensor (Silva and Goldstein, 2013a; Silva and Goldstein, 2013b). The conducting pore of Nav in the slow inactivated state collapses due to asymmetric
movements of S6 segments (Payandeh et al., 2012; Zhang et al., 2012). In contrast, LTI is caused by the highly conserved N-termini of A-FHFs (Dover et al., 2010; Goldfarb, 2012), which obstruct Nav in its open state. The positively charged cationic residues in LTI particles may serve as binding components to block the conducting pore and/or a charged barrier to repel sodium influx.

When we first submitted our paper including the findings described above to The Journal of Neuroscience, one of the reviewers believed that Nav LTI is slow inactivation, and that A-FHFs merely catalyze the entry of Nav into this slow inactivated state with a faster onset. Evidence against this argument is that individual aliphatic and cationic residues in the A-FHF LTI particle are essential in both induction and maintenance of Nav LTI. The mutations in the A-FHF LTI particle that impair Nav accumulative LTI (Figure 8 and 9) also accelerate Nav recovery from LTI (Figure 10). If A-FHFs’ role is only to accelerate the onset of slow inactivation, these mutations should only affect the induction of inactivation. Since these mutations also affect the maintenance of LTI by accelerating the recovery rate, LTI is proved to be a distinctive process from slow inactivation.

6.7 Competition between Two Open-channel Blockers of Nav

My results in Chapter 4.2 show that very similar voltage-dependent sodium channel transitions are required to open the channel and to predispose it to A-FHF-induced LTI. These comparable voltage dependent transitional states of Nav suggest that A-FHFs function as open-channel blockers to induce Nav LTI. Peptide injection experiments demonstrate a reciprocal competition for Nav binding between A-FHF and
Navβ4 derived peptides. Collectively, these findings support that the A-FHF LTI particle is an open-channel blocker, and it can compete with β4 subunit for binding with Nav.

β4 subunit-induced open-channel block has been reported to compete with fast inactivation (Lewis and Raman, 2014). However, β4 subunit only blocks an open channel (Wang et al., 2006). In contrast to other types of inactivation, the associated β4 subunit will be expelled immediately upon repolarization and the freed sodium channels that are locked to an open state by β4 subunit will generate a resurgent current before transitioning to a closed state. This resurgent current is pro-excitatory and facilitates high-frequency firing of neurons (Grieco et al., 2005; Raman and Bean, 1997).

On the contrary, A-FHF-induced Nav LTI is a rather stable state and Nav requires a longer time to recover (Dover et al., 2010). Therefore, A-FHF-mediated open-channel block limits membrane excitability. My collaboration work with Dr. Kumar Venkatesan shows that A-FHFs gradually capture more sodium channels into LTI, progressively raise the threshold of action potentials, delay firings, and thus mediate spike frequency accommodation (SFA) in hippocampal CA1 pyramidal neurons.

The two open-channel blockers have completely opposite functions on membrane excitability due to their dramatically different dissociation rates from Nav. In competition with fast inactivation and LTI, β4 subunit can safeguard some sodium channels from other types of inactivation and facilitate high-frequency firing; whereas A-FHFs can arrest some sodium channels into LTI and mediate SFA. In summary, β4 subunit increases firing frequency, while A-FHFs decreases firing frequency. When both types of blockers are present in cells, it will be interesting to study which blocker outcompetes the
other and what the net effect is on membrane excitability. The mechanisms of the competition between the two open-channel blockers remain elusive—one possibility is that they may share overlapping binding sites within open sodium channels.

**6.8 Research Limitations and Future Directions**

Major limitations of my research include limited cases of EOEE patients, unclear physical mechanisms of FHFs’ modulation of Nav fast inactivation, unavailable mouse models to recapitulate epileptic potentials of EOEE-associated *FHF1* mutation, and no functional validation of the constructed scFv.

**6.8.1 To Screen More Epileptic Patients for Mutations in FHFs**

My collaboration work identifies two EOEE cases with the *FHF1* mutation, and three additional cases with EOEE from unrelated families that carry the identical FHF1 R to H mutation have been characterized (Al-Mehmadi et al., 2016). These five cases collectively strengthen a causal relationship between the gene mutation and phenotypes of EOEE. Our findings may encourage more neurologists to screen for this locus when diagnosing and treating EOEE.

**6.8.2 A New Hypothesis of Physical Mechanisms of Nav Fast Inactivation Modulation**

It remains unknown how FHFs play pro-excitatory roles via modulating Nav. Complete destruction of the binding interface between FHF and Nav abolishes FHF’s pro-excitatory functions (Goetz et al., 2009). However, single residue mutations of the binding interface—the arginine from FHFs (Siekierska et al., 2016), the aspartic acid
(Spampanato et al., 2004), and the histidine (Musa et al., 2015) from Nav—lead to gain-of-functions of sodium channels, enhance membrane excitability, and induce epilepsy or cardio phenotypes. This region within the binding interface seems to restrict membrane excitability by favoring Nav fast inactivation.

How can a residue buried within the FHF/Nav binding interface serve to modify fast inactivation (Musa et al., 2015)? How can the tail of Nav stabilize the fast inactivation state (Cormier et al., 2002)? How can the N-terminal extensions of A-FHFs favor strong modulation of Nav fast inactivation (Dover et al., 2010)? How can epitope tagging of an FHF enhance Nav inactivation modulation (Goetz et al., 2009)? How can the Nav tail have an inhibitory effect on fast inactivation (Mantegazza et al., 2001)?

One hypothesis is that the Nav tail, in the presence of FHFs or not, restricts fast inactivation at resting potential; upon depolarization, a voltage sensor-induced conformational change of the channel drags its tail or tail/FHF complex away to favor and stabilize fast inactivation; increase in the volume or shape of the tail through FHF binding would demand further depolarization and a more dramatic conformational change of the channel to conquer the increased bulkiness of the tail to enable the occurrence of fast inactivation.

In the cases of gain-of-function mutations in the FHF/Nav characterized in epileptic disorders and cardiovascular disease (Musa et al., 2015), each mutation may weaken the tight FHF/Nav interaction and render the Nav tail to become loose and bulky, which would add more challenge to fast inactivation and require further membrane depolarization. This hypothesis may be tested by electrophysiological and biochemical
assays of FHF and Nav variants with various single or double mutations within the FHF/Nav binding interface. For researchers employing structural biology approaches, it will be important to determine whether gain-of-function mutations at the FHF/Nav interface alter the structure of the protein complex.

6.8.3 Additional Construction Strategies for Transgenic Mice

Although the epileptic potential of the EOEE-associated FHF1 mutation at the organismal level has been recapitulated in zebrafish larvae, generating corresponding transgenic mouse models will provide better insights into understanding the etiology of epilepsy and encephalopathy. Currently, embryonic stem cell clones with the Cre-inducible transgene expressing mutant FHF1 proteins (A or B isoform) integrated into the ROSA26 locus have been screened and sent to the Mouse Genetics and Gene Targeting Shared Resource Facility at Mount Sinai to generate transgenic mice, and this will allow conditional expression of mutant FHF1 proteins to avoid lethal phenotypes. By mating the animals with different Cre driver lines, we can identify which types of neurons contribute to the seizure phenotype.

The afflicted patients are heterozygous, whereas we are using knock-in strategy to overexpress the mutant FHF1 proteins in ROSA26 locus. The true genotype may be reconstructed by generating CRISPR derived Fhf1+/R→H mice. In case this strategy results in lethal phenotypes, we are also exploring another strategy “conditional allele unmasking,” a technique that has been used to study the effects of conditional expression of heterozygous RAS oncogenic missense alleles on tumorigenesis (Tuveson et al., 2004). Briefly, mice with one allele that integrates the Cre-inducible exon 3 of Fhf1 with
the missense mutation will be created. The mice can be mated with various neural Cre
driver lines. In the absence of Cre, the mice will be *Fhf1*<sup>+/−</sup>; when mated with Cre driver
lines, the mice will be *Fhf1*<sup>+/R→H</sup>. Using this strategy, we can reconstruct the true
genotype of the EOEE patients, and by mating with different Cre driver lines, we can
investigate which types of neurons contribute to epilepsy. More details of this techniques
were described in Section 5.1.6 of Chapter 5.

6.8.4 Functional Validation of the scFv against A-FHF LTI Particles

Although the constructed EGFP-scFv can stably bind with FHF2A, and the
generated virus can infect primary hippocampal neurons, whether this scFv could block
A-FHF-induced Na<sub>V</sub> LTI has not been validated. It is mysterious that the positive ES
clones did not show any EGFP signal upon Cre induction. However, the viral construct
could still be a potential tool to perform in vivo studies. To block A-FHF LTI particles,
scFv must be expressed in excessive amount to saturate any A-FHFs present in neurons.
If scFv fails to saturate endogenous A-FHFs in neurons, the non-neutralized A-FHFs may
still induce LTI and no noticeable change caused by the svFv could be detected. The viral
construct may enable massive expression of the scFv in infected neurons.
Immunocytochemistry can be performed to detect A-FHFs with its monoclonal antibody.
If the monoclonal antibody fails to recognize A-FHFs when neurons are infected, it may
prove that these endogenous A-FHFs are totally pre-absorbed by the scFv.

A type of rapid-onset Na<sub>V</sub> inactivation with long-term recovery was suggested to
promote dendritic spike attenuation in hippocampal neural dendrites (Colbert et al., 1997;
Jung et al., 1997). Since the characteristics of this type of inactivation resemble A-FHF
mediated Nav LTI, it is possible that A-FHFs mediate hippocampal dendritic attenuation. If the scFv against A-FHFs can by functionally validated, it can be a powerful tool to test this possibility.

6.9 Conclusions

FHF1s modulate membrane excitability via leveraging Nav gating in complex ways. On one hand, FHFs impede Nav fast inactivation and increase membrane excitability. This pro-excitatory effect is enhanced by the EOEE-associated FHF1 mutation. To study the underlying mechanisms of the EOEE, transgenic mice conditionally expressing the mutant FHF1 proteins will be generated.

On the other hand, A-FHFs function as open-channel blockers, relying on the aliphatic and cationic residues in the N-terminus, to progressively capture more sodium channels into a long-term inactivated state. This accumulative reduction of available sodium channels gradually elevates the threshold of action potentials, delays the occurrence of firings, and leads to spike frequency accommodation of hippocampal pyramidal neurons in the brain. To investigate more biological functions of A-FHF-induced Nav LTI, EGFP-scFv to neutralize A-FHF LTI particles has been constructed. This fusion protein can stably bind with FHF2A. More tests are needed to validate whether this scFv can be used to perturb A-FHFs’ functions in vivo.

My research has provided more insights into understanding Nav fast inactivation and LTI mechanisms and functions. These findings may deliver significant diagnostic and therapeutic values for diseases involving dysfunctions of sodium channels, such as epilepsy, arrhythmias, and pain disorders.
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Publications, Presentations, and Awards during Doctoral Study

Academic Publications


Selected Presentations


Awards
Mario Capelloni Doctoral Dissertation Fellowship, Interim Associate Provost and Dean for Academic Affairs, GC, CUNY, 2015-2016

Beatrice Goldstein Konheim Graduate Scholarship in the Life Sciences, Hunter College, CUNY, 2014