NOTE TO USERS

Sodium Channel Mutations Causing Epilepsy

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Masters of Science

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Abstract

Ion channels mediate the electrical properties of neurons and other excitable cells. Mutations in ion channel genes have been linked to several neurological disorders. For example, a rare familial form of epilepsy, generalized epilepsy with febrile seizures plus (*GEFS+*), is associated with mutations in voltage gated sodium channels. We examined how two such mutations (C121W, D188V) alter the functional properties of the channel through voltage-clamp studies in *Xenopus* oocytes and HEK cells respectively. D188V is located in the α subunit and C121W in the auxiliary β1 subunit of the sodium channel.

The C121W mutation causes a 100 fold reduction in efficacy of current modulation as well as a reduction of current amplitude. This may cause increased sodium currents via a negative shift of the steady-state inactivation curve. α-D188V channels recover faster from the inactivated state which causes a resistance to frequency-dependent cumulative inactivation of current amplitude. This may contribute to cellular hyperexcitability resulting in ictal events in the epileptic patient.

Résumé

Les canaux ioniques contribuent à la modulation des propriétés électriques des neurones ainsi que de toutes autres cellules susceptibles d'excitation. Des mutations de canaux ioniques ont été liées à plusieurs troubles neurologiques. Ces conditions pathophysiologiques incluent une certaine forme d'épilepsie, *generalized epilepsy with febrile seizures plus* (GEFS+), associée avec des mutations des canaux sodiques voltage-sensible. Une mutation a été identifiée dans la sous-unité α (D188V) et une autre dans la sous-unité β1 (C121W) de ce canal sodique. Nous avons examiné la modulation des propriétés physiologiques de ce canal sodique par β-C121W et α-D188V, par des études de *voltage-clamp* des oocytes de *Xenopus* et des cellules HEK, respectivement.

Les canaux α-D188V récupèrent plus vite de l'état inactivé, produisant une plus grande résistance à la diminution de l'amplitude du courant suite aux dépolarizations à hautes fréquences. Cette propriété pourrait contribuer à l'hyperactivité cellulaire causant le déclenchement de crises épileptiques. D'autre part, le canal sodique possédant la mutation C121W est 100 fois moins efficace à moduler le courant et l'amplitude de ce courant est réduite. Cette mutation pourrait causer l'hyperactivité cellulaire par un déplacement négatif de la relation d'inactivation homéostatique.

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Introduction

Ion channels mediate the electrical properties of neurons and other excitable cells. Naturally occurring mutations in ion channel genes have been linked to various diseases of excitable tissues, including epilepsy. These disorders are known as *channelopathies*. Generalized Epilepsy with Febrile Seizures Plus (GEFS+), an autosomal dominant, inherited epilepsy, has been linked to different point mutations in voltage-gated sodium channels, one of the most important excitatory channels in the neuron. Considering their pivotal role in the flow of neuronal information, it is not surprising that malfunctioning sodium channels would underlie neurological disorders; nevertheless, much is still not known about how sodium channel mutations alter channel function to cause the observed phenotype. My project addresses this issue by comparing the functional properties of wild type sodium channels to sodium channels with mutations responsible for inherited forms of generalized epilepsy.

Background

The voltage-gated sodium channel

Voltage-gated sodium channels are responsible for the main excitatory current in neurons. They allow the passage of sodium ions through the cellular membrane in response to depolarization of membrane potential. Sodium channels are involved in the initiation and propagation of the action potential, as well as shaping cell firing patterns.

The brain sodium channel is a membrane spanning protein composed of a main, pore-forming α subunit and two auxiliary β subunits (Hartshorne & Catterrall 1981; Hartshorne *et al.*1982) (figure 1). The α subunit is a 260 kDa protein composed of four homologous domains, each with six transmembrane segments (S1-S6) (Catterall 2000) (figure 2). The channel pore is lined by the pore-loop and S6 segment of each domain (Doyle *et al.* 1998; Lipkind and Fozzard 2000) with specific amino acid residues in each of the four pore-loops conferring sodium ion specificity (Terlau *et al.* 1991; Heinemann *et al.* 1992). Each pore loop contributes a single residue (aspartate in domain I, glutamate in domain II, lysine in domain III and alanine in domain IV), which are thought to form an electrostatic ring that is critical for sodium selectivity (reviewed in Yellen 1998). Mutation of this DEKA motif to DEEE confers calcium selectivity to the sodium channel pore (Heinamann et al., 1992), highlighting the importance of these residues in determining ion selectivity.

Sodium Channel Gating

At hyperpolarized membrane potentials, sodium channels are in non-conducting, closed states (Hille, 1992). Sodium channels open from closed states

following sufficient membrane depolarization, allowing sodium ion flow, down its concentration gradient. This sodium influx into the cell causes rapid membrane depolarization. The channels then quickly enter a non-conducting, inactivated state. Channels do not open from the inactivated state. To reopen, channels must first return to the closed state, a process which requires several milliseconds at a repolarized membrane potential. This absolute refractory period sets an upper limit on the rate of action potential firing.

Activation of the sodium channel is achieved through two distinct, but tightly coupled mechanisms: a voltage sensing component which translates membrane depolarization into conformational changes in channel structure and an activation gate which responds by opening the channel pore. The S4 transmembrane segment of each domain contains 2 to 8 positively charged residues that act as the voltage-sensors. According to the most widely held model for activation, the voltage-sensing S4 segments are thought to move outward in response to membrane depolarization, through a "gating pore" formed by the other transmembrane segments. (Catterall 1986; Guy & Seetharamulu 1986; Yang & Horn 1995; Yang *et al.* 1996). However, more recent crystallographic data suggests that the S4 segments may contribute to a voltage-sensing "paddle" that flips from a cytoplasmic to an extracellular position when the membrane is depolarized (Jiang *et al.* 2003). The precise mechanisms by which membrane potential determines the conformation of voltage-gated ion channels is one of the major unresolved challenges in the ion channel structure-function field.

The molecular basis of channel opening has been explored by cysteine availability mutagenesis (Yellen 2002) and by crystallographic analysis (Jiang *et al.*

2002). Together, these data suggest that the S6 segments, which line the inner pore merge together at the intracellular portion of the channel to block the pore, when the channel is in the closed conformation. Following the movement of the voltage sensors, the S6 segments are thought to undergo a spiral-like movement resulting in the opening of the pore (Liu *et al.* 1997; Perozo *et al.* 1999). The channels quickly go from the open state to a fast inactivated state, which terminates ion flux through the pore. This is caused by a *ball-and-chain* mechanism whereby a tethered intracellular blocking particle binds to the opening of the pore, thus occluding the flow of ions. In the sodium channel, the inactivation ball is formed by the intracellular linker between domains III and IV (West *et al.* 1992; Patton *et al.* 1992).

In addition to the conventional fast inactivated state, sodium channels can also enter distinct slow inactivated states, during prolonged membrane depolarizations. In contrast to fast inactivation, which has fast kinetics of onset and recovery, channels enter slow inactivated states slowly during membrane depolarizations, and recovery from slow inactivation is on the order of hundreds of milliseconds to tens of seconds (Ruff et al., 1988; Hille 1992; Ruff, 1996; Wang and Wang, 1997). Fast and slow inactivation are mediated by different parts of the pore; indeed, treatments that remove fast inactivation (enzymes, toxins, and mutations) do not affect slow inactivation

Voltage-gated potassium channels also show fast and slow inactivation known as N-type and C-type inactivation, respectively. The mechanisms involved are thought to be similar to those of sodium channels. N-type (fast) inactivation is caused by occlusion of the inner-pore by the C-terminus whereas C-type (slow) inactivation is

thought to take place on the outer segment of the pore, in the region of the selectivity filter. During slow inactivation, the outermost end of the narrow selectivity filter rearranges, perhaps narrowing, but not collapsing (reviewed by Yellen 1998). Slow inactivation is thought to involve conformational changes in the S4 segment of domain IV (Mitrovic et al., 2000) and the P region (Tomaselli et al., 1995; Balser et al., 1996) (Kiss and Korn, 1998).

Slow inactivation influences membrane excitability, firing properties and spike frequency adaptation (Ruff et al., 1988; Sawczuk et al., 1995; Fleidervish et al., 1996). It has also been proposed to be a mechanism by which the cell may retain traces of previous activity (Toib et al., 1998). Mutations causing a suppression of sodium channel slow inactivation have been shown to cause skeletal muscle channelopathies such as hyperkalemic periodic paralysis type 2 and paramyotonia congenita (Jurkat-Rott et al., 2000; Hayward et al., 1997). In this thesis, I present data suggesting that changes in slow inactivation are responsible for the pathophysiology of the D188V mutant sodium channel linked to GEFS+.

Sodium Channel Molecular Diversity

Molecular cloning has uncovered a surprising diversity in sodium channel subtypes (Table 1). To date, at least ten different α subunits, which are highly conserved across different mammalian species have been identified. The functional properties differ slightly between different channel types. These properties are also influenced by glycosylation, phosphorylation and subunit binding. Further functional diversity is achieved by mRNA splicing. Each subtype exhibits a distinct pattern of regional and subcellular localization (Westenbroek *et al.* 1989).

Auxiliary β subunits

Three distinct sodium channel auxiliary subunits have been identified to date: β1, β2 and β3. The β subunits do not contribute to the ion conducting pore; instead they modulate the α subunit function, expression and localization. The β subunits are not highly homologous, but share a similar topology consisting of a single transmembrane segment with a large extracellular N-terminal domain and a small intracellular C-terminal domain (Isom *et al.* 1995a) (figure 2). In *Xenopus* oocytes, β1 and β3 increase the rate of channel inactivation and also increase the rate of recovery from inactivation as compared to α subunits expressed alone (Isom *et al.* 1992; 1995a; 1995b; Patton *et al.* 1994). This suggests that they play an important role in regulating channel behavior. β subunits also increase sodium channel cell surface expression (Isom *et al.*1992; 1994; 1995a,b), suggesting that they increase channel trafficking to the cell surface and/or stabilize channels in the plasma membrane.

In addition to modulating channel kinetics and expression, the β subunits also act as cell adhesion molecules. All β subunits have a well conserved extracellular immunoglobin (Ig) domain which is structurally homologous to the V-set of the Ig superfamily that includes cell adhesion molecules (CAMs). Indeed, the extracellular portion of β2 is highly homologous to the nCAM contactin (Isom and Catterall 1996). These cell adhesion properties may be important for interactions with extracellular matrix proteins that influence channel localization or cell migration (Isom et al. 1999, Srinivasa et al. 1998).

Epilepsy

Epilepsy is defined as any of various neurological disorders characterized by sudden recurring attacks of motor, sensory, or psychic malfunction with or without loss of consciousness or convulsive seizures. Seizure disorders are common, affecting approximately 1% of the population. Seizures are the result of an imbalance between excitatory and inhibitory mechanisms in the central nervous system resulting in anomalous synchronous network activity. To date over 40 clinical epilepsy syndromes have been identified, affecting over 50 million people world-wide (reviewed in Jacobs et al. 2001). Epileptic ictal events are induced by abnormal focal or generalized synchronized electrical discharges within the central nervous system. Epilepsy syndromes are categorized into three groups: symptomatic, cryptogenic and idiopathic. Symptomatic epilepsies are those for which there is a known cause, such as trauma, tumor, cerebral malformations or metabolic disorders. In cryptogenic epilepsies, the underlying lesion is suspected but unproven. Idiopathic epilepsies are those for which a cause is not known; however, a genetic cause is suspected. Idiopathic generalized epilepsy (IGE) is a common disorder affecting 0.4% of the population. Common idiopathic epilepsy syndromes exhibit complex patterns of inheritance; however, certain rare forms of epilepsy are due to mutations in single genes, facilitating their study. These monogenetic familial epilepsy syndromes may give insights into the underlying causal mechanisms of the more common polygenic idiopathic disorder.

Channelopathies

In recent years, a number of mutations in ion channel genes have been linked to human neurological disorders; these disorders are termed *channelopathies.* Channelopathies were first recognized in 1989 (Riordan *et al.* 1989), with the discovery of cystic fibrosis transmembrane conductance regulator. This family of diseases is most illustrated by diseases of excitable tissue; nevertheless, ion-channel disorders now cover the gamut of medical disciplines, causing significant pathology in virtually every organ system. These are therefore valuable targets for pharmacological intervention.

The prominent role of ion channels in neurological diseases comes as no surprise considering their importance in the normal functioning of the central and peripheral nervous systems. The broad range of ailments associated with ion channel mutations include migraine headaches, deafness, periodic paralysis, malignant hyperthermia, generalized myotonia and epilepsy (reviewed in Ptáček 1998) (Table 2). Channelopathies are responsible for most inherited forms of epilepsy so far identified. These channelopathic epilepsies have been found to be caused by mutations in nicotinic acetylcholine receptors, voltage gated potassium channels, $GABA_A$ receptors and voltage gated sodium channels (reviewed in Ptáček 1998; Gardiner 1999).

Mutations in sodium channel genes have been linked to a form of inherited epilepsy termed *Generalized Epilepsy with Febrile Seizures plus* (GEFS+) (Escayg 2000). The GEFS+ syndrome is subdivided into GEFS+ type I or type II depending

on whether the mutation site is located in the sodium channel auxiliary β1 subunit or the pore-forming α subunit, respectively. GEFS+ is a familial syndrome, which displays an autosomal dominant pattern of inheritance (Wallace *et al.* 1998, Wallace *et al.* 2001). It is characterized by general febrile epilepsies (such as absences, myoclonic seizures, atonic seizures and myoclonic-astatic epilepsy), which display a childhood onset (median 1 year of age). The attacks persist beyond age 6, usually ceasing by mid childhood (median age 11). Heterogeneity of the syndrome results in difficult diagnosis.

GEFS+ was first linked to a point mutation in the voltage-gated sodium channel β1 auxiliary subunit (Wallace et al., 1998). This mutation, consisting of a cysteine to tryptophan switch at position 121, disrupts a critical disulfide bond in the functionally important extracellular immunoglobin loop (figure 2). One aim of my Master's project is to investigate how this mutation alters sodium channel function to cause epilepsy. More recently, several mutations in α subunits have recently been linked to *GEFS+* (Wallace *et al.* 1998, Wallace *et al.* 2001). For example, through gene mapping studies, Guy Rouleau's lab at McGill University has found a novel mutation site in the type 1 voltage gated sodium channel ($Na_V1.1$). It is an aspartate-valine switch at position 188, located in the intracellular loop between segments S2 and S3 of the first domain (figure 2). This amino acid is conserved in almost all sodium channels thus far identified in all species, suggesting it is functionally critical (figure 3). However, the role of this region of the α subunit in sodium channel function has not been elucidated. A second major focus of my Master's work has been to examine how the D188V mutation alters channel function to cause epilepsy.

Rationale

Voltage gated sodium channels play a central role in the flow of neuronal information. Mutations in these channels have been linked to certain forms of idiopathic epilepsy; however, precisely how these mutations produce epilepsy has not been fully worked out. This project investigates two mutations associated with *GEFS+*, to determine how these mutations alter channel function to cause seizures .

GEFS+1 C121Wβ-1 Subunit Functional Assay

The C121W mutation in the β1 subunit was the first sodium channel mutation linked to epilepsy. This substitution is thought to disrupt an extracellular disulfide bond critical for β1 function. Although identifying the genetic link to the disease, the initial study into this mutation did not involve an extensive analysis of how the mutation alters sodium channel function to cause epilepsy. The work described in the first section of my thesis contributed to a thorough study investigating how the C121Wβ1 mutation effects sodium channel expression, function and localization (Meadows et al., 2002).

GEFS+2 D188Vα Subunit Functional Assay

A number of α subunit mutations linked to GEFS+2 have been identified. However, the putative functional changes caused by these mutations have not been determined. The D188 residue, in the intracellular loop between transmembrane segments S2 and S3 in domain I, is conserved in different sodium channels from both vertebrate and invertebrate species suggesting that this residue is functionally critical

(figure 3). Mutation of this residue to valine in the $Na_V1.1$ sodium channel causes *GEFS+* (Wallace et al. 2001). Our aim was to discover what is functionally different between the wild type and mutant proteins that could result in neuronal hyperexcitability and seizures. Our hypothesis was that the mutation modified some functional aspect of the channel resulting in neuronal hyperexcitability. The results of our analysis formed the bulk of a recently published paper (Cossette et al., 2003) in which I was the co-1st author.

Summary

The link between sodium channel mutations and epilepsy has been established. It is now essential to further investigate their effects on ion channels in order to gain insight into epileptogenesis by understanding how the ion channel mutations alter the physiological functioning of the protein. This is what we have set out to do in this thesis .

Materials and Methods

GEFS+1 C121Wβ1 Subunit Functional Assay

Mutagenesis

The C121W mutation was introduced into rat β1 cDNA (pCR2.1 vector) using standard PCR mutagenesis (Barek, 1993) with PWO (Roche Diagnostics, Laval, QC) DNA polymerase. The mutant PCR products were subcloned into the full length rat β1 construct and sequenced to confirm the presence of the mutation and to rule out the introduction of spurious mutations during PCR amplification. C121W mutagenesis was performed by Lori Isom's lab at the university of Michigan.

RNA Synthesis and Oocyte Injection

Rat $Na_V1.2a$ subunit DNA (pSP64T vector), was linearized with *Xho*I, and phenol extracted under RNAase-free conditions. RNA was synthesized using *mMessage mMachine* RNA synthesis kit (Ambion Inc.) with a T7 promoter. The SP6 promoter RNA synthesis kit was used for RNA synthesis of Rat β1 subunit DNA (pSP64T vector) linearized with *Eco*RI. RNA concentrations were estimated from the intensities of bands from RNAase-free agarose gels relative to the intensities of bands of known concentration.

Oocytes were surgically removed from *Xenopus Laevis* frogs, washed twice with OR2 solution (NaCl 82.4mM; KCl 2mM; $MgCl_{2\text{-}6H2O}$ 1mM; Hepes(mwt 238) 5mM), underwent 2 subsequent 1hr collagenase incubations to brake down remaining tissue and transferred to Barth's solution (NaCl 88mM; KCL 1mM; $MgSO_{4\text{-}7H2O}$

0.82mM; $Ca(NO_3)_{2\text{-}4H20}$ 0.33mM; $CaCl_{2\text{-}2H20}$ 0.41mM; $NaHCO_3$ 2.4mM; HEPES(mwt 238) 10mM). Oocytes were injected with 46nl each, of varying α:β RNA molar ratios with glass electrodes. Recordings were performed 2-5 days post-injection.

Electrophysiology

Standard whole-cell 2-electrode voltage clamp recording was performed using *TURBO TEC-10C* amplifier (ALA Scientific Instruments) with *1200Digidata interface* (Axon Instruments) hardware and *pCLAMP* (Axon Instruments) software. A 3M KCl solution was used as intracellular recording solution and Ringer's solution (NaCl 1.2mM; KCl 0.03mM; $CaCl_{2\text{-}2H2O}$ 0.02mM; HEPES (mwt 238) 0.1mM) as extracellular recording solution. All recordings were performed at room temperature.

Whole-cell currents were elicited with a 0mV depolarization from a –90mV holding potential. Inactivation was measured using a two exponential fit ($y = A_1e^{-t/\tau1} + B_2e^{-t/\tau2} + C$) applied to the decaying phase of the current. Proportion of fast current decay was measured by A/(A+B) where A represents the proportion of fast current and B, the slow portion.

Current-voltage relationships were obtained by applying a 15ms depolarization from –60 to 55mV (5mV intervals) from a holding potential of –90mV. Conductance curves were determined from these IV curves by dividing the current amplitude by the test potential minus the reversal potential, then normalized to one. Plots were fitted with a Boltzmann equation (f=1/(1+exp((x-vh)/s))).

Steady-state inactivation curves were obtained by a stimulus protocol which consisted of an initial –90mV holding potential, a 100ms pulse to –100mV (subsequently increasing by 5mV up to –15mV) followed by a 7ms test pulse to 0mV and a final repolarization to –90mV. Plots were also fitted with a Bolztmann equation.

GEFS+2 D188Vα Subunit Functional Assay

Choice of α subunit subtype

D188V was found in NaV1.1. The human clone for this channel was not available, so we proceeded to study D188V in rNaV1.1 which shows a very high degree of homology. We found that this channel could only be heterologously expressed at low levels and only in the *Xenopus* oocyte expression system. Expression was even poorer for mutant channels rendering its functional analysis impossible. Thus, we decided to study the mutation in $rNa_v1.2a$ expressed in HEK cells (human embryonic kidney cells). Our rationale for using rNav1.2a are as follows. rNaV1.2a expresses very well in mammalian cells (such as HEK cells) which are more physiologically valid than the oocyte model for studying sodium channel function. rNaV1.2a shows more than 95% identity to NaV1.1 overall, with 100% identity in the vicinity of the mutation (Goldin et al., 2001) (figure 16). Both Nav1.1 and Nav1.2a are expressed in neurons. rNaV1.2a exhibits electrophysiological properties similar to NaV1.1 when expressed in *Xenopus* oocytes (Smith and Goldin, 1998). A mutation in NaV1.2a can also cause epilepsy in humans (Sugawara, 2001b), with a phenotype that is similar to GEFS+.

Mutagenesis

The D188V mutation was introduced into cDNA encoding the rat Nav1.2a subtype of the sodium channel α subunit, in vector pEUK-CI (Clonetech, Mississauga Ontario) using standard PCR-based mutagenesis. Briefly, a truncated Nav1.2a-pEUK construct was created by digesting Nav1.2a-pEUK with *Sma*I and then circularizing a 6.9 kb fragment consisting of the entire vector and 5' and 3' segments of Nav1.2a. The mutation was introduced into this construct by PCR performed by Patrick Cossette from Guy Rouleau's lab at McGill University. The mutant construct was then cut with *Sma*I and religated with the remainder of the Nav1.2a cDNA, to regenerate the full-length Nav1.2a cDNA, containing the D188V mutation. The entire coding regions of both the wild type and mutant constructs were subsequently resequenced, to confirm the presence of the mutation in only the D188V construct, and to rule out the introduction of spurious mutations in either construct during PCR or other molecular manipulations.

Sodium channel expression and electrophysiological recording

Sodium channel function was studied by transient expression in human embryonic kidney (HEK) cells expressing the α subunit alone or co-expressing α and β1 subunits. Cells were grown to 80% confluence in 60 mm tissue culture dishes in DMEM medium (GIBCO, Burlington, Ontario) and then transfected with 1.5 μg wild type or mutant Nav1.2A-pEUK DNA, along with 2 μg of sodium channel β1 cDNA in vector pcDNA3.1-Zeo (Invitrogen, Burlington, Ontario) and 0.5 μg GFP in vector pEGFP-C1 (Clonetech), using Polyfect transfection reagent (Qiagen, Mississauga Ontario) and the transfection protocol supplied by the manufacturer. Twenty four hours later, the cells were split into 35 mm dishes at densities appropriate for

electrophysiological recording. Recordings were performed 2-4 days after transfection, using the whole cell configuration of the patch clamp recording technique (Hamill et al., 1981). Recordings were at room temperature. Recording pipettes had resistances of 2-4 MΩ, and were filled with an intracellular solution consisting of (in mM): 105 Cs-Aspartate, 10 CsCl, 10 NaCl, 10 EGTA and 10 mM HEPES, pH 7.4 with CsOH. The extracellular bath solution contained (in mM): 130 NaCl, 4 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 5 glucose and 10 HEPES, pH 7.4 with NaOH. Recordings were obtained using an Axopatch 200 amplifier and pCLAMP software (Axon Instruments, Foster City CA). Data was filtered at 5 kHz and sampled at 50 kHz. Cancellation of capacitive currents and series resistance compensation (typically > 70%) were performed using the internal clamp circuitry. Remaining transient and leak currents were subtracted using the P/4 procedure. Other details of whole cell recording were as described elsewhere (Cossette et al., 2003).

The voltage-dependence of channel activation was determined by applying depolarizing test pulses to a range of test potentials, from a holding potential of –90 mV. Peak current amplitude (I_{peak}) was measured at each test potential, and converted to conductance (g) according to $g = I_{peak}/(V_{rev}-V_{test})$, in which V_{test} is the test potential and V_{rev} is the current reversal potential, determined by linear extrapolation of the straight line portion of the falling phase of the current-voltage relationship. The conductance values were normalized with respect to the maximal conductance, plotted as a function of V_{test}, and fit with the Boltzmann equation: $1/(1+\exp((V_{test}-V_{1/2})/k)$, in which $V_{1/2}$ is the midpoint of the curve and k is a slope factor. Steady state inactivation was examined by applying 100 ms long prepulses to a range of prepulse potentials, followed by a test pulse to 0 mV. The peak amplitude of currents evoked

by the test pulses were normalized with respect to the largest currents, plotted as a function of prepulse potential and fit with the Boltzmann equation. Recovery from fast inactivation was assessed by applying an 5 msec conditioning pulse to 0 mV, followed by a recovery interval of varying duration to –80 mV, and a test pulse to 0 mV to determine the fraction of channels available to open. The amplitudes of currents elicited by the test pulse, normalized with respect to currents evoked by the conditioning pulse, were plotted as a function of recovery interval and fit with a single exponential to determine the time course of recovery from inactivation. Recovery from slow inactivation was assessed the same way but with a 1 second conditioning pulse.

Frequency dependent current (cumulative inactivation) was assessed with 100 5-msec pulses from –80 to +10mV at frequencies of 1, 10, 20, 40, 80 or 100 Hz. Currents were normalized with respect to the initial current elicited.

Data analysis, curve fitting and plotting was performed using pCLAMP and SigmaPlot 5.0 (SPSS, Chicago Illinois). Statistically significant differences between wild type and mutant channels were determined by Student's t-test. Groups were considered significantly different when $P < 0.05$.

Results

The following sections describe the results of two projects that constitute my Masters thesis. The first project examined the effects of the C121W mutation in the sodium channel β1 subunit on β1-mediated modulation of the functional properties of

sodium channels expressed in *Xenopus* oocytes. The second project investigated the functional effects of the D188V mutation in the sodium channel α subunit. The goal of these projects was to gain insight into how naturally occurring mutations can change sodium channel function to cause epilepsy.

Project 1 : C121W disrupts functional modulation of sodium channels by β1 in oocytes.

To examine the effects of C121W on sodium channel function we coexpressed wild type or mutant β1 subunits in *Xenopus* oocytes along with the rat Nav1.2a α subunit, and examined the functional properties of the expressed channels using two-electrode voltage clamp recording. Figure 4 shows the effects of rat wild type β1 or C121Wβ1 subunits on the time course of whole cell sodium currents in oocytes. Consistent with previous findings (Isom et al. 1992), coinjection of RNA encoding wild type β1 and $Na_v1.2a$, at equimolar concentrations, resulted in whole cell sodium currents that inactivated ~ 5-times faster than sodium currents in oocytes expressing $Na_v1.2a$ alone (Figure 4A, left-hand traces). In contrast, injection of the same concentration of C121Wβ1 RNA did not result in detectable modulation of current time course (Figure 4A, right-hand traces). Although these data corroborated previous findings (Wallace *et al.* 1998), my results were novel in two respects. First, I found that the C121W mutation did not completely abolish β1 function. Indeed, the mutant β1 subunit fully modulated sodium current time course, but only with injection of ~ 100-fold more RNA than was necessary for functional modulation with β1 (Figure 4A,B). These data indicate that the mutation does not destroy the

determinants required for modulation of sodium channels expressed in oocytes, but instead lowers the efficacy of β1-mediated functional modulation. I also found that mutant C121W-β1 retained its ability to negatively shift the steady-state inactivation curve when expressed at high levels only (figure 5) further supporting the hypothesis that efficacy of modulation is affected by the mutation and thus that it does not completely knock out function.

The other striking and novel effect that I observed for C121Wβ1 was to decrease sodium current amplitude, when expressed at high levels, an effect not observed with wild type β1 expressed at similar concentrations (figure 6). These data suggest that sodium channels associated with the mutant β1 subunit are not as efficiently trafficked to the cell surface and/or are not as stable in the cell membrane as channels associated with wild type β1. Thus, these results raise the intriguing possibility that C121Wβ1 may affect sodium channel expression in vivo.

Summary of Project 1

In summary, these data suggest two main effects of the C121Wβ1 mutation. The first is to greatly reduce but not completely abolish the efficacy of β1-mediated functional modulation of sodium channels. The second, is to disrupt efficient expression of sodium channels on the cell surface. The implications of these findings for epilepsy are adressed in detail in the discussion section of this thesis.

Project 2: Functional analysis of the D188V mutation

The D188V mutation does not alter the time course of sodium channel inactivation.

To determine the effects of the D188V mutation on sodium channel function, we introduced the mutation into cDNA encoding the rat ortholog of the Nav1.2a subtype of the sodium channel α subunit, transfected human embryonic kidney (HEK) cells with wild type or mutant cDNA, and examined the properties of the expressed sodium channels by whole cell patch clamp recording. The $Na_v1.2a$ channel was chosen for this analysis instead of Nav1.1, the channel in which the mutation is found in humans, because Nav1.2a expresses extremely well in HEK cells, whereas the cloned rat $Na_v1.1$ subtype did not produce detectable currents when transfected into HEKs or other mammalian cell lines in preliminary experiments. $Na_v1.2a$ is expressed in brain neurons, is > 95% similar to Nav1.1 (Goldin et al., 2000), is identical to $Na_v1.1$ in the region of the mutation (figure 3), and exhibits properties similar to $Na_v1.1$ when expressed in *Xenopus* oocytes (Smith and Goldin, 1998). In all experiments, we coexpressed wild type or mutant α subunits with the auxiliary β1 subunit, which noncovalently associates with sodium channels in brain neurons and modulates channel function in heterologous expression studies (Isom et al., 1995).

Sodium channel mutations associated with cardiac long QT syndrome (Bennett et al., 1995; Dumaine et al., 1996; Wang et al., 1996), inherited diseases of skeletal muscle (Cannon et al., 1991; Lehmann-Horn, 1991; Cannon and Strittmatter, 1993; Chahine et al., 1994; Mitrovic et al., 1994; Yang et al., 1994; Lerch et al., 1997)

and recently described GEFS+ mutations (Lossin et al., 2002) cause slow or incomplete channel inactivation, suggesting that this is a common mechanism for disorders associated with sodium channelopathies. Decreased inactivation is a plausible physiological mechanism leading to neuronal hyperexcitability, because it would lead to increased or sustained sodium current; therefore, we examined whether the D188V mutation also affected the time course or extent of sodium current inactivation in whole cell recordings from HEK cells. Figure 7A shows that decay of whole cell sodium currents in HEKs expressing either wild type or mutant sodium channels was rapid and nearly complete by the end of test depolarizations to a range of potentials. Current decay was well fit by single exponentials, with time constants that were voltage dependent at negative test potentials and approached an asymptotic value of ~ 0.5 msec with strong depolarizations (Figure 7B). Inactivation time constants for HEK cells expressing wild type or mutant channels were indistinguishable over this entire voltage range. To ensure that our analysis did not miss subtle effects on current time course, we compared the superimposed means of normalized currents elicited by depolarization to 0 mV for cells expressing wild type or mutant channels (figure 7B). Figure 7C shows that the inactivation time courses of these averaged traces are virtually identical. Together, these data indicate that the epileptogenic effects of the D188V are not caused by a change in the time course of sodium channel inactivation. The levels of persistent current at the end of test depolarizations were also indistinguishable between wild type and D188V (Fig. 7D), suggesting that the mutation does not increase neuronal excitability by increasing the level of persistent current, as has been observed for other GEFS+ type 2 mutations (Lossin et al., 2002).

D188V does not affect the voltage-dependence of sodium channel gating.

The range over which sodium channels open and inactivate strongly influences neuronal excitability. For example, a negative shift in the voltage-dependence of activation lowers the action potential threshold, resulting in increased cell excitability. To investigate whether the D188V mutation altered the voltage-dependence of activation, we measured peak currents evoked by test pulses to a range of test potentials and converted the resulting current voltage-relationships (Figure 8A) to activation curves (Figure 8B). Mean activation curves for wild type and mutant channels were virtually identical (Figure 8B), with midpoints of -14 mV. Thus, D188V does not increase cell excitability by altering the voltage range over which sodium channels open.

The fraction of sodium channels available to open at rest also influences cell excitability by determining the maximal sodium current amplitude. Sodium channel availability is large at hyperpolarized membrane potentials, where most sodium channels are in closed resting states, and their availability diminishes progressively at increasingly depolarized potentials, as these channels convert to inactivated states. We determined the voltage-dependence of sodium channel availability from the amplitudes of sodium currents elicited by depolarizations to 0 mV, following 100 msec conditioning pulses to a range of prepulse potentials. Figure 8C shows that the mean normalized availability curves for sodium currents in HEK cells expressing wild type or D188V channels were nearly identical, with midpoints of -45 mV. These data indicate that the D188V mutation does not increase neuronal excitability by altering sodium channel availability.

D188V does not affect sodium current amplitude

Whole cell current amplitudes in HEKs expressing mutant sodium channels were statistically indistinguishable from current amplitudes in cells expressing wild type channels (mean amplitudes at 0 mV: wild type: 1.9 ± 0.3 nA; D188V: 2.1 ± 0.4 nA) (Figure 9), suggesting that the mutation does not affect neuronal excitability by altering the level of functional channels expressed on the cell surface.

D188V alters frequency-dependent cumulative inactivation of sodium channels.

Whole cell sodium currents decrease in amplitude progressively over the course of high frequency trains of channel activity. This frequency-dependent cumulative inactivation is thought to play a significant role in shaping normal neuronal electrophysiology (Jung et al., 1997; Costa et al., 1997), and may also be involved in dampening pathophysiological hyperexcitability associated with seizures. We examined frequency-dependent cumulative inactivation of wild type and D188V sodium currents by applying trains of 5 msec-long test pulses to 0 mV, from a holding voltage of –80 mV, at frequencies ranging from 1 to 100 Hz. Interestingly, at frequencies ≥ 10 Hz, currents in cells expressing mutant sodium channels declined in amplitude significantly less than currents in cells expressing wild type channels (figure 10).

Frequency-dependent cumulative inactivation occurs because channels do not completely recover from inactivation from one pulse to the next. To assess whether the differences between wild type and D188V in cumulative inactivation were caused

by differences in their rates of recovery from fast inactivation, we inactivated channels with a 5 msec depolarizing conditioning pulse, then examined the time course of recovery from inactivation at a hyperpolarized recovery potential. We observed no difference in recovery from fast inactivation between wild type and mutant channels (Figure 11), using this protocol. However, a similar protocol with a longer depolarizing pulse (mimicking a prolonged, high frequency train of brief depolarizing pulses) showed that there was a significant difference between wild type and mutant in the fraction of channels entering the slow inactivated state (figure 12). These data suggest that the mutation causes a decreased entry into slow inactivation. During high frequency trains, this difference in inactivation between wild type and mutant gradually accumulates, resulting in the emergence of a large difference in frequency-dependent rundown of sodium current amplitude.

Summary of Project 2

These data suggest that the D188V mutation causes a specific, selective decrease in frequency-dependent cumulative inactivation of sodium currents. This effect may increase neuronal excitability, thus contributing to the epileptic phenotype in individuals carrying this mutation. These findings complement previous results showing that other sodium channel mutations linked to GEFS+ alter the frequency responses of sodium channels (Spampanato et al., 2001). In demonstrating these effects using a brain sodium channel along with the auxiliary β1 subunit, expressed in a mammalian cell line, the present study represents a significant incremental advance in our understanding of how sodium channel mutations can cause epilepsy. The data presented here suggest one way in which subtle, yet specific, changes in sodium

channel function, caused by genetic variability in amino acid sequence of an ion channel protein, can contribute to the genesis of epileptic seizures.

Discussion

Epilepsy is associated with hyperexcitability of brain neurons. Sodium channels are the principle mediators of intrinsic neuronal excitability, and recent evidence indicates that mutations in sodium channel genes are responsible for some inherited epilepsies. In this thesis, I have examined the effect of two such point mutations on the functional properties of the channel in order to gain insight on how these mutations lead to epilepsy. I discuss the implications of these findings in the following sections.

The C121W mutation in β1 alters sodium channel function and current amplitude.

The C121W β1 mutation was the first sodium channel mutation related to GEFS+ type 1 (Wallace 1998). Although the initial analysis by Wallace et al suggested that the C121W mutation resulted in loss of β1 function, precisely how this causes epilepsy was not clear from the original functional analysis. Therefore, our lab, in collaboration with the lab of Dr. Lori Isom at the University of Michigan, undertook at detailed functional and biochemical analysis of C121Wβ1 (Meadows et al., 2002). My contribution to this project was to investigate functional modulation by wild type and mutant β1 subunits of brain sodium channels expressed in *Xenopus* oocytes. My data led to two novel observations. The first was that the C121W mutation did not completely abolish β1 function, but instead caused an approximately 100-fold reduction in the efficacy of β1-mediated modulation of sodium channel

function in *Xenopus* oocytes. These data, along with subsequent functional and biochemical results (Meadows et al., 2002) suggest that the mutation causes a reduction in the affinity of β1 for sodium channels. In mammalian cell expression systems, cooexpression of β1 shifts sodium channel steady state inactivation to more negative potentials, and slows the rate of channel recovery from fast inactivation. Thus, in terms of epilepsy, the loss of β1 function may increase susceptibility to seizures by increasing sodium channel availability at hyperpolarized membrane potentials and by reduced sodium channel current rundown during high-frequency channel activity. These effects on channel function may destabilize the homeostasis of the neuronal network leading to uncontrolled firing and seizures.

In addition to altering sodium current time course, C121Wβ1 also reduced the amplitude of whole cell currents, suggesting that it reduced the number of functional sodium channels expressed on the cell surface. This may be because sodium channels associated with the mutant β1 subunit were not efficiently trafficked to the cell surface. Thus, the normal β subunit chaperone activity, whereby the protein helps the sodium channel assume its proper conformation within the cell, may be disrupted by the mutation, resulting in a smaller fraction of sodium channel proteins expressed on the cell surface.

C121W in relation to epilepsy

Epilepsy is caused by a destabilization of excitatory and inhibitory activity in neurons that result in unattenuated cellular excitation known as the ictal event. Therefore, we would expect that mutations causing epilepsy would likely cause a

hyperexcitable cell. Reduced functional efficacy of the mutant β1 causes a prolonged sodium current and a rightward shift of the steady-state inactivation curve, these effects are consistent with our predictions. Reduced current amplitude was also observed, this may conceivably contribute to network hyperexcitability if this effect is predominant in inhibitory neurons. Several non-functional Nav1.1 mutants have been linked to a similar disorder, severe myoclonic epilepsy (Claes *et al.* 2000). This is consistent with the hypothesis that a reduced sodium current may result in hyperexcitability. Further studies are required to elucidate the precise mechanism.

The β1-subunit is expressed not only in brain but also in muscle cells. Yet, C121W patients do not suffer from myotonia or periodic paralysis (other sodium channelopathies associated with prolonged currents), even though the skeletal muscle NaV1.4 sodium channel also associates with β1. This may be because the brain is more sensitive to slight physiological alterations in channel properties as compared to muscle cells.

D188V mutation in the α subunit reduces frequency dependent cumulative inactivation

We also examined the functional effects of the α subunit D188V mutation, which is linked to GEFS+ type 2. The main finding of this study was that the mutation causes resistance to current amplitude cumulative inactivation during high frequency trains of depolarizations. In contrast, the mutation did not affect other aspects of channel function. Data from experiments examining recovery from

inactivation suggest that the effects of the mutation on frequency dependent run down are caused by a reduced entry of channels into slow inactivated states.

Although the fraction of channels that become slow inactivated during a single 5 msec long pulse is quite small, over the course of a long pulse train there is a gradual build-up of channels in the slow-inactivated state: those channels which have entered this state during each depolarization do not recover between pulses (since its recovery requires a longer repolarized state) and in each subsequent pulse, more channels are recruited into this state. The result is a gradual build-up of slow-inactivated channels. Mutant channels are less prone to enter the slow inactivated state thereby causing a resistance to frequency dependent current cumulative inactivation. Figure 13 shows a model of the different channel functional states, illustrating this hypothesis.

These findings suggests a possible pattern of functional behavior for GEFS+ mutant channels. Indeed, the R1460H GEFS+ mutation, located in the IV/S4, exhibits a similar run-down of current amplitude during stimulus trains (Spampanato et al. 2001) as well as a threefold increase in the rate of recovery from inactivation (Alekov et al. 2000, 2001). Furthermore, functional analysis of the C121Wβ1 mutation in mammalian cells has demonstrated that this mutation also leads to a channel which is more resistant to high frequency current cumulative inactivation (Meadows et al. 2002). Complete electrophysiological characterization of the other mutants is required to establish how common this mechanism is in the pathophysiology of epilepsy.

D188V and epilepsy

How does the D188V mutation cause epilepsy? During seizures, individual neurons undergo prolonged membrane depolarizations, during which they continuously fire action potentials at high frequency (McNamara, 1994). Cumulative inactivation of sodium currents during this pathological activity would be expected to reduce neuronal excitability and thus dampen neuronal firing. In contrast, neurons expressing sodium channels with the D188V mutation are expected to be more resistant to sustained high frequency firing, and thus more able to sustain and propagate seizure activity in the brain. Although the general idea that decreased sodium current cumulative inactivation will increase neuronal excitability is intuitively obvious, the exact mechanism by which this change in channel function causes seizures in the brain of epileptic patients is less clear. Sodium channels are involved not only in the initiation and propagation of action potentials, but also in synaptic integration (Stuart and Hausser, 2001), boosting of synaptic potentials (Stuart and Sakmann, 1995) and subthreshold electroresponsiveness (Alonso and Llinás, 1989). Sodium channels in different subcellular regions show differing functional properties (Colbert et al., 1997; Jung et al., 1997), and each of the various neuronal sodium channel subtypes exhibits a distinct pattern of regional and subcellular localization (Westenbroek et al., 1989). The protein product of the *SCN1A* gene is localized to the somata of at least some brain neurons (Westenbroek et al., 1989), and thus may play a specialized role in determining neuronal firing patterns. The findings presented here give a starting point for *in vivo* studies to understand the mechanism by which mutations in *SCN1A* lead to epilepsy in humans.

Effects of temperature

GEFS+ patients display febrile seizures, that is seizures induced by elevated body temperatures. The effect of temperature on the physiological properties of the mutant channels is an important factor remaining to be examined. It is possible that sodium channel mutations associated with GEFS+ are temperature sensitive and thus a bigger difference between WT and mutant may be observed at fever-temperatures than at normal resting body temperature. An alternative possibility is that the effects of fever are not specifically on mutant sodium channels, but instead increase the overall excitability of the entire neuronal circuit, which, together with altered sodium channel function cause a destabilizitation of neuronal homeostasis, resulting in seizures. It has been reported that many kinetic properties of sodium channels, such as activation and inactivation time constants, differ at various temperatures (Rosen 2001). This is an important issue, which remains to be examined.

Implications for Therapy

Many of the drugs currently prescribed for the treatment of epilepsy, such as phenytoin, have been discovered through empirical drug testing (reviewed in Ragsdale & Avoli 1998). Upon subsequent analysis, it was found that such drugs may help control seizures by reducing sodium current amplitude during high frequency stimulation. Therefore, it is conceivable that such drugs would be effective in the treatment of GEFS, in which we found a resistance to high frequency run-down of sodium current amplitude. Understanding the structure-function relationship of ion channels involved in diseases will thus help develop new drug therapies based upon their molecular activity on the channel. Therefore such studies are essential in order to

develop new drugs which may offer better seizure control while also targeting specific channel subtypes thus without affecting normal brain function.

Psychopharmacogenetics is a growing field which deals with hereditary effects on variability in drug response. It has been found that different enzymatic and ion channel polymorphisms respond differently to specific therapies (reviewed in Bondy *et al.* 2001). Therefore it is essential to examine the possibility that these specific epilepsy mutations respond best to which drugs in the hopes of offering better seizure control while minimizing the side-effects. It is possible that certain polymorphisms are better molecular targets for drug treatment, an important hypothesis which should be examined.

Conclusion

The first mutation identified related to GEFS+ was a C121W in β1. Oocytes expressing $Na_V1.2$ along with mutant β1 exhibited a decreased rate of inactivation of sodium currents, compared to cells expressing wild-type channels. We have shown that C121Wβ1 protein retains its modulatory effects with much reduced efficacy. More slowly inactivating sodium currents and a negative shift of the steady-state inactivation curve would both contribute to increased cellular excitablity. This is consistent with neuronal hyperexcitability causing epilectic events. As well, we have shown that the mutation causes a reduction in sodium current amplitude when expressed at high levels in *Xenopus* oocytes. If this effect were predominant in inhibitory neurons, it could lead to network hyperexcitability.

We have also shown that the D188V mutation causes a functional channel which exhibits reduced entry into the slow inactivated state. This results in sustained activity during high frequency stimulation, characteristic of epileptic seizures. This functional change has been observed in at least two other GEFS+ mutations, C121W when expressed in mammalian cells (Meadows et al. 2002) and R1460H (Spampanato et al. 2001), suggesting that it may be an important causal factor leading to GEFS+. Investigation of the functional effects of the other GEFS+ mutants is required as well as *in vivo* studies to ascertain this hypothesis.

Precise understanding of how altered sodium channel function causes seizures requires sophisticated modeling incorporating the detailed pattern of $Na_V1.1$ regional and subcellular localization as well as a fundamental understanding of the complex

neuronal and network electrophysiological properties. As well, the effects of these mutations on channel function must be addressed *in vitro*.

This study is an important step in the understanding of the molecular and physiological basis of GEFS+ epileptogenesis which will lead to a greater understanding of more common epileptic syndromes and eventually to the development of better treatments for the disorder.

Appendix

FIGURE 1- *SODIUM CHANNELS CONSIST OF A CENTRAL α SUBUNIT AND AUXILIARY β SUBUNITS.*

The voltage-gated sodium channel is formed by a central, pore-forming α subunit which is sufficient on its own to conduct current. This subunit contains the voltage-sensor, the pore as well as segments involved in inactivation. In the brain, two auxiliary β subunits associate with the α subunit. They are involved in protein folding, cellular localization and modulation of current kinetics.

Fig 1

Sodium channels consist of a central α subunit and auxiliary β subunits.

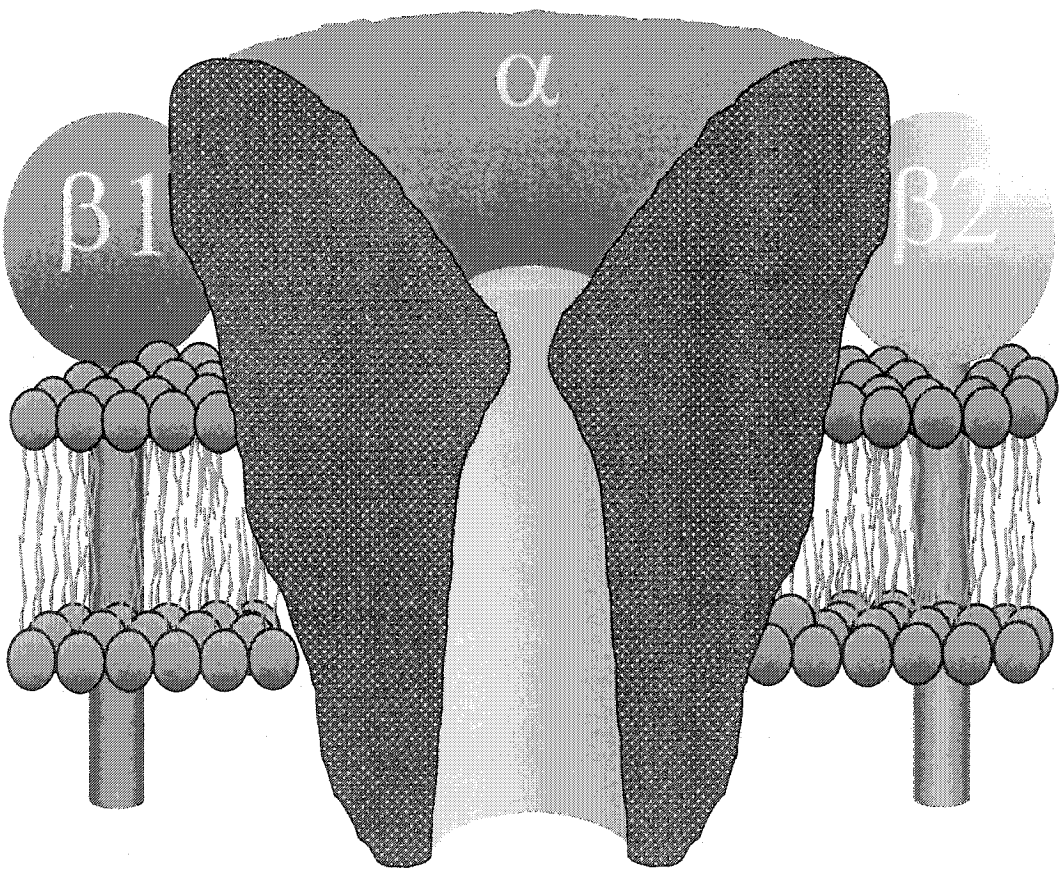
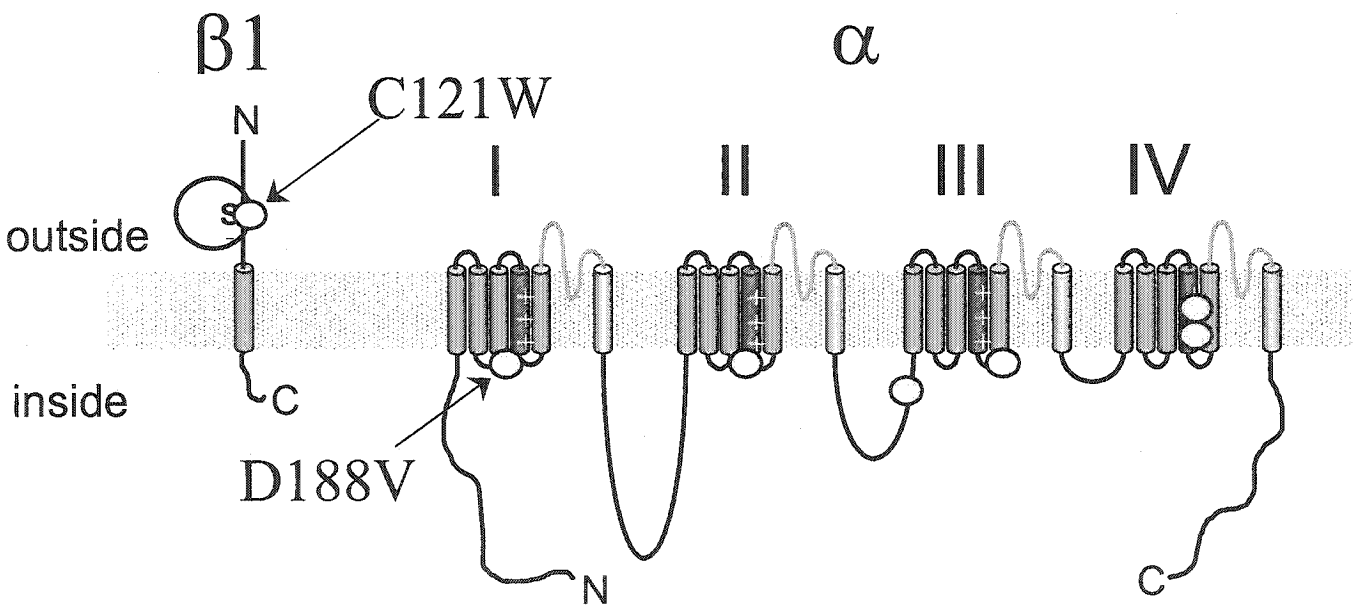


FIGURE 2- *TOPOLOGY OF SODIUM CHANNEL SUBUNITS AND GEFS+ MUTATION SITES.*

The α subunit consists of four homologous domains (I - IV) with six transmembrane segments each (S1-S6). The S4 transmembrane segment contains the voltage-sensor. The region between S5 and S6 is involved in the *ball-and-chain* inactivation mechanism. The β subunits consist of one transmembrane segment and an extracellular Ig loop. The first point mutation (O) associated with GEFS+ was identified in the extracellular portion of the β1 subunit. Subsequently, others have been identified in various parts of the α subunit. This thesis pertains to the C121W and D188V mutations.

Fig 2

Topology of Sodium Channel Subunits and
GEFS+ Mutation Sites



○ GEFS+ mutation sites

FIGURE 3- *ASPARTIC ACID AT POSITION 188 IS CONSERVED IN ALMOST ALL SODIUM CHANNELS.*

The only exception in mammals is the original rat $Na_V1.2a$ clone, where the aspartic acid is replaced by an asparagine at position 188. It is likely that this represents an error during replication of cDNA, since other investigators have cloned the same gene in the rat and found that the aspartic acid is conserved at this position.

Fig 3

SCN1A	F	T	F	L	R	D	P	W	N	W	L
RAT SCN1A	-	-	-	-	-	-	-	-	-	-	-
SCN2A	-	-	-	-	-	-	-	-	-	-	-
RAT SCN2A	-	-	-	-	-	-	-	-	-	-	-
SCN3A	-	-	-	-	-	-	-	-	-	-	-
SCN4A	-	-	-	-	-	-	-	-	-	-	-
SCN5A	-	-	-	-	-	-	-	-	-	-	-
SCN6A	-	S	-	-	G	-	-	-	-	-	-
SCN8A	-	-	-	-	-	-	-	-	-	-	-
SCN9A	-	-	-	-	-	-	-	-	-	-	-
SCN10A	-	-	Y	-	-	-	-	-	-	-	-
SCN11A	-	S	-	-	-	-	-	-	-	-	-
SCN12A	-	S	-	-	-	-	-	-	-	-	-
ELECTRIC EEL	-	-	-	-	-	-	-	-	-	-	-
DROSOPHILA	-	-	Y	-	-	-	A	-	-	-	-
SQUID	-	-	Y	-	-	-	A	-	-	-	-
FLATWORM	-	-	Y	-	-	S	I	-	-	-	-
JELLYFISH	Y	S	Y	-	-	N	S	-	-	-	-

FIGURE 4- *THE C121W MUTANT β1 SUBUNIT RETAINS THE ABILITY TO MODULATE SODIUM CHANNEL FUNCTION IN XENOPUS OOCYTES.*

(A) Typical Na+ currents elicited from oocytes injected with varying α:β RNA ratios. The time bar represents 5 msec. The mutant protein has the ability to modulate the current to the same extent as the wild type protein; however, a much higher proportion of subunit protein is required. (B) WT (○) and C121W (●) β1 Na+ current modulation efficacy. The wild type protein is approximately one hundred fold more efficacious at modulating the sodium current.

Fig 4

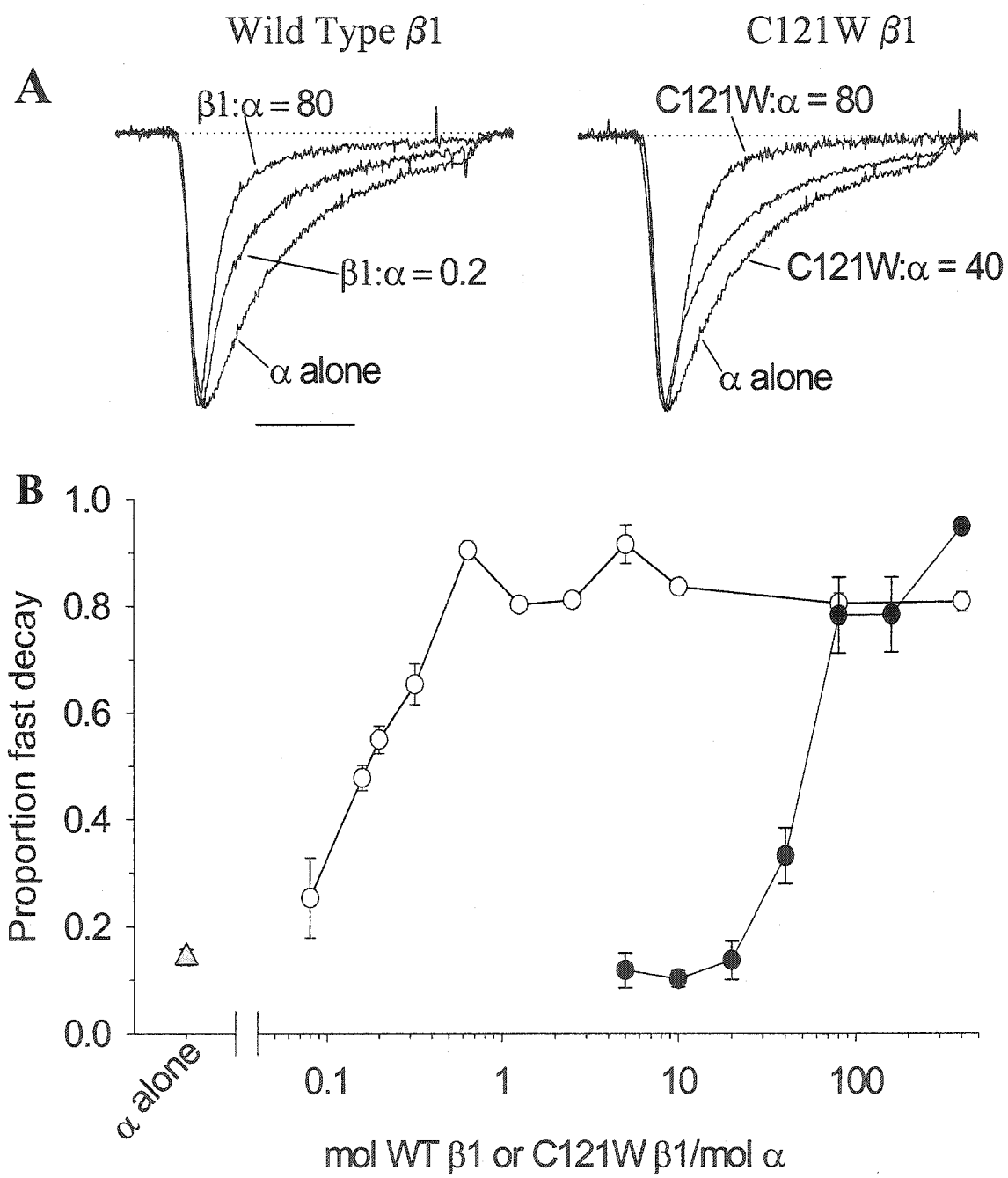

Wild Type β1
C121W β1
A
β1:α = 80
β1:α = 0.2
α alone
C121W:α = 80
C121W:α = 40
α alone
B
Proportion fast decay
1.0
0.8
0.6
0.4
0.2
0.0
α alone
0.1
1
10
100
mol WT β1 or C121W β1/mol α

FIGURE 5- *THE C121W MUTANT β1 SUBUNIT RETAINS THE ABILITY TO MODULATE SODIUM CHANNEL STEADY STATE INACTIVATION IN XENOPUS OOCYTES.*

The X-axis represent the steady-state depolarization in mV, and the Y-axis, the proportional current amplitude resulting from a 0mV depolarization following a conditioning depolarization. Much higher concentrations of C121W β1 RNA (B) are required to achieve complete steady-state inactivation modulation of Na+ currents compared to WT β1 RNA (A) steady-state inactivation.

Fig 5

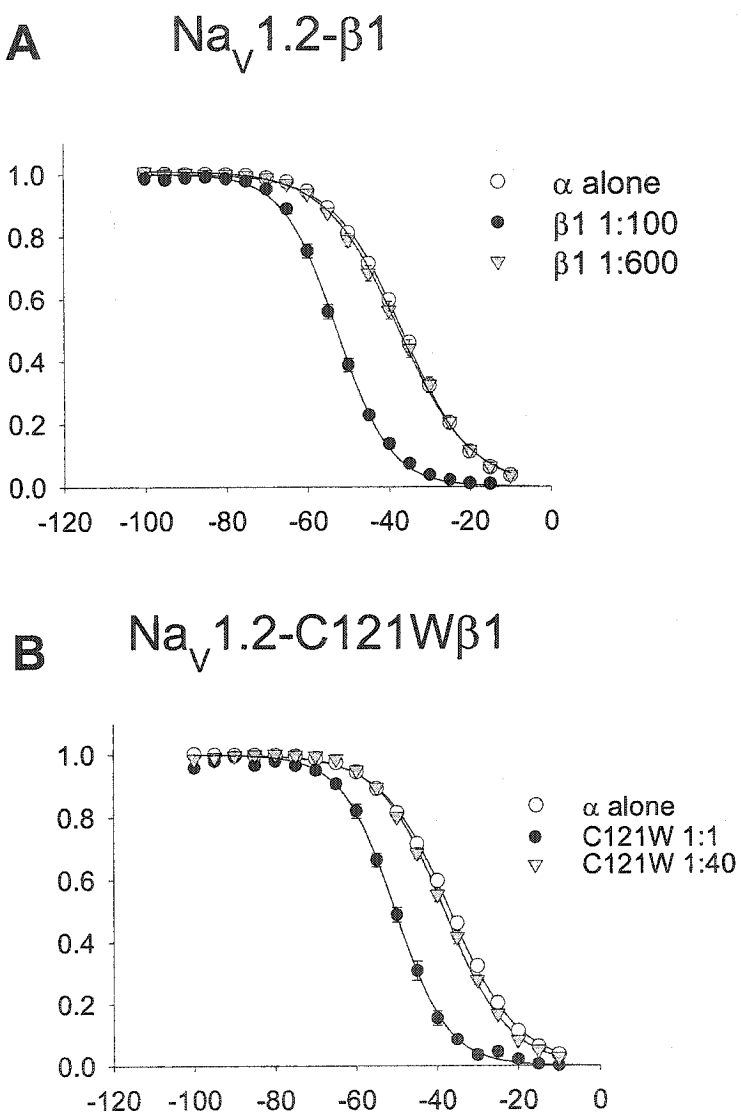

A
$Na_V1.2$-β1
1.0
0.8
0.6
0.4
0.2
0.0
-120
-100
-80
-60
-40
-20
0
α alone
β1 1:100
β1 1:600
B
$Na_V1.2$-C121Wβ1
1.0
0.8
0.6
0.4
0.2
0.0
-120
-100
-80
-60
-40
-20
0
α alone
C121W 1:1
C121W 1:40

FIGURE 6-*C121W CAUSES A DOSE-DEPENDENT DECREASE IN SODIUM CURRENT AMPLITUDE, AN EFFECT NOT OBSERVED WITH WILD TYPE β1.*

Injection of varying α :β RNA ratios from separate RNA preps, into *Xenopus* oocytes from different batches, results in a dose-dependent decrease in sodium current amplitude for mutant channels only. A qualitative difference is consistently seen in each different sample group.

Fig 6

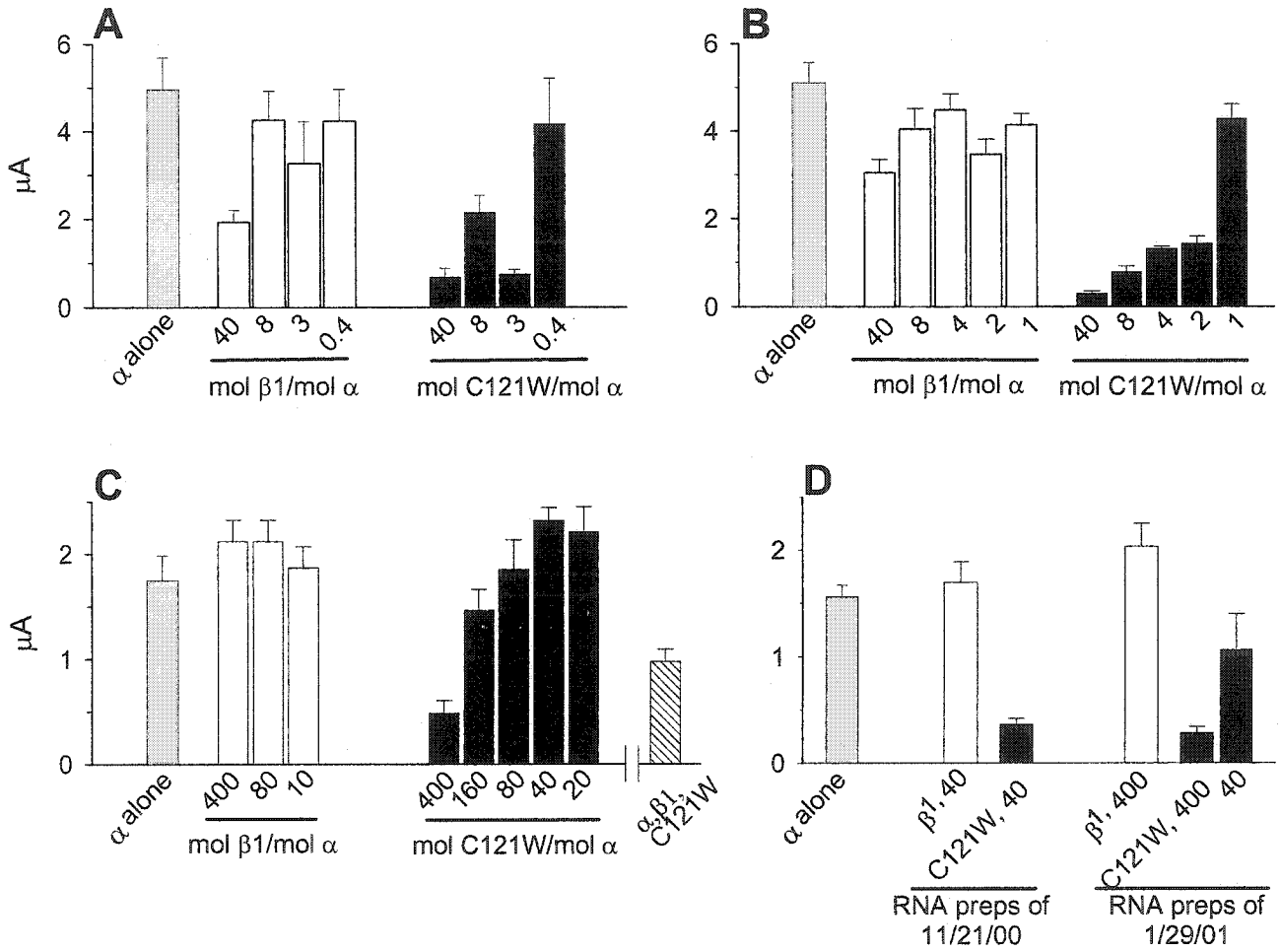

A
μA
6
4
2
0
α alone
40 8 3 0.4
mol β1/mol α
40 8 3 0.4
mol C121W/mol α
B
6
4
2
0
α alone
40 8 4 2 1
mol β1/mol α
40 8 4 2 1
mol C121W/mol α
C
μA
2
1
0
α alone
400 80 10
mol β1/mol α
400 160 80 40 20
mol C121W/mol α
α β1 C121W
D
2
1
0
α alone
β1, 40
C121W, 40
RNA preps of 11/21/00
β1, 400
C121W, 400 40
RNA preps of 1/29/01

FIGURE 7-*D188V DOES NOT AFFECT NA CURRENT TIME COURSE.*

(A) Sample traces in HEK cells expressing $Na_V1.2A$ or $Na_V1.2A$-D188V. In this and subsequent figures, wt and mutant α subunits were coexpressed with β1 subunits. Currents were evoked by –60, -30, -20 and –10 mV depolarizations. (B) Averaged normalized $Na_V1.2A$ and $Na_V1.2A$-D188V current traces induced by a 0mV depolarization. (C) Time constants (τ) for decay of whole cell current as a function of test potential in cells expressing $Na_V1.2A$ or $Na_V1.2A$-D188V. τ values were determined by fitting current decay with a single exponential. (D) D188V does not affect persistent sodium currents. (E) Mean levels of persistent current for wild type (n = 16) and D188V (n = 13) over a range of test potentials. In each cell, persistent current was measured at the end of depolarizing test pulses, and then normalized with respect to the peak current elicited at 0 mV.

Fig 7

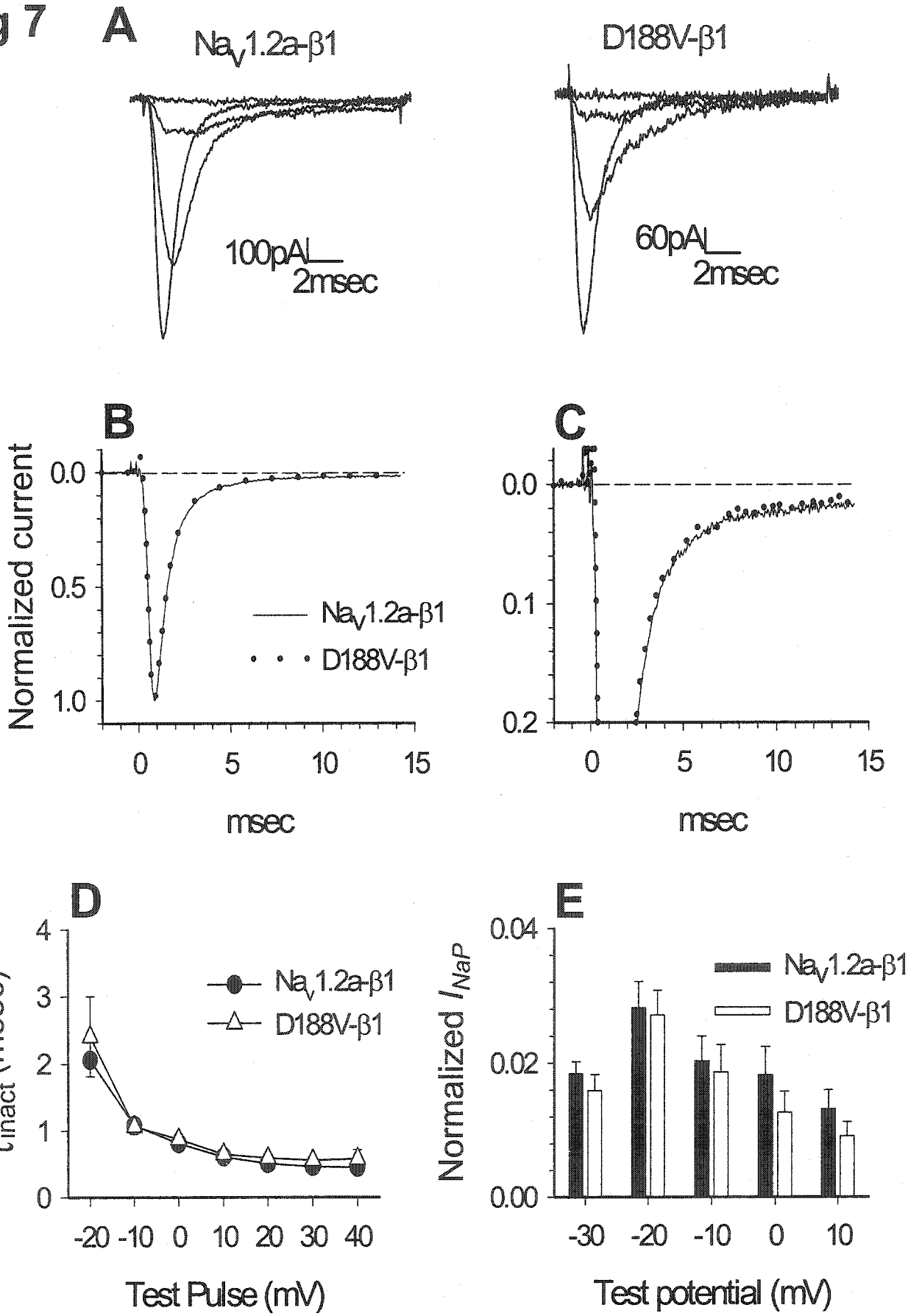
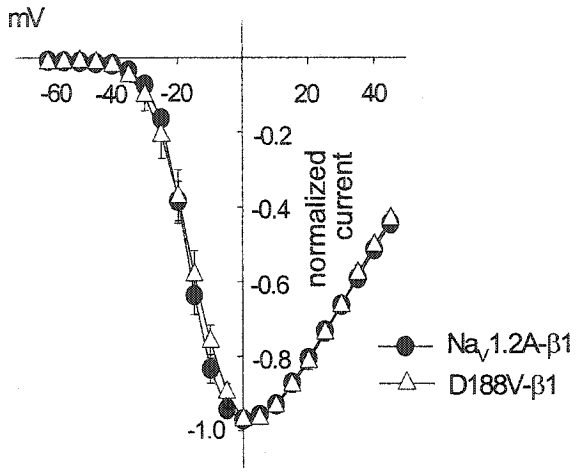

A
Na$_V$1.2a-β1
100pA
2msec
D188V-β1
60pA
2msec
B
Normalized current
0.0
0.5
1.0
Na$_V$1.2a-β1
D188V-β1
0
5
10
15
msec
C
0.0
0.1
0.2
0
5
10
15
msec
D
τ_{inact} (msec)
4
3
2
1
0
Na$_V$1.2a-β1
D188V-β1
-20 -10 0 10 20 30 40
Test Pulse (mV)
E
Normalized I_{NaP}
0.04
0.02
0.00
Na$_V$1.2a-β1
D188V-β1
-30 -20 -10 0 10
Test potential (mV)

FIGURE 8- *D188V DOES NOT AFFECT THE VOLTAGE-DEPENDENCE OF CHANNEL ACTIVATION OR FAST INACTIVATION.*

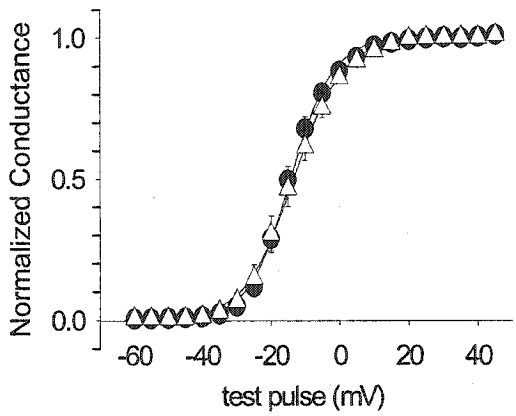
(A) Mean current-voltage relationships. No difference is observed between the wild type and mutant channels. (B) Conductance curves of wild type and mutant proteins are nearly identical. (C) Steady-state inactivation curves determined using 100 msec conditioning pulses of the wild type and mutant channels exhibit no differences.

Fig 8

A


mV
-60
-40
-20
20
40
-0.2
-0.4
-0.6
-0.8
-1.0
normalized current
Na$_V$1.2A-β1
D188V-β1

B


Normalized Conductance
1.0
0.5
0.0
-60
-40
-20
0
20
40
test pulse (mV)

C

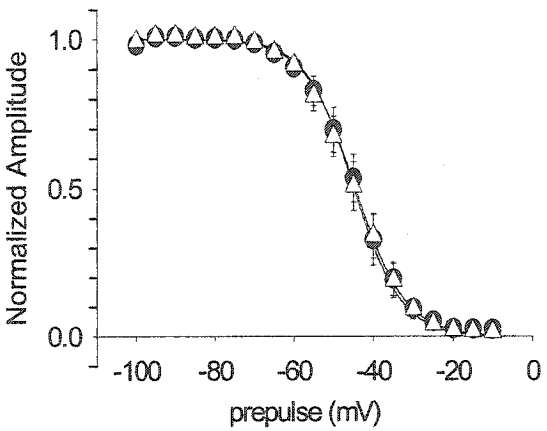

Normalized Amplitude
1.0
0.5
0.0
-100
-80
-60
-40
-20
0
prepulse (mV)

FIGURE 9- *EXPRESSION LEVELS OF WT AND MUTANT CHANNELS WERE INDESTINGUISHABLE.*

(A) Average maximum current evoked by a 0mV test potential from a holding potential of –90mV, show no difference between wild type and mutant channels. (B) Channel density measured by maximum current amplitude evoked by a 0mV test potential from a holding potential of –90m V / whole cell capacitance. No difference is observed between wild type and mutant channels.

Fig 9

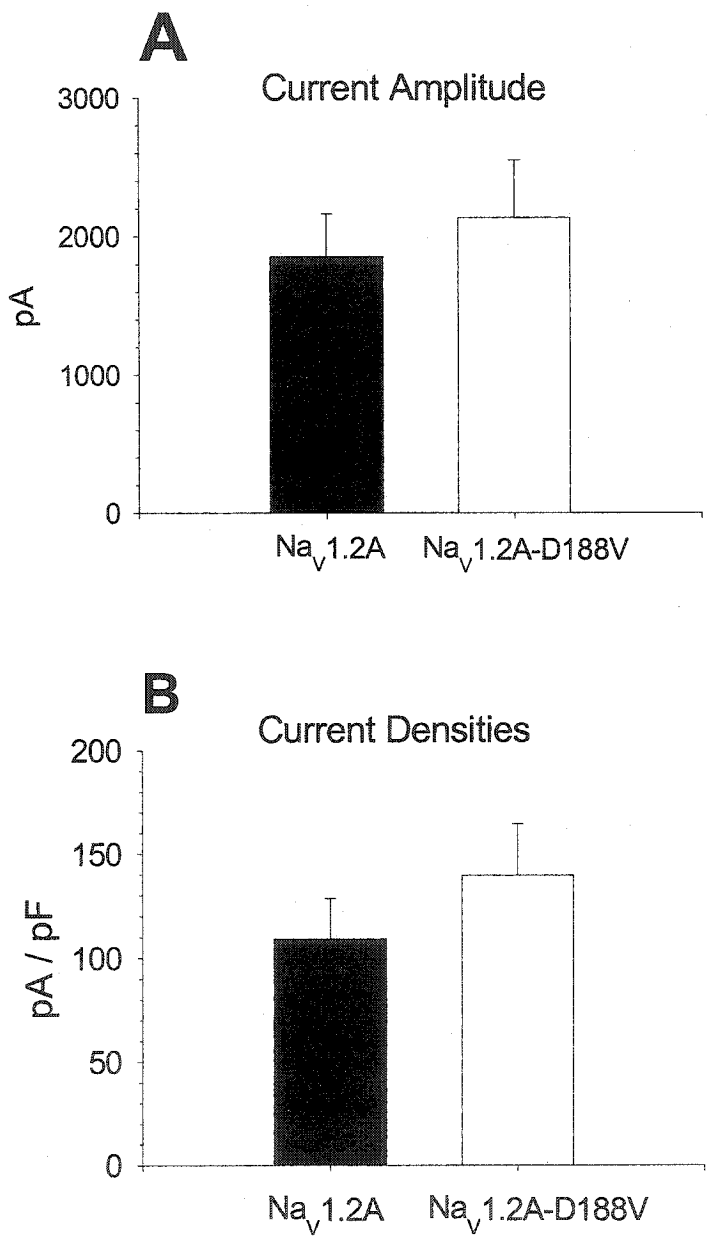

A
Current Amplitude
3000
2000
1000
0
pA
$Na_V1.2A$
$Na_V1.2A$-D188V
B
Current Densities
200
150
100
50
0
pA / pF
$Na_V1.2A$
$Na_V1.2A$-D188V

FIGURE 10- *D188V PREVENTS CURRENT CUMULATIVE INACTIVATION DURING RAPID PULSE TRAINS.*

(A) Typical currents for $Na_V1.2A$ and $Na_V1.2A$-D188V elicited by 5 msec depolarization from –80 to +10 mV, at a frequency of 80Hz. For clarity the 1st, 2nd, 10th and 100th traces of a 100 pulse train are shown. (B-D) Mean current amplitude normalized to the amplitude of the 1st trace for $Na_V1.2A$ and $Na_V1.2A$-D188V during 100 5 msec-long depolarizing pulses from –80 to +10 mV applied at a frequency of 10 (B), 80 (C) or 100 (D) Hz. E: Summary of frequency modulation of current amplitude induced by 100 depolarizing pulses. * corresponds to $p < 0.05$ and ** corresponds to $p < 0.01$, according to Student's t-test.

Fig 10

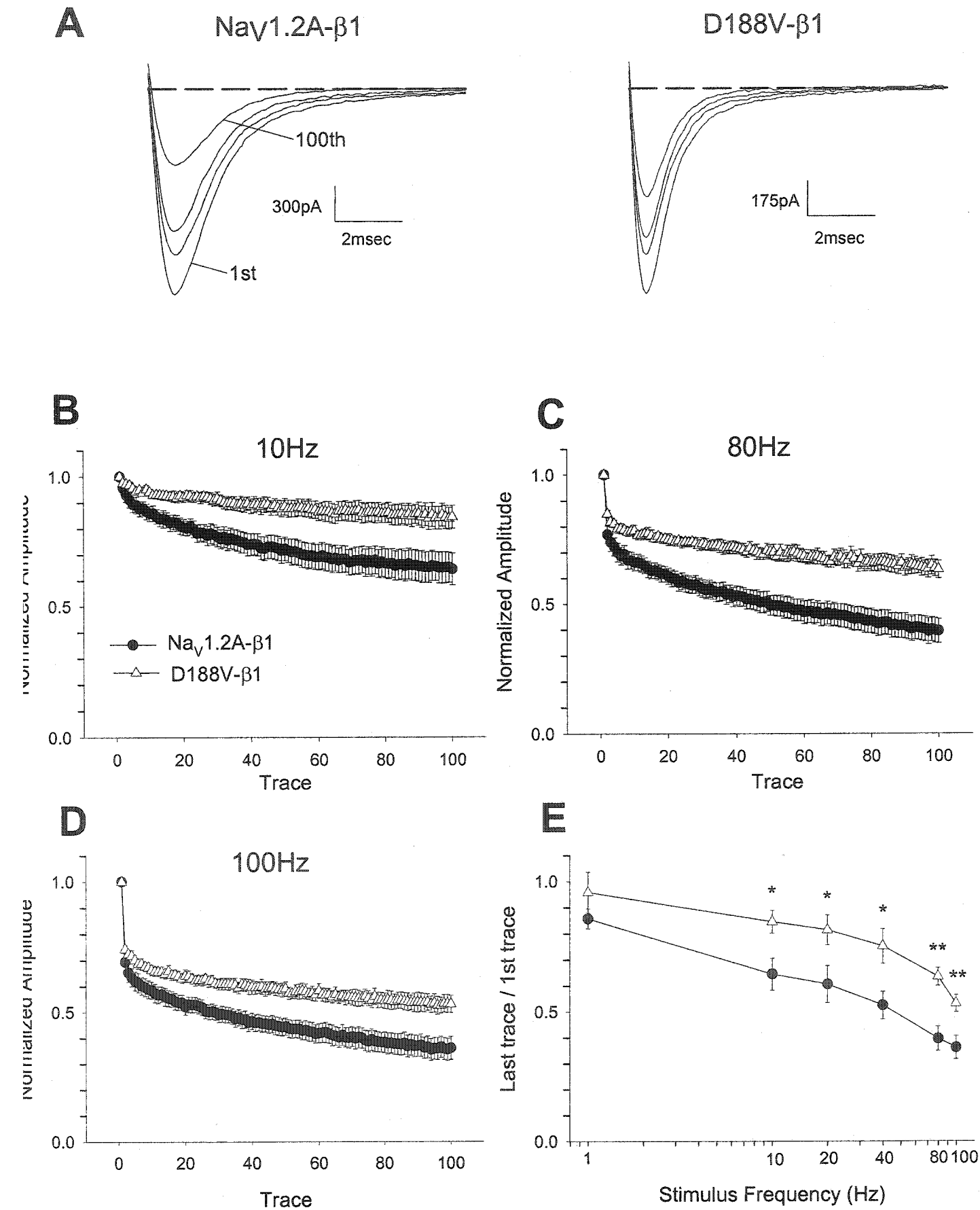

A
Nav1.2A-β1
D188V-β1
100th
1st
300pA
2msec
175pA
2msec
B
10Hz
Normalized Amplitude
Na$_V$1.2A-β1
D188V-β1
Trace
C
80Hz
Normalized Amplitude
Trace
D
100Hz
Normalized Amplitude
Trace
E
Last trace / 1st trace
Stimulus Frequency (Hz)

FIGURE 11- *RATE OF RECOVERY FOR $Na_V1.2$ AND D188V-$Na_V1.2$ ARE NEARLY INDISTINGUISHABLE.*

(A) Proportion of current amplitude recovered from inactivation in relation to recovery time at -90mV for a 5 msec conditioning pulse of 0mV, induced by a test pulse to 0 mV. (B) Recovery rates were fitted with a single exponential.. The plot shows recovery time constants determined at different recpvery potentials.

Fig 11

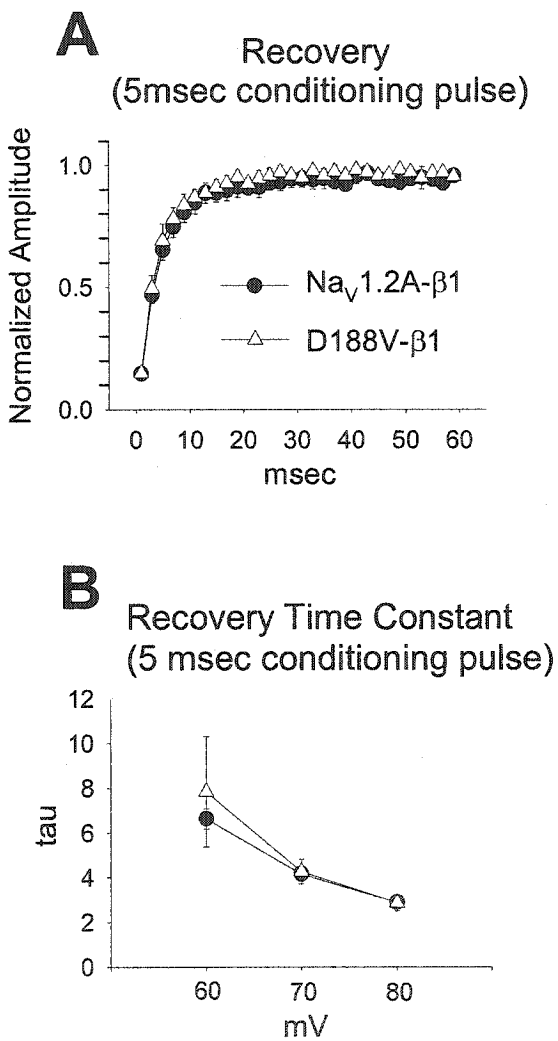

A
Recovery
(5msec conditioning pulse)
Normalized Amplitude
1.0
0.5
0.0
0 10 20 30 40 50 60
msec
Na$_V$1.2A-β1
D188V-β1
B
Recovery Time Constant
(5 msec conditioning pulse)
tau
12
10
8
6
4
2
0
60 70 80
mV

FIGURE 12- *D188V CHANNELS RECOVER FROM INACTIVATION FASTER THAN WT CHANNELS FOLLOWING A LONG CONDITIONING PULSE.*

Normalized current amplitudes of $Na_V1.2A$ or $Na_V1.2A$-D188V elicited by a 0 mV test pulse following a 1sec depolarization to 0 mV and a varying length of recovery time at –80 mV. Mutant channels recover more quickly from prolonged depolarizations compared to wild type channels.

Fig 12

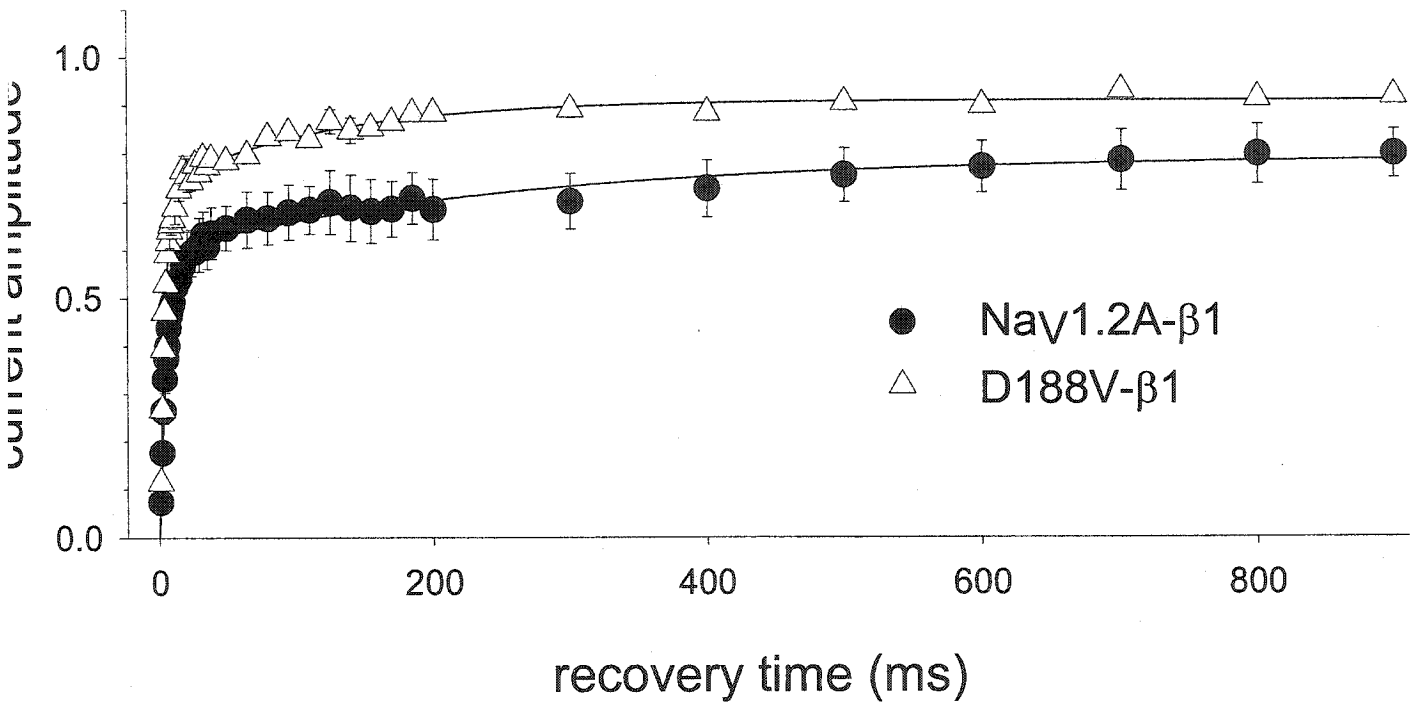

current amplitude
1.0
0.5
0.0
0
200
400
600
800
recovery time (ms)
Nav1.2A-β1
D188V-β1

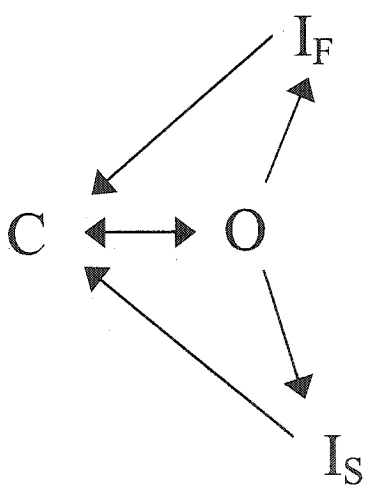
FIGURE 13- *MODEL OF SODIUM CHANNEL KINETIC STATES.*

Channels open (O) from a closed state (C) in response to membrane depolarization. They may then either close or enter either of two inactivated states: fast inactivation (I_F) or slow inactivation (I_S). These states are modulated by distinct molecular mechanisms and are characterized by different recovery time constants. Fast inactivation lasts on the order of milliseconds whereas slow inaction may last up to tens of seconds. D188V-$Na_V1.2$ are less prone to enter the slow inactivated state (dashed arrow).

Fig 13

Model of Sodium Channel Kinetic States

$Na_V1.2$



D188V-$Na_V1.2$

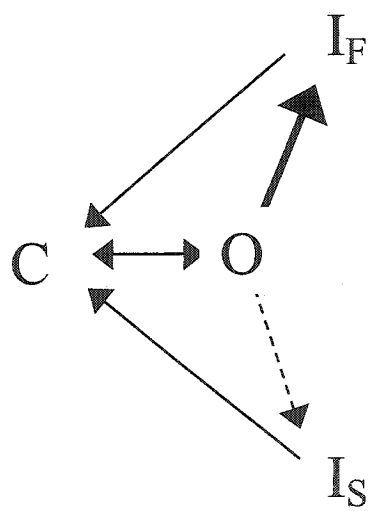


Table 1- Expression profiles of sodium channel α subtypes and corresponding chromosomes.

NaV1.1, NaV1.2, NaV1.3 and NaV1.6 are the predominant alpha sodium channel subtypes expressed in the central nervous system. NaV1.4 is expressed in skeletal muscle, NaV1.5, in the heart and NaV1.7-NaV1.9, in the peripheral nervous system.

Table 1

Sodium channel subtype distribution profile

Sodium channel subtype	Human gene	Expression profile
NaV1.1	SCN1A	CNS
NaV1.2	SCN2A	CNS
NaV1.3	SCN3A	CNS
NaV1.4	SCN4A	Skeletal muscle
NaV1.5	SCN5A	Heart
NaV1.6	SCN8A	CNS
NaV1.7	SCN9A	PNS
NaV1.8	SCN10A	PNS
NaV1.9	SCN11A	PNS

Table 2 - *Naturally occurring mutations of ion channels cause neurological diseases.*

Neurological disorders caused by ion channel mutations are called channelopathies. Affected channels include voltage-gated sodium, potassium and calcium channels which may cause an entire spectrum of disorders including chronic migraines to certain forms of epilepsy. Considering the important role played by ion channels, it is likely that many other channelopathic mutations will be identified.

Table 2

Naturally occurring mutations of ion channels cause neurological diseases

Disorder	Channel Gene
Episodic ataxia with myokymia (EA-1)	KCNA1
Episodic ataxia (EA-2)	CACNL1A4
Hemiplegic migraine	CACNL1A4
Spinocerebellar ataxis (SCA6)	CACNL1A4
Benign neonatal familial convulsions	KCNQ2, KCNQ3
Generalized epilepsy with febrile seizures plus	SCNIB, SCN1A
A.D. nocturnal frontal lobe epilepsy	CHRNA4
Startle disease (hyperekplexia)	GLRA1
Jervell and Lange-Nielson syndrome	KCNQ1

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