Inhibition of voltage-dependent sodium currents by cannabidiol

by

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Abstract

Voltage-gated sodium channels initiate action potentials in excitable tissues. Altering these channels’ function can lead to many pathophysiological conditions. The family of voltage-gated sodium channel genes encodes 10 proteins (including Nav2.1) distributed throughout the central and peripheral nervous systems, cardiac and skeletal muscles. The SCN4A gene encodes the Nav1.4 channel, which is primarily responsible for depolarization of the skeletal muscle fibers. Many mutations in SCN4A are found and associated with the myotonic syndromes and periodic paralyses. These conditions are both considered gain-of-function and can be severely life-limiting with respect to performing everyday tasks. From a broader standpoint, hyperexcitability presents as a significant problem in other tissues besides skeletal muscles. Gain-of-function in sodium channels has been linked to a wide-range of pathophysiological conditions such as inherited erythromelalgia, epilepsy, and arrhythmias. Treating these types of pathologies requires an in-depth understanding of their underlying mechanisms. One way to gain this understanding is to investigate physiological triggers. There is also a dire need for novel ways of reducing the hyperexcitability associated with mutant sodium channels. One promising compound is the non-psychotropic component of the Cannabis sativa plant, cannabidiol. This compound has recently been shown to modulate some of the neuronal sodium channels. Although cannabidiol has shown efficacy in clinical trials, the underlying mechanism of action remains unknown. Sodium channels could be among the molecular targets for cannabidiol.

In my doctoral research: 1) I studied how a single missense mutation, P1158S, in Nav1.4 causes various degrees of gain-of-function (myotonia and periodic paralysis) by using pH changes to probe P1158S gating modifications; 2) I studied the inhibitory effects of cannabidiol on voltage-dependent sodium currents; 3) I investigated the mechanism through which cannabidiol imparts inhibition. Overall, these data reveal novel insights into sodium channel hyperexcitability and pharmacologically targeting of this hyperexcitability using cannabidiol.

Keywords: sodium channel; myotonia; periodic paralysis; cannabidiol; acidosis
To my family, especially my parents,
Ahmad & Mahmonir.

‘Your hand can seize today, but not tomorrow; and thoughts of your tomorrow are nothing but desire. Don’t waste this breath, if your heart isn’t crazy, since "the rest of your life" won’t last forever.’

Omar Khayyam.
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I was lucky to work at Xenon Pharma under the mentorship of three brilliant scientists: Drs. Charles Cohen, JP Johnson, and Samuel Goodchild. Being around Charlie during meetings, I saw firsthand how an outstanding biophysical mind with years of experience in drug discovery thinks about science. From JP, I learned that a hallmark of a great scientist is to care about the patients that may benefit from your research. At Xenon, Sam had the biggest influence on my growth. Sam taught me the importance of deep thinking about science, and that in research, it is better to be conservative than wrong. I will forever be grateful for the many hours I spent with Sam at the lab, office, or lunch rides.

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I have had the pleasure of working with many excellent colleagues; however, a few became close friends. During the early/majority of grad school, Mena Abdelsayed, Colin Peters, and Kaveh Rayani were the ultimate companions I could ask for. Whether it was the long nights at the lab or the trips to various cities or conferences, I will forever cherish every moment (too many to count here) I spent with each of them. More recently, Mohamed Fouda and Ravi Chandra became great friends. These companions were my true inspirations to want to go to the lab every day. Last but not least, I thank my amazing family and long-time friends, whose contributions to my life extend far beyond this thesis.
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<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AP</td>
<td>Action potential</td>
</tr>
<tr>
<td>Cav</td>
<td>Voltage-gated calcium channel</td>
</tr>
<tr>
<td>CBD</td>
<td>Cannabidiol</td>
</tr>
<tr>
<td>GOF</td>
<td>Gain-of-function</td>
</tr>
<tr>
<td>HyperPP</td>
<td>Hypokalemic periodic paralysis</td>
</tr>
<tr>
<td>HypoPP</td>
<td>Hypokalemic periodic paralysis</td>
</tr>
<tr>
<td>Kv</td>
<td>Voltage-gated potassium channel</td>
</tr>
<tr>
<td>LOF</td>
<td>Loss-of-function</td>
</tr>
<tr>
<td>Nav</td>
<td>Voltage-gated sodium channel</td>
</tr>
<tr>
<td>RMP</td>
<td>Resting membrane potential</td>
</tr>
<tr>
<td>SSFI</td>
<td>Steady-state fast inactivation</td>
</tr>
<tr>
<td>THC</td>
<td>$\Delta 9$-Tetrahydocannabinol</td>
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Chapter 1. Introduction

Parts of this chapter describe the work published in (Ghovanloo et al., 2018b; Ghovanloo & Ruben, 2020) with minor modifications and formatting changes to suit the thesis style.

1.1. Voltage-gated sodium channel: structure and function

Ion channels orchestrate an exquisite array of physiological processes, including nerve impulses, muscle contraction, and signaling in all organisms. The electric current in signaling is generated by ion flux across the cellular membrane that is controlled by the opening and closing of ion channels. These channels are permeable to various types of ions, including sodium, potassium, calcium, and chloride (Hille, 2018). The direction of the ion flux is determined by the membrane potential and transmembrane ionic gradients that are established by ion channels and the Na⁺/K⁺ ATPase (Vassalle, 1987). For the generation of a specific type of signaling known as the action potential (AP) to be possible, it is crucial that the ion channels involved are selectively permeable to a specific type of ion and not to others (Hodgkin & Katz, 1949; Hodgkin & Huxley, 1952; Hille, 1975, 2001). A subset of these ion channels is the voltage-gated sodium (Nav) channel superfamily (Figure 1-1) (Catterall et al., 2005; Catterall, 2005).
The primary structures of the subunits of the voltage-gated sodium channels.

Figure 1-1  The primary structures of the subunits of the voltage-gated sodium channels.

A transmembrane folding diagram of the Nav channel. Cylinders represent α-helical segments. Bold lines represent the polypeptide chains of each subunit, with a length approximately proportional to the number of amino acid residues in the brain sodium channel subtypes. The extracellular domains of the β1 and β2 subunits are shown as immunoglobulin-like folds. C, sites of probable N-linked glycosylation; P in red, sites of demonstrated protein phosphorylation by protein kinase A (circles) and protein kinase C (diamonds); blue, pore-lining segments; yellow circles, the outer (EEEE) and inner (DEKA) rings of amino residues that form the tetrodotoxin-binding site and ion selectivity filter; green, S1–S4 voltage sensors; h in blue circle, inactivation particle in the inactivation gate loop; blue circles, sites implicated in forming the inactivation gate receptor. ScTx, scorpion toxin (Catterall & Swanson, 2015).

The sodium current passing through Nav channels initiates action potentials in neurons, skeletal muscles, and cardiac muscles (Figure 1-2). Nav channels are heteromultimeric proteins composed of large ion conducting α-subunits and smaller auxiliary β-subunits (Isom et al., 1992; Patton et al., 1994; Cannon, 2006; Estacion et al., 2010; Catterall, 2012; Calhoun & Isom, 2014; Ghovanloo et al., 2016b; Ghovanloo & Ruben, 2020). The α-subunit is made up of a single transcript that encodes four 6-transmembrane segment domains (Catterall, 2012). Each one of these four structural domains can be divided into two functional sub-domains known as the voltage-sensing domain (VSD) and the pore domain (PD) (Catterall, 2012; Ghovanloo et al., 2016b). These two functional sub-domains are connected through the intracellular S4-S5 linker (Yarov-Yarovoy et al., 2012; Catterall, 2012). The VSD is formed by the first four transmembrane segments of
each domain and the pore is formed by the 5th and 6th segments along with the extracellular pore loop that connects them (Catterall, 2012; Ghovanloo et al., 2016b).

Figure 1-2  Nav channels are predominantly expressed tissue-specifically (The salt-sensing Nav2.1 is not included).

In a simplified model, the sodium channel can exist in three fundamental states: rest, open, and inactivated (Catterall, 2012). During depolarizations that are of sufficient magnitude, sodium channels open, and begin conducting ionic sodium currents. This process is known as activation. Upon activation, while the sodium channel is in its open state, it conducts sodium currents. Any disturbance to this process of sodium current conduction will have downstream effects on excitability. After activation, sodium channels may typically enter a fast-inactivated state, which is initiated by the outward movement of the domain IV-VSD (Figure 1-3). VSD-I-III are associated with translating changes in transmembrane voltage into channel activation through an electromechanical coupling process to a gate at the inner pore. VSD-IV is specialized in its function to control inactivation and is slower to activate with a different voltage dependence to VSD-I-III (Chanda & Bezanilla, 2002; Silva & Goldstein, 2013).
The process of fast inactivation is mediated through the interaction of the domain III-IV linker with another region of the channel (West et al., 1992). Recent cryo-EM structures of eukaryotic sodium channels revealed that fast inactivation may proceed via an allosteric mechanism. This mechanism involves the IFM (isoleucine, phenylalanine, methionine) motif promoting pore closure by squeezing into the space between S6 and S4-S5 restriction ring. Indeed, the skeletal muscle sodium channel’s structure has shown the F residue penetrating into this space, and I and M residues to the edges around this space (Shen et al., 2017; Yan et al., 2017; Pan et al., 2018). The allosteric mechanism of fast inactivation was further elucidated using the structure of the cardiac sodium channel, where outward movement of the third VSD opens an interaction site for the fast inactivation particle. This particle in turn moves into place upon the outward shift of the fourth VSD.
(Ghovanloo & Ruben, 2020; Jiang et al., 2020). This process that happens within milliseconds of activation, blocks the channel pore, and effectively stops current conduction. Therefore, this negative regulation of conductance is a way of controlling excitability.

In addition to fast inactivation, which was discovered by Hodgkin and Huxley (Hodgkin & Katz, 1949; Hodgkin & Huxley, 1952; Hille, 2001), Nav channels have a second slower inactivated state (Vilin & Ruben, 2001). Repetitive or prolonged stimulation can result in slow inactivation. In a physiological setting, this slow inactivation process is vital to limit the frequency of firing and define the length of trains of action potentials to protect cells against excitotoxic injury (Vilin & Ruben, 2001). It is thought that during slow inactivation, two of the opposite S6 segments move towards the pore axis with the other two pointing outwards. This asymmetry causes a structural collapse leading to a slowly reversible inactivation (Gamal El-Din et al., 2013a).

1.1.1. Types of Nav currents

Upon the presence of a sufficiently strong depolarizing stimulus, Nav channels activate. Once activated, a macroscopic transient sodium current is generated, which can be divided into two components: peak and persistent currents. The peak current is known as the large-amplitude inward segment of the sodium transient. The overall sodium current tends to incompletely inactivate in both muscle and nerve cells. This incomplete inactivation results in a small, persistent current component which is only a small percentage (<5%) of the peak current amplitude (Crill, 1996). The persistent current plays a vital role in excitatory cell bodies and dendrites where it increases the size of postsynaptic potentials and boosts the cell threshold during trains of action potentials (Liu & Shipley, 2008).

Some classes of neurons may also generate resurgent currents (Raman & Bean, 1997; Raman et al., 1997; Cannon & Bean, 2010). These currents appear as a rebound inward current that may appear following a voltage pulse or an action potential. Resurgent currents are caused by reopening Nav channels and is dependent on the presence of the β4 subunit, which competes with the inactivation particle at more depolarized potential. At more depolarized potentials, when the fast inactivation particle is in its non-conductive state, the population of channels that have the β4 in its bound state may serve as a
reservoir for subsequent firing. Thus, the unbinding of β4 causes a surge of inward sodium current that depolarizes the membrane. The interactions between Nav1.6 and β4 were studied extensively by (Cannon & Bean, 2010; Patel et al., 2016). Overall, persistent and resurgent currents work together with slow inactivation to generate complex patterns of action potentials firing.

1.1.2. Structural segments of the Nav pore and pharmacological modulation

The general Nav pore structure includes a large external vestibule, a narrow selectivity filter, a large central cavity that is lined by S6 segments that is filled with water, and an intracellular activation gate that is formed by the crossing of S6 segments at the intracellular side of the membrane (Payandeh et al., 2011; Yan et al., 2017; Pan et al., 2018; Jiang et al., 2020).

The first ion channel crystal structures described the architecture of potassium channels (Doyle et al., 1998). The emergence of a multitude of crystal and cryo-EM structures revealed that the overall structure of the pore between Nav and Kv is similar. However, the structure of the ion selectivity filters, and mechanisms of ion conductance are different between the two channels. Potassium channels select potassium by direct interactions between the backbone carbonyls of residues that comprise the selectivity filter. These interactions create 4 ion coordination sites (Catterall, 2012). In potassium channels, no amino acid charged functional groups or water molecules are involved in the selectivity process. However, in Nav channels, the selectivity filter has a high field strength on the extracellular side which is composed of amino acid side chains. This outer vestibule is followed by 2 ion coordination sites that are formed by backbone carbonyls (Figure 1-4) (Payandeh et al., 2011; Yan et al., 2017). These sites allow for passage of sodium ions with 4 water molecules (hydration). These sites would be too large for a dry sodium ion to go through, which would be energetically unfavorable. This indicates that sodium conductance and selectivity are different to that of potassium ions.
Figure 1-4  The pore domain of EeNav1.4 is kept Open at the intracellular gate.

(A) The intracellular gate is kept open. The permeation path, calculated by HOLE, is illustrated by brown dots in the left panel. The pore radii of EeNav1.4 (brown) and NavPaS (purple) are tabulated in the right panel. The side chains of Asp/Glu/Lys/Ala (DEKA) in EeNav1.4 are not well resolved. Therefore, the corresponding radii are shown as a gray dashed line. (B) Conformational changes between the pore domains of EeNav1.4 and NavPaS. For visual clarity, the pore domain segments from the two diagonal repeats of the superimposed structures of EeNav1.4 and NavPaS are shown in each panel. (C) The Gly hinge that demarcates the conformational shifts of S6. The structures of EeNav1.4 and NavPaS are superimposed as in (B). The Cα atoms of the indicated residues are shown as spheres. The labels for EeNav1.4 residues are domain colored. The corresponding NavPaS residues are labeled below in purple. (D) Shifts of the conserved Asn residues on each S6 segment between EeNav1.4, NavPaS, and Cav1.1. Intracellular views are shown. (left) The pronounced conformational shifts of the conserved S6-Asn residues between EeNav1.4 and NavPaS. (right) The corresponding Asn residues in EeNav1.4 and Cav1.1 exhibit similar orientations. (E) The pore domain has four fenestrations. The structure of the pore domain is shown in four perpendicular side views. The fenestrations are highlighted by orange circles. Upper inset: the residues that were characterized to be involved in anesthetic binding are shown as spheres (Yan et al., 2017).
From a functional perspective, both the outer and inner segments of the PD are interaction sites for pharmacological agents. The most selective and well-known Nav blocker is tetrodotoxin (TTX), which comes from symbiotic bacteria in the pufferfish (and some other animals) diet (Hille, 2001; Chau et al., 2011). The sensitivity of sodium channel subtypes to TTX has been used to divide the family into two classes: TTX-sensitive and TTX-resistant. The IC$_{50}$ of TTX-sensitive (Nav1.1-4, Nav1.6-7) channels to TTX is less than 30 nM (Catterall et al., 2005; Gamal El-Din et al., 2013b). The molecular reason underlying differential affinity for TTX in Nav has been attributed predominantly to a single homologous residue difference in the Nav pore-loop. The TTX-resistant channels have a cysteine or serine in this position, instead of a tyrosine or phenylalanine residue in TTX-sensitive channels. A recent study determined that this substitution does not alter the local conformation of the channel. However, lacking an aromatic side chain in this position may cause steric constraints that reduce the TTX affinity (Ghovanloo & Ruben, 2020; Jiang et al., 2020).

TTX is considered a state-independent Nav blocker, a function of its binding-site residing on the outer selectivity filter, which is a more rigid part of the Nav pore-domain. In contrast, most local-anesthetics (LA) are highly state-dependent Nav blockers (may also have high affinity for open state); their binding site is located below the selectivity filter, a more flexible region of the pore-domain. This part of the channel is highly conserved among Navs (Ragsdale et al., 1996). Therefore, it is not surprising that most Nav pore blockers that interact at the LA site impart little subtype selectivity. The flexibility difference between the outer and inner PD has important pharmacological implications.

Because the proportion of channels populating different states is controlled by the membrane potential, the state-dependence of such compounds is often termed voltage-dependence. Many of these compounds also display a phenomenon known as use-dependence, which occurs when the compound apparent potency increases upon higher sodium channel firing frequency stimulations (Gamal El-Din et al., 2018).

Many Nav modulating compounds are used to treat various clinical conditions caused by changes to excitability. In particular they are used as anticonvulsants (ex. Carbamazepine, Phenytoin), as local anesthetics (ex. Lidocaine), and as antiarrhythmics (ex. Mexiletine) (Nuss et al., 1995; Mantegazza et al., 2010; Pless et al., 2013; Ghovanloo et al., 2016a). Because these compounds largely lack selectivity across the sodium
channel superfamily, they may lead to potentially undesirable side-effects. All of these compounds are either neutral or weakly basic. In addition to the two general sites in the PD, recent efforts have culminated in the development of highly selective sodium channel blockers that target DIV-VSD (Ahuja et al., 2015).

1.1.3. Nav distribution and tissue-specific channelopathies

There are multiple sodium channel isoforms expressed in tissues throughout the body. Nav1.1-Nav1.3 are found in the central nervous system (Figure 1-2). Nav1.4 and Nav1.5 are expressed in skeletal and cardiac muscles, respectively (Figure 1-2). Nav1.6 is expressed in both the central and peripheral nervous systems (Figure 1-2). Nav1.7-Nav1.9 are primarily found in the peripheral nervous system (Figure 1-2). The expression pattern of the neuronal Nav channels depends on both the developmental stage, brain region, and cell type. Nav1.3 is expressed predominantly in neonatal brain cells; thus, it is thought to be a key contributor to brain development. In contrast, Nav1.1, Nav1.2, and Nav1.6 are highly expressed in adult brains. Furthermore, Nav1.6 displays greatest expression in unmyelinated axons, whereas Nav1.2 is found in the cell soma (Whitaker et al., 2001; Hu et al., 2009). Although the different isoforms share a similar structure, their gating and response to physiological and pathophysiological modulators can vary widely.

Changes to the gating properties of sodium channels, and subsequently the current passing through them during an action potential can cause potentially fatal abnormalities in electrical signaling. Both gain-of-function (GOF) and loss-of-function (LOF) in sodium channels disrupt electrical signaling. Interestingly, several mutants display both GOF and LOF, leading to multiple disease phenotypes (Makita et al., 2008; Webb & Cannon, 2008).

In the primary sodium channel isoforms of the CNS, namely Nav1.1, 1.2, 1.3, and 1.6, both GOF and LOF elicit epilepsy syndromes (Catterall, 2010; Estacion et al., 2010; Veeramah et al., 2012). These include relatively mild epilepsies, like benign familial neonatal-infantile seizures, or more severe forms, such as Dravet syndrome (Heron et al., 2002; Scalmani et al., 2006; Dravet, 2011) and early-infantile epileptic encephalopathy-13 (O’Brien & Meisler, 2013). In skeletal muscle, Nav1.4, GOF mutants elicit myotonic and paralytic syndromes (e.g. hypokalemic periodic paralysis, hypoPP), causing an inability to relax or contract the muscle, respectively (Cannon, 1996; Ghovanloo et al., 2018a). Long
QT-3 syndrome is due to an increase in the fraction of Nav1.5 cardiac sodium channels that fail to inactivate and, consequently, an increased persistent sodium current throughout the action potential plateau that delays repolarization (Wang et al., 1995). Conversely, mutants that decrease peak Nav1.5 current cause Brugada syndrome and other diseases of conduction (Antzelevitch et al., 2005; Ghovanloo et al., 2020). Lastly, certain GOF mutations in Nav1.7 cause inherited erythromelalgia (Cheng et al., 2010; Shields et al., 2012; Tham et al., 2017; Bankar et al., 2018).

1.2. Clinical phenotypes involved with Nav1.4

Mutations in Nav1.4 are associated with several pathogenic skeletal muscle phenotypes (Figure 1-5). The pathogenicity associated with these mutations can manifest in increased or reduced muscle excitability (Cannon, 2015).

Muscular hyperexcitability typically presents with a distinctive stiffness, known as myotonia. Myotonia results from an involuntary contraction that persists for several seconds after voluntary movement. This after-contraction is caused by a burst of muscle action potentials that lasts for many seconds and is not dependent on motor neuron excitation (Brown & Harvey, 1939; Rudel & Lehmann-Horn, 1985). The severity of myotonic attacks in patients fluctuates with the levels of muscle activity. Myotonia-induced stiffness is often more pronounced after the first forceful movement after long periods of rest, and usually decreases over seconds with continued voluntary movement. This is known as the warm-up phenomenon. In some cases, the myotonic stiffness may paradoxically worsen with repeated muscle contraction. This is known as paramyotonia. Myotonia may be aggravated by external triggers such as cooler temperatures or ingestion of high-potassium food. Muscular myotonia is caused by either a LOF in the muscle chloride channel, ClC-1, or a GOF in Nav1.4 (Cannon, 2015).

A reduction in skeletal muscle contractility can arise from defects in Nav1.4. This is perceived as transient attacks of weakness or a chronic state of permanent myopathic weakness. The most prevalent manifestation of these attacks is a state of periodic paralysis, which happens with episodes of moderate to severe weakness from a sustained depolarization from rest, activating and inactivating Nav1.4. The prolonged Nav1.4 inactivation prevents additional excitability of the muscle fiber (Cannon, 2015).
Similar to myotonia, periodic paralyses-induced attacks of weakness are also usually triggered by external factors, including post-exercise rest. Another factor that could trigger weakness attacks is a diet that is high in carbohydrates, salt, or fasting. Increased levels of emotional stress is also suggested to trigger attacks (Lehmann-Horn et al., 2008; Cannon, 2015).

Periodic paralysis attacks usually onsets over the span of minutes, lasting for several hours, and may go as long as a day or longer. The attacks are followed by a spontaneous recovery. In severe cases, paralytic attacks may hinder the patient’s ability to move any limbs, or even breathe. The onset of symptoms typically begins after the age of forty and worsens with time (Cannon, 2015).

In contrast to the GOF conditions described, LOF in Nav1.4 can result myasthenia or congenital myopathy (Figure 1-5). Myasthenia is characterized by rapid muscle fatigue that lasts from seconds to minutes. Recovery is also rapid. Congenital myopathy is associated with reduced muscle tone that is accompanied by various degrees of weakness (Tsujino et al., 2003; Zaharieva et al., 2016).

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The channelopathies associated with Nav1.4 are considered rare disorders with a prevalence of ~1:100,000 (Horga et al., 2013). The majority of these channelopathies are autosomal dominant and are highly penetrant (Lehmann-Horn & Jurkat-Rott, 1999; Huang et al., 2017). Years of functional studies on SCN4A mutations established the association of particular genotypes to phenotypes (Miller et al., 2004b; Cannon, 2015). These studies
suggest that specific mutations in SCN4A are consistently found to underlie a particular skeletal muscle disorder. Furthermore, most of these mutations are found to be missense changes, although several frameshift, nonsense, and splice-site mutations have also been identified (Zaharieva et al., 2016).

1.2.1. GOF mutations in SCN4A cause myotonic and paralytic syndromes

Missense GOF mutations in SCN4A are associated with three overlapping syndromes. At one end of the spectrum is myotonia, which is manifested in patients showing characteristic symptoms, but without any periodic paralyses. The mutations that cause pure myotonia often do so by altering the kinetics of Nav1.4 (Figure 1-6) (Cannon, 2010a, 2015).

The next syndromes on the spectrum are paramyotonia and hyperPP (triggered by >5 mM plasma [K⁺]), which can manifest in overlapping symptoms (Figure 1-5). The mutations causing these two conditions usually alter both Nav1.4 kinetics, steady-state, and slow inactivation (Figure 1-6) (Cannon, 2010a, 2015).

The last GOF syndrome caused by SCN4A mutations is hypoPP (triggered by <3 mM [K⁺]) (Figure 1-6) (Fontaine, 2008; Ghovanloo et al., 2018a). Unlike the previous conditions that generally increase the ionic sodium current into the cell, hypoPP mutations result in pathogenetic gating pore currents through the VSD. This pathogenic gating current depolarized the membrane potential and renders Nav channels from recovery, and hence prevent further AP firing.

1.2.2. P1158S profile

P1158S is a unique mixed hypoPP and myotonia causing mutation that is located on the intracellular side of Nav1.4 (Sugiura et al., 2000, 2003; Webb & Cannon, 2008; Ghovanloo et al., 2018a). P1158S in Nav1.4 is a missense mutation in the S4-S5 linker of domain (D) III that is the result of a C to T mutation at position 3549 in SCN4A (Sugiura et al., 2003). P1158S is described to cause both myotonia and hypoPP. This mutation was first reported in a Japanese family. The first case report described a 33 year old man who started experiencing paralysis attacks at the age of 13 and myotonia since the age of 25 (Sugiura et al., 2000). The ability of P1158S to give rise to severe GOF (hypoPP) and
more moderate GOF (myotonia) make it a unique clinical SCN4A mutation (Cannon, 2018).

Figure 1-6  Model simulation of myotonia and periodic paralysis.

Simulated Nav1.4 mutant currents. A two-compartment model for skeletal muscle (Cannon et al., 1993), to simulate the sarcolemma and the t-tubule including K+ accumulation, was used to simulate the response to current injection (Cannon, 2015).

1.2.3. Therapeutic approaches against myotonia and periodic paralyses

Treatment for myotonia is focused on reducing the involuntary action potential bursts. It is vital that myotonic patients modify their lifestyles to avoid some triggers including potassium ingestion or cold temperatures. However, there are some commonly used therapeutics to relieve and prevent muscle stiffness (Figure 1-7). These drugs are often anticonvulsants, antiarrhythmics, and local anesthetics (Alfonsi et al., 2007; Trip et al., 2008).

Treating hypoPP is usually achieved using oral potassium ingestion and by avoiding dietary carbohydrates and sodium. During hypokalemia increasing potassium levels could help in reducing membrane depolarization and shifting the resting potential to more negative potentials (in contrast to the hyperpolarization predicted by the Nernst equation, a higher [K+] gradient obtained by low external potassium may cause
paradoxical RMP depolarization (Jurkat-Rott et al., 2009); therefore, extracellular potassium reduction reduces currents that hyperpolarize the RMP, such as the outward component of the inwardly rectifying (Kir) channels. Administering acetazolamide or dichlorphenamide is sometimes useful; however, in some cases these compounds can exacerbate symptoms (Figure 1-7) (Torres et al., 1981; Tawil et al., 2000; Sternberg et al., 2001; Venance et al., 2004). Despite these agents there is still a need for other compounds that would alleviate the hyperexcitability associated with both myotonia and hypoPP.

<table>
<thead>
<tr>
<th>Disorders</th>
<th>Therapies</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMC</td>
<td>Anticonvulsants (phenytoin and carbamazepine)</td>
</tr>
<tr>
<td></td>
<td>Anti-arrhythmics of the class IB (mexiletine and tocainide)</td>
</tr>
<tr>
<td></td>
<td>Anti-arrhythmics class IC (flecainide and propafenone)</td>
</tr>
<tr>
<td></td>
<td>Local anesthetics (acetazolamide and hydrochlorothiazide)</td>
</tr>
<tr>
<td>PAM</td>
<td>Anticonvulsants (phenytoin and carbamazepine)</td>
</tr>
<tr>
<td></td>
<td>Anti-arrhythmics of the class IB (mexiletine and tocainide)</td>
</tr>
<tr>
<td></td>
<td>Anti-arrhythmics class IC (flecainide and propafenone)</td>
</tr>
<tr>
<td></td>
<td>Local anesthetics</td>
</tr>
<tr>
<td>HyperPP</td>
<td>β-adrenergic agonists such as (salbutamol used as an inhalant)</td>
</tr>
<tr>
<td></td>
<td>Glucose/insulin therapy</td>
</tr>
<tr>
<td></td>
<td>Diuretic carbonic anhydrase inhibitors (acetazolamide and dichlorphenamide and thiazides)</td>
</tr>
<tr>
<td>HypoPP</td>
<td>Oral potassium</td>
</tr>
<tr>
<td></td>
<td>Acetazolamide or dichlorphenamide, triamterene, aldosterone antagonists</td>
</tr>
<tr>
<td></td>
<td>Potassium-sparing diuretics</td>
</tr>
</tbody>
</table>

**Figure 1-7** Medication in the different forms of Na$^+ \text{v}$ myotonia and paralyses.

Potassium-aggravated myotonia (PAM), paramyotonia congenita (PMC), hyperkalemic periodic paralysis (HyperPP), hypokalemic periodic paralysis (HypoPP) (Simkin & Bendahhou, 2011).

### 1.3. Sodium channel modulation by pH

Maintaining the physiological pH balance is vitally important to human body function. Under normal physiological conditions, the extracellular pH is maintained at approximately 7.4, with the intracellular pH ranging between 7.2 and 7.4. Under hypoxic
and ischemic conditions, the extracellular pH decreases significantly. During focal ischemia, rabbit brain pH could drop to 6.0 (brain intracellular pH is 7.0) (Meyer, 1990). Similarly, intense physical exercise results in a decrease of muscle extracellular pH to 6.4 (Hermansen & Osnes, 1972; Maruki et al., 1993). In cardiac tissue, myocardial ischemia, including regional and global ischemia, extracellular pH can lower to 6.1 (Cobbe & Poole-Wilson, 1980). The presence of extracellular protons can modulate both the VSD and the PD, depolarizing the voltage-dependence of activation and blocking ionic current, respectively (Khan et al., 2002; Jones et al., 2013b). Acidification decreases peak sodium conductance by protonating the outer vestibule carboxylates (Khan et al., 2002, 2006), and likely by binding to negative charges in the VSDs, which destabilizes the outward conformation of the voltage-sensors (Shi et al., 2014; Peters et al., 2017).

1.3.1. Mechanisms of proton block

We demonstrated additional mechanisms by which neuronal excitability is altered by extracellular acidosis (Ghovanloo et al., 2018b). For instance, in neurons expressing Nav1.1 or Nav1.3, excitability could be decreased during extracellular acidification.

Previous studies in sodium channels determined that mutations in the conserved DEKA and EEDD motifs cause a shift in the pK\textsubscript{a} of proton block in the acidic direction (Terlau et al., 1991; Sun et al., 1997; Khan et al., 2002). Mutating these carboxylates into alanine residues results in an approximately 25% decrease in proton block. This suggests that the interactions between the positively charged H\textsuperscript{+} and pore carboxylates blocks the ion conductance pathway, which subsequently reduces sodium current (Sun et al., 1997). As DEKA and EEDD are conserved across the sodium channel family, it is not surprising that, in all of the sodium channels studied thus far, proton block exists albeit with varying degrees (Vilin et al., 2012a; Peters et al., 2018; Ghovanloo et al., 2018b). This is particularly evident in Nav1.4 where previous studies have shown a reduction in current density that is neither statistically significant nor negligible (Vilin et al., 2012a; Ghovanloo et al., 2018a).

Proton block is not limited to the protonation of carboxylates in the selectivity filter. Previous studies in Nav1.5 identified a cysteine (C373) residue on the outer vestibule of domain I that imparts pH-sensitivity (Khan et al., 2002, 2006). In Nav1.1-1.4 this cysteine is replaced with either phenylalanine or tyrosine residues. During acidosis, C373 gets
protonated, creating a positive charge outside the pore that causes proton block. The presence of this cysteine can in part explain the increased pH-sensitivity observed in Nav1.5 compared to other sodium channels (Jones et al., 2013b).

In addition to C373, two other residues involved in pH response were identified in Nav1.4 and Nav1.5. H880 in Nav1.5 is located in the pore loop of domain II, and P1158 in Nav1.4 is located on the hinge of the intracellular S4-S5 linker of domain III. Both these residues are conserved in Nav1.1-Nav1.5 (Jones et al., 2013b; Ghovanloo et al., 2018a) and are important to the biophysical properties of the respective channels in which they were described. Mutating H880 into a glutamine (Q) residue reduces the pH-sensitive current and shifts the voltage-dependence of activation in Nav1.5. Unlike C373 and H880, both of which directly contribute to proton block in Nav1.5 (Jones et al., 2013b), P1158 in Nav1.4 indirectly contributes to a reduced proton-insensitivity. P1158 is located on the intracellular side of the channel, and we previously showed that mutating this proline to a serine (S) residue increases proton block in Nav1.4 at low pH (Ghovanloo et al., 2018a). This effect may occur by altering the voltage-dependence of gating in domain III.

1.3.2. Mechanisms by which protons modulate gating

In addition to proton block, low pH alters channel gating. The effects of protons on gating were studied in Nav1.5 (Peters et al., 2018). Although the identity of the residues involved in pH-dependent changes in gating are not fully determined, structural studies in bacterial sodium channels and potassium channels suggest that acidic residues play a role (Payandeh et al., 2012; Shi et al., 2014). Interactions of protons at the individual domains typically depolarizes the voltage-dependence, presumably via electrostatic interactions which hinder the outward movement of S4 voltage-sensors. This electrostatic hindrance at domains I-III primarily affects activation, and at domain IV affects fast inactivation. However, the effects of protons on sodium channel gating is strongly subtype-dependent (Vilin et al., 2012a; Peters et al., 2018; Ghovanloo et al., 2018a, 2018b) (Table 1.1).
<table>
<thead>
<tr>
<th>Subtype</th>
<th>Activation Relative to pH7.4</th>
<th>Inactivation Relative to pH7.4</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nav1.1</td>
<td>Depolarized</td>
<td>Unchanged</td>
<td>(Ghovanloo et al., 2018b)</td>
</tr>
<tr>
<td>Nav1.2</td>
<td>Depolarized</td>
<td>Unchanged</td>
<td>(Vilin et al., 2012a; Peters et al., 2013)</td>
</tr>
<tr>
<td>Nav1.3</td>
<td>Depolarized</td>
<td>Unchanged</td>
<td>(Ghovanloo et al., 2018b)</td>
</tr>
<tr>
<td>Nav1.4</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>(Vilin et al., 2012a; Ghovanloo et al., 2018a)</td>
</tr>
<tr>
<td>Nav1.5</td>
<td>Depolarized</td>
<td>Depolarized</td>
<td>(Vilin et al., 2012b)</td>
</tr>
</tbody>
</table>

### 1.3.3. pH response comparison among the Nav subtypes

We characterized the effects of protons on Nav1.1 and Nav1.3. Our findings suggest that the magnitude of the proton-dependent changes in the biophysical properties of these channels is nearly identical (Ghovanloo et al., 2018b). This similarity is consistent with the shared localization of Nav1.1 and Nav1.3 in the cell bodies of neurons, which may suggest similar roles in neuronal excitability, as the expression of these channels are inversely correlated during neonatal development and postnatal weeks (Gordon et al., 1987; Scheinman et al., 1989; Beckh et al., 1989; Whitaker et al., 2001; Yu et al., 2006).

We also found that at low pH, both channel subtypes display a depolarized conductance-voltage relationship, but no effects on steady-state fast inactivation. These results are consistent with the pH-sensitivity reported for rat pyramidal neurons (Tombaugh & Somjen, 1996).

The comparison of proton-sensitivity across Nav1.1-Nav1.5 reveals that activation is more susceptible to pH modulation than inactivation (Table 1.1). There are two potential explanations for this observation that are not mutually exclusive: 1) there is more exposure to extracellular protonation of sites involved in activation, and 2) having one domain controlling fast inactivation instead of three controlling activation decreases the number of protonatable sites and therefore decreases the probability that protons modulate fast inactivation. Testing the first hypothesis requires extensive mutation-based experimentation that should be investigated in future studies. The second hypothesis is based on the classic Hodgkin-Huxley model that describes the sodium conductance in terms of three activation components and a single fast inactivation component \( g_{Na} = m^3 h \) (Hodgkin & Huxley, 1952). Thus, it is conceivable that having more domains controlling activation may increase the likelihood of carboxylate-proton interactions in domains I-III,
which is in part due to having a larger net number of carboxylate-containing residues to protonate (Ghovanloo et al., 2018b).

1.4. Cannabinoids

The cannabis plant, *Cannabis sativa*, contains over 120 active constituents, which are collectively called phytocannabinoids (Morales et al., 2017). Cannabis originated in the Himalayas and was first cultivated in China for seed and fiber production. Early records of using cannabis medicinally can be traced to Sumerians records around 1800 B.C.E., which mention using this plant against a variety of diseases, including convulsions. There are more recent records of cannabis use against epilepsy in Islamic literature (Elsohly, 2007; Russo et al., 2008b).

1.4.1. THC

Over the last century, cannabis consumption became illegal in many parts of the world due to its psychotropic effects; and these legal constraints also disrupted cannabis research. In the 1960s, however, some progress was made on the research front and, several years later, (Devane et al., 1988) determined how Δ9-tetrahydrocannabinol (THC), the key psychotropic phytocannabinoid, imparts psychoactivity. THC discovery led to identifying two cannabinoid receptors, CB1 and CB2. These receptors play many physiological roles in our bodies, including pain response, mood, and memory, among others (Pertwee, 2008; Billakota et al., 2019).

THC has a high affinity for the human CB receptors; its modulation of these receptors trigger psychoactivity. THC serves as an agonist at sub-micromolar concentrations. CB1 receptors are primarily found in the presynaptic terminals of CNS neurons. These receptors are predominantly expressed in cerebral cortex, basal ganglia, limbic structures, and some areas of the midbrain and medulla. CB1 is a G-protein coupled receptor that modulates neurotransmitter release. Endogenous ligands bind to the CB-receptors in the postsynaptic terminals and mediate several forms of long- and short-term synaptic plasticity. CB2 receptors are expressed in the CNS neurons of the brainstem and hippocampus. THC binding to CB2 in these areas can affect neuronal excitability. In addition to CB1 and CB2, THC also interacts with some TRP, calcium, and potassium channels (Lupica et al., 2004; Ross et al., 2008; De Petrocellis et al., 2011). The functional
consequences of modulation of the non-CB receptor targets by THC are not fully understood. However, THC is reported to also be an analgesic, a muscle relaxant, and an anti-inflammatory agent (Russo, 2008; Russo et al., 2008a).

1.4.2. CBD

Cannabidiol (CBD) is another key phytocannabinoid that shares a similar structure and many of the physiological effects of THC without psycho-activity. This major difference is because CBD has little to no affinity for the CB receptors with which THC interacts (Devane et al., 1988). The lack of CBD activity at CB receptors and diversity of reports of efficacy (both anecdotal and clinical trials) in various disorders have resulted in many proteins being proposed as CBD targets, including Navs, Kvs, Cav, TRP channels, GPR receptors, etc. (Ross et al., 2008; De Petrocellis et al., 2011; Patel et al., 2016; Kaplan et al., 2017; Ghovanloo et al., 2018c).

Among these CBD targets, the Nav family is particularly interesting, as abnormalities in the functions of Nav channels are suggested or shown to be associated with many of the conditions for which CBD has therapeutic value, such as Dravet Syndrome (DS) (Dravet, 2011; Devinsky et al., 2017). This condition affects almost every aspect of development in children who suffer from it by causing hundreds of seizures a week. The sheer frequency and intensity of these seizure attacks prevents the child from performing some of the most basic activities, including the ability to talk or walk. Unfortunately, each seizure also has the potential to be lethal (Dravet, 2011; Devinsky et al., 2017).

The first step in understanding the molecular interactions between CBD and Navs suggested that CBD preferentially blocks resurgent currents (Patel et al., 2016). Therefore, if Navs are among the physiological targets for CBD in efficacy against epileptic disorders (specifically Dravet syndrome), then this efficacy might be due to the CBD block of Nav1.6 resurgent currents over peak currents. Nav1.2 and Nav1.6 are presumed to be the predominant Nav isoforms in excitatory neurons; however, the Nav1.2 isoform gets partially replaced by Nav1.6 within the first year of life (Spratt et al., 2019). As Dravet syndrome is a LOF in Nav1.1 (the predominant Nav channel subtype in inhibitory neurons), the inhibition of Nav1.6 resurgent currents would reduce the overall hyperexcitability associated with epilepsy (Patel et al., 2016). Nav1.1 and Nav1.6 exist in
a balance to maintain proper nerve conduction. Therefore, reducing the activity of Nav1.6 excitability would implicate a reduction in Nav1.1 LOF.

1.4.3. Other potential cannabinoid uses

Cannabis constituents are suggested to possess therapeutic effects in a range of other disorders. For instance, in patients with muscular dystrophy, cannabis helps to manage pain and involuntary muscle tightness. In patients that suffer from neuropathic pain, cannabis significantly reduces the intensity of chronic pain and also improve sleep. Cannabis also helps with involuntary muscle tightness and reduces muscle tremors and spasticity (Baker et al., 2000; Pertwee, 2008; Ware et al., 2010; Wilsey et al., 2013; Borgelt et al., 2013; Woodhams et al., 2015; Iannotti et al., 2019).

In addition to the plant-based phytocannabinoids, much effort has gone into studying the endogenous cannabinoids, 2-arachidonoyl glycerol (2-AG) and arachidonoyl ethanolamide (anandamide) which have important physiological implications. Since this dissertation focuses on phytocannabinoids, detailed discussions of endogenous cannabinoids can be found elsewhere (De Petrocellis et al., 2011; Iannotti et al., 2019).

1.5. Conclusion

Many mutations in SCN4A are associated with the myotonic syndromes and periodic paralyses. These conditions can be severely life-limiting with respect to performing everyday tasks. Currently, there is a lack of mechanistic insight on many mutants underlying these conditions. There is little known about how physiological triggers affect the mutants causing these conditions.

From a broader standpoint, hyperexcitability presents as a significant problem in other tissues besides skeletal muscles. GOF in Navs has been linked to a wide range of conditions such as inherited erythromelalgia, epilepsy, and arrhythmias. Treating these types of conditions requires an in-depth understanding of their underlying mechanisms. One way to gain this understanding is to investigate physiological triggers. There is also a dire need for novel ways to reduce the hyperexcitability associated with Navs. One promising compound is CBD.
For my doctoral research I first studied how a single missense mutation (P1158S) in Nav1.4 causes various degrees of GOF (myotonia and periodic paralysis). In aim 1 (Chapter 2), I began by investigating pH-sensitivity in P1158S, as changes in extracellular pH that are associated with physical activity, are a common trigger for both myotonia and periodic paralysis. The results from this project provided new information about additional ways protons interact with Navs. This project also laid the foundation for developing a new *in-vitro/in-silico* assay of Nav1.4 hyperexcitability.

In aim 2 (Chapter 3), I studied the inhibitory effects of the FDA-approved cannabidiol on Nav currents. Although CBD (commercial name: Epidiolex) has shown efficacy in clinical trials, the underlying mechanism for the efficacy was unknown. The findings from project 2 provided important information on how the presence of CBD modulates voltage-dependent sodium currents. These findings laid the foundation of a series of hypotheses about the molecular mechanism of CBD-Nav interactions.

In aim 3 (Chapters 4-5), I investigated the in-depth biophysical (along with other computational and structural means through collaborations) mechanism through which CBD inhibits Nav currents. I also measured CBD effects in the *in-vitro/in-silico* assay developed in aim 1 to model potential therapeutic value against myotonia and periodic paralysis.

These three aims provide a deeper understanding of the mechanism underlying certain aspects of Nav GOF and also unveil the ways through which this GOF could be suppressed by a superhydrophobic compound like CBD.
Chapter 2. A mixed periodic paralysis & myotonia mutant, P1158S, imparts pH-sensitivity in skeletal muscle voltage-gated sodium channels

This chapter describes the work published in (Ghovanloo et al., 2018a) with minor modifications and formatting changes to suit the thesis style.

2.1. Abstract

Skeletal muscle channelopathies, many of which are inherited as autosomal dominant mutations, include myotonia and periodic paralysis. Myotonia is defined by a delayed relaxation after muscular contraction, whereas periodic paralysis is defined by episodic attacks of weakness. One sub-type of periodic paralysis, known as hypokalemic periodic paralysis (hypoPP), is associated with low potassium levels. Interestingly, the P1158S missense mutant, located in the third domain S4-S5 linker of the “skeletal muscle”, Nav1.4, has been implicated in causing both myotonia and hypoPP. A common trigger for these conditions is physical activity. We previously reported that Nav1.4 is relatively insensitive to changes in extracellular pH compared to Nav1.2 and Nav1.5. Given that intense exercise is often accompanied by blood acidosis, we decided to test whether changes in pH would push gating in P1158S towards either phenotype. Our results suggest that, unlike in WT-Nav1.4, low pH depolarizes the voltage-dependence of activation and steady-state fast inactivation, decreases current density, and increases late currents in P1185S. Thus, P1185S turns the normally pH-insensitive Nav1.4 into a proton-sensitive channel. Using action potential modeling we predict a pH-to-phenotype correlation in patients with P1158S. We conclude that activities which alter blood pH may trigger the noted phenotypes in P1158S patients.

2.2. Introduction

Electrical signaling is an integral part of many processes including the heartbeat and voluntary movement. This kind of signaling depends on a group of transmembrane proteins called voltage-gated ion channels (Hodgkin & Huxley, 1952). Voltage-gated sodium channels are responsible for the initiation and propagation of action potentials in most excitable cells. These channels are hetero-multimeric proteins composed of a large
voltage-sensing and pore-forming α-subunit, and smaller β-subunits (Calhoun & Isom, 2014). The α-subunit family is composed of 9 subtypes, which are expressed in different parts of the body (Catterall, 2012). Nav1.4, highly expressed in skeletal muscle, is encoded by the SCN4A gene. Mutations in SCN4A often lead to either gain-of-function or loss-of-function phenotypes (Ghovanloo et al., 2016b).

Although most Nav1.4-mutants depolarize the sarcolemma, this depolarization can result in either hyper- or hypo-excitability (Cannon, 2006). The manifestation of Nav-channelopathies occurs through changes in membrane excitability, and these changes underlie various clinical syndromes. Traditionally, muscular channelopathies are classified as either non-dystrophic myotonias or periodic paralysis (Ryan et al., 2007; Lehmann-Horn et al., 2008). Most of these channelopathies are either sporadic de novo or inherited autosomal dominant mutations (George, 2005).

The phenotypic expression of most muscle channelopathies takes place within the first two decades of life, and many afflicted individuals suffer from life-long symptoms such as muscle stiffness, weakness, or pain (Tan et al., 2011). Despite their high penetrance, these mutations can show clinical variability within different familial groups or even a single family (Tan et al., 2011).

Most gain-of-function mutations in SCN4A result in myotonic syndromes. Myotonia is defined by a delayed relaxation after muscle contraction (Lehmann-Horn & Rudel, 1995; Tan et al., 2011). In myotonia, there is an increase in muscle membrane excitability in which even a brief voluntary contraction can lead to a series of action potentials that can persist for several seconds following the termination of motor neuron activity. This phenomenon is perceived as muscle stiffness (Tan et al., 2011). The global prevalence of non-dystrophic myotonias is estimated at 1: 100,000 (Emery, 1991). Although this condition is not considered lethal, it can be extremely life-limiting due to the multitude of muscle contractility problems it can cause. Given the characteristic hyper-excitability of myotonia, most therapeutics are targeted against membrane excitability (Desaphy et al., 2004; Vicart et al., 2005; George, 2012).

Until recently, loss-of-function Nav1.4-mutants were thought to cause periodic paralysis. This was due to most periodic paralysis-causing mutations neutralizing positively charged residues in voltage sensors. Newer studies described a cation leak with
characteristics similar to the ω-current in Shaker potassium channels. These findings indicate a form of gain-of-function resulting in periodic paralysis (Lehmann-Horn & Jurkat-Rott, 1999; Tombola et al., 2005; Sokolov et al., 2007; Wu et al., 2011). Some channelopathies are triggered by low serum potassium levels and manifest in episodes of extreme muscle weakness. These are known as hypo-kalemic periodic paralyses (hypoPP) (Miller et al., 2004a). The onset of hypoPP occurs between the ages of 15 and 35, and the prevalence of hypoPP is also estimated to be 1: 100,000. HypoPP can be triggered by external factors such as stress, high-sugar diet, and exercise (Miller et al., 2004a). A serum potassium concentration less than 3 mM may trigger hypoPP (Fontaine, 2008).

P1158S in Nav1.4 is a missense mutant in the S4-S5 linker of domain (D) III that is the result of a C to T mutation at position 3549 in SCN4A (Sugiura et al., 2003). P1158S is described to cause both myotonia and hypoPP. The ability to cause two seemingly contrasting syndromes is shared between P1158S and some mutants of the cardiac sodium channel, Nav1.5, which cause both the Brugada and long QT syndromes (Makita et al., 2008). Our group recently showed that physiological triggers may be important in modulating disease phenotype in the E1784K mutant in Nav1.5 (Peters et al., 2016), leading us to hypothesize a possible role of similar triggers in P1158S.

Exercise is a common trigger for both myotonia and hypoPP (Lehmann-Horn & Rudel, 1995; Miller et al., 2004a; Tan et al., 2011). Physical activity results in delayed relaxation in myotonic patients and extreme weakness in hypoPP patients. Exercise is normally accompanied by changes in extracellular pH, which can modulate sodium channel function (Vilin et al., 2012a; Jones et al., 2013b; Peters et al., 2016). We therefore hypothesized that pH alterations may push P1158S towards either myotonia or hypoPP.

Changes in extracellular pH levels modulate the activities of Nav1.2 and Nav1.5; however, Nav1.4 is relatively pH-insensitive (Vilin et al., 2012a). One theory behind the pH-sensitivity of Nav1.5 is the presence of a cysteine (C373) residue on the outer vestibule of DI (Khan et al., 2006; Jones et al., 2013b). The increase in H⁺ concentration results in the protonation of this cysteine, which creates a positive charge outside the pore. This positive charge causes an electrostatic repulsion, blocking sodium ions from going through the pore. This cysteine is missing in Nav1.4 (Khan et al., 2006). However, there are other residues on the extracellular side of Nav1.4 that may get protonated (Khan et al., 2002,
We postulated that one reason for the apparent pH-insensitivity of Nav1.4 relative to other sodium channels might be due to hydrophobic amino acids protecting the protonate-able functional groups from becoming protonated at low pH.

The serine functional group is floppier than the highly restrained proline functional group. Furthermore, previous studies suggest that in Nav1.4, DIII activates before DI and DII (Gosselin-Badaroudine et al., 2012). Also, it is suggested that SCN4A-mutations that disrupt the movement of DIII S4 during recovery can result in hypoPP (Groome et al., 2014). Given the location of P1158S on the DIII S4-S5 linker, along with the nature of the mutation, and because DIII activates first, we predicted that this mutation might lead to the exposure of residues, protected in WT-Nav1.4, to protonation. Here we report the results of homology modeling, patch-clamp experiments, and action potential modeling. These results support our hypothesis and show P1158S imparts pH-sensitivity to Nav1.4 that underlies the dual myotonia/hypoPP phenotype.

2.3. Methods

2.3.1. Cell culture

Chinese Hamster Ovary (CHO) cells were transiently co-transfected with cDNA encoding eGFP and the β1-subunit and either WT-Nav1.4 (GenBank accession number: NM_000334) or P1158S α-subunit. Transfection was done according to the PolyFect transfection protocol. After each set of transfections, a minimum of 8-hour incubation was allowed before plating on sterile coverslips.

2.3.2. Homology modeling

Homology modeling was performed using the Swiss-Model server (Bordoli et al., 2008). The sodium channel structure developed by Ahuja et al., (2015) was used as a template against which the Nav1.4 sequence with and without P1158S were modeled (Arnold et al., 2006; Bordoli et al., 2008). Modeling was done according to the protocol established by Bordoli et al., (2008). PyMOL-pdb viewer was used for optimization and structure visualization.
2.3.3. Electrophysiology

Whole-cell patch-clamp recordings were performed in an extracellular solution containing (in mM): 140 NaCl, 4 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 HEPES or MES (pH6.4). Solutions were adjusted to pH (6.4, 7.0, 7.4, 8.0) with CsOH. Pipettes were filled with intracellular solution, containing (in mM): 120 CsF, 20 CsCl, 10 NaCl, 10 HEPES or MES (pH6.4). All recordings were made using an EPC-9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) digitized at 20 kHz via an ITC-16 interface (Instrutech, Great Neck, NY, USA). Voltage-clamping and data acquisition were controlled using PatchMaster/FitMaster software (HEKA Elektronik, Lambrecht, Germany) running on an Apple iMac. Current was low-pass-filtered at 10 kHz. Leak subtraction was performed automatically by software using a P/4 procedure following the test pulse. Gigaohm seals were allowed to stabilize in the on-cell configuration for 1 min prior to establishing the whole-cell configuration. Series resistance was less than 5 MΩ for all recordings. Series resistance compensation up to 80% was used when necessary. All data were acquired at least 1 min after attaining the whole-cell configuration. Before each protocol, the membrane potential was hyperpolarized to −130 mV to ensure complete removal of both fast inactivation and slow inactivation. All experiments were conducted at 22 °C.

2.3.4. Activation protocols

To determine the voltage-dependence of activation, we measured the peak current amplitude at test pulse potentials ranging from −100 mV to +80 mV in increments of +10 mV for 20 ms. Channel conductance (G) was calculated from peak $I_{Na}$:

$$G_{Na} = \frac{I_{Na}}{V-E_{Na}}$$

(Eq. 1)

where $G_{Na}$ is conductance, $I_{Na}$ is peak sodium current in response to the command potential $V$, and $E_{Na}$ is the Nernst equilibrium potential. Calculated values for conductance were fit with the Boltzmann equation:

$$\frac{G}{G_{max}} = \frac{1}{1+\frac{1}{exp[-ze_{0} (V_{m} - V_{1/2})/kT]}}$$

(Eq. 2)

where $G/G_{max}$ is normalized conductance amplitude, $V_{m}$ is the command potential, $z$ is the apparent valence, $e_0$ is the elementary charge, $V_{1/2}$ is the midpoint voltage, $k$ is the Boltzmann constant, and $T$ is temperature in K.
2.3.5. Steady-state fast inactivation protocols

The voltage-dependence of fast inactivation was measured by preconditioning the channels to a hyperpolarizing potential of \(-130\) mV and then eliciting pre-pulse potentials that ranged from \(-170\) to \(+10\) mV in increments of \(10\) mV for \(500\) ms, followed by a \(10\) ms test pulse during which the voltage was stepped to \(0\) mV. Normalized current amplitudes from the test pulse were fit as a function of voltage using the Boltzmann equation:

\[
\frac{I}{I_{\text{max}}} = \frac{1}{1 + \exp(-zE_0(V_M - V_{1/2})/kT)}
\]  

(Eq. 3)

where \(I_{\text{max}}\) is the maximum test pulse current amplitude.

2.3.6. Late current protocols

Late current was measured between 145 and 150 ms during a \(200\) ms depolarizing pulse to \(0\) mV from a holding potential of \(-130\) mV. Pulses were averaged to increase signal-to-noise ratio.

2.3.7. Use-dependent inactivation protocols

Cells were held at \(-60\) mV and repetitively stimulated with \(2000\), \(20\) ms test pulses to \(0\) mV. Peak currents from test pulses were normalized to the amplitude of the first current in the series and values were plotted versus the time of each pulse in seconds.

2.3.8. Immunocytochemistry

CHO cells co-transfected with the \(\beta1\)-subunit and wild-type Nav1.4 or P1158S -subunits were incubated for \(24\) hours. The transfections were done on glass coverslips. Non-transfected cells were also incubated and were used as negative control. After this incubation period, cells were fixed in \(4\)% paraformaldehyde for \(10\) minutes while being incubated on a shaker. Then, cells were incubated in \(0.1\)% Triton X-100 for another \(10\) minutes. Cells were blocked in \(10\)% goat serum for \(30\) to \(45\) minutes. Following this step, the cells were kept on Parafilm on top of a wet filter paper. Diluted primary anti-Nav1.4 (1:100, Rabbit polyclonal IgG, commercially available via Alomone) antibodies were added. The next day, cells were incubated in PBS for \(10\) minutes. Lastly, secondary IgG2a (1:500) was added. Stained cells were studied using a Zeiss confocal microscope.
2.3.9. Action potential modeling

Skeletal action potential modeling was based on a model developed by Cannon et al., (1993). All action potentials were programmed and run using Python. The modified parameters were based on electrophysiological results obtained from whole-cell patch-clamp experiments (Cannon et al., 1993). The model accounted for activation voltage-dependence, steady-state fast inactivation voltage-dependence, and late sodium currents. The WT pH7.4 model uses the original parameters from the model. As the WT-Nav1.4 was not sensitive to changes in pH, the WT models are identical for all pH values. P1158S models were programmed by shifting the midpoints of activation and fast inactivation from the original Cannon model by the difference between the values in P1158S experiments at a given pH and the average value in WT at all pH values. As persistent current was increased by the P1158S mutant, but was not significantly impacted by changes in pH, the simulated P1158S persistent current was the same at all pH values.

2.3.10. Analysis

Analysis and graphing were done using FitMaster software (HEKA Elektronik) and Igor Pro (Wavemetrics, Lake Oswego, OR, USA). All data acquisition and analysis programs were run on an Apple iMac (Apple Computer). Statistical analysis was performed in JMP version 13. Confocal images were analyzed in ImageJ.

2.3.11. Statistics

A two-factor analysis of variance (ANOVA) was used to compare the mean responses [activation, current density, steady-state fast inactivation, late currents, fast inactivation kinetics, use-dependent inactivation] between the channel variants and pH points. Channel variant, pH, and the way interaction involving the two were considered to be fixed effects in the model. Channel variant had two levels (WT, P1158S) and pH had four levels (pH6.4, 7.0, 7.4, 8.0). A one-factor ANOVA was used to compare the means membrane fluorescence intensities of the two channels and a negative control. Post hoc tests using the Tukey Kramer adjustment compared the mean responses between channel variants across conditions. A level of significance $\alpha = 0.05$ was used in all overall post hoc tests, and effects with p-values less than 0.05 were considered to be statistically significant. All values are reported as means ± standard error of means for n cells.
2.4. Results

2.4.1. P1158S alters the hinge angle of the DIII S4-S5 linker

In the sliding-helix model of voltage-sensor movement during activation, when the potential across the cell membrane becomes depolarized, the voltage-sensor domain (VSD) undergoes an outward movement. This movement pulls on the S4-S5 linker and drags open the pore-domain (PD), leading to an influx of sodium ions (Yarov-Yarovoy et al., 2012). Based on this model we sought to determine whether P1158S alters the S4-S5 topology. We predicted that any topological change might alter the mobility of this linker and impart pH-sensitivity during acidosis.

To test our hypothesis, we performed homology modeling using the Swiss-Model server, accessible via the ExPASy web server, to model the sequence of both WT and P1158S against the Nav1.7 structure (Ahuja et al., 2015). The Nav1.7 structure was used as a skeleton on which the WT and mutant-Nav1.4 sequences were placed (Bordoli et al., 2008). Our results show the critical position of P1158 in Nav1.4 on the hinge of DIII S4-S5 linker (Figure 2-1A-C).

Gain- and loss-of-function mutations may alter the atomic structure of sodium channels by causing connectivity rearrangements and changing interatomic bonds and topological angles (Kapetis et al., 2017). Therefore, we ran the model with the P1158S mutant to test whether there was an effect on the hinge angle of the S4-S5 linker. The hinge angle with proline was 84.9˚; however, this angle was increased to 87.2˚ with the serine mutation (Figure 2-1D-E). This suggests there is less angle strain on the S4-S5 linker when serine is present as opposed to proline. P1158 is conserved across all Nav subtypes, further suggesting its structural importance (Figure 2-1F).
2.4.2. P1158S makes Nav1.4 sensitive to changes in extracellular, but not intracellular pH

We conducted whole-cell patch-clamp experiments to investigate whether the P1158S mutant in the S4-S5 linker increases the sensitivity of Nav1.4 to protons. We examined the effects of pH changes on channel activation in WT and P1158S by measuring peak channel conductance at membrane potentials between -100 and +80 mV (Figure 2-2H). Neither decreasing nor increasing extracellular pH causes significant effects on the midpoint ($V_{1/2}$) or apparent valence ($z$) of activation in WT channels ($p > 0.05$). In the P1158S mutant, however, there are significant shifts in the depolarized direction in the $V_{1/2}$ of activation when extracellular pH is changed from pH8.0 to 6.4 ($p = 0.0011$) (Figure 2-2A-E). We fitted the conductance $V_{1/2}$ of P1158S using the Hill equation and WT using a flat line (Figure 2-2G; Table 2.1). The P1158S mutant has a pKa of 7.27 for the $V_{1/2}$ of activation.
Figure 2-2  Normalized conductance plotted against membrane potential.

(A-D) Show overlaps of WT (blue squares) and P1158S (grey circles) conductance at pH6.4, 7.0, 7.4, and 8.0. (E-F) Show conductance of each WT and P1158S. (G) Shows the voltage-
dependence of activation as a function of pH and fitted with Hill equation (P1158S) or a flat line (WT). (H) Shows pulse protocol.

Table 2-1  Conductance comparison across pH6.4 to 8.0 between WT and P1158S

<table>
<thead>
<tr>
<th>Channel Type-pH</th>
<th>Mean $V_{1/2}$ ± SE (mV)</th>
<th>Mean $z$ ± SE (slope)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1158S-pH6.4</td>
<td>-19.48 ± 2.22$^A$</td>
<td>2.76 ± 0.32</td>
<td>9</td>
</tr>
<tr>
<td>P1158S-pH7.0</td>
<td>-22.11 ± 2.52$^A$</td>
<td>2.68 ± 0.36</td>
<td>7</td>
</tr>
<tr>
<td>P1158S-pH7.4</td>
<td>-29.95 ± 2.35$^B$</td>
<td>4.71 ± 0.34</td>
<td>8</td>
</tr>
<tr>
<td>P1158S-pH8.0</td>
<td>-34.77 ± 2.35$^B$</td>
<td>3.02 ± 0.34</td>
<td>8</td>
</tr>
<tr>
<td>WT-pH6.4</td>
<td>-25.86 ± 2.72</td>
<td>3.15 ± 0.39</td>
<td>6</td>
</tr>
<tr>
<td>WT-pH7.0</td>
<td>-24.87 ± 2.52</td>
<td>3.56 ± 0.36</td>
<td>7</td>
</tr>
<tr>
<td>WT-pH7.4</td>
<td>-24.84 ± 2.52</td>
<td>3.14 ± 0.36</td>
<td>7</td>
</tr>
<tr>
<td>WT-pH8.0</td>
<td>-21.92 ± 2.98</td>
<td>3.16 ± 0.43</td>
<td>5</td>
</tr>
</tbody>
</table>

Letter codes and asterisk indicate statistical significance. Numbers with different letter codes and/or matching asterisks are significantly different.

Maximal conductance density could relate to membrane channel expression and trafficking. We calculated the peak conductance density from the conductance at +20 mV measured by a test pulse and dividing this value by the cell membrane capacitance (nS/pF). We also measured current density from the ratio of peak current amplitude to the cell membrane capacitance (pA/pF). Representative current traces are shown in (Figure 2-3A-H). Although the conductance and current densities of WT channels were decreased at lower pH, these decreases were not significant (p > 0.05); however, the P1158S conductance (p = 0.0014) and current (p < 0.0001) densities were significantly decreased at lower extracellular pH’s of 6.4 and 7.0 compared to 7.4 and 8.0 (Figure 2-3I; Table 2.2). To study the effects of proton block in WT and P1158S, we measured peak current amplitudes over a series of depolarizing pulses while perfusing extracellular solutions with pH8.0 to pH6.4 (Figure 2-3J). Peak current reduction at low pH was larger in P1158S than WT (Figure 2-3K).
Figure 2-3  Current and conductance densities measured in pA/pF and nS/pF, respectively.

(A-H) Sample macroscopic sodium currents elicited by depolarizations between -100 and +80 mV.  
(I) Average current (left Y-axis) and conductance (right Y-axis) densities of P1158S and WT at extracellular pH between 6.4 and 8.0. The solid bars represent current density and patterned bars represent conductance density. ** indicate p < 0.01, * indicate p < 0.05 (P1158S at pH6.4 and 7.0 are significantly different from pH7.4 and 8.0; P1158S at physiological pH of 7.4 is significantly different from WT at pH7.4). (J) Sample proton block trace. Maximal current amplitude is plotted over a series of depolarizing pulses. External solution pH is 8.0 at the start of the trace. pH6.4 gets perfused at the pulses indicated by arrows. (K) Sample current amplitude at pH8.0 and 6.4 in WT and P1158S.

Table 2-2  Peak conductance and current densities

<table>
<thead>
<tr>
<th>Channel Type-pH</th>
<th>Mean density ± SE (pA/pF)</th>
<th>n</th>
<th>Mean Conductance ± SE (nS/pF)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1158S-pH6.4</td>
<td>45.7 ± 19.5^A</td>
<td>9</td>
<td>1.06 ± 0.45^A</td>
<td>9</td>
</tr>
<tr>
<td>P1158S-pH7.0</td>
<td>70.4 ± 20.7^A</td>
<td>8</td>
<td>1.68 ± 0.48^A</td>
<td>8</td>
</tr>
<tr>
<td>P1158S-pH7.4</td>
<td>192.0 ± 20.7^B</td>
<td>8</td>
<td>3.40 ± 0.48^B</td>
<td>8</td>
</tr>
<tr>
<td>P1158S-pH8.0</td>
<td>167.6 ± 22.1^B</td>
<td>7</td>
<td>3.86 ± 0.51^B</td>
<td>7</td>
</tr>
<tr>
<td>WT-pH6.4</td>
<td>77.2 ± 26.1</td>
<td>5</td>
<td>1.69 ± 0.57</td>
<td>5</td>
</tr>
<tr>
<td>WT-pH7.0</td>
<td>88.0 ± 26.1</td>
<td>5</td>
<td>1.90 ± 0.57</td>
<td>5</td>
</tr>
<tr>
<td>WT-pH7.4</td>
<td>110.2 ± 18.5^*</td>
<td>10</td>
<td>2.22 ± 0.40^*</td>
<td>10</td>
</tr>
<tr>
<td>WT-pH8.0</td>
<td>94.0 ± 23.9</td>
<td>6</td>
<td>1.78 ± 0.52</td>
<td>6</td>
</tr>
</tbody>
</table>

Letter codes and asterisk indicate statistical significance. Numbers with different letter codes and/or matching asterisks are significantly different.

After activation, the DIII-DIV linker, known to be the fast inactivation gate, binds to the inside of the channel within milliseconds and blocks the flow of sodium through the
pore (West et al., 1992). We measured the voltage-dependence of fast inactivation using a standard pre-pulse voltage protocol (Figure 2-4H). Normalized current amplitudes were plotted as a function of pre-pulse voltage (Figure 2-4A-F). Like activation, the voltage-dependence of steady-state fast inactivation was depolarized by low extracellular pH in P1158S ($p = 0.0027$). Also, similar to activation, the WT $V_{1/2}$ of fast inactivation was not significantly affected by changes in extracellular pH ($p > 0.05$) (Table 2.3). We fit fast inactivation $V_{1/2}$ as a function of pH with either a Hill curve (P1158S) or a flat line (WT) (Figure 2-4G). The P1158S $V_{1/2}$ of fast inactivation has a pKa of 7.63.
Figure 2-4  Voltage-dependence of steady-state fast inactivation as normalized current plotted against membrane potential.

(A-D) Show the voltage-dependence of fast inactivation of WT (blue squares) and P1158S (grey circles) at pH6.4, 7.0, 7.4, and 8.0. (E-F) Show voltage-dependence of fast inactivation of each WT
and P1158S. (G) Shows the voltage-dependence of steady-state fast inactivation as a function of pH and fitted with a Hill equation (P1158S) or a flat line (WT). (H) Shows voltage protocol.

Table 2-3  Steady-state fast inactivation comparison across pH6.4 to 8.0 between WT and P1158S

<table>
<thead>
<tr>
<th>Channel Type-pH</th>
<th>Mean $V_{1/2} \pm SE$ (mV)</th>
<th>Mean $z \pm SE$ (slope)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1158S-pH6.4</td>
<td>-70.18 ± 3.16(^A)</td>
<td>-2.96 ± 0.25</td>
<td>6</td>
</tr>
<tr>
<td>P1158S-pH7.0</td>
<td>-70.95 ± 2.74(^A)</td>
<td>-2.53 ± 0.21</td>
<td>8</td>
</tr>
<tr>
<td>P1158S-pH7.4</td>
<td>-74.52 ± 2.58(^B)</td>
<td>-2.63 ± 0.20</td>
<td>9</td>
</tr>
<tr>
<td>P1158S-pH8.0</td>
<td>-84.54 ± 3.16(^B)</td>
<td>-2.41 ± 0.25</td>
<td>6</td>
</tr>
<tr>
<td>WT-pH6.4</td>
<td>-68.41 ± 3.16</td>
<td>-3.74 ± 0.25</td>
<td>6</td>
</tr>
<tr>
<td>WT-pH7.0</td>
<td>-67.65 ± 2.74</td>
<td>-3.32 ± 0.21</td>
<td>8</td>
</tr>
<tr>
<td>WT-pH7.4</td>
<td>-67.77 ± 2.58</td>
<td>-2.49 ± 0.20</td>
<td>9</td>
</tr>
<tr>
<td>WT-pH8.0</td>
<td>-67.44 ± 2.74</td>
<td>-3.27 ± 0.21</td>
<td>8</td>
</tr>
</tbody>
</table>

Letter codes indicate statistical significance. Numbers with different letter codes are significantly different.

We also measured the open-state fast inactivation time constant, which did not differ significantly between channels or pH points ($p > 0.05$) (Figure 2-5; Table 2.4).

Figure 2-5  Open-state fast inactivation time constants.

Numbers are from across all conditions at: -30, -10, 0, +10 mV.
Table 2-4 Open-state fast inactivation time constants across four voltages

<table>
<thead>
<tr>
<th>Channel Type-pH</th>
<th>-30 mV $\tau \pm$ SE (ms)</th>
<th>-10 mV $\tau \pm$ SE (ms)</th>
<th>0 mV $\tau \pm$ SE (ms)</th>
<th>+10 mV $\tau \pm$ SE (ms)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1158S-pH6.4</td>
<td>1.85 ± 0.27</td>
<td>0.95 ± 0.20</td>
<td>0.66 ± 0.09</td>
<td>0.59 ± 0.13</td>
<td>7</td>
</tr>
<tr>
<td>P1158S-pH7.0</td>
<td>2.26 ± 0.41</td>
<td>0.96 ± 0.11</td>
<td>0.80 ± 0.05</td>
<td>0.69 ± 0.06</td>
<td>4</td>
</tr>
<tr>
<td>P1158S-pH7.4</td>
<td>1.48 ± 0.14</td>
<td>0.78 ± 0.05</td>
<td>0.63 ± 0.04</td>
<td>0.56 ± 0.04</td>
<td>13</td>
</tr>
<tr>
<td>P1158S-pH8.0</td>
<td>1.26 ± 0.10</td>
<td>0.67 ± 0.05</td>
<td>0.56 ± 0.06</td>
<td>0.54 ± 0.08</td>
<td>7</td>
</tr>
<tr>
<td>WT-pH6.4</td>
<td>2.06 ± 0.40</td>
<td>0.98 ± 0.17</td>
<td>0.72 ± 0.06</td>
<td>0.60 ± 0.05</td>
<td>6</td>
</tr>
<tr>
<td>WT-pH7.0</td>
<td>1.62 ± 0.34</td>
<td>0.77 ± 0.10</td>
<td>0.62 ± 0.06</td>
<td>0.52 ± 0.04</td>
<td>6</td>
</tr>
<tr>
<td>WT-pH7.4</td>
<td>2.41 ± 0.37</td>
<td>1.22 ± 0.21</td>
<td>0.89 ± 0.20</td>
<td>0.77 ± 0.17</td>
<td>8</td>
</tr>
<tr>
<td>WT-pH8.0</td>
<td>2.00 ± 0.20</td>
<td>1.05 ± 0.15</td>
<td>0.76 ± 0.07</td>
<td>0.68 ± 0.10</td>
<td>7</td>
</tr>
</tbody>
</table>

Total sodium current can be divided into two components: peak (INaP) and late (INaL) currents. Whereas INaP refers to the maximum amount of sodium ions going through the channels during the open state, the smaller INaL is a manifestation of destabilized fast inactivation. We show representative normalized current traces for both channels across all conditions (Figure 2-6A-D). We measured the percentage of INaL by dividing the maximum INaL between 145 ms and 150 ms by INaP. Our results indicated that WT INaL is not significantly sensitive to changes in pH ($p > 0.05$). In contrast, P1158S showed a channel effect with a greater fraction of late current compared to WT ($p = 0.0041$) (Figure 2-6E; Table 2.5). This is an important result because previous studies suggested that late sodium current may be the basis of repetitive action potential firing and, consequently, myotonia in skeletal muscle fibers (Cannon et al., 1993; Cannon, 2006, 2010b, 2015).
Figure 2-6  Late sodium current.

(A-D) Representative normalized current traces of late currents in WT and P1158S at extracellular pH between 6.4 and 8.0. The inset in panel (A) shows the pulse protocol. (E) Shows average late
sodium current as a percentage of peak sodium current for WT and P1158S at extracellular pH between 6.4 and 8.0. ** indicate p < 0.01 (P1158S is significantly different from WT).

Table 2-5  Late sodium current percentage across pH6.4 to 8.0

<table>
<thead>
<tr>
<th>Channel Type-pH</th>
<th>Mean %INaL ± SE (%INaL)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1158S-pH6.4</td>
<td>7.67 ± 1.42^A</td>
<td>9</td>
</tr>
<tr>
<td>P1158S-pH7.0</td>
<td>8.96 ± 1.81^A</td>
<td>4</td>
</tr>
<tr>
<td>P1158S-pH7.4</td>
<td>4.13 ± 1.21^A</td>
<td>6</td>
</tr>
<tr>
<td>P1158S-pH8.0</td>
<td>5.18 ± 1.46^A</td>
<td>6</td>
</tr>
<tr>
<td>WT-pH6.4</td>
<td>2.66 ± 1.03^B</td>
<td>4</td>
</tr>
<tr>
<td>WT-pH7.0</td>
<td>3.33 ± 1.03^B</td>
<td>5</td>
</tr>
<tr>
<td>WT-pH7.4</td>
<td>4.34 ± 0.67^B</td>
<td>5</td>
</tr>
<tr>
<td>WT-pH8.0</td>
<td>2.57 ± 0.95^B</td>
<td>4</td>
</tr>
</tbody>
</table>

Letter codes indicate statistical significance. Numbers with different letter codes are significantly different.

To investigate the effects of pH on inactivation in WT and P1158S, we compared use-dependent current reduction at all four pH points. The maximal voluntary contraction frequencies in the soleus muscle, biceps branchii, and deltoid muscle are: ~11, ~23, and ~29 Hz, respectively (Hermansen & Osnes, 1972; Kukulka & Clamann, 1981; Bellemare et al., 1983; St Clair Gibson, 2004). We chose a 45 Hz pulse stimulation frequency to emulate physical activity. This frequency was chosen as some studies have shown that artificial stimulation rates can increase firing rates in muscles (St Clair Gibson, 2004). Our results suggest that, although WT current decay does not significantly differ with changes in extracellular pH (p > 0.05), P1158S shows a significant acceleration of use-dependent inactivation when extracellular pH is reduced from pH8.0 to 6.4 (p = 0.0142) (Figure 2-7; Table 2.6).
Figure 2-7  Use-dependent inactivation.

(A-D) Show normalized current decay plotted as a function of time. The inset in panel (A) shows the pulse protocol. (E-H) Show normalized current decay fitted with an exponential curve.
Table 2-6 Use-dependent inactivation comparison between WT and P1158S

<table>
<thead>
<tr>
<th>Channel Type-pH</th>
<th>Mean τ ± SE (s)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1158S-pH6.4</td>
<td>0.132 ± 0.057\textsuperscript{A}</td>
<td>4</td>
</tr>
<tr>
<td>P1158S-pH7.0</td>
<td>0.725 ± 0.238</td>
<td>8</td>
</tr>
<tr>
<td>P1158S-pH7.4</td>
<td>1.643 ± 0.309</td>
<td>3</td>
</tr>
<tr>
<td>P1158S-pH8.0</td>
<td>1.753 ± 0.539\textsuperscript{B}</td>
<td>5</td>
</tr>
<tr>
<td>WT-pH6.4</td>
<td>0.416 ± 0.128</td>
<td>3</td>
</tr>
<tr>
<td>WT-pH7.0</td>
<td>0.849 ± 0.090</td>
<td>6</td>
</tr>
<tr>
<td>WT-pH7.4</td>
<td>0.639 ± 0.290</td>
<td>4</td>
</tr>
<tr>
<td>WT-pH8.0</td>
<td>0.711 ± 0.290</td>
<td>4</td>
</tr>
</tbody>
</table>

Letter codes indicate statistical significance. Numbers with different letter codes are significantly different.

During physical activity, accumulation of lactate and CO\textsubscript{2} reduces extracellular pH because of acid efflux from muscle cells (Aalkjaer & Peng, 1997). These changes in blood acidity play a vital role in blood flow homeostasis (Aalkjaer & Peng, 1997). However, this acidosis begins on the intracellular side of muscle cells. To test whether the P1158S responses to pH changes are due to protonation on the intracellular side of the channel, we performed whole-cell voltage-clamp experiments in which the pipette solution pH was lowered to 6.4, while buffering the extracellular bath pH at 7.4. The results of these experiments were compared to the experiments with both intra- and extracellular solutions at pH7.4. We found that intracellular acidosis does not significantly alter activation, steady-state fast inactivation, current density, or late current levels in P1158S (p > 0.05) (Figure 2-8; Table 2.7). Thus, the observed pH-dependence in the previous experiments are due to extracellular modifications.
Figure 2-8   Effects of changing intracellular pH to 6.4.

(A-B) Representative current traces of P1158S with both intra- and extracellular pH at 7.4, and intracellular pH 6.4 and extracellular pH 7.4, respectively. (C) Shows the voltage-dependence of activation for P1158S at intracellular pH 7.4 (blue squares) and intracellular pH 6.4 (grey circles). (D) Shows the voltage-dependence of steady-state fast inactivation for P1158S at intracellular pH 7.4 (blue squares) and intracellular pH 6.4 (grey circles). Insets show voltage protocols. (E) Shows average current density for P1158S channels at intracellular pH 7.4 and 6.4. (F) Shows late sodium current as a percentage of peak sodium current for WT and P1158S channels.

Table 2-7   Intracellular acidosis biophysical parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>P1158S IpH7.4</th>
<th>n</th>
<th>P1158S IpH6.4</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conductance V_{1/2} ± SE (mV)</td>
<td>-29.95 ± 2.41</td>
<td>8</td>
<td>-31.67 ± 2.58</td>
<td>7</td>
</tr>
<tr>
<td>Conductance z ± SE (slope)</td>
<td>4.71 ± 0.56</td>
<td>8</td>
<td>3.34 ± 0.60</td>
<td>7</td>
</tr>
<tr>
<td>SSFI V_{1/2} ± SE (mV)</td>
<td>-74.52 ± 2.43</td>
<td>9</td>
<td>-77.78 ± 2.75</td>
<td>7</td>
</tr>
<tr>
<td>SSFI z ± SE (slope)</td>
<td>-2.63 ± 0.24</td>
<td>9</td>
<td>-3.10 ± 0.27</td>
<td>7</td>
</tr>
<tr>
<td>Current density ± SE (pA/pF)</td>
<td>282.7 ± 45.3</td>
<td>10</td>
<td>222.3 ± 50.7</td>
<td>8</td>
</tr>
<tr>
<td>%INaL ± SE (%INaL)</td>
<td>4.91 ± 1.72</td>
<td>6</td>
<td>6.92 ± 2.43</td>
<td>3</td>
</tr>
</tbody>
</table>

2.4.3. P1158S does not alter channel trafficking or expression

Our patch-clamp results indicated the average conductance (p = 0.0395) and current (p = 0.0092) densities of P1158S mutants were significantly greater than that of WT at physiological pH (Table 2-2). Therefore, we sought to determine whether there are any differences in the trafficking and expression between WT and P1158S. We performed immunocytochemistry and used confocal microscopy to visualize and quantify channel
distribution and, thus, channel trafficking (Figure 2-9A-C). The non-specific fluorescence in negative control was subtracted from the WT and P1158S conditions in all analyses. Pixel intensity grey value was used as a function of the length of cells (distance) from the middle confocal plane image.

For a typical CHO cell with a length of about 14 µm, both WT and P1158S were localized within the 0 to 4 µm and 9 to 14 µm ranges. These two ranges correspond to the outer cellular edges, indicating that most of the channels seem to be concentrated in the proximity of cell membranes (Figure 2-9D-E). To quantify expression, we measured the average pixel intensity across all confocal planes per cell area, for all cells in all conditions. We found no significant difference between WT and P1158S channel expression (p > 0.05) (Figure 2-9F).

Figure 2-9 Immunocytochemistry measurements of channel trafficking and expression.

(A-C) Representative images of WT, P1158S, and negative control cells taken from the middle confocal plane. (D-E) Show the distribution of channels, measured by using pixel grey value, across the cell. Both WT and P1158S channels are localized around the edges of cells, in proximity to cell membrane (distances 0-4 µm, and 9-14 µm). (F) Average pixel intensity across all confocal images per cell area (n = 3 per condition).
2.4.4. pH Alterations Can Push P1158S into Periodic Paralysis or Myotonia

We used an action potential model to simulate how the pH-dependent electrophysiological shifts in P1158S may affect phenotype (Cannon et al., 1993). We ran the simulations at a stimulus of 50 µA/cm². A single action potential at physiological pH7.4 required a shorter stimulus duration in P1158S (1.2 ms) than WT (2.0 ms) (Figure 2-10A). The repolarization of P1158S was slightly prolonged compared to WT due to the presence of larger late sodium current. This late current also led to an increase in the T-Tubule potassium concentrations in P1158S (Figure 2-10B). The long duration simulation pulse started at 50 ms and stopped at 350 ms. During this pulse, the WT channels activated at 50 ms. WT channels fired only a single action potential. The channels remained inactivated until the stimulus was removed at 350 ms (Figure 2-10C). Unlike the WT channels, the action potential morphology changed as a function of pH in P1158S. At the beginning of the stimulus, in pH8.0 models, P1158S mutants displayed an action potential spike, followed by a period during which the membrane potential remained depolarized at -30 mV even after the stimulus was removed (Figure 2-10D). This inability to repolarize holds the sodium channels in an inactivated state, and is consistent with the periodic paralysis phenotype (Cannon, 2015). As with pH8.0, P1158S also displayed a periodic paralysis-like action potential at pH7.4 (Figure 2-10D). At pH7.0, P1158S showed a continuous train of action potentials for most of the stimulus duration. After the stimulus was removed, there was an after-discharge consistent with myotonic action potentials, which then degenerated into periodic paralysis (Cannon, 2012, 2015) (Figure 2-10D). At pH6.4, the P1158S-mutants showed a continuous train of action potentials for the entire stimulation period. After the stimulus was removed, P1158S displayed a progressive repolarization of the membrane potential during a myotonic burst (Cannon, 2012, 2015) (Figure 2-10D). Overall, during extreme acidosis, at pH6.4, P1158S only displayed the myotonic phenotype (Figure 2-10E); however, at the less extreme pH of 7.0, P1158S displayed both myotonia and periodic paralysis (Figure 2-10F). At pH7.4 and pH8.0, P1158S only showed periodic paralysis (Figure 2-10G-H).
Figure 2-10  Skeletal muscle action potential modeling.

(A) Shows single action potential simulations of WT and P1158S channels at the physiological pH of 7.4. The stimulation pulse of 50 µA/cm² was applied for 2.0 ms (WT) and 1.2 ms (P1158S). (B) Shows the T-Tubule potassium concentration in WT and P1158S. The potassium concentration is proportional to amount of sodium late currents. (C-D) Show long stimulation pulse from 50-350 ms in WT, and P1158S at all four pH points. (E-H) Show P1158S at pH 6.4, 7.0, 7.4, and 8.0.

2.5. Discussion

Periodic paralysis and myotonia represent two life-limiting conditions. Interestingly, they fall at the opposite extremes of the disease-spectrum caused by Nav1.4-related channelopathies. P1158S is a single missense mutant that can cause both conditions. We
sought to understand how replacing one amino acid might cause both myotonia and hypoPP. Our investigations began with a quest to find common ground between myotonia and periodic paralysis. Because exercise is a common trigger for both conditions, we focused on physiological changes caused by exercise. These changes include variations in cytosolic calcium levels, blood sugar, pH, body temperature, etc. Previous studies characterized P1158S with respect to its temperature sensitivity. A study by Sugiura et al. (2003) suggested that the voltage-dependence of activation in P1158S hyperpolarizes upon cooling (Sugiura et al., 2003). Furthermore, P1158S can also disrupt slow inactivation at cold temperatures (Webb & Cannon, 2008).

P1158 is fully-conserved in all mammalian Nav subtypes, the eukaryotic cockroach sodium channel (NavPaS), and the electric eel sodium channel (EeNav1.4) (Shen et al., 2017; Yan et al., 2017). The mutation of this vital proline to leucine (P1308L) in Nav1.7 has been implicated in causing inherited erythromelalgia (IEM). IEM is characterized by intermittent burning pain and skin redness in hands and feet. This condition is usually triggered by warmth or exercise (Cheng et al., 2010b). Therefore, P1308L in the neuronal Nav1.7 shares some of the triggers with P1158S in skeletal muscles.

We examined whether exercise-induced acidosis can modulate P1158S. Although it is reported that during exercise the skeletal muscle tissue pH may drop to as low as pH6.4, our previous study found that Nav1.4 is relatively unaffected by extracellular acidosis compared to Nav1.2 and Nav1.5 (Hermansen & Osnes, 1972; Vilin et al., 2012a). The pH-independent current was suggested to protect skeletal muscle activity during exercise-induced acidosis (Khan et al., 2006).

We hypothesized that the mutation of P1158 to a serine could lead to exposure of protonate-able residues to protons. To test this hypothesis, we took a bottom-up approach. We started with a homology model to understand where the conserved P1158 is located on a molecular level. The model also enabled us to determine that there is a hinge-angle difference in the S4-S5 linker with serine instead of proline. Our next step was to find out how much this angle difference would affect the electrophysiology of Nav1.4. The patch-clamp experiments indicated that P1158S causes Nav1.4 to become a pH-sensitive sodium channel, with similar pH-sensitivity to Nav1.5 (Vilin et al., 2012a). During extracellular acidosis, the voltage-dependence of activation and steady-state fast inactivation depolarized, current density decreased, and late currents were larger.
Previous studies on Nav1.5-mutants, S1787N and S1103Y, have shown that intracellular acidosis can modulate channel gating leading to arrhythmias (Plant et al., 2006; Hu et al., 2015). To determine whether intracellular pH changes can also modify the gating characteristics of P1158S in Nav1.4, we repeated experiments under acidic intracellular conditions. We found intracellular acidosis to have minimal effects. This suggests the pH-dependence in P1158S is due to the unmasking of protonate-able extracellular residues by the mutation.

Changes in the sodium channel expression in NG108-15 cells can alter current and conductance densities, leading to downstream changes in action potential generation (Liu et al., 2012). We observed a difference in current and conductance densities between WT and P1158S, raising the possibility that the mutant might alter channel trafficking and expression. Our confocal microscopy results eliminated this possibility, leaving the mutant effect on channel gating as the most parsimonious explanation for differences in current and conductance densities.

Lastly, to understand how the pH-dependent changes in P1158S can cause the described phenotypes, we ran action potential simulations. These simulations predict that during alkalosis, such as while hyperventilating (Johnson, 2017), P1158S can cause periodic paralysis. However, in the presence of a long stimulus, extreme acidosis can trigger myotonia in P1158S. This is interesting because it is suggested that a pH decrease in muscle cells could alleviate paralytic attacks in some patients (Kuzmenkin et al., 2002). Furthermore, P1158S requires a shorter stimulation period to activate compared to WT-Nav1.4. The simulated action potential patterns in P1158S are consistent with the phenotypes associated with periodic paralysis- and myotonia-specific mutants (Cannon, 2012, 2015).

The molecular mechanism of Nav1.5-proton interactions suggest two mechanisms of proton block. The first mechanism involves the selectivity filter. There are four residues in the P-loop that compose the selectivity filter (DEKA), which along with another four carboxylate residues (EEDD) cause sodium permeation (Terlau et al., 1991; Favre et al., 1996; Balser et al., 1996). These residues are fully conserved across the sodium channel superfamily. The carboxylates of these residues can get protonated during acidosis. The second mechanism involves the protonation of C373. The pH-insensitivity of Nav1.4 has
been attributed to the absence of C373, and the presence of the homologous Y401 in its place (Khan et al., 2006; Jones et al., 2013b).

In addition to the two modes of proton block, a third mode of action exists where protons interact with channel voltage-sensors (Jones et al., 2013a). We propose that the interaction of protons with the channel voltage-sensors can occur in Nav1.4. Based on the sliding-helix model (Yarov-Yarovoy et al., 2012), our results suggest that the mechanism through which P1158S imparts pH-sensitivity in Nav1.4 might involve a change in mobility of the DIII S4-S5 linker. Indeed, gating current studies indicate that in Nav1.4, DIII activates prior to DI and DII, suggesting that changes in this linker’s structure could have downstream effects on the mobility of the rest of the channel (Gosselin-Badaroudine et al., 2012). As a result, the outer vestibule residues that are normally hidden from protons during acidosis, could become exposed, and thus mediate pH-sensitivity. This idea is at least partly supported by the upward conformation of VSDIII in the EeNav1.4 cryo-EM structure (Yan et al., 2017). Future experiments should test this idea using fluorescently labeled residues within the voltage-sensors (Chanda & Bezanilla, 2002; Peters et al., 2017).

Cations at the extracellular side of the sodium channel VSD modulate gating charges and fast inactivation, hence the presence of protons impedes the immobilization of gating charges (Jones et al., 2013a). Previous studies on Nav1.5 indicate a destabilized fast inactivation at low pH. This suggests that protons disrupt charge immobilization through a direct interaction with the extracellular carboxylates of DIII and DIV VSDs (Jones et al., 2013b, 2013a). Interestingly, unlike cardiac tissue, the skeletal muscle pH changes more frequently, which may have contributed to the evolution of the pH-insensitive current in Nav1.4 (Hermansen & Osnes, 1972; Khan et al., 2002, 2006). We suggest that the P1158S mutant likely exposes the carboxylates of the DIII VSD, pushing the Nav1.4 gating properties towards Nav1.5. The identity and exact location of these carboxylates need to be determined in future studies.

In conclusion, we characterized a naturally occurring mutation that increases pH-sensitivity in Nav1.4. From a clinical perspective, we identified pH as a potential trigger for periodic paralysis and myotonia in patients with P1158S. Any activity that alters the blood pH balance in P1158S patients could trigger the mutant phenotypes.
2.6. Acknowledgments

This work was supported by grants from Natural Science and Engineering Research Council of Canada and the Canadian Foundation for Innovation, and Rare Disease Foundation and the BC Children’s Hospital Foundation to Dr. Peter C. Ruben.

Mohammad-Reza Ghovanloo collected, assembled, analyzed, and interpreted the data. Mena Abdelsayed and Colin H. Peters assisted in analysis, interpretation, and performed action potential modeling. Mohammad-Reza Ghovanloo wrote the manuscript. Dr. Peter C. Ruben conceived the experiments and revised the manuscript critically for important intellectual content.
Chapter 3. Inhibitory effects of cannabidiol on voltage-dependent sodium currents

This chapter describes the work published in (Ghovanloo et al., 2018c) with minor modifications and formatting changes to suit the thesis style.

3.1. Abstract

Cannabis sativa contains many related compounds known as phytocannabinoids. The main psychoactive and non-psychoactive compounds are Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD), respectively. Much of the evidence for clinical efficacy of CBD-mediated anti-epileptic effects has been from case reports or smaller surveys. The mechanisms for CBD’s anticonvulsant effects are unclear and likely involve non-cannabinoid receptor pathways. CBD is reported to modulate several ion channels, including sodium channels (Nav). Evaluating therapeutic mechanisms and safety of CBD demands a richer understanding of its interactions with central nervous system targets. Here, we used voltage-clamp electrophysiology of HEK-293 cells and iPSC neurons to characterize the effects of CBD on Nav channels. Our results show that CBD inhibits hNav1.1-1.7 currents, with an IC$_{50}$ of 1.9–3.8 μM, suggesting that this inhibition could occur at therapeutically relevant concentrations. A steep Hill slope of ~3 suggested multiple interactions of CBD with Nav channels. CBD exhibited resting-state blockade, became more potent at depolarized potentials, and also slowed recovery from inactivation, supporting the idea that CBD binding preferentially stabilizes inactivated Nav channel states. We also found that CBD inhibits other voltage-dependent currents from diverse channels, including bacterial homomeric Nav channel (NaChBac) and voltage-gated potassium channel subunit Kv2.1. Lastly, the CBD block of Nav was temperature-dependent, with potency increasing at lower temperatures. We conclude that CBD’s mode of action likely involves (1) compound partitioning in lipid membranes, which alters membrane fluidity affecting gating, and (2) undetermined direct interactions with sodium and potassium channels, whose combined effects are loss of channel excitability.
3.2. Introduction

The cannabis plant is composed of over 100 compounds known as phytocannabinoids (Lerner, 1963). Among these phytocannabinoids, CBD, is of great interest due to its lack of potency on CB1 and CB2 receptors that are thought to mediate psychotropic activity. Interactions with these receptors by yet another cannabinoid, THC, at sub-micromolar concentrations cause the well-known ‘cannabis’ effects (Pertwee, 2008). Recently, reports of the use of CBD as an anticonvulsant agent have been rapidly increasing (Devinsky et al., 2017); however, to date, there is no consensus on a well-defined mode of action for the CBD-mediated anti-epileptic effects.

As CBD has a lower affinity for the endocannabinoid receptors than THC (Straiker et al., 2018), several studies suggest that the anticonvulsant effects of THC and CBD in maximal electroshock (ED$_{50}$ ~120 mg/kg, brain concentration ~ 22 µM) and pilocarpine models occur via different mechanisms (Deiana et al., 2012; Devinsky et al., 2014). Whereas the THC activity is mostly on the CB1 receptor, the anticonvulsant effects of CBD are not. These findings have inspired the growth of CB1- and CB2-independent-focused research. Many mechanisms have been proposed for the action of CBD on different systems. CBD acts as an agonist on human TRP channels (3-30 µM), and specifically on TRPV1 (Ledgerwood et al., 2011; Ibeas Bih et al., 2015), which is in part responsible for calcium channel modulation (Blumenfeld et al., 2009). CBD also inhibits heterologously expressed Cav3.1, Cav3.2, and native neuronal T-type voltage-gated calcium channels (Ross et al., 2008). These low-voltage activated channels also can be blocked by other anti-epileptic drugs such as zonisamide (Porter & Jacobson, 2013). In addition to Cav channels, CBD inhibits persistent and resurgent sodium currents generated by WT-Nav1.6 channels, and the exacerbated versions of these currents in GOF epilepsy-causing Nav1.6-mutants (Patel et al., 2016). A recent human clinical trial has indicated that CBD (dosed at 20 mg/kg) is efficacious against drug-resistant seizures in Dravet syndrome (Devinsky et al., 2017). Previous investigations of the cannabis-mediated blockade of Nav channels have determined that CBD (10 µM) significantly decreases action potential frequency in rat CA1 hippocampal neurons and Nav current density in human neuroblastoma cells and mouse cortical neurons (Hill et al., 2014). Other studies have used dissociated hippocampal cultures derived from embryonic day 18 rats to measure
toxicity and neuroprotective responses to CBD. These studies indicate that the EC$_{50}$ of CBD is within the 1-4 µM range, while causing neurotoxicity at 33 µM (Kinney et al., 2016).

The transient sodium current through Nav channels initiates action potentials in neurons, skeletal muscles, and cardiac muscles. Any changes to the gating properties of these channels, and subsequently the current passed through them during an action potential can cause extremely life-limiting conditions that can sometimes be lethal. Both GOF and LOF in sodium channels disrupt electrical signaling (Catterall, 2012; Ghovanloo et al., 2016b, 2016a, 2018a).

In the primary sodium channel isoforms of the CNS, Nav1.1, 1.2, 1.3, and 1.6, both GOF and LOF elicit epilepsy syndromes (Catterall, 2010; Estacion et al., 2010; Veeramah et al., 2012; Ghovanloo et al., 2016b). These include relatively mild epilepsies, like benign familial neonatal-infantile seizures, and more severe forms, such as Dravet syndrome (Heron et al., 2002; Scalmani et al., 2006; Dravet, 2011) and early-infantile epileptic encephalopathy-13 (O’Brien & Meisler, 2013).

Compounds that inhibit Nav current have been used extensively for clinical treatment of all of the above hyperexcitability disorders (George, 2005). In this study, we sought to characterize, in detail, the biophysical effects of CBD on peripheral and CNS sodium channel isoforms.

### 3.3. Methods

#### 3.3.1. Cell culture

Suspension Human Embryonic Kidney (HEK-293) cells were used for automated patch-clamp experiments. HEK-293 cells were either stably transfected using inducible protein expression - T-REx™ system, or transiently transfected using Lipofectamine 2000 with sodium α-subunit cDNA constructs. The human SCN1B cDNA construct was co-transfected into each cell line. The stable cell lines were maintained under Zeocin (5 µg/ml) and G418 (1 mg/ml) selection in DMEM (Dulbecco's Modified Eagle's Medium), 10% FBS (Fetal Bovine Serum) and 2mM L-Glutamine media. The hNav1.1 F1763A and the hNav1.6 N1768D mutations were generated using the QuickChange site-directed mutagenesis kit (Agilent Technologies). GABAergic iPSC neurons were purchased from Cellular Dynamics International, (Madison, WI, USA). The cells were seeded at 20-40%
confluency onto glass cover slips and cultured up to 4 weeks according to user’s guide provided by the vendor. All cells were incubated at 37 °C/5% CO₂. All cell culture reagents were purchased from ThermoFisher Scientific, Waltham, MA, unless otherwise noted.

3.3.2. Automated patch-clamp

Automated patch-clamp recording was used for all experiments unless otherwise noted. HEK cell lines stably expressing the full-length cDNAs coding each Nav α-subunit were generated. The human β1-subunit was co-expressed in each of internally generated cell lines. GenBank accession numbers for α-subunits were: Human Nav1.1 (NM_006920), hNav1.2 (NM_021007), hNav1.3 (AF225987), hNav1.4 (NM_000334), hNav1.5 (AC137587; SCN5A), hNav1.6 (NM_014191), mNav1.6 (NM_001077499) and hNav1.7 (NM_002977). Sodium currents were measured in the whole-cell configuration using a Qube-384 (Sophion A/S, Copenhagen, Denmark) automated voltage-clamp system. To determine inhibition of the inactivated-state, the membrane potential was maintained at a holding-potential (HP) where inactivation of the channel subtype is complete. Membrane holding voltages used for subtype selectivity experiments were: -60 mV (hNav1.5 and hNav1.7), -45 mV (hNav1.1, hNav1.2, hNav1.3, hNav1.4, hNav1.6, and mNav1.6). In the protocol used to assess inhibition for hNav1.5 and hNav1.7 a brief hyperpolarization to a negative (V_hold = -150 mV) voltage for 20 ms was applied at 1 Hz to recover fast inactivated channels but not compound bound channels and then a 20 ms test pulse to 0 mV was applied to quantify the fractional availability of channels. For hNav1.1, hNav1.2, hNav1.3, hNav1.4, and hNav1.6 the recovery period was 60 ms applied at a frequency of 0.05 Hz. Intracellular solution contained (in mM): 120 CsF, 10 NaCl, 2 MgCl₂, 10 HEPES, adjusted to pH7.2 with CsOH. The extracellular recording solution contained (in mM): 145 NaCl, 3 KCl, 1 MgCl₂, 1.5 CaCl₂, 10 HEPES, adjusted to pH7.4 with NaOH. For some studies, a flipped Na⁺ gradient was used where the extracellular solution contained (in mM): 125 choline chloride, 1 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES/NaOH, pH7.4, and the intracellular solution contained (in mM): 115 NaF, 15 CsCl₂, 5 CsF, 3 Na2ATP, 0.3 Na2GTP, 2 MgCl₂, 0.1 CaCl₂, 10 EGTA, 10 HEPES/CsOH, pH7.2. Liquid junction potentials calculated to be ~7 mV were not adjusted for. Currents were low pass filtered at 5 kHz and recorded at 25 kHz sampling frequency. Series resistance compensation was applied at 100% and leak subtraction enabled. The Qube-384 temperature controller was used to manipulate recording chamber temperature.
for certain experiments. The rest of the measurements were obtained at room temperature
which corresponds to 27 ± 2 °C at the recording chamber. Appropriate filters for cell
membrane resistance (typically >500 MΩ), Series resistance (<10 MΩ) and Nav current
magnitude (>500 pA at a test pulse from a resting HP of -120 mV) were routinely applied
to exclude poor quality cells. Vehicle (0.5% DMSO) controls were run on each plate to
enable correction for any compound independent decrease of currents over time.
Baselines were established after 20 minutes in vehicle. Fractional inhibition was measured
as current amplitude from baseline to maximal inhibition after 20-minute exposure to test
compound unless otherwise noted. Maximal inhibition was established by application of
300 nM tetrodotoxin (TTX) to each well at the end of the experiment apart from for Nav1.5
for which 30 uM tetracaine was used. Normalized mean inhibition data were fit to the Hill-
Langmuir equation:

\[ Y = \frac{[C]^h}{[IC_{50}]^h+[C]^h} \]  
(Eq. 1)

to estimate the half maximal inhibitory concentration (IC\textsubscript{50} value); where Y is the
normalized inhibition, C the compound concentration, IC\textsubscript{50} the concentration of test
compound to inhibit the currents 50%, and h the Hill coefficient. Data analysis was
performed using Analyzer (Sophion A/S, Copenhagen, Denmark) and Prism (GraphPad
Software Inc., La Jolla, CA, USA) software. IC\textsubscript{50} values on the Qube-384 automated
voltage-clamp platform were generated from Hill equation fits to pooled data at each
concentration so do not have standard errors.

3.3.3. Manual patch-clamp

Whole-cell patch-clamp recordings were obtained using an Axopatch 200B patch-
clamp amplifier (Molecular Devices, Sunnyvale, CA) controlled and recorded using
pClamp8 (Molecular Devices, Sunnyvale, CA). The recording pipette intracellular solution
contained (in mM): 120 CsF, 10 NaCl, 2 MgCl\textsubscript{2}, 10 HEPES, adjusted to pH7.2 with CsOH.
Pipette resistances were 2-5 MOhm. The extracellular recording solution contained (in
mM): 145 NaCl, 3 KCl, 1 MgCl\textsubscript{2}, 1.5 CaCl\textsubscript{2}, 10 HEPES, adjusted to pH7.4 with NaOH.
Currents were low-pass-filtered at 5 kHz and recorded at 25 kHz sampling frequency using
a Digidata1440 (Molecular Devices, Sunnyvale, CA). Series resistance compensation was
applied at 60-80%. Experiments were performed at room temperature 19-20 °C. Liquid
junction potentials calculated to be ~7 mV were not adjusted for.
3.3.4. Compound preparation

CBD was purchased from Cayman Chemicals and THC was purchased from Toronto Research Chemicals in powder form. Powdered CBD and THC were dissolved in 100% DMSO to create stock. The stock was used to prepare drug solutions in extracellular solutions at various concentrations with no more than 0.5% total DMSO content.

3.3.5. Manual patch-clamp IC\textsubscript{50} measurements

IC\textsubscript{50} measurements were made while holding the membrane voltage at -45 mV. To activate sodium channels, a 60 ms pre-pulse to -150 mV was used to recover channels from fast-inactivation, followed by a 10 ms pulse to -20 mV to open the channel. Using this protocol at a 1 Hz pulse rate, the membrane voltage is maintained at -45 mV 97% of the time. The peak of the inward sodium current at -20 mV was measured and a stable baseline was established prior to perfusion with compound, after which increasing concentrations of compound were perfused and resulting currents measured after equilibrium was obtained.

3.3.6. Activation protocols

To determine the voltage-dependence of activation, we measured the peak current amplitude at test pulse potentials ranging from -100 mV to +80 mV in increments of +5 mV for 100 ms. Channel conductance (G) was calculated from peak I\textsubscript{Na}:

\[ G_{\text{Na}} = \frac{I_{\text{Na}}}{V - E_{\text{Na}}} \]  \hspace{1cm} (Eq. 2)

where \( G_{\text{Na}} \) is conductance, \( I_{\text{Na}} \) is peak sodium current in response to the command potential \( V \), and \( E_{\text{Na}} \) is the Nernst equilibrium potential. Calculated values for conductance were fit with the Boltzmann equation:

\[ \frac{G}{G_{\text{max}}} = \frac{1}{1 + \exp\left(\frac{V_{\text{m}} - V_{1/2}}{k}\right)} \]  \hspace{1cm} (Eq. 3)

where \( G/G_{\text{max}} \) is normalized conductance amplitude, \( V_{\text{m}} \) is the command potential, \( V_{1/2} \) is the midpoint voltage and \( k \) is the slope.
3.3.7. Steady-state fast-inactivation protocols

The voltage-dependence of fast-inactivation was measured by preconditioning the channels from -120 to +10 mV in increments of 5 mV for 100 ms, followed by a 10 ms test pulse during which the voltage was stepped to -20 mV. Normalized current amplitudes from the test pulse were fit as a function of voltage using the Boltzmann equation:

\[ \frac{I}{I_{\text{max}}} = \frac{1}{1 + \exp\left(\frac{V_{1/2} - V_m}{k}\right)} \]  
(Eq. 4)

where \( I_{\text{max}} \) is the maximum test pulse current amplitude.

3.3.8. State-Dependence protocols

To determine state-dependence, potency was measured from four different holding-potentials (-100, -90, -80, -70 mV). The protocol started with a holding-potential of -100 mV followed by 180 x 20 ms depolarizing pulses to 0 mV at 1 Hz. Then, the holding-potential was depolarized by 10 mV, and the 180-pulse protocol was repeated until -70 mV was reached.

3.3.9. Recovery from inactivation protocols

Recovery from inactivation was measured by holding the channels at -120 mV, followed by a depolarizing pulse to 0 mV, then the potential was returned to -120 mV. This was followed by a depolarizing 10 ms test pulse to 0 mV to measure availability. Recovery from inactivation was measured after pre-pulse durations of 300 ms and 10 s and fit with a bi-exponential function of the form:

\[ \text{SpanFast} = (Y_0 - \text{Plateau}) \times \text{PercentFast} \times 0.01 \]  
(Eq. 5)

\[ \text{SpanSlow} = (Y_0 - \text{Plateau}) \times (100 - \text{PercentFast}) \times 0.01 \]  
(Eq. 6)

\[ Y = \text{Plateau} + \text{SpanFast} \times \exp(-K_{\text{Fast}} \times t) + \text{SpanSlow} \times \exp(-K_{\text{Slow}} \times t) \]  
(Eq. 7)

where \( t \) is time in seconds, \( Y_0 \) is the Y intercept at \( t=0 \), \( K_{\text{Fast}} \) and \( K_{\text{Slow}} \) are rate constants in units the reciprocal of t, \( \text{PercentFast} \) the fraction of the Y signal attributed to the fast decaying component of the fit.
3.3.10. {\color{red} Kinetics at varying temperatures }

The kinetics of CBD block were measured at three temperatures: 20, 28, and 33 °C. The channels were held at -45 mV followed by a recovery pulse to -150 mV for 60 ms. The blocked sodium current was normalized to vehicle and subsequently fit with a single exponential function:

\[
Y = (Y_0 - \text{Plateau}) \times \exp(-K \times t) + \text{Plateau} \quad \text{(Eq. 8)}
\]

3.3.11. {\color{red} Action potential modeling }

Neuronal action potential modeling was based on a modified Hodgkin-Huxley model (Hodgkin & Huxley, 1952). The model was modified to match the properties of cortical pyramidal cells (Yi et al., 2017). The modified parameters were based on electrophysiological results obtained from whole-cell patch-clamp experiments in this study. The model accounted for activation voltage-dependence, steady-state fast-inactivation voltage-dependence, and peak sodium currents.

3.3.12. {\color{red} Homology modeling }

Homology modeling was performed using the Swiss-Model server. The cryo-EM sodium channel structure of Navpas was used as a template against which the hNav1.1 sequence was modeled (Bordoli et al., 2008; Shen et al., 2017). Modeling was done according to the protocol established by Bordeli et al., (2008).

3.3.13. {\color{red} Molecular docking }

The homology model of hNav1.1 and SMILES structure of CBD were imported into Chimera. The structures were energetically minimized, and the lowest energy structure was determined as the most likely molecular interaction.

3.3.14. {\color{red} Immunocytochemistry }

HEK cells stably transfected with the β1-subunit and hNav1.1 α-subunits were incubated for 24 hours with 0 (vehicle) and 5 µM CBD. After this incubation period, cells were fixed in 4% paraformaldehyde for 10 minutes while being incubated on a shaker.
Then, cells were incubated in 0.1% Triton X-100 for a further 10 minutes. Cells were blocked in 10% goat serum for 30 to 45 minutes. Following this step, diluted primary anti-hNav1.1 (1:100, Mouse monoclonal IgG, commercially available via UC Davis/NIH NeuroMab Facility) antibodies were added. The next day, cells were incubated in PBS for 10 minutes. Lastly, secondary Alexa Fluor 546 IgG (1:500) and Hoe33342 (nuclear stain) were added. Stained cells were studied using a Nikon confocal microscope. Images were analyzed using ImageJ.

3.3.15. Lipid binding assessment

Made using the TRANSILXL Brain Absorption Kit (ADME Cell) as per manufacturer's instructions.

3.3.16. Statistics

A one-factor analysis of variance (ANOVA) was used to compare the mean responses. Post hoc tests using the Tukey Kramer adjustment compared the mean responses between channel variants across conditions. A level of significance $\alpha = 0.05$ was used in all overall post hoc tests, and effects with $p$-values less than 0.05 were considered to be statistically significant. All values are reported as means ± standard error of means (SEM) for $n$ cells.

3.4. Results

3.4.1. CBD is an inhibitor of human sodium currents

Previous reports suggested that Nav currents are inhibited by micromolar concentrations of CBD but lacked detailed concentration-response data and defined potencies. We sought to determine whether CBD has any selectivity across the sodium channel superfamily. Thus, we measured the concentration-dependence of inhibition of inactivated-state sodium channels by CBD. Our results demonstrate that CBD inhibits hNav1.1-1.7 with low micromolar potency (Figure 3-1A, D). To construct the concentration-response, individual cells were exposed to single concentrations of CBD, then normalized inhibition at each concentration was pooled and fit with a Hill Langmuir equation, providing $IC_{50}$ and Hill-slopes (Figure 3-1A). Representative current traces at
approximately IC\textsubscript{50} concentrations for each subtype tested are shown in (Figure 3-1C). Interestingly, the sodium current inhibition has very steep Hill-slopes of $\geq 2$ across all subtypes (Figure 3-1A, D). This indicates that CBD does not likely inhibit the channel currents through a 1:1 binding mechanism, but instead suggests multiple interactions contribute to this inhibition. Although there is some variation in the IC\textsubscript{50} values obtained on each subtype, the steep Hill-slopes make IC\textsubscript{50} values extremely sensitive to small changes in concentration, which could account in part for this variation of $\sim 2$-fold in IC\textsubscript{50}. Similar to the hNav channels, CBD also inhibited the mouse Nav1.6 (mNav1.6) current suggesting these human IC\textsubscript{50} values hold in rodent isoforms (Figure 3-1A, D).

Figure 3-1  CBD & THC inhibits Nav currents.

(A) The IC\textsubscript{50} curves of CBD block on hNav1.1-1.7 and mNav1.6. The chemical structure of CBD is shown on the top left corner. (B) THC IC\textsubscript{50} of hNav1.2 compared with CBD. The pulse protocols used, and chemical structure of THC are shown on the bottom right. Channels were exposed to each compound for 20 min. (C) Representative current traces at IC\textsubscript{50} in each sodium channel. Traces are taken from the concentrations that are closest to IC\textsubscript{50}. (D) Table of IC\textsubscript{50} and Hill-slope fitted parameters ($n = 3$-$15$ cells exposed at each concentration, SE quoted are errors of the fit).

3.4.2. THC inhibition of Nav currents

The chemical structures of CBD and THC are very similar, with the sole difference being the closure of a ring on THC as opposed to a free hydroxyl group in CBD. Given
that this difference is the structural basis for the functional differences between CBD and THC, we tested THC against hNav1.2. Our results suggest that although the potency of the sodium current inhibition between THC and CBD is similar, the Hill-slope associated with THC is less steep (Figure 3-1B, D). This may indicate that THC has some differences in the mechanism of sodium current inhibition than CBD.

3.4.3. CBD prevents Nav channels from opening

We next examined the effects of CBD on Nav channel activation by measuring peak channel conductance at membrane potentials between -120 and +30 mV. We show the effects of 3.3 µM CBD on peak sodium current densities (Figure 3-2A) and a plot of conductance (Figure 3-2B). About ~90% of the sodium conductance was inhibited. The normalized conductance is plotted against membrane potential, showing that CBD does not induce large changes in either the midpoint (V_{1/2}) or apparent valence (slope, k) of activation of the available sodium channels (p>0.05) (Figure 3-2C). Therefore, exposure to CBD at this concentration prevents channels from conducting; however, this exposure does not alter the voltage-dependence of activation.

We next measured the voltage-dependence of fast-inactivation. The normalized current amplitudes at the test-pulse is shown as a function of pre-pulse voltages (Figure 3-2D). The current at the test-pulse was inhibited by over 90%; however, unlike activation, the voltage-dependence of steady-state fast-inactivation of the remaining current was hyperpolarized by 8.1 mV (p=0.0002). This indicates CBD increased the propensity for channels to inactivate over the 500 ms pre-pulse in channels that were not inhibited from opening from rest, suggesting that CBD stabilizes the inactivated-state of sodium channels.

It was previously shown that 1 µM CBD inhibits the persistent and resurgent sodium currents in form of epilepsy caused by hNav1.6 GOF mutation, N1768D, which displays a non-inactivating component (Patel et al., 2016). We also found that CBD inhibits the resurgent current induced by including 200 µM β4-peptide to the intracellular solution. Figure 3-2E shows that 5 µM CBD inhibits the majority of resurgent currents, which is consistent with CBD preventing channels from opening (Figure 3-2A). Next, we sought to establish the concentration-dependence of CBD inhibition of the persistent current of the inactivation-deficient N1768D mutant. We found that persistent currents were inhibited at
slightly lower concentrations than the peak currents, suggesting there may be interactions between CBD and the open or inactivated-states over the course of a 100 ms depolarization (Figure 3-2F).

Figure 3-2  CBD effects on activation, steady-state fast-inactivation (SSFI), resurgent, & persistent currents.

(A) Conductance difference in hNav1.1 in vehicle and 3.3 µM CBD concentration (Veh: \(V_{1/2} = -42.5 \pm 0.8\), slope = 3.1 ± 0.7, n = 11; CBD: \(V_{1/2} = -39.6 \pm 1.8\), slope = 4.6 ± 1.5, n = 5). (B) Average current density of hNav1.1 in vehicle and CBD (Veh: current density = -75.3 ± 8.9 pA/pF, n = 11; CBD: current density = -6.8 ± 3.0 pA/pF, n = 5). (C) Voltage-dependence of activation as normalized conductance plotted against membrane potential (Vehicle: \(V_{1/2} = -41.9 \pm 0.4\) mV, Slope = 3.6 ± 0.4, n = 7; CBD: \(V_{1/2} = -40.3 \pm 1.0\) mV, Slope = 7.2 ± 0.9, n = 5). (D) Voltage-dependence of SSFI as normalized current plotted against membrane potential (Vehicle: \(V_{1/2} = -69.7 \pm 0.2\) mV, Slope = 5.0 ± 0.2, n = 12; CBD: \(V_{1/2} = -77.8 \pm 0.3\) mV, Slope = 5.8 ± 0.3, n = 5). (E) Resurgent current block in hNav1.6 (Veh: resurgent density = -33.9 ± 4.8 pA/pF, n = 11; CBD: resurgent density = -7.3 ± 1.2 pA/pF, n = 23; TTX: resurgent density = -3.3 ± 0.7 pA/pF, n = 31). (F) IC\(_{50}\) of CBD block of peak and persistent currents in hNav1.6 mutant (N1768D) (Peak: IC\(_{50}\) = 10.0 ± 0.7 µM, slope = 2.0 ± 0.4, n = 3-11; Persistent: IC\(_{50}\) = 6.4 ± 1.0 µM, slope = 1.3 ± 0.2, n = 3-9).

3.4.4. CBD stabilizes inactivated-states of Nav channels

We used a protocol to examine state-dependent inhibition across a range of holding-potentials where channel inactivation varies (Kuo & Bean, 1994). We first held channels at a holding-potential of -100 mV where channels are almost all in the resting-state, while pulsing the channels 180 times at 1 Hz to allow CBD to reach equilibrium. Then, we depolarized the holding-potential by 10 mV three more times and repeated the
pulse train at each voltage (Figure 3-3A). We show the fractional block of sodium currents from the last pulse (180th) from each holding-potential (Figure 3-3B). Figure 3-3C shows a plot of the inverse of the apparent IC50 fit with a 4-state binding model that used parameters obtained from the Boltzmann fit of the voltage-dependence of steady-state fast-inactivation. This established that the apparent potency is directly related to the proportion of inactivated channels at different holding-potentials. Our results demonstrate that CBD inhibits the sodium current from both rest and inactivated-states; however, the potency of CBD is about 10-fold greater for inhibiting inactivated compared to resting-states (Figure 3-3C).

To assess the time-dependence and degree of stabilization of the inactivated-state we then measured the recovery from inactivation of hNav1.6 in the presence of 3.7 µM CBD (Figure 3-1A). This was done after depolarizing pre-pulse durations of 300 ms and 10 s, from a holding-potential of -120 mV. These pre-pulse durations correspond to inactivation recovery from fast and slow inactivated-states, respectively. The mean normalized recovery following the pre-pulse in CBD and control conditions were plotted and fit with a bi-exponential function (Figure 3-3D-E). The τ_slow and fraction of the recovery fit with τ_slow are plotted in (Figure 3-3F), which shows that CBD increases the fraction of recovery that is slow and the time constant of the slow component of recovery from inactivation from 300 ms to 10 s. This indicates that CBD slows the recovery from inactivation supporting the hypothesis that CBD stabilizes the inactivated-states (Figure 3-3D-F).
Figure 3-3  State-dependent inhibition of Nav current by CBD & effects on recovery from inactivation.

(A) Pulse protocol showing 180 pulses run at 1 Hz at each holding-potential and representative current traces. (B) CBD potency at four holding-potentials at pulse 180 (3 min) (IC₅₀ (µM): -100 mV = 12.7 ± 1.0, -90 mV = 10.3 ± 0.5, -80 mV = 6.7 ± 0.4, -70 mV = 2.9 ± 0.6; n = 2-6). (C) Apparent Kd at different voltages was well fit with a 4-state model invoking different potencies for resting and inactivated-state block. (D-E) Recovery from inactivation in 3.7 µM CBD at: 300 ms (Veh: τFast = 0.00173 s, τSlow = 0.0688 s, n = 35; CBD: τFast = 0.00654 s; τSlow = 0.516 s; n = 3) and 10 s (Veh: τFast = 0.0715 s, τSlow = 0.696 s; n = 33; CBD: τFast = 0.272 s; τSlow = 8.72 s; n = 3). (F) The slow components of recovery from inactivation in vehicle and CBD at 300 ms and 3 s are shown on the left Y-axis, and the fraction of slow to fast component of recovery from inactivation is shown on the right Y-axis.

3.4.5. CBD potency is increased at lower temperatures

Binding kinetics are typically responsive to changes in temperature, with higher temperatures increasing the rates of compound equilibration. To further investigate the mode of CBD interaction with sodium channels, we measured the observed rates of equilibration (time constant observed, τobs) of inhibition by fitting single exponential decays to inhibition of currents at three different temperatures. We examined the kinetics at concentrations above the IC₅₀ to ensure a clear inhibition signal window to define the τobs (Figure 3-4A-C). Channels were held at -45 mV and pulsed at 1 Hz following a recovery pulse to -150 mV for 60 ms and CBD was rapidly applied to the cells at different concentrations (Figure 3-4A). The fraction of inhibition was normalized against the
response in vehicle and plotted against the time elapsed after CBD addition which was set at $t = 0$. The inhibition was then fit with a single exponential function to obtain $\tau_{obs}$ and plotted against concentration. We show $\tau_{obs}$ saturated at a minimum with increasing concentrations, counter to the prediction of a single 2-state ligand binding reaction, which predicts a continually increasing $\tau_{obs}$ with increasing compound concentrations (Figure 3-4D). This suggests a rate-limiting step in the inhibitory pathway that is not dependent on the concentration of CBD in bulk solution as would be expected for binding to a specific inhibitory site on the channel. Interestingly, the kinetics of CBD inhibition were also found to be more rapid at cooler temperatures (Figure 3-4D). This is the opposite of what is expected for a classic inhibitor in a 2-state binding model, where the binding and unbinding rate constants, $k_{on}$ and $k_{off}$, are intrinsically temperature-dependent. These results further suggest that CBD does not inhibit conductance through a direct interaction with a single specific binding-site on the channel. We also found that the potency of CBD is increased at the lower temperatures (Figure 3-4E-F).

Figure 3-4  Lower temperatures increase kinetics & potency of CBD inhibitory effects.

(A-C) The kinetics of CBD block at 20, 28, and 33 °C at 50.0, 25.0, 12.5, and 6.3 µM (20 °C: 6.3 µM = 51.0 ± 0.6 s, 12.5 µM = 42.2 ± 0.3 s, 25.0 µM = 21.6 ± 0.4 s, 50.0 µM = 23.3 ± 0.3 s; 28 °C: 6.3 µM = 162.6 ± 0.9 s, 12.5 µM = 87.7 ± 0.2 s, 25.0 µM = 42.8 ± 0.1 s, 50.0 µM = 30.4 ± 0.4 s; 33 °C: 6.3 µM = 299.0 ± 9.5 s, 12.5 µM = 137.0 ± 0.5 s, 25.0 µM = 79.2 ± 0.3 s, 50.0 µM = 84.1 ± 0.3 s, n = 10-14). The variability at the lower concentration of 6.3 µM at 33 °C is larger due to the
3.4.6. CBD does not inhibit via interactions at the classic local-anesthetic pore binding-site

As CBD inhibition shares a characteristic of classic pore blockers (preference for the inactivated-state), we created a pore mutation in the local-anesthetic receptor site in hNav1.1 (F1763A) to determine whether the CBD potency was affected. The F1763 residue is part of a well-established binding-site for many of the most common local-anesthetics, including Tetracaine (TTC) (Ragsdale et al., 1994). To avoid any impacts on potency being caused by the shifts in stability of inactivation in this mutant (WT-hNav1.1 inactivation $V_{1/2} = -62.0 \pm 0.4$ mV, Slope = $7.2 \pm 0.4$, n = 3; F1763A inactivation $V_{1/2} = -49.3 \pm 0.1$ mV, Slope = $6.4 \pm 0.1$, n = 17), we measured inhibition from a holding potential of -45 mV where both channels were >50% inactivated. To validate the F1763A-mutant channels, we also measured the potency of TTC, and compared the results against WT-hNav1.1, which showed a drop in potency (Figure 3-5A). For CBD, the F1763A-mutant channels only caused a slight drop in potency of approximately 2-fold (Figure 3-5B). Molecular docking using a homology model of hNav1.1 based on the eukaryotic cockroach cryo-EM structure (Navpas) (Figure 3-6) suggested that CBD may in fact bind close to F1763. This suggests that F1763 is not a primary determinant of CBD inhibition, but does not rule out the possibility that CBD may interact with other pore residues.

To determine if CBD could also inhibit other non-human Nav channels, we tested it on the bacterial homomeric Nav channel (NaChBac) (Figure 3-5C). Interestingly, we found that NaChBac is also blocked by CBD, though with a slightly greater potency (Figure 3-5C). Unlike mammalian Nav channels, NaChBac does not fast inactivate (Ren et al., 2001). We hypothesized that if the previously observed state-dependence of CBD block in hNav channels is dependent upon the fast-inactivation process, then NaChBac should not show such a state-dependence. The potency measured at -100 and -55 mV suggest that state-dependence also exists in NaChBac; moreover, this effect may be even more pronounced than in hNav channels (Figure 3-5D). This may implicate that CBD is not dependent upon, or selective amongst, different modes of Nav inactivation.
Our findings in the homo-tetrameric NaChBac raises the question of whether CBD might also inhibit voltage-gated potassium channels, which are also homo-tetrameric. Our results indicate that CBD also inhibits the Kv2.1 current. Current traces are shown (Figure 3-5E). These findings, along with the previous reports of CBD modulation of calcium channel currents support the idea that CBD is a poly-pharmacological inhibitor of voltage-dependent ionic currents (Ross et al., 2008).

**Figure 3-5** CBD block of F1763A-mutant, NaChBac, and Kv2.1.

(A) TTC block of hNav1.1 (hNav1.1, IC\textsubscript{50} = 1.6 ± 0.1 µM, slope = 0.7 ± 0.03; F1763, IC\textsubscript{50} ~ 44.1 µM) with and without the F1763A mutation. (B) F1763A causes a slight decrease in CBD potency in hNav1.1 (hNav1.1: IC\textsubscript{50} = 2.5 ± 0.2 µM, slope = 2.0 ± 0.2, n = 2-6; F1763A: IC\textsubscript{50} = 4.8 ± 0.2 µM, slope = 4.1 ± 0.6, n = 3-811 cells exposed at each concentration). (C) Shows a comparison of the CBD inhibition of hNav1.6, NaChBac, and Kv2.1 (hNav1.6: IC\textsubscript{50} = 3.0 ± 0.1 µM, slope = 3.1 ± 0.2, n = 2-6; NaChBac: IC\textsubscript{50} = 1.5 ± 0.2 µM, slope = 2.8 ± 0.9, n = 1-4; Kv2.1: IC\textsubscript{50} = 3.7 ± 0.8 µM, slope = 1.1 ± 0.2, n = 1-5 11 cells exposed at each concentration). (D) Shows the state-dependent block of NaChBac tested at -55 and -100 mV (-100 mV: IC\textsubscript{50} = 1.5 ± 0.2 µM, slope = 2.8 ± 0.9, n = 1-4; -55 mV: IC\textsubscript{50} = 0.24 ± 0.05 µM, slope = 2.8 ± 0.9, n = 1-311 cells exposed at each concentration). (E) Current traces associated with the channels shown in (C).
3.4.7. CBD Does Not Alter Trafficking

As CBD non-specifically inhibits voltage-dependent currents, we sought to establish whether incubation of CBD could affect the channel trafficking to the cell surface over the time scale of our voltage-clamp experiments. To address this question, we assessed membrane channel distribution in cells incubated overnight with CBD. The results indicate that overnight incubation in 5 µM CBD does not alter sodium channel trafficking and therefore would not affect trafficking on timescales of the voltage-clamp experiments (<20-minute exposure) (Figure 3-7A-C).
Figure 3-7  Analysis of overnight CBD incubation.

(A) Confocal images of stained HEK cells from the middle confocal plane. The nuclei are stained with Hoe33342 (blue) and hNav1.1 is stained with Alexa Fluor-546 (red) (n = 3). (B) Pixel intensity grey value was used as a function of the length of cells (distance) from the middle confocal plane image. For a typical HEK cell with a diameter of about 13 µm, the majority of both vehicle and CBD incubated hNav1.1 channels were localized within the 0 to 3 µm and 11 to 13 µm ranges. These two ranges correspond to the outer cellular edges, indicating that most of the channels seem to be concentrated in the proximity of cell membranes. (C) We functionally tested both conditions using whole-cell voltage-clamp. The sodium currents of the CBD-incubated channels were inhibited. In the figure, maximal inactivating current density of HEK cells stably transfected with hNav1.1 is
shown (Veh: current density = 280.1 ± 67.5 pA/pF, n = 5; CBD: current density = 18.2 ± 3.5 pA/pF, n = 6). The comparison is between vehicle and 5 µM CBD following overnight incubation. Representative current traces are shown.

3.4.8. CBD inhibits nav currents in h-iPSC neurons

To determine whether our observations in HEK cells translate to native neuronal voltage-dependent currents, we measured the effects of CBD on human iPSC neurons. First, we established that potency established with manual patch-clamp methodology, using continuous perfusion of compound, correlated with Qube data by measuring the potency of sodium current inhibition of HEK cells expressing hNav1.2. Figure 3-8A shows the mean normalized concentration response plot which gave an IC$_{50}$ value similar to the Qube value for hNav1.2. The slightly increased potency in the manual assay can be explained by the temperature-dependence data (Figure 3-4), as manual voltage-clamp was performed at around 20 °C (room temperature) and the Qube IC$_{50}$ values were established at 28 °C (Figure 3-1A). Consistent with our previous results in HEK cells, both the neuronal sodium and potassium currents were blocked ~50 % by 1 µM CBD as shown by the representative families of current traces in (Figure 3-8D-E). CBD also caused a hyperpolarization of ~16 mV in the steady-state inactivation in the remaining available Nav channels in iPSCs (p=0.0031), which was similar to the shifts observed in hNav1.1 in HEK cells (Figure 3-2D; Figure 3-8B). We also measured the rate time constant of open-state fast inactivation at -20 mV, which did not differ significantly before and after CBD perfusion (p>0.05) (Figure 3-8C).
Figure 3-8  CBD inhibits human iPSC neuronal Nav, & Kv currents.

(A) Concentration-response relationship for CBD inhibition of hNav1.2 channels obtained using manual patch clamp (IC$_{50}$ = 1.3 ± 0.1 µM, slope = 2.1 ± 0.4, n = 3-6). (B) Plot of normalized SSFI after a 100 ms pre-pulse before (V$_{1/2}$ = -50.0 ± 0.4 mV, slope = 6.4 ± 0.4, n = 3) and after (V$_{1/2}$ = -66.1 ± 0.7 mV, slope = 8.1 ± 0.6, n = 3) perfusion. (C) Open-state fast-inactivation time constants shown on log scale on the Y-axis at -20 mV for vehicle (-20 mV: 1.3 ± 0.1 ms, n = 3) and CBD (-20 mV: 1.3 ± 0.4 ms, n = 3) in iPSC neurons. (D) Representative current-voltage relationship recorded from iPSC neurons with a KF based internal solution before 1 µM perfusion of CBD and (E) after perfusion. Inset in the panel are zoomed-in view of currents at the test-pulse used to assess availability.

3.4.9. CBD reduces neuronal excitability in a Hodgkin-Huxley model of cortical neuron

To test the effect of IC$_{50}$ concentrations of CBD on neuronal excitability, we used a modified version of the Hodgkin-Huxley model to simulate a cortical neuron’s excitability (Hodgkin & Huxley, 1952; Yi et al., 2017). In the CBD condition, the sodium and potassium conductances were reduced by 50%. The activation and inactivation V$_{1/2}$ and slopes were taken from the results shown in (Figure 3-2C-D). In our simulations, the channels were given a series of stepwise current injections with increasing intensities at each step for 100 ms. Each 100 ms step was followed by a 50 ms recovery period in which no current injection was applied (Figure 3-9A). Our results suggest that the peak amplitude of the first action potential (AP) in CBD is smaller than vehicle. This is consistent with the reduction of peak sodium conductance caused by CBD. Furthermore, in the CBD condition
we observed that threshold for the first AP after depolarization was reduced (Figure 3-9B). The overall effect of CBD on the action potential morphology is that at all current injection intensities, CBD reduces the number of action potentials, leading to a net loss of excitability. These results are consistent with previous studies using current clamp recordings, and may in part theoretically explain the reported efficacy of CBD in treating conditions including epilepsy and pain (Khan et al., 2018).

![Figure 3-9](image)

**Figure 3-9**  CBD reduces excitability in an action potential model & schematic representation of CBD’s mode of action.

(A) Simulation of the effects of CBD on action potential morphology over a series of increasing current injection intensities. (B) Zoomed-in simulation of action potentials from the first interval shown in (A). (C) Proposed mode of action of CBD involving interactions with both the membrane, resting and inactivated sodium channels.

### 3.5. Discussion

CBD has a weak affinity for CB receptors thus its anticonvulsant properties are attributed to modulations of other targets, including ion channels. Our study suggests that the Nav channel family is amongst the possible ion channel targets of CBD.

Our results in this study suggest that CBD as a sodium channel inhibitor is non-selective; therefore, we performed some of our experiments on single sodium channel subtypes, including the temperature-dependence and channel trafficking assays. We
believe that due to the non-specific nature of the interactions, the results obtained from single subtypes will be generalizable to the whole sodium channel superfamily. This idea is supported by the consistencies we observed between HEK cells that express only a single sodium channel subtype, and iPSC neurons, which host several sodium channel subtypes in a native environment.

3.5.1. Mechanisms of sodium current inhibition

The initial results obtained from the selectivity experiments on hNav channels suggested that CBD is indeed a sodium current inhibitor. However, this inhibition is relatively non-selective in nature and has a steep average Hill-slope, suggesting multiple interactions. Like CBD, we found that THC also inhibits hNav1.2, albeit with a less steep Hill-slope. Our results indicated that CBD prevents the activation of sodium channels from rest, while also stabilizing the inactivated-states of these channels without altering the voltage-dependence of activation. This inhibition has similarities to that reported for amphiphilic compounds (Lundbæk et al., 2004; Lundbæk, 2005). It is suggested that amphiphilic compounds tend to localize at the solution-bilayer interface. This occurrence is made possible by having the polar group of the amphiphile residing at the interface with the hydrophobic region, which then gets inserted into the core of the bilayer. This partitioning into the lipid bilayer alters the biophysical properties of the membrane by reducing stiffness, changing phase preference and curvature. The net effect of these alterations to the membrane is an increased preference for the bilayer-embedded Nav channel in its inactivated-state (Lundbæk, 2005).

The similarity of CBD to amphiphiles, along with the multi-modal interaction relationship between this compound and the voltage-dependent sodium currents suggested three potential mechanisms culminating in inhibition: 1) multiple direct interactions between CBD and Nav channels, 2) CBD alteration of membrane biophysical properties, or 3) a combination of direct interactions with Nav channels and changes in the membrane fluidity.
3.5.2. Evidence for CBD effects on sodium channels through altering membrane fluidity

Contrary to what is expected for classic pseudo-second order bimolecular blocking scheme, CBD inhibition curves displayed a Hill-slope ~3 and compound was fastest to equilibrate and most potent at lower temperatures, indicating that CBD does not follow a classic blocking scheme (Figure 3-4). One potential explanation for the decreased potency at 33 °C is that higher temperatures increase the thermal fluctuations of the sodium channels, which become faster, and consequently, it becomes harder for CBD to find interaction sites that may reside in thermally volatile regions of the structure. A second potential explanation is that because the fluidity of the membrane changes as a function of temperature (Otto et al., 1984), higher CBD concentrations are required to have the same reduction of stiffness at 33 °C where the membrane is more fluid than at lower temperatures, and the relative reduction in stiffness is more pronounced.

3.5.3. Evidence for direct binding of CBD to the channels to cause inhibition

The observation of an inactivated-state preference, which is a characteristic of Nav pore blocking local-anesthetics, led us to investigate the CBD block of a hNav1.1-mutant (F1763A) with a mutation in the local-anesthetic pore binding-site. We found that CBD’s inhibitory effects were minimally affected in the F1763A mutants. This suggests that binding at this site is not via similar interactions to local-anesthetics (Ragsdale et al., 1994; Pless et al., 2013), although molecular docking supports the possibility that CBD could interact in the pore of hNav1.1.

We found that CBD also inhibits a non-mammalian sodium channel, NaChBac, and the voltage-gated potassium channel, Kv2.1. Interestingly, the CBD block of NaChBac was slightly more potent than the mammalian orthologs. This increased potency might be due to the presence of 4-fold symmetry in these channels and thus, the 4-time repeating of a domain that may be involved in inhibition. Indeed, the equivalent F residue in the pore of homo-tetrameric NaChBac that contributes to local-anesthetic binding (Lee et al., 2012) (F200) is repeated 4-times, as opposed to the single F1763 in the monomeric hNav1.1, where the F1763A mutation in Nav1.1 induced a small drop in potency. These results offer some support to the hypothesis that, in addition to altering membrane properties, CBD
might also have direct interactions with Nav channels that alter conductance. Additionally, our observation that membrane Nav channel distribution was unchanged in cells after a 24-hour incubation with CBD suggested there were no effects on trafficking.

3.5.4. CBD inhibits heterologously expressed and native hNav currents

To assess whether the observed CBD inhibitory effects on voltage-dependent currents in HEK cells could be replicated in the native neuronal environment, we assessed inhibition of currents in iPSC neurons and saw significant inhibition of both Nav and Kv currents (Figure 3-8). The potency was comparable to the effects seen in HEK cells stably expressing Nav channels indicating the HEK cell is a valid expression system to characterize Nav inhibition by CBD.

3.5.5. Mechanism of sodium current inhibition by CBD

We found CBD to be highly lipid bound in a TRANSIL brain lipid binding assay (Table 3.1). This supported the possibility that the observed effects might be due to alterations to the biophysical properties of the membrane. However, consistent with previous studies (Longhi et al., 2011), we found that phenytoin, a well-established non-selective Nav blocker also had high lipid binding (98.5%, Table 3-1), and it is assumed that phenytoin’s efficacy against seizures is via direct Nav channel pore block (Ragsdale et al., 1996; Remy et al., 2003; Mantegazza et al., 2010). The reported brain exposure levels required for efficacy in rodent maximal electroshock seizure models was also comparable between CBD and phenytoin supporting the possibility that CBD could at least in part mediate its efficacy through sodium channel inhibition (Table 3-1) (Consroe & Wolkin, 1977; Demonaco & Lawless, 1983; Ragsdale et al., 1996; Grothenhermen, 2003; Beyreuther et al., 2007; Mantegazza et al., 2010; Deiana et al., 2012; Shandra et al., 2013). This similarity in lipid binding however raises the question of why the two compounds inhibit through different mechanisms. When comparing other physicochemical properties between the compounds it is striking that the calculated LogD of CBD and phenytoin are 6.6 and -0.7, respectively (ChEMBL database). In contrast to TRANSIL which measures lipid binding, the LogD is derived by measuring the partitioning of small molecules between octanol and water at pH7.4, and depends on a variety of structural interactions, including hydrogen bonds and Van der Waals interactions. However, since
octanol is structurally different from membrane phospholipid bilayers, which contain both polar and non-polar moieties, it cannot model these interactions well. Consequently, it is possible for compounds to have similar TRANSIL (lipid binding) numbers and different LogD, as found for these compounds. This may suggest that despite having similar lipid binding percentages (Table 3-1), the much lower LogD of phenytoin underlies its action as a classic sodium channel blocker that can enter the pore. In contrast, CBD’s very high LogD could result in its accumulation deeper inside the bilayer hydrophobic regions that leads to a greater effective change in the membrane stiffness than phenytoin and underlies CBD’s different mode of current inhibition compared with phenytoin. This disconnect between LogD and lipid binding fractions is further supported by considering another anti-epileptic sodium channel pore blocker, carbamazepine, that has a lower lipid binding percentage (83.7%) than phenytoin, while having a higher LogD (2.0) than phenytoin (Table 3-1). We therefore suggest that high lipid binding alone is not a sufficient property for a compound to alter membrane stiffness, which results from a combination of lipid binding with other physico-chemical properties unique to CBD over phenytoin and carbamazepine. Overall, our results suggest that CBD has a non-selective inhibitory effect on voltage-dependent sodium currents. The results presented in this study suggest that this mechanism likely involves a combination of: direct interactions with channel hydrophobic regions, possibly the pore, and changing the membrane bilayer flexibility through lipid accumulation, as illustrated by the cartoon in (Figure 3-9C).

Table 3-1  The comparison of CBD to phenytoin and carbamazepine.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cannabidiol</th>
<th>Phenytoin</th>
<th>Carbamazepine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain Lipid Binding - TRANSIL (%)</td>
<td>99.6</td>
<td>98.5</td>
<td>83.7</td>
</tr>
<tr>
<td>Plasma Protein Binding (%)</td>
<td>~95-99*</td>
<td>95*</td>
<td>~70*</td>
</tr>
<tr>
<td>IC50 on Nav Channels (µM)</td>
<td>1-4</td>
<td>~10*</td>
<td>~25*</td>
</tr>
<tr>
<td>ED50 MES (mg/kg)</td>
<td>~120*</td>
<td>8.2-17.5*</td>
<td>7.5*</td>
</tr>
<tr>
<td>Efficacious Plasma Level (µM)</td>
<td>6.4-8.3*</td>
<td>4-10*</td>
<td>5-12*</td>
</tr>
<tr>
<td>Brain Concentration (µM)</td>
<td>21.9*</td>
<td>86*</td>
<td>9.7*</td>
</tr>
<tr>
<td>Brain / IC50 (Ratio)</td>
<td>5.5-21.9</td>
<td>8.6</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Parameters with asterisk were taken from literature (Hooper et al., 1975; Consroe & Wolkin, 1977; Demonaco & Lawless, 1983; Ragsdale et al., 1996; Kuo et al., 1997; Sun et al., 2002; Grotenhermen, 2003;
Results from previous studies along with our findings in this study leads us to conclude that the underlying mechanism for the reported efficacy of CBD against seizures likely involves many systems beyond just CB receptors (Ledgerwood et al., 2011), GPR receptors (Kaplan et al., 2017), or ion channels (Ross et al., 2008; Patel et al., 2016). Dravet syndrome is primarily caused by a heterozygous LOF of Nav1.1 in the brain (Parihar & Ganesh, 2013). As such, clinically used sodium channel blockers are often considered pro-convulsant in Dravet syndrome (Brunklaus et al., 2012; Nabbout et al., 2013), presumably because they inhibit the reduced Nav1.1 current even further. This raises the question of why CBD, as a non-selective Nav inhibitor, would act as an anti-convulsant in Dravet syndrome. A recent study, however, unexpectedly demonstrated efficacy with a non-selective sodium channel blocker in a Dravet mouse model, suggesting that Nav inhibition can be protective (Anderson et al., 2017). In addition, another study in a Dravet mouse model suggested that the efficacy of CBD against Dravet syndrome may be mediated through antagonism at GPR55 receptors independently of Nav channels (Kaplan et al., 2017).

The promiscuity of CBD interactions that have been reported with multiple targets suggests that this compound, like many natural products, is a pleiotropic compound with a complex poly-pharmacology. It is therefore unlikely that any efficacy against epilepsy is related solely to inhibitory effects on Nav channels; however, our study suggests that inhibition of Nav currents could occur at therapeutically relevant concentrations, and thus might contribute to efficacy against seizures.

### 3.6. Acknowledgments

This work was supported by a Mitacs fellowship to Mohammad-Reza Ghovanloo, Dr. Peter C. Ruben received funding from Agrima Botanicals Inc and NSERC, and Xenon Pharmaceuticals Inc provided funding to Mohammad-Reza Ghovanloo, Dr. Noah G. Shuart, Janette Mezeyova, Dr. Richard A. Dean, and Dr. Samuel J. Goodchild.

Mohammad-Reza Ghovanloo assembled data, performed patch-clamp experiments, immunocytochemistry, homology modeling, molecular docking, action potential modeling, data analysis, figure making, wrote manuscript, and contributed to data
interpretation. Dr. Noah G. Shuart collected and analyzed electrophysiological data. Janette Mezeyova performed cell culture. Dr. Richard A. Dean made mutations. Dr. Samuel J. Goodchild performed electrophysiological data collection, assembly, analysis, interpretation and manuscript writing. Drs. Samuel J. Goodchild and Peter C. Ruben conceived the experiments and revised the manuscript critically.
Chapter 4. Mechanism and effects of the skeletal muscle Nav1.4 inhibition by cannabidiol

This chapter describes the work submitted for publication and posted as a pre-print (Ghovanloo et al., 2020b) with minor modifications and formatting changes to suit the thesis style.

4.1. Abstract

*Cannabis sativa* contains active constituents called phytocannabinoids. Some phytocannabinoids are psychotropic and others are not. The primary non-psychotropic phytocannabinoid is cannabidiol (CBD), which is proposed to be therapeutic against many conditions, including muscle spasms. Mechanisms have been proposed for the action of CBD on different systems, involving multiple targets, including the voltage-gated sodium channel (Nav) family, which are heavily implicated in many of the conditions CBD has been reported to relieve. In this study, we investigated the modulatory mechanism of CBD on Nav1.4. Based on previous results, we tested the hypothesis that CBD mechanism of action involves: 1) modulation of membrane elasticity, which indirectly contributes to Nav inhibition; and 2) physical block of the Nav pore. We first performed molecular dynamic (MD) simulations to visualize CBD effects and localization inside the membrane, and then performed NMR to verify the MD results, showing CBD localizes below membrane headgroups. Then, we performed a gramicidin-based fluorescence (GFA) assay that showed CBD alters membrane elasticity. Next, we used site-directed mutagenesis in (F1586A) and around (WWWW) the Nav1.4 pore. Removing the local anesthetic binding site with F1586A reduced CBD block of $I_{Na}$. Occluding the fenestrations with WWWW blocked CBD access from the membrane into the Nav1.4 pore. However, stabilization of inactivation, via CBD-induced changes in membrane elasticity persisted, in WWWW. To investigate the potential therapeutic value of CBD against some Nav1.4 channelopathies, we used a pathogenic variant of Nav1.4, P1158S, known to cause myotonia and periodic paralysis. We found CBD reduces excitability in both wild-type and the mixed myotonia/periodic paralysis variant. Our *in-vitro/in-silico* results suggest that CBD may have therapeutic value against myotonia. Because Nav1.4 is crucial to skeletal muscle contraction, we used rat diaphragm myography and found the presence of saturating levels of CBD reduces skeletal muscle contraction.
4.2. Introduction

The cannabis plant, *Cannabis sativa*, contains over 120 active constituents, collectively known as phytocannabinoids (Morales *et al.*, 2017). Some phytocannabinoids mediate psychotropic effects, whereas others do not (Morales *et al.*, 2017). The primary non-psychotropic phytocannabinoid is cannabidiol (CBD). The structure of CBD is nearly identical to the main psychotropic compound isolated from cannabis, ∆9-tetrahydrcannabinol (THC) (Morales *et al.*, 2017). The only structural difference between the two compounds is the presence of a free hydroxyl in CBD in place of a closed ring in THC. This structural difference underlies THC’s high affinity for the human cannabinoid receptors, CB1 and CB2, thought to mediate the euphoria associated with using cannabis (Devinsky *et al.*, 2017). In contrast to THC, CBD has little to no affinity for CB receptors (Pertwee, 2008). However, CBD has been suggested to be a potentially therapeutic compound against a variety of different conditions, including muscle spasms, pain, and seizures. Some reports of CBD efficacy are anecdotal, whereas others have been experimentally and clinically substantiated (Devinsky *et al.*, 2017). CBD showed therapeutic efficacy in a recent phase III human clinical trial against Dravet syndrome (Devinsky *et al.*, 2017), a severe form of childhood epilepsy and received FDA approval for its treatment.

Reports of CBD efficacy, along with its low affinity for CB receptors, have inspired studies on its CB-independent actions. Many mechanisms and targets have been proposed for the action of CBD (Ross *et al.*, 2008; De Petrocellis *et al.*, 2011; Patel *et al.*, 2016; Kaplan *et al.*, 2017; Ghovanloo *et al.*, 2018c). The voltage-gated sodium channel (Nav) family is among these suggested targets (Patel *et al.*, 2016; Ghovanloo *et al.*, 2018c), in part because Nav underpin many of the conditions for which CBD is, or is suggested, to be efficacious.

Sodium currents through Nav initiates action potentials (AP) in neurons, myocardium, and skeletal muscles. Nav are hetero-multimeric proteins composed of a large ion conducting and voltage-sensing α-subunit and smaller β-subunits (Cannon, 2006; Ghovanloo *et al.*, 2016b; Ghovanloo & Ruben, 2020). The α-subunit is a single transcript that includes four 6-transmembrane segment domains. Each structural domain can be divided into two functional sub-domains: the voltage-sensing domain (VSD) and
the pore-domain (PD) (Ghovanloo et al., 2016b). These functional sub-domains are connected through the intracellular S4-S5 linker (Yarov-Yarovoy et al., 2012). The Nav pore is the site of interaction for many pharmacological blockers (Lee et al., 2012; Gamal El-Din et al., 2018). The pore is surrounded by four intra-bilayer fenestrations whose functional roles remain speculative (Pan et al., 2018).

Variants of the Nav subtype predominantly expressed in skeletal muscles, Nav1.4, are associated with contractility dysfunction. Most Nav1.4 variants depolarize the sarcolemma; this depolarization can result in either hyper- or hypo-excitability in phenotypes (Cannon, 2006). Hyperexcitable muscle channelopathies are classified as either non-dystrophic myotonias or periodic paralyses (Lehmann-Horn et al., 2008). Most of these channelopathies arise from sporadic de-novo or autosomal dominant mutations in SCN4A (Ghovanloo et al., 2018a).

The majority of gain-of-function (GOF) Nav1.4 variants result in myotonic syndromes, which are defined by a delayed relaxation after muscle contraction (Lehmann-Horn & Rudel, 1995; Tan et al., 2011). In myotonia, there is an increase in muscle membrane excitability in which even a brief voluntary contraction can lead to a series of APs that can persist for several seconds after motor neuron activity is terminated, a condition that is perceived as muscle stiffness (Tan et al., 2011). The global prevalence of non-dystrophic myotonias is ~1/100,000 (Emery, 1991). This condition is not considered lethal, but it can be life-limiting due to the multitude of problems it can cause, including stiffness and pain (Vicart et al., 2005).

A cationic leak (gating-pore current in the VSD), with characteristics similar to the ω-current in Shaker potassium channels, causes periodic paralyses (Jiang et al., 2018). This mechanism indicates that periodic paralyses can be caused by a severe form of GOF in Nav1.4 (Tombola et al., 2005; Wu et al., 2011). A subset of periodic paralyses is triggered by low serum [K+] and results in episodes of extreme muscle weakness. These are known as hypo-kalemic periodic paralyses (hypoPP) (Miller et al., 2004a). hypoPP onsets between the ages of 15–35. The prevalence of hypoPP is also ~1/100,000. A serum [K+] less than 3 mM (normal concentration=3.5–5.0) may trigger hypoPP (Fontaine, 2008).
There are few therapeutics for these skeletal muscle dysfunctions, and treating myotonias and periodic paralyses mostly relies on drugs developed for other conditions, including local-anesthetics (LA). Myotonia treatment is focused on reducing the involuntary AP bursts (Desaphy et al., 2004; Vicart et al., 2005); hypoPP treatment is mostly focused on restoring serum [K⁺] (Torres et al., 1981; Tawil et al., 2000; Sternberg et al., 2001; Venance et al., 2004). Thus, there remains a need for compounds that alleviate the hyperexcitability associated with both myotonia and hypoPP. Interestingly, non-euphoric plant cannabinoids enhance muscle quality and performance of dystrophic mdx mice (Iannotti et al., 2019).

In this study, we first sought to delineate the mechanisms by which CBD may affect Nav1.4. We previously described the inhibitory effects of CBD on some neuronal Nav subtypes, which prompted us to predict a possible mechanism in which CBD both directly (direct interaction with channel) and indirectly (modulating membrane elasticity) inhibits Nav (Ghovanloo et al., 2018c). In the present study, we explored whether CBD accumulates in the membrane, which could alter membrane elasticity, and, while residing inside the membrane, enters the Nav1.4 fenestrations and blocks the channel pore (Gamal El-Din et al., 2018). Next, we explored the effects of CBD on a mixed periodic paralysis and myotonia Nav1.4 mutation (P1158S) (Webb & Cannon, 2008; Ghovanloo et al., 2018a) to determine whether it could alleviate the mutant phenotypes. Finally, we sought to survey whether saturating levels of CBD can reduce skeletal muscle contractility, which it does.

4.3. Methods

4.3.1. Rat diaphragm preparation

Four 4-week old male Sprague Dawley rats (Charles River, Raleigh site) were euthanized. The rat phrenic hemi-diaphragm preparation was isolated according to the method described by Bulbring (1946) (BULBRING, 1946). A fan-shaped muscle with an intact phrenic nerve was isolated from the left side and transferred to a container with Krebs’ solution (NaCl 95.5, KCl 4.69, CaCl₂ 2.6, MgSO₄.7H₂O 1.18, KH₂PO₄ 2.2, NaHCO₃ 24.9, and glucose 10.6 mM) and aerated with carbogen (95% oxygen and 5% carbon dioxide). All experimental protocols were approved by the Animal Care and Use
Committees (Xenon Pharmaceuticals). Contraction experiment was performed using a Radnoti Myograph system.

4.3.2. Molecular docking

Docking of CBD into the cryo-EM structure of hNav1.4 (PDB ID: 6AGF was carried out using Autodock Vina (Trott & Olson, 2010). CBD was downloaded in PDB format from Drugbank (Wishart et al., 2018). To dock CBD into Nav1.4 a large search volume of 32 Å x 44 Å x 26 Å was considered, that enclosed nearly the whole of the pore domain and parts of VSD. This yielded the top 9 best binding poses of CBD ranked by mean energy score.

4.3.3. MD simulations system preparation

We ran two different sets of MD simulations, a first consisting of CBD interacting with model POPC membranes, and a second consisting of CBD interacting with the hNav1.4 channel embedded in its POPC/solution environment.

First, a homogenous lipid bilayer consisting of 188 POPC molecules was prepared using the CHARMM-GUI Membrane builder (Jo et al., 2008; Wu et al., 2014; Lee et al., 2016). Three different systems were created: one with two CBD molecules, each one placed in each leaflet of the bilayer (System 1), one with three CBDs, all of them placed in the upper leaflet (System 2) and one with six CBDs, of which three were placed in the upper leaflet and three in the lower leaflet (System 3). CBD was placed manually into the bilayer, with the polar headgroup of CBD facing the lipid headgroups. Lipid molecules with at least one atom within 2 Å of a CBD were manually deleted. A control simulation without any CBD was also prepared (System 0). The system was hydrated by adding two ~25 Å layers of water to both sides of the membrane. Lastly, 150 mM NaCl was added (30 Na⁺ and 30 Cl⁻). The simulation systems are summarized in Table 4-1. This system was defined as the lipid-CBD

Second, hNav1.4 and the best docked position of CBD obtained from Autodock Vina was used as a starting structure. The starting system was embedded into POPC lipid bilayer. The system was hydrated by adding two ~25 Å layers of water to both sides of the
membrane. Lastly, the system was ionized with 150 mM NaCl. This system is defined as the Nav1.4-CBD-lipid system.

Table 4-1 Details of the simulation systems.

<table>
<thead>
<tr>
<th>System</th>
<th>Number of CBD molecules</th>
<th>Number of lipid molecules</th>
<th>Number of water molecules</th>
<th>Number of ions (Na/Cl)</th>
<th>Total number of atoms</th>
</tr>
</thead>
<tbody>
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<td>#0</td>
<td>0</td>
<td>188</td>
<td>11377</td>
<td>30/30</td>
<td>59383</td>
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<td>171</td>
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4.3.4. MD simulations

The CHARMM36 forcefield was used to describe the protein, lipid bilayer, and the ions (Klauda et al., 2010). CBD was parameterised using the SWISS-PARAM software (Zoete et al., 2011). The TIP3P water model was used to describe the water molecules. The systems were minimised for 5000 steps using steepest descent and equilibrated with constant number of particles, pressure and temperature (NPT) for at least 450 ps for the lipid-CBD system and 36 ns for the Nav1.4-CBD-lipid system, during which the position restraints were gradually released according to the default CHARMM-GUI protocol. During equilibration and production, a time step of 2 fs was used, pressure was maintained at 1 bar through Berendsen pressure coupling, temperature was maintained at 300 K through Berendsen temperature coupling with the protein, membrane and solvent coupled and LINCs algorithm (Hess et al., 1997) was used to constrain the bonds containing hydrogen. For long range interactions, periodic boundary conditions and particle mesh Ewald (PME) were used. For short range interactions, a cut-off of 12 Å was used. Finally, unrestrained production simulations were run for 150 ns for each of the lipid-CBD system and 10 ns for the Nav1.4-CBD-lipid system, using Parinello-Rahaman pressure coupling (Parrinello & Rahman, 1981) and Nose-Hoover temperature coupling (Nosé, 1984). Simulations were performed using GROMACS 2018.4 (Abraham et al., 2015).
4.3.5. ABMD simulations

Adiabatic biased molecular dynamics (ABMD) (Marchi & Ballone, 1999) simulations were performed using GROMACS 2018.4 (Abraham et al., 2015) patched with Plumed-2.5.1 (Tribello et al., 2014) to study the entrance pathway of CBD into its docking site in hNav1.4. ABMD is a simulation method in which a time dependent biasing harmonic potential is applied to drive the system towards a target system along a predefined collective variable. Whenever the system moves closer towards the target system along the collective variable, the harmonic potential is moved to this new position, resulting in pushing the system towards the final state. The bias potential was applied along the distance between the center of masses of CBD and F1586. Two types of biasing potentials were considered: one along the y-component of distance and other along all components of distance.

4.3.6. ²H NMR lipid analysis

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC-d31, sn-1 chain perdeuterated) was obtained from Avanti Polar Lipids (Alabaster, AL). The POPC-d31:CBD sample was prepared with ~50 mg lipid and 3.4 mg of CBD for a ratio of POPC/CBD 8:2. The two samples, pure POPC-d31 and POPC-d31:CBD (8:2), were dissolved in Bz/MeOH 4:1 (v/v) and freeze-dried. After hydration with excess amounts of deuterium-depleted water (ddw), five freeze-thaw-vortex cycles were done between liquid nitrogen (−196°C) and 60°C to create multilamellar dispersions (MLDs).

Deuterium ²H NMR experiments were performed on a TacMag Scout spectrometer at 46.8 MHz using the quadrupolar echo technique. The spectra were produced from ~20,000 two-pulse sequences. 90° pulse lengths were set to 3.1 µs, inter-pulse spacing was 50 µs, dwell time was 2 µs, and acquisition delays were 300 ms. Data were collected using quadrature with Cyclops eight-cycle phase cycling. The spectra were dePaked to extract the smoothed order parameter profiles of the POPC sn-1 chain in the presence or absence of CBD. Samples were run at 20, 30, and 40°C, left to equilibrate at each temperature for 20 mins before measurements were taken.
4.3.7. Cell culture

Chinese Hamster Ovary (CHOK1) cells were transiently co-transfected with cDNA encoding eGFP and the β1-subunit and either WT-Nav1.4 (GenBank accession number: NM_000334) or any of our mutant α-subunits. Transfection was done according to the PolyFect transfection protocol. After each set of transfections, a minimum of 8-hour incubation was allowed before plating on sterile coverslips.

4.3.8. Gramicidin-fluorescence membrane elasticity assay

1,2-dierucyl-sn-glycero-3-phosphocholine (DC_{22:1}PC) were from Avanti Polar Lipids (Alabaster, AL). CBD was from Sigma-Aldrich (St. Louis, MO). 8-Aminonaphthalene-1,3,6-trisulfonate (ANTS) was from Invitrogen Life Technologies (Grand Island, NY). Gramicidin D was from (Sigma Aldrich).

GFA: Large unilamellar vesicles (LUVs) were made from DC_{22:1}PC as described previously (Rusinova et al., 2015). Briefly, phospholipids in chloroform and gA in methanol (1000:1 lipid:gA weight ratio) were mixed. Quench rates were obtained by fitting the quench time course from each mixing reaction with a stretched exponential (Ingólfsson et al., 2010):

\[
F(t) = F(\infty) + (F(0) - F(\infty)) \cdot \exp\left\{-\left(t / \tau_0 \right)^\beta\right\} \tag{Eq. 1}
\]

and evaluating the quench rate at 2 ms (the instrumental dead time is \(~1.5\) ms):

\[
k(t) = \left(\beta / \tau_0\right) \cdot \left(t / \tau_0\right)^{[\beta-1]} \bigg|_{2\text{ms}} \tag{Eq. 2}
\]

To test drug effects on the lipid bilayer CBD was equilibrated with the LUVs for 10 min at 25 °C before acquiring quench time courses. Each measurement consisted of (4 – 8) individual mixing reactions, and the rates for each mixing reaction were averaged and normalized to the control rate in the absence of drug.
4.3.9. Patch-clamp

Whole-cell patch-clamp recordings were performed in an extracellular solution containing (in mM): 140 NaCl, 4 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 HEPES or MES (pH6.4). Solutions were adjusted to pH6.4 and 7.4 with CsOH. Pipettes were filled with intracellular solution, containing (in mM): 120 CsF, 20 CsCl, 10 NaCl, 10 HEPES. In some experiments lower sodium concentration of 1 mM (intracellular) was used to boost driving force, and hence current size. All recordings were made using an EPC-9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) digitized at 20 kHz via an ITC-16 interface (Instrutech, Great Neck, NY, USA). Voltage-clamping and data acquisition were controlled using PatchMaster/FitMaster software (HEKA Elektronik, Lambrecht, Germany) running on an Apple iMac. Current was low-pass-filtered at 10 kHz. Leak subtraction was performed automatically by software using a P/4 procedure following the test pulse. Giga-ohm seals were allowed to stabilize in the on-cell configuration for 1 min prior to establishing the whole-cell configuration. Series resistance was less than 5 MΩ for all recordings. Series resistance compensation up to 80% was used when necessary. All data were acquired at least 1 min after attaining the whole-cell configuration. Before each protocol, the membrane potential was hyperpolarized to −130 mV to ensure complete removal of both fast inactivation and slow inactivation. All experiments were conducted at 22 ± 2 °C. Analysis and graphing were done using FitMaster software (HEKA Elektronik) and Igor Pro (Wavemetrics, Lake Oswego, OR, USA). All data acquisition and analysis programs were run on an Apple iMac (Apple Computer).

Some cDNA constructs produced small ionic currents. To ensure, the recorded currents were indeed construct-produced currents and not endogenous background currents, untransfected cells were patched and compared to transfected cells. The untransfected CHOK1 cells, which were exclusively used for cDNA expression, produced no endogenous sodium currents.

4.3.10. Activation protocol

To determine the voltage-dependence of activation, we measured the peak current amplitude at test pulse potentials ranging from −100 mV to +80 mV in increments of +10 mV for 20 ms. Channel conductance (G) was calculated from peak $I_{Na}$.
where $G_{Na}$ is conductance, $I_{Na}$ is peak sodium current in response to the command potential $V$, and $E_{Na}$ is the Nernst equilibrium potential. Calculated values for conductance were fit with the Boltzmann equation:

$$\frac{G}{G_{\text{max}}} = \frac{1}{1 + \exp\left[-ze_0(V_m - V_{1/2})/kT\right]} \quad \text{(Eq. 4)}$$

where $G/G_{\text{max}}$ is normalized conductance amplitude, $V_m$ is the command potential, $z$ is the apparent valence, $e_0$ is the elementary charge, $V_{1/2}$ is the midpoint voltage, $k$ is the Boltzmann constant, and $T$ is temperature in K.

### 4.3.11. Steady-state fast inactivation protocol

The voltage-dependence of fast inactivation was measured by preconditioning the channels to a hyperpolarizing potential of $-130$ mV and then eliciting pre-pulse potentials that ranged from $-170$ to $+10$ mV in increments of $10$ mV for $500$ ms, followed by a $10$ ms test pulse during which the voltage was stepped to $0$ mV. Normalized current amplitudes from the test pulse were fit as a function of voltage using the Boltzmann equation:

$$\frac{I}{I_{\text{max}}} = \frac{1}{1 + \exp\left(-ze_0(V_m - V_{1/2})/kT\right)} \quad \text{(Eq. 5)}$$

where $I_{\text{max}}$ is the maximum test pulse current amplitude.

### 4.3.12. Persistent currents protocol

Persistent current was measured between $145$ and $150$ ms during a $200$ ms depolarizing pulse to $0$ mV from a holding potential of $-130$ mV. Pulses were averaged to increase signal-to-noise ratio.

### 4.3.13. Recovery from fast inactivation protocol

Channels were fast inactivated during a $500$ ms depolarizing step to $0$ mV, and recovery was measured during a $19$ ms test pulse to $0$ mV following a $-130$ mV recovery.
pulse for durations between 0 and 4 s. Time constants of fast inactivation recovery showed two components and were fit using a double exponential equation:

\[ I = I_{ss} + \alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2) \quad (\text{Eq. 6}) \]

where \( I \) is current amplitude, \( I_{ss} \) is the plateau amplitude, \( \alpha_1 \) and \( \alpha_2 \) are the amplitudes at time 0 for time constants \( \tau_1 \) and \( \tau_2 \), and \( t \) is time.

### 4.3.14. Isothermal titration calorimetry

The peptide with the following sequence: SYIIISFLIVVNM (from Nav1.4 DIV-S6, residues 1580-1592) was synthesized by GenScript. It was solubilized in DMSO and diluted to a final concentration of 1 mM with the final buffer containing by percentage: 10% DMSO, 60% acetonitrile, 30% ITC buffer. Acetonitrile was required to solubilize the peptide. The ITC buffer contained 50 mM HEPES pH7.2 and 150 mM KCl. CBD and Lidocaine were each solubilized in DMSO and diluted to a final concentration of 40 mM and 100 mM, respectively in the same final buffer as the peptide. Each titrant was injected into the peptide containing sample cell 13 times each with a volume of 3 µM with the exception of the first injection which was 0.4 µM. Stirring speed was set at 750 rpm.

### 4.3.15. Action potential modeling

Skeletal AP modeling was based on a model developed by Cannon et al., (1993). All APs were programmed and run using Python. The modified parameters were based on electrophysiological results obtained from whole-cell patch-clamp experiments (Cannon et al., 1993). The model accounted for activation voltage-dependence, SSFI voltage-dependence, and persistent \( I_{Na} \). The WT pH7.4 model uses the original parameters from the model. P1158S models were programmed by shifting parameters from the original Cannon model by the difference between the values in P1158S experiments at a given pH/CBD (Cannon et al., 1993; Ghovanloo et al., 2018a).

### 4.3.16. Statistics

A one-factor analysis of variance (ANOVA) was used to compare the mean
responses. Post-hoc tests using the Tukey Kramer adjustment compared the mean responses between channel variants across conditions. A level of significance $\alpha=0.05$ was used in all overall post-hoc tests, and effects with p-values less than 0.05 were considered to be statistically significant. All values are reported as means ± standard error of means (SEM) for $n$ recordings/samples. Power analysis with $\alpha=0.05$ was performed to yield sufficient $n$ size for each experiment. Analysis was performed in JMP version 14.

4.4. Results

4.4.1. Molecular dynamics (MD) simulations predict CBD accumulates in the hydrophobic region of phospholipid bilayers

We previously determined that CBD non-selectively inhibits voltage-dependent sodium and potassium currents with a steep average Hill-slope of ~3, which suggested multiple interactions. Contrary to what is expected for classic pseudo–second order bimolecular blocking schemes, we found CBD was fastest to equilibrate and most potent at lower temperatures (Ghovanloo et al., 2018c). These findings, together with CBD’s stabilizing effects on neuronal Nav inactivation and CBD’s high LogP of ~5.9, led us to explore whether CBD alters membrane elasticity, which indirectly could inhibit Nav currents (Ghovanloo et al., 2018c), similar to what has been suggested for amphiphilic compounds (Lundbæk et al., 2004; Lundbæk, 2005). To test this hypothesis, we performed MD simulations of CBD (in mM concentration in membrane) on 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) lipid membranes in the hundreds of nanoseconds range (Figure 4-1A-E). MD results indicate that in both symmetrical (i.e. the same number of CBD molecules in both leaflets of the membrane) and asymmetrical (i.e. CBD in a single leaflet), there are no substantial changes in the area per lipid.

Figure 4-1C shows CBD density estimates as a function of membrane leaflet coordinate, where the lipid bilayer is centered at 0 (membrane thickness of ~4 nm is unchanged across conditions). These results show, in symmetrical conditions, that there are two density peaks in both negative and positive coordinate ranges with an almost perfect overlap, and that CBD localizes in the area between the lipid headgroups and the membrane center, close to the lipid headgroup region. In the asymmetric condition, with 3 CBD molecules initially placed in the leaflet to the right, there is only a single peak in the positive coordinate range. The MD results show that CBD molecules tend to reside in the
leaflet to which they originally were added, where they interact with, and detach rapidly from polar residues at the bilayer/solution interface and occasionally, move toward the water molecules outside the lipid. However, CBD then quickly moves back down into the lipid. This suggests, within the hundreds of nanoseconds timeframe of our simulations, that CBD does not diffuse across the two leaflets but, instead, tends to localize in the leaflet where it was initially placed. This could be a product of the oxygen atoms in CBD keeping it from diffusing across leaflets, and the CBD’s hydrophobic tail keeping it from getting too close to water molecules outside the membrane.
Figure 4-1  Effects of CBD on POPC membrane, via MD simulations and $^2$H NMR.
(A-B) The effects of CBD on POPC membrane area per lipid and lipid diffusion. System 0 is control, System 1 is 2 CBD molecules in symmetry (1 in each leaflet), System 2 is 3 CBD molecules in asymmetry (only in a single leaflet), System 3 is 6 CBD molecules in symmetry (3 in each leaflet) (at 300 K, 26.9 °C). (A) Area per lipid and (B) mean square displacement as a function of time are not affected by CBD. (C) Distribution of CBD into the membrane across a range of conditions. The distribution of phosphate groups is shown as solid lines, the distribution of CBD as dotted lines. The bilayer thickness remains ~4 nm in presence and absence of CBD. (D) Order parameter of lipid acyl chains estimated from the MD simulations. (E) Snapshot of a CBD molecule in the POPC leaflet extracted from the MD simulations. The zoomed-in image shows localization of CBD molecule below the leaflet headgroup. (F) NMR spectra collected at 20°C. (G) De-packed traces. (H) Order parameter calculation from NMR.

4.4.2. $^2$H NMR verifies the MD predictions regarding localization

Next, we performed acyl chain order parameter calculations (from MD) which suggested that CBD causes a slight ordering of the membrane methylenes in the plateau region of the palmitoyl chain (C3-C8) (Figure 4-1D). Overall, the MD results suggest that CBD preferentially localizes under the phosphate heads, close to carbons 3-7 of the aliphatic chains of the POPC molecules (Figure 4-1E).

We tested MD predictions regarding CBD localization using NMR(Lafleur et al., 1989) with POPC-d31 and POPC-d31/CBD at a 4:1 ratio in deuterium depleted water at three different temperatures (20, 30, and 40°C) (Figure 4-1F-H; Figure 4-2). The NMR results were in striking agreement with the MD predictions of the changes in acyl chain order parameters and suggested that CBD causes an ordering of the C2-C8 methylenes, and a slight disordering from C10-C15, in a temperature-dependent manner.

![Figure 4-2](image)

$^2$H NMR at different temperatures.

(A-C) Order parameters associated with POPC membranes at 20, 30, and 40 °C.
4.4.3. CBD alters bilayer elasticity in gramicidin-based fluorescence assay (GFA)

To explore CBD’s possible effects on lipid bilayer properties (membrane elasticity) that were predicted from the MD and NMR experiments, we tested CBD’s effects on lipid bilayer properties at concentrations where CBD has acute effects on Nav channels using a gramicidin-based fluorescence assay (GFA) which takes advantage of the gramicidin channels’ unique sensitivity to changes in bilayer properties (Andersen & Koepp, 2007). The GFA is based on the gramicidin channels’ permeability to Tl⁺, a quencher of the water-soluble fluorophore 8-aminonaphthalene-1,3,6-trisulfonate (ANTS), which can be encapsulated in large unilamellar vesicles (LUVs) that have been doped with gramicidin. The rate of Tl⁺ influx, the rate of fluorescence quench, is a measure of the time-averaged number of gA channels in the LUV membrane (Ingólfsson et al., 2010). Molecules that alter the thickness and elasticity of the LUV membrane will alter the lipid bilayer contribution to the free energy of dimerization, and thus the free energy of dimerization:

\[
\frac{[D]}{[M]^2} = K^{M\rightarrow D} = \exp \left\{ \frac{\Delta G_{\text{protein}}^{M\rightarrow D} + \Delta G_{\text{bilayer}}^{M\rightarrow D}}{k_B T} \right\},
\]

which will produce a shift in the gramicidin monomer\(\leftrightarrow\)dimer equilibrium (Figure 4-3A). Changes in this equilibrium will result in changes in the rate of Tl⁺ influx (fluorescence quench). CBD reduced the Tl⁺ influx rates in concentration-dependent manner (Figure 4-3). For comparison, we also show results obtained with Triton X-100, from (Ingólfsson et al., 2010), which increased the quench rates demonstrating that these two molecules have opposite effects on the membrane, and we conclude that CBD increases bilayer stiffness or thickness, whereas Triton X-100 decreases bilayer stiffness or thickness. Given that CBD has minimal effects in bilayer thickness (Figure 4-1), we conclude that CBD indeed alters lipid bilayer elasticity.
CBD alters lipid bilayer properties in gramicidin-based fluorescence assay (GFA).

(A) Cartoon representation of gramicidin monomers in each leaflet coming together (dimerizing) to form cationic channels. The dimerization of the gramicidin channels is directly related to membrane elasticity. These properties are used to assay compound (e.g. CBD) effects on membrane elasticity. (B) Fluorescence quench traces showing Ti⁺ quench of ANTS fluorescence in gramicidin-containing DC_{22:1}PC LUVs with no drug (control, black) and incubated with CBD for 10 min at the noted concentrations. The results for each drug represent 5 to 8 repeats (dots) and their averages (solid white lines). (C) Single repeats (dots) with stretched exponential fits (red solid lines). (D) Fluorescence quench rates determined from the stretched exponential fits at varying concentrations of CBD (red) and TX100 (purple, from (Ingólfssson et al., 2010)) normalized to quench rates in the absence of drug. Mean ± SD, n = 2 (for CBD).

4.4.4. CBD interacts with the Nav local-anesthetic site

We previously found that CBD displays an approximately 10-fold state-dependence in Nav inhibition, a property similar to classic pore-blockers (Bean et al., 1983; Kuo & Bean, 1994; Ghovanloo et al., 2018c), which has also been observed with bilayer-modifying molecules (Lundbæk, 2005). Therefore, we tested CBD inhibition from the inactivated-state in a Nav1.1 pore-mutant (F1763A–LA mutant) (Ghovanloo et al., 2018c) and the results suggested a relatively small ~2.5-fold drop in potency (Ghovanloo et al., 2018c). To further explore possible CBD interactions at the pore, we performed molecular docking using the human Nav1.4 cryo-EM structure (Pan et al., 2018). Figure 4-4A-B show CBD docked onto the Nav1.4 pore, supporting a possible interaction at the LA site.
To test the docking result, we mutated the LA Nav1.4 F1586 into alanine (A) and performed voltage-clamp. Figure 4-4C-F show biophysical characterization of F1586A compared with WT-Nav1.4. We found that both channels have similar biophysical properties and, most importantly, the inactivation voltage-dependences were almost identical (p>0.05) (Figure 4-4F), suggesting that at any given potential both F1586A and Nav1.4 would have the same availability; therefore, pharmacological experiments could be performed using the same voltage-protocols on both channels.

In contrast to neuronal Navs that have inactivation midpoints ($V_{1/2}$) of ~65 mV in neurons with resting membrane potentials (RMP) that are also ~65 mV, Nav1.4 has a $V_{1/2}$ of ~67 mV in skeletal muscle fibers with an RMP of ~90 mV. This indicates that, whereas neuronal Navs are half-inactivated at RMP, Nav1.4 is almost fully available at RMP. Therefore, we measured lidocaine (positive control) and CBD inhibition of Nav1.4 from rest (-110 mV holding-potential to 0 mV, test-pulse at 1 Hz) to be closer to physiological conditions (Figure 4-4G-H). Our results suggest that 1.1 mM (resting IC$_{50}$ on Nav1.4 (Nuss et al., 1995)) lidocaine blocks ~60% of $I_{Na}$ in WT, and ~20% in F1586A (p=0.020). 10 µM CBD blocks ~45% $I_{Na}$ in WT and ~25% in F1586A (p=0.037). Hence, there is a 3-fold difference between lidocaine’s inhibition of WT vs. F1586A, and a smaller 1.5-fold difference for CBD inhibition. This suggests that while CBD may interact with the Nav pore similar to lidocaine, CBD’s interaction at the pore is likely not as critical a determinant of its $I_{Na}$ inhibition compared with lidocaine.
Figure 4-4  Inhibition of Nav1.4 pore by CBD, F1586A reduces inhibition.
(A) Side-view of CBD docked into the pore of the human Nav1.4 structure. The structure is coloured by domain. DIV is coloured in deep blue. (B) Zoomed-in side-view, F1586 is coloured in yellow. (C-D) Representative families of macroscopic current traces from WT-Nav1.4 and F1586A. (E) Voltage-dependence of activation as normalized conductance plotted against membrane potential (Nav1.4: \( V_{1/2} = -19.9 \pm 2.7 \, \text{mV}, z = 2.8 \pm 0.3; \) F1586A: \( V_{1/2} = -22.4 \pm 2.2 \, \text{mV}, z = 3.0 \pm 0.3; n = 5-7 \)). (F) Voltage-dependence of SSFI as normalized current plotted against membrane potential (Nav1.4: \( V_{1/2} = -66.9 \pm 2.8 \, \text{mV}, z = -2.6 \pm 0.3; \) F1586A: \( V_{1/2} = -63.3 \pm 3.0 \, \text{mV}, z = -3.5 \pm 0.3; n = 8-9 \)). (G-H) Lidocaine/CBD inhibition of Nav1.4 and F1586A from -110 mV (rest) at 1 Hz (Lidocaine-Nav1.4: Mean block = 60.6 ± 2.3%; Lidocaine-F1586A: Mean block = 24.6 ± 9.3%; CBD-Nav1.4: Mean block = 42.4 ± 6.4%; CBD-F1586A: Mean block = 25.3 ± 4.8%; n = 3-5). Sample traces before and after compound perfusion are shown.

4.4.5. CBD interacts with DIV-S6

Because CBD’s \( I_{\text{Na}} \) inhibition was less dependent on interactions in the LA site than a well-established pore-blocker like lidocaine, we investigated whether CBD interacts with the DIV-S6 (which includes F1586) or if it is inert, using isothermal titration calorimetry. We then compared CBD interactions to lidocaine. We found that both lidocaine and CBD appear to interact with the protein segment, though the nature of this interaction seems to differ (Figure 4-5). Figures 4.5A-B show sample ITC heat traces. Our results suggested that in the presence of protein, lidocaine titration causes an endothermic interaction. However, when the protein is absent, lidocaine titration into blank buffer causes exothermicity. In contrast, CBD titration in both blank buffer and in the presence of protein resulted in endothermicity. Interestingly, the magnitude of CBD’s heats of interaction were ~4-fold larger in the protein condition compared to the blank condition. This was in contrast to lidocaine that showed comparable heats of interaction magnitudes in both conditions, but in different directions. To quantify interactions of both lidocaine and CBD, we subtracted the heats from runs with both protein and ligand subtracted from only ligand (blank). The subtracted heats show a similar trend between lidocaine and CBD (Figure 4-5C-D). These results suggest that both lidocaine and CBD interact with the protein segment; however, the nature of this interaction is different possibly due to a variation in physicochemical properties.
CBD interactions with DIV-S6, using isothermal titration calorimetry (ITC).

(A) Representative ITC traces for titration of 100 mM lidocaine into 1 mM peptide or blank buffer. The heat signal, once the binding is saturated is the same as the blank if the blank was only measuring the interaction if lidocaine with the solution. The blank measures 3 interactions, interactions between solute molecules, solute and lidocaine and lidocaine and lidocaine. As more lidocaine is added with each injection the solution is changed. This makes the heat of interaction with in-blank trace different from beginning to end. This change is due to a change in amount of lidocaine in the solution. Because this change is progressive with each injection and that the injections into the peptide are the same volume, subtraction was used as a means to quantify lidocaine peptide interaction. The purpose of this experiment was to visualize heats of interaction between the protein and ligand, not binding of protein with ligand, in which case protein configuration becomes important. (B) Representative ITC traces for titration of 40 mM CBD into 1 mM peptide or blank buffer. (C) The blank condition subtracted heat of titration in protein condition is shown for lidocaine, and (D) CBD. A peak heat of 968.0 ± 23.4 kcal*mol⁻¹ was seen for lidocaine titration and a peak heat of 1022.2 ± 160.6 kcal*mol⁻¹ was seen for the CBD titration (n = 3-4).

4.4.6. CBD may penetrate into the pore through fenestrations

LAbs block bacterial Navs in their resting-state by entering the pore through fenestrations in a size-dependent manner (i.e. smaller LAbs get through more readily) (Gamal El-Din et al., 2018). Here, we sought to determine whether it is possible to block CBD’s access to the human Nav1.4 pore from the lipid phase of the membrane by occluding fenestrations. We previously found that CBD is highly lipid-bound (99.6%) (Ghovanloo et al., 2018c), and our MD results show that it preferentially localized in the hydrophobic part of the membrane, just below the lipid headgroups. Therefore, we
reasoned that once CBD partitions into the membrane, it will have access to the Nav pore through the intramembrane fenestrations. To test this idea, we scrutinized the docking pose of CBD in the human Nav1.4 and observed its localization close to the fenestrations (Figure 4-6A; Figure 4-7A-D).

Next, we identified 4 residues (DI-F432, DII-V787, DIII-I1280, and DIV-I1583) that partially or fully occluded the fenestrations when mutated to tryptophan (W), as predicted by computational mutagenesis and structural minimization (partial versus full occlusion is due to structural asymmetry of mammalian Navs) (Figure 4-6B-C).

We measured resting-state block of 1.1 mM lidocaine, 350 µM flecainide, and 10 µM CBD from -110 mV on our WWWW construct. Our results suggest that lidocaine (p>0.05) and flecainide (p>0.05), but not CBD (p<0.05) blocked the WWWW mutant the same as WT (after compound has reached equilibrium) (Figure 4-6D). This is an interesting result considering that CBD is larger than lidocaine, but slightly smaller than flecainide. CBD was inert with respect to block of WWWW, relative to WT-Nav1.4, which suggests that CBD interacts with Nav1.4 via the fenestrations.

To visualize the possible pathway CBD follows through Nav1.4 fenestrations and into the pore at an atomistic resolution, we performed MD simulations in which we encouraged CBD to detach from its binding-site (see Methods, Figure 4-6E-G; Figure 4-7E). These results demonstrate that CBD can enter its binding-site in the pore through the fenestration without major reorganization of the channel structure.
CBD interactions with and through Nav fenestrations.

Figure 4-6

**CBD Docks Inside Fenestrations**

**Full Occlusion**

**Partial Occlusion**

**CBD Pathway Through Fenestration – Side View**

**CBD Pathway Through Fenestration – Top View**

**Domain II**

**Domain III**

**Domain IV**

**CBD Path**

**Domain I**

**Figure 4-6**

CBD interactions with and through Nav fenestrations.
(A) Side-view of CBD docked into the human Nav1.4 structure. The structure is coloured by domain (matched color to domain is shown in panel (F)), CBD is represented in purple. (B) Side-view of all four sides of human Nav1.4 (coloured by domain). Nav1.4 fenestrations are highlighted in red, along with the position of respective residues that were mutated into tryptophans (W). (C) Computational mutagenesis of fenestrations results 2 full and 2 partial occlusions (paralleled domains). (D) Lidocaine (1.1 mM) inhibition of Nav1.4 and WWW from -110 mV (rest) at 1 Hz (Nav1.4: Mean block = 60.6 ± 2.3%; WWW: Mean block = 53.6 ± 11.7%), flecainide (350 µM) inhibition (Nav1.4: Mean block = 64.6 ± 6.0%; WWW: Mean block = 76.4 ± 11.3%), and CBD (10 µM) inhibition (Nav1.4: Mean block = 42.4 ± 6.4%; WWW: Mean block = 6.4 ± 1.3%; n = 3-5 panel-wide). Traces before and after compound perfusion are shown. (E) CBD pathway through the Nav1.4 fenestration from side view, as predicted by MD simulations, red and blue correlate to CBD being inside and outside the fenestration, respectively. (F) CBD pathway from top view of the channel. (G) Progressive snapshots of the movement of CBD over time from inside to outside the channel.
CBD in hNav1.4 (PDB ID – 6AGF) in the best position energy wise (-7.9 kcal/mol)

CBD in hNav1.4 (PDB ID – 6AGF) in the second and third best position energy wise (-7.8 kcal/mol)

Figure 4-7  Nav1.4 fenestration interactions with CBD.
(A-D) Shows CBD posed in the human Nav1.4 structure using molecular docking. (E) RMSD of the fenestration residues as a function of time in the absence (black) and the presence of CBD passing through the fenestration (red and green, two different simulation parameter sets). The similar RMSD profiles show that CBD’s passage does not distort the structural integrity of the fenestration.

4.4.7. CBD does not affect Nav1.4 activation but stabilizes the inactivated state

We previously characterized the effects of CBD on Nav1.1 gating (Ghovanloo et al., 2018c). We found that CBD at ~IC₅₀ reduced channel conductance, did not change the voltage-dependence of activation, but produced a hyperpolarizing shift in steady-state fast inactivation (SSFI), and slowed recovery from fast (300 ms) and slow (10 s) inactivation (Ghovanloo et al., 2018c). Together with CBD’s inhibition of resurgent sodium currents (Patel et al., 2016; Ghovanloo et al., 2018c), these results suggested that CBD prevents the opening of Navs, but the channels that can open, activate with unchanged voltage-dependence and are more likely to inactivate. The overall effect is a reduction in excitability (Ghovanloo et al., 2018c). Here, we hypothesized that CBD’s non-selectivity in I₅Na inhibition suggests non-selectivity in modulating Nav gating (i.e. CBD imparts similar gating modulation across Nav subtypes). To test this idea, we assessed Nav1.4 activation in presence and absence of 1 µM CBD by measuring peak channel conductance at membrane potentials between −100 and +80 mV (Figure 4-8A). CBD did not significantly alter V₁/₂ or apparent valence (z) of activation (p>0.05). Normalized Nav1.4 currents as function of membrane potential are shown in Figure 4-8B. These results indicate that, as with Nav1.1, CBD does not alter Nav1.4 activation.

Next, we examined the voltage-dependence of SSFI using a standard 200 ms pre-pulse voltage protocol. Normalized current amplitudes were plotted as a function of pre-pulse voltage (Figure 4-8C). These results mimicked our previous observations in Nav1.1 (Ghovanloo et al., 2018c), in that CBD left-shifted the Nav1.4 inactivation curve (p<0.05).

To measure recovery from inactivation, we held Nav1.4 at -130 mV to ensure that the channels were fully available, then pulsed the channels to 0 mV for 500 ms and allowed different time intervals at -130 mV to measure recovery as a function of time. As previously observed in Nav1.1, CBD slowed the Nav1.4 recovery from inactivation (p<0.05), suggesting that it takes longer for CBD to come off the channels than the time it takes the channels to recover from inactivation (Figure 4-8D). Collectively, these results
support our hypothesis that CBD non-selectively modulates Nav gating, and further suggests how CBD may reduce Nav1.4 excitability.

**Figure 4-8**  Effects of CBD (1 μM) on Nav1.4 gating.

(A-B) Voltage-dependence of activation as normalized conductance plotted against membrane potential (Control: $V_{1/2} = -19.9 \pm 4.2 \text{ mV}$, $z = 2.8 \pm 0.3$; CBD: $V_{1/2} = -14.3 \pm 4.2 \text{ mV}$, $z = 2.8 \pm 0.3$; $n = 5$) and normalized activating currents as a function of potential. (C) Voltage-dependence of SSFI plotted against membrane potential (Control: $V_{1/2} = -64.1 \pm 2.4 \text{ mV}$, $z = -2.7 \pm 0.3$; CBD: $V_{1/2} = -72.7 \pm 3.0 \text{ mV}$, $z = -2.8 \pm 0.4$; $n = 5-8$). (D) Recovery from fast inactivation at 500 ms (Control: $\tau_{\text{fast}} = 0.0025 \pm 0.00069 \text{ s}$, $\tau_{\text{slow}} = 0.224 \pm 0.046 \text{ s}$; CBD: $\tau_{\text{fast}} = 0.0048 \pm 0.00081 \text{ s}$, $\tau_{\text{slow}} = 0.677 \pm 0.054 \text{ s}$; $n = 5-7$).

**4.4.8. CBD hyperpolarizes SSFI in Nav1.4-WWWW**

To determine a possible association between membrane elasticity and stabilized inactivation, we measured effects of CBD before and after compound perfusion in the WWWWWW mutant, in a matched-pair manner. Although CBD did not inhibit peak $I_{\text{Na}}$, it hyperpolarized the SSFI curve ($p<0.05$), suggesting CBD’s modulation of membrane elasticity is at least in part responsible for stabilizing Nav inactivation (**Figure 4-9**). This is an interesting finding because our GFA results suggest that CBD increases bilayer stiffness or thickness, and previous studies suggest that compounds such as Triton X-100 that reduce this stiffness or thickness also hyperpolarize the Nav SSFI curve (Lundbæk *et al.*, 2004).
Figure 4-9  CBD stabilizes inactivation in the fenestration-occluded construct.

(A-B) Show voltage-dependence of SSFI before and after (A) control (extracellular (ECS) solution) and (B) CBD (10 µM) in WWWW construct (Before control: V_{1/2} = -54.7 ± 5.1 mV, After control: V_{1/2} = -54.2 ± 5.4 mV, Before CBD: V_{1/2} = -48.8 ± 8.8 mV, After CBD: V_{1/2} = -72.7 ± 5.7 mV, n = 3-6). The ECS experiment was performed to ensure that hyperpolarization shifts in the CBD condition are not due to possible confounding effects associated with fluoride in the internal (CsF) solutions. (C-D) Show representative families of inactivating currents before and after perfusion. CBD does not block peak currents but shifts the SSFI curve to the left. (D) Show averaged shift in the midpoint of SSFI before and after perfusion.

4.4.9. CBD effects on a pH-sensitive mixed myotonia/hypoPP Nav1.4-mutant, P1158S (DIII-S4-S5)

Because CBD is therapeutic against seizure disorders (Devinsky et al., 2017), typically considered neuronal GOF conditions, we examined whether CBD may similarly ameliorate a skeletal muscle GOF condition (Cannon, 2015). We recently discovered that the P1158S mutation in Nav1.4 increases the channel’s pH-sensitivity (Ghovanloo et al., 2018a). The P1158S gating displays pH-dependent shifts that, using AP modeling, are predicted to correlate with the phenotypes associated with this variant. Therefore, the relationship between pH and P1158S could be used as an in-vitro/in-silico assay of Nav1.4 hyperexcitability (to model moderate to severe GOF). Here, we used this assay to investigate CBD’s effects on skeletal muscle hyperexcitability. We tested effects of 1 µM CBD (pK_a=9.64) on P1158S at pH6.4 (myotonia-triggering) and pH7.4 (hypoPP-triggering). Figure 4-10 shows CBD effects on P1158S at low and high pH. Interestingly,
the lack of selectivity in CBD gating modulation by CBD also exists in P1158S at both pHs. CBD did not change activation (p>0.05), but hyperpolarized inactivation (p<0.05) and slowed recovery from inactivation (p<0.05) (Figure 4-10A-F). Consistent with previous results where CBD inhibited persistent $I_{\text{Na}}$ (Patel et al., 2016; Ghovanloo et al., 2018c), CBD also reduced the exacerbated persistent $I_{\text{Na}}$ associated with P1158S at pH7.4 (p<0.05) (Figure 4-10G). Persistent $I_{\text{Na}}$ reduction could not be detected at pH6.4 (p>0.05) (Figure 4-10H) because both low pH (Peters et al., 2018; Ghovanloo et al., 2018a, 2018b) and CBD reduce current amplitudes to levels where differences in amplitudes could not be resolved above background noise.
Figure 4-10 Effects of CBD (1 µM) on the gating of a mixed mutant, P1158S, in pH7.4 and 6.4.

(A-B) Voltage-dependence of activation as normalized conductance plotted against membrane potential, at pH7.4 (Control: \(V_{1/2} = -30.0 \pm 3.3\) mV, \(z = 3.1 \pm 0.2\); CBD: \(V_{1/2} = -32.7 \pm 3.6\) mV, \(z = 2.9 \pm 0.2\); \(n = 7-8\)) and pH6.4 (Control: \(V_{1/2} = -23.0 \pm 3.3\) mV, \(z = 2.9 \pm 0.2\); CBD: \(V_{1/2} = -21.1 \pm 3.3\) mV, \(z = 2.5 \pm 0.2\); \(n = 8\)). (C-D) Voltage-dependence of SSFI plotted against membrane potential at pH7.4 (Control: \(V_{1/2} = -73.2 \pm 2.6\) mV, \(z = 2.9 \pm 0.2\); CBD: \(V_{1/2} = -83.0 \pm 2.6\) mV, \(z = 3.0 \pm 0.3\); \(n = 7\)) and pH6.4 (Control: \(V_{1/2} = -68.4 \pm 3.0\) mV, \(z = 2.7 \pm 0.4\); CBD: \(V_{1/2} = -81.7 \pm 2.3\) mV, \(z = 2.7 \pm 0.3\); \(n = 8\)). (E-F) Recovery from fast-inactivation at 500 ms at pH7.3 (Control: \(\tau_{\text{Fast}} = 0.0018 \pm 0.006\) s, \(\tau_{\text{Slow}} = 0.15 \pm 0.6\) s; CBD: \(\tau_{\text{Fast}} = 0.24 \pm 0.07\) s; \(\tau_{\text{Slow}} = 2.5 \pm 0.6\) s; \(n = 6-7\)) and pH6.4 (Control: \(\tau_{\text{Fast}} = 0.065 \pm 0.04\) s, \(\tau_{\text{Slow}} = 0.75 \pm 0.4\) s; CBD: \(\tau_{\text{Fast}} = 0.13 \pm 0.07\) s; \(\tau_{\text{Slow}} = 0.62 \pm 0.1\) s; \(n = 4-7\)). (G-H) Persistent currents measured from a 200 ms depolarizing pulse to 0 mV from a holding potential of \(-130\) mV at pH7.4 (Control: Percentage = 4.4 ± 1.2%; CBD: Percentage = 1.0 ± 0.2%; \(n = 4\)) and pH6.4 (Control: Percentage = 4.4 ± 2.1%; CBD: Percentage = 5.4 ± 1.2%; \(n = 5-6\)).

4.4.10. AP model predicts CBD reduces myotonia, but not hypoPP in the P1158S-pH assay

We modeled the gating changes from the patch-clamp experiments with WT and P1158S (both control and CBD) into the Cannon AP model (Cannon et al., 1993). We ran the simulations using a 50 µA/cm² stimulus. The simulation pulse started at 50 ms and stopped at 350 ms (Figure 4-11). During this pulse, the WT channels activated at 50 ms and fired a single AP. The channels remained inactivated until the stimulus was removed at 350 ms, and then the potential recovered back to RMP (Figure 4-11A). As expected, CBD reduced the AP amplitude (Figure 4-11B), similar to CBD effects observed in different neuron types (Khan et al., 2018; Ghovanloo et al., 2018c). At pH6.4, P1158S displayed a continuous train of APs for the entire stimulation period. After the stimulus was removed, P1158S showed a progressive series of after-depolarizations of the membrane potential, characteristic of a myotonic burst (Figure 4-11C) (Cannon, 2015). Interestingly, the CBD-mediated shifts at pH6.4 in P1158S reduced the simulated AP amplitudes for the entirety of the pulse duration (and delayed onset of first AP, consistent with CBD preventing Nav opening) and abolished the post-pulse myotonic after-depolarizations (Figure 4-11D). At pH7.4, P1158S fired a single AP, followed by a period where membrane potential remained depolarized around -35 mV, even post-stimulus termination (Figure 4-11E). This inability to repolarize holds the Nav channels in an inactivated state, and is consistent with the periodic paralysis phenotype (Cannon, 2015). In contrast to the myotonic phenotype, CBD did not alleviate the hypoPP phenotype in our P1158S-pH in-vitro/in-silico assay (Figure 4-11F), consistent with its slowing of recovery from fast inactivation.
Pulse Protocol

300 ms

P1158S-pH – Skeletal Muscle Hyperexcitability Assay

Paralytic

Myotonic

Nav1.4 - pH 7.4 - Control

Nav1.4 - pH 7.4 - CBD

P1158S - pH 6.4 - Control

P1158S - pH 6.4 - CBD

P1158S - pH 7.4 - Control

P1158S - pH 7.4 - CBD
4.4.11. CBD reduces rat diaphragm muscle contraction

To survey and determine whether CBD reduces skeletal muscle contractions, we surgically removed rat diaphragm muscles and measured muscle contractions evoked by phrenic nerve stimulation. In Figure 4-12A-B, we show images of the diaphragm, cut into a hemi-diaphragm. We used electrodes to stimulate the phrenic nerve and measured the muscle contraction using a force transducer, at a saturating concentration of 100 µM of CBD, reasoning that if CBD reduces muscle contraction, a saturating concentration should provide a large enough response to detect any potential reduction in contraction. Our results suggested that CBD reduces the contraction amplitude to ~60% of control (p<0.05) (Figure 4-12C). Next, we sought to determine whether a selective block of Nav channels also reduces skeletal muscle contraction using 300 nM tetrodotoxin (TTX), a saturating concentration of this potent blocker of selected Nav channels (IC₅₀ ~10-30 nM on TTX-sensitive channels (Hille, 2001)). TTX also reduced contraction to ~20% of control (p<0.05) (Figure 4-12C). The remaining ~20% contraction could be due to stimulation of voltage-gated calcium channels in transverse membranes that directly interact with ryanodine-sensitive calcium release in the SR that can initiate contraction (Catterall, 1991, 2011; TANABE et al., 1993). Representative traces of muscle contraction in control, CBD, and TTX are shown in Figure 4-12D-F. These results show that a selective inhibition of Nav reduces skeletal muscle contraction, suggesting that CBD’s reduction of muscular contraction could be due, at least in part, to its effect on Nav (Ghovanloo et al., 2018c). Therefore, our molecular in-vitro and in-silico data could have some physiological relevance.
Figure 4-12  Effects of CBD on rat diaphragm contraction.

(A) Image of dissected rat diaphragm muscle. (B) Image of rat diaphragm cut into a hemi-diaphragm, which was placed between electric plates that were used for electric stimulation. The subsequent muscle contractions were measured using a force transducer. (C) Quantification of normalized of muscle contractions in CBD and TTX (Percentage of normalized contraction: Control = 100 ± 5.3%; CBD = 60.6 ± 3.5%; TTX = 28.9 ± 5.3%; n = 6-9). (D-F) Sample contraction traces across all three conditions.

4.5. Discussion

4.5.1. Pathway and mechanism of Nav1.4 inhibition by CBD

Although CBD holds therapeutic promise (Ross et al., 2008; Patel et al., 2016; Devinsky et al., 2017; Kaplan et al., 2017; Ghovanloo et al., 2018c; Pumroy et al., 2019; Fouda et al., 2020) and has been approved for two seizure disorders, its mechanisms of action remain largely unknown. We previously described the effects of CBD on Nav, which are among its proposed targets (Ghovanloo et al., 2018c). CBD’s effects on neuronal Nav resemble the properties described for both amphiphilic compounds and traditional pore-blockers. That study provided foundational hypotheses about CBD’s mechanism of action on Nav. Here, we tested those ideas using a combination of ex-vivo, in-vitro, and in-silico techniques.

Amphiphiles, those molecules possessing both lipophilic and hydrophilic properties, often display non-selective modulatory effects on seemingly unrelated targets
(Lundbæk et al., 2004; Lundbæk, 2005; Kapoor et al., 2019). The apparent diversity of targets is a by-product of amphiphiles modulating membrane elasticity (Lundbæk et al., 2004; Lundbæk, 2005; Kapoor et al., 2019). This modification is achieved by amphiphiles localizing at the solution–bilayer interface, which is made possible by having the compound’s polar group residing at the interface with the hydrophobic region, which then gets inserted into the bilayer core. This partitioning into the lipid bilayer alters membrane elasticity, and changes phase preference and curvature (Lundbæk et al., 2004; Lundbæk, 2005; Kapoor et al., 2019). The net effect of these alterations to the membrane for the bilayer-embedded Nav channel is a stabilized inactivated state (Lundbæk et al., 2004; Lundbæk, 2005; Kapoor et al., 2019).

We used MD simulations to ‘visualize’ CBD localization and its effects on membrane elasticity. Interestingly, the MD prediction regarding localization, independently confirmed by NMR measurements of CBD in lipid vesicles, suggested CBD positioning between C8-C10. Our gramicidin-based functional assay suggested that CBD slightly changes membrane elasticity. This result is consistent with our previous findings, including CBD’s temperature-dependence (CBD effects were enhanced at lower temperatures), stabilized Nav inactivation, and non-selectivity (Ghovanloo et al., 2018c). Together, these findings suggest that CBD inhibition of Nav currents (and possibly other ionic currents) is, at least in part, mediated through changing lipid bilayer elasticity.

In this study, we further found that CBD had the opposite effect to Triton X-100 in GFA. Also, the magnitude change of quench rate was different between the two compounds at a given concentration; however, both compounds similarly hyperpolarized the Nav inactivation. These findings suggest that there could be at least two, maybe three mechanisms involved. The exact mechanisms through which CBD’s presence alter the lipid/Nav interactions should be further investigated in future studies.

The modulated receptor hypothesis suggests that resting-state block occurs when a compound enters from the lipid phase of the membrane into the LA binding site, whereas rapid open-state block happens when a compound enters the open pore from the cytosol (Hille, 1977a; Hondeghem & Katzung, 1984). Pore-blockers can reach their binding site from the cytosolic side when the activation gate is open. A recent study showed that compounds can have direct access from the membrane phase to the LA site through channel fenestrations, culminating in resting-state block (Gamal El-Din et al., 2018).
We previously found that some characteristics of CBD inhibition of Nav are similar to classic pore-blockers (Ghovanloo et al., 2018c; Fouda et al., 2020). Here, we tested CBD interactions inside the Nav1.4 LA site at rest. Destabilizing the LA site by the F1586A mutation, reduced CBD block of Nav1.4. This result is particularly notable since the LA site becomes a more favourable interaction site when the channel adopts a more inactivated state (a key reason for LAs’ strong state-dependence) (Ghovanloo & Ruben, 2020). Therefore, CBD’s reduced block in F1586A at rest could support the idea that CBD interacts with Nav at the pore. However, this does not indicate that the pore is the primary determinant of CBD inhibition, especially when CBD is compared to a traditional blocker like lidocaine, which is more affected by the F1586A mutation.

Next, we reasoned that, if CBD blocks the pore, a likely path to reach the pore from the lipid phase would be through the Nav fenestrations (based on MD results, high LogP, and high lipid binding partitioning). We found that our fenestration-occluded Nav1.4-WWWW construct abolished resting-state block by CBD but not lidocaine or flecainide. This could be a consequence of the differences in hydrophobicity (and size/shape) between these three compounds. Because CBD is several orders of magnitude more hydrophobic than either lidocaine or flecainide, it may preferentially reach the pore through the fenestrations, whereas lidocaine and flecainide can reach the pore also from the cytoplasmic phase even if access through the fenestrations is blocked. This interpretation is consistent with the modulated receptor hypothesis (Figure 4-13).

Finally, we determined that, while CBD’s $I_{Na}$ block occurs through its interactions inside the Nav1.4 pore, its stabilization of inactivation at least in part arises from modulating membrane elasticity. Both mechanisms contribute to CBD’s overall inhibition of Nav currents.
Figure 4-13 Comparison between some of the relevant physicochemical properties of the compounds used in this study.

(A) Chemical structures of the compounds used in this study. (B) Three dimensional structures of the compounds. (C) Volume (Å³) and area (Å²) for each compound was calculated using UCSF Chimera. LogP values are obtained from ChEMBL database. CBD, lidocaine, and flecainide all interact with the LA site inside the Nav pore. TTX interacts with the outer selectivity filter of the Nav pore. CBD is several times more hydrophobic than the other compounds. CBD is larger than lidocaine and slightly smaller than flecainide.

4.5.2. Possible applications for CBD for skeletal muscle

Skeletal muscle hyperexcitability disorders have historically received less attention than disorders in other tissues, including the brain. Drugs most commonly used for myotonia include compounds developed for other conditions, such as anti-convulsants and anti-arrhythmics (Alfonsi et al., 2007; Trip et al., 2008), which may cause unwanted, off-target side-effects. Hence, another therapeutic approach has been lifestyle modifications. For instance, myotonic patients may modify their lifestyles to avoid triggers like potassium ingestion or cold temperatures. Treatment of hypoPP is usually achieved using oral potassium ingestion and by avoiding dietary carbohydrates and sodium. During hypokalemia, increasing K⁺ levels may reduce membrane depolarization and shift the resting potential to more negative potentials. Acetazolamide or dichlorphenamide may be useful; however, these compounds can exacerbate symptoms (Torres et al., 1981; Tawil et al., 2000; Sternberg et al., 2001; Venance et al., 2004). There is a need for new treatments for these conditions.
Cannabinoids have long been used to alleviate muscular problems (Baker et al., 2000; Borgelt et al., 2013). In this study, we show CBD reduces skeletal contraction in rat diaphragm muscle. As CBD is a poly-pharmacology compound, we cannot state with certainty that the observed contraction reduction is due to $I_{\text{Na}}$ inhibition alone, but as demonstrated with the TTX results, $I_{\text{Na}}$ block is sufficient to reduce contraction, meaning that CBD’s activity at Nav1.4 could be a part of the mechanism in this reduction. Another caveat is that we cannot exclude the possibility of a phrenic nerve independent (i.e. direct muscle) stimulation resulting in muscle contraction in our myography experiments. The overall mechanism suggested by our results is summarized in Figure 4-14.

To explore a possible use for CBD in myotonia and hypoPP, we tested it in an in-vitro/in-silico assay. Our results suggest that CBD may alleviate the myotonic but not the hypoPP phenotype. One caveat is that these predictions are based in part on computer simulations. However, from a theoretical perspective, most Nav1.4 mutations that cause myotonia do so by changing conventional channel gating (e.g. activation, inactivation, persistent currents); hypoPP mutants are due to pathogenic gating pore currents associated with the VSD, so it is conceivable for a compound like CBD to alleviate myotonic behavior, but not hypoPP.

In conclusion, our results suggest that CBD inhibition of Nav has at least two components: altered membrane elasticity and pore block. Nav1.4 inhibition could contribute to CBD reducing skeletal muscle contractions and may have potential therapeutic value against myotonia (Figure 4-14). From a broader perspective, our proposed mechanism may hold true for other compounds that are similar to CBD in modulating Navs or other channels with similar structures.
Figure 4-14  Pathway of skeletal muscle inhibition via Nav1.4.

This is a cartoon representation of the mechanism and pathway through which CBD inhibits Nav1.4. Once CBD is exposed to the skeletal muscle, given its high lipophilicity, the majority of it gets inside the sarcolemma. Upon entering the sarcolemma, it localizes in the middle regions of the leaflet, and travels through the Nav1.4 fenestrations into the pore. Inside the pore mutation of the LA F1586A reduces CBD inhibition. CBD also alters the membrane elasticity, which promotes the inactivated state of the Na\(+\) channel, which adds to the overall CBD inhibitory effects. The net result is a reduced electrical excitability of the skeletal muscle, which - at least in part - contributes to a reduction in muscle contraction.

4.6. Acknowledgements

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Mohammad-Reza Ghovanloo assembled data, performed patch-clamp experiments, assisted in ITC experiments, action potential modeling/simulations, functional assay development, data analysis, figure making, wrote manuscript, data interpretation, and assisted to conceiving of experiments. Koushik Choudhury and Tagore S. Bandaru performed MD simulations and docking. Dr. Mohamed A. Fouda performed myography. Kaveh Rayani performed ITC, assisted in mutagenesis, and various experimental conceptualizations. Dr. Radda Rusinova performed GFA. Tejas Phaterpekar performed NMR. Karen Nelkenbrecher performed diaphragm preparation. Abeline R. Watkins helped with NMR. Dr. Damon Poburko assisted with myography. Drs. Jenifer Thewalt, Olaf S. Andersen, Lucie Delemotte, Samuel J. Goodchild, and Peter C. Ruben conceived the experiments and revised the manuscript critically.

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Chapter 5. Cannabidiol interactions with voltage-gated sodium channels

This chapter describes work submitted for publication. A purely structural version of this work is posted as a pre-print (Sait et al., 2020). My contributions to this work were added after the pre-print posting. This chapter presents a modified version of this study, with a focus on key structural findings and my own electrophysiological contributions.

5.1. Abstract

Voltage-gated sodium (Nav) channels are targets for a range of pharmaceutical drugs developed for treatment of various conditions. Cannabidiol (CBD), the non-psychoactive compound isolated from cannabis plants, was recently approved for treatment of two types of epilepsy associated with sodium channel mutations. In this study, we first used high resolution X-ray crystallography to demonstrate the detailed nature of the interactions of CBD inside the bacterial NavMs. Our results show CBD binds at a novel site at the interface of the fenestrations and the central hydrophobic cavity of the channel. Binding at this site blocks the transmembrane-spanning sodium ion translocation pathway, providing a molecular mechanism for channel inhibition. Next, we performed electrophysiological studies on NavMs that show CBD blocks this channel, similar to other Nav channels tested previously. In summary, this study provides new insight into a possible mechanism for CBD with Nav channels.

5.2. Introduction

Nav channels are responsible for the upstroke of action potentials in excitable tissues. The members of the human Nav channels are predominantly expressed in a tissue-specific manner and share many functional characteristics. Mutations in these channels are responsible for a variety of different conditions (Catterall et al., 2005; Catterall, 2014).

CBD is a highly hydrophobic compound that is recently shown to be a Nav inhibitor. Furthermore, in addition to Nav channels, CBD is suggested to target a variety of different targets (Ross et al., 2008; Patel et al., 2016; Kaplan et al., 2017; Ghovanloo et al., 2018c;
Fouda et al., 2020). We previously characterized the effects of CBD on human Nav channels (Ghovanloo et al., 2018c). Our findings in that study suggested that one of the CBD pathways of Nav inhibition is via interactions inside the Nav pore. We also recently showed that CBD’s mechanism of Nav inhibition has at least two components, lipid membrane modulation and direct pore block. The Nav subtype that we described these mechanisms in was the skeletal muscle variant, Nav1.4. In that study, we used extensive functional and simulations to show CBD movement through Nav1.4 fenestrations and into the pore (Ghovanloo et al., 2020b).

In the present study, we sought to structurally show CBD interactions inside the Nav pore. This was to gain additional insights into CBD’s binding pose inside the pore. To do this, we performed X-ray crystallography of the prokaryotic NavMs with CBD. We provide a crystal structure of CBD-NavMs (Bagnéris et al., 2014; Naylor et al., 2016; Sula & Wallace, 2017) at 2.25 Å. The high-resolution crystallography provides a means of understanding the CBD interactions with Nav channels.

5.3. Methods

5.3.1. Protein expression and purification

The NavMs (Uniprot ID: A0L5S6) and NavMs proteins were expressed and purified as previously described (Sula & Wallace, 2017), with the following modifications: the bound protein was eluted in a buffer containing 20 mM Tris, pH 7.5, 300 mM NaCl, 0.5 M imidazole and 0.52% Hega10. The Histag was removed by thrombin cleavage overnight at 4 °C. The protein sample was loaded onto a Superdex 200 column and eluted with 20 mM Tris, pH 7.5, 300 mM NaCl, and 0.52% Hega10 buffer. Protein samples were pooled and concentrated to 10 mg/ml using a 100 kDa cut-off Amicon concentrator and stored at a concentration of 10 mg/ml at -80 °C.

5.3.2. Crystallisation, data collection, and structure determination

1 μl of CBD (100 mM) in 100% DMSO was added to 50 μl of the purified protein solution to produce a final protein concentration of ~10 mg/ml containing 2 mM CBD and 2.5% v/v DMSO. The best crystals were grown at 4 °C via the sitting drop vapour diffusion method using a 2:1 ratio of the protein and reservoir solutions containing 0.1 M lithium
sulphate, 0.1 M HEPES, pH 7, and 40% v/v PEG200. The apo NavMs crystals were grown under the same condition as the crystals of the CBD complex, but without the DMSO and drug. Crystals were flash-frozen, with the PEG200 acting as the cryo-protectant.

Data were collected on beamline P13 at the Electron Synchrotron (DESY, Germany); on beamline Proxima1 at the Soleil Synchrotron (France), and on beamlines IO3, IO4, and I24 at the Diamond Light Source (UK). Hundreds of crystals were screened, and full data sets were collected from more than 40 crystals. Diffraction images were integrated and scaled using XDS (Kabsch et al., 2010) and then merged with Aimless (Evans & Murshudov, 2013) using the CCP4 suite of programmes (Winn et al., 2011). The structure was determined from the crystals which diffracted to the highest resolution (2.20 Å for the apo protein, and 2.25 Å for the CBD complex). Because of the small but significant variations in the unit cell dimensions and resolution between different crystals of the same type produced under the same conditions, as we have seen previously (Naylor et al., 2016; Sula & Wallace, 2017), datasets from different crystals were not merged.

The structure determinations by molecular replacement were as previously described using Phaser with the full-length wildtype NavMs structure (PDB 5HVX) as the search model (McCoy et al., 2007; Sula & Wallace, 2017). Model building was carried out using Coot (Emsley et al., 2010). Refinement was done using REFMACS. The structure quality was checked using PROCHECK and MolProbity, which indicated that 99.2% of the residues were in allowed conformations. Figures were created in CCP4mg, unless otherwise noted (Laskowski et al., 1993; Chen et al., 2010; McNicholas et al., 2011).

5.3.3. Electrophysiology compound preparation

Powdered CBD (Toronto Research Chemicals) was dissolved in 100% DMSO to create stock. The stock was used to prepare drug solutions in extracellular solutions at different concentrations with no more than 0.5% total DMSO content.

5.3.4. Cell culture

Chinese Hamster Ovary (CHOK1) cells were transiently co-transfected with cDNA encoding eGFP, the β1-subunit, and NavMs α-subunit (https://www.addgene.org/100004/). Transfection was done according to the PolyFect
transfection protocol. After each set of transfections, a minimum of 8-hour incubation was allowed before plating on sterile coverslips. All cells were incubated at 37 °C/5% CO₂.

5.3.5. Electrophysiology

Whole-cell patch-clamp recordings were performed in an extracellular solution containing (in mM): 140 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES (pH 7.4). Solutions were adjusted to pH 7.4 with CsOH. Pipettes were filled with intracellular solution, containing (in mM): 120 CsF, 20 CsCl, 10 NaCl, 10 HEPES. All recordings were made using an EPC-9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) digitized at 20 kHz via an ITC-16 interface (Instrutech, Great Neck, NY, USA). Voltage-clamping and data acquisition were controlled using PatchMaster software (HEKA Elektronik, Lambrecht, Germany) running on an Apple iMac. Current was low-pass-filtered at 10 kHz. Leak subtraction was performed automatically by software using a P/N procedure following the test pulse. Gigaohm seals were allowed to stabilize in the on-cell configuration for 1 min prior to establishing the whole-cell configuration. Series resistance was less than 5 MΩ for all recordings. Series resistance compensation up to 80% was used when necessary. All data were acquired at least 1 min after attaining the whole-cell configuration. Before each protocol, the membrane potential was hyperpolarized to −180 mV to ensure complete removal of inactivation. All experiments were conducted at 22 ± 2 °C. Analysis and graphing were done using FitMaster software (HEKA Elektronik) and Igor Pro (Wavemetrics, Lake Oswego, OR, USA). All data acquisition and analysis programs were run on an Apple iMac (Apple Computer).

5.3.6. Statistics

Variables are presented as means ± standard error and were normally distributed. T-test was used to compare the responses. A level of significance α = 0.05 was used in all overall tests, and effects with p-values less than 0.05 were considered to be statistically significant.
5.4. Results

5.4.1. CBD binding site in sodium channels

The key reason for using NavMs for this study is that they provide, to date, the highest resolution (~2.2-2.5 Å) views of any Nav channel (Naylor et al., 2016; Sula & Wallace, 2017), especially of the transmembrane and drug binding regions. This feature results in obtaining detailed views of the protein molecular structures with drugs bound to them.

We found that the CBD binding site is located in a hydrophobic pocket present in each subunit that runs perpendicular to the channel direction (Figure 5-1A-B). These features are known as fenestrations and located in the transmembrane segments (Figure 5-1C). These fenestrations are located just below the selectivity filter, which are the features originally proposed by (Hille, 1977b) as sites for ingress of hydrophobic drugs in the channel interior. CBD is located at the end of the fenestrations that lies closest to the central pore and protrudes into (and blocks) the central pore cavity. This site is in agreement with the MD simulations showing CBD pathway into the Nav1.4 cavity (Ghovanloo et al., 2020b). Inside the NavMs pore, there is enough room for 4 CBD molecules, but 1 CBD molecule is sufficient to block sodium conduction. The CBD interaction site is very close to the locations of the binding sites that have been identified for other analgesics and hydrophobic compounds in both NavMs and NavAb (Sula & Wallace, 2017; Gamal El-Din et al., 2018).
5.4.2. Functional CBD inhibition of NavMs

To investigate whether CBD functionally inhibits NavMs, we performed whole-cell voltage-clamp of transiently transfected cells. We previously determined that CBD imparts little selectivity in inhibiting various voltage-dependent sodium (inhibition of human channels from inactivated-state range: ∼2–4 μM) and potassium currents, including the bacterial sodium channel NaChBac. We also found that CBD inhibition of Nav channels has a steep Hill-slope (∼2) from both resting- and inactivated-states, indicating that relatively small magnitude fold differences in CBD potency are a product of the slope (Ghovanloo et al., 2018c). Here, we found that CBD inhibits NavMs less potently and with slightly shallower Hill slope than other Nav channels previously studied (Ghovanloo et al., 2018c; Fouda et al., 2020) (Figure 5-2). The moderate variation in CBD inhibition potency between human Navs and NavMs is consistent with previous reports using other Nav channels.
blockers (Bagnéris et al., 2014). Overall, these results show that CBD inhibits NavMs similarly to other Nav channel and, thus, the proposed interaction inside the pore from the NavMs X-ray structure is functionally relevant.

![A: Fractional Block](image1)

![B: Concentration vs. Fractional Block](image2)

![C: CBD Inhibition of NavMs](image3)

**Figure 5-2  CBD inhibition of NavMs.**

(A) Block was measured after ~6 minutes wash and incubation in CBD. IC\textsubscript{50} measurement was done from CBD inhibition data obtained from whole cell voltage-clamp recordings and fit with the Hill Langmuir equation. The IC\textsubscript{50} for CBD's inhibition of NavMs is 19.4 ± 3.1 µM with a Hill slope of 1.5 ± 0.4 (the S.E. values quoted are errors of the fit, n = 7 panel-wide). (B) Sample traces before and after 10 µM CBD perfusion are shown. (C) Bar graphs showing percentage of peak sodium current remaining over time after control/CBD perfusion at 10 µM (p = 0.0150).
5.5. Discussion

This study has demonstrated the structural nature of the interactions of CBD and a Nav channel, showing that CBD binding blocks the transmembrane pathway for sodium ion translocation through the membrane (Naylor et al., 2016), and hence provides a potential mechanism for the functioning of CBD in Nav channels. This further suggests a possible molecular basis for the medicinal effects of CBD in the treatment of epilepsies, as Nav channels have been shown to be causally related to various types of human epilepsy, with disease-related mutations interfering with sodium ion transmembrane flux. The CBD binding site is a novel site, near to, but not coincident with, known analgesic binding sites in sodium channels; binding at this site would effectively block Nav channel functioning. This binding site is located at the pore end of the transmembrane fenestrations which enable the ingress of hydrophobic molecules into the channel lumen, hence indicating this may also provide the pathway for CBD to enter and block the channels.

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Dr. Bonnie A. Wallace performed conceptualization; Drs. Bonnie A. Wallace, Altin Sula, and Lily Goodyer Sait designed structural experiments, purified and crystallized protein, collected crystal data, undertook structure solutions and analyses and Dr. Altin Sula did PDB depositions. Mohammad-Reza Ghovanloo performed all functional experiments, analysis, and related figure-making. Dr. Peter C. Ruben conceived electrophysiology experiments. All co-authors contributed to writing.

I received explicit permission from our collaborators to use the information generated by their respective groups in my thesis.
Chapter 6. General discussion

6.1. Key findings, conclusions, and implications

The overall goal of this thesis was to gain additional knowledge about how hyperexcitability is imparted upon Nav channels and to find new ways of pharmacologically targeting this hyperexcitability.

6.1.1. Aim 1

Skeletal muscle hyperexcitability can impose serious limitations on a patient’s quality of life. Mutations that cause hyperexcitability in Nav1.4 are plentiful, and their biophysical consequences are complex. Although a given mutation may alter only single component of the channel gating, many clinically relevant mutations tend to alter multiple aspects of channel biophysics (Ghovanloo et al., 2016b). For instance, a single missense mutation may right-shift activation (LOF) and also exacerbate persistent currents (GOF). The overall channotype (channel sequence variation profile) is a mixture of both defects (Klassen et al., 2011). The most intriguing aspect of P1158S is that its channotype culminates in multiple degrees of hyperexcitability.

The results from aim 1 describe how P1158S increases the pH-sensitivity of Nav1.4. These results are particularly notable in that P1158S is located on the intracellular side of the channel, and the interactions between protons and Nav seem to be predominantly confined to the extracellular side of the channel. This suggests that the P1158S effect on pH-sensitivity is likely indirect in nature. This observation is further supported by the patch-clamp experiments with reduced intracellular pH showing no pH-mediated shifts in the P1158S gating.

Aim 1 highlights the complexity of pH-Nav interactions and also illuminates the importance of investigating residues that could both directly (directly get protonated) or in directly (alter gating, potentially exposing secondary residues to protons) mediate pH-sensitivity. Additionally, aim 1 introduces an in-vitro/in-silico hyperexcitability assay of Nav1.4 which is a useful tool to investigate potential therapeutics against myotonia and periodic paralysis (Chapter 2).
6.1.2. Aim 2

CBD is a highly hydrophobic compound with a very complex profile. Numerous studies from researchers across scientific disciplines suggest a wide range of molecular targets for CBD. The diversity of targets has suggested potential therapeutic value against a variety of disorders, many of which seem unrelated to one another. For instance, in addition to the noted hyperexcitability disorders, CBD has also been suggested to possess antibiotic properties (van Klingerent & ten Ham, 1976; Kosgodage et al., 2019). This has created a reputation in which CBD is perceived as both a panacea and somewhat of a ‘snake oil’.

This CBD reputation has at least two possible explanations. First, CBD indeed interacts with the diverse molecular targets suggested by the existing literature. Second, the experimental assays in use are inadequate to investigate CBD effects; therefore, any given proposed molecular target could be a false positive.

One way to rectify this apparent is to investigate CBD interactions with a molecular target that is involved in seemingly different disorders. The Nav superfamily fits this description given that, depending on the tissue, Navs underlie a broad range of disorders.

In aim 2, the main goal was to determine the effects of CBD on different Navs, determine whether CBD has any selectivity, and discover how CBD modulates Nav gating. The results in aim 2 provide the first in-depth description of CBD effects on voltage-dependent sodium currents. The lack of selectivity, high hydrophobicity, high lipid partitioning, and increased apparent potency at lower temperatures (along with faster kinetics; this indicates that the CBD effects would be less pronounced at higher temperatures, such as regular body temperature compared to the lower temperatures in in-vitro experiments) resulted in concluding that CBD likely affects the membrane as much as it likely modulates the Nav gating directly (Chapter 3). These conclusions laid the foundation for a series of hypothesis regarding CBD mechanisms of action.

6.1.3. Aim 3

The hypotheses generated in aim 2 were tested in aim 3. The extensive mutational work and MD simulations suggested that CBD both directly interacts inside the Nav pore to physically block sodium conduction, and also alters the cell membrane elasticity to
stabilize the Nav inactivated state. Aim 3 also describes the pathway of CBD passage through the intralipid Nav fenestrations into the LA site, inside the pore (Chapter 4). We recently further verified this pathway with our collaborators using X-ray crystallography (Chapter 5), showing the CBD pose crystallized inside the pore of the bacterial NavMs (Sait et al., 2020).

From a broader perspective and beyond CBD’s inhibition of Navs, the mechanisms presented in aim 3 could hold true for a variety of membrane-bound protein targets (Ibeas Bih et al., 2015; Ghovanloo et al., 2018c; Fouda et al., 2020). For instance, CBD’s modulation of membrane elasticity would conceivably alter the biophysical properties of any given ion channel or receptor. Furthermore, the presence of intralipid fenestrations or fenestration-like structures in a given protein target could mediate CBD passage into the heart of the target, altering the protein’s normal function. Although these predictions should be tested on a case by case basis, the results from aim 3 provide a theory that could, at least in part, explain the diversity of CBD targets proposed in the literature.

From a pharmacological perspective, the CBD results presented in aims 2 and 3 are the first (to our knowledge) detailed mechanistic description of how a superhydrophobic compound inhibits Navs. In aim 2, we discovered that CBD does not affect Nav open-state inactivation. The results in both aims 2 and 3 also suggest that CBD prevents Navs from opening. These results, along with the finding that occluding the fenestrations abolishes block, prompts us to propose that CBD does not interact with the open-state of the channel. This prediction is consistent with the overall trend that has been proposed for LAs that are charged or neutral with low LogDs (Hille, 2001; Ghovanloo et al., 2018c) and suggests that, as the drug becomes more hydrophobic, it tends to interact more with resting- and inactivated-states (Figure 6-1). One caveat to the scheme proposed in (Figure 6-1) is that it is based on a single ultra-hydrophobic compound. To determine whether this scheme holds true for other compounds with similar physicochemical properties to CBD, other compounds must be tested.
Figure 6-1  Proposed channel blocking scheme for a superhydrophobic compound.

Hypothesis for block by LAs, based on: (Hille, 1977b, 2001; Hondeghem & Katzung, 1977). (A-B) The first two models are based on previous studies. (A) Nav states and transitions with charged drug molecules. Charged (hydrophilic) drug may come and go only while the gate is open. (B) Neutral (hydrophobic) drug can bind and unbind even if when the gate is closed. Therefore, two pathways exist for drug to reach its receptor in the pore. The hydrophilic pathway is closed when the gate is closed. (C) The third model is based on our results from our studies on CBD and Nav channels. The star indicates drug. We propose as the drug becomes more hydrophobic, its interaction with the channel transitions from only the open-state (O) to only interactions with rest- (R) and inactivated-states (I).

From a clinical perspective, in aim 3 (Chapter 4), we showed that CBD reduces skeletal muscle contraction. Furthermore, CBD may hold therapeutic value against myotonia, as suggested by the in-vitro/in-silico hyperexcitability assay of Nav1.4. This
prediction must be further validated and tested in future studies using animals-based and clinical assays.

6.2. Potential limitations

One of the primary limitations of these studies is that the majority of experiments were performed using heterologous expression systems: CHOK1 and HEK-293 cells. CHO cells have small background currents and are good for studying channels with smaller currents, while HEK cells typically last longer and are more durable during experiments. Navs are typically modulated by many proteins and accessory subunits which could be missing in heterologous systems. However, despite their limitations, heterologous systems work like blank canvases that are excellent for purely biophysical studies such as those presented here.

To verify the CBD effects observed in heterologous systems in a more physiologically relevant system, human iPSC-derived neurons were used in aim 2 to reproduce CBD inhibition of both sodium and potassium currents. Although iPSC cells are generally considered more physiological than either CHO or HEK cells, they do not fully recapitulate intact human cells. For instance, iPSC-derived cells are often immature and lack various structures. Furthermore, these cells are inadequate for studying the biophysical properties of a given channel. This is particularly evident when studying neuronal Navs. To determine which Nav isoform is predominantly (or solely) expressed in a given iPSC-derived in neuron is rather challenging without the aid of highly selective Nav modulators. Therefore, even with extensive pharmacological tools, experiments with these cells require elaborate pharmacological cocktails which may introduce confounding effects.

The nature of patch-clamp experiments is also a limitation. A typical patch-clamp experiment involves the overexpression of a given ion channel to many times greater than physiological levels. This overexpression is often done using transient transfections. This process involves co-transfection of 2-3 cDNAs. In these experiments, α subunit, β subunit (to boost expression and make gating more physiological), and eGFP (reporter) were used. Because only fluorescent cells were patched, both α subunit and eGFP were successfully co-transfected in all of the presented data; however, it was not possible to be sure the β subunit was also present in all cells. Stably transfected cells reduce some of
these limitations. However, perhaps the biggest limitation to whole-cell patch-clamp is that, upon rupturing the contact point between the tip of the pipette and cell membrane, all of the intracellular components of the cell are dialyzed out.

Another caveat to these studies is that many conclusions are based on computer simulations. In the case of AP modeling, all of the simulations were based on functional data, and the MD simulations were performed according to well-established parameterizations and protocols. To ensure that simulation-based limitations are minimal, all simulations were either intertwined with functional data (e.g. in-vitro/in-silico hyperexcitability assay of Nav1.4) or were functionally tested (e.g. fenestration and lipid NMR experiments).

### 6.3. Final remarks

The structural similarities in the Nav family is a major challenge in developing selective Nav-targeting therapeutics. Most small molecules that inhibit Nav channels are non-selective in nature, a function of the binding site in the pore of the channel in which key residues are conserved across the orthologues. Despite this lack of selectivity, these molecules have found wide ranging clinical efficacy in disorders of excitability. However, these compounds typically have a narrow therapeutic index, presumably due to their non-selective inhibition. The results described in this thesis suggest that CBD, as a Nav inhibitor would suffer from the same limitations as traditional Nav blockers. Furthermore, because of its hydrophobicity, it modulates a wider range of targets than traditional Nav blockers. These properties could cause enough off-target effects to render CBD useless against Nav hyperexcitable conditions, including the conditions associated with the skeletal muscle. However, the strongest argument for CBD as a Nav-related therapeutic is its clinically proven efficacy in Dravet Syndrome. This efficacy is likely the result of CBD modulating multiple targets, and not just one particular set of Nav channels.

In conclusion, the work presented in this thesis describes a new pathway of pH-sensitivity, a novel assay to investigate drug effects against Nav1.4 hyperexcitability, CBD effects and mechanism of action on Nav and membrane, and implicates CBD as a possible therapeutic for myotonia. Finally, this work could be a first steppingstone into determining whether CBD or other similar compounds could develop or contribute to the development of other promising therapeutics.
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